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BIOSYNTHESIS OF SHIKONIN IN CALLUS CULTURES OF *LITHOSPERMUM ERYTHRORHIZON**

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; callus; biosynthesis; shikonin.

Abstract—Administration of various supposed precursors to the callus cultures of *Lithospermum erythrorhizon* grown on the Linsmaier–Skoog medium supplemented with IAA and kinetin established that the constituent shikonin is formed via shikimic acid, *p*-hydroxybenzoic acid, *m*-geranyl-*p*-hydroxybenzoic acid and geranylhydroquinone. In a strain of callus culture lacking the capacity to synthesize shikonin and in callus cultures which have had this capacity but lost it due to cultivation on a medium supplemented with 2,4-D, substances up to *m*-geranyl-*p*-hydroxybenzoic acid in the biosynthetic sequence have been detected. Although illumination with white light also arrested shikonin production, traces of pigment were still formed presumably because light did not reach the innermost part of the callus cultures.

INTRODUCTION

While studying the biosynthesis of shikonin-type naphthoquinones in plants of the Boraginaceae, Zenk *et al.* [1] administered several precursors to *Plagiobothrys arizonicus* (A. Gray) Greene, which contains alkannin the enantiomer of shikonin (**1**) in the leaves, and clarified that its skeleton is formed from two molecules of mevalonic acid (MVA) and *p*-hydroxybenzoic acid (**2**), biosynthesized in turn from phenylalanine via cinnamic acid. On administration of potential precursors to the root systems of *Lithospermum erythrorhizon* Sieb. et Zucc. and *Alkanna tinctoria* Tausch., they, however, found that the incorporation of these substances into shikonin (**1**) or alkannin was too low to carry out the necessary degradation of the isolated pigments. Later, Sankawa *et al.* [2] also administered MVA and other labelled compounds to *L. erythrorhizon* and found that MVA was not incorporated; they concluded that the exogeneous MVA had decomposed prior to arrival at the site of biosynthesis.

In the meantime, Tabata *et al.* [3, 4] have succeeded in callus induction from the seedlings of *L. erythrorhizon* on the Linsmaier–Skoog basal medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin at 25° in the dark and obtained a callus producing a series of shikonin-type pigments when 2,4-D was replaced by 3-indoleacetic acid (IAA). Furthermore, through clonal selection, they obtained cell lines of high pigment reproducibility (strain M-231a and M-18) and a cell line lacking the ability to produce the pigment (strain M-386). They observed that pigment production was suppressed by substitution of 2,4-D for IAA or by illumination of the callus cells with a white light and recognized that the photodegradation product of FMN was involved [5] in the latter case. In order to clarify the biosynthetic pathway to shikonin (**1**), we administered various presumed precursors to the above-mentioned cell cultures of *L. erythrorhizon*, donated by Professor Tabata.

RESULTS AND DISCUSSION

At first, *p*-hydroxybenzoic acid (**2**) and MVA were fed to the pigment-producing callus cultures (strain M-231a) in order to confirm that shikonin (**1**) was formed by the same pathway as that leading to alkannin. The tritium-labelled **2** used in this experiment was synthesized, starting from methyl isovanillate (**3**). Reaction of **3** and 1-phenyl-5-chlorotetrazole in the presence of K_2CO_3 gave the phenyltetrazolyl ether (**4**), which was demethylated with BCl_3 to the corresponding phenol (**5**). Hydrogenolysis of **5** over Pd–C followed by alkali hydrolysis yielded *p*-hydroxybenzoic acid (**2**). Parallel work-up of **5** with D_2 over Pd–C and the subsequent alkaline hydrolysis gave **2**-[3-^{2}H]. After confirming the position of deuterium in **2** by ^{1}H NMR and MS, substance **5** was subjected to hydrogenolysis with $^3H_2$ over Pd–C and the product was hydrolysed with alkali to give *p*-hydroxybenzoic acid-[3-^{3}H].

The callus cells of *L. erythrorhizon* (strain M-231a) were subcultured on Linsmaier–Skoog agar medium supplemented with IAA (10^{-6} M) and kinetin (10^{-5} M) at 25° in the dark. *p*-Hydroxybenzoic acid-[3-^{3}H] and MVA-[2-^{14}C] were administered in turn to the callus cultures grown for two weeks after subculturing. After cultivation for three weeks further, the callus cells were extracted with $CHCl_3$. Alkaline hydrolysis of the $CHCl_3$ extract afforded radioactive shikonin (**1**), which was further converted into the leucoacetate (**6**) by reductive

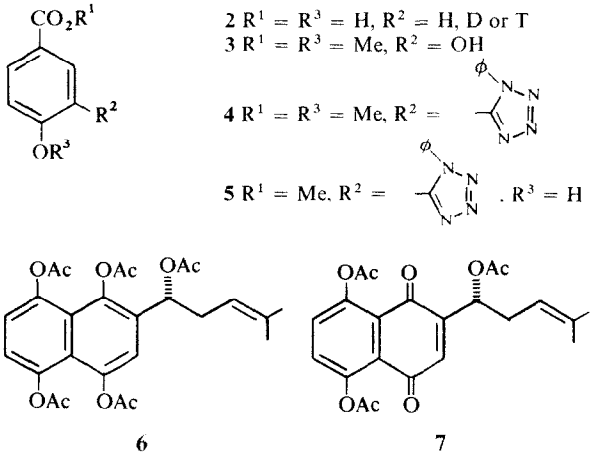
* Part 8 in the series "Quinones and Related Compounds in Higher Plants". For Part 7 see Inoue, K., Shiobara, Y., Chen, C.-C., Sakuyama, S. and Inouye, H. (1979) *Yakugaku Zasshi* **99**, in press.

Table 1. Incorporation of *p*-hydroxybenzoic acid (2) and MVA into shikonin (1)

Precursor	Shikonin (1)			3,6-Dihydroxyphthalic acid	Acetone
	Wt (mg) Total act. (dpm)	Wt (mg) Sp. act. (dpm/mM)	Incorp. (%) Sp. incorp. (%)	% Total act. of shikonin (1)	% Total act. of shikonin (1)
p-Hydroxybenzoic acid-[3-^{3}H] (2)	0.38 1.11×10^9	4.11 2.84×10^8	0.37* 0.071	91.8	—
MVA-[2-^{14}C]	2.07 2.22×10^8	3.30 5.25×10^7	0.27 0.33	4.56	44.5

* If the isotope effect is disregarded, half of the tritium labelling in 2 would be lost during the incorporation into 1. The incorporation ratio was calculated on the basis of this assumption.

acetylation, so as to avoid the quenching effect on the determination of the radioactivity. The labellings of **1** were located by the following degradative reactions [6]. The triacetate (**7**) derived from an aliquot of **1** was subjected to ozonolysis. The resulting 3,6-dihydroxyphthalic acid and acetone were converted into the anhydride and 2,4-dinitrophenylhydrazone, respectively, and their radioactivities were measured. The results of these experiments (Table 1) indicate that **2** is incorporated into the hydroquinone portion of **1** and that two molecules of MVA-[2-^{14}C] are also incorporated into **1** labelling C-1′ and C-5′ positions of the side chain. Thus **1**, like alkannin, is biosynthesized from *p*-hydroxybenzoic acid (**2**) and MVA.



Scheme 1.

We next attempted to prove the occurrence of *m*-geranyl-*p*-hydroxybenzoic acid (**8**) and geranylhydroquinone (**9**) as intermediates after **2** in shikonin biosynthesis. We first tried to detect these substances by trapping experiments. *m*-Geranyl-*p*-hydroxybenzoic acid (**8**) was prepared by the condensation of the Na salt of methyl *p*-hydroxybenzoate with geranyl bromide in toluene followed by alkaline hydrolysis [7]. Geranylhydroquinone (**9**) was synthesized according to the literature [8].

The callus cells (strain M-18) administered with *p*-hydroxybenzoic acid-[3-^{3}H] (**2**) were cultured for ten days and extracted with $CHCl_3$. The extract, after the addition of the carriers **8** and **9**, was fractionated to reisolate substances **8** and **9** and to isolate fatty acid esters of shikonin (**1**). The esters of **1** were hydrolysed and led to the leucoacetate (**6**), as mentioned above. Substance **8** was purified as such while **9** was converted to the crystalline *p*-nitrobenzoate. The radioactivity and the incorporation ratio of substances obtained in this experiment clearly indicate that the labelling of 2-[3-^{3}H] was trapped, as expected, in both *m*-geranyl-*p*-hydroxybenzoic acid (**8**) and geranylhydroquinone (**9**) (Table 2). Accordingly, we next fed **8** and **9**, both labelled with tritium, to the callus tissues. The labelled substances were synthesized in the following ways. *m*-Geranyl-*p*-hydroxybenzoic acid-[8′-^{3}H] (**8**): *m*-Geranyl-*p*-hydroxybenzoic acid methyl ester (**10**), after acetylation, was oxidized with an equimolar amount of SeO_2 [9] to give the allyl alcohol (**11**). The introduction of an oxygen atom into the C-8′ position was confirmed by NOE between the C-6′ proton and the C-8′ methylene protons. The allyl bromide (**12**), derived from the allyl alcohol (**11**) by bromination with PBr_3, was reduced with $LiAlH_4$ to give *m*-geranyl-*p*-hydroxybenzyl alcohol (**13**,) which was converted into the aldehyde (**14**) by Collins oxidation. Substance **14**, after acetylation, was subjected to Jones oxidation and subsequent alkaline hydrolysis to give *m*-geranyl-*p*-hydroxybenzoic acid (**8**). Reduction of the allyl bromide (**12**) with $LiAl^2H_4$ gave *m*-geranyl-*p*-hydroxybenzyl alcohol-[1‴-$^2H_2$,8′-^{2}H] (**13**). The labelling was determined by ^{1}H NMR and MS. Finally, the bromide (**12**) was reduced with $LiAl^3H_4$ to give *m*-

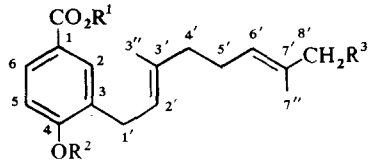
Table 2. Detection of *m*-geranyl-*p*-hydroxybenzoic acid (**8**) and geranylhydroquinone (**9**) by dilution analysis

	p-Hydroxybenzoic acid-[3-^{3}H] (**2**)	*m*-Geranyl-*p*-hydroxybenzoic acid (**8**)	Geranylhydroquinone (**9**)	Shikonin (**1**)
Total act. (dpm)	3.70×10^8	4.64×10^4	8.08×10^3	3.86×10^5
Incorp. (%)		0.013*	0.0022*	0.104*

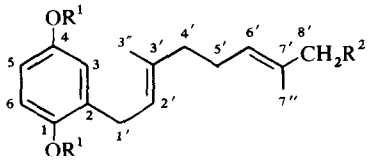
* The incorporation ratios into these substances are calculated by assuming the loss of half of the tritium labelling in **2**.

geranyl-*p*-hydroxybenzyl alcohol-[1‴-$^3H_2$,8′-^3H] (**13**), which after conversion into **14**-[1‴-^3H,8′-^3H] by the same procedure as in the case of the model compound, led to *m*-geranyl-*p*-hydroxybenzoic acid-[8′-^3H] (**8**). Geranylhydroquinone-[8′-^3H] (**9**): Unlabelled geranylhydroquinone (**9**), after acetylation, was oxidized with an equimolar amount of SeO_2 to yield the allyl alcohol (**15**) as the main product along with the corresponding 8′-aldehyde. The position of the introduced oxygen was confirmed by the NOE experiment. The allyl alcohol (**15**) was brominated with PBr_3 to yield the bromide (**16**), which was reduced with $LiAl^2H_4$ giving rise to geranylhydroquinone-[8′-^2H] (**9**). After confirming the position of label in this compound by ^1H NMR and MS, the bromide (**16**) was reduced with $LiAl^3H_4$ to yield **9**-[8′-^3H].

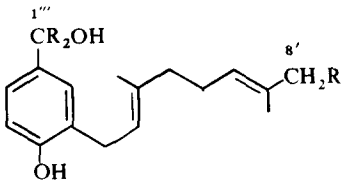
shikonin (**1**). Their lower incorporation into **1** in comparison with those of MVA and **2** would probably be ascribable to the poor state of the callus tissues used in this experiment; with regard to the specific incorporations, they were comparable with those of **2**. Thus both *m*-geranyl-*p*-hydroxybenzoic acid (**8**) and geranylhydroquinone (**9**) are the biosynthetic intermediates for shikonin (**1**). From these results and those of Zenk *et al.*, it seems that shikonin (**1**) is formed as shown in Scheme 3 via shikimic acid (**17**), phenylalanine (**19**), cinnamic acid (**20**), *p*-hydroxybenzoic acid (**8**) and geranylhydroquinone (**9**). The side chain moiety would be introduced by the reaction of geranyl pyrophosphate with *p*-hydroxybenzoic acid (**2**), though the detailed mechanism of the condensation still needs to be examined. The detailed biosynthetic sequence after **9** also needs elucidating. The



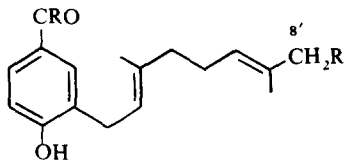
8 $R^1 = R^2 = H$, $R^3 = H$, D or T
10 $R^1 = Me$, $R^2 = R^3 = H$
11 $R^1 = Me$, $R^2 = Ac$, $R^3 = OH$
12 $R^1 = Me$, $R^2 = Ac$, $R^3 = Br$



9 $R^1 = H$, $R^2 = H$, D or T
15 $R^1 = Ac$, $R^2 = OH$
16 $R^1 = Ac$, $R^2 = Br$



13 R = H, D or T



14 R = H, D or T

Scheme 2.

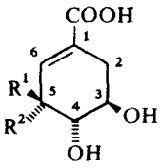
Both labelled compounds **8** and **9**-[8′-^3H] were administered to the callus tissues (strain M-18) which were suspended in the Linsmaier–Skoog medium after cultivation on the agar medium for two weeks. The tissues were worked up as before. Compound (**1**) from both experiments was purified as the leucoacetate (**6**). Aliquots of **6** from both experiments were subjected to ozonolysis and the resulting radioactive acetone was purified as its 2,4-dinitrophenylhydrazone. The results of these experiments (Table 3) indicate that both substances **8** and **9** were almost specifically incorporated into sequence in Scheme 3 is similar to that in the biosynthesis of ubiquinones, for which the intermediacy of polyprenylphenol formed by the decarboxylation of polyprenyl-*p*-hydroxybenzoic acid has been established. In the same way, *o*-geranylphenol is also a likely intermediate in shikonin biosynthesis, although the possibility of the direct formation of **9** from **8** by a concerted oxidative decarboxylation cannot be ruled out.

We next turned our attention to determine the stage at which pigment biosynthesis is blocked in the callus tissues cultured under conditions [3] which turn off

Table 3. Incorporation of *m*-geranyl-*p*-hydroxybenzoic acid (**8**) and geranylhydroquinone (**9**) into shikonin (**1**)*

Precursor	Wt (mg) Total act.	Shikonin (**1**) Wt (mg) Sp. act. (dpm/mM)	 Incorp. (%) Sp. incorp. (%)	Acetone % Total act. of shikonin (**1**)
m-Geranyl-*p*-hydroxybenzoic acid-[8-^3H] (**8**)	7.50 1.56×10^7	2.06 2.75×10^5	0.013 0.048	92.5
Geranylhydroquinone-[8-^3H] (**9**)	7.50 1.22×10^8	5.89 4.44×10^6	0.074 0.081	86.2

* The incorporation ratio was calculated after subtracting the radioactivity of the recovered precursor from the total activity administered.



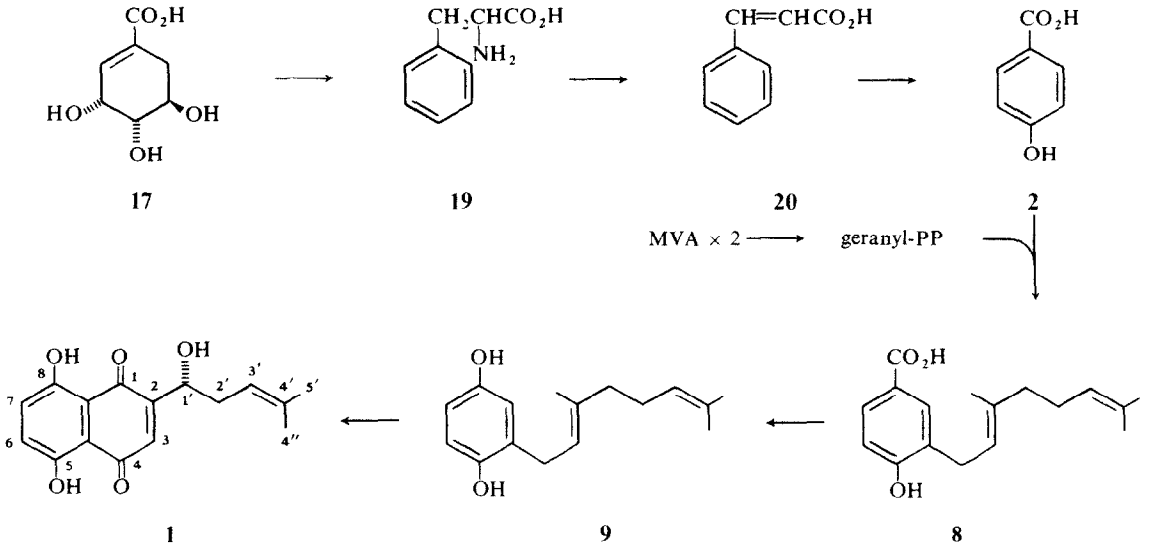
17 R^1 = H or T, R^2 = OH
18 R^1 = OH, R^2 = H or T

Scheme 3.

pigment production or in the cell line originally lacking the ability to produce pigment. Shikimic acid (**17**)*, a primary precursor of shikonin (**1**), was labelled at the 5-position with tritium and administered to the respective cell cultures. The incorporation of **17** into the now established biosynthetic intermediates **2**, **8** and **9** was examined by dilution analysis.

Shikimic acid-[5-^{3}H] (**17**) was first administered to the callus tissues (strain M-18) which had lost the ability to produce pigment through three transfers at intervals of 4 weeks to the Linsmaier–Skoog agar medium supplemented with kinetin (10^{-5} M) as well as 2,4-D (10^{-6} M) instead of IAA (strain M-18 (2,4-D)). After cultivation for 10 days, the callus tissues were extracted with $CHCl_3$. Carriers of **2**, **8** and **9** as well as a mixture of fatty acid esters of **1** isolated from the root of *L. erythrorhizon* were added to the $CHCl_3$ extract and the whole was fractionated by the conventional method to reisolate each of these substances. The substances were purified: **2** as its methyl ester, **8** as such and **9** as its *p*-nitrobenzoate. Fatty acid esters of **1** were converted into the leucoacetate (**6**). The shikimic acid-[5-^{3}H] (**17**) was fed to the callus lacking the capacity of pigment formation (strain M-386), by using cells grown for a week after subculturing. The callus tissues were extracted with $CHCl_3$ after cultivation for 10 days. Carriers **2**, **8** and **9** as well as fatty acid esters of **1** were added to the $CHCl_3$ extract and worked up as described above.

Shikimic acid-[5-^{3}H] (**17**) was then administered to the callus tissues with high pigment-producing capacity (strain M-231a) cultured under a white light (5000 lx) for a week (designated as M-231 (light)) and they were cultured under the same conditions for 10 days further.



17 **19** **20** **2** **1** **9** **8**

Scheme 4. Biosynthetic pathway of shikonin (**1**).

Under these conditions, as Tabata *et al.* [5] reported, the callus was expected to produce substances up to **2**. On the other hand, the callus still contained a small amount of fatty acid esters of **1** which had been formed before subculturing. Accordingly, only carriers **8** and **9** were added to the $CHCl_3$ extract of the callus cultures and then each substance was isolated and purified in the usual way. As a parallel experiment, shikimic acid-[5-^{3}H] (**17**) was administered to the callus cells (strain M-231a) grown in the dark for a week after subculturing. Being cultured in the dark for 10 days, the cells were worked up as in the case of M-231a (light). It follows (Table 4) that substances up to **8** were formed in both strain M-18 (2,4-D) and M-386, while **9** and **1** were not. It is also evident from Table 4 that all the compounds **8**, **9** and **1** were detected in the experiment with strain M-231a (light), though the amounts of the substances were halved at each stage as compared with the parallel experiment carried out in the dark. Therefore, it may be assumed that a trace of pigment is formed in an innermost part of the callus where the light cannot reach.

* Shikimic acid-[5-^{3}H] (**17**) [10] used in the present experiment was prepared by the NaB^3H_4 reduction of methyl 5-dehydroshikimate followed by alkaline hydrolysis. The resulting **17**-[5-^{3}H] was contaminated with undesirable product 5-epishikimic acid-[5-^{3}H] (**18**); nevertheless, we designated it as shikimic acid-[5-^{3}H] (**17**) and administered it to the plant as it was, because it is unlikely that the contaminating epishikimic acid (**18**) is metabolized with retention of the labelling. The ratio of shikimic acid (**17**) to its isomer (**18**) in the labelled mixture was assumed to be 9:11 from the integration ratio. [The C-6 proton of shikimic acid (**17**) appears at δ 6.73 as a double triplet (J = 4.0 and 2.0 Hz) while that of 5-epishikimic acid (**18**) at δ 6.58 as a triplet (J = 3.0 Hz)] of the C-6 proton signals of the unlabelled mixture.

Table 4. Dilution analysis of the precursors of shikonin biosynthesis in several callus cultures fed with shikimic acid-[5-^{3}H] (**17**)*

		M-18 (2,4-D)	M-386	M-231a (light)	M-231a
Shikimic acid-[3-^{3}H] (**17**)†	Total act. (dpm)	4.91×10^8	4.30×10^8	2.25×10^8	2.25×10^8
p-Hydroxybenzoic acid (**2**)	Total act. (dpm)	7.80×10^4	1.71×10^5		
	Incorp. (%)	0.016	0.04	—	—
m-Geranyl-*p*-hydroxybenzoic acid (**8**)	Total act. (dpm)	1.82×10^4	1.92×10^4	7.40×10^3	1.94×10^4
	Incorp. (%)	0.0037	0.0045	0.0033	0.0086
Geranylhydroquinone (**9**)	Total act. (dpm)	287>	136>	3.80×10^3	6.10×10^3
	Incorp. (%)	0	0	0.0017	0.0027
Shikonin (**1**)	Total act. (dpm)	10	0	1.19×10^4	3.12×10^4
	Incorp. (%)	0	0	0.0053	0.0139

* The incorporation ratios of **17** into **8**, **9** and **1** were calculated by assuming the loss of half of the tritium-labelling.
† Shikimic acid-[5-^{3}H] (**17**) administered was not recovered.

EXPERIMENTAL

Mps are uncorr. Unless otherwise specified, ^{1}H NMR spectra were obtained at 60 MHz in $CDCl_3$ using TMS as an int. stand. TLC experiments of non-radioactive materials were carried out with Si gel GF_{254}, while those of radioactive ones with Si gel 60 F_{254} and the solvent systems were $CHCl_3$–MeOH (19:1, solvent 1), toluene–HCO_2Et–HCO_2H (5:4:1, solvent 2), C_6H_6–EtOAc (4:1, solvent 3). I_2 and UV light (254 nm) were used for visualization. Radioactive spots were monitored using an Aloka radioscanner. Si gel (Mallinckrodt) was used for column chromatography. Scintillation counting was conducted with each sample dissolved in toluene (10 ml) containing PPO (50 mg) and POPOP (3 mg). Specific activities are the values before dilution. Unless otherwise stated, callus cultures were grown at 25° in the dark and labelled precursors were administered to the cells grown for 2 weeks on Linsmaier–Skoog agar medium supplemented with IAA 10^{-6} mol and kinetin 10^{-5} mol (IAA medium) (20 ml) in each of ten 100 ml flasks after being transferred.

Preparation of p-*hydroxybenzoic acid*-[3-^{3}H]. A mixture of Me isovanillate (4.2 g), 1-phenyl-5-chlorotetrazole (6.2 g), K_2CO_3 (7 g) and Me_2CO (100 ml) was refluxed for 18 hr. After the addition of H_2O (100 ml), the mixture was cooled with ice. The resulting crystals were recrystallized from EtOAc to give 3-phenyltetrazolyloxy-4-methoxybenzoic acid Me ester (**4**) as plates (7.0 g), mp 157–157.5°. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1715, 1600, 1536, 1505, 1280, 1130, 905. ^{1}H NMR: δ 3.86 (*s*, CO_2Me), 3.89 (*s*, OMe), 7.08 (1H, *d*, J = 9.2 Hz, C-5), 7.50–7.90 (5H, *m*), 8.02 (2H, *dd*, J = 9.2 and 2.0 Hz, C-6), 8.05 (1H, *d*, J = 2.0 Hz, C-2). (Found: C, 59.16; H, 4.24; N, 17.08. $C_{16}H_{14}O_4N_4$ requires: C, 58.89; H, 4.32; N, 17.17%). To a soln of **4** (1.15 g) in CH_2Cl_2 (50 ml) cooled to −78° in a dry ice–Me_2CO bath was added a 20% BCl_3–CH_2Cl_2 soln (10 ml) and the reaction was kept at −78° for 1 hr. After standing at room temp. for 4 days, the reaction was washed with 10% $NaHCO_3$ and H_2O, dried and evapd *in vacuo*. The residue was chromatographed on Si gel (100 g) with C_6H_6–EtOAc (9:1) as eluent. Fractions indicating a spot of R_f 0.40 on TLC (solvent 1) were combined and concd. Recrystallization of the residue from EtOAc gave 3-phenyltetrazolyloxy-4-hydroxybenzoic acid Me ester (**5**) as needles (0.72 g), mp 157.5–158°. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3200, 1715, 1605, 1540, 1430, 1290. ^{1}H NMR: δ 3.87 (*s*, CO_2Me), 7.18 (1H, *d*, J = 8.5 Hz, C-5), 7.59–8.14 (7H, *m*, arom. H). (Found: C, 57.97; H, 3.77; N, 17.81. $C_{15}H_{12}O_4N_4$ requires: C, 57.69; H, 3.87; N, 17.94%). **5** (100 mg) was hydrogenated in EtOH (10 ml) over 10% Pd–C at room temp. until uptake of $^2H_2$ ceased. The reaction product was chromatographed on Si gel (10 g) starting with C_6H_6 as eluent and increasing the percentage of EtOAc. The C_6H_6–EtOAc (97:3) eluates afforded, after recrystallization from EtOH, Me *p*-hydroxybenzoate as needles (23.1 mg), mp 131–132°. Alkaline hydrolysis of this substance gave *p*-hydroxybenzoic acid (**2**) as plates, mp 212–214°, which were identical with an authentic sample in TLC (solvent 2) and mmp. ^{1}H NMR (Me_2CO-d_6): δ 6.93 (1H, *d*, J = 9.0 Hz, C-5), 7.95 (1H, *d*, J = 2.0 Hz, C-2), 7.95 (1H, *dd*, J = 9.0 and 2.0 Hz, C-6). MS *m/e*: 139 (M^+). Reaction of **5** (100 mg) in EtOH (10 ml) over 10% Pd-C with $^3H_2$ (2 Ci) for 1 hr and then with a large excess of H_2 for a further 1 hr and the subsequent work-up, as in the case of the preliminary experiment, gave Me *p*-hydroxybenzoate-[3-^{3}H] as needles (20.9 mg). An aliquot (16 mg) of this substance was converted into *p*-hydroxybenzoic acid-[3-^{3}H] (**2**) plates (9.54 mg, sp. act. 4.03×10^{11} dpm/mmol).

Administration of p-*hydroxybenzoic acid*-[3-^{3}H] (**2**) *to strain* M-231a *and isolation of radioactive shikonin* (**1**). A sample of **2** (0.38 mg, sp. act. 4.03×10^{11} dpm/mmol) dissolved in EtOH–H_2O (1:9) (10 ml) was administered to the callus cultures (strain M-231a). Three weeks after the precursor application, the cell mass (fr. wt 13.5 g) was extracted with $CHCl_3$ (50 ml × 4) under reflux and the extract was dried and concd *in vacuo*. The residue (38.7 mg) mixed with 2% NaOH (10 ml), after standing at 35° overnight, was cooled, acidified with N HCl and extracted with Et_2O (30 ml × 4). The extract was washed with H_2O, dried and concd. Column chromatography of the residue on Si gel (5 g) gave radioactive shikonin (**1**) as red needles (4.11 mg). An aliquot (2.05 mg) of this substance diluted with the carrier (**1**) (78.52 mg) was left standing with Zn powder (80 mg), Ac_2O (1.5 ml) and Py (1.5 ml) at room temp. overnight. Ice H_2O was poured into the reaction and extracted with Et_2O. The Et_2O layer was washed with N HCl, 5% $NaHCO_3$ and H_2O, dried and concd. The residue was recrystallized from EtOH giving radioactive shikonin leucoacetate (**6**) as needles (68.3 mg).

Ozonolysis of radioactive shikonin triacetate (**7**). A stream of O_3 was passed through a soln of **7** derived from radioactive **1** (2.06 mg) diluted with the carrier (**1**) (118.3 mg) in CH_2Cl_2 (20 ml) cooled to −70° in a dry ice–MeOH bath for 9 hr. After standing overnight at room temp., the mixture was diluted with H_2O (10 ml) and concd *in vacuo* to *ca* 10 ml. The soln, after

heating at 50° for 30 min, was filtered to remove insoluble materials and washed with Et_2O. The aq. layer was concd *in vacuo* and the residue was heated at 150–170° under red. pres. (2 mm Hg) to give radioactive 3,6-dihydroxyphthalic anhydride as a yellow sublimate (5.33 mg), which was diluted with the carrier (27.43 mg) and repeatedly sublimed to constant activity. The radioactivity was determined by the combustion method.

Administration of MVA-[2-^{14}C] *to strain* M-231a, *isolation and the successive degradation of radioactive shikonin* (**1**). DL-MVA-DBED salt-[2-^{14}C] (2.07 mg, sp. act. 2.88×10^{10} dpm/mmol) was dissolved in H_2O (10 ml) and administered to the callus cultures by the method described above. After 3 weeks, the cell mass (fr. wt 12.5 g) was worked up to give radioactive **1** (3.30 mg) as red needles, an aliquot was converted to the leucoacetate (**6**). Another aliquot of **1** (2.08 mg) diluted with the carrier (121.3 mg) was acetylated and then subjected to ozonolysis. After the conventional work-up, the ozonide was mixed with H_2O (10 ml) and steam distilled to introduce the distillate into a soln of 2,4-dinitrophenylhydrazine–H_2SO_4. The resulting orange yellow ppt. was collected, washed with H_2O and subjected to PLC (solvent, C_6H_6) to give yellow needles (14.3 mg) of Me_2CO–2,4-dinitrophenylhydrazone (2,4-DNP), which, after dilution with the carrier (35.2 mg), were recrystallized from EtOH to constant activity. Insoluble materials in the residue of the steam distillation were filtered off and the filtrate was concd *in vacuo*. The residue was heated at 150–170° under red. pres. (2 mm Hg) to give 3,6-dihydroxyphthalic anhydride (8.65 mg) as a yellow sublimate, which, after dilution with the carrier (23.77 mg), was repeatedly sublimated to constant activity. The radioactivity of this substance was determined by the combustion method.

Synthesis of m-*geranyl*-p-*hydroxybenzoic acid* (**8**). Na (1.2 g) was added to a soln of Me *p*-hydroxybenzoate (7.12 g) in dry toluene (50 ml) and the mixture was refluxed under N_2 for 5 hr. Geranyl bromide (10.3 g) was then added dropwise to the ice-cooled soln over a 2 hr period. The reaction mixture was stirred at room temp. for a further 16 hr. The resulting ppt. was filtered off and the filtrate was extracted with 2.5 N NaOH (30 ml × 3). The combined extracts were acidified with 2 N H_2SO_4 under ice-cooling and extracted with Et_2O (100 ml × 3). The Et_2O extract was dried and concd. The residue (1.43 g) was chromatographed on Si gel (150 g) with C_6H_6–EtOAc (98:2) as eluent and fractions giving a single spot on TLC (solvent 3) were combined and concd to give *m*-geranyl-*p*-hydroxybenzoic acid Me ester (**10**) as an oil (915 mg). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 2930, 1705, 1605, 1440, 1280, 1120. ^{1}H NMR: δ 1.61 (3H, *s*, C-7′), 1.68 (3H, *s*, C-8′), 1.78 (3H, *s*, C-3″), 2.00–2.25 (4H, *m*, C-4′ and 5′), 3.41 (2H, *d*, J = 7.0 Hz, C-1′), 3.90 (3H, *s*, CO_2Me), 5.23 (1H, *m*, C-6′), 5.36 (1H, *t*(*br*), J = 7.0 Hz, C-2′), 5.50–6.50 (1H, phenolic OH), 6.87 (1H, *d*, J = 9.0 Hz, C-5), 7.83 (1H, *dd*, J = 9.0 and 2.5 Hz, C-6), 7.86 (1H, *d*, J = 2.5 Hz, C-2). (Found: C, 75.07; H, 8.68. $C_{18}H_{24}O_3$ requires: C, 74.97; H, 8.39%). A soln of **10** (720 mg) in a mixture of 2.5 N NaOH and MeOH (1:1) (40 ml) was stirred at 60° for 5 hr. The soln was acidified with 2 N H_2SO_4 under ice-cooling and extracted with Et_2O (50 ml × 3). The Et_2O extract was dried and concd. The residue was recrystallized from cyclohexane to give *m*-geranyl-*p*-hydroxybenzoic acid (**8**) as needles (644 mg), mp 67–69°. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400–2650, 1685, 1605, 1280. ^{1}H NMR: δ 1.61 (3H, *s*, C-7″), 1.69 (3H, *s*, C-8′), 1.78 (3H, *s*, C-3″), 2.00–2.25 (4H, *m*, C-4′ and 5′), 3.42 (2H, *d*, J = 7.0 Hz, C-1′), 4.87–5.50 (2H, C-2′ and 6′), 6.83 (1H, *d*, J = 9.0 Hz, C-5), 7.89 (1H, *dd*, J = 9.0 and 2.0 Hz, C-6), 7.90 (1H, *d*, J = 2.0 Hz, C-2), 7.50–8.07 (1H, phenolic OH). (Found: C, 74.29; H, 8.03. $C_{17}H_{22}O_3$ requires: C, 74.42; H, 8.08%).

Administration of p-*hydroxybenzoic acid*-[3-^{3}H] (**2**) *to callus cultures* (*strain* M-18) *and the dilution analysis of* m-*geranyl*-p-*hydroxybenzoic acid* (**8**) *and geranylhydroquinone* (**9**). A sample of **2** (0.66 mg, sp. act. 7.74×10^{10} dpm/mmol) was administered in the same way as described above to the callus cells (strain M-18) grown for a week after subculturing. Ten days after the precursor application, the cell mass (fr. wt 12.3 g) was extracted with $CHCl_3$ (30 ml × 4) under reflux. The $CHCl_3$ extract was dried and concd *in vacuo*. The residue was mixed with **8** (24.0 mg) and **9** (23.5 mg) and chromatographed on Si gel (10 g) using $CHCl_3$ as eluent and 15 ml fractions were collected. The residue from fractions 2–4 was worked up as described above to give radioactive **1** (2.81 mg), which after dilution with the carrier (48.0 mg), was converted into shikonin leucoacetate (**6**) (28.4 mg) of constant radioactivity. PLC (solvent 3) of the residue of fractions 6–9 gave a band (R_f 0.40), which afforded radioactive **9** (21.4 mg). Work-up of **9** with *p*-nitrobenzoyl chloride and Py gave radioactive geranylhydroquinone bis-*p*-nitrobenzoate which was recrystallized as needles of constant activity. PLC (solvent 3, double developments) of the residue of fractions 13–18 gave radioactive **8** (17.05 mg) as needles. This substance was recrystallized from cyclohexane to constant activity.

Preparation of m-*geranyl*-p-*hydroxybenzoic acid*-[8′-^{3}H] (**8**). Substance **10** (2.36 g) was acetylated (Ac_2O, Py) to give an oil (2.76 g). A soln of the acetate (2.14 g) in EtOH (50 ml) was mixed with freshly sublimated SeO_2 (713 mg) and refluxed for 3.5 hr. After being cooled, the mixture was diluted with H_2O (100 ml) and extracted with Et_2O (100 ml × 3). The Et_2O layer was washed with 10% $NaHCO_3$ and H_2O, dried and concd. The residue was chromatographed on Si gel (120 g). Elution was carried out with C_6H_6 and different proportions of C_6H_6–EtOAc. The C_6H_6–EtOAc (96:4) eluates afforded the allyl alcohol (**11**) (556 mg) as an oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3470, 2900, 1740, 1705, 1430, 1280, 1180, 1160, 1105. ^{1}H NMR: δ 1.62 (3H, *s*, C-3″), 1.69 (3H, *s*, C-7″), 2.04–2.23 (5H, *m*, C-4′, 5′ and OH), 2.28 (3H, *s*, CO_2Me), 3.27 (2H, *d*, J = 7.0 Hz, C-1′) 3.87 (3H, *s*, CO_2Me), 3.93 (2H, *s*(*br*), C-8′), 5.23 (1H, *t*, J = 7.0 Hz, C-2′), 5.36 (1H, *m*, C-6′), 7.10 (1H, *d*, J = 9.0 Hz, C-5), 7.93 (1H, *dd*, J = 9.0 and 2.0 Hz, C-6), 7.95 (1H, *d*, J = 2.0 Hz, C-2). (Found: C, 69.05; H, 7.69. $C_{20}H_{26}O_5$ requires: C, 69.34; H, 7.57%). Over a 1.5 hr period, PBr_3 (1.0 ml) was added dropwise to a soln of **11** (500 mg) in dry Et_2O (15 ml) and Py (0.3 ml) under N_2. After stirring for a further 1 hr, the reaction was quenched with ice H_2O and extracted with Et_2O (15 ml × 3). The Et_2O extract was washed with 10% $NaHCO_3$ and H_2O, dried and concd to give the allyl bromide (**12**) (466 mg) which showed a single spot (R_f 0.60) on TLC (solvent 3). $LiAlH_4$ (32.4 mg) was added to an ice-cooled soln of **12** (103 mg) in dry THF (5 ml) under N_2 and the mixture was stirred for 1 hr. Subsequently, the reaction was warmed to 50–60° and stirred for a further 5 hr. After cooling to room temp., the reaction was quenched by adding EtOAc (1 ml) under ice-cooling and inorganic materials were dissolved by adding 1 N NaOH (5 ml). The mixture, after dilution with H_2O (15 ml), was extracted with Et_2O (10 ml × 3). The Et_2O extract was washed with H_2O, dried and concd. PLC (solvent 3) (R_f 0.24) of the resulting residue gave *m*-geranyl-*p*-hydroxybenzyl alcohol (**13**) (51.5 mg) as an oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 2900, 1600, 1485, 1430, 1370, 1255. ^{1}H NMR: δ 1.60 (3H, *s*, C-7′), 1.68 (3H, *s*, C-8′), 1.73 (3H, *s*, C-3″), 1.98–2.22 (4H, *m*, C-4′ and 5′), 2.33 (1H, *s*(*br*), OH), 3.33 (2H, *d*, J = 7.5 Hz, C-1′), 4.53 (2H, *s*, C-7), 5.12 (1H, *m*, C-6′), 5.32 (1H, *t*(*br*), J = 7.0 Hz, C-2′), 6.15 (1H, *s*(*br*), phenolic OH), 6.72 (1H, *d*, J = 9.0 Hz, C-5), 7.08 (1H, *dd*, J = 9.0 and 2.0 Hz, C-6), 7.08 (1H, *d*, J = 2.0 Hz, C-2). (Found: C, 78.68; H, 9.23. $C_{17}H_{24}O_2$ requires: C, 78.42; H, 9.29%). **13** (100 mg) and CrO_3–Py complex (600 mg) were dissolved in Py (15 ml) and stirred at room temp. for 1 hr. The reaction was diluted with H_2O (50 ml) and extracted with Et_2O (30 ml × 3). The Et_2O extract was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, dried and concd *in vacuo*. PLC (solvent 3)

(R_f 0.48) of the residue gave the aldehyde (**14**) as an oil (86.2 mg). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3300, 2900, 1670, 1585, 1480, 1430, 1370, 1270, 1090. ^1H NMR: δ 1.61 (3H, *s*, C-7''), 1.69 (3H, *s*, C-8'), 1.77 (3H, *s*, C-3''), 2.00–2.25 (4H, *m*, C-4' and 5'), 3.44 (2H, *d*, J = 7.5 Hz, C-1'), 5.10 (1H, *t*(*br*), J = 7.0 Hz, C-6'), 5.35 (1H, *t*(*br*), J = 7.5 Hz, C-2'), 6.58 (1H, *s*(*br*), phenolic OH), 6.95 (1H, *d*, J = 9.0 Hz, C-5), 7.68 (1H, *dd*, J = 9.0 and 2.0 Hz, C-6), 7.69 (1H, *d*, J = 2.0 Hz, C-2), 9.87 (1H, *s*, CHO). (Found: C, 78.81; H, 8.61. $C_{17}H_{22}O_2$ requires: C, 79.03; H, 8.58%). Work-up of **14** with Ac_2O and Py gave the acetate as an oil (78 mg), a 70 mg aliquot of which was treated with excess Jones reagent in Me_2CO (5 ml) for 1 hr at room temp. The reaction was quenched by adding MeOH (1 ml), diluted with H_2O (15 ml) and extracted with Et_2O (15 ml × 3). The Et_2O layer was dried and concd. A soln of the residue in MeOH (1 ml) was mixed with 10% NaOH (5 ml) and stirred for 6 hr at 35°. The mixture was acidified with 1 N HCl under ice-cooling and extracted with Et_2O (15 ml × 3). The Et_2O extract was dried and concd. The residue was recrystallized from cyclohexane to give *m*-geranyl-*p*-hydroxybenzoic acid (**8**) (39.6 mg) as needles, which were identical with an authentic sample in TLC (solvent 3), IR and ^1H NMR spectra.

Next, *m*-geranyl-*p*-hydroxybenzoic acid-[8'-^{2}H] (**8**) was synthesized in the following way starting from the above described allyl bromide (**12**). $LiAl^2H_4$ (20 mg) was added to an ice-cooled soln of **12** (96 mg) in dry THF (5 ml) and stirred for 1 hr. After stirring for a further 5 hr at 50°, the reaction was worked up as described above to give *m*-geranyl-*p*-hydroxybenzyl alcohol-[1'''-$^2H_2$,8'-^{2}H] (**13**) (52.6 mg), which was converted in the same way as above into *m*-geranyl-*p*-hydroxybenzoic acid-[8'-^{2}H] (**8**), needles (18.3 mg). ^1H NMR: δ 1.61 (3H, *s*, C-7''), 1.69 (2H, *s*, C-8'), 1.78 (3H, *s*, C-3''), 2.00–2.25 (4H, *m*, C-4' and 5'), 3.42 (2H, *d*, J = 7.0 Hz, C-1'), 4.90–5.58 (2H, *m*, C-2' and 6'), 6.63 (1H, *s*(*br*), phenolic OH), 6.84 (1H, *d*, J = 9.0 Hz, C-5), 7.89 (1H, *dd*, J = 9.0 and 2.0 Hz, C-6), 7.89 (1H, *d*, J = 2.0 Hz, C-2). MS *m/e*: 275 (M^+). Next, **8**-[8'-^{3}H] was synthesized in an analogous way as **8**-[8'-^{2}H]. Namely, $LiAlH_4$ (16 mg) was added to an ice-cooled soln of **12** (100 mg) in dry THF and stirred for 1 hr. $LiAl^3H_4$ (2 mg, sp. act. 2.22 × 10^{11} dpm/mmol) was added to the mixture under ice-cooling and stirred for 20 min. After stirring at 50° for a further 5 hr, the reaction was worked up in the same way as described in the cold run to give **13**-[1'''-$^3H_2$, 8'-^{3}H] (38.6 mg). This substance, after dilution with the carrier (**13**) (20 mg), was treated by the usual way giving rise to *m*-geranyl-*p*-hydroxybenzoic acid-[8'-^{3}H] (**8**) (7.88 mg, sp. act. 5.71 × 10^8 dpm/mmol).

Preparation of geranylhydroquinone-[8'-^{3}H] (**9**). Freshly sublimated SeO_2 (950 mg) was added to a soln of geranylhydroquinone diacetate (2.85 g), a colourless oil, obtained by the acetylation of **9** in EtOH (50 ml) and refluxed for 1.5 hr. H_2O was added to the reaction and extracted with Et_2O (100 ml × 3). The Et_2O extract was washed with 200 ml each of 10% $NaHCO_3$ and H_2O, dried and concd. The resulting residue was separated by column chromatography on Si gel (150 g) using $CHCl_3$ as eluent into the oxidation products and the unreacted starting material. The oxidation products were further subjected to chromatography on Si gel (100 g) using C_6H_6–$CHCl_3$ (1:1) as eluent and 100 ml fractions were collected. Fractions 7 and 8 gave the allyl aldehyde as an oil (68 mg). Fractions 10–15 provided the allyl alcohol (**15**) as an oil (565 mg). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 2900, 1745, 1480, 1360, 1160, 1000. ^1H NMR: δ 1.65 (6H, *s*, C-3'' and 7''), 1.99–2.20 (4H, *m*, C-4' and 5'), 2.15–2.60 (1H, OH), 2.28 (6H, *s*, CO_2Me × 2), 3.23 (2H, *d*, J = 7.0 Hz, C-1'), 3.95 (2H, *s*(*br*), C-8'), 5.23 (each 1H, *t*, J = 7.0 Hz, C-2' and 6'), 6.90–7.03 (3H, *m*, arom. H). (Found: C, 69.53; H, 7.59. $C_{20}H_{26}O_5$ requires: C, 69.34; H, 7.57%). Over a 1 hr period, PBr_3 (0.4 ml) was added dropwise under N_2 to a soln of **15** (280 mg) in dry Et_2O (5 ml) and Py (0.1 ml) which was cooled to $-10° \sim -5°$. After stirring for a further 1 hr, the reaction was quenched by adding H_2O and extracted with Et_2O (10 ml × 3). The Et_2O extract was washed with 15 ml each of 5% $NaHCO_3$ and H_2O, dried and concd to give the bromide (**16**) as a slightly yellowish oil (21.5 mg), which showed a spot (R_f 0.62) on TLC (solvent 3). $LiAl^2H_4$ (20 mg) was added to an ice-cooled soln of **16** (70 mg) in dry THF (5 ml) under N_2. Work-up of the mixture in a similar manner as in the conversion of **12** into **13** and following chromatography on Si gel (10 g) using C_6H_6 as eluent gave geranylhydroquinone-[8'-^{2}H] (**9**) as an oil (22 mg), which was identical with an authentic unlabelled sample in TLC (solvent 3) and IR. ^1H NMR: δ 1.60 (3H, *s*, C-7''), 1.71 (5H, *s*, C-3'' and 8'), 2.00–2.20 (4H, *m*, C-4' and 5'), 3.27 (2H, *d*, J = 7.0 Hz, C-1'), 5.12 (2H, *s*(*br*), OH × 2), 5.28 (each 1H, *t*, J = 7.0 Hz, C-2' and 6'), 6.53–6.68 (3H, *m*, arom. H). MS *m/e*: 247 (M^+). $LiAlH_4$ (6 mg) was added to an ice-cooled soln of **16** (80 mg) in dry THF (5 ml) under N_2 and stirred for 1 hr. Then, $LiAl^3H_4$ (2.5 mg, sp. act. 4.29 × 10^{11} dpm/mmol) was added to the mixture and stirred for 15 min. After stirring for a further 5 hr, the reaction was conventionally worked up to give geranylhydroquinone-[8'-^{3}H] (**9**) (15.0 mg, sp. act. 4.00 × 10^9 dpm/mmol).

Administration of m-*geranyl*-p-*hydroxybenzoic acid*-[8'-^{3}H] (**8**) *to the callus cultures* (*strain* M-18) *and isolation of radioactive shikonin* (**1**). A soln of *m*-geranyl-*p*-hydroxybenzoic acid-[8'-^{3}H] (**8**) (7.88 mg, sp. act. 5.71 × 10^8 dpm/mmol) in EtOH (1 ml) and H_2O (9 ml) with a few drops of Tween 80 was administered to the callus cultures. After a period of 2 weeks, the callus cells (fr. wt 16.45 g) were extracted with $CHCl_3$. The $CHCl_3$ extract, after the addition of carrier **8** (10.4 mg), was worked up as described above to give radioactive **1** (2.06 mg), which was converted into the leucoacetate (**6**). Radioactive **8** (8.93 mg, 8.02 × 10^5 dpm/mmol) was recovered from the $CHCl_3$ extract of the cells. Substance **6** was subjected to ozonolysis to yield radioactive Me_2CO, which was purified as the 2,4-DNP.

Administration of geranylhydroquinone-[8'-^{3}H] (**9**) *to callus cultures* (*strain* M-18) *and isolation of radioactive shikonin* (**1**). A soln of geranylhydroquinone-[8'-^{3}H] (**9**) (10.0 mg, sp. act. 4.00 × 10^9 dpm/mmol), EtOH (1 ml), Tween 80 (a few drops) and H_2O (9 ml) was administered to callus cultures (strain M-18). After a period of 2 weeks, the callus cells (fr. wt 28.0 g) were extracted with $CHCl_3$. The $CHCl_3$ extract, after the addition of carrier **9** (14.2 mg), was worked up as described above to give radioactive **1** (5.89 mg). The radioactivity was determined after conversion of **1** into the leucoacetate (**6**). On the other hand, radioactive **9** (15.9 mg, 4.12 × 10^7 dpm) was recovered from the $CHCl_3$ extract. Substance **6** was subjected to ozonolysis to yield radioactive Me_2CO, which was converted into the 2,4-DNP.

*Administration of shikimic acid** *to the callus cultures* (*strain* M-18) *grown in the presence of* 2,4-D. Callus mass (strain M-18) was transferred to L-S agar medium supplemented with 2,4-D (10^{-6} mol) and kinetin (10^{-5} mol) and cultured for a month. After 3 repetitions of this procedure, the callus cultures completely lost the ability of the pigment production. This cell line (strain M-18 (2,4-D)) was used for the following experiment. A soln of shikimic acid-[5-^{3}H] (**17**) (3.16 mg, sp. act. 2.70 × 10^{10} dpm/mmol) in H_2O (10 ml) was administered to the callus cells which were grown for a week after transferring. After a period of 10 days, the callus mass (fr. wt 10.2 g) was extracted with $CHCl_3$ (20 ml × 4) under reflux. The $CHCl_3$ extract was

* The radioactivity of [^{3}H]-shikimic acid (**17**) administered was calculated from that of the mixture of this substance and 5-epishikimic acid (**18**) on the basis of the above-described percentage of [^{3}H]-**17** in the mixture.

dried and concd *in vacuo*. The residue, after mixing with **2** (21.8 mg), **8** (19.3 mg), **9** (20.4 mg) and a mixture of fatty acid esters of **1** (31.5 mg) obtained from the C_6H_6 extract of the roots of *L. erythrorhizon*, was chromatographed on Si gel (15 g) using $CHCl_3$ as eluent and 15 ml fractions were collected. Fractions 2–5, 7–8 and 9–17 were combined, concd and worked up in a similar manner as in the case of the dilution analysis of **8** and **9** after the administration of **2**-[3-^{3}H] to give **1** (12.7 mg), **8** (16.1 mg) and **9** (16.6 mg), respectively. **1** and **9** were converted into leucoacetate (**6**) and the *p*-nitrobenzoate, respectively. After eluting 20 fractions with $CHCl_3$, the column was eluted with MeOH and the eluate, after concn, was methylated (CH_2N_2–Et_2O) and subjected to PLC (solvent 3) to yield Me *p*-hydroxybenzoate (18.5 mg).

Administration of shikimic acid to the callus cultures (strain M-386). A soln of **17**-[5-^{3}H] (2.77 mg, 2.70×10^{10} dpm/mmol) in H_2O (10 ml) was administered to callus cells grown on IAA medium for a week after transferring. After 10 days, the cells (fr. wt 19.5 g) were extracted with $CHCl_3$. **2** (25.5 mg), **8** (31.4 mg), **9** (30.1 mg) and a mixture of the fatty acid esters of **1** (30.7 mg) were added to the $CHCl_3$ extract and worked up the same as above to give the Me ester of **2** (21.7 mg), **8** (28.1 mg), **9** (26.8 mg) and **1** (12.6 mg). **1** and **9** were converted into the leucoacetate (**6**) and the *p*-nitrobenzoate, respectively.

Administration of shikimic acid to callus cultures (strain M-231a *(light)*). Callus cultures (strain M-231a) were transferred to five 100 ml flasks containing IAA medium and illuminated with a white light (5000 lx) for a week. A soln of **17**-[5-^{3}H] (1.45 mg, 2.70×10^{10} dpm/mmol) in H_2O (5 ml) was administered to the cells and cultured under the illumination for 10 days further. The cell mass (fr. wt 9.4 g) was extracted with $CHCl_3$ under reflux (30 ml × 4). Substances **8** (18.4 mg) and **9** (12.6 mg) were added to the extract and worked up the same as in the case of the dilution analysis of the same substances described above to give **8** (14.1 mg), **9** (18.7 mg) and **1** (1.61 mg). For counting the radioactivity, **1** and **9** were converted into the leucoacetate (**6**) and *p*-nitrobenzoate, respectively. As a control experiment, an aq. soln of the same amount of **17**-[5-^{3}H] was administered to callus cells (strain M-18) and cultured in the dark for 10 days. The cell mass (fr. wt 9.9 g) was worked up in the same way as described in the experiment under illumination and **8**, **9** and **1** were isolated.

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REFERENCES

1. Schmid, H. V. and Zenk, M. H. (1971) *Tetrahedron Letters* 4151.
2. Sankawa, U., Ebizuka, Y., Shibata, S. and Yamazaki, K. (1976) *Shoyakugaku Zasshi* **30**, 183.
3. Tabata, M., Mizukami, H., Hiraoka, N. and Konoshima, M. (1974) *Phytochemistry* **13**, 927.
4. Mizukami, H., Konoshima, M. and Tabata, M. (1978) *Phytochemistry* **17**, 95.
5. Mizukami, H. (1976) Ph.D. Thesis, Kyoto University.
6. Majima, R. and Kuroda, C. (1922) *Acta Phytochem.* **1**, 43.
7. Kaczka, E. A., Shunk, C. H., Pichter, J. W., Wolf, F. J., Gasser, M. M. and Folkers, K. (1956) *J. Am. Chem. Soc.* **78**, 4125.
8. Inouye, H., Tokura, K. and Tobita, S. (1968) *Chem. Ber.* **101**, 4057.
9. Bhalerao, U. T. and Rapoport, H. (1971) *J. Am. Chem. Soc.* **93**, 4835.
10. Leduc, M. M., Dansette, P. M. and Azerard, R. G. (1970) *Eur. J. Biochem.* **15**, 428.