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ENZYME ACTIVITIES IN CELL-FREE EXTRACTS OF SHIKONIN-PRODUCING *LITHOSPERMUM ERYTHRORHIZON* CELL SUSPENSION CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; cell culture; naphthoquinones; shikonin; chorismate mutase; shikimate dehydrogenase.

Abstract—*Lithospermum erythrorhizon* cell cultures excrete large quantities of naphthoquinone pigments, *viz.* shikonin derivatives, which have made enzymatic studies impossible so far. This paper describes methods for the removal of shikonin derivatives from the cells with liquid paraffin during culture growth and for the preparation of active enzyme extracts. Chorismate mutase and shikimate dehydrogenase activities were used as indicators for the preparation of active extracts. The efficacy of the methods developed was proved by the demonstration of the enzymatic formation of *m*-geranyl-*p*-hydroxybenzoic acid from geranylpyrophosphate and *p*-hydroxybenzoic acid in cell-free extracts of *L. erythrorhizon* cultures, the first detection of a key enzyme of shikonin biosynthesis.

INTRODUCTION

Cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) are capable of producing large amounts of shikonin derivatives (see [1] for a review), which show antibacterial [2], antiinflammatory and wound-healing [3] activities. By the selection of stable high-yielding strains [4] and the adoption of a two-stage culture system [5, 6], the yield of shikonin derivatives could be increased to 4 g per l. medium in two weeks. This biotechnological process is now used for the industrial production of shikonin in Japan [1].

The biosynthetic pathway leading to alkannin and its enantiomer shikonin has been elucidated by Schmid and Zenk [7] and by Inouye *et al.* [8] and various physical and chemical factors influencing the production of shikonin have been investigated by Tabata *et al.* [9–14]. It has also been shown that shikonin formation is linked to the development of characteristic swellings of the rough endoplasmic reticulum, suggesting the ER as the site of the biosynthesis [15]. Biochemical studies on the regulation of shikonin biosynthesis, however, have been hampered by the technical difficulty of detecting the enzymes involved in this pathway. Shikonin derivatives, which have been used for dyeing silk since ancient times, bind irreversibly to proteins and are powerful enzyme inactivators. Previous attempts in this laboratory to demonstrate the activity of enzymes related to shikonin biosynthesis have therefore been unsuccessful.

This paper describes methods for the removal of shikonin derivatives from pigment-producing *L. erythrorhizon* cell suspension cultures and for the preparation of active enzyme extracts. The activity of chorismate mutase (EC 5.4.99.5) and of shikimate dehydrogenase (EC 1.1.1.25), both sensitive to inactivation by inhibitors present in shikonin-producing cultures, were used as parameters for the successful preparation of active enzyme extracts. The application of this method has made it possible, for the first time, to detect the activity of geranylpyrophosphate:*p*-hydroxybenzoate geranyltransferase, a key enzyme in shikonin biosynthesis.

RESULTS AND DISCUSSION

Demonstration of enzyme inhibition in extracts of shikonin-producing cultures

Enzyme extracts were prepared from two cell lines, the shikonin-producing strain M18 in M9 medium and the shikonin-free strain LY in LS medium [4–6]. DTT (0.2 mM) was used as the sole enzyme-protective reagent. Extracts from shikonin-producing cells were strongly coloured, suggesting the presence of phenolic or quinonoid compounds in these extracts. When alkaline buffers were used, the extracts showed the typical purple colour of shikonin bound to proteins. Activities of the primary metabolic enzymes chorismate mutase and shikimate dehydrogenase were barely detectable in these extracts, whereas both enzymes were present in preparations from shikonin-free cultures (Table 1). Mixtures of shikonin-producing and shikonin-free cells in a ratio of 1:1 also yielded extracts with a very low activity (see Table 1), showing that even the enzymes present in the shikonin-free cells were inactivated by contact with substances from the shikonin-producing cells.

From the data presented in Table 2, it appears that some enzyme inhibitors were still present in the crude cell-free extract from shikonin-producing cultures. When an extract from shikonin-producing cells (Table 2; **B**) was added to an active enzyme preparation from shikonin-free cells (**A**), the resulting mixture (**C**) showed an activity much less than the mean value between the activities of **A** and **B**. The inhibitor seems to be a low-molecular weight, soluble

Table 1. Inactivation of primary metabolic enzymes in cell-free extracts of shikonin-producing cell cultures of *Lithospermum erythrorhizon*

Enzyme activity (nKat/mg protein)	Extract from shikonin-free culture	Extract from shikonin-producing culture	Extract from mixture of shikonin-producing and shikonin-free culture (1:1)
Shikimate dehydrogenase	3.29 (100%)	0.04 (1.2%)	0.32 (9.7%)
Chorismate mutase	0.376 (100%)	0.027 (7.2%)	0.032 (8.5%)

Extracts were prepared, gel-filtered and assayed as described in the Experimental. The homogenization buffer contained DTT (0.2 mM) as the sole enzyme-protective reagent. The protein contents were 1.15 mg/ml (shikonin-free culture), 0.80 mg/ml (shikonin-producing culture) and 1.05 mg/ml (mixture of cells from both cultures).

Table 2. Shikimate dehydrogenase activity in extracts from shikonin-free and shikonin-producing cells, before and after gel-filtration through Sephadex G-25

Crude enzyme extract from:	Shikimate dehydrogenase activity (nKat/mg protein)	Gel-filtered extract from:	Shikimate dehydrogenase activity (nKat/mg protein)
A: shikonin-free cells	2.43 (100%)	**D**: shikonin-free cells	3.29 (135.4%)
B: shikonin-producing cells	0.02 (0.8%)	**E**: shikonin-producing cells	0.04 (1.6%)
C: mixture of extracts **A** and **B** (1:1)	0.31 (12.8%)	**F**: mixture of extracts **D** and **E** (1:1)	1.57 (64.6%)

Extracts were prepared and assayed as described in the Experimental. Protein contents of the extracts were: **A** = 1.63 mg/ml, **B** = 1.15 mg/ml, **D** = 1.11 mg/ml and **E** = 0.80 mg/ml.

compound, which can be removed by gel-filtration. When a gel-filtered extract from a shikonin-producing culture (**E**) was mixed with an extract from a shikonin-free culture (**D**), the activity of the mixture (**F**) corresponded approximately to the mean value between the activities of **D** and **E**. However, even after gel-filtration the extract of the shikonin-producing cells (**E**) was inactive, suggesting that an irreversible enzyme inhibition had taken place prior to filtration. These findings may be explained by assuming that shikonin derivatives, which are water-insoluble, cause the irreversible inhibition by binding to enzyme protein during homogenization, whereas an additional water-soluble inhibitor can be removed by gel filtration.

Removal of shikonin from cell cultures with liquid paraffin

Shikonin derivatives are excreted by *L. erythrorhizon* cells through the plasma membrane to accumulate on the outside of the cell wall [15]. Experiments carried out in the Bioscience Research Centre of Mitsui Petrochemical Industries showed that the pigments may be removed from the cells during culture growth by the addition of liquid paraffin to the liquid medium [Y. Fujita, personal communication]. We have investigated whether this method might yield cells accumulating only small amounts of shikonin derivatives on the cell wall while actively secreting these pigments, and whether the enzyme inactivation described above could be avoided if such cells were used for the preparation of extracts.

Cells of strain M18 were grown in 30 ml of the M9 medium overlaid with different amounts of liquid paraffin, and the cell growth as well as the shikonin accumulation in the cells and in the paraffin layer were observed. As Fig. 1 shows, growth was not affected by the presence of 10 ml paraffin, and production of shikonin derivatives was only slightly reduced. Most of the shikonin derivatives were found in the paraffin layer, whereas the cells were nearly free of these pigments in the early growth phase. Even after 9 days of culture, the cells contained only 1.4% of the total amount of shikonin produced. Similar results were obtained when 5 ml of paraffin were added per culture flask (data not shown), while the addition of 20 ml paraffin reduced shikonin production considerably, probably owing to an inhibition of the gas exchange between the medium and the air.

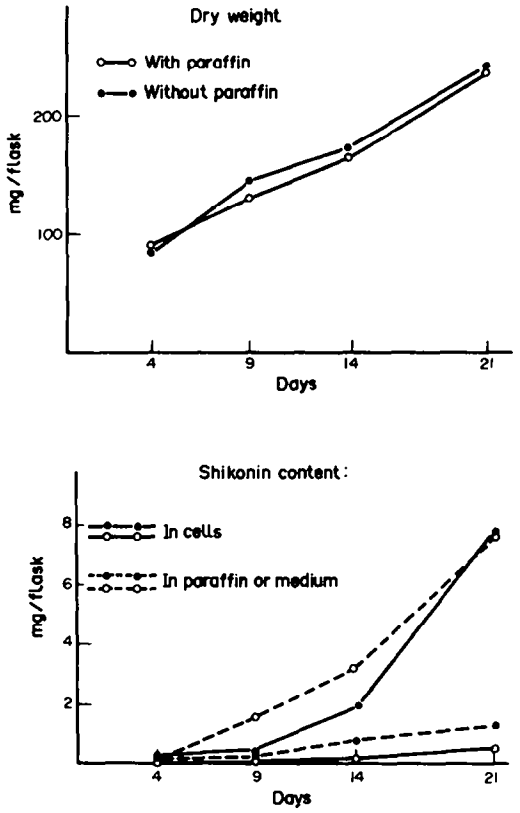

Fig. 1. Growth and shikonin accumulation of *L. erythrorhizon* cell suspension cultures in the presence of paraffin. Cells of strain M18 were grown in 100 ml Erlenmeyer flasks, containing 30 ml of M9 medium or 30 ml of M9 medium plus 10 ml of liquid paraffin, respectively.

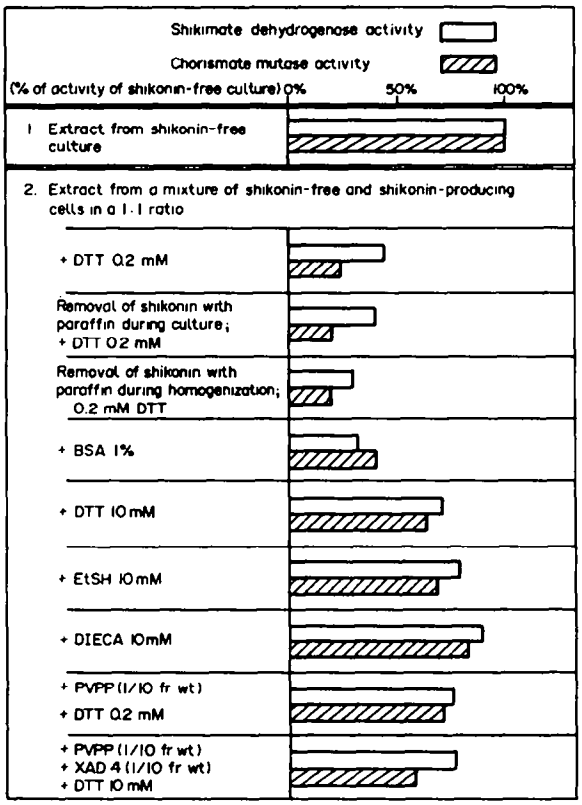

Fig. 2. Influence of enzyme-protective reagents on enzyme activities in cell-free extracts of *L. erythrorhizon* cell cultures. Shikonin-producing cells were grown in the absence of paraffin unless indicated otherwise. Extracts were prepared, gel-filtered and assayed as described in the Experimental. In different experiments, the activities found in extracts of shikonin-free cell cultures were used for comparison. 100% activity corresponded to *ca* 3.5 nKat/mg protein shikimate dehydrogenase activity and 0.4 nKat/mg protein chorismate mutase activity.

Thus, this method proved to be useful for obtaining almost shikonin-free cells from shikonin-producing cultures. In contrast to other plant cell culture systems [16–18], the final yield of shikonin derivatives was not increased by the continuous removal of these metabolites. However, when the extracts of cultures grown in the presence of paraffin were examined for the activity of chorismate mutase and shikimate dehydrogenase, an enzyme inhibition was observed as in the case of shikonin-containing cells (see Fig. 2). It was not clear whether this effect was due to a small amount of shikonin remaining in the cells, or to another inhibitor not removable by paraffin. Therefore, further technical improvements had to be made in order to obtain active enzyme extracts.

Effect of enzyme-protective reagents

Figure 2 shows the influence of different enzyme-protective reagents on the activities of shikimate dehydrogenase and chorismate mutase in extracts prepared from mixtures of shikonin-producing and shikonin-free cultures. Cells in an early growth phase, i.e. with a rather low shikonin content, were used in these experiments in order to obtain an easily measurable enzyme activity.

Attempts to trap shikonin with paraffin during homogenization were unsuccessful, and also the inclusion of 1% bovine serum albumin (BSA) did not improve the enzyme activities significantly. However, the addition of high concentrations of thiol reagents, such as mercaptoethanol (EtSH), dithiothreitol (DTT) and diethyldithiocarbamate (DIECA), resulted in extracts with much higher activity, and polyvinylpolypyrrolidone (PVPP) was similarly effective. An additional inclusion of the polystyrene resin Servachrome XAD 4 to absorb more lipophilic substances did not increase the activity any further.

Subsequently, enzyme activities were compared in extracts from a shikonin-producing culture, a shikonin-free culture and a mixture of cells from both cultures in a ratio of 1:1, all prepared using PVPP and 10 mM DTT (Table 3). The shikonin-producing culture showed much slower growth than the non-producing one, which may explain the lower activity of the measured primary metabolic enzymes. The extract obtained from the mixture of both cell types showed an activity similar to the mean value between the activities of the two cell types (Table 3), in contrast to the results obtained using low concentrations of DTT only (Table 1). This suggested that enzyme inhibition had largely been overcome.

Table 3. Enzyme activities in extracts of shikonin-producing and shikonin-free cells, prepared with PVPP and 10 mM DTT

Enzyme activity (nKat/mg protein)	Extract from shikonin-free culture	Extract from shikonin-producing culture	Extract from mixture of shikonin-producing and shikonin-free culture (1:1)
Shikimate dehydrogenase	2.58 (100%)	1.33 (51.6%)	2.16 (83.7%)
Chorismate mutase	0.346 (100%)	0.256 (74.0%)	0.315 (91.0%)

Cells were grown in the absence of paraffin. Extracts were prepared, gel-filtrated and assayed as described in the Experimental. The homogenization buffer contained PVPP (1/10 of fr. wt of cells) and DTT (10 mM). The protein contents were 0.60 mg/ml (shikonin-free culture), 0.62 mg/ml (shikonin-producing culture) and 1.01 mg/ml (mixture of cells from both cultures).

Detection of geranylpyrophosphate: p-*hydroxybenzoate geranyl-transferase activity*

A key step in shikonin biosynthesis is the prenylation of *p*-hydroxybenzoic acid with a C_{10} side chain derived from mevalonic acid yielding *m*-geranyl-*p*-hydroxybenzoic acid [8]. Using the methods developed in the enzymatic experiments described above, this reaction was demonstrated in cell-free extracts of *L. erythrorhizon* cell suspension cultures. The extracts were prepared: (a) from cultures grown in the presence of liquid paraffin in order to remove most of the potentially inactivating shikonin; (b) using slightly acidic buffers in order to avoid solubilization of residual shikonin in the form of its anion; (c) using PVPP and 10 mM DTT as protective reagents. The extracts were incubated with [^{14}C]*p*-hydroxybenzoic acid, geranylpyrophosphate and magnesium chloride. After termination of the reaction with formic acid, the reaction mixture was applied to thin-layer chromatography. Radio scanning revealed the rapid formation of a compound co-chromatographing with authentic *m*-geranyl-*p*-hydroxybenzoic acid (Fig. 3). This reaction was dependent on the presence of intact enzyme, geranylpyrophosphate and magnesium (Table 4).

To confirm the identity of the enzymatic product, 10 nmol (0.5 μCi) of [^{14}C]*p*-hydroxybenzoic acid were incubated with GPP, $MgCl_2$ and enzyme extract as described in the Experimental. TLC of an aliquot of the reaction mixture revealed that 97% of the [^{14}C]*p*-hydroxybenzoic acid had been converted into its geranylated derivative. The product was extracted with ether after acidification of the reaction mixture. This isolated product behaved as the authentic substance in TLC on silica gel using the two solvent systems A and B.

A sample of the authentic *m*-geranyl-*p*-hydroxybenzoic acid was converted into its methyl ester by treatment with diazomethane in ether for 10 min. An aliquot of the enzymatic product was methylated in the same way, and the chromatographic behaviour of the resulting substance in TLC was identical with the authentic ester in two solvent systems (C and D). Another sample of the authentic acid was treated with diazomethane in dry ethanol for 3 hr to yield *m*-geranyl-*p*-methoxybenzoic acid methyl ester. The enzymatic product, after alkylation in the same way, also behaved as this substance in TLC using the solvent systems E and F.

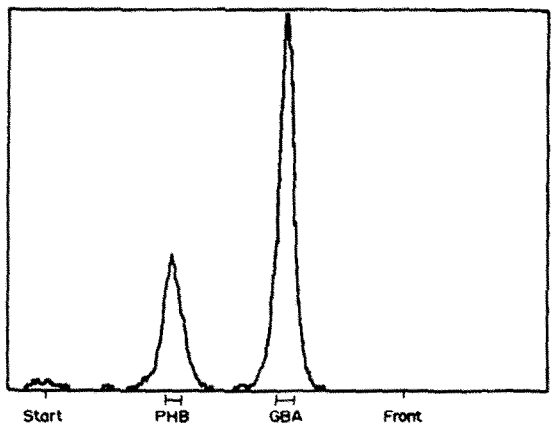

Fig. 3. Detection of *m*-geranyl-*p*-hydroxybenzoic acid formed by cell-free extracts of *L. erythrorhizon* cell cultures. Radio scan of a thin layer chromatogram of an incubation containing in 100 μl: [^{14}C]*p*-hydroxybenzoic acid, 1 nmol (50 nCi); GPP, 200 nmol; $MgCl_2$, 1000 nmol; crude enzyme extract, 80 μl (40 μg protein). Incubation for 30 min at 30°. PHB = UV-detectable spot of authentic *p*-hydroxybenzoic acid. GBA = UV-detectable spot of authentic *m*-geranyl-*p*-hydroxybenzoic acid.

Table 4. Formation of *m*-geranyl-*p*-hydroxybenzoic acid in cell-free extracts of *L. erythrorhizon* cell suspension cultures

Incubation mixture	Formation of *m*-geranyl-*p*-hydroxybenzoic acid (pmol)	Relative activity (%)
Complete	387	100
$-Mg^{2+}$	<5	<1.3
−GPP	<5	<1.3
Heat-denaturated extract	<6	<1.6

The complete incubation mixture (50 μl) contained: [^{14}C]*p*-hydroxybenzoic acid, 0.5 nmol (25 nCi); geranylpyrophosphate, 100 nmol; $MgCl_2$, 500 nmol; crude enzyme extract, 30 μl (= 15 μg protein).

Subsequently, unlabelled *m*-geranyl-*p*-hydroxybenzoic acid (*ca* 1.5 μmol) was prepared enzymatically as described in the Experimental. The product was extracted with ether and purified by TLC. The isolated substance was identical with authentic *m*-geranyl-*p*-hydroxybenzoic acid in TLC (solvent A) and in HPLC. The authentic substance and the enzymatic product were converted into their respective methyl esters by treatment with diazomethane in ether for 60 min. Both esters were shown to be identical by TLC in solvent A, by HPLC and by capillary GC (see Experimental). The mass spectra obtained by GC-MS analysis of both esters showed identical molecular peaks and fragmentation patterns: GC-MS (*m*-geranyl-*p*-hydroxybenzoic acid methyl ester) 70 eV, *m/z* (rel. int.): 288 [M^+] (8), 245 (11), 187 (24), 165 (22), 123 (100), 69 (35). The enzymatic product observed, therefore, was confirmed as *m*-geranyl-*p*-hydroxybenzoic acid, an intermediate in shikonin biosynthesis [8].

EXPERIMENTAL

Radiochemicals. [Carboxyl-^{14}C]*p*-hydroxybenzoic acid (50 Ci/ mol) was obtained from CEA, France.

TLC solvent systems. A = $CHCl_3$–MeOH–CH_3COOH (100:2:1); B = hexane–MeCOEt (6:4); C = toluene–EtOAc (9:1); D = hexane–MeCOEt (8:2); E = toluene; F = hexane–MeCOEt (9:1).

HPLC. Column TSK-Gel ODS 120 A 10 μm (Toyo Soda, Japan), 150 × 4.6 mm; solvent system $CHCl_3$–MeOH–HOAc (75:25:0.3); flow rate 1.5 ml/min; detection: absorption at 257 nm.

Gas chromatography. 25 m × 0.31 mm fused silica column coated with cross-linked, bonded methyl silicone (OV-101) 0.53 μm thick. Temp. programmed 80–240° at 10°/min. Detection: FID.

GC-MS. The GC column described above was adapted to an Hitachi M-80 spectrometer and the 70 eV spectra were recorded.

Cell cultures. Callus cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. were derived from germinating seeds [9]. By selection from heterogeneous callus cultures, strain M18 capable of producing shikonin derivatives and strain LY incapable of producing these pigments were obtained [4]. Cell suspension cultures were initiated and maintained as described elsewhere [12].

To induce shikonin formation, cells of the strain M18 (inoculum size: 1.1 g) were transferred from LS medium into 100 ml Erlenmeyer flasks containing 30 ml of the production medium M9 [5, 6] supplemented with 10^{-6} M IAA and 10^{-5} M kinetin. When paraffin was included, 5 ml of liquid paraffin (ampoule quality, Merck) were added to the medium prior to sterilization unless stated otherwise. To obtain shikonin-free cultures, cells of the strain LY were cultured in LS medium containing the same hormones.

Shikonin content. Determined in cells as described elsewhere [10]. To determine the content of shikonin derivatives in the paraffin layer, the paraffin was separated from the aq. layer and filled up with hexane to 50 ml. An aliquot of this soln was extracted twice with 5 ml 2.5 N KOH, and the absorption of the combined KOH phase was measured at 620 nm.

Preparation of cell-free extracts. Cells (5 g) were suspended in KPi buffer (10 ml, 0.1 M, pH 6.5), containing 10 mM DTT and 0.5 g PVPP unless stated otherwise. They were ruptured in a Potter homogenizer and centrifuged at 10000 *g* for 10 min. The supernatant was gel filtered through Sephadex G25 (PD 10 column), which had been equilibrated with Tris–HCl buffer (0.1 M, pH 7.5) containing the same concentration of DTT as the homogenization buffer.

Protein content. Determined according to the method of Bradford [19].

Shikimate dehydrogenase and chorismate mutase. Determined according to the methods of Sanderson [20] and of Chu and Widholm [21], respectively.

Enzymatic formation of m-*geranyl*-p-*hydroxybenzoic acid.* Unless stated otherwise, the incubation mixture (50 μl) contained: [carboxyl-^{14}C]*p*-hydroxybenzoic acid, 0.5 nmol (25 nCi); geranylpyrophosphate, 100 nmol; $MgCl_2$, 500 nmol; and enzyme extract from shikonin-producing cultures, 30 μl (= 15 μg protein). After incubation for 30 min at 30°, the reaction was terminated by cooling to 0° and addition of 5 μl formic acid. *p*-Hydroxybenzoic acid and *m*-geranyl-*p*-hydroxybenzoic acid (0.1 mg each) were added as carriers, and the soln was directly subjected to TLC on silica gel in solvent system A. Chromatograms were examined with a TLC radio scanner.

For the derivatization of the enzymatic product, [^{14}C]*p*-hydroxybenzoic acid (10 nmol = 0.5 μCi), GPP (2 μmol) and $MgCl_2$ (10 μmol) were incubated with 800 μl enzyme extract (total vol. 1 ml) for 90 min at 30°. After acidification with 100 μl 4 N HCl, the product was extracted twice with 4 ml Et_2O.

Preparative isolation of enzymatically formed m-*geranyl*-p-*hydroxybenzoic acid.* In a total vol. of 2 ml, 3.6 μmol *p*-hydroxybenzoic acid, 7.2 μmol GPP and 24 μmol $MgCl_2$ were incubated with 0.85 mg of the enzyme protein obtained from the pellet of 100000 *g* centrifugation of an enzyme extract from a shikonin-producing culture [22] at 37° for 140 min. The reaction was terminated by addition of 100 μl formic acid, and the product was extracted with 4 × 7 ml Et_2O. The combined Et_2O layer was evapd and the residue was applied to TLC (silica gel) in solvent A. The UV-detectable spot of *m*-geranyl-*p*-hydroxybenzoic acid was scraped off and eluted with MeOH. The eluate was filtered and evapd.

Chemical synthesis of m-*geranyl*-p-*hydroxybenzoic acid.* Performed according to the method of Inouye *et al.* [8].

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