BIOORGANIC CHEMISTRY 3, 72–80 (1974)

Biosynthesis of an Antibiotic, Siccanin

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Received April 6, 1973

The biosynthetic pathway of the antibiotic siccanin (**1**) is based on the experiments using cell-free systems and intact cell systems of *Helminthosporium siccans* Drechsler. It involves (a) formation of *trans*-γ-monocyclofarnesol (**5**) from mevalonic acid lactone or farnesyl pyrophosphate; (b) coupling reaction of the terpenic precursor with orsellinic acid; (c) oxidative conversion of presiccanochromenic acid (**8**), into siccanochromenic acid (**9**), followed by decarboxylation to siccanochromen-A (**10**); and (d) epoxy-olefin type cyclization of siccanochromen-B (**11**) to siccanin (**1**).

INTRODUCTION

Siccanin (**1**), an antibiotic, was isolated from the cultured broth of *Helminthosporium siccans*, a parasitic organism of rye grass, *Lolium multifolium* Lam (*1*). Siccanin exhibits a significant inhibitory activity against *Tricophyton interdigitale*. The structure was previously determined by three-dimensional X-ray diffraction analysis (*2*). Recently we reported the chemistry and the physicochemical properties of some chemically transformed derivatives including siccanochromen-E (**2**), which was found as a minor cometabolite of *H. siccans* (*3*). Siccanin (**1**) and siccanochromen-E (**2**) were assumed to be derived biogenetically by combination of sesquiterpene and orsellinate entities. Some biogenetically similar triprenyl phenols—such as grifolin, tauranin, ascochlorin or a series of antibiotic LL-Z 1272, and mycophenolic acid (*4*)—are known among fungus metabolites (*5*).

The unique ring junctions—*cis*/*syn*/*cis*—of siccanin (**1**) have encouraged its biogenetic study, especially on the cyclization mechanism leading to the ring junctions. During the biosynthetic study of siccanin (**1**), a number of triprenyl phenol derivatives were detected by autoradiograms and color reaction with diazotized benzidine salt solution on tlc plates and then isolated by column chromatography. These derivatives included siccanochromen-A (**10**), -B (**11**) (*6*, *7*), and -E (**2**) (*3*, *8*); siccanochromenic acid (**9**); and presiccanochromenic acid (**8**) (*8*). These natural products structurally and biogenetically correspond to a series of diprenyl phenol derivatives that are isolated from hashish, such as cannabiochromen, cannabigerolic acid, and cannabichromenic acid (*9*). The diprenyl phenol derivative **3**, a minor metabolite isolated from *H. siccans*, corresponds structurally and, presumably, biogenetically to cannabigerolic acid isolated from hashish. The structure of **3** was unequivocally confirmed by chemical synthesis (*10*).

By comparing the structures of the minor metabolites of *H. siccans*, it was possible to postulate a biogenetic pathway leading to siccanin (**1**): (a) coupling reaction of *trans*-γ-monocyclofarnesyl pyrophosphate (**4**), which is a cyclization product of *trans,trans*-farnesyl pyrophosphate, with orsellinic acid to give presiccanochromenic acid (**8**); (b) oxidative conversion of **8** to siccanochromenic acid (**9**); (c) decarboxylation of **9** to siccanochromen-A (**10**); (d) epoxidation of the exocyclic methylene to produce siccanochromen-B (**11**); and (e) epoxy-olefin type cyclization (*11, 12*) to siccanin (**1**).

Biosynthetic studies using both the cell-free extracts and the growing cells of this fungus were initiated to support the aforementioned hypothesis. The use of the cell-free extract greatly simplified the study of this multistep biosynthetic pathway.

Enzymatic Formation of trans-*γ-Monocyclofarnesol* (**5**)

The cell-free system was prepared by disrupting the cultured mycelia of *H. siccans* with washed sea sand in 0.1 *M* phosphate buffer (pH 7.22, containing 0.1 *M* $MgCl_2$) and centrifuging at 11,000 *g*, 0°C. *dl*-[2-^{14}C]-Mevalonic acid lactone was incubated aerobically with the supernatant fraction in the presence of ATP at 36°C for 3 hr. Products were isolated by extraction with ether after saponification. The nonsaponifiable fraction was found to contain from 30 to 60% of the added radioactivity. Over 80% of the radioactivity was located at a position with R_f 0.11 on a silica gel tlc plate developed with benzene. This active compound **5**, as well as its corresponding acetate **6** and aldehyde **7** (a product of MnO_2 oxidation), showed tlc and radio-gas chromatography behavior identical to *trans*-γ-monocyclofarnesol (**5**), its corresponding acetate **6** and aldehyde **7**. Isolation of *trans*-γ-monocyclofarnesol as a minor constituent of *H. siccans* has been previously reported (*13*). The structure of the alcohol **5** was confirmed by chemical synthesis. The double-bond isomers (*trans*-α- and -β-monocyclofarnesol or the *cis*-isomers) and farnesol (and its pyrophosphate ester) were not observed in the incubation mixture. Incubation of *trans,trans*-[4,8,12-^{14}C]-farnesyl pyrophosphate with the same cell-free system without ATP also produced *trans*-γ-monocyclofarnesol (**5**).

Coupling Reactions with Aromatic Substrates (*14*)

Incubation of *dl*-[2-^{14}C]-mevalonic acid lactone or *trans,trans*-[4,8,12-^{14}C]-farnesyl pyrophosphate in the presence of the aromatic cosubstrate, orsellinic acid, under the same conditions as described above yielded a prenyl phenol derivative instead of *trans*-γ-monocyclofarnesol (**5**). The product showed the same behavior as that of siccanochromen-A (**10**) on tlc plates as well as on radio-gas chromatograms. The conversion was about 30% in the case of mevalonic acid lactone and quantitative in the case of farnesyl pyrophosphate. To test the substrate specificity of this enzyme activity for condensation reaction of prenyl pyrophosphate with phenolic compound, incubations were carried out using cosubstrates such as 2,4-dihydroxy-6-*n*-propylbenzoic acid, orcinol, α- and β-resorcylic acid, and resorcinol in the same incubation conditions, but no coupling products were found to be formed.

Formation of Chromen Derivative

The formation of siccanochromen-A (**10**) from mevalonic acid or farnesyl pyrophosphate and orsellinic acid by the above enzyme preparation suggested the involvement of the enzyme activities that catalyze oxidative conversion of the prenyl phenol **8** into siccanochromenic acid (**9**), followed by decarboxylation of **9** to give siccanochromen-A (**10**). When unlabeled presiccanochromenic acid (**8**) was incubated aerobically with the cell-free preparation mentioned above, siccanochromen-A (**10**) was obtained in 40% yield along with recovered **8** (50%) and **9** (4%). Anaerobic incubation, however, afforded no transformation products, indicating that oxygen is required for the formation of the chromen ring.

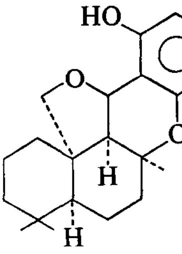
Decarboxylation

The cell-free preparation used in the above experiments contains an enzyme that catalyzes the decarboxylation of siccanochromenic acid (**9**). The siccanochromenic acid (**9**) obtained from *H. siccans* was found to be a 2:1 diastereomeric mixture of **9** and episiccanochromenic acid. These compounds differ in the configuration of the methyl group on the chromen ring. The major isomer corresponds to the naturally occurring siccanochromens. The ratio of the diastereomers was readily estimated by NMR spectral comparison of the signals of the olefinic proton on the chromen ring with those of the chemically derived chromenic acid **9** obtained by DDQ treatment (*15*) of **8**. Incubation of this diastereomeric mixture with cell-free systems of *H. siccans* gave a mixture of siccanochromen-A (**10**) and epi-siccanochromen-A in quantitative yield. Although there are several hypotheses for the mechanism of chromen ring formation (*16*), it is possible to postulate as follows: Since **8** yields isomerically pure **9** and **10** by enzyme reaction and since all known siccanochromens except **9** are isomerically pure at the asymmetric center of the chromen ring, the epimeric mixture of **9** isolated from *H. siccans* may be formed through an unstable intermediate such as **12**. A solvolytic cyclization of intermediate **12** during the isolation process would afford the epimeric mixture of siccanochromenic acid (**9**). Orsellinic acid and 2,4-dihydroxy-6-*n*-propylbenzoic acid were also decarboxylated in appreciable yield, but presiccanochromenic acid (**8**) and α- and β-resorcylic acids were not decarboxylated by this enzyme system.

Prenylation and successive decarboxylation of the aromatic carboxylic acid are reminiscent of the corresponding steps in the biosynthesis of ubiquinone (*17*, *18*).

Cyclization to Siccanin (*19*)

To examine the intermediary roles of siccanochromenic acid (**9**) and siccanochromen-A (**10**) and -B (**11**) each was heated in a sealed tube with deuterium oxide in the presence of a catalytic amount of triethylamine to yield the deuterated compounds **9**, **10**, and **11**, respectively. NMR spectra of the deuterated compounds showed that approximately 50, 80, and 70% of the aromatic protons were exchanged, respectively. Tritium-labeled substances (specific activity = 17.3 Ci/mole for **9**, 56.3 Ci/mole for **10**, and 63.8 Ci/mole for **11**), were similarly prepared using tritiated water and were fed to the 3-day-old culture of *H. siccans* at a concentration of 4–5 μCi/100 ml of culture solution. Incubation was continued aerobically for an additional 48 hr with shaking at 120 rpm, 26.5°C. The products were extracted and separated by silica gel column chromatography. Siccanin was purified by repeated crystallization with unlabeled siccanin until a constant specific activity was obtained. It was found that 22 to 25% of the added radioactivity was incorporated into siccanin in the case of tritium-labeled siccanochromenic acid (**9**), 26 to 43% in the case of siccanochromen-A (**10**), and about 70% in the case of siccanochromen-B (**11**). Washed cell suspensions (4-day-old mycelia) in 0.1 *M* phosphate buffer (pH 7.62) and in 0.1 *M* citrate phosphate buffer (pH 5.6), to which were added tritium-labeled siccanochromenic acid (**9**), siccanochromen-A (**10**), and siccanochromen-B (**11**), were incubated aerobically for 24 hr with shaking



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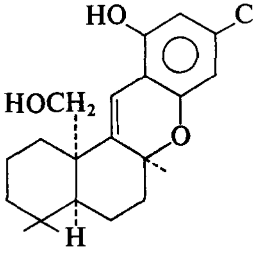

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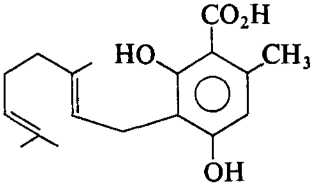

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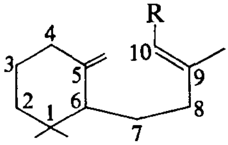
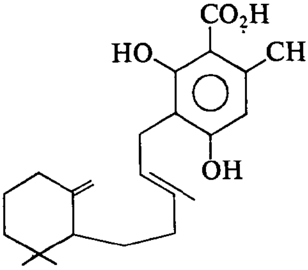


4 $R = CH_2OP_2O_6H_3$
5 $R = CH_2OH$
6 $R = CH_2OCOCH_3$
7 $R = CHO$



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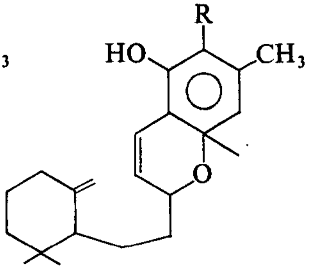
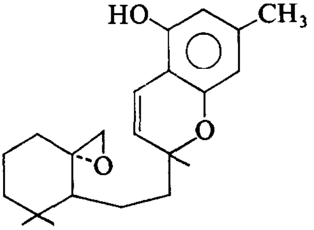
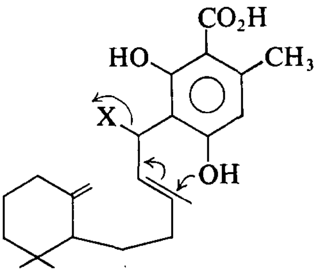


9 $R = CO_2H$
10 $R = H$



11



12

at 120 rpm, 26.5°C. It was found that 2.3 to 4.7%, 3.6 to 5.7%, and 16 to 39% of the added radioactivity was incorporated into siccanin from **9**, **10**, and **11**, respectively. It was confirmed that no siccanin was formed by nonenzymatic acid treatment (BF_3-etherate in dry benzene) of **11** or by the incubation with preheated mycelia. Siccanochromen-B (**11**) was also incubated with the cell-free supernatant, but no transformation products were obtained.

EXPERIMENTAL METHODS

Fermentation of H. siccans. *H. siccans* was cultured in a 500-ml flask, containing 100 ml of a solution of 3% peptone, 4% glucose, 0.5% potassium bisulphate, and 0.25% magnesium sulphate (pH 5.6). It was precultured for 4 days, and then one crop of precultured mycelia was again inoculated into a 500-ml flask and cultured at 120 rpm, 26.5°C.

Preparation of cell-free system. The cultured mycelia were collected, washed with 0.1 *M* phosphate buffer (pH 7.22, containing 0.1 *M* $MgCl_2$), and mechanically disrupted by hand in a mortar with an equal weight of sea sand and an equal volume of buffer for 15 min at 0°C. The disrupted mycelia were centrifuged at 200 *g* for 5 min, and the supernatant was again centrifuged at 11 000 *g* for 20 min.

Fractionation of cell-free supernatant. The fractionation of each enzyme activity was attempted. The 11 000 *g* supernatant fraction used so far in this study was further centrifuged at 105 000 *g* for 1 hr at 0°C. Each experiment was reexamined using both 105 000 *g* supernatant and 105 000 *g* precipitate fractions in the same procedures. It was found that cyclase activity for the transformation of farnesyl pyrophosphate to *trans*-γ-monocylofarnesyl pyrophosphate was located in the 105 000 *g* precipitate fraction, and other activities (prenylation, chromenation, and decarboxylation) were located in the 105 000 *g* supernatant fraction.

Enzymatic formation of trans-*γ-monocyclofarnesol.* A typical experiment is shown as follows: *dl*-[2-^{14}C]-Mevalonic acid lactone (270 000 dpm) and ATP (1 mg) were aerobically incubated with the cell-free preparation (1 ml) at 36°C for 3 hr. Then 1 *N* KOH–MeOH solution (1 ml) was added to the incubated medium, which was hydrolyzed overnight at room temperature. The products were extracted with ether and washed with water, dried over Na_2SO_4, and evaporated to dryness. [4,8,12-^{14}C]-Farnesyl pyrophosphate was also treated by the same procedure as above but without ATP.

Identification of trans-*γ-monocyclofarnesol.* The enzymatically formed products, obtained by the above procedure, as well as its acetate and aldehyde were chromatographed on silica gel plates ($AgNO_3$-impregnated silica gel plates and liquid-paraffin-impregnated silica gel plates using various solvent systems). The typical R_f values of *trans*-γ-monocyclofarnesol and the related substances are listed in Table 1. Labeled and unlabeled *trans*-γ-monocyclofarnesols and the combined mixture all had the same R_f values. This sample was also analyzed by radio-gas chromatography (RIO-2E Radioisotope Detector Connected with Gas Chromatograph Shimadzu-GC-4APTF, 1.5% OV-17 on Gaschrom; 4 mm × 1.5 m column; column temperature 120°C; ratio of flow rate, 12.8 ml/min:85.8 ml/min). The retention time (R_t) was 10.2 min (the relative retention time to *trans*-farnesol was 0.74). Relative retention times of related compounds on gas chromatograph (Gas Chromatograph Shimadzu 4APTF, 1.5% OV-17 on Shimalite W, 4 mm × 1.5 m column) were as follows: *trans,trans*-farnesol 1.00, *cis,trans*-farnesol 0.78, *trans*-α-monocyclofarnesol 0.90, *cis*-α-monocyclofarnesol 0.70, *trans*-β-monocyclofarnesol 1.01, *cis*-β-monocyclofarnesol 0.83, and *trans*-γ-monocyclofarnesol (**5**) 0.80 (as for nomenclature, see Table 1).

Enzymatic formation of siccanochromen-A (**10**). *dl*-[2-^{14}C]-Mevalonic acid lactone (270 000 dpm), ATP (1 mg), and oresellinic acid (1 mg) were aerobically incubated

TABLE 1

TYPICAL R_f VALUES OF *trans*-γ-MONOCYCLOFARNESOL AND RELATED COMPOUNDS

Compounds	Silica gel plates[a]		10% $AgNO_3$-impregnated silica gel plates[b]			5% Liquid-paraffin-impregnated silica gel plates[c];
	Benzene 100%	Benzene 20, acetone 1	Benzene 100%	Benzene 20, acetone 1	Benzene 10, acetone 1	acetone 65, water 35
t-γ-Monocyclofarnesol (5)	0.11	0.36	0.11	0.25	0.48	0.54
t-β-Monocyclofarnesol[d]		0.32		0.31		
c-β-Monocyclofarnesol[d]		0.40		0.36		
t-α-Monocyclofarnesol[d]		0.33		0.28		
c-α-Monocyclofarnesol[d]		0.38		0.34		
t,*t*-Farnesol	0.10	0.36	0.03		0.11	0.56
c,*t*-Farnesol	0.14	0.39	0.03		0.16	0.56
Geraniol	0.10	0.29	0.05			0.79
t-γ-Monocyclofarnesyl acetate	0.49		0.80		0.97	0.29
t-γ-Monocyclofarnesal	0.28		0.60		0.94	0.47
t,*t*-Farnesyl acetate			0.20		0.79	0.27
c,*t*-Farnesyl acetate			0.27		0.84	0.27

[a] Silica gel G plates (DC-Fertigplatten Kieselgel Merck) were used.

[b] Silica gel plates were dipped in 10% $AgNO_3$ solution in $EtOH$–CH_3CN (9:1) and dried.

[c] Silica gel plates were dipped in 5% liquid paraffin solution in hexane and dried (*21*).

[d] The names of α- and β-monocyclofarnesol were used for the double-bond isomer of **5**, in which the olefin linkages are located at $\Delta^{4,5}$ and $\Delta^{5,6}$, respectively. *t* and *c* in these cases show geometry of $\Delta^{9,10}$ double bond.

with the cell-free preparation (1 ml) at 36°C for 3 hr. Proteins were filtered off by adding acetone (1 ml) to the incubated medium and heating. Products were extracted from the filtrate with ether, dried over Na_2SO_4, and evaporated to dryness. Autoradiographs of the products on various silica gel tlc plates were analyzed and identified as siccanochromen-A (**9**) and *trans*-γ-monocyclofarnesol (**5**). The ratios of siccanochromen-A (**10**) to *trans*-γ-monocyclofarnesol (**5**) were dependent on the age of disrupted mycelia.

[4,8,12-^{14}C]-Farnesyl pyrophosphate (10 000 dpm) and orsellinic acid (0.5 ~ 1 mg) were incubated with the cell-free system prepared from 40- to 50-hr-old mycelia. Products were isolated in the same manner. After incubation, radioactivity was quantitatively transferred to a neutral organic solvent fraction, and the products were found to have exclusively the same R_f values as those of siccanochromen-A (**9**) in various tlc systems (Table 2).

The identity of this active compound as siccanochromen-A (**9**) was confirmed by autoradiograph on tlc plates under various conditions as well as by radio-gas chromatography (R_t 5.2 min on the same instrument and under the conditions of preceding section, column temperature 220°C. In comparison, the R_t of siccanin (**1**) was 9.8 min).

TABLE 2

TYPICAL R_f VALUES OF SICCANOCHROMEN-A AND RELATED COMPOUNDS

	Silica gel plates[a]			10% $AgNO_3$-impregnated silica gel plates[b];	5% Liquid-paraffin-impregnated silica gel plates[c];
Compounds	Benzene 100%	Chloroform 100%	Benzene 20, acetone 1	benzene 100%	acetone 65, water 35
Siccanochromen-A (**10**)	0.46	0.42	0.58	0.31	0.34
Siccanochromen-A acetate	0.69	0.74		0.83	0.20
Siccanin (**1**)	0.19	0.24	0.47	0.48	0.43

[a] Silica gel G plates (DC-Fertigplatten Kieselgel Merck) were used.

[b] Silica gel plates were dipped in 10% $AgNO_3$ solution in EtOH–CH_3CN (9:1) and dried.

[c] Silica gel plates were dipped in 5% liquid paraffin solution in hexane and dried (*21*).

Enzymatic transformation of presiccanochromenic acid (**8**) *into siccanochromen-A* (**10**). Presiccanochromenic acid (**8**) (17 mg in 0.2 ml MeOH solution) was added to the cell-free system (17 ml) prepared from 50-hr-old mycelia and aerobically incubated at 36°C, 120 rpm for 3 hr. Products were extracted in the same manner as that used for siccanochromen-A and separated into acid and neutral fractions. The former fraction contained recovered starting material (50%) and siccanochromenic acid (**9**) (4%), and the latter fraction contained siccanochromen-A (**10**) (40%). The NMR spectrum of the acetate of this siccanochromen-A sampled showed the same signals as those of natural siccanochromen-A (**10**). The NMR spectrum of the acetylated methyl ester of this enzymatically obtained siccanochromenic acid (**9**) indicated the presence of only one isomer. Only one doublet for the vinylic proton adjacent to the asymmetric center of the chromen ring (5.45 ppm) was present. In contrast, treatment of methyl presiccanochromenate with DDQ in benzene yielded a mixture diastereomeric at the chromen ring, and the acetate of this chemically derived methyl siccanochromenate showed NMR signals at 5.45 and 5.47 ppm (adjacent protons on the asymmetric center of chromen ring) in 1:1 ratio. On the other hand, the methyl acetate of naturally occurring siccanochromenic acid (**9**) showed those signals in a 2:1 ratio.

The same amount of presiccanochromenic acid (**8**) was an aerobically incubated (oxygen was removed by replacing air with argon gas three times). Products were isolated in the same manner. Starting material was recovered in this case.

Enzymatic decarboxylation of siccanochromenic acid (**9**) *into siccanochromen-A* (**10**). Siccanochromenic acid (**9**) (22 mg) was aerobically incubated with the cell-free system (20 ml) prepared from 50-hr-old mycelia at 36°C, 120 rpm for 3 hr. Products were isolated in the same manner as the one in the preceding section to yield siccanochromen-A (**10**). The NMR spectrum of **10** isolated from this procedure showed the same olefinic proton signals at 5.31 and 5.33 ppm as those of starting material in a ratio of 2:1.

Exchange of aromatic protons with ^2H *and* ^3H. To examine the extent of exchange of aromatic protons and stability of labels, siccanochromen-A (**10**), -B (**11**), siccanochromenic acid (**9**), presiccanochromenic acid (**8**), and siccanin (**1**) were treated with deuterium oxide in the presence of triethylamine. A solution of each substance (10 mg) in DMF (0.15–0.20 ml) was heated in the presence of deuterium oxide (40 μl, purity 99.8%) and triethylamine (10 μl) in a sealed tube at 100°C for 65 to 70 hr. Products were extracted with ether, washed with water, dried over Na_2SO_4, and evaporated to dryness. NMR and mass spectra were taken to estimate the extent of incorporation. The aromatic proton of siccanochromen-A (**10**), -B (**11**), and siccanin (**1**) were exchanged with deuterium in 70 ~ 80% yield and siccanochromenic acid (**9**) in 50% yield. On the other hand, presiccanochromenic acid (**8**) was decarboxylated to a large extent, and these labels were labile to washing.

Tritium oxide (specific activity, 90 Ci/mole) was used in place of deuterium oxide. The specific activities of the isolated siccanochromen-A (**10**), -B (**11**), and siccanochromenic acid (**9**) were 56.3, 63.8, and 17.3 Ci/mole, respectively.

Incorporation of siccanochromens into siccanin (**1**). Siccanochromenic acid (**9**) and siccanochromen-A (**10**) and -B (**11**) were fed as a 4–5 μCi/10 μl MeOH solution to a 3-day-old culture of a growing cell system that contained 100 ml of culture solution in a 500-ml flask. Incubation was continued aerobically for 48 hr with shaking at 120 rpm, 26.5°C. The incubated medium was filtered. The mycelial fraction was extracted with hot acetone several times. Acetone was evaporated at reduced pressure and combined with the filtrate fraction and extracted with ether. Crude products were separated by silica gel column chromatography with benzene and purified by repeated crystallization from MeOH in the presence of the carrier substance until a constant specific activity was obtained.

ACKNOWLEDGMENT

The authors are grateful to Professor S. Okuda of this Institute for his encouragement and interest in this work and to Dr. A. Kawaguchi for his gift of [4,8,12-^{14}C]-farnesyl pyrophosphate. Thanks are also due to Professors S. Shibata and U. Sankawa and Mr. T. Komiya, Department of Pharmaceutical Sciences of this University, for their gift of aromatic substances. We also thank Professor Y. Satoh of Kyoritsu College of Pharmacy for his kind assistance in obtaining the radio-gas chromatograms.

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