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THE BIOSYNTHESIS OF METHYLCYCLOPENTANE MONOTERPENOIDS—II.

NEPETALACTONE*†‡

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Abstract—Radioactivity from mevalonate-2-^{14}C is incorporated into nepetalactone and into several terpene hydrocarbons by *Nepeta cataria* L. plants. A convenient chemical degradation procedure using alkaline hydrogen peroxide was developed to determine the distribution of label in carbon atoms 3 and 8. The Kuhn–Roth oxidation of nepetalactone was used to determine the extent of labeling in carbon atoms 8 and 9. A limited randomization of radioactivity between carbons 3 and 8 and also carbons 6 and 9 of nepetalactone was observed.

INTRODUCTION

Nepeta cataria L. plants produce the feline attractant[1] nepetalactone (Fig. 1 (I)) chemically related to a large family of methylcyclopentane monoterpenoids[2–7] which are attracting increased attention. Little is known concerning the physiological role of these compounds in nature. The unusual attraction of cats to nepetalactone has been studied by Todd.[8] Several of the methylcyclopentane monoterpenoids isolated from the Argentine ant (*Iridomyrmex humulis*),[9] the Australian ant (*I. detectus*)[10] and the Southern walking-stick (*Anisomorpha buprestoides*)[11, 12] have been found to be arthropod defense chemicals. It has been shown that nepetalactone is an insect repellent and suggested that it may protect plants from phytophagous insects.[13] Elucidation of the structures and stereochemistry of many of the methylcyclopentane monoterpenoids by comparisons with nepetalactone and its degradation products has given this compound a central role in the chemistry of this interesting group of natural products.[2–7]

* For the first paper of this series see H. AUDA, H. R. JUNEJA, E. J. EISENBRAUN, G. R. WALLER, W. R. KAYS and H. H. APPEL, *J. Am. Chem. Soc.* **89**, 2416 (1967).

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[3] E. J. EISENBRAUN and S. M. MCELVAIN, *J. Am. Chem. Soc.* **77**, 3383 (1955).
[4] G. W. K. CAVILL and H. D. LOCKSLEY, *Australian J. Chem.* **10**, 352 (1957).
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[6] J. H. RICHARDS and J. B. HENDRICKSON, *The Biosynthesis of Steroids Terpenes and Acetogenins*, pp. 221–224. Benjamin, New York (1964).
[7] G. W. K. CAVILL, *Rev. Pure Appl. Chem.* **10**, 169 (1960).
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[10] L. M. ROTH and T. EISNER, *Ann. Rev. Entomol.* **7**, 107 (1962).
[11] J. MEINWALD, M. S. CHADHA, J. J. HURST and T. EISNER, *Tetrahedron Letters* 29 (1962).
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[13] T. EISNER, *Science* **146**, 1318 (1964).

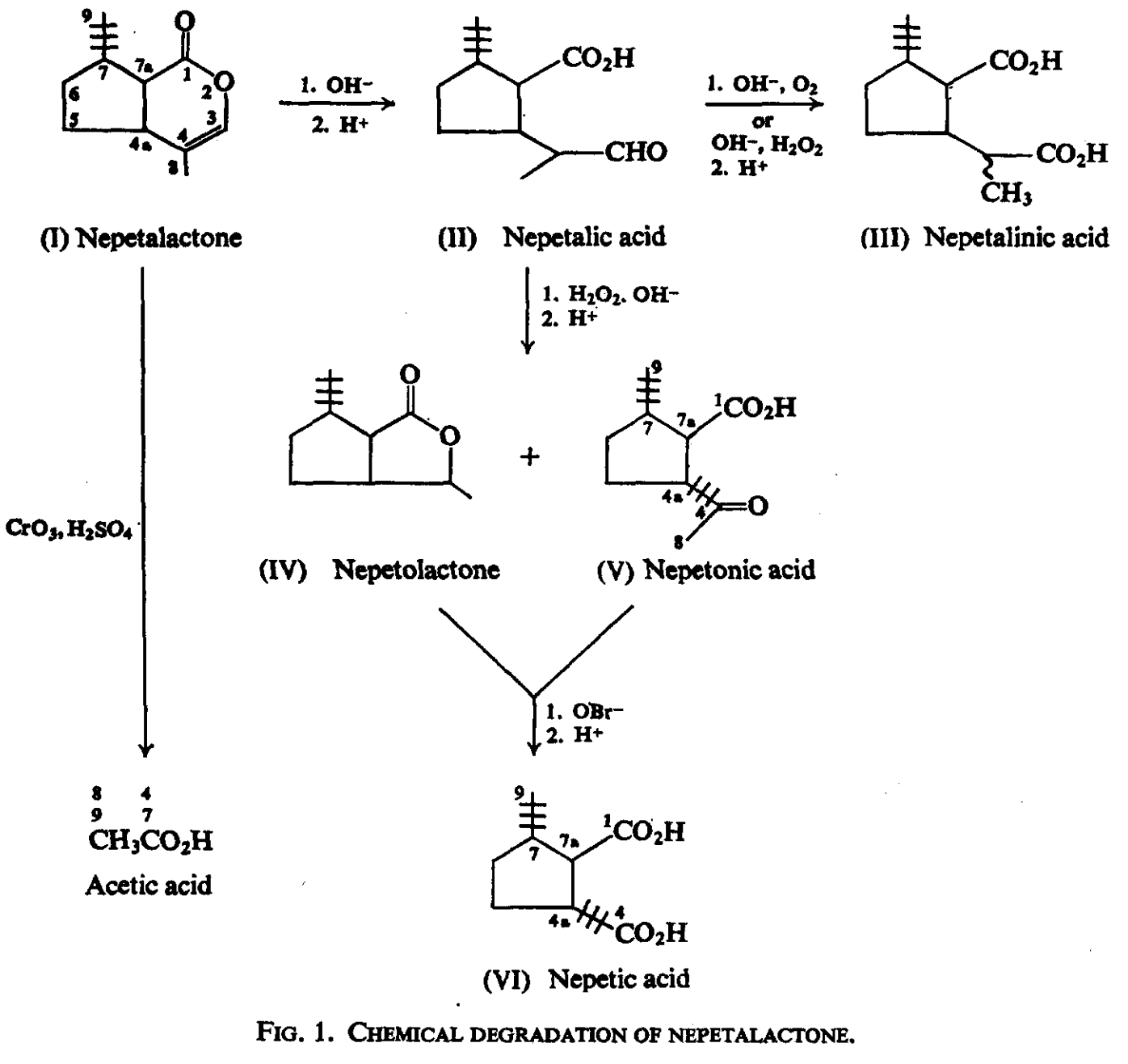


FIG. 1. CHEMICAL DEGRADATION OF NEPETALACTONE.

From the structure of nepetalactone it seems likely that an isoprenoid biosynthesis pathway would be utilized in its formation. Preliminary communications by Regnier *et al.*[14] and Meinwald *et al.*[15] tend to support this concept. This paper reports studies on the incorporation of mevalonic acid-2-^{14}C into nepetalactone.

RESULTS AND DISCUSSION

The essential oil from the leaves, stems, and flowers of mature *Nepeta cataria* L. plants contains 80–95% nepetalactone (I) and epinepetalactone,[16] 2–10% caryophyllene, dihydronepetalactone and humulene and 1–5 per cent of a mixture of other compounds.[17] These parts of the plant yield approximately 0·2 per cent of this steam-volatile oil on a fresh-weight basis. Plants 1 month old were found to contain 0·15 per cent of nepetalactone and 0·01 per cent of a mixture of mono- and sesquiterpene hydrocarbons.[17] It appears that there is a continuous increase of these compounds which reaches a maximum at senescence.

There is considerable variation in the absolute content of nepetalactone and the terpene

[14] F. E. REGNIER, E. J. EISENBRAUN and G. R. WALLER, *Abstracts* 150*th Mtg Am. Chem. Soc.*, Sect: C, p. 166. Atlantic City, New Jersey, September, 1965.
[15] J. MEINWALD, G. M. HAPP, J. LABOWS and T. EISNER, *Science* **151**, 79 (1966).
[16] F. E. REGNIER, E. J. EISENBRAUN and G. R. WALLER, *Phytochem.* **9**, 1271 (1967).
[17] F. E. REGNIER, G. R. WALLER and E. J. EISENBRAUN, *Phytochem.* **9**, 1281 (1967).

hydrocarbons between the upper parts of the plant and the root system. Roots (693 g) yielded 5×10^{-4} per cent (4 mg) of nepetalactone and 4×10^{-6} per cent (0·03 mg) of the terpene hydrocarbons. This yield of nepetalactone is 3000 times smaller than was obtained from an equivalent weight of leaves and stems. The ratio of nepetalactone to the terpene hydrocarbons (125:1) differs considerably from that found in the upper parts of the plant.

Mevalonate-^{14}C as a Terpenoid Precursor

When mevalonate-2-^{14}C was tested as a precursor for the biosynthesis of nepetalactone and terpene hydrocarbons by *N. cataria* L. plants of different ages, radioactivity was incorporated into these compounds. The extent of incorporation varied with time and with the parts of the plant. The incorporation of radioactivity from mevalonate-2-^{14}C in young plants (approx. 4 cm tall) into nepetalactone and the terpene hydrocarbons as a function of time is

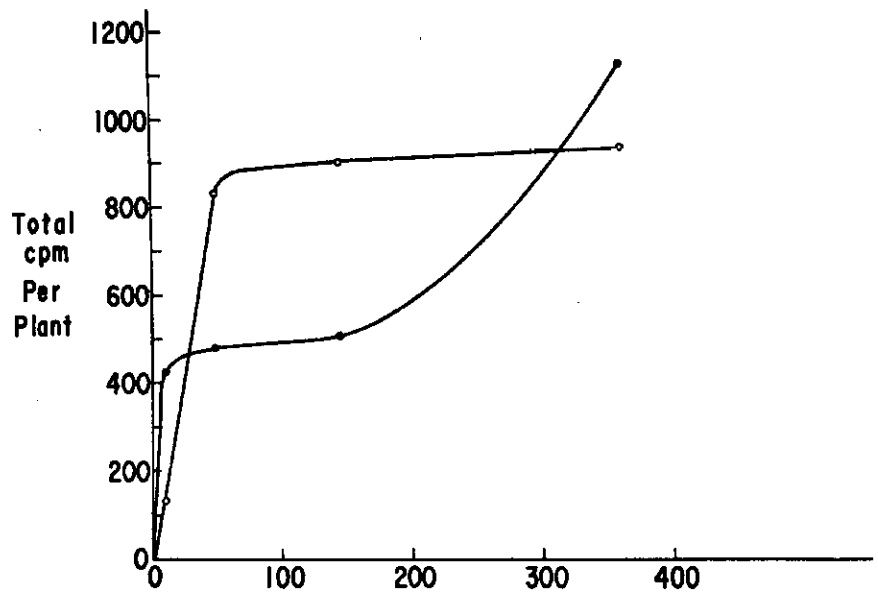


FIG. 2. INCORPORATION OF RADIOACTIVITY FROM MEVALONATE-2-^{14}C INTO NEPETALACTONE AND TERPENE HYDROCARBONS PRODUCED BY *Nepeta cataria* L. PLANTS.

0·32 μc of mevalonate-2-^{14}C was injected into the stem of plants weighing 1·1–2·6 g. At appropriate intervals four plants were harvested and the nepetalactone and terpene hydrocarbons isolated and their radioactivity measured on a TLC plate as described under Methods. ○—Terpene hydrocarbons; ●—Nepetalactone.

shown in Fig. 2. The initial rapid incorporation of carbon 14 into the terpene hydrocarbons was observed to level off at about 50 hr and remain constant for the duration of the experiment. In contrast, there was an initial rapid period of incorporation of carbon 14 into nepetalactone which leveled off at about 12 hr, remained constant for nearly 125 hr and then began to increase again. It is possible that this peculiar phenomenon results from a lower rate of synthesis in old leaves than in young leaves as observed by Battaile and Loomis in *Mentha pulegium*.[18] The specific activity of nepetalactone isolated from new leaves is 6·3 times that isolated from old leaves of the same plant (Table 1). Possible explanations for the higher specific activity of the material from new leaves are: (1) there may be greater metabolic activity which would cause an increased rate of synthesis in the new leaves or (2) the specific activity of the material in old leaves may be lowered by dilution with material formed prior to the experiment. It is impossible to reach a definite conclusion until more information on the metabolism of nepetalactone is available.

[18] J. BATTAILE and W. D. LOOMIS, *Biochim. Biophys. Acta* **51**, 545 (1961).

TABLE 1. INCORPORATION OF RADIOACTIVITY FROM MEVALONIC ACID-2-^{14}C INTO NEPETALACTONE PRODUCED BY *N. cataria* L. PLANTS GROWN HYDROPONICALLY

Precursor	Nepetalactone: Plant part	Nepetalactone: Radioactivity recovered (cpm/mg)	Nepetalactone: Ratio radioactivity in young leaves to old leaves
Mevalonic acid-2-^{14}C sp. act. 2·0 μc/μmole (1·5 μmoles administered by injecting 0·21 μmole per day for 7 days)	Young leaves	257	6·3
	Old leaves	41	

To establish the extent that mevalonate-2-^{14}C was being metabolized to $^{14}C_2O$, the CO_2 released by the plants after administration of the radioisotope was collected and counted (Fig. 3). The increase of respiratory $^{14}CO_2$ was linear with respect to time up to 30 hr for plants exposed to 12-hr days, after which no further increase in evolution of $^{14}CO_2$ was observed. A total of 0·14 per cent of the administered mevalonate-2-^{14}C was evolved as respiratory CO_2 during the 50-hr experiment. Only about 25 per cent as much $^{14}CO_2$ was released by plants grown entirely in the dark (Fig. 3). These results make it seem unlikely that the carbon 14 incorporated into the terpenes resulted from expired $^{14}CO_2$. It may be concluded that metabolism of mevalonate-2-^{14}C occurred at a faster rate when the plants were exposed to light.

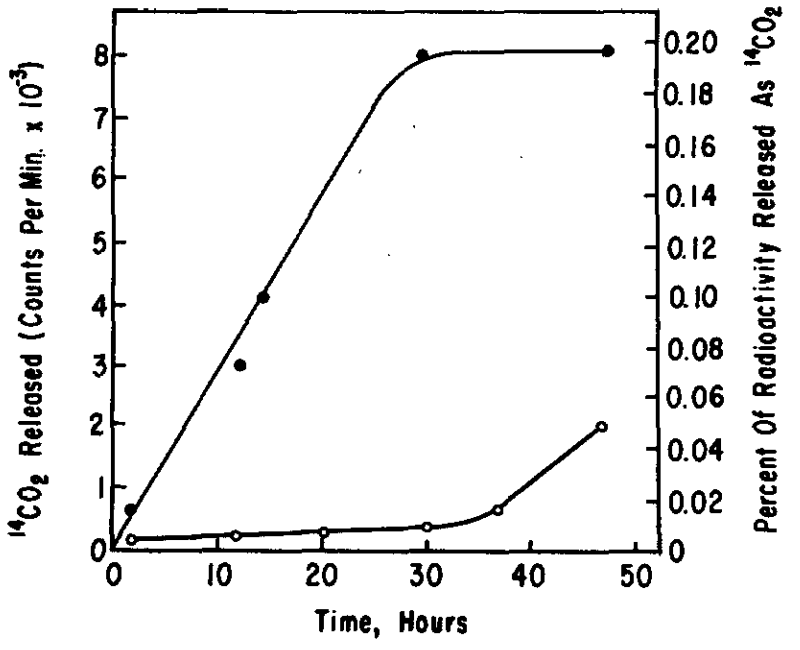


FIG. 3. $^{14}CO_2$ RELEASED BY YOUNG *Nepeta cataria* PLANT AFTER ADMINISTRATION OF MEVALONATE-2-^{14}C.

1·9 μc of mevalonate-2-^{14}C were administered to the plant by injection into the stem. The plant was maintained in a closed chamber. The CO_2 was collected in a NaOH trap and counted using a liquid scintillation spectrometer. ●–●—12-hr day plants; ○–○—dark-grown plants.

A preliminary investigation of the site of synthesis of nepetalactone indicated that the roots were not required. Plants with excised roots were dipped into an aqueous solution containing 1·9 μc of the sodium salt of mevalonic acid-2-^{14}C (sp. act., 1·0 mc/mmole). After 24 hr nepetalactone and the terpene hydrocarbons were isolated by TLC and their radioactivity measured. The results indicated that 0·01 per cent of the administered radioactivity

was recovered in nepetalactone and 0·03 per cent in the terpene hydrocarbons. These results were similar to those obtained using whole plants (Fig. 2), consequently, it may be concluded that roots are not required for the biosynthesis of these terpenoids.

The rate of uptake of mevalonate-2-^{14}C by *N. cataria* L. can be influenced by the method used in administering the compound. Uptake by plants with excised roots was the most rapid, with detectable radioactivity appearing in the growing tips within 15 min. From stem injections, about 2 hr were required for translocation to the upper growing tips and from the crown (the base from which the stems protrude) injections, about 12 hr were required.

Since mevalonate-2-^{14}C was rapidly metabolized by *N. cataria* a study of its distribution into other compounds produced by the mature plant was made using the method of Schwenk and Werthessen.[19] The results obtained in a 96-hr experiment showed that of the radioactivity administered approximately 43 per cent was recovered in the crude lipid extract and that 2–6 per cent of this amount was present in the fatty acid portion whereas 15–18 per cent was present in the sterol fraction.

Chemical Degradation of Nepetalactone to Remove Carbons 3, 8 *and* 9

From the structure of nepetalactone (I) it could be predicted that radioactivity from mevalonate-2-^{14}C would be incorporated into carbons 3 and/or 8 and 6 and/or 9 if an isoprenoid biosynthetic pathway is operative. The amount of labeling in these positions was determined by two degradative routes (Fig. 1). Labeling in positions 8 and 9 was determined by subjecting nepetalactone-^{14}C to Kuhn–Roth C-methyl determination. Incorporation of radioactivity in positions 3 and/or 8 was deduced by a combination of the alkaline hydrogen peroxide oxidation and haloform reaction.

In the alkaline hydrogen peroxide degradation, nepetalactone in crude "catnip oil" was converted to nepetalic acid ((II), potassium salt) by 10% KOH (Fig. 1). The contaminating neutral components of the essential oil which were still present after alkaline hydrolysis were removed by extraction with ether. When the volume of the solution was small (0·1 ml) and the surface area of the reaction vessel large, as much as 50 per cent of the nepetalic acid was converted to the nepetalinic acids (III). Purging the solution with nitrogen during hydrolysis eliminated this undesirable side-reaction which probably was caused by molecular oxygen. When small-scale reactions were run, the isolation of crystalline nepetalic acid (II) was not attempted. The alkaline hydrogen peroxide degradation of nepetalic acid to nepetolactone (IV) and nepetonic acid (V) was achieved by adding 30 per cent aqueous hydrogen peroxide to the nepetalactone hydrolysate following extraction of the neutral components. The reaction rate was increased with elevated temperatures and with increased base concentration. The oxidation of nepetalic acid (II) was complete in 10 min at 27°, as shown by GLC analyses, while 50–60 min were required at 0°. As the reaction temperature was increased to 54°, degradation of nepetonic acid (V) formed in the initial reaction increased as the concentration of base in the reaction was increased from 10 to 30 per cent. However, the concentration of hydrogen peroxide did not seem to be extremely critical in these reactions. Nepetolactone (IV) was separated from the acidic degradation products by extraction of ether solutions of this mixture with saturated aqueous sodium bicarbonate. Both nepetolactone (IV) and nepetonic acid (V) were converted to nepetic acid (VI) in approximately 80 per cent yield by treatment with alkaline solutions of sodium hypobromite.

The results presented in Table 2 show the distribution of the label in nepetalactone formed

[19] E. SCHWENK and N. T. WERTHESSEN, *Arch. Biochem. Biophys.* **40**, 334 (1952).

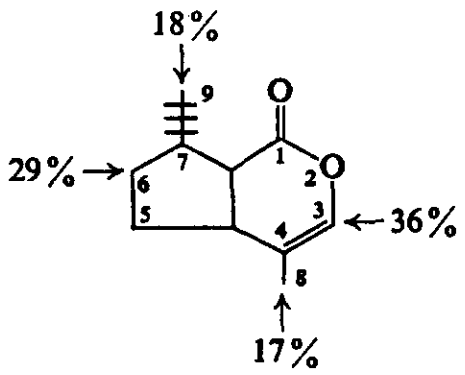
TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN NEPETALACTONE FORMED FROM DL-MEVALONATE-2-^{14}C IN *Nepeta cataria* L.

Compound isolated			
Name	Specific activity (mμc/mmole)	Radioactivity (%)	Carbon atoms*
Nepetalactone	0·33	100	All
Nepetonic acid	0·21	64	All but C-3
Nepetic acid	0·15	47	All but C-3, 3-4 and C-8
Acetic acid	0·16	35	From carbons 8,4 and 9,7

(Experiment duration was 30 days.)

* Numbering system refers to carbon skeleton of nepetalactone (Fig. 1).

from mevalonate-2-^{14}C. The difference between the per cent of radioactivity found in nepetonic acid (V) and nepalactone (I), 36 per cent, is the amount of radioactivity in carbon 3. The difference between the per cent of radioactivity found in nepetic acid (VI) and nepetonic acid (V), 17 per cent, is the amount of radioactivity in carbon 8. The amount of radioactivity recovered in the acetate from a Kuhn–Roth determination which represents carbons (4 and 8) and (7 and 9) is 35 per cent of that in the original nepetalactone. By difference* then the amount of radioactivity in carbons (7 and 9) is 18 per cent which is in agreement with the 17 per cent found in carbon 8 using the alkaline hydrogen peroxide degradation procedure. Also by difference† it can be predicted that carbon 6 contains about 30 per cent of the radioactivity. These results provide evidence for limited randomization (i.e. about 35 per cent) of the carbon 14 label in nepetalactone formed from mevalonate-2-^{14}C by flowering *N. cataria* plants. This result might be expected since a time lapse of 30 days between the administration of mevalonate-2-^{14}C and harvesting the plants occurred. These data support those presented



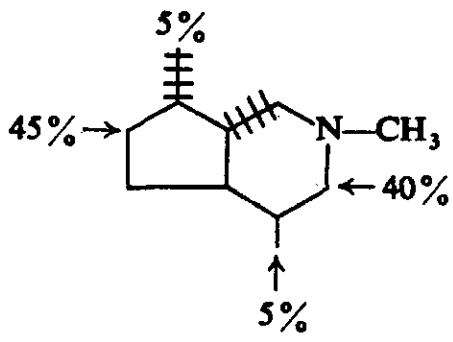
(I) Nepetalactone

for the biosynthesis of β-skytanthine (VII) from mevalonate-2-^{14}C by mature, 3-yr-old *Skythanthus acutus* M. plants by Auda *et al*,* Yeowell and Schmid[20] showed that randomization of carbon 14 occurred in plumieride (VIII) formed biosynthetically from mevalonate-2-^{14}C. Carbon atoms 3 and 15 each contained 25 per cent of the radioactivity and carbon 7 contained 44 per cent. It can be assumed that carbon atom 10 of plumieride (VIII) would contain about 6 per cent of the radioactivity which is only ⅓ of that found at the equivalent carbon atom 9 for nepetalactone. Results on the biosynthesis of verbenalin

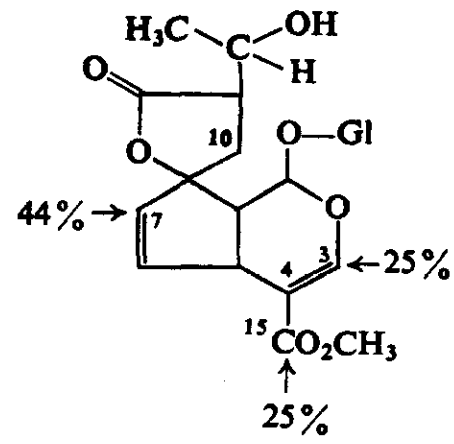
* It is assumed that the acetate obtained from the Kuhn–Roth oxidation of nepetalactone is formed in equal amounts from carbons (4 and 8) and (7 and 9).

† The amount of label estimated to be present in carbon 6 is obtained by subtracting the amount estimated to occur in carbons (7 and 9) from that found in nepetic acid (Table 2).

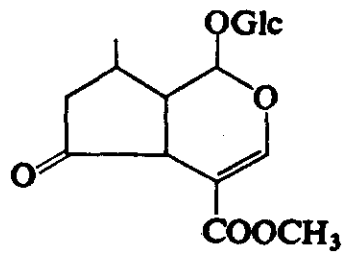
[20] D. A. YEOWELL and H. SCHMID, *Experentia* **20**, 251 (1964).



(VII) β-Skytanthine



(VIII) Plumieride



(IX) Verbenalin

(IX) produced by *Verbina officinalis* seedlings by Hüni *et al.*[21] show a similar labeling pattern to that found for plumieride.‡ It was suggested[20] for plumieride that randomization might occur after ring closure of the methylcyclopentanoid ring and that carbon atoms 3 and 15 probably became equivalent aldehyde groups. Randomization of the terminal methyl label in the isoprenoid protion of certain indole alkaloids has been reported[22, 24] and the proposed mechanisms are similar to that suggested for plumieride. The biosynthesis of indole alkaloids from the methylcyclopentane monoterpenoids is also supported by labeling experiments using geranylpyrophosphate-2-^{14}C[23–26] and geraniol-2-^{14}C.[27, 28]§ For the review of methylcyclopentane monoterpenoids as intermediates in the biosynthesis of indole alkaloids see Taylor[29] and Battersby.[26] It seems logical that randomization might occur at the monoterpenoid level in the indole alkaloids and plumieride; particularly since the biosynthesis of plumieride must proceed through the formation of a carboxyl group (carbon 15) and the biological oxidation might be expected to proceed in a stepwise direction involving alcohol,

‡ Results presented by E. J. Eisenbraun, A. G. Horodysky and G. R. Waller, *6th Ann. Mtg Plant Phenolics Group North America*, Austin, Texas, April 6–8, 1966, indicated that a non-randomized distribution of carbon 14 occurred in verbenalin produced by mature *Verbena officinalis* plants.

§ We have administered geranylpyrophosphate-1-^{14}C and geranol-1-^{14}C to mature *N. cataria* plants and found that the former compound was not translocated and the latter only poorly; however, some incorporation of ^{14}C into nepetalactone was observed.

21 J. E. S. Hüni, H. Hiltebrand, H. Schmid, D. Gröger, S. Johne and K. Mothes, *Experentia* **22**, 656 (1966).
22 F. McCapra, T. Money, A. I. Scott and I. G. Wright, *Chem. Commun.* **1**, 537 (1965).
23 H. Goeggel and D. Argoni, *Chem. Commun.* **1**, 538 (1965).
24 A. R. Battersby, R. T. Brown, R. S. Kapel, A. O. Plunkett and J. B. Taylor, *Chem. Commun.* **2**, 47 (1966).
25 A. R. Battersby, R. T. Brown, J. A. Knight, J. A. Martin and A. O. Plunkett, *Chem. Commun.* **2**, 346 (1966).
26 A. R. Battersby, *The Chemistry of Natural Products, IUPAC Symp.*, Vol. 4. Stockholm, Sweden, June 26–July 2, 1966, in press.
27 P. Loew, H. Goeggel and D. Arigoni, *Chem. Commun.* **2**, 347 (1966).
28 E. S. Hall, F. McCapra, T. Money, K. Fukumoto, J. R. Hanson, B. S. Mootoo, G. T. Phillips and A.I. Scott, *Chem. Commun.* **2**, 348 (1966).
29 W. I. Taylor, *Science* **153**, 954 (1966).

aldehyde and finally acid. In nepetalactone, carbon atom 8 (equivalent to carbon 15 of plumieride) remains as a methyl carbon, and the chance for randomization of label via a similar type of intermediate involving methyl groups (as carbon atoms 3 and 8) exists at the geraniol or geranylpyrophosphate state. In contrast to the experiments showing randomization, the results obtained by Birch *et al.*[30] from the incorporation of mevalonate-2-^{14}C into the terpenoid side-chain of mycelianamide indicated that 80 per cent of the radioactivity was not randomized and it was suggested that the degree of randomization observed was more likely a result of the chemical degradative procedure than a lack of specificity during biosynthesis.

A major difference between the biosynthesis of nepetalactone (I) and β-skytanthine, (VII) plumieride, (VIII) or verbenalin (IX) is the evidence for the high amount of carbon 14 located in carbon 9. The fact that the percent carbon 14 located in carbon 9 is about equal to that found in carbon 8 suggests that some randomization can occur at the isopentenylpyrophosphate level. This finding needs further study before it is completely understood since the evidence to date tends to support the mechanism of isomerization of isopentenylpyrophosphate proposed first by Agranoff *et al.*[31] and later established by Shah *et al.*[32] In this mechanism the methylene carbon atom of isopentenylpyrophosphate is protonated, the proton from carbon atom 2 is discharged into the medium and dimethylallylpyrophosphate is formed. In the reverse reaction a proton is added stereospecifically at carbon atom 2 of dimethylallylpyrophosphate. Hence this isomerization would not result in a randomization of label originally present in the methylene group of isopentenylpyrophosphate.

The metabolic relationship between mevalonate-2-^{14}C and nepetalactone-^{14}C in flowering *N. cataria* L. plants has been examined. A limited randomization of the carbon 14 was found in carbons (3 and 8) and (6 and 9) of nepetalactone. The pathway for the biosynthesis of nepetalactone is not well understood; however biogenesis routes involving iridodial as an intermediate have been proposed.[33, 34] It is the only methylcyclopentane monoterpenoid studied where evidence has been obtained for a significant amount of randomization of label from mevalonate-2-^{14}C to occur; however, the long experiment duration probably has an influence on the distribution of label since degradation of nepetalactone-^{14}C could occur and some of the degradation products could be incorporated into the monoterpenoid.

Histological Examination of Nepeta cataria *Leaves*

Microscopic examination of leaf cross-sections prepared from leaves at three different stages of development show (Fig. 4) several types of glandular structures. The glands occur on both sides of the leaf. These glands are multicellular, resting between hairs arising from the cuticle or epidermis. They appear to originate from a single cell and change in shape with development. In the early stage (Fig. 4A) no stalk is visible; in the intermediate stage (Fig. 4B) formation of a stalk with a globular head is seen. In the late stage (Fig. 4C) the globular structure is expanded and the stalk is enlarged. These stages of development are similar to those found in the glandular trichomes of *Hemizonia minthornii* reported by Carlquist.[35]

The essential oil of the plant is probably accumulated in these glands. These glands have a very fragile membrane since the lightest brushing with the finger-tip produces a pronounced

[30] A. J. BIRCH, M. KOCOR, N. SHEPPARD and J. WINTER, *J. Chem. Soc.* 1502 (1962).
[31] B. W. ARGANOFF, H. EGGERER, U. HENNING and F. LYNEN, *J. Biol. Chem.* **235**, 326 (1960).
[32] D. H. SHAH, W. W. CLELAND and J. W. PORTER, *J. Biol. Chem.* **240**, 1946 (1965).
[33] G. W. K. CAVILL and P. L. ROBERTSON, *Science* **149**, 1337 (1965).
[34] E. J. EISENBRAUN, G. R. WALLER and H. H. APPEL, manuscript in preparation.
[35] S. CARLQUIST, *Am. J. Botany* **45**, 675 (1958).

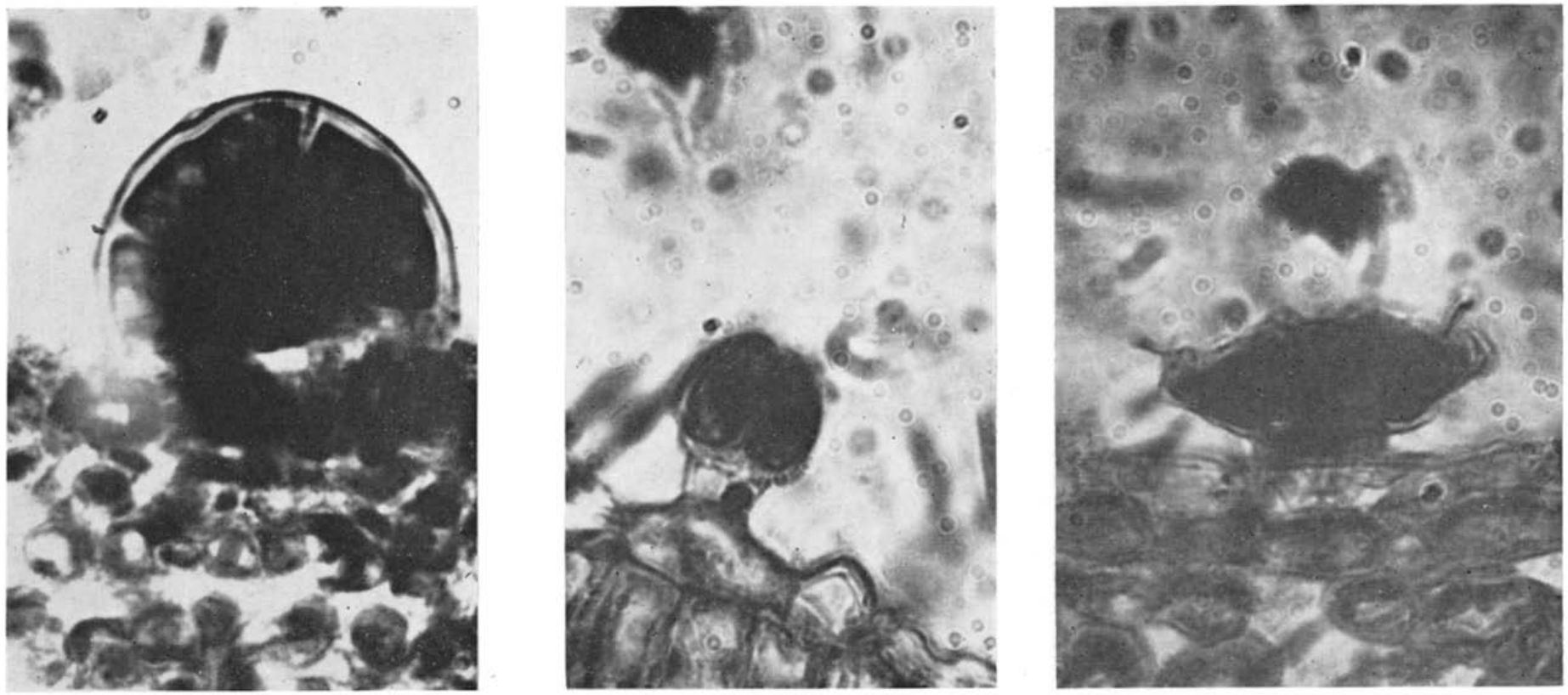


FIG. 4. *Nepeta cataria* L. TRANSVERSE (CROSS) SECTIONS OF LEAF TISSUE.

(A) Early stage ×1125; (B) Intermediate stage ×562·5; (C) Later stage ×562·5.

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odor of catnip oil. A crude homogenate of *N. cataria* leaves using mevalonate-2-^{14}C as the substrate has proven effective in incorporating radioactivity into nepetalactone and other monoterpenes. Experiments are in progress to determine what role these glands play in the biosynthesis of nepetalactone.

EXPERIMENTAL PROCEDURE

Plant Material

Nepeta cataria L. plants were grown in the greenhouse of the Horticulture Department either from seeds or cuttings. Additional oil of catnip was obtained from catnip plants grown in fields near Fairbury, Nebraska, and East Lansing, Michigan. The plants used contained a ratio of nepetalactone to epinepetalactone of greater than 97:3.[16]

Isolation of the Essential Oils

The plants were steam distilled for 30 min, using an all-glass system. The distillate was saturated with NaCl and extracted with ethyl ether. The dried ($MgSO_4$) ether solution was concentrated at atmospheric pressure. The essential oil was stored at $-15°$.

An alternate procedure for the isolation of the essential oil without the use of steam employed hexane extraction. The dried ($MgSO_4$) hexane extract was filtered through a 1:1 Super-Cel-MgO column. The quality of the essential oil thus obtained was fully as good as that obtained by steam distillation, but more lipid material was extracted.

Chromatographic Separation of Nepetalactone and the Terpene Hydrocarbons

The separation of nepetalactone and the terpene hydrocarbons from the other components of the essential oil obtained from the *N. cataria* L. plants was accomplished by using GLC and TLC.

1. *Gas–Liquid chromatography.* GLC was performed on a Beckman Model GC-2A or Perkin-Elmer Model 801 gas chromatograph equipped with a thermal conductivity detector or a hydrogen flame detector, a Bristol 1-mV recorder and Disc Instruments, Incorporated disc integrator. Analysis for nepetalactone and the terpene hydrocarbons was performed: (*a*) on 6 m × 3 mm stainless steel columns packed with 20% Apiezon L on 60–80 mesh Chromosorb W (acid washed) and held at 225° to (*b*) on a 75 m × 0·5 mm stainless steel open tubular column coated with Apiezon L and held at 160°.

Analysis of the degradation products (Fig. 1) was performed on the latter column with a helium flow rate of 2 ml/min. Nepetalactone was identified by comparison of its retention time with that of a standard[16,17] and by using combination mass spectrometry–gas chromatography.[17] Nepetalic acid (II) and nepetalinic acids (III) with known absolute configuration and stereochemistry were available from earlier studies[3,36] and were also freshly prepared. Peak areas were measured by electromechanical integration and quantitation of amounts made by comparison with standard curves obtained with pure compounds.

2. *Thin-layer chromatography.* Commercially available plates (20 × 5 × 0·4 cm) were coated with a 0·5 mm layer of silica gel G and activated at 110° for 30 min.

Qualitative comparisons of samples were made by applying an ether solution of the essential oil (10–100 μg) as a single spot. Comparable amounts of authentic standards were placed beside the unknowns for a direct comparison of R_f values. The plates were developed with a hexane:acetone:ethanol (40:10:4) mixture and the developed spots were located with iodine vapor.

Labeled Compound Used

For all experiments except those where the nepetalactone was degraded chemically, DL-mevalonic acid-2-^{14}C (N,N-dibenzylethylenediamine salt) obtained from Nuclear Research Chemicals, Orlando, Florida, was converted to the free acid (specific activity 1·7 mc/mmole) by adjusting the pH of an aqueous solution to 10 with 1 N NaOH and extracting the dibenzylethylenediamine with ethyl ether. The mevalonate-2-^{14}C was purified by preparative paper chromatography using Whatman No. 1 paper and 85% isopropyl alcohol (R_f 0·48). Its purity was further checked in 1-butanol:acetic acid:water (4:1:1) and only one spot was observed.

For the biosynthesis of nepetalactone used for chemical degradation chromatographically pure DL-nevalonic acid-2-^{14}C (N,N-dibenzylethylenediamine salt) obtained from New England Nuclear Corp., Boston, Massachusetts, was used. Chromatography was performed using Whatman No. 1 paper and 2-propanol: ammonium hydroxide:water (80:5:15) as the solvent.

The location of radioactivity was made with a Nuclear-Chicago Actigraph III paper strip scanner.

[36] R. B. BATES, E. J. EISENBRAUN and S. M. MCELVAIN, *J. Am. Chem. Soc.* **80**, 3420 (1958).

Administration of Labeled Compounds

An aqueous solution of the labeled compound was injected into the stems of a plant. At appropriate time intervals the plants were harvested and stored at −15° until used.

Isotopic Analysis

Four methods were used for determining the radioactivity in the terpene components. The counting technique used was dependent on the specific activity of the isolated material and the method used in the isolation of the compound. Samples of low specific activity were subjected to TLC and the radioassay was accomplished by (*a*) external scanning of the plate with a Nuclear-Chicago Model 1032 4 Pi Actigraph III Chromatogram Scanner; (*b*) autoradiography; and (*c*) scraping the plates clean at the appropriate areas and direct counting of absorbent and compound in a liquid scintillation spectrometer (Tri-Carb, Packard Instrument Co., Downers Grove, Ill.). The scintillation solvent was composed of 58·7% toluene, 39·3% absolute ethanol and 2% water. The phosphors were 0·5% 2,5-diphenyloxazole and 0·02% *p-bis*-2-(5-phenyloxazolyl)-benzene. This system had an efficiency of 44 per cent and iodine vapor used to detect the compounds had no measurable effect. Quenching and absorption of labeled compounds by silica gel G were tested by the internal standard method using benzoic acid-^{14}C and the results showed that no loss occurred in counting efficiency at the concentrations used; consequently, no correction factors were used.

The components separated by gas chromatography were assayed for radioactivity with a Nuclear-Chicago Model 4998 continuous gas flow monitoring system. This instrument had a counting efficiency of 18 per cent at a detector temperature of 225° using 100 ml/min of methane as the counting gas.

Chemical Degradation of Nepetalactone

1. *Nepetalic acid (II) from oil of catnip.* The essential oil was extracted with excess 10% NaOH. The alkaline solution was acidified and extracted twice with equal volumes of ether. After drying ($MgSO_4$) and concentrating under diminished pressure, the residue was dissolved in a minimal quantity of warm light petroleum (b.p. 40–60°). Nepetalic acid slowly crystallized from the solution after dry-ice cooling.

2. *Alkaline hydrogen peroxide degradation of nepetalic acid.* A solution containing 1 g of nepetalic acid (II) in 10 ml of 10% KOH was treated with 5 ml of 30% H_2O_2. The temperature was maintained at 27°, with stirring, for 1 hr. The solution was then treated with several mg of PtO_2 and stirred for an additional 15 min. After acidification and extraction with ether, the extracts were dried ($MgSO_4$) and concentrated. The samples were prepared for gas chromatography by esterifying the acids with CH_2N_2.

3. *Separation of nepetolactone (IV) from degradation products.* The ether extract from the alkaline H_2O_2 degradation of nepetalic acid was extracted with saturated $NaHCo_3$ solution to remove acidic material. The remaining ether layer contained nepetolactone (IV). This compound was not further purified.

4. *Nepetic acid (VI).* A solution of 10 mg of nepetonic acid (V) or nepetolactone (IV) in 0·5 ml 10% NaOH was treated with 0·5 ml of 10% Br_2–20% KBr solution by dropwise addition over 1 hr. After acidification the solution was evaporated to dryness at 0·1 mm pressure. Distillation at 200° (0·1 mm) yielded nepetic acid (VI).

5. *Kuhn–Roth oxidation of nepetalactone.*[37] Nepetalactone (20 mg) was oxidized with CrO_3—H_2SO_4 under reflux for 90 min. The reaction mixture was steam distilled until 50 ml were collected, and the distillate neutralized with 0·07 N NaOH. The 14 mg of sodium acetate obtained on evaporation was purified by chromatography on a Celite column.

Preparation of Nepeta cataria *leaf tissue for histological examination.*

Green leaves of *N. cataria* in various stages of development were fixed in formalin–acetic acid–ethanol, dehydrated in a *t*-butyl alcohol series and embedded in paraffin. Transverse serial sections were cut at 10 μ and stained in safranin-fast green. Microscopic examinations were made with high dry and oil immersion objectives. Black and white micro-photographs were made at magnifications of 562·5× and 1125×.

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[37] E. J. EISENBRAUN, S. M. MCELVAIN and B. F. AYCOCK, *J. Am. Chem. Soc.* **76**, 607 (1954).