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BIOSYNTHESIS OF MONOTERPENOIDS BY *SANTOLINA CHAMAECYPARISSUS* L.*†

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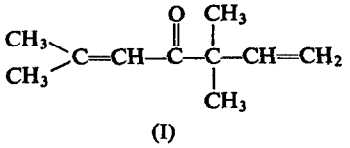
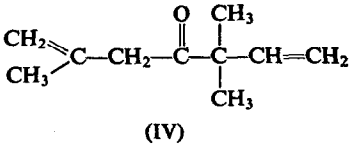
Abstract—Radioactivity from mevalonate-2-^{14}C was shown to be incorporated into β-pinene, myrcene and three unidentified monoterpenes in *Santolina chamaecyparissus* L. Artemisia ketone (3,3,6-trimethyl-1,5-heptadien-4-one), regarded as a non head-to-tail monoterpenoid, did not incorporate *significant* label from mevalonate-2-^{14}C.

A REINVESTIGATION of the steam-volatile oil of the ornamental shrub *Santolina chamaecyparissus* L. has shown that the largest component is 3,3,6-trimethyl-1,5-heptadien-4-one (I) also known as artemisia ketone.[1] Ketone I, first isolated from *Artemisia annua* L., has been regarded as an atypical monoterpenoid in which the tail of one isoprene unit is linked to one of the central carbon atoms of the other in contrast with the usual head-to-tail arrangement.[2]



(I)



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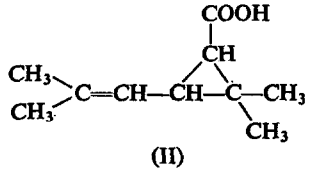

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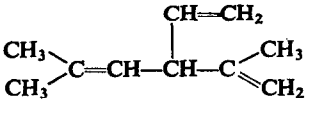

(III)

STRUCTURES OF: ARTEMISIA KETONE (I); CHRYSANTHEMIC ACID (II); 2,5-DIMETHYL-3-VINYLHEXA-1,4-DIENE (III); ISOARTEMISIA KETONE (IV).

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[1] L. H. ZALKOW, D. R. BRANNON and J. W. UECKE, *J. Org. Chem.* **29**, 2786 (1964).

[2] J. H. RICHARDS and J. B. HENDRICKSON, *The Biosynthesis of Steroids, Terpenes and Acetogenins*, p. 211, Benjamin, New York (1964).

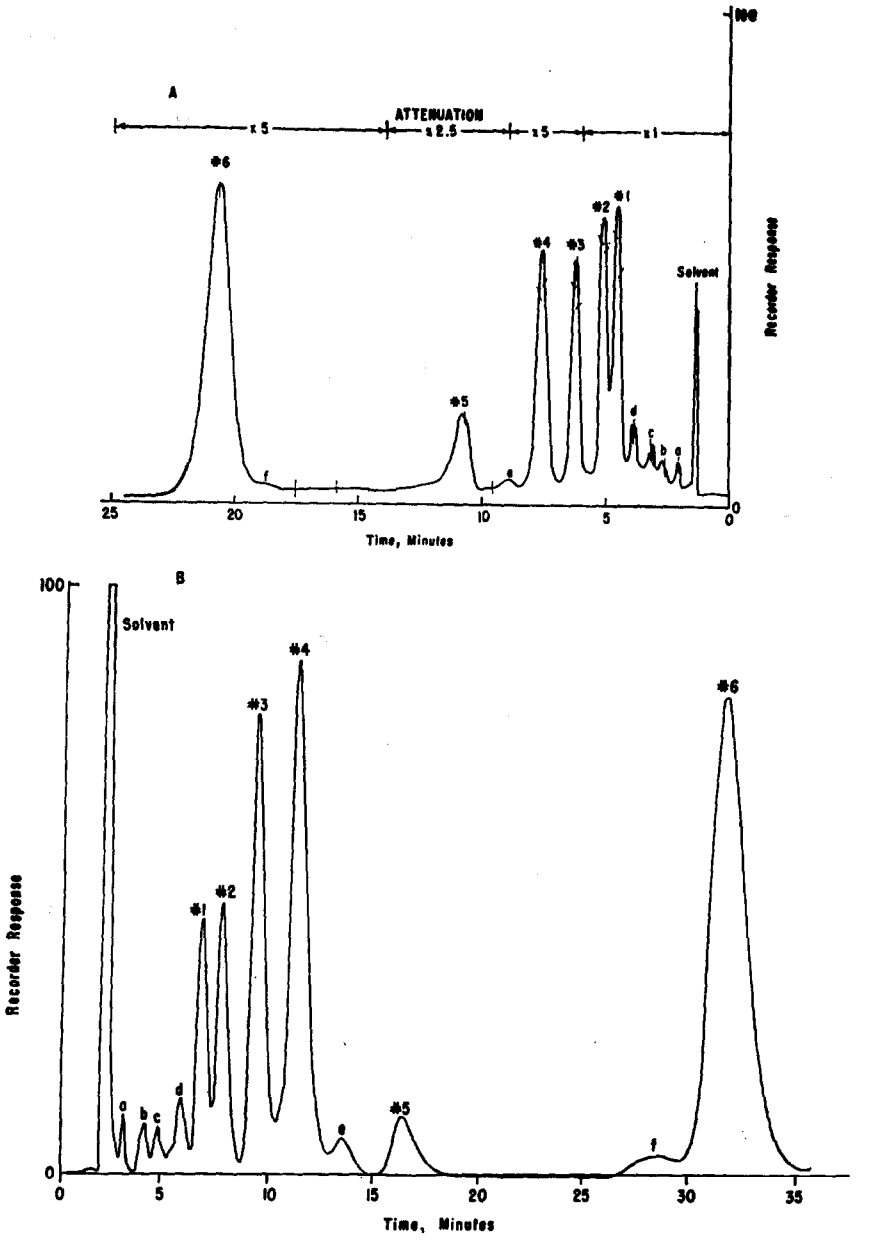

FIG. 1. MASS SPECTROMETRIC–GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF *Santolina chamaecyparissus* L. OIL.

A (top). Mass spectrometer–gas chromatograph tracing. The column dimensions were a 10-ft × ¼-in. glass packed with 6% LAC 728 on acid-washed Chromosorb W. The column temperature was kept at 70°, injection port at 150°, separators at 250° and ion source at 310°. The He flow rate was 50 ml/min. One μl. of oil was injected. Slash (/) marks indicate the places where mass spectra were taken. B (bottom). Hydrogen flame gas chromatograph tracing. Column used and amount of oil injected was the same as in A above. The column temperature was kept at 70°, injection port at 150°, and detector at 225°; the He flow rate was 50 ml/min, the H_2 was 12 psi and the air was 30 psi. The attenuation on the Barber–Colman Gas Chromatograph was 300.

Bates and Paknikar[3] have proposed a biogenetic scheme involving isoprene units for the production of artemisia ketone, chrysanthemic acid (II) and 2,5-dimethyl-3-vinylhexa-1,4-diene (III). Chrysanthemic acid has been shown to be derived from mevalonic acid.[4] 2,5-Dimethyl-3-vinylhexa-1,4-diene has been identified in the essential oil from the flowers of *S. chamaecyparissus* L.[5] Earlier it was proposed that artemisia ketone could be derived from Δ^3-carene[6] or from limonene by methyl migration[7] and ring opening.

In earlier experiments in this laboratory the essential oil from *S. chamaecyparissus* plants previously fed with mevalonate-2-^{14}C was analyzed by gas radiochromatography. The peak which corresponded to artemisia ketone contained radioactivity and overlapped component *5*, minor component *f* and a minor component of uncertain identity (but with a retention time longer than artemisia ketone) visible only on the Ucon Polar columns. Further investigation using an improved gas-chromatography system gave further resolution showing a strongly radioactive minor component (Peak No. *5*, Fig. 1) previously obscured by the artemisia ketone peak. The present communication describes further experiments showing that artemisia ketone was poorly labeled from mevalonate-2-^{14}C whereas five monoterpene hydrocarbons incorporated significant radioactivity from the same precursor.

RESULTS

Gas Chromatographic Analysis

The detector response obtained when about 1 mg of *Santolina chamaecyparissus* oil was injected on the LAC column with the hydrogen flame detector instrument and the combination mass spectrometer–gas chromatograph is shown in Fig. 1. The major components were eluted from the Ucon column in the same order except that components 1 and 2 were not separated. The retention times are slightly shorter on the MS–GC tracing; this may be due to the vacuum which is placed on the column as the effluent passes through the molecular separators.[8–10] Component *5* was barely separated from component *6* (artemisia ketone) on the Ucon columns. A number of minor components (not shown in Fig. 1) were eluted when either column was heated to a higher temperature after elution of the ketone; they comprised about 25 per cent of the oil as measured by the total hydrogen flame response.

Component *1* had the same retention time as β-pinene on LAC, Ucon Polar and Apiezon L columns. Component *3* corresponded with myrcene and minor component *c* with α-pinene using these three columns.

Mass Spectrometry

Mass spectra were taken of the compounds as they were eluted from the LAC column (Fig. 1). Table 1 summarizes the pertinent mass spectral data. The mass spectra of components *1* and *3* at both 20 eV and 70 eV were identical with those of β-pinene and myrcene respectively and the spectrum of minor component *c* at 20 eV was the same as that of α-pinene. The mass spectra of components *2*, *4* and *5* all showed parent ions at m/e = 136

[3] R. B. Bates and S. K. Paknikar, *Tetrahedron Letters* 1453 (1965).
[4] M. P. Crowley, P. J. Godin, H. S. Inglis, M. Snarey and E. M. Thain, *Biochim. Biophys. Acta* **60**, 312 (1962).
[5] A. F. Thomas and B. Willhalm, *Tetrahedron Letters* 3775 (1964).
[6] G. R. Clemo, *Perfumery Essent. Oil Record* **41**, 435 (1950).
[7] P. Crabbé, *Rec. Chem. Prog.* (*Kresge Hooker Sci. Lib.*) **20**, 189 (1959).
[8] G. R. Waller, *Proc. Okla. Acad. Sci.* (In press).
[9] R. Ryhage, *Anal. Chem.* **36**, 759 (1964).
[10] R. Ryhage, S. Wikstrom and G. R. Waller, *Anal. Chem.* **37**, 435 (1965).

and fragmentation patterns resembling published data for monoterpene hydrocarbons.[11,12] In view of possible variations due to instrument parameters it was deemed inadvisable to attempt to identify them by comparison with mass spectral data in the literature. Minor component *f* may be isoartemisia ketone (IV) because repetitive spectrum scans over this time showed no compound that did not possess a parent ion at m/e = 152. However, since compound *f* was not completely separated from artemisia ketone in the MS–GC work, it is not possible to be certain of its identity at this time.

TABLE 1. MOLECULAR IONS AND BASE PEAKS OF MASS SPECTRA OF COMPONENTS OF *S. chamaecyparissus* L. OIL

Components	20 eV		70 eV		Identified with standard
	Base peak (m/e)	M^+	Base peak (m/e)	M^+	
Minor *b*	43	110	—	—	—
Minor *c*	93	136	—	—	α-Pinene
Minor *d*	121	136	—	—	—
1	93	136	93	136	β-Pinene
2	93	136	93	136	—
3	93	136	93	136	Myrcene
4	93	136	93	136	—
5	136	136	121	136	—
6	83	152	83	152	Artemisia ketone

Preliminary Labeling Experiments with Acetate-1-^{14}C and Mevalonate-2-^{14}C

Preliminary experiments were conducted by injecting 10 μc of acetate-1-^{14}C and mevalonate-2-^{14}C into 3-month-old *S. chamaecyparissus* plants. The essential oil was isolated 2, 4 and 7 days after injection. The per cent of the administered radioactivity recovered in the essential oil was 0·05, 0·08 and 0·04 per cent for the 2-, 4- and 7-day acetate-1-^{14}C experiment and 1·25, 0·7 and 0·5 per cent for the mevalonate-2-^{14}C experiment. All of the samples obtained in the acetate experiments had too little radioactivity for determining the specific activity of the components by gas radiochromatography so our attention was henceforth devoted to biosynthesis studies using mevalonate-2-^{14}C as a precursor.

Further Labeling Experiments with Mevalonate-2-^{14}C

Three groups of five 3-month-old plants were each injected with a total of 22·4 μc of mevalonate-2-^{14}C. The essential oil was isolated 40 hr, 88 hr and 192 hr following injection. Total ^{14}C activity of the essential oil was determined by liquid scintillation counting (Exp. 5, Table 2).

Samples of the oils were analyzed using the gas-chromatograph continuous-flow counter. The results are given in Table 3 (Exp. 5). Results from another experiment using 8-month-old plants (Exp. 4) are also shown in Table 3. The general labeling pattern obtained from the two experiments was similar in that compound *5* possessed the highest specific activity but the extent of incorporation was much lower in the older plants. In addition one variable was noted—that compound *5* (a terpene hydrocarbon of mass 136) tended to become more

[11] R. RYHAGE and E. VON SYDOW, *Acta. Chem. Scand.* **17**, 2025 (1963).
[12] A. F. THOMAS and B. WILLHALM, *Helv. Chim. Acta* **47**, 475 (1964).

extensively labeled after time lapses in the older plant whereas the opposite appeared to occur in the younger plants. This phenomenon was also true to a lesser extent for the mixture of compounds *1* and *2*.

While β-pinene and myrcene and components *2*, *4* and *5* showed considerable radioactivity, only a small amount of activity was detected in the artemisia ketone peak (Exp. 5).* Radioactivity was detected in some of the minor higher-boiling components eluted after the ketone (about 25 per cent of the total activity). These were not studied further as a satisfactory resolution of the mixture of these components had not been achieved.

TABLE 2. INCORPORATION OF RADIOACTIVITY FROM MEVALONATE-2-^{14}C INTO *S. chamaecyparissus* L. OIL*

Duration of experiment (hr)	Fresh weight of plants (g)	Total radioactivity in oil (mμc)	Incorporation (%)
40	52	151	0·67
88	58	113	0·50
192	49	109	0·49

* These data are from Exp. 5 which is reported on in more detail in Table 3.

TABLE 3. INCORPORATION OF MEVALONATE-2-^{14}C INTO *S. chamaecyparissus* L. MONOTERPENES

Experiment number	Duration of experiment (hr)	Specific activity (μcm/mole) *1* (β-pinene)	*2* (mass=136)	*3* (myrcene)	*4* (mass=136)	*5* (mass=136)
Exp. 5* (3-month-old plants)	40	0·41	0·60	0·14	0·61	1·5
	88	0·29	0·52	0·10	0·30	1·0
	192	0·25	0·28	0·08	0·39	0·93
Exp. 4† (8-month-old plants)	12	0·11		0·014	0·04	0·19
	24	0·18		0·056	0·16	0·43
	96	0·29		0·058	0·17	0·47
	336	0·26		0·04	0·13	0·66

* The LAC GLC column was used.

† The Ucon GLC column was used. Compounds *1* and *2* were not resolved on this column but since both possess a molecular weight of 136 a specific activity representing the mixture is shown.

To establish clearly whether the artemisia ketone obtained in these experiments was labeled, the specific activities of the artemisia ketone peaks collected from the gas chromatograph (Exp. 5) were determined with the liquid scintillation spectrometer. The specific activities were found to be about 1–2 per cent of those of the monoterpene hydrocarbons (Table 4). Further purification of the gas-chromatograph eluate from the 40-hr experiments by preparative thin-layer chromatography caused a four-fold reduction in specific activity

* In Exp. 4 where the Ucon GLC column was used there was 129 ± 23 dpm/mg detected in the artemisia ketone peak but since peak *5* and the ketone were not completely resolved this radioactivity was considered to be the peak *5* compound.

(Table 4). Another purification using thin-layer chromatography yielded artemisia ketone* from the 40-hr experiment that contained 4 ± 4 dpm/mg. Thus, it may be concluded that the artemisia ketone was not significantly labeled in these biosynthesis experiments using mevalonate-2-^{14}C as a potential precursor.

TABLE 4. RADIOACTIVITY OF PURIFIED ARTEMISIA KETONE

Experiment (hr)	Treatment			
	Eluate collected from GLC		Eluate collected after TLC separation	
	(dpm/mg)	(μc/mmole)	(dpm/mg)	(μc/mmole)
40	130	0·0089	30	0·002
88	100	0·0069	22	0·0015
192	90	0·0062		

DISCUSSION

Biosynthesis experiments with young *Santolina chamaecyparissus* plants using acetate-1-^{14}C and mevalonate-2-^{14}C as a precursor of compounds found in the essential oil showed that mevalonate was 10–20 times more efficient. In experiments of 12–336 hr duration with *S. chamaecyparissus* plants no significant incorporation of ^{14}C radioactivity from mevalonate-2-^{14}C into artemisia ketone was detected. In biosynthesis experiments of 40–192 hr duration the five ten-carbon terpene hydrocarbons present as major components of the steam-volatile oil, including myrcene and β-pinene, were found to contain radioactivity. The specific activities of the identified monoterpenes were from 300–1500 times the minimum specific activity of artemisia ketone detectable† under the conditions employed. The specific activities of the TLC eluates in columns 3 and 5 of Table 4 correspond to an isotope dilution of about 2×10^6. These results indicate that artemisia ketone may not be biosynthesized from isoprene units in *S. chamaecyparissus*, however, more definitive evidence is needed before the isoprenoid postulation can be eliminated. Studies to elucidate the biosynthesis of artemisia ketone are continuing in our laboratory.

The specific activities of the terpene hydrocarbons were generally found to be lower in the experiments of longer duration. This may indicate that degradation occurs in the plant but since the hydrocarbons are volatile, the possibility of loss of labeled molecules‡ by evaporation and resulting dilution with unlabeled material cannot be ruled out.

It would not seem likely that compartmentalization could be the explanation for the lack of radioactivity appearing in artemisia ketone in the mevalonate-2-^{14}C experiments; however,

* The artemisia ketone isolated from preparative thin-layer chromatography was determined to be higher than 99 per cent purity by GLC on the hydrogen flame instrument. The impurities were the monoterpene hydrocarbon (compound Nos. *1*, *2*, *3*, and *4*). At the 0·5–1·0 per cent impurity level it can readily be estimated that they contribute from 2–8 dpm/mg to the radioactivity found in the purified artemisia ketone, thereby, possibly accounting for all of the radioactivity found.

† Based on 4 dpm/mg.

‡ This is not meant to imply a preferential loss of labeled molecules but that loss of the terpenes might occur continuously throughout the life of the plant; consequently, terpene loss would be expected to continue throughout the course of the biosynthesis to establish a "steady-state" condition and after all carbon-14 labeled precursors have disappeared from the pool then only unlabeled terpene molecules would be synthesized.

this possibility cannot be ignored since Goodwin[13] has shown that compartmentalization plays a large role in determining the disposition of mevalonate. If compartmentalization did occur then the terpene precursors, when once formed, leak into compartments where they could be secondarily transformed, as for example the terpenes of pines which are made in glandular cells and secreted into the resin ducts where secondary transformations can then occur.[14] Alternatively to considering compartmentalization the existence of an enzyme under metabolic control which was specifically involved in conversion of a terpenoid precursor to the ketone might be postulated. Further study is required to assess the possible significance of these physiological factors in the biosynthesis of artemisia ketone.

EXPERIMENTAL

Materials

Santolina chamaecyparissus L. plants were propagated from cuttings in the greenhouse of the Horticulture Department. Two- to three-month-old cuttings were used in the biosynthesis experiments unless otherwise specified.

Mevalonate-2-^{14}C. The N,N'-dibenzylethylenediamine salt of DL-mevalonic acid-2-^{14}C was obtained from New England Nuclear Corporation. For application to the plants the salt (3·78 mc/mmole of mevalonic acid) was used without prior treatment. The mevalonic acid-2-^{14}C was 99·6 per cent pure as determined by paper chromatography in ethanol:ammonia:water (80:5:15) and subsequent analysis of the paper strip in the Nuclear Chicago 4π Actigraph III scanner.

Monoterpene standards. α-Pinene, β-pinene, myrcene and *d*-limonene were obtained from Hercules Powder Company, Wilmington, Delaware.

Administration of labeled compounds. Solutions of labeled mevalonate were applied to plant stems in 1–3 μl portions at punctures made with a syringe needle in the outer tissues. The solutions were taken up by the plant within 5–15 min. A series of applications was made at each of several sites on each plant followed by applications of distilled water.

Isolation of the essential oil. A steam distillation-continuous ether extraction apparatus was employed. The ethereal solution of the oil was dried over anhydrous magnesium sulfate. The oil was stored at $-15°$ in a dilute solution in ether until used.

Methods

Gas–liquid chromatography. For analytical work a modified Barber–Colman Model 5000 gas chromatograph equipped with a hydrogen flame detector was used with 3 m × 6 mm glass columns of 20% Ucon Polar on Chromosorb W at 100°, 20% Apiezon L on silanized Gas-Pack S at 125° and 6% LAC 728 on acid-washed Chromosorb W at 70°. For radioisotope analysis with the continuous-flow counter a Beckman GC 2A gas chromatograph with a 3 m × 6 mm stainless-steel column of 20% LAC 728 on acid-washed Chromosorb W at 70° was used. When *S. chamaecyparissus* oil was chromatographed, the column temperature was increased to 190° after elution of artemisia ketone to elute minor components of long retention time. For quantitative determination the peak areas were measured with a planimeter and compared with those of the peaks obtained by injecting known amounts of *d*-limonene.

Mass spectrometry. The prototype (6) of the LKB* Mass Spectrometer–Gas Chromatograph Model 9000 was used to obtain mass spectra of the eluate from the LAC 728 glass column. The gas–liquid chromatography columns are interchangeable between the Barber–Colman Model 5000 and this instrument. The separator temperature was 250° and the source temperature 310°. Spectra were taken at 20 eV and 70 eV at a scan speed of m/e 12–200 in 1·25 sec.

Isotype analysis. A Packard Tricarb† Model 3003 liquid scintillation spectrometer was used to determine ^{14}C activity of solutions. Appropriate corrections were made for quenching when necessary by using the external standard supplied with the instrument.

Activity of the eluted peaks from the gas chromatograph was determined by means of a Nuclear-Chicago Model 8200 Proportional Gas Flow Counter equipped with an 85-ml counting chamber. The total effluent (40 cm^3/min) from the gas-chromatograph detector was fed into the counting chamber through an inlet line

* LKB Instruments, Inc., Rockville, Maryland.

† Packard Instrument Co., La Grange, Illinois.

[13] T. W. GOODWIN, In *Biosynthetic Pathways in Higher Plants* (edited by J. B. PRIDHAM and T. SWAIN), p. 57. Academic Press, New York (1965).

[14] J. BONNER and J. E. VARNER, *Plant Biochemistry*, p. 674. Academic Press, New York (1965).

maintained at 225°. Reagent grade methane at a flow rate of 40 cm^3/min was used as the diluent gas, and the temperature of the counting chamber was 225°. The instrument was calibrated using *n*-octane-2-^{14}C with a specific activity of 24 mμc/mmole.

Purification of artemisia ketone. Samples of the oil from the biosynthesis experiments were injected on the Beckman GC 2A gas chromatograph using the 20% LAC column at 100°. The artemisia ketone peaks were collected in a −78° trap and ethereal solutions were applied to Silica Gel G thin-layer plates. The thin-layer chromatograms were irrigated with benzene–ethanol (98:2) and the artemisia ketone band located by staining a portion with iodine vapor. The sections (leading, center, tailing) of the unstained artemisia ketone bands were eluted with ether. The purity of the artemisia ketone thus obtained was estimated by injecting a sample of the samples obtained from preparative TLC on the analytical gas chromatograph.

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