Biosynthesis of Gibberellins

IV. BIOSYNTHESIS OF CYCLIC DITERPENES FROM TRANS-GERANYLGERANYL PYROPHOSPHATE*

ISHAIAHU SHECHTER AND CHARLES A. WEST
From the Division of Biochemistry, Department of Chemistry, University of California, Los Angeles, California 90024

(Received for publication, January 22, 1969)

SUMMARY

A 14C-diterpene alcohol (copalol) was isolated as its 4-(4'-nitrophenylazo)benzoyl ester from a soluble enzyme system which had been prepared from a cell-free extract of mycelia of the fungus Gibberella fujikuroi and incubated with 2-14C-mevalonate, ATP, and MgCl2. Copalol was identified as trans-(−)-labda-8(16),13-dien-15-all from its properties in comparison with those of the enantiomeric alcohol synthesized from manool. 14C-Geranylgeraniol and 14C-(−)-kaurene were also identified as diterpenes formed in these incubation mixtures. All three diterpenes were also found as products when trans-14C-geranylgeranyl pyrophosphate was used as the substrate in place of 2-14C-mevalonate and ATP.

A substance with the properties expected for 14C-copalyl pyrophosphate was also extracted with collidine from these incubation mixtures with either 2-14C-mevalonate or trans-14C-geranylgeranyl pyrophosphate as the substrate. The structure of the purified product was established as copalyl pyrophosphate by showing (a) that it yielded copalol on treatment with alkaline phosphatase and (b) that the doubly labeled product was formed from trans-14C-geranylgeranyl 32P-P-pyrophosphate with the same 14C:32P ratio as the substrate.

Copalyl pyrophosphate was converted to kaurene by a soluble enzyme preparation from G. fujikuroi. The plant growth retardant tributyl-2,4-dichlorobenzylphosphonium chloride (Phosfon) at 1 mM concentration inhibited this conversion, whereas two other retardants, β-chloroethyltrimethylammonium chloride, and Phosfon for tributyl-2,4-dichlorobenzylphosphonium chloride.

The formation of (−)-kaurene (I), a cyclic diterpene hydrocarbon, is of particular interest in connection with the biosynthesis of the plant growth-regulating gibberellins. Kaurene was first shown to be a precursor of gibberellic acid in cultures of the fungus, Gibberella fujikuroi (Fusarium moniliforme in the imperfect stage), by Cross, Galt, and Hanson (1). The biosynthesis of kaurene from mevalonate and trans-geranylgeranyl pyrophosphate has been shown in cell-free preparations of the endosperm of immature seed of wild cucumber (Echinocystis macrocarpa) (2,3). Its formation from mevalonate has also been reported in cell-free systems from castor bean seedlings (4) and immature pea seeds and fruits (5,6).

It was suggested on theoretical grounds that geranylgeraniol or a geranylgeraniol derivative might first be converted by a proton-initiated cyclization to a stable bicyclic intermediate which could, in turn, serve as the precursor to several types of diterpene hydrocarbons by additional cyclization steps (7,8). Thus, the pyrophosphate ester of the bicyclic alcohol (II) was included as a proposed intermediate in the formation of kaurene from geranylgeranyl-PP in E. macrocarpa endosperm (2), even though there was no direct experimental evidence for its existence. The work described in this paper documents the production of the diterpene alcohol (II), which has been given the trivial name copalol, and its pyrophosphate ester, in cell-free extracts of G. fujikuroi. Furthermore, the role of copalyl pyrophosphate as a precursor of kaurene in G. fujikuroi and E. macrocarpa enzyme preparations, and of kaurene and other cyclic diterpene hydrocarbons of the same stereochemical family in castor bean (Ricinus communis) seedling extracts, has been established.

A preliminary account of this work has been presented.2

1 For convenience (−)-kaurene is referred to as kaurene. The trivial name copalol is introduced to refer to (−)-labda-8(16),13-dien-15-all; copalol refers to the trans isomer throughout this paper unless otherwise specifically mentioned. Geranylgeraniol and geranylgeranyl-PP are used to indicate the all trans isomer of 3,7,11,15-tetramethylhexadecane-2,0,10,14-tetraen-1-ol and its pyrophosphate ester, respectively. The following trivial designations are used for the plant growth retardants: Amo 1618 for 2'-isopropyl-4'-trimethylammonium chloride)·5'-methylphenyl piperidine-1-carboxylate, CCC for β-chloroethyltrimethylammonium chloride, and Phosfon for tributyl-2,4-dichlorobenzylphosphonium chloride.

Experimental Procedure

The N,N'-dibenzylethylenediammonium salt of 2-14C-DL-mevalonic acid was obtained from New England Nuclear. DL-Mevalonolactone was obtained from Mann. ATP and Amo 1618 were produced by Calbiochem. Phosfon (technical grade) was donated by the Research and Development Department of the Virginia-Carolina Chemical Corporation, Richmond, Virginia. CCC (technical solution, 11.8% CCC by weight) was donated by the Agricultural Division of the American Cyanamid Company, Princeton, New Jersey. Bacterial alkaline phosphatase came from Worthington. 4-(4'-Nitrophenylazo)benzoyl chloride was synthesized by the procedure of Hecker (9). The orange-red derivative, after crystallization from warm carbon tetrachloride, melted sharply at 135.5° (uncorrected).

[Diagram of compounds]

14C-Geranylgeranyl-PP—This substrate was prepared biosynthetically from 2-14C-DL-mevalonate incubated with E. macrocarpa endosperm in the presence of added ATP, MgCl₂, and Amo 1618, and was purified according to procedures previously published (9).

Preparation of Cell-free Extracts

G. fujikuroi—Stock cultures of G. fujikuroi (Lilly 1) were maintained on freshly prepared potato dextrose agar. The liquid culture medium contained glucose (80 g), KH₂PO₄ (1.0 g), MgSO₄ (0.50 g), NH₄NO₃ (1.0 g), and trace elements (2.5 ml) made up to a total volume of 1 liter with water. The trace elements solution contained in 100 ml: FeSO₄.7H₂O (0.10 g), MgSO₄ (0.50 g), NH₄NO₃ (1.0 g), and trace elements (2.5 ml) made up to a total volume of 1 liter with water. The trace elements solution contained in 100 ml: FeSO₄.7H₂O (0.10 g), CuSO₄ (0.015 g), MnSO₄·H₂O (0.010 g), ZnSO₄·H₂O (0.16 g), and ammonium molybdate (0.010 g). Inocula were prepared by transfer from a slant into 100 ml of sterile culture medium in 150-ml flasks. Ten milliliters of the cell suspension resulting after 40 hours of growth with shaking at room temperature were transferred to 1 liter of fresh medium in 2-liter flasks. Growth was permitted to proceed for 48 hours at room temperature while the flask was shaken. At the end of this period the mycelium were harvested by filtration and washed three times with 1 liter of water.

The washed cells were suspended in 20 ml of 0.10 M sodium phosphate, pH 7.6, and placed in a cylindrical Lucite container which was sealed and frozen in Dry Ice. The frozen suspension was passed through the Sagers’ press (12) under high pressure. An additional 10 ml of buffer were added to the crushed cell mass and the suspension was centrifuged at 20,000 × g for 15 min. The supernatant fraction was separated from the pellet and recentrifuged at 105,000 × g for 60 min. The resulting clear, yellow supernatant fraction (S₁₀₀) was utilized as the source of fungal enzymes for the experiments reported. All centrifugation steps were conducted at 4° and the extract was kept at ice bath temperatures. Extracts were prepared fresh just prior to use. Such extracts typically contained 6 to 8 mg per ml of protein as determined by the method of Bessey, Lowry, and Love (18).

E. macrocarpa—Cell-free extracts of the endosperm of E. macrocarpa seed were prepared as previously described (2). Extracts which had been centrifuged at 105,000 × g (S₁₀₀) were used in the experiments reported.

K. commune—Seeds of castor bean (Baker Hybrid 66 kindly donated by the Baker Castor Oil Company, Plainview, Texas) were germinated in darkness for 60 to 72 hours at 30–32°. Seedlings (minus roots) were mixed with 1.5 ml of suspension medium (0.050 M Tris bicine buffer, pH 7.3, and 0.010 M 2-mercaptoethanol) and 0.25 g of Polycar A (a water-insoluble
polyvinylpyrrolidone supplied by General Aniline and Film Corporation, New York) per g of fresh weight of seedlings. This mixture was homogenized (Virtis 23 homogenizer for 2 min at three-quarters maximum speed) and the resulting homogenate was squeezed through four layers of cheesecloth. This filtrate, pH 6.7 to 6.8, was centrifuged at 105,000 x g for 60 min at 4°. One milliliter of the supernatant solution (S100) contains 2.5 to 3 mg of protein.

Assay of 14C-Lipids Formed in G. fujikuroi Extracts

Incubations with 2-14C-Mevalonate as Substrate—The N,N'-dibenzylethenediammonium salt of 2-14C-DL-mevalonic acid in the amounts indicated in individual experiments was incubated with G. fujikuroi S100 preparation in the presence of added 1 mM MgCl2 and 2 mM ATP under an N2 atmosphere in glass-stopped tubes at 30°. An equal volume of acetone was added after 1 hour to stop the enzymatic reactions. The mixture was then extracted with n-hexane (3:1) equivalent to 0.50 of the original volume of the aqueous phase. The combined benzene-acetone layers were washed with an equal volume of water before the organic layer was evaporated to a volume of approximately 0.1 ml under a stream of nitrogen.

Incubations with 14C-Geranylgeranyl-PP or 14C-Copalyl-PP as Substrate—The amount of substrate specified in individual experiments was incubated with the S100 preparation and 1 mM MgCl2 under an N2 atmosphere in glass-stopped tubes at 30°. After 1 hour, 0.01 ml of bacterial alkaline phosphatase (0.26 e.u.) was added and the incubations were continued for an additional 10 min. A volume of acetone equivalent to the volume of the reaction mixture was added to stop the enzymatic reactions, and the extraction with benzene-acetone was carried out as described above.

Assay of Benene-extractable 14C Products by Thin Layer Chromatography—The residual 0.1 ml of the benzene-acetone extract was applied to an appropriate previously coated silica gel plate (F-254 supplied by Brinkmann Instruments, Inc.). The general types of 14C-lipids present were assayed on untreated silica gel plates developed first with n-hexane to 15 cm from the origin followed by development in the same direction with benzene-ethyl acetate (9:1) to 10 cm. 14C-Hydrocarbons migrate to the region ahead of the second solvent front, while C18 and C20 monoa-lcohols are found approximately midway between the origin and the second solvent front (14). Silica gel plates impregnated with AgNO3 in ethanol-acetonitrile (9:1) were utilized in the further resolution of the alcohol or hydrocarbon fraction. Development with n-hexane-benzene (7:3) was used to separate hydrocarbons, while development with n-hexane-ethyl acetate-isopropanol (2:1:1) resolved the alcohols (9). Reversed phase silica gel plates coated with silicone and developed with methanol-water (5:1) were used to classify monoalcohols according to chain length as previously described (3).

The radioactivity of materials adsorbed on silica gel plates was determined by counting in a Packard model 2008 liquid scintillation spectrometer appropriate portions of silica gel suspended in scintillation fluid containing 4 g of 2,5-diphenyloxazole and 50 mg of p-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene. Nonradioactive reference standards were detected on the plain or reversed phase plates by exposure to iodine vapor or on plain or AgNO3-impregnated plates by spraying with an ethanolic solution of p-anisaldehyde and sulfuric acid (15).

The 14C fraction prepared from mycelia harvested from a 1-liter culture of G. fujikuroi was incubated with 2-14C-DL-mevalonate (2 mM; 3.82 x 10-8 cpm per μmole), 3.33 mM ATP, and 1 mM MgCl2 in a total volume of 30 ml. The incubation was conducted at 30° under an N2 atmosphere for 24 hours. At the end of this period 30 ml of acetone was added to stop the enzymic reactions, and the reaction mixture was extracted three times with 15-ml portions of benzene-acetone (3:1). The combined benzene-acetone extracts were reduced in volume and applied to the origin of a preparative silica gel plate which was then developed with benzene-ethyl acetate (3:1). A scan revealed three major zones of radioactivity along the plate, Zone A near the origin, Zone B (alcohols) in the middle of the plate, and Zone C (hydrocarbons) near the solvent front. The gel corresponding to each of these zones was scraped separately from the plate and the radioactive material was eluted, Zone A with acetone, Zone B with ethyl acetate, and Zone C with n-hexane. The radioactive alcohol fractions (Zone B) from 25 incubations carried out in this manner were pooled for further processing.

A AgNO3-impregnated silica gel column was prepared as follows. Powdered silica gel (J. T. Baker Chemical Company) was mixed with 5% of its weight of silver nitrate. The mixture was stirred with sufficient acetonitrile to cover the gel. The solvent was then removed by evaporation under reduced pressure in a rotary evaporator heated to 55°. The dry silver nitrate-silica gel mixture was suspended in n-hexane and packed into a column, 1 x 25 cm. The column was washed with n-hexane-ethyl acetate (4:1) just prior to adding the combined fractions containing the radioactive alcohols. The profile of radioactivity emerging from the column with respect to the fraction number after development with n-hexane-ethyl acetate (4:1) is shown in Fig. 2. Fractions 12 through 28 (3.64 x 106 cpm) were pooled and further chromatographed on a column, 1 x 25 cm, of untreated silica gel developed with benzene-ethyl acetate (19:1). This served primarily to remove any small amounts of silver nitrate carried along from the first column. The fractions comprising the single peak of radioactive material emerging from the latter column (3.38 x 106 cpm) were pooled and evaporated in a stream of N2 to a small volume.

The residual oil was evaporated to dryness from a benzene solution three times to remove traces of water, and finally it was dissolved in 3 ml of benzene. Anhydrous pyridine (0.1 ml) and 4-(4'-nitrophenylazo)benzoyl chloride (50 mg) were added, and the mixture was permitted to stand overnight. The solution was then washed with 2 ml of 0.5 M sulfuric acid and twice with 1 ml of water. The benzene solution was applied to a column, 1 x 10 cm, of silica gel and development was continued with benzene. The first 5-ml portion of eluate was discarded, and the next 25 ml, containing the red-orange product, were collected. The 4-(4'-nitrophenylazo)benzoyl ester was further purified on a preparative silica gel plate, 20 x 20 cm, impregnated with 5% AgNO3 (0.5-mm layer). After development of the plate to 15 cm with n-hexane-benzene (1:1), four red-orange bands could be seen; however, the single radioactive band on the plate corresponded to only one of these (Rf 0.00). The radioactive band was eluted by scraping away the gel from either side and then permitting benzene to flow through the gel until all of the red-orange derivative had passed into a paper wick attached to one end of the remaining gel zone. The derivative was extracted from the paper with several portions of benzene. The solvent
was removed and the product was crystallized from warm carbon tetrachloride solution (8.55 mg; 2.51 × 10⁶ cpm).

The product had a melting point of 136° (uncorrected). It gave a single spot in which radioactivity and color were coincident on an AgNO₃-impregnated silica gel plate developed with n-hexane-benzene (1:1). The position on the plate also coincided with that of the 4-(4'-nitrophenylazo)benzoyl ester of synthetic trans-(-)-labda-8(16),13-dien-15-ol at Rᵢ 0.60 (Fig. 3).

The free alcohol was recovered from a portion of the ester by the hydrolysis procedure of Hecker (9).

**Biosynthesis of Pyrophosphate Esters and Their Purification from G. fujikuroi Extracts**

The Sₘₐₜ fraction derived from mycelia of a 1-liter culture of *G. fujikuroi* was incubated with 2-[¹⁴C]-DL-mevalonate (0.167 mM; 5.4 × 10⁶ cpm per mg), 3.33 mM ATP, and 1 mM MgCl₂ in a total volume of 30 ml at 30° under an atmosphere of N₂. After 1 hour, 1 g of EDTA-4Na was added to stop the enzymatic reaction. The pyrophosphate esters were then purified from the incubation mixture according to the procedure which has been described for geranylgeranyl-PP (3). A profile of the radioactivity eluted from the DEAE-Sephadex A-25 column is shown in Fig. 4. Appropriate pools of fractions (according to radioactivity) were lyophilized to dryness. Analyses of these pooled fractions are presented under “Results.”

**Physical Measurements**

Samples for the determination of optical rotation were weighed on a Cahn Electrobalance and dissolved in 1.00 ml of benzene. Optical rotations were measured in a Perkin-Elmer model 341 spectropolarimeter, with a cell with a 10-cm light path. Nuclear magnetic resonance spectra were measured in a Varian spectrometer, with either a heated inlet or direct insertion of the sample on the probe.

**RESULTS**

**Isolation and Identification of Copalol**—Thin layer chromatographic analyses of the benzene-extractable [¹⁴C]-labeled products formed from 2-[¹⁴C]-DL-mevalonate in cell-free extracts of *G. fujikuroi* revealed three major regions of radioactivity. The region marked A in Fig. 1 appeared to be a mixture of trans-geranylgeraniol and trans-farnesol and [¹⁴C]-geranylgeraniol. The presence of geranylgeraniol in these reaction mixtures was confirmed from the proton nuclear magnetic resonance spectrum of material purified from Fractions 62 to 70. The radioactive material associated with the region marked C in Fig. 1 cochromatographed with authentic kaurene. This identification was confirmed from the proton magnetic resonance spectrum of the [¹⁴C]-hydrocarbon recovered from the larger scale incubations which agreed in all respects with that of authentic (-)-kaurene. The chromatographic behavior of the radioactive material marked as B in Fig. 1 was not identical with any of the [¹⁴C] metabolites formed from [¹⁴C]-mevalonate in either the *E. macrocarpa* endosperm of *R. communis* cell-free extracts previously investigated in conjunction with kaurene synthesis. However, B did cochromatograph with other C₃₀ alcohols in the reversed phase.
system capable of separating the isoprenoid alcohols on the basis of chain length. This information suggested that it was probably a diterpene alcohol. It was further concluded that $B$ probably had fewer ethylenic double bonds than geranylgeraniol since it moved much further in the AgNO$_3$-silica gel system. It seemed possible that $B$ might be the alcohol $II$, and thus related to the predicted intermediate in the cyclization of geranylgeranyl-PP to kaurene. Therefore, the enantiomeric alcohol (+)-labda-8(16),13-dien-15-ol (III) was synthesized from mannoyl (V) for comparison with $B$. The radioactivity of $B$ chromatographed with the slower moving (trans) isomer of III on a silica gel plate developed with benzene-ethyl acetate (9:1) and on a AgNO$_3$-silica gel plate developed with n-hexane-ethyl acetate-isopropyl ether (2:1:1).

Further support for this postulated structure of $B$ came from the observation that incubation of $^{14}$C-geranylgeranyl-PP with the S$_{106}$ fraction of $G$. fujikuroi in the presence of MgCl$_2$ led to the production of $B$ and $C$ components identical in chromato-
graphic properties with those formed from 2-$^{14}$C-mevalonate (Fig. 1) as substrate. As expected, radioactive material re sembling geranylgeraniol, but none resembling (trans)-farnesol, was also present.

In order to establish the structure of this new metabolite conclusively, it seemed desirable to isolate sufficient quantities in a pure state for the determination of its physicochemical properties. Therefore, the isolation of $B$ from the metabolites formed from mevalonate, ATP, and MgCl$_2$ in cell-free extracts prepared from $G$. fujikuroi was undertaken as described under "Experimental Procedure." Table I summarizes the recovery of the metabolite (copalol) during various stages of the purification. The amounts recovered are estimated from the radioactivity on the assumption that 4 moles of mevalonate are incorporated into each mole of a metabolite of molecular formula C$_{30}$H$_{54}$O. The AgNO$_3$-silica gel column (Fig. 2) was crucial in resolving the new metabolite from other radioactive alcohols formed in the incubation mixtures as discussed above. In all subsequent steps of the purification the radioactivity seemed to be associated with a single component. However, the formation of the 4-(4'-nitrophenylazo)benzoyl ester was required to facilitate the resolution of at least three nonradioactive substances from the metabolite.

The yield of purified ester of 8.85 mg corresponds to 4.74 mg (16.3 $\mu$moles) of an alcohol of molecular weight 290 (C$_{30}$H$_{54}$O).

### Table I

**Purification of copalol**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Radioactivity associated with copalol $^{a}$</th>
<th>Amount of copalol*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{cpm} \times 10^{4}$</td>
<td>$\mu$moles</td>
</tr>
<tr>
<td>1. Fraction B (from AgNO$_3$-silica gel column)</td>
<td>3.64</td>
<td>23.6</td>
</tr>
<tr>
<td>2. Silica Gel G column fractions...</td>
<td>3.38</td>
<td>21.9</td>
</tr>
<tr>
<td>3. Crude 4-(4'-nitrophenylazo)benzoyl derivative from silica gel column...</td>
<td>2.86</td>
<td>18.6</td>
</tr>
<tr>
<td>4. Pure 4-(4'-nitrophenylazo)benzoyl derivative from preparative AgNO$_3$-silica gel plate...</td>
<td>2.51</td>
<td>16.3</td>
</tr>
</tbody>
</table>

* Calculated from the radioactivity associated with copalol with the assumption that 4 moles of mevalonate are incorporated into 1 mole of copalol (C$_{30}$H$_{54}$O).

Thus, approximately 2.2% of the available isomer of mevalonate is converted to the purified product. The observed specific activity of the product of 2.84 $\times 10^{5}$ cpm per mg agrees well with the value of 2.92 $\times 10^{5}$ cpm per mg calculated on the assumption that the product consists entirely of the monoester of an alcohol of formula C$_{30}$H$_{54}$O derived from 4 moles of mevalonate of specific activity 3.82 $\times 10^{4}$ cpm per $\mu$mol.

All of the properties of the isolated ester and the alcohol liberated from it strongly support the assignment of Structure II to the alcohol. The melting point of 136° for the ester is in good agreement with that of the ester of III which was found to melt at 135.5°. Fig. 3 illustrates the cochromatography of the isolated $^{14}$C ester and the synthetic ester of III on an AgNO$_3$-silica gel plate. In this case, as well as in several other systems tested, the two could not be distinguished. The specific rotations of the isolated ester were $[\alpha]^{20}_{D} -15.1 \pm 2^{2}$ and $[\alpha]^{23}_{D} -16.7 \pm 2^{2}$, while the ester of III gave values of $[\alpha]^{20}_{D} +14.5 \pm 2^{2}$ and $[\alpha]^{23}_{D} +16.2 \pm 2^{2}$. Thus, these values are consistent with an enantiomeric relationship for the two compounds.

The mass spectrum of the ester of the biosynthetic alcohol measured by direct insertion of the sample showed the expected parent peak at $m/e$ 543 and two additional high field peaks at $m/e$ 525 and 513 corresponding to the loss of one and two methyl groups. A strong peak was also present at $m/e$ 272 which would be expected by the elimination of the allylic ester from Structure II. The mass spectrum of the ester of III measured under similar conditions showed the presence of the same peaks with virtually identical relative peak heights.

The proton nuclear magnetic resonance spectrum of the ester of the biosynthetic alcohol had the following characteristics (with the suggested structural features responsible for them in parentheses): a sharp 3-proton peak at 9.32 $\tau$ (C-20 angular methyl); two sharp 3-proton peaks at 9.21 $\tau$ and 9.16 $\tau$ (C-18 and C-19 gem dimethyl); a sharp 3-proton peak at 8.21 $\tau$ (al-
lysid C-17 methyl); a single proton peak at 5.43 \( \tau \) and a 2-proton doublet at 5.09 \( \tau \) with J value of 7 cps, which overlaps a single proton peak at 5.16 \( \tau \) (the two resonance peaks at 5.43 \( \tau \) and 5.16 \( \tau \) represent the 2 protons of the exocyclic methylene at C-16, and the 2-proton doublet at 5.09 \( \tau \) is assigned to the C-15 hydrogens), a triplet 1-proton peak at 4.60 \( \tau \) with J value of 7 cps (vinyl proton at C-14). The analogous spectrum of the ester of the synthetic alcohol III was identical in all respects.

Hydrolysis of the ester of the biosynthetic alcohol by the procedure of Hecker (11) yielded an oil which cochromatographed with III in several solvent systems. The mass spectra of the two free alcohols were also virtually identical in both positions and relative heights of the peaks. In neither of them was a peak seen at m/e 290 corresponding to a molecular ion. The peak of highest m/e observed was at 272. This peak would be expected to result from the facile elimination of water from aliphatic alcohols such as II or III.

From all of these lines of evidence, the structure of the isolated biosynthetic alcohol is clearly indicated as II. The trivial name copalol is suggested for this substance because of its relationship to the natural product copalic acid which was isolated from the copal of Hymenaea courbaril L. by Nakano and Djerassi (16).

Isolation and Identification of Copalyl Pyrophosphate—The predicted intermediate in the cyclization of geranylgeranyl-PP to kaurene and other cyclic diterpenes is copalyl-PP rather than copalol. It was considered likely that copalol was present in the incubation mixtures as a result of the action of phosphatases on the pyrophosphate ester in the same manner as trans-farnesol and geranylgeraniol were believed to be released from their pyrophosphates. Therefore, an investigation of the presence of phosphorylated forms of copalol produced from 2-\(^{14}\)C-mevalonate in incubation mixtures was undertaken. The procedure used was patterned after the method developed for the isolation of geranylgeranyl-PP from E. macrocarpa endosperm (3). Fig. 4 illustrates the separation of radioactive components achieved on the DEAE-Sephadex A-25 anion exchanger resin column utilized as the final step in the purification. The radioactivity in Fractions 4 and 5, which emerged as the salt gradient was first applied, is due to some unconstrained mevalonate carried through in the collidine extracts. The small amount of radioactivity in Fraction 11 is converted by treatment with alkaline phosphatase to a benzene-extractable form which cochromatographs with trans-farnesol. The radioactivity in Fractions 14 to 18 was also converted by treatment with alkaline phosphatase to a benzene-extractable form. This hydrolysis product behaved as a \( C_{20} \) alcohol in the reversed phase thin layer system. Approximately 85% of the radioactivity migrated with copalol and 15% with trans-geranylgeraniol in the thin layer system with AgNO\(_3\)-silica gel plates. The material in column Fractions 14 to 18 did not serve as a substrate for the formation of diterpene hydrocarbons (see below). On the basis of this information, the radioactive material in these fractions was tentatively identified as a mixture of the monophosphates of copalol and trans-geranylgeraniol.

Treatment of the last radioactive peak to be eluted from the column (Fractions 20 to 22) with alkaline phosphatase also gave rise to benzene-extractable radioactive components which cochromatographed with \( C_{20} \) alcohols in the reversed phase thin layer system. In the AgNO\(_3\)-silica gel system, 97.5% of these radioactive hydrolysis products migrated as copalol, while 1.5% migrated as geranylgeraniol and the other 1.0% remained at the origin of the plate. The radioactive material in Fractions 20 to 22 did serve as a substrate for the formation of diterpene hydrocarbons (see below). Therefore, this final radioactive peak was tentatively identified as a mixture of primarily copalyl-PP with a small amount of geranylgeranyl-PP.

Several experiments of the type just described have been run with the same qualitative results. However, the relative proportions of the radioactive products varied considerably. In some cases derivatives of trans-farnesol predominated, while in others a higher proportion of derivatives of geranylgeraniol were found in the \( C_{20} \) products than in the experiment cited above. Although it has not been tested in a critical way, there seems to be a general correlation between the age of the culture at the time the enzyme extract is prepared and the type of product found. Farnesol derivatives predominated in extracts prepared from seemingly younger cultures, and derivatives of the \( C_{20} \) alcohols in extracts prepared from somewhat older cultures. In a few cases of older cultures, the extracts converted mevalonate primarily to kaurene and accumulated very little if any of the phosphorylated derivatives extracted by the above procedure.

A more conclusive identification of copalyl-PP in these incubation mixtures has been made from the following observations. The radioactive alcohol released by treatment with alkaline phosphatase of tentatively identified \(^{14}\)C-copalyl-PP was purified and converted to its 4-(4'-nitrophenylazo)benzoyl ester. This ester cochromatographed with the 4-(4'-nitrophenylazo)benzoyl ester of the reference alcohol III. Furthermore, a mass spectrum of the ester from copalyl-PP hydrolysis product was identical with that of the ester of copalol isolated directly from the incubation mixture as the free alcohol. This conclusively identified the alcohol portion of the derivative as copalol.

In order to establish the presence of pyrophosphate in the suspected copalyl-PP, a sample of doubly labeled \(^{14}\)C-geranylgeranyl \(^{32}\)P-PP was biosynthesized from \( \gamma^{32}\)P-ATP and 2-\(^{14}\)C-mevalonate in E. macrocarpa endosperm preparations and purified as previously described (3). This doubly labeled material (\(^{14}\)C counts per min/\(^{32}\)P counts per min = 46.0) was then used as a substrate for the formation of copalyl-PP in \( S_{100} \) preparations of G. fujikuroi. The legend of Fig. 5 lists the conditions of the incubation.

![Fig. 4. Chromatography of phosphate esters produced from 2-\(^{14}\)C-mevalonate in G. fujikuroi extracts. The phosphate esters were produced and extracted as described under "Experimental Procedure." The radioactivity in fractions eluted from the DEAE-Sephadex A-25 column (C) is plotted as a function of the fraction number. The dashed line indicates the approximate molarity of \( \text{NH}_4\text{HCO}_3 \) in the eluting solvent as development progressed.](http://www.jbc.org/)
The incubation mixture was extracted with collidine and the phosphorylated derivatives were chromatographed on DEAE-Sephadex A-25 in the usual way. Fig. 5 shows the results of radioassays for $^{32}$P and $^{14}$C in the fractions obtained from this column. The small peak centered in Fractions 32 and 33 contained primarily unreacted substrate. The main peak, centered in Fractions 38 and 39, is believed to be copalyl-PP since (a) it yielded only $^{14}$C-copalol after alkaline phosphatase treatment and (b) it served as a substrate for the formation of kaurene when added to G. fujikuroi extract in the presence of Amo 1618 (see below). The average ratio of $^{14}$C counts per min to $^{32}$P counts per min over the region of this major product was 46.3, in very close agreement with the ratio of 16.0 in the substrate supplied.

Thus, the presence of 2 phosphate residues per copalol is indicated, a finding which almost certainly identifies the metabolite as copalyl-PP.

**Metabolic Role of Copalyl-PP**—The role of copalyl-PP as a precursor of diterpene hydrocarbons has been tested in three different enzyme systems. $^{14}$C-Copalyl-PP derived from 2$^{14}$C-mevalonate in G. fujikuroi extracts and isolated as described in the preceding section was used as the substrate. The results of typical experiments in which it was incubated in the presence of 1 mM MgCl$_2$ with S$_{10}$ extracts from either G. fujikuroi or E. macrocarpa endosperm or R. communis seedlings are presented in Table II. From 75 to 88% of the radioactivity added could be recovered as a hydrocarbon product after a 1-hour incubation in each case. The hydrocarbon formed with the G. fujikuroi and E. macrocarpa enzymes was identified as kaurene by careful co-chromatography with authentic kaurene in a AgNO$_3$-silica gel thin layer system developed with $n$-heptane-benzene (7:3). Kaurene had been previously shown to be the only hydrocarbon formed in these extracts from geranylgeranyl-PP. The diterpene hydrocarbons formed from copalyl-PP in the R. communis extract had the same mobilities in the AgNO$_3$-silica gel system as (+)-stachene, (+)-sandaracopimaradiene, kaurene, and trachylobane. These four hydrocarbons, the so-called C group, are known to be products formed from $^{14}$C-geranylgeranyl-PP metabolism in S$_{10}$ preparations of R. communis. A fifth incompletely identified diterpene hydrocarbon called casbene is also produced from mevalonate or geranylgeranyl-PP; however, there was no evidence for the formation of casbene from copalyl-PP.

It was noted earlier under "Results" that material tentatively identified as a mixture of the monophosphate esters of copalol and geranylgeranyl did not serve as a precursor to kaurene in the G. fujikuroi extracts under the conditions of the experiments in Table II. It was further shown that the free alcohol copalol was not transformed to kaurene or any other hydrocarbon after 3-hour incubations with the fungal extract either in the presence of 1 mM MgCl$_2$ alone or 1 mM MgCl$_2$ plus 2 mM ATP. Thus, the pyrophosphoryl group appears to be necessary for cyclization to proceed.

A second series of incubations of $^{14}$C-copalyl-PP with the various enzyme preparations were conducted in the presence of 0.1 mM Amo 1618, a plant growth retardant. Amo 1618 at this concentration is known to inhibit completely the conversion of geranylgeranyl-PP to kaurene in E. macrocarpa (17) and to the C group of diterpene hydrocarbons in R. communis (4). In the present experiments summarized in Table II, no inhibition by Amo 1618 of the conversion of $^{14}$C-copalyl-PP to the diterpene hydrocarbons was observed. These results point to the step from geranylgeranyl-PP to copalyl-PP as the probable site of inhibition of Amo 1618 in these systems.

A comparison was made of the effects of Amo 1618 and two other plant growth retardants, CCC and Phosfon, on the con-
kaurene produced in a given incubation was greatly favored when ATP and its degradation products were not expected to be re-inhibiting the conversion of copalyl-PP to kaurene even though suspected that the higher levels of ATP might be in some way relative high initial concentrations of ATP were used. It was actants in this step. The experiment described in Table IV was performed to test this proposal. 14C-Geranylgeranyl-PP was incubated with G. fujikuroi extract in the presence of 1 μM ATP and levels of added ATP ranging from 0 to 6.6 mM. It can be seen that the added ATP had little effect on the total conversion of geranylgeranyl-PP to copalol plus kaurene, but it did drastically alter the fraction of copalol in the products of conversion from 18% in the absence of ATP to 90% in the presence of 6.6 mM ATP.

**DISCUSSION**

Structure II is assigned to copalol on the basis of a comparison of its properties with those of the enantiomeric reference compound trans-(-)-labda-8(16):13-dien-15-ol (III), which was synthesized from manool (V). The evidence for this structural assignment was discussed under “Results.” We have chosen to call the compound of Structure II copalol because of its relationship to the naturally occurring copalic acid (IV) (16). The evidence for this structural assignment was discussed under “Results.”

The incubation mixtures contained geranylgeranyl-PP (30,000 cpm), MgCl₂ (1 mM), enzyme extract (1.0 ml of S₁₀₀₀₀ preparation), and growth retardants as indicated. Incubations were for 1 hour at 30°C. At this time alkaline phosphatase (0.01 ml) was added, and the incubations were continued for 10 min before extraction of the products for analysis.

### TABLE IV

**Effect of ATP on conversion of geranylgeranyl-PP to copalol and kaurene in G. fujikuroi extracts**

<table>
<thead>
<tr>
<th>ATP added (mM)</th>
<th>Geranylgeranyl-PP formed (cpm)</th>
<th>Copalol formed (cpm)</th>
<th>Kaurene formed (%)</th>
<th>Total lipids recovered (cpm)</th>
<th>Total conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8,490</td>
<td>3,125</td>
<td>12</td>
<td>14,020</td>
<td>25,635</td>
</tr>
<tr>
<td>1.0</td>
<td>8,900</td>
<td>7,375</td>
<td>27</td>
<td>9,480</td>
<td>25,755</td>
</tr>
<tr>
<td>6.6</td>
<td>12,885</td>
<td>14,532</td>
<td>50</td>
<td>1,900</td>
<td>29,220</td>
</tr>
</tbody>
</table>

* Per cent of the total radioactive lipids recovered present in this form.

**TABLE III**

**Effects of growth retardants on conversions of geranylgeranyl-PP and copalyl-PP to kaurene in extracts of G. fujikuroi**

The incubation mixtures contained geranylgeranyl-PP (13,000 cpm) or copalyl-PP (10,000 cpm), MgCl₂ (1 mM), enzyme extract (1.0 ml of S₁₀₀₀₀ preparation), and growth retardants as indicated. Incubations were for 1 hour at 30°C. At this time alkaline phosphatase (0.01 ml) was added, and the incubations were continued for 10 min before extraction of the products for analysis.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Addition</th>
<th>Geranylgeranyl-PP formed (cpm)</th>
<th>Copalol formed (cpm)</th>
<th>Kaurene formed (%)</th>
<th>Total lipids recovered (cpm)</th>
<th>Total conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geranylgeranyl-PP</td>
<td>None</td>
<td>4,866</td>
<td>820</td>
<td>6.5</td>
<td>7,026</td>
<td>55.5</td>
</tr>
<tr>
<td>Geranylgeranyl-PP</td>
<td>Amo 1618, 1 mM</td>
<td>11,830</td>
<td>125</td>
<td>1.1</td>
<td>32</td>
<td>0.3</td>
</tr>
<tr>
<td>Geranylgeranyl-PP</td>
<td>Phosfon, 1 mM</td>
<td>8,076</td>
<td>10</td>
<td>0.1</td>
<td>27</td>
<td>0.3</td>
</tr>
<tr>
<td>Geranylgeranyl-PP</td>
<td>CCC, 1 mM</td>
<td>11,732</td>
<td>140</td>
<td>97.5</td>
<td>190</td>
<td>1.5</td>
</tr>
<tr>
<td>Copalyl-PP</td>
<td>None</td>
<td>6,406</td>
<td>3,651</td>
<td>36</td>
<td>10,117</td>
<td>36</td>
</tr>
<tr>
<td>Copalyl-PP</td>
<td>Amo 1618, 1 mM</td>
<td>6,916</td>
<td>2,724</td>
<td>28</td>
<td>9,640</td>
<td>28</td>
</tr>
<tr>
<td>Copalyl-PP</td>
<td>Phosfon, 1 mM</td>
<td>3,733</td>
<td>141</td>
<td>4</td>
<td>3,874</td>
<td>141</td>
</tr>
<tr>
<td>Copalyl-PP</td>
<td>CCC, 1 mM</td>
<td>6,584</td>
<td>2,642</td>
<td>28</td>
<td>9,526</td>
<td>28</td>
</tr>
</tbody>
</table>

* Per cent of the total radioactive lipids recovered present in this form.

+ Conversion to copalol and kaurene.
pyrophosphate ester to (-)-kaurene in both G. fujikuroi and E. macrocarpa endosperm extracts. In R. communis seedling extracts, (-)-sandaracopimaradiene, (+)-stachene, and trachylobane in addition to (-)-kaurene are formed from copalyl-PP. The intermediary role of copalyl-PP in these biosyntheses can be visualized as shown in Fig. 6. Ruzicka (7) and Wenkert (8) predicted on theoretical grounds the involvement of a bicyclic intermediate of this general type in the biosynthesis of polycyclic diterpenes. Birch (19), Dev (20), and Upper and West (2) have discussed this possibility more specifically in relation to the polycyclic diterpenes considered in the present work. In general terms, it has been suggested that the first stage of cyclization involves an over-all proton addition to the terminal double bond at C-14 of geranylgeranyl-PP, a series of electronic shifts from the allylic pyrophosphate group to (-)-kaurene and its enantiomer. Hansen has presented evidence, for example, that the alcohol corresponding to this pyrophosphate is converted to rosenonolactone (VI) when fed to cultures of Trichoderma roseum (21). Thus, it would appear that two different modes of enzymic cyclization of geranylgeranyl-PP must be involved in this case.

It seems likely that copalyl-PP is the precursor not only of the diterpenes discussed here, but also of the large family of diterpenes which have A and B rings with the same sterical arrangement of substituents at the A/B ring fusion and at C-9 as copalol. Certain additional diterpenes with modified carbon skeletons can also be visualized as arising from copalyl-PP by further cyclization coupled with substituent migrations. Among the diterpenes frequently encountered are also another group which have the opposite absolute stereochemical arrangement of substituents at the A/B ring fusion and at C-9. This group is most likely derived by cyclizations or other modifications of these bicyclic diterpenol pyrophosphates. Kaurene has been shown to be a precursor of gibberellin acid in G. fujikuroi cultures (1). Thus, the sequence geranylgeranyl-PP to copalyl-PP to kaurene must be a part of the gibberellin biosynthesis pathway in the fungus and other gibberellin-synthesizing systems such as the endosperm of E. macrocarpa. Kende et al. showed that the plant growth retardants Amo 1618 or CCC were capable of blocking the production of gibberellic acid when...
added to the culture medium of \textit{G. fujikuroi} (22, 23). Phosfon, another plant growth retardant tested, was not effective, but evidence was presented that this was due to rapid destruction of Phosfon by the fungus. Subsequently, Dennis, Upper, and West (17) found both Amo 1618 and Phosfon, but not CCC, to be effective inhibitors of the enzymic conversion of geranylgeranyl-PP to kaurene in \textit{E. macrocarpa} endosperm extracts. It was suggested that the observed inhibition of this reaction and the resultant reduction of gibberellin biosynthesis might explain the physiological actions of the growth retardants. The results of Table III show that all three retardants, Amo 1618, CCC, and Phosfon, at 1 mM concentration block the formation of kaurene from geranylgeranyl-PP in \textit{G. fujikuroi} extracts. Furthermore, it can be seen that neither Amo 1618 nor CCC at this relatively high concentration has appreciable inhibitory activity on the transformation of copalyl-PP to kaurene. Thus, an inhibitory site of action of Amo 1618 and CCC in the fungal extracts is more precisely located at the step leading from geranylgeranyl-PP to copalyl-PP. The same site of inhibition of Amo 1618 was also shown in both \textit{E. macrocarpa} endosperm and \textit{R. communis} seedling extracts. Phosfon is less specific in its action in the fungal extracts since it blocks both cyclization steps. It is interesting that Phosfon is inhibitory in the cell-free extract since its addition to the whole culture did not block gibberellic acid production. This is consistent with the suggestion that the lack of activity is due to rapid degradation of Phosfon by the whole culture rather than an inherent inactivity of Phosfon in the gibberellin biosynthesis pathway of \textit{G. fujikuroi}.

The mechanism of the effect of high concentrations of ATP in the incubation mixture leading to the accumulation of copalol at the expense of kaurene (see Table IV) is not understood. It may be a direct effect of ATP or one of its metabolites on the enzymic catalysis of the copalyl-PP to kaurene transformation. Alternatively, ATP may act indirectly. For example, the divalent metal ion concentration may be lowered by the sequestering action of ATP to one which permits the first step of cyclization to proceed at an unchanged rate while the second step is inhibited. Whatever the explanation, the utilization of higher levels of ATP had the important practical consequence of increasing the yield of copalol formed from mevalonate in the experiments whose objective was the isolation of copalol for structural investigations.

\textbf{Acknowledgment—}We wish to acknowledge the generous gift of γ-3P-ATP from Dr. Claus Leitzmann.

\textbf{REFERENCES}
