PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

Alcohol Dehydrogenase Activity Converts 3″-Hydroxy-geranylhydroquinone to an Aldehyde Intermediate for Shikonin and Benzoquinone Derivatives in *Lithospermum erythrorhizon*

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(Received 16 May 2020; Accepted 10 August 2020)

Shikonin derivatives are red naphthoquinone pigments produced by several boraginaceous plants, such as *Lithospermum erythrorhizon*. These compounds are biosynthesized from *p*-hydroxybenzoic acid and geranyl diphosphate. The coupling reaction that yields *m*-geranyl-*p*-hydroxybenzoic acid has been actively characterized, but little is known about later biosynthetic reactions. Although 3″-hydroxy-geranylhydroquinone produced from geranylhydroquinone by CYP76B74 has been regarded as an intermediate of shikonin derivatives, the next intermediate has not yet been identified. This study describes a novel alcohol dehydrogenase activity in *L. erythrorhizon* cell cultures. This enzyme was shown to oxidize the 3″-alcoholic group of (*Z*)-3″-hydroxy-geranylhydroquinone to an aldehyde moiety concomitant with the isomerization at the C2′–C3′ double bond from the *Z*-form to the *E*-form. An enzyme oxidizing this substrate was not detected in other plant cell cultures, suggesting that this enzyme is specific to *L. erythrorhizon*. The reaction product, (*E*)-3″-oxo-geranylhydroquinone, was further converted to deoxyshikonofuran, another meroterpenoid metabolite produced in *L. erythrorhizon* cells. Although nonenzymatic cyclization occurred slowly, it was more efficient in the presence of crude enzymes of *L. erythrorhizon* cells. This activity was detected in both shikonin-producing and nonproducing cells, suggesting that the aldehyde intermediate at the biosynthetic branch point between naphthalene and benzo/hydroquinone ring formation likely constitutes a key common intermediate in the synthesis of shikonin and benzoquinone products, respectively.

Keywords: Alcohol dehydrogenase • Benzoquinone • Cell cultures • *Lithospermum erythrorhizon* • Naphthoquinone pigment • Shikonin • Specialized metabolism.

Introduction

Shikonin is a red naphthoquinone pigment produced by some boraginaceaeous plants, including *Lithospermum erythrorhizon* and species belonging to the genera *Arnebia*, *Anchusa*, *Alkanna*, *Echium* and *Onosma*. Shikonin derivatives exist as esters of low-molecular-weight fatty acids in the root bark of these plants (**Fig. 1A**). Because these naphthoquinone derivatives have various biological activities, including anti-inflammatory, antibacterial and hemostatic activities, the dried roots of *L. erythrorhizon* and other shikonin-containing plants are widely used as crude drugs in European and Asian countries (reviewed in Yazaki 2017). Recently, shikonin derivatives were found to inhibit the main protease of the pandemic virus SARS-CoV-2 (Jin et al. 2020). Shikonin derivatives have also been used for over 2,000 years as natural dyes to stain clothes, yielding a purple color with Al-containing mordants. *Lithospermum erythrorhizon*, however, has been classified as an endangered species, leading to the establishment of cell suspension culture systems for the industrial production of shikonin derivatives (Tabata and Fujita 1985).

Studies of the biosynthesis of shikonin have utilized biochemical methods, including enzymatic analysis, and feeding radio-labeled compounds to cells. The shikonin biosynthetic pathway, especially its latter half, has not yet been determined completely (Inouye et al. 1979, Okamoto et al. 1995, Yazaki et al. 1997, Lange et al. 1998, Mühlenweg et al. 1998, Wang et al. 2019, Yazaki et al. 2001). More recent developments in sequencing technology and other omics techniques have led to multiple omics studies and genome analysis of shikonin biosynthesis (Takanashi et al. 2019, Tang et al. 2020, Ueoka et al. 2020). These studies have identified candidate genes encoding enzymes putatively involved in shikonin biosynthesis, such as

Plant Cell Physiol. 61(10): 1798–1806 (2020) doi:10.1093/pcp/pcaa108, Advance Access publication on 18 August 2020, available online at https://academic.oup.com/pcp

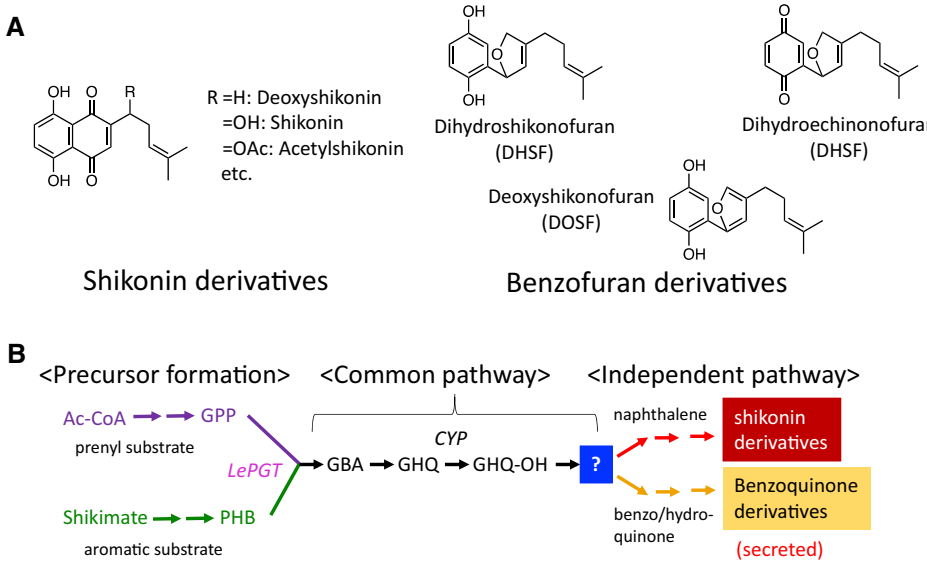

Fig. 1 Shikonin and benzoquinone derivatives in *L. erythrorhizon*. Chemical structures of shikonin derivatives and representative benzo/hydroquinone derivatives described in this study (A). Current understanding of shikonin and benzo/hydroquinone biosynthetic pathways in cultured *L. erythrorhizon* cells (B)

P450s, dioxygenases, alcohol dehydrogenases and flavin-linked oxidoreductases. However, the types of enzymes responsible for individual biosynthetic reactions, especially those in the latter half of the shikonin biosynthetic pathway, remain largely unknown. Conventional biochemical methods may result in the detection of enzyme activity and the identification of reaction products in *L. erythrorhizon* cells. Fresh materials, such as cultured cells and hairy roots capable of producing large quantities of shikonin derivatives, may provide a better alternative than intact plants.

Methods by which shikonin biosynthesis is regulated in cultured *L. erythrorhizon* cells have been described (Yazaki 2017). Shikonin is a C_{16} compound built from two key precursors, *p*-hydroxybenzoic acid (PHB) and geranyl diphosphate (GPP), which are coupled by a PHB-specific geranyltransferase (PGT) to yield *m*-geranyl-*p*-hydroxybenzoic acid (**Fig. 1B**) (Yazaki et al. 2002, Ohara et al. 2013). This intermediate is converted to geranylhydroquinone (GHQ) by an as-yet-unknown mechanism, and GHQ is hydroxylated to 3″-hydroxy-geranylhydroquinone ((Z)-3″-OH-GHQ) by a P450 enzyme (Yamamoto et al. 2000a). An RNAi-mediated technique showed that this hydroxylated metabolite is an intermediate of shikonin derivatives (Wang et al. 2019). Cultured cells maintained in the Linsmaier–Skoog (LS) medium (Linsmaier and Skoog 1965) do not produce shikonin derivatives because the concentration of ammonium ion in the medium is high. Rather, these cells produce benzoquinone and hydroquinone derivatives, including the C_{16} compounds deoxyshikonofuran (DOSF), dihydroshikonofuran (DHSF) and dihydroechinofuran (DHEF) (**Fig. 1A**) (Yazaki et al. 1986, Yazaki et al. 1987, Fukui et al. 1992). Cells cultured in M9 medium, however, produce shikonin derivatives along with these benzo/hydroquinone derivatives. Regulation of the branch reactions, toward either benzo/hydroquinones or shikonins, has not been clarified, and the intermediate at this branch position has not been determined. The intermediate after (Z)-3″-OH-GHQ and the enzyme families that mediate the biosynthetic reaction remain unclear. To identify the next biosynthetic intermediate, this study analyzed the activities of enzymes in cell-free extracts of cultured *L. erythrorhizon* cells that utilize (Z)-3″-OH-GHQ as their substrate.

Results

Enzyme assay

Both shikonin and benzoquinone derivatives are highly oxidized metabolites. Thus, several types of oxidative enzymes, such as P450s, alcohol dehydrogenases, polyphenol oxidases and flavin-linked oxidoreductase, are thought to be involved in the biosynthetic pathway from 3″-hydroxy-geranylhydroquinone ((Z)-3″-OH-GHQ) to shikonin/benzoquinone derivatives (**Fig. 1B**). Because shikonin is a strong inhibitor of many enzymes, such as chorismate mutase, prenyltransferase (Heide and Tabata 1987) and P450 (Yamamoto et al. 2000a), a crude cell-free extract (ca. 200 μg protein) was prepared from *L. erythrorhizon* cells cultured in modified LS medium for 6 d, conditions that do not result in the production of shikonin derivatives. Incubation of this cell-free extract for 30 min at 30°C with 0.2 mM of chemically synthesized (Z)-3″-OH-GHQ in the presence or the absence of 1 mM $NADP^+$ yielded two peaks, compounds **A** and **B**, corresponding to new enzymatic reaction products (**Fig. 2**). The production of compound **B** was enhanced in the presence of $NADP^+$ (**Fig. 2A**), whereas both compounds were detected in the absence of $NADP^+$. Heat treatment of the cell-free extract abolished the conversion of (Z)-3″-OH-GHQ to compounds **A** and **B** (**Fig. 2C**). Neither compound was detected in the absence of substrate (**Fig. 2D**), and no other compounds were produced. Incubation of the microsomal fraction of the cell-free extract with (Z)-3″-OH-GHQ yielded only trace amounts of

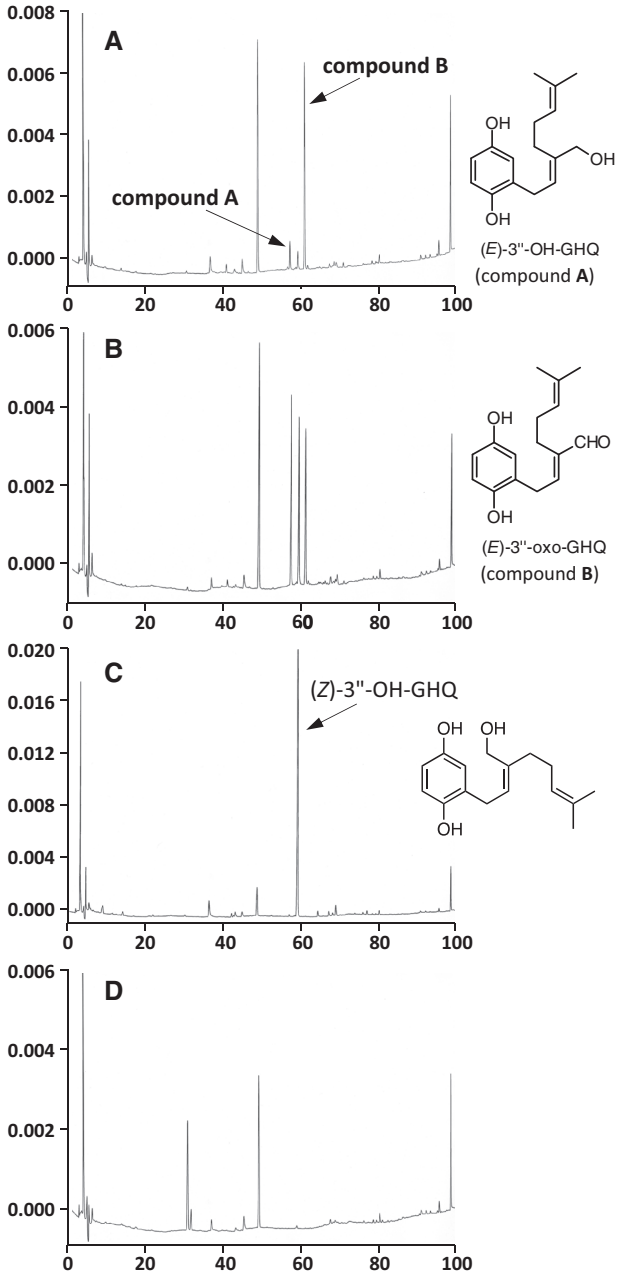

Fig. 2 Conversion of (*Z*)-3″-OH-GHQ by crude cell-free extracts of *L. erythrorhizon* cells cultured in the modified LS medium. Incubation of 0.2 mM (*Z*)-3″-OH-GHQ with ca. 200 μg protein for 30 min at 30°C in the presence (A) or absence (B) of 1 mM $NADP^+$ as a cofactor. (C) Incubation of 0.2 mM (*Z*)-3″-OH-GHQ with heat-denatured crude cell-free extracts and 1 mM $NADP^+$. (D) Incubation of crude extract without the substrate.

compounds **A** and **B**, suggesting that the synthesis of these compounds was catalyzed by a soluble, not by a membrane-associated, enzyme(s).

Isolation of the enzymatic reaction products

To determine the structures of compounds **A** and **B**, 60 μmol (15.72 mg) of chemically synthesized (*Z*)-3″-OH-GHQ and 60 μmol $NADP^+$ were incubated with $(NH_4)_2SO_4$-fractionated enzymes prepared from 160 g of cells cultured in modified LS medium. The reaction was terminated by the addition of acid, the mixture was partitioned with ethyl acetate and the organic phase was evaporated in vacuo. Separation of the residue by preparative high performance liquid chromatograpy (HPLC) yielded 4.1 mg of compound **A** and 3.8 mg of compound **B**.

Determination of the structures of the enzymatic reaction products

High-resolution (HR)-electrospray ionization (ESI)-mass spectropetry (MS) analysis showed that the molecular formula of compound **A** was $C_{16}H_{22}O_3$. The proton signal at δ 4.03 and the carbon signal at δ 66.84 on ^1H and ^{13}C NMR spectra indicated the presence of a hydroxymethyl group at the side chain. These spectral data were almost same as those of synthetic (*Z*)-3″-OH-GHQ, except for protons and carbons around the C2′–C3′ double bond, suggesting that **A** is a stereoisomer of (*Z*)-3″-OH-GHQ. The (2′*E*)-configuration of **A** was confirmed by observation of cross-peaks for 1′-H_2/4′-H_2 and 2′-H/CH_2OH in the 2D NOESY spectrum (Fig. 2, Supplementary Data 1).

HR-ESI-MS analysis showed that the molecular formula of compound **B** was $C_{16}H_{20}O_3$. The proton signal at δ 9.42 and the carbon signal at δ 195.41 on ^1H and ^{13}C NMR spectra for **B** indicated the presence of a formyl group. These spectral data were in good accordance with those of synthetic (*E*)-3″-oxo-GHQ (Fig. 2, Supplementary Data 1). The (2′*E*)-configuration of **B** was confirmed by the observation of NOESY correlations for 1′-H_2/4′-H_2 and 2′-H/CHO.

Spectral data of compounds **A** and **B** are shown in the Materials and Methods sections, and the structures of compounds **A** ((*E*)-3″-oxo-GHQ) and **B** ((*Z*)-3″-OH-GHQ) are shown in Fig. 2 (insert).

Partial purification of enzymes

To clarify the cofactor requirement for the enzymatic reaction, the supernatant of cell homogenate was fractionated by $(NH_4)_2SO_4$ precipitation from 0% to 30% and from 30% to 90%. Each fraction was dissolved in Tris-HCl buffer (pH 8.0) and desalted, and ca. 50 μg protein was incubated with 20 μmol (*Z*)-3″-OH-GHQ in the presence or absence of $NADP^+$. Although the cell-free extract shown in Fig. 3A appeared to contain low-molecular-weight cofactors, fractions prepared from $(NH_4)_2SO_4$ precipitates required $NADP^+$ for the conversion of (*Z*)-3″-OH-GHQ (Fig. 3B, C). The activity was clearly concentrated in the 30–90% $(NH_4)_2SO_4$ fraction, suggesting that passage of cell debris-removed supernatant through PD-10 would be insufficient to remove low-molecular-weight cofactors.

Time course of enzymatic reactions

(*E*)-3″-OH-GHQ and (*E*)-3″-oxo-GHQ can be synthesized from (*Z*)-3″-OH-GHQ by two possible pathways: (i) the substrate is first isomerized and then dehydrogenated or (ii) the substrate is first dehydrogenated and then isomerized. The amount of (*E*)-3″-oxo-GHQ was higher than that of (*E*)-3″-OH-GHQ, suggesting that the second pathway was more likely (Fig. 3). This hypothesis was strongly supported by time-course experiments,

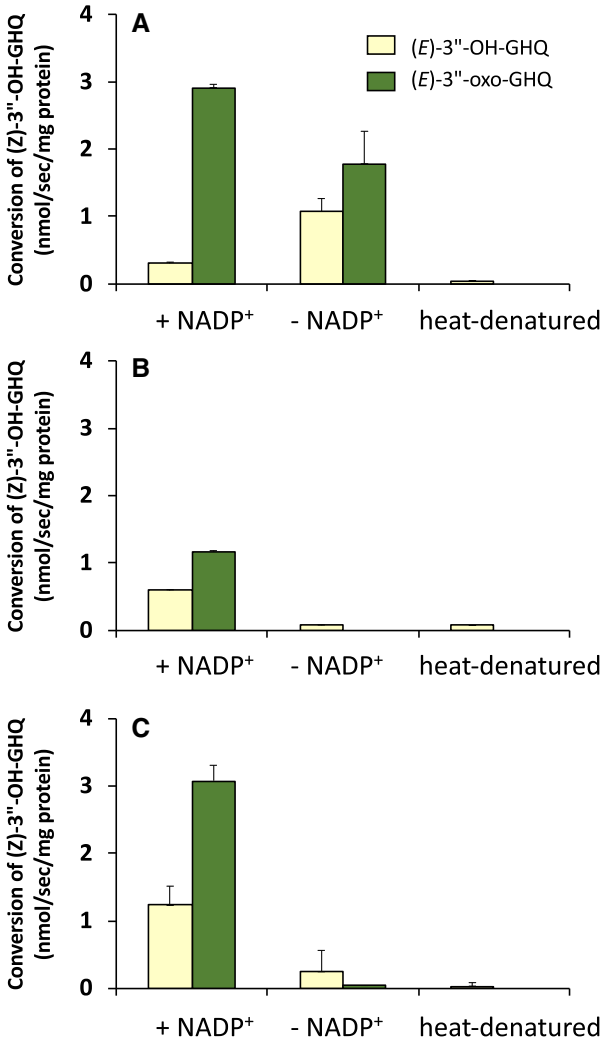

Fig. 3 Partial purification of enzymes from *L. erythrorhizon* cells. The supernatant of cell homogenate was fractionated by $(NH_4)_2SO_4$ precipitation from 0% to 30% and from 30% to 90% saturation and desalted by PD-10, and ca. 50 μg protein were incubated with 20 μmol (*Z*)-3″-OH-GHQ in the presence or absence of $NADP^+$, respectively. Yellow box: (*E*)-3″-OH-GHQ, green box: (*E*)-3″-oxo-GHQ. (A) Crude cell-free extracts, (B) 0–30% saturated $(NH_4)_2SO_4$ precipitates and (C) 30–90% saturated $(NH_4)_2SO_4$ precipitates.

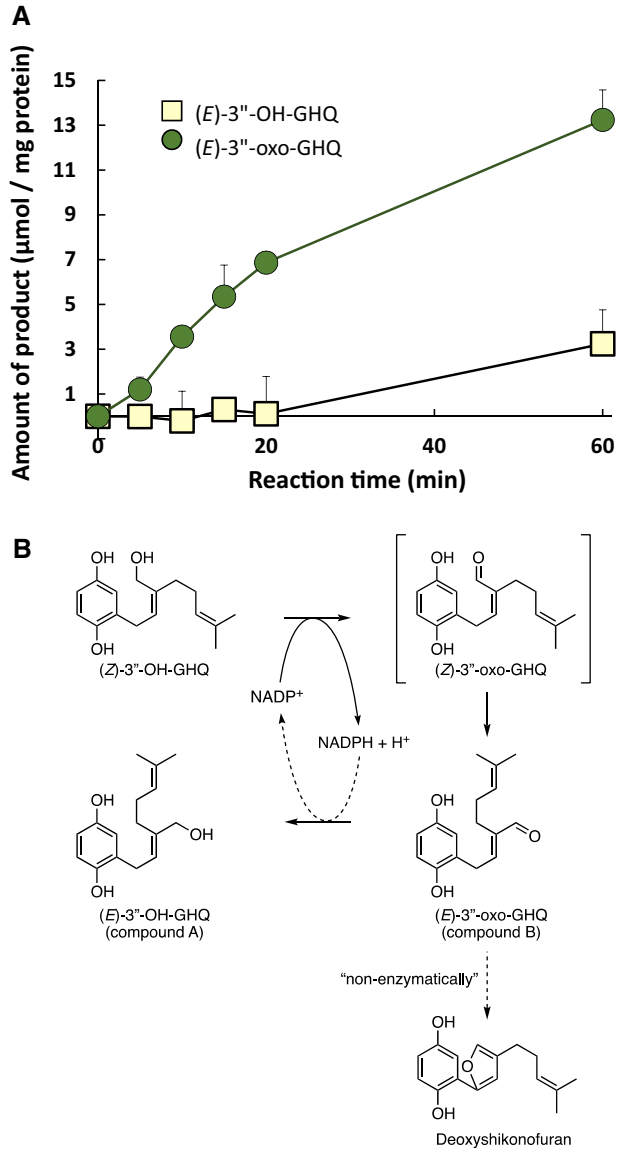

Fig. 4 Time-course of the production of (*E*)-3″-oxo-GHQ (green circles) and (*E*)-3″-OH-GHQ (yellow squares) during enzymatic reactions with (*Z*)-3″-OH-GHQ as the substrate (A). Schematic representation showing the enzymatic formation of (*E*)-3″-oxo-GHQ and (*E*)-3″-OH-GHQ from (Z)-3″-OH-GHQ (B). Nonenzymatic conversion to deoxyshikonofuran is also shown.

showing that (*E*)-3″-oxo-GHQ was produced initially and then reduced to (*E*)-3″-OH-GHQ (**Fig. 4A**). More importantly, during the chemical synthesis of putative biosynthetic metabolites, (*Z*)-3″-oxo-GHQ was very unstable and, once produced, was readily isomerized to its *E*-form nonenzymatically (see **Supplementary Data 1**). The enzymatic conversion of (*E*)-3″-oxo-GHQ to (*E*)-3″-OH-GHQ was found to be reversible, and the oxidation velocity of (*E*)-3″-OH-GHQ in the presence of 1 mM $NADP^+$ (1.02 ± 0.05 nmol/s/mg protein) was similar to the reduction velocity of (*E*)-3″-oxo-GHQ in the presence of 1 mM NADPH (1.54 ± 0.15 nmol/s/mg protein). NADPH formed by the oxidation of the alcohol might be used for the reduction in the aldehyde (**Fig. 4B**). No other compound was detected in this study.

Detailed characterization of the enzyme reaction

Many oxidoreductases have been reported to act on alcohol groups of donor substrates. These include geraniol dehydrogenases, which require $NADP^+/NAD^+$ as cofactors (Hassan et al. 2012), and cinnamyl alcohol dehydrogenases grouped in flavoproteins (Lauvergeat et al. 1995). The oxidoreductase in *L. erythrorhizon* cells catalyzing (*Z*)-3″-OH-GHQ dehydrogenation requires $NADP^+$ as its sole cofactor but could not use NAD^+ (**Fig. 5**). Although shikonin production is strongly inhibited by illumination (Tabata et al. 1974, Yamamoto et al. 2002), the oxidoreductase activity detected in the present study was not inhibited by light irradiation (**Supplementary Fig. S1**). Under the same conditions, about 60% of FAD (0.01 mM in 100 mM

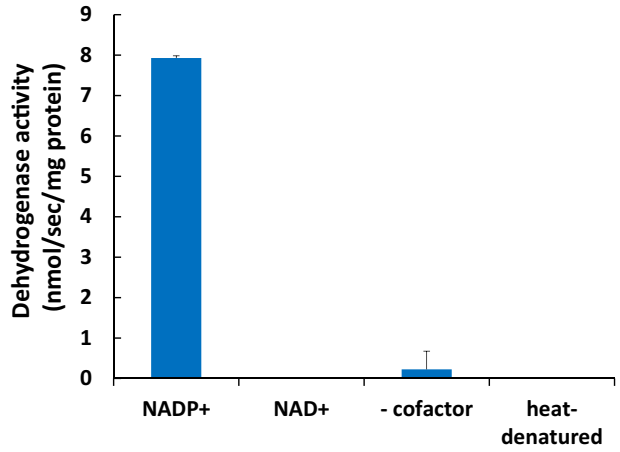

Fig. 5 Cofactor requirement of (Z)-3″-OH-GHQ dehydrogenase.

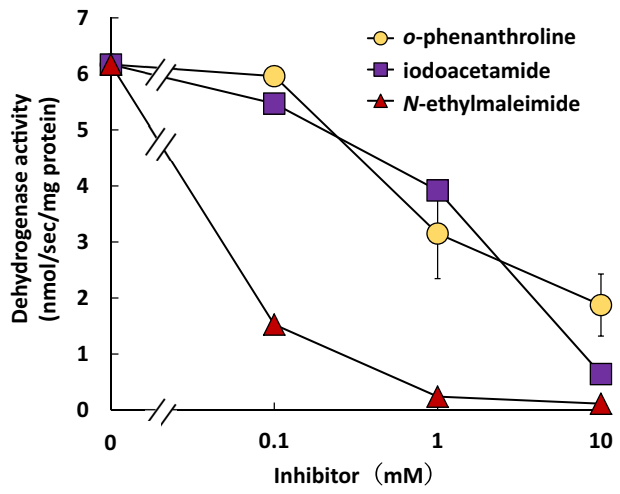

Fig. 6 SH reagents inhibit (Z)-3″-OH-GHQ dehydrogenase activity. Dose dependence of three inhibitors, *o*-phenanthroline, indoacetamide and *N*-ethylmaleimide, is shown.

Tris-HCl, pH 8.0) was decomposed. Moreover, this activity was inhibited by sulfhydryl (SH) reagents, such as *o*-phenanthroline, iodoacetamide and *N*-ethylmaleimide (**Fig. 6**). These results suggested that the enzyme that oxidizes (Z)-3″-OH-GHQ in *L. erythrorhizon* cells is an ADH-type oxidoreductase. This dehydrogenase had an optimum pH of about 8.5 (**Supplementary Fig. S2**) and K_m values of 19.89 ± 1.27 and 5.08 ± 0.19 μM for (Z)-3″-OH-GHQ and $NADP^+$, respectively, comparable to or lower than those of other plant ADHs (Hallahan et al. 1995, Hassan et al. 2012).

The substrate specificity of the dehydrogenase was assessed by monitoring the consumption of $NADP^+$. Use of geraniol, a common monoterpene alcohol, as a substrate resulted in an almost 10-fold greater consumption of $NADP^+$ than the use of (Z)-3″-OH-GHQ as the substrate (**Fig. 7**). However, the production of (E)-3″-oxo-GHQ was almost the same in the presence or absence of geraniol (**Fig. 7**, insert). An unknown enzyme(s) that reacts with geraniol and $NADP^+$, but is unrelated to shikonin biosynthesis, may be present in the $(NH_4)_2SO_4$-precipitated fraction. The dehydrogenase did not recognize other

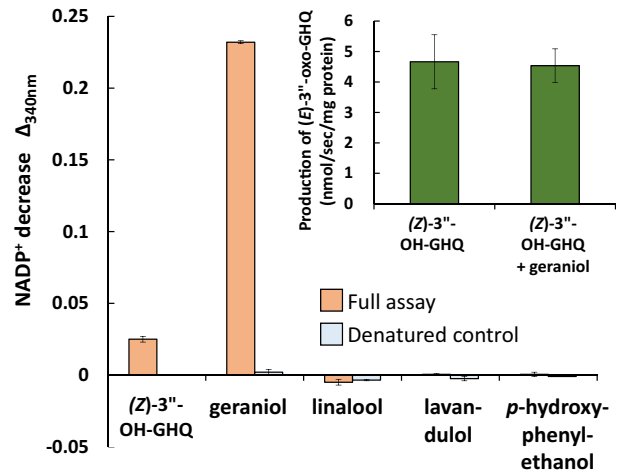

Fig. 7 Substrate specificity of dehydrogenase assayed by monitoring the consumption of $NADP^+$. The heat-denatured enzyme was used as a control. Insert: (Z)-3″-OH-GHQ dehydrogenase activity in the presence or absence of geraniol.

monoterpene alcohols, such as linalool and lavandulol, and the aromatic alcohol, *p*-hydroxyphenylethanol, as substrates.

Testing of the plant species specificity of the enzymatic activity responsible for the oxidation of (Z)-3″-OH-GHQ showed that this enzyme activity was to be specific for *L. erythrorhizon*, as it was present only in cultures of *L. erythrorhizon* cells, but not in cultures of *Sophora flavescens* cells, which produce prenylated flavonoids (Yamamoto et al. 1991), and in callus cultures of *Angelica keiskei*, which do not produce any prenylated polyphenols (Yamamoto et al., unpublished data). By contrast, intact *A. keiskei* cells were found to accumulate prenylated polyphenols, including xanthoangelol and bergapten (Akihisa et al. 2003, Caesar and Cech 2016). These species-specific results indicate that (Z)-3″-OH-GHQ dehydrogenase is closely correlated with shikonin/benzoquinone biosynthesis.

Formation of deoxyshikonofuran

Potentially, an aldehyde form of GHQ is a precursor common to both shikonin and benzo/hydroquinone derivatives. To investigate further conversion, 1 mM (E)-3″-oxo-GHQ was incubated in 100 mM Tris-HCl buffer (pH 7.0) with proteins prepared from *L. erythrorhizon* cells cultured in modified LS medium, as well as with heat-denatured proteins and in the absence of proteins. All of these incubations yielded a compound having a retention time and UV spectrum identical to those of deoxyshikonofuran, as confirmed by LC–MS analysis (ESI-positive mode, *m/z*: 259.13) (**Fig. 8A, B**). Incubation with *L. erythrorhizon* proteins yielded more deoxyshikonofuran than that resulting from its nonenzymatic formation from (E)-3″-oxo-GHQ in buffer solution, suggesting that the cell extract contains an enzyme that catalyzes the furan ring closure of the aldehyde compound. Testing of the protein extract of *L. erythrorhizon* cells grown in the M9 medium also showed the formation of deoxyshikonofuran. To detect new intermediates in the pathway between (E)-3″-oxo-GHQ and shikonin, these newly generated peaks were compared with various chemically synthesized

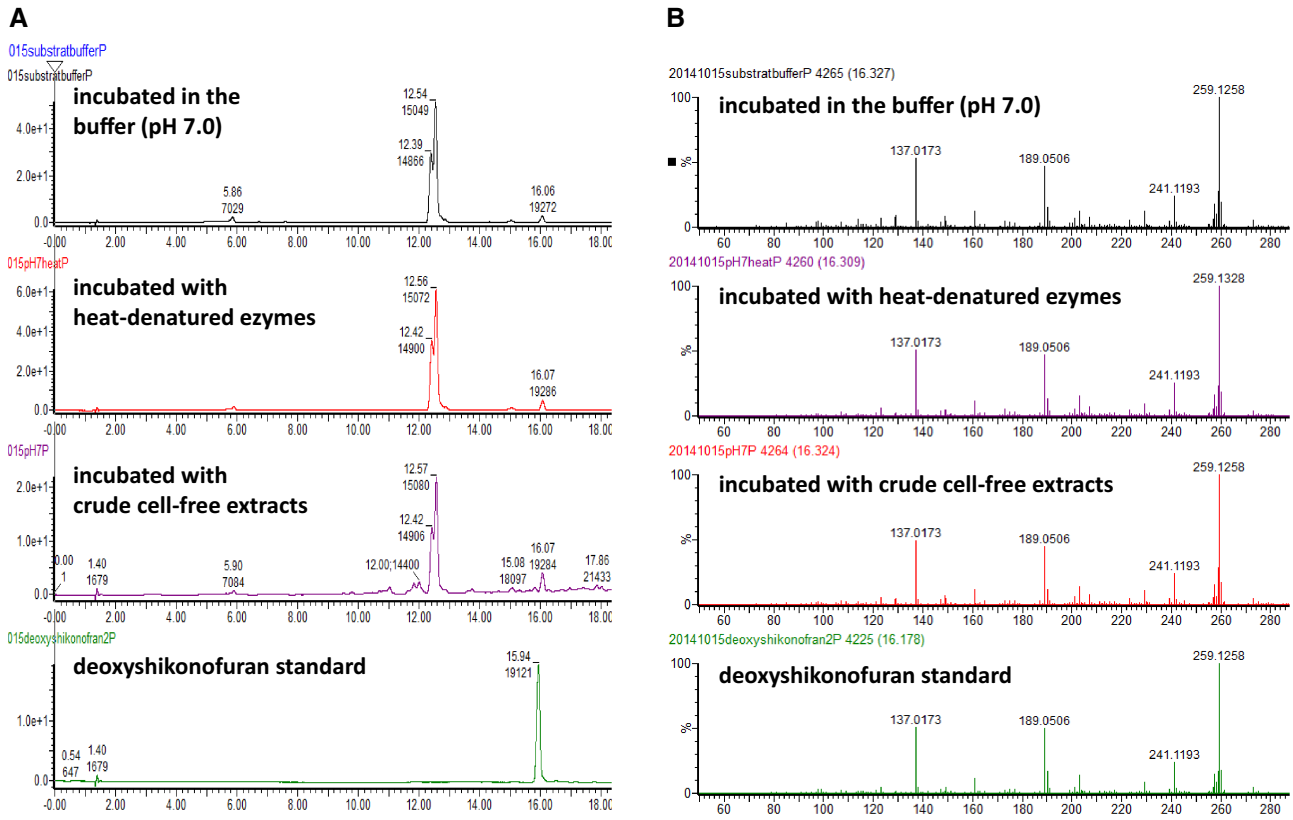

Fig. 8 Nonenzymatic deoxyshikonofuran formation from (*E*)-3″-oxo-GHQ. (*E*)-3″-oxo-GHQ was incubated in the buffer (pH 7.0) with the heat-denatured enzyme or crude cell-free extracts from shikonin-free cells. (A) Chromatograms of the samples at 280 nm and (B) ESI-mass spectra (positive mode) of the compound in **A** eluted at 16.0 min.

naphthalene derivatives (**Supplementary Data 1**), but no identical peaks were detected.

Discussion

Lithospermum erythrorhizon cells are cultured on an industrial scale to produce large quantities of shikonin derivatives. Due to their high productivity, yielding over 10% shikonin per dry weight, cells cultured in M9 medium are thought to produce only shikonin derivatives. However, these cells produce and secrete appreciable amounts of benzoquinone and hydroquinone derivatives represented products, such as dihydroechinofuran, that can be effectively trapped by the overlay of liquid paraffin on the medium (Fukui et al. 1992). These benzo/hydroquinone derivatives are also produced by cells cultured in LS medium, whereas shikonin is not (Yamamoto et al. 2000c) (**Supplementary Fig. S3**). Because the benzo/hydroquinone derivatives are C_{16} compounds synthesized from GHQ, they share a pathway common to naphthalene (shikonin) derivatives from PHB and geranyl diphosphate to the C_{16} intermediates. The point at which the common pathway diverges to produce either naphthalene or benzoquinone derivatives is therefore of great interest. The pathway leading up to the synthesis of (*Z*)-3″-OH-GHQ is thought to be common to shikonin and benzoquinone derivatives. The present study found that cultured *L. erythrorhizon* cells have a strong enzyme activity, oxidizing (*Z*)-3″-OH-GHQ to yield an aldehyde compound, with the latter being the key intermediate that forms the branch point between naphthalene and benzo/hydroquinone derivatives.

Although some of these benzo/hydroquinone derivatives were isolated from intact *L. erythrorhizon* (Yoshizaki et al. 1982, Yamamoto et al. 2000c, Liao et al. 2020), some benzoquinone derivatives, such as echinofuran B (**Supplementary Fig. S3**), are unstable, rapidly decomposing during the drying process even after purification. To maintain stability, echinofuran B should be stored in an organic solvent like ethyl acetate. Because of the chemical features of this compound, its presence in the crude drug (dried roots) of *L. erythrorhizon* is probably difficult to detect. Fresh roots of intact *L. erythrorhizon* plants or cultured hairy roots may be more appropriate for evaluating the biosynthetic ability of those benzo/hydroquinone metabolites, as these metabolites cannot be detected after drying but before extraction.

To our knowledge, the enzymatic reaction product (*E*)-3″-OH-GHQ had not been previously detected in *L. erythrorhizon*. This may have been due to its instability or the presence of putative 'metabolon' in shikonin and benzo/hydroquinone biosynthesis. Feeding experiments using radio-labeled phenylalanine detected no putative intermediate between GHQ and deoxyshikonin (Okamoto et al. 1995). This type of metabolon has been reported in ubiquinone biosynthesis (Gin and Clarke

2005), which shares a striking similarity with shikonin biosynthesis. In ubiquinone biosynthesis, a membrane-bound PHB: prenyltransferase COQ2, which is a homolog of *L. erythrorhizon* PHB:geranyltransferase (LePGT) (Yazaki et al. 2002), provides the membrane anchor, to which other soluble biosynthetic enzymes involved in ubiquinone biosynthesis, from coq3 to coq9, bind to form an enzyme complex, blocking the detection of the release of other biosynthetic intermediates. Similarly, during shikonin biosynthesis, other proteins interacting with LePGT may form a similar enzyme complex, blocking the release of intermediates and effectively converting these intermediates to the final product as shikonin derivatives.

The enzyme activity detected in the present study was surprisingly strong and specific to the geranyl moiety. A comparison between white shikonin-free cells grown in modified LS medium and red shikonin-producing cells grown in M9 medium showed that their oxidation activities were 6.28 ± 0.02 and 3.36 ± 0.13 nmol/s/mg of protein, respectively. This difference was likely due to shikonin derivatives being generally strong inhibitors of most enzymes (Heide and Tabata 1987, Yamamoto et al. 2000a). Moreover, this activity was specific to *L. erythrorhizon*. Enzymatic characterization strongly suggested that this reaction is catalyzed by an alcohol dehydrogenase. These findings may enable the identification of a gene responsible for this oxidation reaction.

The distance between the aromatic ring and the aldehyde group in (*E*)-3″-oxo-GHQ suggests that furan ring closure can occur in the presence or absence of enzyme. Although we detected the formation of deoxyshikonofuran from this aldehyde metabolite, this (*E*)-form is disadvantageous for naphthalene ring formation. A rearrangement of the groups around the C2′–C3′ double bond is likely necessary, and the coexistence of oxidoreductases or isomerases with the alcohol dehydrogenase may help clarify the mechanism of naphthalene ring formation.

Recent developments in analytical technology have led to the determination of *L. erythrorhizon* genome sequences (Tang et al. 2020), multiple omics studies of this plant (Takanashi et al. 2019), the establishment of an efficient stable transformation method (Tatsumi et al. 2020) and the identification of a cytosol-localized geranyl diphosphate synthase (Ueoka et al. 2020). These findings may help in identifying genes involved in each step of the biosynthesis of shikonin and benzo/hydroquinone derivatives. Moreover, a conventional biochemical approach, involving the characterization of native enzyme activities in this plant species, is indispensable to understand the correct biosynthetic pathways involved in the formation of these specialized metabolites. Combinations of molecular biological applications and biochemical characterization may clarify the entire biosynthetic routes of these unique secondary metabolites in *L. erythrorhizon*.

Materials and Methods

Chemical synthesis of standard substances

The enzymatic reaction products, (*Z*)-3″-OH-GHQ and (*E*)-3″-oxo-GHQ, and other putative intermediates of shikonin and benzoquinone (hydroquinone) derivatives were synthesized chemically, as described in **Supplementary Data 1**.

Other chemicals

Deoxyshikonofuran was isolated from *L. erythrorhizon* cell suspension cultures as described (Yazaki et al. 1986). $NADP^+$, NAD^+ and FAD were purchased from Oriental Yeast (Tokyo, Japan). Other chemicals were from Fujifilm Wako (Osaka).

Cell cultures

Lithospermum erythrorhizon cells of strain M18TOM (Yamamoto et al. 2000b) were grown on modified LS 1% agar medium (Mühlenweg et al. 1998) containing 1 μM indole-3-acetic acid (IAA) and 10 μM kinetin. Callus tissues were cultured in the dark in test tubes containing 10 ml medium at 23°C and subcultured at intervals of 1 month. For the experiments, the cells cultured for 1 month were inoculated into modified LS liquid medium containing 3% sucrose and the same growth regulators as above and agitated on a rotary shaker (80 rpm) at 23°C in the dark for 7 d. To induce shikonin production, cells cultured for 1 month in the modified LS medium were inoculated into the M9 medium (Fujita et al. 1981) containing 3% sucrose, the same plant hormones and 4.3 g liquid paraffin (Deno et al. 1987, Heide and Tabata 1987) and cultured for 5–10 d, as mentioned above.indole-3-acetic acid (IAA)

Callus cultures of *S. flavescens* were grown in the dark at 23°C on the Murashige–Skoog (MS) (Murashige and Skoog 1962) 0.3% gelrite medium containing 1 μM 2,4-dichlorophenoxyacetic acid and 1 μM kinetin and subcultured at 1-month intervals (Yamamoto et al. 1991). Callus cultures of *A. keiskei* were induced from plants grown in the experimental field of the Faculty of Life Sciences, Toyo University. Surface-sterilized stems of these plants were inoculated onto the MS 0.3% gelrite medium containing 100 μM 1-naphthaleneacetic acid and 1 μM 6-benzylaminopurine and incubated at 23°C in the dark. Callus tissues were subcultured in the same condition at 1-month intervals for >5 years.

Enzyme preparation

All procedures were performed at 4°C. Aliquots of 20 g of *L. erythrorhizon* cells cultured in modified LS medium were ground in a mortar containing 20 ml of 100 mM K-phosphate buffer (pH 6.5), 10 mM dithiothreitol, 2 g of polyvinylpolypyrrolidone and sea sand. The homogenate was centrifuged at 35,870 × *g* for 20 min to remove cell debris, and the supernatant was passed through a PD-10 column (GE Healthcare, Chicago, USA) equilibrated with 100 mM Tris-HCl buffer (pH 7.5) to obtain cell-free extracts. The supernatant was centrifuged at 160,000 × *g* for 30 min, and the resulting microsomal pellet was resuspended twice in 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM sodium ascorbate, recentrifuged at 160,000 × *g* for 30 min and finally resuspended in 2 ml of the same buffer. In 3″-hydroxy-geranylhydroquinone dehydrogenase assays, the 35,870 × *g* supernatant of cell homogenate was fractionated using solid $(NH_4)_2SO_4$. The precipitate obtained at 30–90% salt saturation was collected by centrifugation at 35,870 × *g* for 20 min, dissolved in 10 ml of 100 mM Tris-HCl buffer (pH 8.0) and desalted by gel filtration on PD-10 equilibrated with the same buffer. To extract enzymes from shikonin-producing cells, the liquid paraffin in the culture flask was diluted with 10 ml of *n*-hexane and removed with a pipet (Yamamoto et al. 2000a). The cells were collected by suction filtration, briefly washed with 100 ml of *n*-hexane and 100 ml of distilled water and prepared as above.

Protein content

Protein contents were determined according to the method of Bradford (1976) with BSA as standard protein.

Enzyme assays

The standard dehydrogenase assay solution consisted of 200 nmol (*Z*)-3″-OH-GHQ dissolved in 10 μl of methanol containing 200 nmol $NADP^+$, 2 μmol sodium ascorbate, about 20 μg of protein and 100 mM Tris-HCl buffer (pH 8.0), in a total volume of 200 μl. The reaction was initiated by the addition of (*Z*)-3″-OH-GHQ to the mixture; after incubation for 30 min at 30°C, the reaction was terminated by the addition of 200 μl of acetonitrile containing 0.2 μg 2-naphthol as an internal standard. The mixture was centrifuged at 4,900 × *g* for 5 min, and the supernatant was filtered with Minisart RC 4 (Sartorius Stedim Biotech, Goettingen, Germany) for HPLC analysis. The effect of light on dehydrogenase

activity was evaluated in an FLI-2000 growth chamber (EYELA, Tokyo, Japan). The enzyme solution in glass test tubes was irradiated with fluorescent light (212 μmol/m^2/s) for 30–60 min at 26°C, and the enzyme activities were assayed. Aluminum foil-covered tubes were used as the control. Substrate specificity was measured by monitoring the reduction in NADP$^+$ using a DU720 UV–VIS spectrophotometer (Beckman Coulter, Brea, USA) equipped with a 50-μl microcell folder. The reaction mixture was incubated for 15 min at 30°C, the reaction was terminated by the addition of acetonitrile, and the absorbance at 340 nm was measured. All assays were performed in duplicate.

HPLC analysis

The amount of reaction products was determined using an Alliance HPLC-PDA system (2695 Separation Module and 2996 Photodiode Array Detector, Waters, Milford, USA), equipped with a SHODEX C18M4E column (5 μm, 4.6 mm × 250 mm; SHOWA DENKO, Tokyo, Japan) in an oven at 40°C. The column was developed with a linear gradient of 0–100% methanol in H_2O containing 1% acetic acid in 100 min at a flow rate of 1.0 ml/min. The quantities were calculated from the peak area at 280 nm monitored by Empower Chromatography Data Software (Waters, USA). LC–MS analysis was performed on a UPLC system (ACQUITY UPLC system; Waters) coupled with an ACQUITY PDA eλ detector and a SYNAPT G2 MS detector (Waters) and an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 mm × 50 mm; Waters). The mobile phase consisted of solvent A (0.1 % HCOOH containing H_2O) and solvent B (0.1 % HCOOH containing methanol) at a flow rate of 0.1 min/ml under the following gradient condition: a 0–25 min linear gradient from 30% (v/v) to 100% (v/v) solvent B and 25–28 min isocratic with 100% (v/v) solvent B. The injection volume was 5 μl, and the column temperature was 40°C. Mass spectra were obtained in positive mode (m/z = 50–600) with the following settings: capillary cone, 2.4 kV; extraction cone, 5.0 V; sampling cone, 40 V; source temperature, 120°C; desolvation temperature, 450°C; and desolvation gas, 800 l/h.

Nonenzymatic formation of deoxyshikonofuran from (*E*)-3″-oxo-GHQ (200 nmol) dissolved in 10 μl of methanol was incubated for 10 min at 30°C with 190 μl of 100 mM Tris-HCl buffer (pH 7.0), 190 μl of crude *L. erythrorhizon* cell-free extracts in modified LS medium or 190 μl of heat-denatured enzymes (10 min, 90°C). A 10-μl aliquot of 5 M HCl was added to each, followed by extraction three times with 200 μl ethyl acetate each. The extracts were evaporated in vacuo, dissolved in 200 μl methanol and then analyzed by LC–MS.

Identification of products

To isolate reaction products, 60 μmol (15.72 mg) (*Z*)-3″-OH-GHQ, 60 μmol NADP$^+$ and 600 μmol sodium ascorbate were incubated at 30°C for 90 min with 61 mg of enzymes (total volume: 60 ml) prepared from shikonin-free cells cultured for 7 d in the modified LS medium. The reaction was terminated by the addition of 1.5 ml concentrated HCl, and the products were extracted three times with 20 ml ethyl acetate each. The combined organic phase was washed with 20 ml H_2O, dehydrated with Na_2SO_4 and evaporated in vacuo. The residue was dissolved in 0.9 ml methanol and separated by preparative HPLC on a Shimadzu LC-10A system (Shimadzu, Kyoto, Japan), consisting of a SHODEX C18M20E column (SHOWA DENKO), developed with 40:60:0.1 methanol:H_2O:HCOOH at a flow rate of 6 ml/min and an oven temperature of 40°C, with spectrophotometric detection at 280 nm. This reaction yielded 4.1 mg of compound **A** ((*E*)-3″-OH-GHQ) and 3.8 mg of compound **B** ((*E*)-3″-oxo-GHQ). The spectral data of these two compounds are shown below.

(*E*)-3″-OH-GHQ (compound **A**): ^{1}H NMR (acetone-d_6, 500 MHz) δ: 1.61, 1.66 (each 3H, *d*, J = 1 Hz, *gem*-CH_3), 2.14 (2H, br. *q*, J = 8 Hz, 5′-H_2), 2.21 (2H, *m*, 4′-H_2), 3.34 (2H, *d*, J = 7.5 Hz, 1′-H_2), 3.66 (1H, br. *t*, J = 5 Hz, OH), 4.03 (2H, br. *d*, J = 5 Hz, CH_2OH), 5.19 (1H, *t sept*, J = 7.5, 1 Hz, 6′-H), 5.59 (1H, br. *t*, J = 7.5 Hz, 2′-H), 6.49 (1H, *dd*, J = 8.5, 3 Hz, 5-H), 6.61 (1H, *d*, J = 3 Hz, 3-H), 6.65 (1H, *d*, J = 8.5 Hz, 6-H), 7.55, 7.56 (each 1H, *s*, phenolic OH × 2). ^{13}C NMR, (acetone-d_6, 125 MHz) δ: 17.73, 25.86 (*gem*-CH_3), 27.93 (C5′), 28.40 (C1′), 28.83 (C4′), 66.84 (CH_2OH), 113.76 (C5), 116.26 (C6), 117.16 (C3), 124.11 (C2′), 125.36 (C6′), 129.32 (C2), 131.91 (C7′), 140.91 (C3′), 148.64 (C1), 151.29 (C4). NOE: 1′-H—4′-H, 2′-H—CH_2OH. HRMS (ESI positive) m/z: 263.1643 (MH$^+$, $C_{16}H_{23}O_3$ requires 263.1642), 285.1461 (MNa$^+$, $C_{16}H_{22}O_3$Na requires 285.1461).

(*E*)-3″-oxo-GHQ (compound **B**): ^{1}H NMR (acetone-d_6, 500 MHz) δ: 1.59, 1.66 (each 3H, *d*, J = 1 Hz, *gem*-CH_3), 2.09 (2H, br. *q*, J = 7.5 Hz, 5′-H_2), 2.38 (2H, *t*, J = 7.5 Hz, 4′-H_2), 3.66 (2H, *d*, J = 7.5 Hz, 1′-H_2), 5.18 (1H, *t sept*, J = 7.5, 1 Hz, 6′-H), 6.57 (1H, *dd*, J = 8.5, 3 Hz, 5-H), 6.65 (1H, *d*, J = 3 Hz, 3-H), 6.70 (1H, *t*, J = 7.5 Hz, 2′-H), 6.72 (1H, *d*, J = 8.5 Hz, 6-H), 7.68, 7.89 (each 1H, br. *s*, phenolic OX × 2), 9.42 (1H, *s*, CHO). ^{13}C NMR, (acetone-d_6, 125 MHz) δ: 17.72 (7′-CH_3), 24.73 (C4′), 25.84 (7′-CH_3), 27.90 (C5′), 30.40 (C1′), 114.81 (C5), 116.62 (C6), 117.46 (C3), 124.67 (C6′), 126.58 (C2), 132.63 (C7′), 143.80 (C3′), 148.78 (C1), 151.47 (C4), 153.82 (C2′), 195.41 (CHO). NOE: 1′-H—4′-H, 2′-H—CHO. HRMS (ESI positive) m/z: 261.1487 (MH$^+$, $C_{16}H_{21}O_3$ requires 261.1485), 283.1306 (MNa$^+$, $C_{16}H_{20}O_3$Na requires 283.1305).

Supplementary Data

Supplementary data are available at PCP online.

Funding

Grant-in-Aid for Scientific Research by JSPS (19H05638 to K.Y.) and Grant for Mission Research by RISH, Kyoto University (to K. Y.).

Acknowledgment

The authors thank Ms. Noriko Machida for technical support.

Disclosures

The authors have no conflicts of interest to declare.

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