BIOGENESIS OF *l*-METHYLISOBORNEOL AND *l*-CARBOXYMETHYLISOBORNEOL FROM *d*-CAMPHOR IN SOIL[1]

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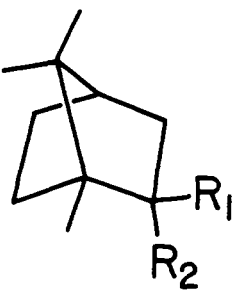
ABSTRACT.—*l*-2-Methylisoborneol (**1**) is a common volatile constituent of soil considered to be of microbial origin. *d*-[G-^{3}H]Camphor (**2**), a monoterpene ketone also identified as a soil constituent, was converted to 2-methylisoborneol (**1**), borneol (**3**) and isoborneol (**4**) in fresh garden soil, but not in sterile garden soil. An additional metabolite of [G-^{3}H] camphor was identified as 2-carboxymethylisoborneol (**5**), suggesting a pathway for the formation of methylisoborneol (**1**) involving condensation of camphor (**2**) with an acetate moiety to yield carboxymethylisoborneol (**5**), followed by decarboxylation. The intermediate role of carboxymethylisoborneol (**5**) in the biosynthesis of methylisoborneol (**1**) was demonstrated directly with [^{3}H]carboxymethylisoborneol.

l-2-Methylisoborneol (*l*-1,2,7,7-tetramethyl-*exo*-bicyclo [2.2.1]heptan-2-ol) (**1**), a volatile terpenoid-like compound with a camphoraceous odor (1), has been isolated from garden soil, and is considered to be a metabolite of soil microorganisms (2). The production of methylisoborneol (**1**) in cultures of several *Actinomycetales* has, in fact, been demonstrated (3–7). The *de novo* biosynthesis of this unusual compound by soil microorganisms is thus assumed, and it has been suggested that (**1**) may be derived via the elimination of a C_4-fragment from a rearranged eudesmane sesquiterpene (3). However, it seemed equally plausible that the common monoterpene ketone *d*-camphor (**2**) might serve as a precursor of methylisoborneol (**1**), camphor (**2**) also having been isolated from soil (2). As an extension of our studies on the biosynthesis of camphor in higher plants



1, R_1=OH, R_2=CH_3 **4**, R_1=OH, R_2=H
2, R_1=R_2=$=O$ **5**, R_1=OH, R_2=CH_2CO_2H
3, R_1=H, R_2=OH **6**, R_1=OH, R_2=$CH_2CO_2CH_3$

FIG. 1. Structures of various bornane derivatives. The configurations illustrated are stereochemically correct (12).

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(8–11), we examined the possible involvement of camphor in the formation of methylisoborneol and related compounds in soil.

EXPERIMENTAL

MATERIALS.—*d*-[G-^{3}H]Camphor (24.3 Ci/mol) (**2**) was prepared by CrO_3 oxidation (13) of *d*-[G-^{3}H]borneol (**3**) which had been previously obtained by $^3H_2$ exposure (8). The ketone was purified by tlc (R_f=0.46) on silica gel G with hexane-ethyl acetate (4:1 v/v) as developing solvent (system I); the chemical and radiochemical purity was verified as 99+% by radio-glc. For use as a substrate, this material was suspended in water with the aid of Tween-20 (20 μg/μmol) and sonication.

l-2-Methylisoborneol (**1**) was prepared from *d*-camphor (**2**) by Grignard reaction with methylmagnesium bromide under standard conditions described elsewhere (1, 14, 15). The crude reaction mixture, after workup, was treated with $NaBH_4$ in methanol to reduce the residual camphor to a mixture of *l*-isoborneol and *d*-borneol and thus facilitate the purification of methylisoborneol by preparative tlc (silica gel G, system I, R_f methylisoborneol=0.42, R_f isoborneol=0.35, R_f borneol=0.28).

The methyl ester of *l*-2-carboxymethylisoborneol (**6**) was prepared from *d*-camphor (**2**) by Reformatsky reaction with methyl bromoacetate under conditions described for the preparation of related hydroxyterpenylacetate esters (16, 17). Separation of the reaction mixture by tlc on silica gel with hexane-ether (3:2, v/v) (system II) yielded pure methyl ester (**6**) (R_f=0.31). Hydrolysis of the methyl ester in 10% alcoholic KOH afforded the corresponding hydroxy acid (**5**) and a significant quantity of what appeared to be elimination product (probably the 2-enoic acid as evidenced by the appearance of bands at 3080 cm^{-1} and 3010 cm^{-1} in the ir). The [G-^{3}H]hydroxy acid (**5**) (24.3 Ci/mol) was similarly prepared from *d*-[G-^{3}H]camphor, and it was suspended in H_2O as described above for use as a substrate.

1,2-Campholide was obtained by Baeyer-Villager oxidation of *d*-camphor (18); other monoterpene standards were obtained from Aldrich Chemical Co. or ICN Pharmaceuticals, Inc.

INCUBATION PROCEDURES AND ISOLATION OF PRODUCTS.—Soil obtained from a local garden (250 g dry weight at 125° as determined on a representative sample) was diluted in an Erlenmeyer flask with 120 ml distilled H_2O in order to make a thick slurry. *d*-[G-^{3}H]Camphor (30 μCi) was added, and the flask was sealed with a cotton plug and shaken on an oscillating shaker for 24 h at 25°.

At the end of the incubation period, 100 ml of 0.1M sodium phosphate buffer (pH 5.0) was added, and the mixture was extracted with vigorous mechanical stirring with 3 x 30 ml portions of ether. Internal standards, 30 mg each of *d*-camphor, *d*-borneol, *l*-isoborneol, *l*-methylisoborneol, *d*-1,2-campholide, *d,l*-camphoroquinone and *l*-2-carboxymethylisoborneol, were added to the ether extract, which was then concentrated under vacuum. The concentrate was then washed repeatedly with aqueous saturated $NaHCO_3$ to remove acidic metabolites. The ether extract was next concentrated further to about 5 ml and subjected to micro-steam distillation (19) to obtain the volatile substances. The volatile fraction was then separated by tlc (system I), and compounds both more polar than camphor and less polar than camphor were isolated for radio gas-liquid chromatographic analysis. To facilitate the analysis of components more polar than camphor (see text), this fraction was reduced with $LiAlH_4$ in ether. Excess hydride was decomposed with water, and the ether soluble fraction was again subjected to tlc (system I); individual components (e.g., borneol, isoborneol, methylisoborneol) were isolated.

The aqueous $NaHCO_3$ extract from above was acidified, and the acidic metabolites were recovered by ether extraction. This fraction was methylated with CH_2N_2 in ether and subjected to tlc (system II).

Experiments with *d*-[G-^{3}H]carboxymethylisoborneol as substrate (15 μCi/250 g soil) were carried out exactly as described above. In all cases, autoclaved soil was used as a control.

ANALYTICAL PROCEDURES.—Vacuum steam distillation-solvent (hexane) extraction of garden soil (7.5 kg), followed by combined gas chromatographic-mass spectrometric analysis of the volatile oil (~2 parts per 10^6 parts soil), was carried out as described by Buttery and Garibaldi (2). Retention data and spectra were compared to those of authentic samples.

Thin-layer chromatography was done on 1-mm layers of silica gel G activated at 120° for 3 h. Solvent systems are indicated elsewhere in the appropriate sections in the text. Chemical bands were located under uv light after the developed plates were sprayed with a solution of 2,7-dichlorofluorescein in ethanol. Either silica gel scraped from the plate was transferred directly to a scintillation vial for determination of radioactivity, or the products were eluted from the gel with ether for further analysis.

Radio gas-liquid chromatography was performed on a Varian gas chromatograph attached to a model 7357 Nuclear Chicago radioactivity monitor. The monitor was externally calibrated with [^{3}H]toluene for each analysis, and the radioactivity in each separate component was determined by triangulation of the resulting chromatographic peaks. Gas-liquid chromatography columns were: 10 ft x 0.125 in. o.d. stainless steel, packed with 12% Carbowax

4000 on 80/100-mesh Gas Chrome Q; and 6 ft x 0.125 in. o.d. stainless steel, packed with 10% SE-30 on 80/100-mesh Gas Chrome Q. Other chromatographic conditions are indicated under the appropriate figure.

Radioactivity in liquid samples and in thin-layer fractions was determined in a counting solution consisting of 0.4% (w/v) Omifluor (New England Nuclear) dissolved in 30% ethanol in toluene with a Packard model 3255 liquid scintillation spectrometer. The counting efficiency for ^3H was 34%, and all assays were done with a s.d. less than 3%.

RESULTS AND DISCUSSION

Incubation of soil with *d*-[G-^3H]camphor (30 μCi) for 24 h, followed by separation of the various fractions as described in the EXPERIMENTAL section, provided the following distribution of radioactivity: ether-soluble, steam-volatile metabolites (6.24 μCi); ether-soluble, acidic metabolites (0.72 μCi); ^3H_2O (2.52 μCi, by lyophilization); water-soluble metabolites (3.12 μCi, after removal of ^3H_2O); insoluble material (0.24 μCi). Approximately 50% of the administered label was recoverable after 24 h, the remaining label presumably being lost as [^3H]camphor by vaporization to the atmosphere. As it seemed likely that the labeled insoluble material and water-soluble metabolites, as well as the ^3H_2O, resulted from the oxidative degradation of [^3H]camphor by well-known microbial pathways (20, 21), these products were not examined further.

Thin-layer chromatography (system I) of the ether-soluble, steam-volatile fraction indicated the presence of a considerable quantity of residual [^3H]camphor (5.30 μCi). Metabolites chromatographically less polar than camphor (i.e. $R_f > 0.46$ with tlc system I) were negligible, whereas the fraction containing metabolites more polar than camphor (R_f 0.0–0.46) was labeled (0.98 μCi). Radio-glc of this fraction on the Carbowax 4000 column indicated the presence of at least eight components, including those chromatographically coincident with methylisoborneol, isoborneol, borneol, 1,2-campholide and camphoroquinone. To more readily distinguish between methylisoborneol, isoborneol and borneol, and the previously established (20, 21) diketone and lactone metabolites of camphor, this fraction was treated with $LiAlH_4$ in ether; thereby the diketones and lactones, and their congeners were converted to compounds more polar than borneol (i.e., diols, triols, etc.). The $LiAlH_4$-reduced material was again subjected to tlc (system I), and the labeled components chromatographically coincident with methylisoborneol (0.14 μCi), isoborneol (0.11 μCi) and borneol (0.33 μCi) were isolated for further analysis by radio-glc on two columns of widely differing selectivity (SE-30 and Carbowax 4000). For both borneol and isoborneol, a single radioactive component, chromatographically coincident with the appropriate authentic standard, was demonstrated, and CrO_3 oxidation of each presumptive alcohol yielded [^3H]camphor, thus confirming the identification. Similarly, radio-glc of the putative methylisoborneol revealed the presence of a single radioactive component, coincident with the authentic coinjected standard (fig. 2a). To confirm the identification of the [^3H]methylisoborneol, the labeled material was further diluted with authentic *l*-2-methylisoborneol and sublimed to a constant specific activity of 0.175 mCi/mol (Fisher-Johns mp = 164–165° (1) [lit = 163–165°(1)]. These results clearly indicated that camphor was reduced to borneol and isoborneol and converted to methylisoborneol in soil. Several other presumed microbial degradation products of camphor, such as 1,2-campholide (20, 21), were noted, but they were not identified. None of the above metabolites were detected when [G-^3H]camphor was incubated with sterile (autoclaved) soil under identical conditions.

Of several possible mechanisms for the transformation of *d*-camphor

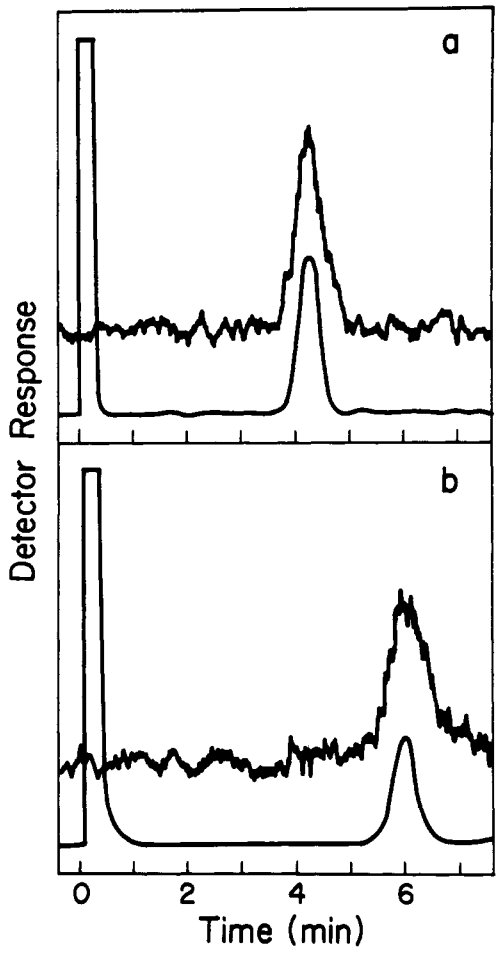

FIG. 2. (a) Radio gas-liquid chromatogram of the methylisoborneol isolated from soil that had been incubated with *d*-[G-^{3}H]camphor. The top tracing is the response of the radioactivity monitor attached to the gas-liquid chromatograph. The smooth bottom tracing is the flame ionization detector response obtained from an authentic coinjected standard of *l*-2-methylisoborneol. The chromatographic column (Carbowax 4000, described in the EXPERIMENTAL section) was held at 145° with an argon flow rate of 120 cm^3/min. (b) Radio gas-liquid chromamatogram of the methyl ester of carboxymethylisoborneol isolated from soil that had been incubated with *d*-[G-^{3}H]camphor. The top and bottom tracings correspond to the response of the radioactivity monitor and the flame ionization detector, respectively, and the coinjected standard was an authentic sample of the methyl ester of *l*-2-carboxymethyliosborneol. The gas chromatographic column (SE-30, described in the EXPERIMENTAL section) was held at 150° with an argon flow rate of 120 cm^3/min.

to *l*-methylisoborneol, the condensation of camphor with an acetate moiety, followed by decarboxylation, appeared to be the most viable in that both reaction types have ample biochemical precedent in the terpenoid field (22). Such a reaction scheme implies carboxymethylisoborneol (**5**) as an intermediate, and this compound was sought among the ether-soluble, acidic metabolites formed when soil was incubated with [G-^{3}H]camphor. Methylation of the acidic metabolite fraction (0.72 μCi) followed by tlc (system II) allowed the isolation of a

labeled component (0.08 μCi) chromatographically coincident ($R_f = 0.31$) with the methyl ester of carboxymethylisoborneol (**6**). Radio-glc of the isolated material confirmed the presence of a single radioactive product, coincident with the authentic standard (fig. 2b). Other labeled acidic metabolites, both more polar and less polar than methyl ester **6**, were noted on tlc, but these components either did not elute from the radio-glc under the conditions employed, or they contained too little radioactivity to permit further analysis. None of the acidic metabolites were formed when sterile soil was incubated with [G-^{3}H]camphor.

To examine further the possible role of *l*-2-carboxymethylisoborneol (**5**) in the formation of *l*-2-methylisoborneol (**1**), *l*-2-[^{3}H]carboxymethylisoborneol was prepared, and an aliquot (15 μCi) was incubated with soil as before. Radio chromatographic analysis of the ether-soluble, steam-volatile metabolites revealed the presence of labeled methylisoborneol (0.37 μC, sublimed to constant specific activity and mp as before); thus the intermediacy of carboxymethylisoborneol in the pathway was supported. Although gas chromatographic-mass spectrometric analysis of the volatile oil obtained from our soil sample revealed the presence of both camphor (0.7% of oil) and methylisoborneol (0.5% of oil), carboxymethylisoborneol (analyzed as the methyl ester) was present at too low a level (<0.1% of oil) to permit adequate characterization of this presumed intermediate.

While the present results clearly do not preclude other possible pathways for the biogenesis of methylisoborneol, they do suggest that camphor present in soil can be converted to methylisoborneol via condensation with acetate followed by decarboxylation. The origin of camphor in soil is presently uncertain, but, as the camphane (bornane) monoterpenes are minor volatile constituents of many species of both higher and lower plants, this source seems likely.

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