periments; the amounts of propene formed (Table I) exceed this detection limit by factors of 10^2–10^3; (2) the allene content (≤0.5%, ir) of the purified cyclopropene samples corresponded to a maximum partial pressure of 10^{-4} atm in assay gas mixtures; the maximal initial rate of propene production calculated[15] for this value is several orders of magnitude less than observed rates, and in fact the amounts of propene formed exceeded the maximal allene contamination by factors of 10 or more; and (3) traces of allyl chloride and allyl amine carried over in the collection of crude cyclopropene were no longer detectable (FID GC, ir) after purification and can also be disregarded by stoichiometry alone, although there is no evidence that either compound can undergo reduction by nitrogenase. Nonhydrocarbon products of nonreductive decomposition of cyclopropene due to contact with the aqueous assay mixture cannot be an important source of propene formation because this should lead to an increased rate of such formation as a function of preincubation time, whereas experimentally the opposite was observed (C. E. McKenna, unpublished data).

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(22) Recipient of 1976 Sigma Xi Student Research Award, U.S.C.

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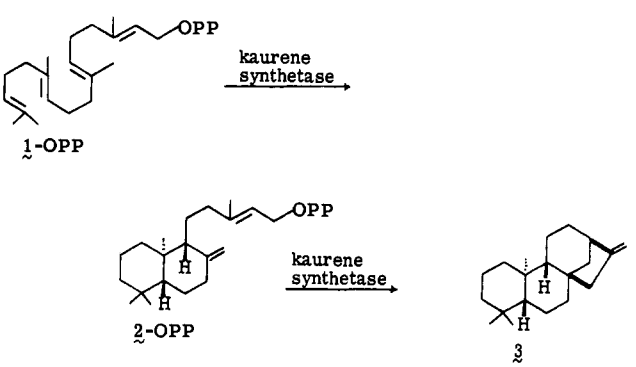
Enzymatic Cyclization of (*R,S*)-14,15-Oxidogeranylgeranyl Pyrophosphate to 3α- and 3β-Hydroxykaurene[1]

Sir:

The first step in the biosynthesis of the majority of the known diterpene natural products from *all-trans*-geranylgeranyl pyrophosphate (**1**-OPP), is a double cyclization to the labdane nucleus, a process apparently initiated by enzymatic protonation of the 14,15-double bond.[2] Thus, the tetracyclic diterpene kaurene (**3**), a biosynthetic precursor of the gibberellin plant growth regulators,[2,3] is formed from **1**-OPP by way of the bicyclic intermediate, copalyl pyrophosphate (**2**-OPP).[4] Although many members of the gibberellin family and certain other diterpenes possess a hydroxyl group at C_3,[5] this functional group is apparently introduced by an oxygenation reaction subsequent to the cyclization stages.[2,3,6] In contrast, the characteristic C_3 hydroxyl group present in most cyclic triterpenes and sterols is a direct outcome of the enzymatic cyclization of (3*S*)-2,3-oxidosqualene.[7] We wish to report that the (*R,S*)-14,15-epoxide (**4**-OPP) of geranylgeranyl pyrophosphate is cyclized to a mixture of 3α- and 3β-hydroxykaurene (**5** + **6**)[1] by soluble enzyme preparations containing the diterpene cyclase, kaurene synthetase.[8,9]

all-trans-Geranylgeraniol (**1**-OH)[10] was rendered radioactive (MnO_2 oxidation; NaB^3H_4 reduction) and converted to the known (*R,S*)-14,15-epoxide (**4**-OH, 47 mCi/mm)[8b] by means of regioselective terminal hypobromination of **1**-OAc.[12] Phosphorylation of **4**-OH was accomplished with the modified Cramer procedure,[13] the pyrophosphate (**4**-OPP) being obtained in 20% yield after purification by ion exchange chromatography on Dowex 1-X8[14] and elution from an Amberlite XAD-2 column to separate ammonium formate.[15] The ammonium pyrophosphate was characterized by its specific activity (46 mCi/mm), typical thin layer chromatographic (TLC) behavior,[16a] and reconversion to **4**-OH (75%, identification by TLC mobility)[16b] by treatment with bacterial alkaline phosphatase.

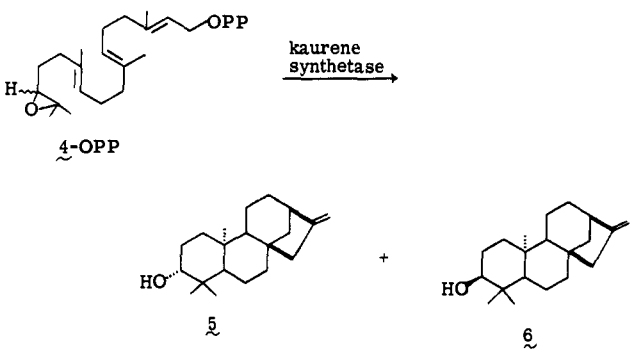
Scheme I



Soluble preparations of endosperm homogenates from immature *Echinocystis macrocarpa* (wild cucumber) seed are known to contain kaurene synthetase activity.[17] A series of preliminary experiments was performed with these preparations as the source of enzyme. The epoxy pyrophosphate is converted in substantial amounts to an extractable, radioactive fraction (X) which runs ahead of the epoxy alcohol (**4**-OH) on TLC[16c] to the region expected for hydroxy diterpenes. Smaller amounts of extractable radioactivity were associated with the triol (from epoxide and pyrophosphate hydrolysis) and one or two very small radioactive fractions were seen at positions intermediate between reference markers of the triol and epoxy alcohol. No distinct radioactive peak was associated with the epoxy alcohol itself. Heat-inactivated enzyme preparations did not catalyze the formation of X. Treatments with bacterial alkaline phosphatase at the end of the initial incubation period did not increase the amounts of X extracted. The plant growth retardant Amo-1618, which strongly inhibits the enzymatic cyclization of **1**-OPP,[18] greatly reduced incorporation into X. Under appropriate TLC conditions,[16b] X is resolved into two closely running components (4:1 ratio), the more polar and predominant of which migrated with authentic 3 α-hydroxykaurene (**5**).[19]

Preparative incubations were carried out with 7.3 g of lyophilized endosperm from *E. macrocarpa* which was reconstituted in 235 ml of cold pH 7.4 buffer (0.1 M Tris and 0.01 M KH_2PO_4). Magnesium chloride hexahydrate (0.47 mmol) and epoxy pyrophosphate (2.1 mg, 4.2 μmol, 420 × 10^6 dpm) were then added. After 15 h at 30° the incubation was terminated by addition of 230 ml of acetone, and the products were separated by extraction with benzene. Purification by preparative TLC[16b] afforded radioactive product (161 × 10^6 dpm, 38%) with R_f 0.5–0.6. The radioactivity was separated into a slightly more polar major fraction (96.6 × 10^6 dpm, 23%, ~260 μg) and a less polar minor fraction (20.7 × 10^6 dpm, 5%, ~60 μg) by means of high pressure liquid chromatography (Corasil type II column, 0 → 20% ethyl acetate/hexane gradient). The major and minor products were identified as 3α- and 3β-hydroxykaurene (**5** and **6**),[1] respectively, by direct comparisons with authentic specimens prepared from the natural diterpene abbeokutone.[20,21]

Scheme II



The two enzymatic products exhibited gas–liquid chromatographic (GLC) retention times coincident with those of the hydroxykaurene reference samples, albeit under conditions (3% OV-17 at 190 or 210°) which effected only slight separation of the two isomers. Since the area of the GLC peaks from the enzymatic products was approximately the same as that from the reference samples (separate injection of ~0.2 μg), the peaks observed for the former are judged to contain the majority of the radioactive material present. The virtual superimposability of the mass spectra (GC/MS, 3% OV-17, 190 or 210°) of the major and minor products with those of 3α- and 3β-hydroxykaurene, respectively, lends further support to the structural assignments.[22] The substantial differences in the relative intensities of the peaks in the mass spectra of the two hydroxy kaurene epimers enhances the significance of these comparisons.[23]

Additional evidence in favor of the proposed structures was secured by crystallization of **5** and **6** in the presence of the major and minor products, respectively, the specific activity remaining essentially constant, or increasing somewhat, over the course of three recrystallizations. In contrast, three recrystallizations of **5** in the presence of the minor product resulted in a 90% decrease in specific activity. A satisfactory 220-MHz NMR spectrum of the major product (~150 μg in C_6D_6) was obtained by means of Fourier transform spectroscopy. Although a few minor extraneous absorptions are present, the spectrum nevertheless displays all of the absorptions characteristic of 3α-hydroxykaurene: δ 4.97, 4.93 (2 s, $=CH_2$), 2.97 (t, J = 7 Hz, C*H*OH), 2.65 (s, $C_{13}H$), 2.04 (s, $C_{14}H_2$), 1.86 (d, J = 10 Hz), 0.97, 0.86, 0.77 (3 s, CH_3). A 30-μg portion of the major product was subjected to chromic acid oxidation and the product identified as 3-oxokaurene by mass spectral comparisons.

3α-Hydroxykaurene (**5**) presumably arises from enzyme-catalyzed cyclization of the (14*R*) enantiomer of **4**-OPP by way of 3α-hydroxycopalyl pyrophosphate. The isolated yield of **5** is, therefore, 46% (based on one enantiomer). The formation of 3β-hydroxykaurene (**6**, axial OH) is remarkable. Since a control experiment established that **5** is not converted to **6** in the incubation medium, the 3β isomer is apparently produced by cyclization of the 14*S* enantiomer of **4**-OPP from either a boat–chair conformation (inversion at C_{15}) or a chair–chair conformation (retention at C_{15}). The K_m value for **4**-OPP cyclization is estimated as 9 μM which is not very different from the apparent K_m value of 1.5 μM for the normal substrate **1**-OPP. Furthermore, **1**-OPP is a competitive inhibitor of the cyclization of **4**-OPP with a K_i value nearly equal to its K_m value as a substrate. These results indicate that the same enzyme is involved in the cyclization of both substrates. Also, the initial rate of cyclization of the epoxy pyrophosphate is comparable to that of **1**-OPP under similar conditions.

The utilization of the epoxide analogue as a substrate for conversion to substances with the TLC mobility of hydroxy diterpenes is also observed with diterpene cyclase preparations from *Ricinus communis* (castor bean) seedlings[24] and mycelia of the gibberellin-producing fungus *Fusarium moniliforme*.[15] The *R. communis* enzyme preparation, which synthesizes a family of related diterpenes from **1**-OPP via **2**-OPP, catalyzes the formation of at least three radioactive fractions with expected TLC mobilities of 3-hydroxyditerpenes analogous to those obtained from **1**-OPP.[16c,24] The general characteristics of the production of these substances are similar to those observed with *E. macrocarpa*. Control experiments established that neither the triol nor the epoxy alcohol are converted to these products in the incubation medium. The only diterpene hydrocarbon synthesized from **1**-OPP in the *F. moniliforme* preparation is kaurene; the epoxy pyrophosphate is converted by this preparation to products with the TLC mobilities of **5** and **6**.[16b] Thus, the epoxy pyrophosphate and 3-hydroxycopalyl pyrophosphate, the presumed bicyclic intermediate, appear to be acceptable substrates for the diterpene cyclase enzymes from a number of sources.

A microsomal oxidase preparation from *E. macrocarpa* endosperm which catalyzes the oxidation of kaurene through a succession of steps to 7β-hydroxykauren-19-oic acid,[26] converts 3α-hydroxykaurene to unidentified oxidized metabolites. The apparent K_m values for kaurene and 3α-hydroxykaurene in these oxidations are very similar (about 0.5 μM).[27]

Related to the present results is the finding that epoxides serve as alternative substrates for the enzymes which catalyze the hydration of fumarate and various unsaturated fatty acids.[28] The remarkable tolerance of the diterpene cyclase and oxidase enzymes to the presence of an additional epoxide or hydroxyl function in the normal substrate noted in this investigation suggests the possibility of an alternative biosynthetic pathway (**1**-OPP → **4**-OPP → **5** and/or **6**) to the gibberellins and other functionalized diterpenes. The enzymatic cyclization of the epoxy pyrophosphate (**4**-OPP) to 3β-hydroxykaurene (**6**, axial OH) adds credibility to the proposal that naturally occurring 3α-hydroxy-triterpenes (axial OH), and by extension other axially hydroxylated terpenes, might arise in nature by direct cyclization of (3*R*)-2,3-oxidosqualene, or other appropriate epoxide precursors.[29,30]

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(23) Of 132 peaks registered in both spectra, 72 differed in relative intensity by more than 3% of the base peak. In both spectra the base peak comprised 3.8% of the total ionization.
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Direct Observation by Raman Spectroscopy of the Coexistence of a Variety of Ion-Paired Species in Liquid Ammonia Solutions of Alkali Metal Salts

Sir:

Spectroscopic studies have provided indirect evidence[1] for the existence, in electrolyte solutions, of contact, solvent-shared and solvent-separated ion-pairs.[2] It has further been suggested that the relative concentration of each type of ion-pair is determined by the bulk dielectric constant, ϵ, of the solvent.[3,4] For liquid ammonia ϵ is in the region within which the different types of ion-pair may coexist.[4] We present the first direct evidence, from the Raman spectra of multiatomic anionic species, that various types of ion-pair do coexist in liquid ammonia solution. We illustrate this evidence by giving some details of the spectra of sodium and potassium cyanide solutions and the way they change with changing concentration, temperature, and cation. The spectra of other anions which show comparable changes are discussed briefly.

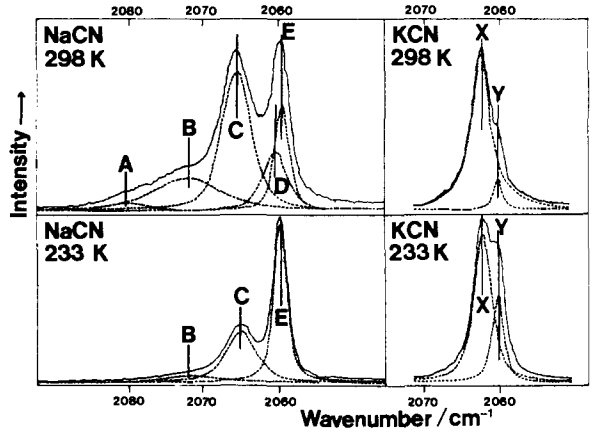

Figure 1. Resolved bands in the C-N stretching region of the Raman spectra of liquid ammonia solutions of NaCN and KCN (parallel polarization).

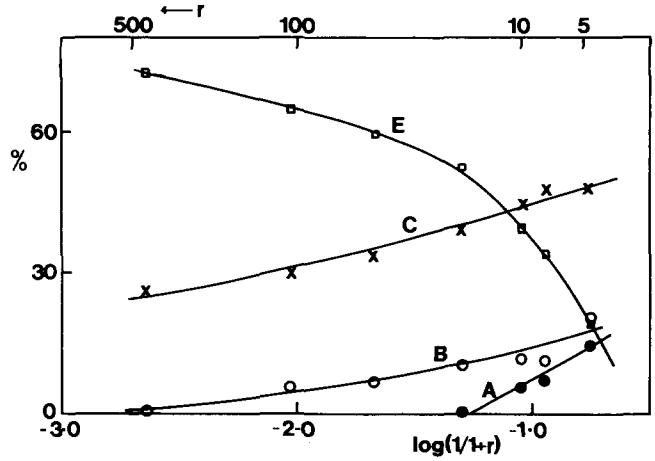

Figure 2. The variation with concentration (expressed as $\log(1/(1+r))$ of resolved component areas (expressed as percent of the total band area) for liquid ammonia solutions of NaCN at 233 K.

The isolated cyanide ion should give a single Raman-active band at ca. 2050 cm^{-1} corresponding to the C-N stretching vibration. The most striking feature of the solution spectra is that more than one band is observed in all cases. Resolved spectra for one concentration of each of the salt solutions at two different temperatures are illustrated in Figure 1.

At least four (polarized) components (not necessarily all visible in the same spectrum) are clearly required to obtain a satisfactory fit of the anion's spectrum with sodium cyanide solutions. These appear at ca. 2083 (A), 2072 (B), 2067 (C), and 2059 (E) cm^{-1}, and all are comparatively sharp.[5] The half-width, w, is between 2 and 9 cm^{-1} and is much less than is observed for comparable bands with aqueous salt solutions. The variation of relative intensity of each component with changes in concentration and temperature are shown in Figures 2 and 3. (r = number of moles of solvent/number of moles of solute). With decreasing concentration components A, B, and C decrease in intensity with respect to E, the lowest frequency component. E becomes the predominant feature of the spectrum at lowest concentration. Similar intensity-concentration trends occur at all temperatures studied. The effect of decreasing temperature is similar to the effect of decreasing concentration and E is the predominant feature of the spectra obtained at the lowest temperature.

The Raman spectra of KCN solutions are simpler, and can be resolved into only two components, X at ca. 2062 and Y at ca. 2060 cm^{-1}. Similar concentration and temperature trends apply to the intensities, with the component at the lower frequency, Y, becoming the more dominant feature at low tem-