ENZYMATIC SYNTHESIS OF CAMPHOR FROM NERYL PYROPHOSPHATE BY A SOLUBLE PREPARATION FROM SAGE (*SALVIA OFFICINALIS*)[1]

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SUMMARY: Camphor, a bicyclic monoterpene ketone, is one of the major components of the volatile oil of sage (*Salvia officinalis*). A soluble enzyme preparation obtained from homogenates of young sage leaves converts the acyclic precursor neryl pyrophosphate to borneol in the presence of Mg^{++}, and in the presence of NAD, dehydrogenates the borneol formed to camphor. The biosynthetic products were identified via the synthesis of derivatives and radiochromatographic analysis. The results presented strongly suggest that camphor is derived by the cyclization of neryl pyrophosphate to borneol followed by dehydrogenation of this bicyclic alcohol. This is the first report on the enzymes involved in the biosynthesis of camphor.

Camphor (1,7,7-trimethylbicyclo[2.2.1]-2-heptanone) is a bicyclic monoterpene ketone produced by a number of plant species including *Salvia*, where it appears to function as a phytotoxin inhibiting the growth of competing grasses (1). Ruzicka and associates (2), in formulating the biogenetic isoprene rule, suggested a scheme for the formation of the camphane (bornane) family of monoterpenes in which neryl pyrophosphate is first cyclized to an α-terpinyl cation, which then undergoes internal addition of the positive center to the double bond to yield the camphane nucleus (i.e., a bornyl cation). The labeling pattern of camphor derived from exogenous precursors such as [2-^{14}C]mevalonic acid and [2-^{14}C]geraniol in intact tissue is consistent with such an hypothesis (3, 4); however, a cell-free biosynthetic system has not been reported. In this communication we describe a soluble enzyme preparation obtained from sage (*Salvia officinalis*) leaves that converts

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neryl pyrophosphate to borneol and then dehydrogenates this bicyclic alcohol to camphor.

MATERIALS AND METHODS

[1-^{3}H]Neryl pyrophosphate and (±)-[3-^{3}H]α-terpineol (both 204 Ci/mole) were prepared as described previously (5).

Young, rapidly expanding sage leaves (5) were powdered in liquid N_2, and the frozen powder was ground in a Ten-Broeck homogenizer with an equal weight of insoluble polyvinylpyrrolidone (Polyclar AT) in cold 0.1 M sodium phosphate buffer (pH 6.5) containing 0.25 M sucrose, 1 mM dithioerythritol, 10 mM ascorbic acid, 10 mM $Na_2S_2O_5$, and 0.5 mM EDTA. The homogenate was slurried with an equal tissue weight of polystyrene resin (Amberlite XAD-4), and particulate matter was removed by centrifugation at 27,000*g*, followed by centrifugation at 105,000*g* for 90 min. The soluble supernatant was fractionated by addition of $(NH_4)_2SO_4$ at 0°C. The precipitate obtained between 20-60% saturation was suspended in a minimum volume of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.5 mM dithioerythritol and 1 mM ascorbic acid, and dialyzed against this same buffer overnight. The protein content of this preparation, which was used as the enzyme source, was determined by the method of Lowry *et al.* (6).

In the enzyme assays, centrifuge tubes containing the enzyme, appropriate amounts of additions and cofactors, and an aliquot of substrate (1.5×10^6 cpm, 20 nmoles) in a final volume of 1 ml 0.1 M sodium phosphate buffer (pH 8.0) were incubated (sealed under N_2) at 30°C in a shaking water bath for up to 3 hr. At the end of the incubation period, each sample was extracted with diethyl ether (3 × 1.3 ml), and internal standards ((±)-borneol, (±)-camphor, etc.) were added to each extract. These extracts were either analyzed directly by thin-layer chromatography (1 mm layer of Silica Gel G with hexane:ethyl acetate (4:1, v/v) as developing solvent (system A)), or were treated with OsO_4 (2 mg OsO_4 plus 50 µl pyridine) overnight. The osmic esters were decomposed to diols with aqueous $NaHSO_3$, and the ether-soluble materials recovered and analyzed by thin-layer chromatography as above. The appropriate regions were located and the silica gel from these regions was either transferred directly to a counting vial and the ^{3}H content assayed by liquid scintillation spectrometry (5), or the radioactive products were eluted from the gel with diethyl ether for further analysis.

The preparation and chromatographic analysis of trimethylsilyl ethers, acetates, and benzoates has been described before (5). Two-phase chromic acid oxidation of borneol (50 mg in ether) was carried out by published procedures (7). The yield of camphor in this semi-micro preparation was about 90%, and the product was purified by thin-layer chromatography (system A). Bornyl phenylurethane was prepared by treating borneol (50 mg) in hexane with phenylisocyanate (0.3 g). The insoluble diphenylurea was removed by filtration and bornyl phenylurethane was recrystallized to constant specific activity from hexane. Camphor oxime was prepared by refluxing camphor (50 mg) in ethanol: pyridine (20:1) with hydroxylamine-HCl (50 mg), and the resulting products were partitioned between ether and H_2O. Evaporation of the ether phase afforded the crude oxime which was purified by thin-layer chromatography (silica gel, hexane:ethyl acetate (7:3, v/v)), or was recrystallized to constant specific activity from aqueous ethanol. Camphor was reduced to isoborneol with excess $NaBH_4$ in methanol overnight at 20°C. Decomposition of the reaction mixture with dilute HCl followed by ether extraction yielded the product, which was analyzed by thin-layer chromatography (system A). Gas-liquid chromatography columns were 10 ft. × 0.125 in. o.d. stainless-steel packed with 12% carbowax

4000 on 80/100 mesh Gas Chrome Q, and 8 ft. × 0.125 in. o.d. stainless-steel packed with 5% OV-1 on 60/80 mesh Gas Chrome Q. Other chromatographic conditions used are described under the appropriate figures.

RESULTS AND DISCUSSION

Soluble enzyme preparations obtained from young sage leaves convert the acyclic precursor [1-^{3}H]neryl pyrophosphate to several cyclic monoterpenes including α-terpineol and 1,8-cineole (5). Thin-layer chromatographic analysis of the biosynthetic products formed under the conditions of the assay (pH 6.5, 2 mM $MnCl_2$, 10 mM $MgCl_2$) revealed the presence of a minor component that had the chromatographic properties of borneol. Although the volatile oil of sage does contain borneol and a significant quantity of the corresponding ketone camphor (10-15%) (5), suggesting the possibility that borneol might be formed in the cell-free preparation, the yield of the product was too low to permit adequate identification. In an attempt to improve the specific activity of this preparation, the 105,000*g* supernatant was fractionated by $(NH_4)_2SO_4$ precipitation and the fraction precipitating between 20-60% saturation was assayed at pH 8.0 in the presence of 10 mM $MgCl_2$. Thin-layer chromatographic analysis of the ether-soluble products obtained under such conditions revealed a ten-fold increase in the yield of the borneol-like product. OsO_4 treatment did not alter the chromatographic properties of this unknown, indicating that it did not contain unsaturation. Neither did $LiAlH_4$ reduction change the properties of the product, confirming the absence of an epoxide or carbonyl function. The isolated product was shown to be coincident with borneol when subjected to radio gas-liquid chromatography on two columns of widely differing polarity (Figure 1a).

Benzoylation of the unknown, followed by thin-layer chromatographic analysis of the products, revealed the presence of a single radioactive component, coincident with bornyl benzoate (Figure 1b). Radio gas-liquid chromatography of this derivative indicated that all of the radioactivity was coincident with authentic bornyl benzoate (Figure 1c). Synthesis of the ace-

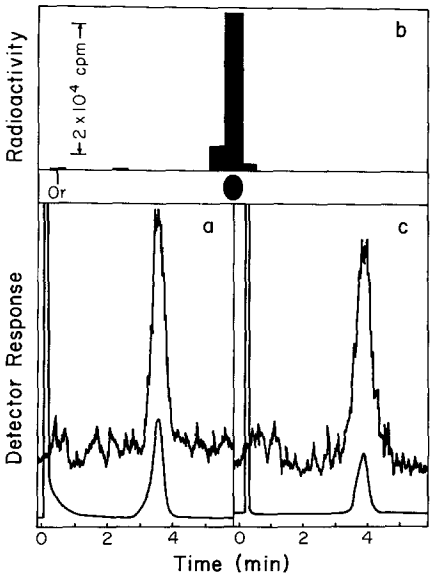

Figure 1. (a) Radio gas-liquid chromatogram of borneol isolated from an extract of sage leaves that had been incubated with [1-^{3}H]neryl pyrophosphate. The smooth bottom tracing is the flame ionization detector response obtained from co-injected authentic (±)-borneol. The shoulder at the leading edge of the peak is due to isoborneol. The top tracing shows the radioactivity recorded by a model 7357 Nuclear-Chicago radioactivity monitor attached to the gas chromatograph. Gas-liquid chromatography was performed on the OV-1 column described in the Methods section, held at 100°C with an argon flow rate of 150 cm^3/min. Chromatography on the carbowax column gave similar results. (b) Radio thin-layer chromatogram of the benzoylated products of the borneol described in 1a above. Thin-layer chromatography was performed on Silica Gel G with hexane:diethyl ether (9:1, v/v) as the developing solvent. The bar graph represents ^{3}H contained in 1 cm sections of gel. The standard indicated is authentic bornyl benzoate. Or is the origin. (c) Radio gas-liquid chromatogram of the bornyl benzoate fraction isolated from the thin-layer chromatogram shown in Figure 1b. The bottom tracing is the detector response obtained from co-injected bornyl benzoate. The top tracing shows radioactivity. Gas-liquid chromatography was performed on the OV-1 column held at 220°C with a flow rate of 150 cm^3/min.

tate and the trimethylsilyl ether of the unknown gave similar results; a single radioactive product, coincident with the appropriate borneol derivative on both thin-layer and gas-liquid chromatographic analyses. The bornyl phenylurethane was also prepared and recrystallized to constant specific activity. To confirm the identification, the labeled borneol was oxidized with chromic acid. Thin-layer chromatography (system A) of the resulting products revealed the presence of a single radioactive component, coincident with cam-

phor. Isolation of this radioactive material, followed by radio gas-liquid chromatography, showed that the product coincided with authentic camphor. Furthermore, the oxime of the oxidized product was coincident with camphor oxime on both radio thin-layer and radio gas-liquid chromatographic analyses. Thus, the labeled product derived from neryl pyrophosphate was identified as borneol.

$MgCl_2$ was required for borneol synthesis, and the amount of borneol formed increased as the $MgCl_2$ concentration was increased up to about 20 mM, beyond which little further stimulation of activity was observed (Table I). The rate of conversion of neryl pyrophosphate to borneol increased linearly with an increase in protein concentration up to about 3 mg/ml for a 2 hr assay

Table I: Cofactor Requirements for the Conversion of Neryl Pyrophosphate to Borneol and Camphor by the Soluble Preparation from *Salvia officinalis*.

Additions	Product Formed	
	Borneol	Camphor
	(cpm × 10^{-4})	
None	0.12	0.07
5 mM $MgCl_2$	3.13	0.41
10 mM $MgCl_2$	5.96	0.85
20 mM $MgCl_2$	6.79	0.98
40 mM $MgCl_2$	6.98	1.04
40 mM $MgCl_2$ (boiled enzyme)	0.03	0.06
20 mM $MgCl_2$, 1.5 mM NADP	4.87	3.01
20 mM $MgCl_2$, 1.5 mM NAD	3.38	11.85
20 mM $MgCl_2$, 1.5 mM NAD (boiled enzyme)	0.02	0.07
1.5 mM NADP	0.09	0.09
1.5 mM NAD	0.06	0.11

Each reaction mixture containing the additions indicated, 2.8 mg protein (precipitated at 20-60% saturation with $(NH_4)_2SO_4$), 20 μM [1-^{3}H]neryl pyrophosphate (1.5 × 10^6 cpm), 0.5 mM dithioerythritol, 1 mM ascorbic acid, and 10 mM NaF in a total volume of 1.0 ml 0.1 M sodium phosphate buffer (pH 8.0), was incubated anaerobically at 30°C for 3 hr.

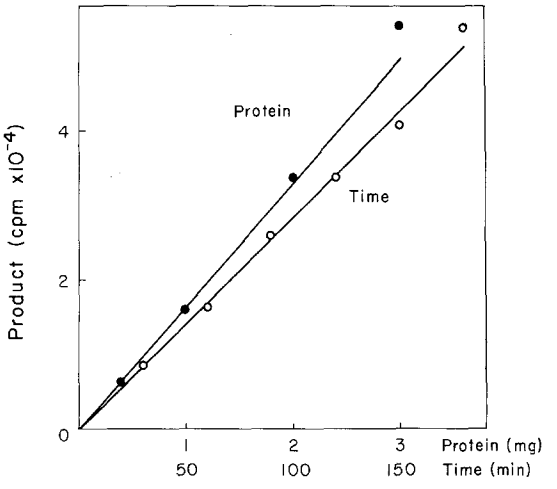

Figure 2. Effect of protein concentration and time on the conversion of neryl pyrophosphate to borneol by the soluble preparation from *S. officinalis*. Conditions and assay procedures are described under Table I. For the time-course experiment the protein level was 2 mg/ml, and for the protein dependence experiment the assay time was 2 hr.

(Figure 2). The rate of the reaction was linear up to at least 3 hr at the 2 mg/ml protein level (Figure 2).

Although neryl pyrophosphate was readily converted to borneol, a specific search of the enzymatic products for the diastereomeric isoborneol revealed that this alcohol was not formed in detectable yield. A small amount of α-terpineol was formed by the cell-free preparation. However, synthetic (±)-[3-^{3}H]-α-terpineol was not converted to borneol by the enzyme preparation, indicating that α-terpineol was not an intermediate in borneol biosynthesis.

In addition to α-terpineol, another minor radioactive component was formed by the enzyme extract when incubated with [1-^{3}H]neryl pyrophosphate. This component had the same R_f as camphor, and the OsO_4 treatment did not influence the chromatographic properties of this compound, indicating the absence of unsaturation. On the other hand, $NaBH_4$ reduction did alter the properties of the product, suggesting the presence of a carbonyl function. These results indicated that a dehydrogenase might be present in the preparation that converts borneol to camphor. To examine this possibility, assays

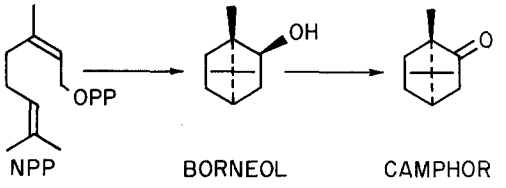

Figure 3. Proposed pathway for the biosynthesis of camphor from neryl pyrophosphate. The (+)-isomers of borneol and camphor are shown.

were conducted in the presence of NAD and NADP. Thin-layer chromatographic analysis of the products isolated from the incubation mixture revealed that, in the presence of the oxidized pyridine nucleotides, much higher levels of the camphor-like product were formed (Table I). NAD was the preferred cofactor. The camphor-like product was isolated and subjected to radio gas-liquid chromatography on two different columns. In both cases, all of the radioactivity was coincident with an authentic camphor standard. Treatment of the product with hydroxylamine-HCl, followed by isolation of the products and thin-layer chromatographic analysis, revealed only one radioactive region, coincident with camphor oxime. Subsequent radio gas-liquid chromatography showed all of the radioactivity to be coincident with authentic camphor oxime, and the oxime was recrystallized to constant specific activity. Additionally, treatment of the camphor with excess $NaBH_4$ gave rise to isoborneol as predicted (8), and the identity of this reduction product was confirmed by radio thin-layer and radio gas-liquid chromatographic analyses. Thus, camphor was formed in the cell-free preparation.

NAD or NADP, alone, did not allow camphor formation from neryl pyrophosphate; Mg^{++} was required (Table I). These results strongly suggested that camphor was derived from the biosynthetic borneol. In fact, [^{3}H]borneol isolated from the reaction mixture was shown to undergo dehydrogenation directly to camphor in the presence of NAD, and Mg^{++} was not required under these conditions (data not shown).

The data presented clearly show that the soluble enzyme preparation

reported here contains both a cyclase and a dehydrogenase, and these results strongly suggest that camphor is synthesized by dehydrogenation of borneol formed by the cyclization of neryl pyrophosphate (Figure 3). α-Terpineol is not an intermediate in borneol biosynthesis, and it is possible that the cyclization of neryl pyrophosphate to the bicyclic alcohol is direct, and does not involve any free intermediates. We are now in the process of purifying and characterizing these new enzymes in order to examine this biosynthetic pathway in greater detail.

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