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4-HYDROXYBENZOATE PRENYLTRANSFERASES IN CELL-FREE EXTRACTS OF *LITHOSPERMUM ERYTHRORHIZON* CELL CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; biosynthesis; shikonin; prenyltransferases; methyl jasmonate; ubiquinone; geranyldiphosphate:4-hydroxybenzoate geranyltransferase; solanesyldiphosphate:4-hydroxybenzoate solanesyltransferase.

Abstract—Aromatic prenylation reactions of 4-hydroxybenzoate (4HB) are involved in the biosynthesis of ubiquinones and of shikonin, a naphthoquinone pigment derived from 4HB and geranyldiphosphate (GPP) in *Lithospermum erythrorhizon*. The enzymic prenylation of 4HB with GPP and with solanesyldiphosphate (SPP) was measured in cell-free extracts from *L. erythrorhizon* cell cultures. The conversion of GPP was induced by methyl jasmonate, an inducer of shikonin biosynthesis, whilst the conversion of SPP was not, suggesting that the two reactions are carried out by different enzymes. Either reaction was found both in the microsomal fraction and in the organellar membrane fraction. The activity of the 4HB geranyltransferase in the microsomal fraction could be separated from the 4HB solanesyltransferase by partial purification using DEAE Sephacel and Heparin Sepharose affinity columns. The results indicate the presence of two distinct enzymes, GPP:4-HB geranyltransferase and SPP:4-HB solanesyltransferase. The enzymes are apparently quite specific for the chain length of the isoprenoid precursors, which is in contrast to the broad substrate specificity of the polyprenyldiphosphate:4-HB polyprenyltransferase from *Escherichia coli*. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

A key step in the biosynthesis of the naphthoquinone pigment shikonin in cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) is the prenylation of 4-hydroxybenzoate (4HB) with geranyldiphosphate (GPP) to 3-geranyl-4-hydroxybenzoate (GBA, see Scheme 1) [1]. This reaction is carried out by GPP:4HB geranyltransferase [2] and can be induced by the plant signal transducer methyl jasmonate [3]. The enzyme appears to be localized in the endoplasmatic reticulum [4].

A similar prenylation of 4HB, but with long chain prenyldiphosphates, is encountered in ubiquinone biosynthesis [5]. This reaction has been examined in cell-free extracts of *Escherichia coli* [6], in yeast [7] as well as in higher plants [8, 9] and in animals cells [10]. We have recently reported the cloning and the characterization of the polyprenyldiphosphate:4HB polyprenyltransferase (4HB polyprenyltransferase) from *E. coli* [11]; the overexpressed enzyme showed a broad substrate specificity for the chain length of the prenyldiphosphate substrate, accepting not only solanesyldiphosphate (SPP), but also farnesyldiphosphate (FPP) and GPP. In eukaryotic cells, this enzyme has mostly been described as an enzyme of the inner mitochondrial membrane [8, 10]; recently, however, evidence has been presented that it is localized also, or even predominantly, in the endoplasmatic reticulum [9, 12].

We then wanted to examine in *L. erythrorhizon* whether the prenylation of 4HB in shikonin and in ubiquinone biosynthesis is carried out by a single prenyltransferase or whether two prenyltransferases with different specifities can be distinguished. Furthermore, whether the respective prenyltransferases are localized in the endoplasmatic reticulum or the mitochondrial membrane.

RESULTS AND DISCUSSION

Analysis of endogenous ubiquinones

Different organisms produce ubiquinones with different lengths of the prenyl side chain. In order to establish which polyprenyldiphosphate may be the preferred precursor for ubiquinone biosynthesis in *L. erythrorhizon*, we identified the predominant ubiquinones in this material.

HPLC analysis of methanolic extracts of lyophilized

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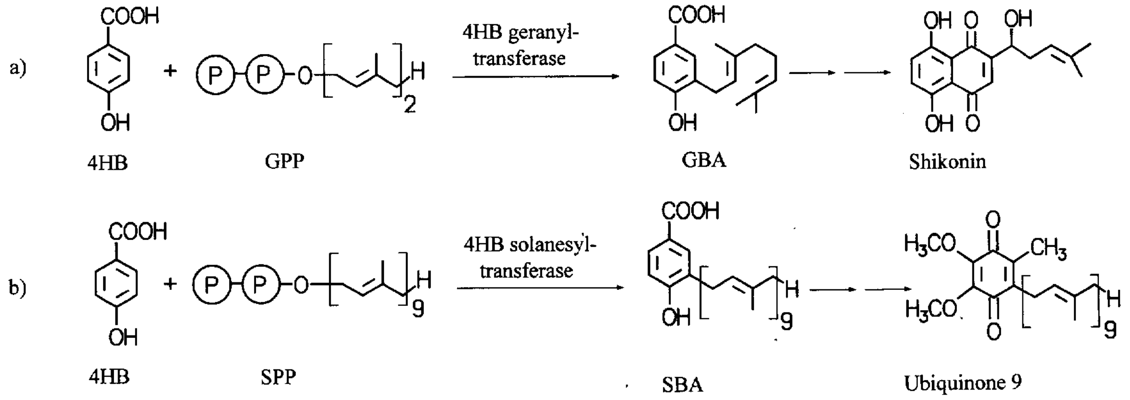

Scheme 1. Prenylation of 4-hydroxybenzoate in the biosynthesis of (a) shikonin and (b) ubiquinone 9.

cells revealed several peaks, which, by their retention times and their absorption at 275 nm, might represent ubiquinones (Fig. 1). The 10 largest peaks were isolated by HPLC, and their identity as ubiquinones was checked by UV spectroscopy [13], by UV spectroscopy after reduction with $NaBH_4$ to the respective ubiquinols [14] and by TLC separation and detection with reduced methylene blue [14] (see Experimental).

Only the two largest peaks (see Fig. 1) proved to be ubiquinones. By comparison with authentic ubiquinones in HPLC and TLC, these were identified as ubiquinone 9 (Q9, C_{45} side chain) and ubiquinone 10 (Q10, C_{50} side chain), respectively. The content of these substances was determined as 54.3 $\mu g\ g^{-1}$ dry wt for Q9 and 63.8 $\mu g\ g^{-1}$ dry wt for Q10. This suggests that SPP (C_{45}) and decaprenyldiphosphate (C_{50}) are the natural substrates of the 4HB prenyltransferase involved in ubiquinone biosynthesis in *L. erythrorhizon* cell cultures. For the following investigations of this prenyltransferase reaction, SPP was chosen as substrate, since this substance was readily available.

Detection of GPP:4HB geranyltransferase (4HB geranyltransferase) and SPP:4HB solanesyltransferase (4HB solanesyltransferase) activity

In crude extracts of *L. erythrorhizon* cell cultures, the enzymic conversion of GPP and 4HB into GBA (see Scheme 1) could be detected using the HPLC assay described previously [15]. When GPP was replaced by SPP, the enzymic formation of 3-solanesyl-4-hydroxybenzoate (SBA, see Scheme 1) was observed, using [^{14}C]4HB as substrate. The radioactive product was identified by HPLC (see Experimental), showing an identical retention time to an authentic substance, which had been produced from 4HB and SPP with the cloned and overexpressed polyprenyldiphosphate:4HB polyprenyltransferase (4HB polyprenyltransferase) from *E. coli* [11].

The quantitative determination of SBA was originally hampered by its hydrophobicity, which led to substance losses during sample preparation for HPLC. For quantitative determination of the 4HB solanesyltransferase activity, the reaction product

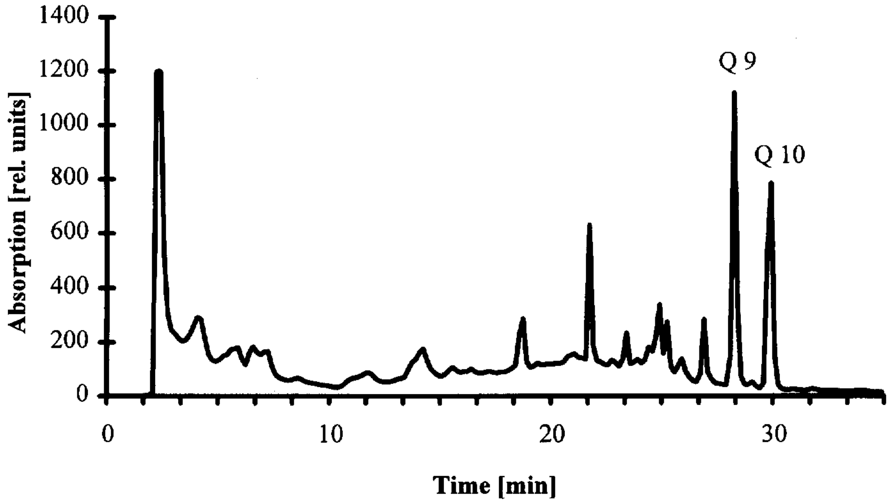

Fig. 1. HPLC analysis of methanolic extracts of *L. erythrorhizon* cell cultures (detection: UV, 275 nm). The largest peaks were identified as ubiquinone 9(Q9) and ubiquinone 10(Q10), respectively (explanations in text).

[^{14}C]SBA was, therefore, twice extracted from the acidified reaction mixture with *n*-heptane, which resulted in near-quantitative extraction. Since the unreacted [^{14}C]4HB is not *n*-heptane-soluble, the organic layer could be conveniently assayed for SBA by direct scintillation counting. Product formation was dependent on SPP and active protein (data not shown).

The same assay procedure could be used for 4HB geranyltransferase activity, taking into account that the more polar product GBA was only extracted with a yield of 60% by *n*-heptane under these conditions, as established with an authentic reference substance.

Intracellular localization of the prenyltransferase activities

4HB geranyltransferase activity is likely to be localized in the ER [4], whereas 4HB solanesyltransferases have been described to residue in the inner mitochondrial membrane [8, 10], although the latter hypothesis has recently been questioned [9, 12]. We therefore separated these two cellular membrane systems by subjecting the crude extract to differential centrifugation as described in the Experimental. The purity of the fractions was estimated by measurement of NADH:cytochrome *c* oxidoreductase as a marker enzyme for ER [16] and ubichinol:cytochrome *c* oxidoreductase as a marker enzyme for inner mitochondrial membrane [17] (see Experimental).

As shown in Table 1, the fraction containing the microsomes showed very little contamination with the mitochondrial marker. However, *ca* 10% of the total microsomal marker appeared as a contamination in the mitochondrial membrane fraction. This separation result appeared satisfactory for investigating the localization of the prenyltransferase activities.

When the activities of 4HB geranyltransferase and 4HB solanesyltransferase were measured, 4HB geranyltransferase activity was found in both fractions, although the major part (*ca* 70%) of this enzyme was associated with the microsomes (Table 2). The total activity of the 4HB solanesyltransferase was equally distributed between organellar and microsomal fractions. This suggests that 4HB solanesyltransferase, involved in ubiquinone biosynthesis, is not restricted to the mitochondrial membrane, but also localized in the ER, as suggested by the results of other studies [9, 12]. Unexpectedly, some of the 4HB geranyltransferase activity was found in the organellar membrane fraction; it cannot be distinguished by our experiments whether this activity resides in the inner mitochondrial membrane, as the marker enzyme, or in other membranes contained in the organellar membrane fraction (e.g. outer mitochondrial membrane).

Specific induction of 4HB geranyltransferase by methyl jasmonate

In order to obtain evidence that the 4HB geranyltransferase reaction and the 4HB solanesyltransferase reaction are in fact carried out by two independent enzymes, we attempted to induce specifically the former enzyme with methyl jasmonate, which is an important signal transducer in plant secondary metabolism [18] and inducer of shikonin biosynthesis [3]. Addition of this substance to the *L. erythrorhizon* cell cultures resulted in a 20-fold increase in the total

Table 1. Activities of marker enzymes and prenyltransferases in different membrane fractions of *L. erythrorhizon* cell cultures

	Product formation (fmol sec^{-1})			
	Per g cells (fr. wt)		Per mg protein	
Enzyme	Microsomal fraction	Organellar membrane fraction	Microsomal fraction	Organellar membrane fraction
NADH:cytochrome *c* oxidoreductase (ER)	2100 (91%)	220 (9%)	8830	1530
Ubiquinol:cytochrome *c* oxidoreductase (mitochondria)	<10 (<3%)	330 (97%)	<40	2270
4HB geranyltransferase	19.2 (71%)	7.9 (29%)	81.8	14.5
4HB solanesyltransferase	2.3 (53%)	2.0 (47%)	9.2	54.8
+Methyl jasmonate:				
4HB geranyltransferase	375.4 (69%)	172.6 (31%)	1940	1368
4HB solanesyltransferase	3.7 (59%)	2.6 (41%)	19.0	20.5

Microsomes and organellar membranes were obtained by differential centrifugation as described in the Experimental. Where indicated, methyl jasmonate (100 μM) was added 28 hr prior to harvest of the cells. Data are mean values of two replicates.

Table 2. Conversion of different prenyldiphosphates by 4HB prenyltransferases from *L. erythrorhizon* cell cultures and from *E. coli*

	Product formation (pmol^{-1} sec^{-1} mg^{-1} protein)		
	L. erythrorhizon		*E. coli*
Substrate	Solubilized microsomal protein	Purified 4HB geranyl transferase	Overexpressed 4HB polyprenyl transferase
GPP	61.70	14 418	4.61
	(100%)	(100%)	(100%)
FPP	0.85	100.8	0.72
	(1.4%)	(0.7%)	(15.6%)
GGPP	0.20	<5.8	0.26
	(0.3%)	(<0.04%)	(5.6%)
SPP	0.44	<5.8	0.16
	(0.7%)	(<0.04%)	(3.5%)
Without prenyl diphosphates	<0.06	<5.8	<0.008
	(<0.1%)	(<0.04%)	(<0.2%)

Enzyme fractions were prepared from cells induced with methyl jasmonate, and enzyme assays were carried out as described in the Experimental. Preparation of the membrane fraction from *E. coli* overexpressing 4HB polyprenyltransferase is described in ref. [11].

activity of 4HB geranyltransferase, which is involved in shikonin biosynthesis (Scheme 1). In contrast, the total activity of the 4HB solanesyltransferase changed only by a factor of *ca* 1.5 (Table 1). This indicates that two independent prenyltransferases are operating in the cell, one for shikonin biosynthesis and one for ubiquinone formation.

Separation of 4HB geranyltransferase from 4HB solanesyltransferase

To provide further proof that the 4HB geranyltransferase and the 4HB solanesyltransferase activities found in the microsomes are due to specific enzymes, we attempted to separate these two activities by chromatographic purification. Both activities could be actively solubilized from the microsomes using digitonin as a detergent. Chromatography on DEAE Sephacel could not separate the two prenyltransferases. However, semi-affinity chromatography on a HiTrap Heparin column yielded a 230-fold increase in the specific activity of 4HB geranyltransferase, and this purified 4HB geranyltransferase was virtually free from 4HB solanesyltransferase activity (Table 2). This again indicates that the two prenyltransferase reactions are carried out by different enzymes. Unfortunately, demonstration of purified 4HB solanesyltransferase activity in the other fractions of the HiTrap Heparin column was not possible, due to the low activity of this enzyme.

FPP and geranylgeranyldiphosphate (GGPP) were used for comparison in the examination of these enzyme fractions (Table 2). The purified 4HB geranyltransferase accepted, besides GPP, also FPP as substrate to a very minor extent, but not GGPP or SPP. The conversion of GGPP and SPP observed in the solubilized microsomal protein may therefore be attributed to the 4HB solanesyltransferase, which was then removed by chromatography on the HiTrap Heparin column.

An overexpressed 4HB polyprenyltransferase from *E. coli* was examined under the same assay conditions as the *Lithospermum* enzyme fractions for product formation from various prenyldiphosphates (Table 2). This enzyme shows a broad substrate specificity although octaprenyldiphosphate is its predominant natural substrate. It is able to accept also short-chain prenyldiphosphates. As we have reported earlier, it can even convert GPP with very high reaction velocity, though the k_m value for this unnatural substrate is eight times higher than for SPP [11].

Comparing the specificity of the *E. coli* enzyme with that of the *Lithospermum* fractions, it can be concluded that a specific, highly active 4HB geranyltransferase exists in *L. erythrorhizon*, which is likely to be responsible for shikonin biosynthesis. A second enzyme, 4HB solanesyltransferase, apparently exists for ubiquinone biosynthesis. Since we did not succeed in the purification of this enzyme, no definite judgement can be made of its substrate specificity. The data obtained with the solubilized microsomal fraction (Table 2) suggest, however, that the 4HB solanesyltransferase of *L. erythrorhizon* does not accept short-chain prenyldiphosphates such as FPP to the same extent as the *E. coli* 4HB polyprenyltransferase, i.e. this *Lithospermum erythrorhizon* enzyme may have a higher specificity for long-chain prenyldiphosphates than the *E. coli* enzyme.

4HB geranyltransferase is an important regulatory enzyme of the secondary metabolism in cell cultures of *L. erythrorhizon* [3, 15], which are used industrially for the production of shikonin [1]. In the current attempt to clone the structural gene of this enzyme, the DNA sequences obtained will have to be distinguished from the gene for 4HB solanesyltransferase, which apparently is a distinct enzyme and which can also be found in the endoplasmatic reticulum.

EXPERIMENTAL

Chemicals. [^{14}C]4HB (12 mCi mmol^{-1}), GGPP, Q9 and Q10 were from Sigma. GPP was synthesized according to ref. [19], and FPP and SPP were a gift from Prof. Ogura (Sendai, Japan). SPP was also synthesized according to ref. [20]. The SPP, commercially available from American Radiolabelled Chemicals, turned out to be unsuitable for this investigation, probably due to the presence of *cis*-configurated double bonds; in contrast to SPP from other sources, it was not accepted as substrate by 4HB prenyltransferases, neither from *Lithospermum* nor from the established, cloned and overexpressed *E. coli* enzyme [11]. Me jasmonate was from Serva/cytochrome *c* and methylene blue were from Merck. All other chemicals were of the highest purity available.

Cell culture. Suspension cultures of *L. erythrorhizon* were maintained in LS liquid medium containing 10^{-6} M indole-3-acetic acid and 10^{-5} M kinetin. The cultures were maintained in the dark at 25° in 500-ml round flasks (160 ml medium) on a rotary shaker at 100 rev min^{-1}. Strain TOM, which was used for all experiments, was derived from M18 strain [8] by continuous selection for high shikonin production. Cells were subcultivated at 2-week intervals. For 4HB geranyltransferase induction, methyl jasmonate was added to the medium 26–30 hr before harvest to give a concn of 100 μM; flasks were subsequently transferred to a reciprocal shaker at 110 strokes min^{-1}.

Ubiquinone analysis. *Lithospermum* cells (20 g fr. wt) were lyophilized and extracted ×2 with 15 ml $CHCl_3$–MeOH (3:1). To the combined extracts, H_2O and *n*-hexane (30 ml each) were added. After vigorous shaking, the organic layer was evapd and the residue was subjected to HPLC analysis on a Multospher RP-18 5 μm column (250 × 4 mm) with a Perisorb RP-18 30 μm precolumn (40 × 4 mm), both from Chromatography Service (Langenwehe, Germany). A linear gradient of MeOH–H_2O (4:1) to MeOH–*iso*-PrOH (3:2) was used for sepn. The flow rate was 1 ml min^{-1}, and substances were detected at 275 nm. TLC analysis for ubiquinones was performed using RP-18 F_{254} plates (Merck), developed with MeOH–*iso*-PrOH (3:2). Ubiquinones were visualized by UV and methylene blue [13]. Spots were eluted and further characterized by their UV spectra (240–340 nm) before and after reduction with $NaBH_4$ [14] to the respective ubiquinols.

Enzyme extraction and cell fractionation. Harvested cells (30 g fr. wt) were suspended in 30 ml 0.1 M K-Pi buffer (pH 6.5) containing 10 mM DTT and 5% PVPP. Cells were disrupted with an Ultra-Turrax (3 × 3 sec). The brew was filtered through 2 layers of nylon gauze (40 μm). The filtrate was centrifuged at 800 *g* for 1 min. A 1 ml aliquot of the supernatant was homogenized with a Potter homogenizator to give crude extract. The residual 29 ml of the supernatant were centrifuged at 10 000 *g* for 15 min to give an organellar pellet which was subsequently resuspended in 6 ml 0.1 M K-Pi buffer (pH 6.5) containing 0.6 M KCl and centrifuged again under identical conditions to remove microsomal contaminations. The resulting pellet was resuspended in 6 ml 0.1 M K-Pi buffer (pH 6.5) using a Potter homogenizer and centrifuged again at 100 000 *g* for 90 min. The pellet was resuspended in 1 ml 0.1 M K-Pi buffer (pH 6.5) to give the organellar membrane fr. (mitochondrial and plastidal membranes and matrix proteins). The 10 000 *g* supernatant was centrifuged at 100 000 *g* for 90 min; the pellet was resuspended in 200 μl 0.1 M K-Pi buffer (pH 6.5) to give the microsomal fr. (ER, golgi apparatus/tonoplast, plasma membrane).

Protein determination. The concn of protein was determined by the method of ref. [21], using BSA as standard protein.

Marker enzyme assays. NADH:cytochrome *c* oxidoreductase, a marker for the endoplasmatic reticulum [16], was assayed [22] under specific inhibition of ubiquinol:cytochrome *c* oxidoreductase with antimycin A and KCN. The antimycin A sensitive ubiquinol:cytochrome *c* oxidoreductase is a marker enzyme for the inner mitochondrial membrane [17] and was determined with NADH as electron donor by measuring the complete cytochrome *c* oxidoreductase activity in the absence of inhibitors and subtracting the activity determined for NADH:cytochrome *c* oxidoreductase.

Prenyltransferase assay. The assay mixt. contained in a final vol. of 100 μl: 10 nmol respective prenyldiphosphate, 6 nmol ring-UL-[^{14}C]4HB (150 000 dpm), 5 μmol $MgCl_2$, 10 vol% DMSO, 100 nmol KF, 2.5 μmol K-Pi (pH 7.8) and enzyme protein. Assay mixts were incubated for 90 min (37°) and the reaction was stopped by addition of 3 μl HCO_2H. After extraction with 2 × 500 μl *n*-heptane, 900 μl of the combined organic layers were assayed by scintillation counting. A correction was made for the radioactivity detected in assays without enzyme protein.

HPLC identification of SBA. After termination of the enzymic reaction by addition of HCO_2H, pptd protein with adsorbed [^{14}C]SBA was collected by centrifugation and extracted ×2 with 500 μl Me_2CO. This soln was analysed in the HPLC system described for ubiquinone analysis with a linear gradient of MeOH–HCO_2H (199:1) to MeOH–*iso*-PrOH–HCO_2H (79:120:1).

Partial purification of 4HB geranyltransferase. A microsomal fr. was obtained from 650 *g* cell material, pretreated with Me jasmonate, as described above. The microsomal pellet was resuspended in 140 ml 0.1 M Tris buffer (pH 7.5) containing 2 mM DTT, 1 M KCl and 10% (w/v) glycerol. After stirring for 1 hr (4°), the soln was centrifuged at 100 000 *g* for 90 min (4°). The pellet was resuspended in 140 ml 0.5 M Bis–Tris buffer (pH 7.0) containing 2 mM DTT, 2 M KCl, 10% (w/v) glycerol and 8 mM digitonin and stirred for 1 hr (4°).

Residual membrane fragments were removed by centrifugation at 100 000 *g* for 90 min (4°). The supernatant, containing solubilized microsomal proteins, was passed over a Sephadex G-25 column (50 × 80 mm), equilibrated with 0.05 M Bis–Tris (pH 7.0) containing 2 mM DTT, 10% (w/v) glycerol and 1.5 mM digitonin. The eluate was passed over a DEAE Sephacel column (75 × 26 mm). Prenyltransferases did not bind to the material and were collected in the eluate. This fr. is termed solubilized microsomal protein in Table 2.

The eluate from the DEAE column was passed over Sephadex G-25, equilibrated with 0.01 M K-Pi buffer (pH 6.8) containing 2 mM DTT, 50 mM KCl and 1.5 mM digitonin, and applied to a HiTrap Heparin Sepharose column 1 ml). Elution was carried out with equilibration buffer, supplemented with 0.2 mg ml^{-1} Na heparinate. Frs containing 4HB geranyltransferase activity were pooled. This pool is termed purified 4HB geranyltransferase in Table 2.

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