Biosynthesis of Cyclic Diterpenes in Extracts from Seedlings of *Ricinus communis* L. I. Identification of Diterpene Hydrocarbons Formed from Mevalonate*

David R. Robinson† and Charles A. West

ABSTRACT: Soluble enzyme preparations from 60-hr-old seedlings of castor bean (*Ricinus communis* L.) convert mevalonic acid in the presence of adenosine triphosphate into a mixture of hydrocarbons and more polar materials. The hydrocarbon fraction consists of at least five diterpenes along with small amounts of a material tentatively identified as squalene. Milligram quantities of the five diterpene hydrocarbons biosynthesized from mevalonate in this system were purified for characterization studies. Three of these substances were identified as (+)-beyerene (I), (+)-sandaracopimaradiene (II), and (−)-kaurene (III) from their spectral and other physical properties in comparison with those of authentic reference compounds. A fourth substance, which was not isolated in a chemically pure state, was tentatively identified as trachylobane of uncertain configuration from a comparison of its chemical and chromatographic properties with those of a sample of authentic (−)-trachylobane (IV). A structure based on spectral properties is proposed for the fifth product casbene, which appears to be structurally related to the naturally occurring hydrocarbon cembrene (V).

Many types of cyclic diterpenes have been isolated and characterized structurally from various plant sources, especially from the resinous exudates and inclusions of the *Coniferae* (McCrindle and Overton, 1965). These compounds occur occasionally as hydrocarbons, but more frequently they are oxidized and sometimes conjugated with other residues. A number of cyclic diterpene alkaloids are known. For the most part the functional role of diterpenes in the development of the plants which produce them is unknown. However, (−)-kaurene (III) has been shown to be a normal precursor of the plant growth regulating gibberellins (Cross *et al.*, 1964) which may be regarded themselves as diterpenes or diterpenoid compounds.

The mode of biosynthesis of diterpenes has been the subject of considerable speculation but relatively little experimental investigation. Studies of the metabolic pathways involved in diterpene synthesis in cell-free systems have been limited to a very few cases. (−)-Kaurene is synthesized from mevalonate and *trans*-geranylgeranyl pyrophosphate in soluble, cell-free preparations of endosperm tissue from developing seeds of *Echinocystis macrocarpa* (Graebe *et al.*, 1965; Upper and West, 1967). In this case (−)-kaurene was the only diterpene hydrocarbon detected. The particulate fraction from the same tissue catalyzed the further conversion of (−)-kaurene into a number of oxidized derivatives (Dennis and West, 1967). The synthesis of kaurene from mevalonate has also been reported in extracts from immature seeds of *Pisum sativum* (Anderson and Moore, 1967), and *Cucurbita pepo* (Graebe, 1969).

Investigations initially aimed at examining extracts of 2–3-day-old castor bean seedlings (*Ricinus communis* L.) for their capacity to synthesize (−)-kaurene from mevalonate soon led to the realization that at least five diterpene hydrocarbons were formed in this tissue. This presented an opportunity for the study of the biosynthesis of a number of apparently related diterpenes in extracts from one organism, and if the compounds were cyclic, as seemed probable, the system would be of potential value for the direct testing of many of the specific proposals put forward by Ruzicka and by Wenkert (Ruzicka *et al.*, 1953; Wenkert, 1955). Furthermore, it was felt that this system from the germination stage of plant growth could be important in discovering the biological functions of diterpenes. This paper presents the evidence for the identification of four of the products of the castor bean seedling system as (+)-beyerene (I), (+)-sandaracopimaradiene (II), (−)-kaurene (III), and trachylobane (IV) (see Chart I). A tentative structure is proposed for a fifth product which has been assigned the trivial name of casbenes. Some of the characteristics of the enzymes responsible for the formation of these diterpenes are described in a second paper (Robinson and West, 1970).

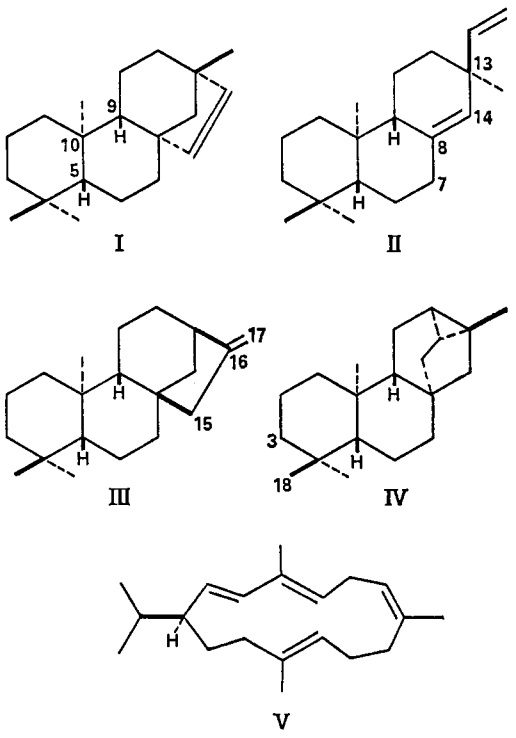
Materials

Seeds of *R. communis* were the genetically dwarf hybrids 66 and 33 and were gifts of the Baker Castor Oil Co., Plainview, Texas. Insoluble polyvinylpyrrolidone ("Polyclar AT") was obtained from General Aniline and Film Corp. (*RS*)-2-[^{14}C]Mevalonic acid as the *N*,*N*′-dibenzylethylenediamine salt was obtained from Volk Radiochemical Co. (2.82 μCi/μmole of acid) and from New England Nuclear Corp. (3.28

* From the Division of Biochemistry, Department of Chemistry, University of California, Los Angeles, California 90024. *Received July 23, 1969.* The investigation was supported by U. S. Public Health Service Grant GM-07065. This is a part of the work described in a dissertation submitted by D. R. Robinson in partial satisfaction of the requirements for the degree of Doctor of Philosophy, University of California, Los Angeles, California, 1967. Preliminary communications of part of the work have been made: Robinson and West (1967) and West *et al.* (1967).

† Present address: Milstead Laboratory of Chemical Enzymology, Shell Research Limited, Sittingbourne, Kent, England.

CHART I



I (+)-Beyerene
II (+)-Sandaracopimaradiene
III (−)-Kaurene
IV (−)-Trachylobane
V (+)-Cembrene

TABLE I: Relative Mobilities of Authentic Diterpene Hydrocarbons on Silver Nitrate–Silica Gel Thin-Layer Chromatograms.

Compounds[a]	$R_{kaurene}$[b]
Kaurane	1.22
Trachylobane	1.22
Kaurene	1.00
Phyllocladene	1.00
Pimaradiene	0.96
Atiserene	0.91
Isopimaradiene	0.88
Sandaracopimaradiene	0.83
Isophyllocladene	0.69
Isokaurene	0.41
Beyerene	0.40
Isoatiserene	0.31

[a] Kaurene and isokaurene were made from kaurene. Atiserene and isoatiserene were derived from trachylobane. [b] Mobility relative to kaurene on 3% silver nitrate impregnated silica gel thin layers developed with *n*-hexane–benzene (7:3). The R_F of kaurene varied between 0.65 and 0.75.

μCi/μmole of acid). Silica gel G for thin-layer chromatography was obtained from E. Merck, A. G., Darmstadt, through Brinkmann Instruments, and silicic acid for lipid chromatography, Bio-Sil HA, minus 325 mesh, from Calbiochem.

We thank the following investigators for their generous gifts of authentic diterpene hydrocarbons: Dr. O. E. Edwards, National Research Council, Ottawa, Canada, for pimaradiene, isopimaradiene, and sandaracopimaradiene; Dr. L. H. Briggs, University of Auckland, New Zealand, for phyllocladene, isophyllocladene, and isokaurene; Dr. S. Dev, National Chemical Laboratory, Poona, India, for beyerene; Dr. G. Ourisson, Université de Strasbourg, France, for trachylobane; Dr. B. E. Cross, Imperial Chemical Industries, Welwyn, England, for kaurene. Where necessary, the hydrocarbons were purified before use by silver nitrate impregnated silica gel thin-layer chromatography.

Methods

Enzyme Preparation. Castor bean seeds were freed from their coats and germinated between moist cheesecloth in darkness at 30–32° for 60–72 hr. Chilled, whole seedlings were mixed with the extraction medium consisting of (1) 50 mM Tris plus 50 mM $KHCO_3$ (pH 7.3), containing 10 mM 2-mercaptoethanol, at 1.5–2.0 ml/g of fresh weight of seedlings, plus (2) insoluble polyvinylpyrrolidone at 0.2 g/g of fresh weight of seedlings. The mixture, of up to 70 ml of total volume, was then homogenized for 2 min with a Virtis 23 homogenizer operating at three-quarters' maximum line voltage. The homogenate was pressed through several layers of cheesecloth and the filtrate was centrifuged at 12,000*g* for 5 min. The floating lipid layer was removed and the supernatant solution was further centrifuged either at 78,000*g* for 2 hr or at 105,000*g* for 1 hr (Spinco Model L ultracentrifuge). These final supernatant fractions had a pH of 6.7–6.9 and contained 10–15 mg of protein/ml as measured by the Folin–Ciocalteu method (Layne, 1957). They served as a source of enzymes for the metabolism of both [^{14}C]mevalonate and [^{14}C]geranylgeranyl pyrophosphate to diterpene hydrocarbons.

Thin-Layer Chromatography. Fractionations of the lipophilic radioactive products from small-scale incubations of the enzyme preparation with 2-[^{14}C]mevalonic acid were achieved by chromatography on thin layers of silica gel and silica gel impregnated with silver nitrate. Glass plates (5 × 20 cm) were coated to 0.25 mm with slurries of silica gel G and water or a solution of silver nitrate (at 3% of the weight of the silica gel) in water. The layers were dried at 120° and stored over desiccant.

Materials on the plates were detected by heating after treatment of the plates with 10% phosphomolybdic acid in methanol, or, alternatively, where recovery of materials was required, by iodine staining for plain silica gel plates and by fluorescence under ultraviolet light after treatment with a solution of 0.1%, 2′,7′-dichlorofluorescein in methanol for silver nitrate impregnated plates.

Table I illustrates the usefulness of silver nitrate–silica gel thin-layer chromatography for the separation of diterpene hydrocarbons.

Detection and Measurement of Radioactivity. The location

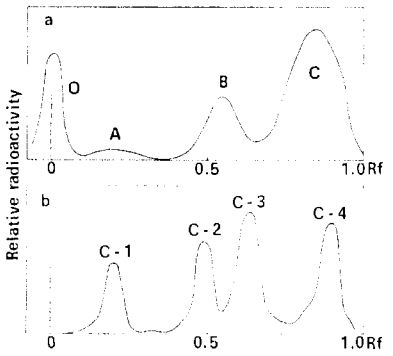


FIGURE 1: Thin-layer fractionation of the benzene-soluble radioactive materials formed from mevalonic acid. A standard enzyme preparation (0.90 ml) was incubated for 2 hr at 30° with $MgCl_2$, 1 μmole; $MnCl_2$, 1 μmole; ATP (neutralized), 2 μmoles; (*RS*)-2-[^{14}C]-mevalonic acid (as the dibenzylethylenediamine salt), 48 nmoles, 2.0×10^5 dpm; and water to 1.00 ml. The reaction was terminated by heating at 100° for 3 min and the precipitated material after sedimenting by centrifugation was extracted with acetone. The acetone was diluted with water and the mixture was extracted with benzene. Typically, under these conditions 20–30 nmoles of mevalonate was converted into benzene-soluble radioactive materials. The concentrated benzene extract was chromatographed on a silica gel plate developed in *n*-hexane, after which the plate was scanned (a). Fraction C so obtained was eluted from the silica gel with hexane and rechromatographed on a 3% silver nitrate–silica gel plate developed with *n*-hexane–benzene (7:3), and the plate was scanned (b).

of radioactive materials on thin-layer chromatograms was determined by means of a Vanguard Autoscanner 880. Materials adsorbed to silica gel or silver nitrate–silica gel were assayed quantitatively for radioactivity either by transfer of an aliquot of an eluate or by direct transfer of the silica gel to 10 or 15 ml of liquid scintillation solution (4 g of 2,5-diphenyloxazole and 50 mg of *p*-bis-2′-[5′-phenyloxazolyl]-benzene/l. of toluene). The presence of small amounts of silica gel did not affect the counting efficiency for hydrocarbons; for more polar compounds the efficiency was reduced by 10–15%. For the assay of radioactive materials in solution (usually in benzene or hexane), 0.2–0.4-ml aliquots were added directly to the scintillation solution. Carbon-14 disintegrations were measured at an efficiency of 76% with a Nuclear-Chicago 720 liquid scintillation spectrometer.

Catalytic Hydrogenation. Reaction mixtures under an atmosphere of hydrogen at 30–35 lb/in.^{2} were heated at 40–50° and stirred continually. For readily hydrogenatable compounds such as kaurene the solvent was cyclohexane with 5% palladium on charcoal as catalyst and reaction times were from 15 min to 2 hr. The saturated product was recovered by chromatography of the reaction mixture over 0.3 g of 5% silver nitrate impregnated silicic acid and elution with cyclohexane. Where compounds of unknown structure were hydrogenated, the reaction mixture was chromatographed over plain silicic acid, and the eluate was examined by silver nitrate–silica gel thin-layer chromatography in order that partially hydrogenated compounds might be detected. Kaurene reacted quantitatively under these conditions and the product, 16,17-dihydrokaurene (kaurane), was crystallized from ethanol and characterized by its melting point and its nuclear magnetic resonance and mass spectra.

More vigorous conditions of hydrogenation were achieved by using platinum dioxide catalyst in acetic acid with reaction times of from 2 to 24 hr. Products were extracted with benzene from the acetic acid diluted with water and were analyzed by silver nitrate–silica gel thin-layer chromatography.

Preparation of Isokaurene from Kaurene. Kaurene, 20 mg dissolved in 1.5 ml of glacial acetic acid, was heated under reflux for 90 min (Briggs *et al.*, 1950). The benzene-extractable reaction products were chromatographed on a preparative silver nitrate–silica gel layer developed with *n*-hexane–benzene (1:1) and the slower moving component was eluted and crystallized from ethanol. It was characterized as isokaurene (Δ-15 isomer of III) by nuclear magnetic resonance and mass spectrometry and by comparison with an authentic sample. Yields were routinely between 50 and 75%.

Formation of Cyclopropyl Derivatives of Kaurene and Isokaurene. The method used was based on that of Simmons and Smith (1959) for the addition of methylene to double bonds; the zinc–copper couple was purified just before use by the method of Shank and Shechter (1959). Products were purified by silver nitrate–silica gel chromatography with *n*-hexane and crystallized by evaporation from hexane–ethanol (1:1). The yields of 16,17- and 15,16-methylenekaurane (characterized by nuclear magnetic resonance and mass spectrometry) were approximately 10%.

Determination of Physical Parameters. Melting points were taken with a Fisher–Johns melting point apparatus; they were not corrected. Nuclear magnetic resonance spectra were determined with a 60-Mc Varian A60 spectrometer coupled with a Varian C-1024 time-averaging computer where appropriate. Samples were dissolved in 10–25 μl of carbon tetrachloride and inserted into capillary tubing. Tetramethylsilane was included as the internal reference. All chemical shifts are reported in terms of δ (tetramethylsilane = 0 ppm). Infrared spectra were determined in carbon disulfide or carbon tetrachloride solution with a Perkin–Elmer 421 grating spectrophotometer. Mass spectra were obtained with an Associated Electrical Industries MS-9 mass spectrometer employing both heated inlet and direct insertion. Optical rotations were determined in chloroform at the sodium D line with a Perkin–Elmer 141 polarimeter. Samples were at room temperature, about 25°, and were held in a 10-cm cell of 1-ml capacity. Optical densities of aqueous solutions were measured with a Hitachi Perkin–Elmer 139 spectrophotometer.

Results

Fractionation of the Lipophilic Reaction Products. Chromatography of the concentrated benzene extract from reaction mixtures in which 2-[^{14}C]mevalonate served as the substrate on plain silica gel developed with *n*-hexane typically led to the separation of three radioactive hydrocarbon fractions, A, B, and C, from more polar radioactive materials, designated fraction 0, as shown in a radioactivity scan in Figure 1a. Further chromatography of fraction 0 revealed that it consisted of many radioactive components; however, most of the radioactivity was associated with two components which were tentatively identified as *trans*-geranylgeraniol and *trans*-farnesol on the basis of cochromatography on plain silica gel developed with benzene–ethyl acetate (9:1).

Fraction A was always relatively small. It was found to cochromatograph with squalene on plain silica gel developed with *n*-hexane and on silver nitrate–silica gel developed with

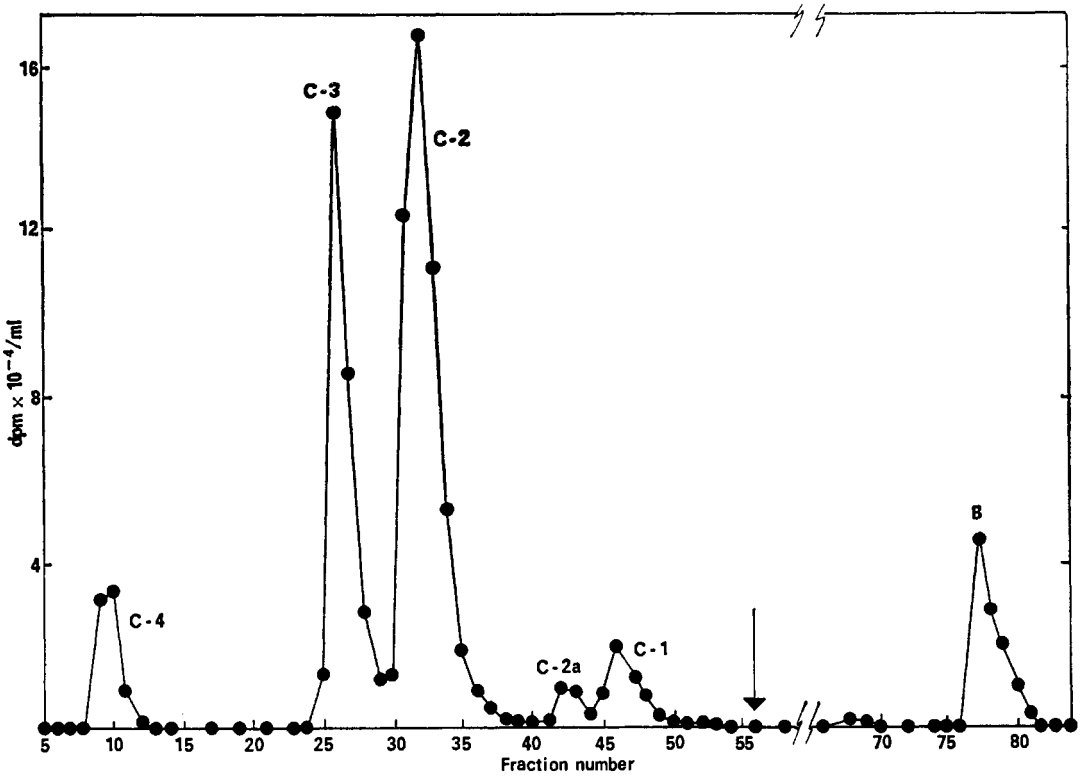


FIGURE 2: Radioactivity elution profile from the chromatography of the hydrocarbons obtained from the large-scale incubation. A solution of the radioactive hydrocarbons in *n*-hexane was applied to a column of 5% silver nitrate impregnated silicic acid which was then developed with a nonlinear gradient of increasing concentrations of benzene in *n*-hexane. At the point marked by the arrow (fraction 56) the benzene was replaced by ethyl acetate. Assignments of the peaks were made on the basis of subsequent silver nitrate–silica gel thin-layer chromatography.

benzene–ethyl acetate (9:1). Consistent with the tentative identification of this component as squalene are the observation of its formation from mevalonate in large amounts (to the almost total exclusion of fractions B and C) in particulate 1000*g* supernatant enzyme preparations, and the later finding that geranylgeranyl pyrophosphate does not serve as a precursor for fraction A although it is converted into fractions B and C.

Fraction B remained as a single radioactive peak upon rechromatography on silver nitrate–silica gel layers developed with 1-propanol–*n*-hexane (2:23), R_F 0.50; diethyl ether–benzene–*n*-hexane (1:1:3), R_F 0.65; and on tetradecane-coated silica gel developed with acetonitrile–1-butanol (1:1), R_F 0.50.

Fraction C was resolved into four radioactive components, C-1, C-2, C-3, and C-4, in order of increasing mobility, by rechromatography on silver nitrate–silica gel developed with *n*-hexane–benzene (7:3) (Figure 1b).

Large-Scale Biosynthesis of Hydrocarbons. Several observations suggested that the B and C hydrocarbons were diterpenes; *e.g.*, the properties on thin-layer and gas–liquid partition chromatography were similar to known diterpene hydrocarbons and the synthesis of the C hydrocarbons was inhibited by the growth retardant Amo 1618 [2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine-1-carboxylate], a compound which prevents the formation of kaurene from both mevalonate and geranylgeranyl pyrophosphate (Dennis *et al.*, 1965). Furthermore, there were early indications that all of the hydrocarbons could be formed from geranylgeranyl pyrophosphate itself.

Proper identification of the hydrocarbons required larger quantities than could be obtained from standard mevalonate incubations. Direct extraction of the hydrocarbons from germinated seeds proved to be impractical and, therefore, biosynthesis on a large scale was undertaken.

Seedlings (10,000) of Baker hybrid 66 (7.75 kg) were processed as described under Methods to yield 7.5 l. of 78,000*g* supernatant material. Ten 800-ml portions of this enzyme extract were incubated with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ATP, and 285 μM (*RS*)-2-[^{14}C]mevalonic acid (total mevalonate 2.28 mmoles, 38.2 × 10^6 dpm) at 30° for 10–14 hr, a period of time shown in pilot experiments to result in maximum yields of hydrocarbons with enzyme preparations supplied with excess mevalonate. At the conclusion of incubation, each batch was swirled in a boiling-water bath for 10 min. The resulting precipitate was sedimented at 2000*g* and the supernatant fraction was discarded. The precipitate was extracted five times with a total of 1 l. of acetone. The acetone extract was then thoroughly mixed with 1.6 l. of benzene and 0.5 l. of water. The lower aqueous phase was separated and further extracted with 0.5 l. of benzene. The combined organic phases from each batch were then washed with water and concentrated under reduced pressure. Chromatography and radioassay of aliquots of the extracts showed that the ten incubation mixtures had yielded a total of 8.0 × 10^6 dpm of polar materials and 6.7 × 10^6 dpm of hydrocarbons. This repre-

TABLE II: Yields, Melting Points, Molecular Weights and Specific Optical Rotations of the Isolated Hydrocarbons.

Component	mg[a]	Mp (°C)	Mol Wt[b]	$[\alpha]_D^{25}$ (deg)
B	3.2	−20 (approximately)	272	−50
C-1	1.5	(Oil)	272	+40
C-2	9.7	43	272	+14
C-2a	0.5			
C-3	8.6	49–51	272	−75
C-4	1.2			

[a] Estimated from radioactivity measurements. [b] Determined by mass spectrometry.

TABLE III: Bioassay of the Hydrocarbon Components on the Dwarf-5 Mutant of *Zea mays*.[a]

Test Sample	μg/Plant[b]	Number of Plants	Average Length of 2nd + 3rd Leaf Sheaths (mm)
Control		10	47
(−)-Kaurene	50	10	77
B	30	3	33
C-1	50	3	50
C-2	50	5	50
C-3	50	8	75
C-4	50	5	43

[a] Test compounds were applied in 0.01-ml volumes of acetone to the opened first leaf blade of 5-day-old seedlings. Control seedlings were treated with acetone alone. After 7 days further growth under continuous light the sheaths of the second and third leaves were measured. [b] The amounts of B and the C components applied were estimated from radioactivity measurements. Component C-4 was not chemically pure.

sented a conversion of 402 μmoles or 35% of the (*R*)-mevalonate supplied and was equivalent to 27 mg of diterpene hydrocarbon product.

The concentrated benzene extracts were combined and mixed with 20 g of silicic acid and the solvent was then removed by vacuum evaporation. The resulting dry powder was washed with 200 ml of *n*-hexane. The hexane eluate, containing 13.2×10^6 dpm, was concentrated to 3.0 ml and applied to a 27×1.7 cm column of silicic acid (30 g) prewashed with *n*-hexane. The column was eluted with 200 ml of *n*-hexane; 7.2×10^6 dpm was detected in the eluate between 40 and 60 ml, and additional radioactivity, 0.13×10^6 dpm, was eluted between 60 and 70 ml. However, this latter radioactivity coincided with a bright yellow pigment, presumably a cartoenoid, and was therefore not further processed. No further radioactivity was eluted by *n*-hexane.

The 40–60 ml of *n*-hexane eluate was concentrated to 3.0 ml and applied to a column similar to the one above except for the utilization of 5% by weight of silver nitrate impregnated silicic acid. The adsorbent was prewashed successively with *n*-hexane–ethyl acetate (3:1), *n*-hexane–benzene (1:1), and *n*-hexane. Elution of the column was begun by running a mixture of *n*-hexane–benzene (1:4) into 1 l. of *n*-hexane held in a constant volume mixing chamber (Hirsch and Ahrens, 1958). Fractions (5 or 10 ml) were collected at a flow rate of 1 ml/min. After 430 ml of effluent had been collected, the solvent in the upper reservoir was replaced by ethyl acetate and elution was continued. A 0.1-ml aliquot of each fraction was assayed for radioactivity and the amount of radioactivity per milliliter was plotted against the fraction number (Figure 2). Fractions comprising the major peaks were pooled on the basis of qualitative assays of the column fractions by silver nitrate–silica gel thin-layer chromatography. The peaks designated C-2 and C-2a had similar chromatographic mobilities in the standard system. The samples, all yellow oils after concentration, were further purified on 1-mm preparative silica gel layers developed with *n*-hexane. After location by brief exposure of the plates to iodine vapor and by scanning for radioactivity, the materials were eluted with *n*-hexane. Evaporation of solvent yielded colorless oils. Components C-2 and C-3 were readily crystallized by evaporation from ethanol solution. Each component gave a single coincident spot on silver nitrate–silica gel thin-layer chromatography. Component C-4, however, consisted of about 0.1 ml of oil on concentration indicating severe contamination with other nonradioactive hydrocarbons. The preparative thin-layer chromatography of component C-2a resulted in conversion of more than 90% of the radioactivity into polar materials. Therefore no attempt was made to characterize this product.

Table II gives the recoveries and, where obtained, the melting points, molecular weights, and specific optical rotations of the hydrocarbons.

Hydrocarbon Identification. COMPONENT C-3. Several lines of evidence suggested the identity of component C-3 with kaurene (III), even before the more extensive examination of the material from the large-scale biosynthesis. Cochromatography of the radioactivity of C-3 with authentic kaurene was observed on thin layers of silica gel developed with *n*-hexane and silver nitrate–silica gel developed with *n*-hexane–benzene (7:3). In addition, the specific radioactivity of a mixture of C-3 with authentic kaurene did not change significantly through five successive crystallizations from ethanol.

The sample of C-3 from the large-scale synthesis melted at 49–51° and had a specific rotation of −75° as compared with values of 51 and −72°, respectively, reported for (−)-kaurene (Briggs *et al.*, 1963). The same mass spectral degradation patterns were obtained for C-3 and kaurene. The proton nuclear magnetic resonance spectrum of C-3 also exhibited the same chemical shifts as that of kaurene (major features included three quaternary methyl groups at 0.81, 0.85, and 1.02 ppm, and two olefinic protons centered at 4.70 ppm). Finally, the infrared spectra of C-3 and kaurene were found to be identical. Component C-3 was also assayed for its gibberellin-like biological activity on the dwarf-5 mutant of *Zea mays* and was found to possess activity equivalent to that of (−)-kaurene (Phinney *et al.*, 1964) (Table III).

COMPONENT C-1. The mobility of component C-1 on thin layers was found to be very similar to that of isokaurene (Δ-15 isomer of III). For example, cochromatography occurred on silica gel developed with *n*-hexane (R_F 0.33) and on tetradecane-coated silica gel developed with 1-butanol–acetonitrile (1:1) (R_F 0.34). However, in spite of this similarity, C-1 was shown not to be isokaurene from the determination of the specific radioactivity of isokaurene samples obtained from successive recrystallizations in the presence of radioactive C-1. Most of the radioactivity was lost from the isokaurene after three crystallizations.

The nuclear magnetic resonance spectrum of component C-1 provided the most structural information and the following assignments of the principal features were made: four quaternary methyl groups at 0.73, 0.82, 0.86, and 0.98 ppm; two olefinic protons as an AB quartet centered at 5.51 ppm (J = 6 cps). These chemical shifts were the same as those reported in the literature for beyerene (I) (also known as hibaene or stachene) (*e.g.*, Kapadi and Dev, 1964). Direct comparisons were made possible after the donation of a sample of authentic (+)-beyerene by Dr. S. Dev. Both the nuclear magnetic resonance spectrum and the mass spectral degradation pattern of beyerene were identical with those of component C-1. The specific optical rotation of C-1 was measured as +40° and that of the beyerene sample as +35°. Cochromatography in the different systems was also completely consistent and it was concluded that component C-1 was identical with (+)-beyerene (I).

COMPONENT C-2. The molecular weight of component C-2 by mass spectrometry was 272, consistent with an empirical formula of $C_{20}H_{32}$. With this information, the following tentative assignments in the nuclear magnetic resonance spectrum were made: four quaternary methyl groups at 0.80, 0.84, 0.86, and 1.01 ppm; one olefinic proton, unsplit at 5.15 ppm; three olefinic protons exhibiting complex splitting between 4.66 and 6.00 ppm; two allylic methylene protons at 2.16 ppm, and one at 2.00 ppm; and approximately 13 nonallylic methylene protons. It was noted that these data were consistent with the pimaradiene (13-epimer of II) or sandaracopimaradiene (II) structures. The suspicion was strengthened by literature reports of the methyl group shifts of the pimaradienes (*e.g.*, Wenkert *eta l.*, 1965) and confirmed by the identities of the mass spectral degradation patterns of C-2 with the published patterns of pimaradiene (Wenkert *et al.*, 1965) and sandaracopimaradiene (*e.g.*, Audier *et al.*, 1966). In addition, it was found that partial catalytic hydrogenation of C-2 to a product of mol wt 274 occurred under mild conditions with palladium–charcoal catalyst, whereas complete hydrogenation required catalysis by platinum dioxide in acetic acid. These characteristics have also reported for the pimaric acids (*e.g.*, Edwards and Howe, 1959).

The availability of samples of pimaradiene, sandaracopimaradiene and isopimaradiene (Δ-7 isomer of II) from Dr. O. E. Edwards enabled direct comparisons to be made. As expected, the mass spectral degradation patterns of pimaradiene and sandaracopimaradiene were identical with the pattern of C-2. The nuclear magnetic resonance and infrared spectra of C-2 were identical with the spectra of sandaracopimaradiene and were clearly different from the corresponding spectra of pimaradiene and isopimaradiene. Further, the melting point of C-2 was 43° and the specific optical rotation +14° as compared with values of 38 and −12° reported for (−)-sandaracopimaradiene (Johnson *et al.*, 1966) and 24–27 and +100° for (+)-pimaradiene (Erdtman and Westfelt, 1963). Thus, component C-2 was concluded to be (+)-sandaracopimaradiene (II). This is the first report of the natural occurrence of the (+)-enantiomer.

COMPONENT C-4. The large-scale synthesis did not result in a sample of sufficient purity for the physical properties of component C-4 to be determined. The material of interest, estimated to be approximately 1 mg from radioactivity measurements, could not be separated from the contaminating oil either by thin-layer chromatography or by crystallization from ethanol.

Component C-4 migrated with kaurene on thin layers of 3% silver nitrate–silica gel, 25% silver nitrate–silica gel, and 25% ammoniacal silver nitrate–silica gel (prepared with concentrated ammonium hydroxide solution in place of water, Wood and Snyder, 1966), all developed with *n*-hexane. These adsorbents caused successively decreasing mobility in reference to kaurane of diterpene hydrocarbons containing double bonds. Thus, it appeared that C-4 was saturated.

In spite of the apparent lack of unsaturation, however, C-4 was very reactive toward bromine. Treatment of [^{14}C]-kaurane with bromine in carbon tetrachloride solution in the light at room temperature for 1 hr led to the conversion of only 25% of the radioactivity into eight distinguishable components, all of which migrated more slowly than kaurane on silica gel thin layers. However, similar treatment of C-4 gave complete conversion into radioactive materials migrating more slowly than kaurane. Complete reaction under these conditions was also observed with the model cyclopropane-containing compounds 15,16- and 16,17-methylenekaurane. The possibility was considered that C-4 contained a cyclopropane ring. Evidence consistent with this was obtained in the following way. A sample of C-4 was subjected to conditions of hydrogenation with platinum dioxide catalyst in acetic acid for 24 hr. The radioactive material was extracted into carbon tetrachloride solution and treated with bromine as described above. Chromatography of the reaction mixture on silica gel developed with *n*-hexane showed that 9% of the recovered radioactivity migrated with kaurane, whereas a similar sample of C-4 not subjected to hydrogenation conditions reacted completely with bromine. This result could be explained by the hydrogenolytic cleavage of a cyclopropane ring or the proton-catalyzed cleavage of a cyclopropane ring generating a double bond followed by catalytic reduction. In both cases the saturated, noncyclopropane-containing product would be only partially reactive toward bromine and that portion which remained unreacted would exhibit the same chromatographic properties as kaurane.

Trachylobane (IV) is the hydrocarbon parent of a group of naturally occurring diterpenes which contain a cyclopropane ring (Hugel *et al.*, 1965b). When a sample of (−)-trachylobane was made available by Dr. G. Ourisson a direct comparison of C-4 with this compound was undertaken. The two materials showed cochromatography on silver nitrate–silica gel layers developed with *n*-hexane, as expected. It was noted where C-4 had been treated with bromine that an essentially reproducible spectrum of five or six radioactive products was obtained on silica gel chromatograms developed with *n*-hexane, and the following experiment was designed to take advantage of this property in a more critical comparison with trachylobane. A sample of C-4 containing 9000

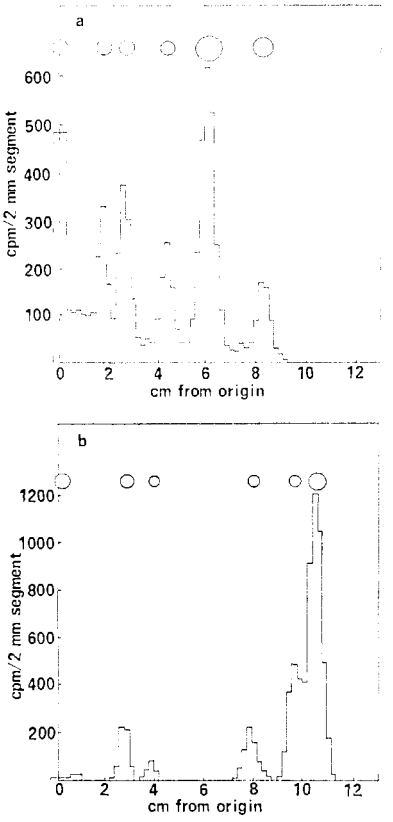


FIGURE 3: Thin-layer chromatography of the products obtained by treatment of component C-4 and trachylobane with bromine or acetic acid. (a) A mixture of component C-4 and trachylobane was allowed to react with bromine in carbon tetrachloride solution for 1 hr in light. The reaction mixture was applied to a silica gel thin-layer plate which was then developed to 15 cm with *n*-hexane. Materials derived from trachylobane were revealed by iodine vapor (represented by the open circles) and radioactive materials derived from C-4 were revealed by liquid scintillation counting of 2-mm wide segments of the gel layer. (Kaurane chromatographed simultaneously in an adjacent position migrated to 12.5 cm.) (b) A mixture of component C-4 and trachylobane was treated with hot glacial acetic acid for 90 min. The benzene extract of the diluted reaction mixture was applied to a silver nitrate–silica gel thin-layer plate which was then developed to 15 cm with benzene–*n*-hexane (3:7). Materials derived from trachylobane were revealed in ultraviolet light after spraying with a solution of 2′,7′-dichlorofluorescein (open circles). Radioactivity was determined as in (a), above. (Kaurane chromatographed simultaneously in an adjacent position migrated to 10.5 cm suggesting that the fastest moving material in the reaction mixture was unreacted trachylobane.)

cpm was prepared from a standard incubation with high specific activity mevalonate. The mass in the sample was insufficient to be detected by the usual visualization agents after thin-layer chromatography. This sample was mixed with 50 μg of the authentic trachylobane and the mixture was treated with bromine in carbon tetrachloride as described above. After chromatography of the reaction mixture on silica gel developed with *n*-hexane, five discrete, circular spots were observed upon staining with iodine vapor, all of which migrated more slowly than a reference sample of kaurane chromatographed in an adjacent position on the same place. The precise positions of these spots were noted, the gel layer was divided into 2-mm segments and the measured radioactivity in each segment was plotted in the form of a histogram (Figure 3a). In every case, the visible derivatives of authentic trachylobane coincided exactly with the radioactive derivatives of component C-4. It was observed in separate experiments that the spectra of products obtained on treatment of 15,16- and 16,17-methylenekaurane with bromine were distinctly different from one another and from the trachylobane-derived spectrum.

TABLE IV

m/e	Relative Intensity
278 (parent, P)	100
279 (P + 1)	25
280 (P + 2)	16
281 (P + 3)	4

Treatment of component C-4 with hot glacial acetic acid gave rise to at least four hydrocarbons which could be separated by silver nitrate–silica gel chromatography. In an experiment analogous to that of treatment with bromine, a mixture of 9000 cpm of C-4 and 45 μg of trachylobane was refluxed in glacial acetic acid for 90 min. The reaction mixture was diluted with water and extracted with benzene. After chromatography of the benzene extract on silver nitrate–silica gel developed with *n*-hexane–benzene (7:3), five spots, including one coincident with trachylobane, were revealed with 2′,7′-dichlorofluorescein. Measurement of radioactivity in gel segments showed that in every case radioactive material derived from C-4 coincided with visible material derived from trachylobane (Figure 3b).

These two experiments in which a total of nine derivatives of trachylobane and of C-4 were shown to cochromatograph are considered to be sufficient for a preliminary identification of component C-4 as trachylobane. Although there is no direct experimental evidence, it is likely that C-4 is the (−)-enantiomer as illustrated in view of the particular enantiomeric forms of the other biosynthesized hydrocarbons beyerene, kaurene, and sandaracopimaradiene and their possible biogenetic relationships (Robinson and West, 1970), and also because the oxidized derivatives of trachylobane that occur in nature have this particular configuration (Hugel *et al.*, 1965b). The fact that copalyl pyrophosphate of known absolute configuration has been shown to give rise to C-4 (Shechter and West, 1969) is further evidence for the stereochemistry of that product as (−)-trachylobane.

COMPONENT B. The strong adsorption of component B to silver nitrate–silica gel thin layers suggested the presence of several double bonds in the molecule. The mass spectrum showed the molecular weight to be 272 which is consistent with an empirical formula of $C_{20}H_{32}$. Hydrogenation with platinum dioxide catalyst in acetic acid gave a material which exhibited a mobility equivalent to kaurane on silver nitrate–silica gel thin layers. The mass spectrum of this material included a major peak at *m/e* 278 representing the addition of six hydrogens, presumably to three double bonds. However, the presumed heavy isotope masses were anomalous (see Table IV). The mass spectra of eleven other diterpenes that were examined in the course of this work all possessed P + 1 peaks

of 23 to 25% of the intensity of the parent and P + 2 peaks of 3 or 4%; in no case was a P + 3 peak visible. These values are consistent with the natural occurrence of 1.1 atom % of ^{13}C, the prime contributor to the P + 1 and P + 2 species in hydrocarbons. Since the peak of *m/e* 280 cannot by itself represent the parent of hydrogenated component B, the situation could arise from a mixture of two compounds of mol wt 278 and 280 with the parent 280 peak having an intensity of about 13% of the parent 278 peak. These results may be explained by the presence in component B of four double bonds, one of which is hydrogenated only with difficulty, or by the hydrogenolytic opening of a ring, a possibility favored from evidence presented below.

The nuclear magnetic resonance spectrum of component B was characterized by the following features: two quaternary methyl groups at 0.90 and 1.05 ppm (evidently not of the *gem*-dimethyl arrangement as found in tricyclic and tetracyclic diterpenes such as pimaradiene and kaurene where the chemical shifts are invariably 0.03 to 0.05 ppm apart); three methyl groups attached to trisubstituted double bonds, two at 1.53 and one at 1.62 ppm; three olefinic protons in two broad overlapping bands centered at 4.80 and 4.93 ppm; ten allylic methylene protons between 1.92 and 2.25 ppm; two nonallylic methylene protons at 1.20 and 1.32 ppm; a doublet of about one proton at 5.16 and 6.66 ppm which at high resolution appears to be part of an AB quartet.

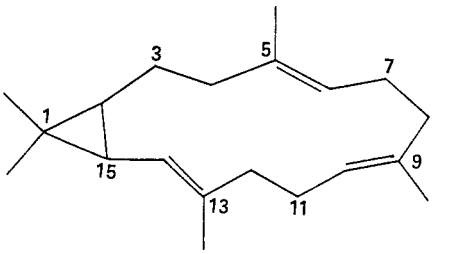
A structure for component B compatible with the above data is illustrated (Chart II). This is similar to the structure assigned to cembrene (V) (Dauben *et al.*, 1965; Kobayashi and Akiyoshi, 1963). The principal difference is in the inclusion of a cyclopropane ring in place of a double bond. The arrangement of the double bonds in the proposed structure is that which could be derived directly from an all-*trans*-geranylgeranyl pyrophosphate precursor with the fewest modifications. Methyl groups attached to *cis*- or *trans*-trisubstituted double bonds in acyclic molecules absorb characteristically at 1.67 and 1.59 ppm (Bates and Gale, 1960). However, in cyclic terpenoids, particularly those with two or more double bonds, the rule rarely holds. Accordingly, in the case of cembrene, it has not been possible to assign configurations to the double bonds from the nuclear magnetic resonance data.

The absence of a clear splitting pattern for the three olefinic protons in the nuclear magnetic resonance spectrum of component B is consistent in the proposed structure with the broad absorptions shown by the corresponding protons in geranylgeraniol (Fedeli *et al.*, 1966) and cembrene (Kimland and Norin, 1968).

The cyclopropane ring is so located in the proposed structure in view of the apparent absence of *gem*-dimethyls either in the form of an isopropyl group, or attached to a double bond, or of the essentially unrestricted form as in cyclohexane ring systems. A cyclopropane ring would be expected to influence the chemical shifts of attached methyl group protons. For example, the introduction of a cyclopropyl group adjacent to the C-17 methyl group of kaurane (to form 15,16-methylenekaurane) alters the chemical shift of the methyl group from 0.95 to 1.13 ppm. The same absorption of 1.13 ppm is shown by the methyl group attached to the cyclopropane ring of trachylobane (Hugel *et al.*, 1965a).

The proposed structure has two cyclopropane protons but the nuclear magnetic resonance data indicate only one clearly (as an AB quartet corresponding to the proton at C-2 of the proposed structure). Published data show that cyclopropane protons can absorb over a very wide range and it is possible that a second proton could have been obscured by the excess of tetramethylsilane used as a trigger for the time-averaging computer. The second cyclopropane proton would exist presumably as a triplet, the small peaks of which could easily be masked. Insufficient material was available for a more detailed examination of this region.

CHART II



The presence of a cyclopropane ring in component B would readily explain the anomalous parent peaks observed in the mass spectrum of the hydrogenated material (discussed above). Further indirect evidence was the observation that as much as 95% of the fully hydrogenated material, which possessed mobility equivalent to kaurane on silver nitrate–silica gel chromatograms, reacted with bromine. This observation is consistent with the expectation that the hydrogenated material would be principally the compound with the cyclopropane ring intact. It is recalled that compounds such as kaurane react with bromine under the same conditions to the extent of only 25% or less.

Major features of the mass spectrum of component B may be explained in accordance with the proposed structure; for example, loss of CH_3 to give *m/e* 257, cleavage at the two allylic bonds to give fragments of *m/e* 136, and loss of methyl from one of these fragments to give the base peak of *m/e* 121. Further assignments become increasingly speculative, however, in the absence of additional structural information.

A satisfactory infrared spectrum of component B was not obtained. The sample had become contaminated and insufficient for repurification. The ultraviolet absorption curve showed chiefly end absorption with no appreciable absorption at 245 nm. The conjugated double bonds of cembrene give an extinction coefficient of 18,000 at 245 nm (Dauben *et al.*, 1965).

The data available are completely consistent with the proposed structure for component B; however, evidence is lacking for positioning the double bonds and assigning their configuration.

Discussion

The natural occurrence of cyclic diterpenes in *R. communis* has apparently not been reported previously, although many other members of the *Euphorbiaceae* are rich sources. Attempts in the present work to isolate diterpene hydrocarbons from seedlings indicated that the endogenous levels were comparatively low; however, the metabolism of mevalonate in extracts

under the conditions employed was largely directed toward the formation of diterpenes.

The chemical, physical, and spectral properties of the isolated components C-1, C-2, and C-3 in comparison both with published data and directly with the properties of authentic reference compounds, leave little doubt that these materials are (+)-beyerene (I), (+)-sandaracopimaradiene (II), and (−)-kaurene (III), respectively. Kaurene has been isolated from a number of *Gymnosperms*, including the New Zealand kauri, *Agathis australis* (Aplin *et al.*, 1963). It has also been isolated from culture filtrates of the fungus *Gibberella fujikuroi* which produces gibberellins (Cross *et al.*, 1962, 1963). The implication that (−)-kaurene is an obligate precursor of the gibberellin family of plant hormones (Cross *et al.*, 1964) suggests that it should be of widespread or universal occurrence in higher plants. Many oxidized derivatives of (−)-kaurene are also known as natural products.

(+)-Beyerene is a natural constituent of the wood of *Erythroxylon monogynum* (Kapadi and Dev, 1964; Murray and McCrindle, 1964); other species have provided a number of oxidized derivatives. Neither (+)-sandaracopimaradiene itself nor any oxidized derivatives have been reported previously as natural products, although the enantiomeric hydrocarbon has been isolated from *Dacrydium colensoi* (Corbett and Smith, 1967) and *Xylia dolabriformis* (Laidlaw and Morgan, 1963); also, oxidized derivatives of the latter are known. An acid of the (−)-pimaradiene structure (13-epimer of II) having the same A/B ring stereochemistry as (+)-sandaracopimaradiene has been isolated from *Aralia cordata* (Shibata *et al.*, 1967). Trachylobane itself has not been previously reported as a natural product; however, trachyloban-18-ol, trachyloban-18-oic acid, and trachyloban-3-ol-18-oic acid have been identified as products in the resinous seed pods of *Trachylobium verrucosum* (Hugel *et al.*, 1965b). These substances have the stereochemical configuration of (−)-trachylobane (IV).

No identification was made of the products of bromine treatment of trachylobane. However, addition of bromine to cyclopropane rings can give a variety of products (Gordon, 1967) and a number of substitution reactions of diterpene hydrocarbons with bromine are known to occur (Briggs *et al.*, 1965). From previous studies on proton-catalyzed isomerization of trachylobane (*e.g.*, Appleton *et al.*, 1966; Hugel *et al.*, 1965a) it is suggested that the derivatives observed on acid treatment are, in order of increasing mobility (see Figure 3b), isoatiserene, isokaurene, and/or beyerene and atiserene, with kaurene forming the shoulder to this last peak. The material migrating slightly less than trachylobane is of unknown nature. The approaches used here for the comparison of C-4 with trachylobane might be useful in many situations. The nature of the derivatives need not be known but the spectrum of products should be sufficiently complex to be characteristic of the compound in question and should be generated in a reasonably reproducible manner.

The identification and characterization of component B (casbene) rests on somewhat tenuous grounds, since this compound appears not to have been isolated previously. The data presented are consistent with the structure proposed in the text; however, additional spectral and chemical information is clearly required. Reports of diterpenes possessing 14-membered carbocyclic rings are becoming increasingly numerous (*e.g.*, Rowland *et al.*, 1964; Kimland and Norin, 1968). All of the compounds appear to possess the carbon skeleton of the hydrocarbon cembrene (V) which itself is found in certain pine species (Dauben *et al.*, 1965; Kobayashi and Akiyoshi, 1963).

The structural and stereochemical relationships of the A and B rings of (+)-beyerene, (+)-sandaracopimaradiene, and (−)-kaurene are evident. All possess the characteristic *trans-anti* configuration of the substituents at C-5, C-9, and C-10 with hydrogens at C-5 and C-9 β oriented and the methyl at C-10 α oriented. The trachylobane isolated may well have the same configuration in these rings. This suggests a close biogenetic relationship among the "C" group of hydrocarbons. The structure proposed for casbene would presumably be derived from a quite different mechanism. The accompanying paper discusses some features of the enzymic formation of these diterpenes and some speculations concerning their function in the developing plant (Robinson and West, 1970).

References

Anderson, J. D., and Moore, T. C. (1967), *Plant Physiol. 42*, 1527.

Aplin, R. T., Cambie, R. C., and Rutledge, P. S. (1963), *Phytochemistry 2*, 205.

Appleton, R. A., McAlees, A. J., McCormick, A., McCrindle, R., and Murray, R. D. H. (1966), *J. Chem. Soc. (C)*, 2319.

Audier, H. E., Bory, S., Fétizon, M., and Anh, N. T. (1966), *Bull. Soc. Chim. Fr.*, 4002.

Bates, R. B., and Gale, D. M. (1960), *J. Am. Chem. Soc. 82*, 5749.

Briggs, L. H., Cain, B. F., Cambie, R. C., Davis, B. R., Rutledge, P. S., and Wilmshurst, J. K. (1963), *J. Chem. Soc.*, 1345.

Briggs, L. H., Cambie, R. C., Rutledge, P. S., and Stanton, D. W. (1965), *J. Chem. Soc.*, 6212.

Briggs, L. H., Cawley, R. W., Loe, J. A., and Taylor, W. L. (1950), *J. Chem. Soc.*, 955.

Corbett, R. E., and Smith, R. A. J. (1967), *J. Chem. Soc. (C)*, 300.

Cross, B. E., Galt, R. H. B., and Hanson, J. R. (1964), *J. Chem. Soc.*, 295.

Cross, B. E., Galt, R. H. B., Hanson, J. R., Curtis, P. J., Grove, J. F., and Morrison, A. (1963), *J. Chem. Soc.*, 2937.

Cross, B. E., Galt, R. H. B., Hanson, J. R., and Klyne, W. (1962), *Tetrahedron Lett.* 145.

Dauben, W. G., Thiessen, W. E., and Resnick, P. R. (1965), *J. Org. Chem. 30*, 1693.

Dennis, D. T., Upper, C. D., and West, C. A. (1965), *Plant Physiol. 40*, 948.

Dennis, D. T., and West, C. A. (1967), *J. Biol. Chem. 242*, 3293.

Edwards, O. E., and Howe, R. (1959), *Can. J. Chem. 37*, 760.

Erdtman, H., and Westfelt, L. (1963), *Acta Chem. Scand. 17*, 1826.

Fedeli, E., Capella, P., Cirimele, M., and Jacini, G. (1966), *J. Lipid Res. 7*, 437.

Gordon, A. J. (1967), *J. Chem. Ed. 44*, 461.

Graebe, J. E. (1969), *Planta 85*, 171.

Graebe, J. E., Dennis, D. T., Upper, C. D., and West, C. A. (1965), *J. Biol. Chem. 240*, 1847.

Hirsch, J., and Ahrens, E. H. (1958), *J. Biol. Chem. 233*, 311.

Hugel, G., Lods, L., Mellor, J. M., and Ourisson, G. (1965a),

Bull. Soc. Chim. Fr., 2894.
Hugel, G., Lods, L., Mellor, J. M., Theobald, D. W., and Ourisson, G. (1965b), *Bull. Soc. Chim. Fr.*, 2882, 2888.
Johnson, P., Sheppard, R. C., Stehr, C. E., and Turner, S. (1966), *J. Chem. Soc. (C)*, 1847.
Kapadi, A. H., and Dev, S. (1964), *Tetrahedron Lett.* 2751.
Kimland, B., and Norin, T. (1968), *Acta Chem. Scand. 22*, 943.
Kobayashi, H., and Akiyoshi, S. (1963), *Bull. Chem. Soc. Jap. 36*, 823.
Laidlaw, R. A., and Morgan, J. W. W. (1963), *J. Chem. Soc.*, 644.
Layne, E. (1957), *Methods Enzymol. 3*, 447.
McCrindle, R., and Overton, K. H. (1965), *Advan. Org. Chem. 5*, 47.
Murray, R. D. H., and McCrindle, R. (1964), *Chem. Ind.* (London), 500.
Phinney, B. O., Jefferies, P. R., Katsumi, M., and Henrick, C. A. (1964), *Plant Physiol. Suppl., 39*, 27.
Robinson, D. R., and West, C. A. (1967), *Fed. Proc. 26*, 454.
Robinson, D. R., and West, C. A. (1970), *Biochemistry 9*, 800.
Rowland, R. L., Rodgman, A., Schumacher, J. N., Roberts, D. L., Cook, L. C., and Walker, W. E. (1964), *J. Org. Chem. 29*, 16.
Ruzicka, L., Eschenmoser, A., and Heusser, H. (1953), *Experientia 9*, 357.
Shank, R. S., and Shechter, H. (1959), *J. Org. Chem. 24*, 1825.
Shechter, I., and West, C. A. (1969), *J. Biol. Chem. 244*, 3200.
Shibata, S., Mihashi, S., and Tanaka, O. (1967), *Tetrahedron Lett.* 5241.
Simmons, H. E., and Smith, R. D. (1959), *J. Am. Chem. Soc. 81*, 4256.
Upper, C. D., and West, C. A. (1967), *J. Biol. Chem. 242*, 3285.
Wenkert, E. (1955), *Chem. Ind.* (London), 282.
Wenkert, E., Afonso, A., Beak, P., Carney, W. J., Jeffs, P. W., and McChesney, J. D. (1965), *J. Org. Chem. 30*, 713.
West, C. A., Oster, M., Robinson, D., Lew, F., and Murphy, P. (1967), *in* Biochemistry and Physiology of Plant Growth Substances, Wightman, F., and Setterfield, G., Ed., Ottawa, Runge, p 313.
Wood, R., and Snyder, F. (1966), *J. Am. Oil Chem. Soc. 43*, 53