THE BIOSYNTHESIS OF NEPETALACTONE

By

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CHAPTER I

INTRODUCTION

This dissertation reports studies on the biosynthesis of the methylcyclopentane ring system in higher plants. The acyclic monoterpene nepetalactone was chosen for study because the plant which produces it, Nepeta cataria L., is readily available and an extensive amount of work has been done on the chemical degradation of this compound. Nepetalactone is the principle ingredient of catnip oil and is a strong feline attractant.

This research had three main objectives: (A) to determine the composition of the essential oil of Nepeta cataria L., (B) to develop a method for the analysis and study the distribution of nepetalactone isomers from different Nepeta species, and (C) to investigate the biosynthesis and chemical degradation of nepetalactone.

Investigations of the composition of catnip oil were undertaken as a prelude to biosynthetic studies. The primary objective of this study was the identification of compounds which might have a structural precursor-product relationship to nepetalactone. The limited amount of essential oil and its complexity necessitated the use of the combination gas chromatograph-mass spectrometer for identifications.

Observations made during the study of the composition of the essential oils of Nepeta species indicated that there were large

variations in the ratio of nepetalactone to epinepetalactone in different samples. The possibility that variations in the stereochemistry of the methylcyclopentane ring system could be of value in the study of the biosynthesis of nepetalactone or phylogenetic classification led to the development of techniques for the quantitation of nepetalactone and epinepetalactone. This new procedure was used in the analyses of essential oils from several different Nepeta species.

From the isoprenoid structure of nepetalactone, it seemed likely that an isoprenoid biosynthetic pathway might be involved in its formation. The precursor used in these studies was mevalonic acid-2-^{14}C, a known isoprenoid precursor. Since the plant Nepeta cataria L. also produces the sesquiterpene, caryophyllene, observations on its biosynthesis were made. The de novo rate of formation of these two compounds and their site of synthesis was also studied.

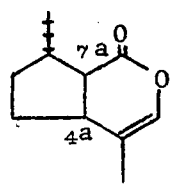
Degradation of biosynthetically formed nepetalactone-^{14}C was accomplished by modifying the reactions used in the proof of structure of this compound. These modifications necessitated a study of the reactions of carbonyl compounds with alkaline hydrogen peroxide solutions.

CHAPTER II

LITERATURE REVIEW

Nepeta cataria L. (family Labiatae) is a rugged plant which is native to Europe and Asia. This plant was first introduced into North America as a decorative house plant. It grows to 2 or 3 feet and has gray-green, heart-shaped leaves with a hairy under surface. Rubbing the leaves of this plant produces the very characteristic minty odor of catnip oil.

The feline attracting power of Nepeta cataria L. and nepetalactone (I)



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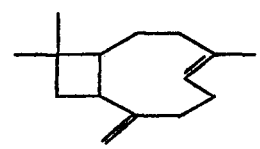


II

has been recognized for sometime. Todd (1) has postulated that nepetalactone (I) mimics a pheromone of the cat which elicits specific courtship displays. The following evidence was given to support this hypothesis:

1. The catnip response and courtship display are very similar.
2. Field observations indicate that catnip-like displays occur at sites known to have been marked by the urine of tom cats.

3. Catnip-like activity has been found in ether extracts of tom cat urine.

4. Cross fatigue of the catnip response and courtship display appears to occur.

5. The "central neural substrate" of the catnip response and courtship behavior appear to be intimately related.

6. Mounting activity of two male snow leopards was provoked by catnip responses of two females.

7. There is no correlation between distribution of plants having catnip-like activity and that of cats which are sensitive to them.

8. There is a striking difference in responses to catnip between lions and tigers which may be due to the unknown mechanism which reproductively isolated these two species in nature (but not in captivity).

It is possible that there are other pheromones which induce similar responses in other animals.

Eisner (2) has recently reported that nepetalactone (I) is an insect repellant for a wide variety of insects, a biological phenomenon also associated with some of the insect produced methylcyclopentane monoterpenoids. Caryophyllene (II) was also observed to be a potent repellant by Skaife (3). Many insect repellants, such as cinnamic alcohol, geraniol, clove oil and linalyl acetate, are quite pleasant to the human nose. Ants are not affected by the foul-smelling skatole. This suggests that there is no relationship between olfactory response of the human and that of insects.

These findings tend to indicate that a large number of volatile compounds produced by plants are of practical value as insect repellants. It was suggested by Eisner (2) that nepetalactone (I) protects Nepeta cataria L. by repelling phytophagous insects.

A great deal of research has been done on essential oils in general. Essential oils are defined as those aromatic steam volatile substances of natural origin which are of an oily nature (4). These volatile oils are widespread in nature. It has been estimated that twenty-nine per cent of all angiosperms and gymnosperms produce essential oils (4). Of these essential oil producing families, 44% are tropical, 7% are tropical-subtropical, 2% are subtropical, 3% are subtropical-temperate, 18% are temperate and 25% have a wide climatic range.

The early work of McNair (4,5) on the physical properties of 398 different essential oils showed a definite correlation of specific gravity and refractive index with phylogenetic evolution and environmental origin of samples. Tropical oils were found to have higher refractive indicies than oils from temperate regions. These workers claim that plants of the same genus which could not be sharply separated on a morphological basis were easily differentiated on the basis of the constituents in the essential oils.

In recent years it has become increasingly evident that the methylcyclopentane ring system is widespread in nature with the isolation of many compounds containing this carbon skeleton. The methylcyclopentanoids are composed of three basic groups: glycosides, alkaloids, and oxygenated monoterpenes. The function(s) of these compounds in nature is not known, with the exception of the insect produced monoterpenoids which have been found to be Arthopod defense chemicals and in some cases antibiotics.

The methylcylopentanoid glycosides (Figure 1) are composed of verbenalin (III) (6-13), loganin (IV) (14-17), asperuloside (V) (18-22), catalposide (VI) (23-25), monotropein (VII) (26,27), plumieride (VIII) (28-31) and aucubin (IX) (32-35). The sugar moiety in these compounds has been found to be β-D-glucose. Hydrolysis with mineral acids converts the aglycone portion of most of these compounds to a black polymer.

Skytanthine (X) (36-39), tecomanine (XI) (40-42), and actinidine (XII) (43) are some of the members of the methylcyclopentanoid alkaloids. These liquid alkaloi s are all of plant origin and are of particular interest to the biochemist. The isoprenoid skeleton of actinidine (XII) suggests that the pyridine nucleus of this compound arises from an isoprenoid precursor.

The oxygenated monoterpenes are of both plant and insect origin. Anisomorphal (XIII) (44), iridodial (XIV) (45,46), iridomyrmecin (XV) (47,48) and isoiridomyrmecin (XVI) (49) are defense chemicals of a number of different species of ants. The iridomyrmecins are produced by anal glands of some ants and represent over 1% of their body weight (50). The monoterpenoids of plant origin consist of nepetalactone (I) (51-55) and genipin (XVII) (56). Genipin is more like the aglycone portion of the glycosides than the other monoterpenoids of this group.

Investigation of the chemical nature of "Oil of Catnip" began in the Department of Pharmaceutical Chemistry at the University of Wisconsin under the direction of Professor Kremers (51). Approximately 85% of this essential oil was found to be alkali soluble. Acidification of the alkaline extract precipitated a viscous oil which slowly crystallized on standing. Subsequent studies dealt specifically with

the structure of this acidic compound for which the name nepetalic acid (XVIII) (Figure 2) was proposed. Pyrolysis of nepetalic acid (XVIII) gave the unsaturated lactone, nepetalactone (I). This compound possessed physical properties identical to the original lactone of the crude oil.

Meyer (57) showed that either chromic acid or permanganate oxidation of nepetalic acid (XVIII) resulted in the formation of a number of acidic oxidation products. Acetic acid and two other acids with melting points of 85° and 250° were among the products isolated.

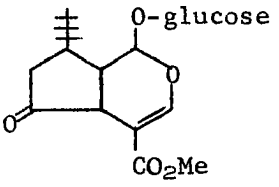
McElvain *et al.* (51) were the first workers to report the oxidative degradation of nepetalic acid (XVIII) to nepetonic acid (XIX) by alkaline hydrogen peroxide. Nepetonic acid (XIX) was isolated in a yield of 67% of the theoretical value from this reaction. It was later reported (52) that when nepetonic acid (XIX) was prepared from nepetalic acid (XVIII) which had been pyrolyzed and rehydrolyzed, that the yield of XIX dropped to 40% of the theoretical. The decreased yield of nepetonic acid (XIX) was attributed to epimerization of the 7a ring position (see nepetalactone (I)) during pyrolysis. By hydrogenation of nepetonic acid (XIX) these workers obtained a new acid, nepetolic acid (XX). The failure of this compound to form a lactone upon distillation gave an indication that the ring substituents were in the *trans* configuration at the 4a and 7a positions. An investigation (52) of the acidic residues remaining after preparation of nepetonic acid (XIX) from nepetalic acid (XVIII) by alkaline hydrogen peroxide oxidation, led to the isolation of two epimeric nepetalinic acids (XXI, m.p. 85° and XXIa, m.p. 117°). The separation of XXI and XXIa is dependent upon the insolubility of the barium salt of XXI in water.

When nepetonic acid was treated with sodium hypoiodite an 80% yield of nepetic acid (XXII) was observed (51). This dicarboxylic acid distilled unchanged and was later assigned a trans orientation of the carboxyl groups (54,55). Acetic anhydride was found to convert this acid to an anhydride XXIII, which yielded a new nepetic acid (XXIIa) upon hydrolysis.

It was recognized (52) that the alkaline hydrogen peroxide degradation of nepetalic acid (XVIII) always produced a small quantity of the γ-lactone nepetolactone (XXIV). This lactone was ultimately assigned the cis-trans configuration (55).

In 1942 McElvain reported the presence of a sesquiterpene in oil of catnip. It was proposed that this compound was caryophyllene (II) on the basis of the physical properties and the melting point of the dihydrochloride derivative. This compound had previously been isolated from oil of cloves by Ruzicka (58).

Figure 1. Structures of some methylcyclopentane monoterpenoids



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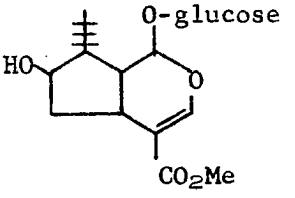

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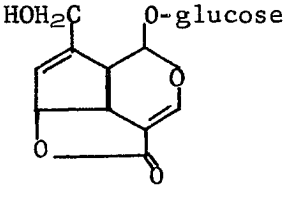

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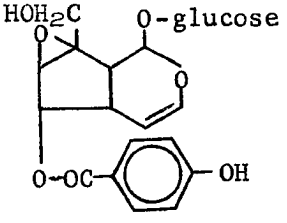

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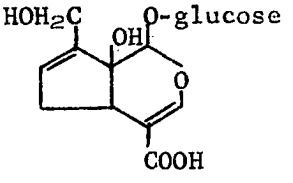

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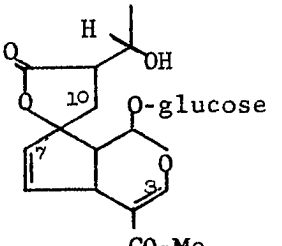

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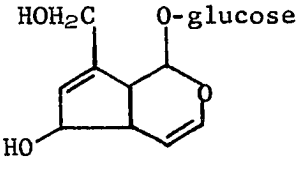

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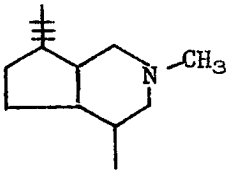

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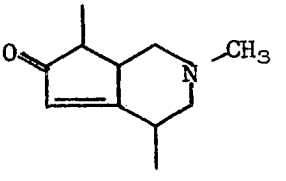

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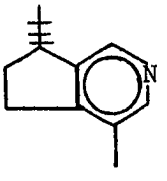

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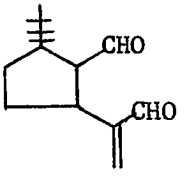

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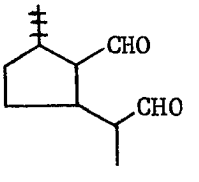

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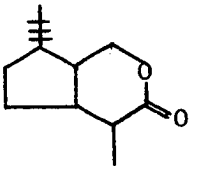

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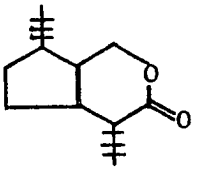

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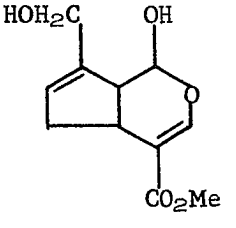
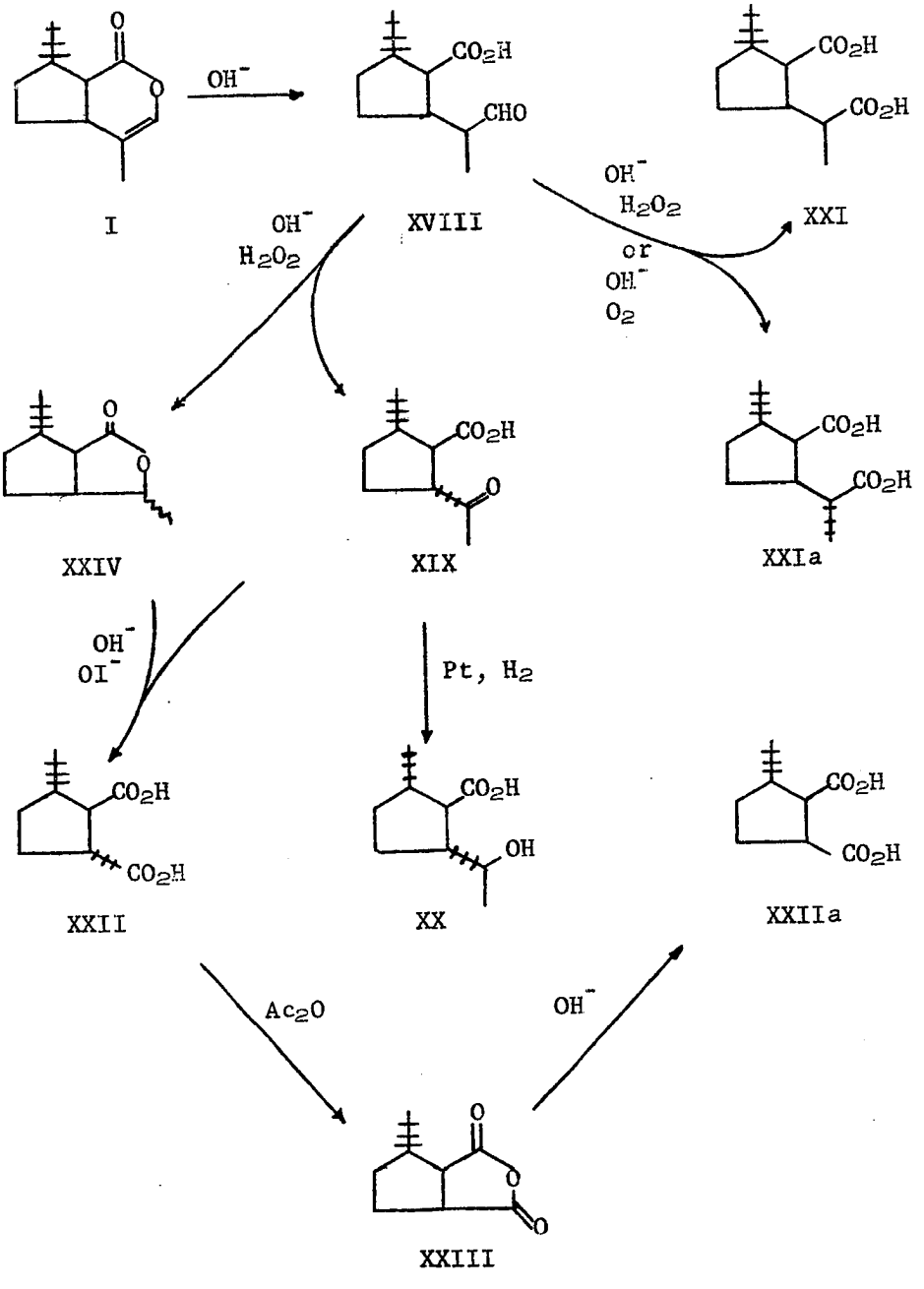


XVII

Figure 2. Chemical degradation of nepetalactone



CHAPTER III

COMPOSITION OF THE ESSENTIAL OIL OF *NEPETA cataria* L.

Catnip oil was first studied by Umney and Bennett (59) in 1905. The oil examined was of Sicilian origin and was reported to contain 22.2% menthol (XXV) and 3.3% menthyl acetate (XXVI) (Figure 3). Kremers and McElvain (51) later found the major constituent of this oil to be an alkali extractable enol-lactone for which the name nepetalactone (I) was proposed. McElvain (60) subsequently separated the non-alkali soluble portion of the oil into a number of fractions by distillation. The neutral fraction of the oil was reported to contain 42% nepetalactone (I), 14% caryophyllene (II), 36% nepetalic anhydride (XXVII), 3% of an ether ($C_{14}H_{24}O$), and 2% of an ester ($C\ H_{14}O_2$).

Several reports of work on other species of *Nepeta* have appeared in the literature. Gupta *et al.* (61) have recently studied the essential oil of *Nepeta leucophylla*. This oil was reported to contain 16.6% thymoquinone (XXVIII), 16.2% nepetalic acid (XVIII), 6.2% phenylethyl acetate (XXIX), 6.2% phenylethyl isovalerate (XXX), 5.2% 4-*tert.*-butyl-1-methyl-benzene (XXXI), 3.9% ethyl benzoate (XXXII), and 22.4% sesquiterpenoid alcohols. The components of this oil mixture were separated by a combination of bicarbonate extraction and adsorption chromatography on alumina.

An investigation of the essential oil of *Nepeta ciliaris* was

conducted by Talwar (62). The essential oil of this plant was reported to contain 6.6% α-pinene (XXXIII), 4.6% phenylethyl acetate (XXIX), 18% pinocarvone (XXXIV), 7.2% myrtenal (XXXV), 27.5% of a tricyclic sesquiterpene and 27% of a new acetal ester.

The structural formulas for the constituents of the essential oils of the different Nepeta species are found in Figure 3.

This chapter reports the results of the analysis of the essential oil of Nepeta cataria L. by analytical gas liquid chromatography (GLC) and the combination mass spectrometry-gas chromatography (MS-GC).

APPARATUS AND REAGENTS

Apparatus. Preparative gas chromatographic separations were achieved on an Aerograph Autoprep gas chromatograph equipped with a 0-1 millivolt Leeds and Northrop Model H recorder, a thermal conductivity cell, and a 10' x 3/8" aluminum column packed with 20% Apeizon L on Chromosorb W.

MS-GC analyses were performed on an instrument as described by Ryhage (63) except that the gas chromatographic record was obtained by continuous registration of the total ion current collected on a plate in the analyzer tube (64). A 20' x 5/32" coiled glass column packed with 2% dimethyl polysiloxane (JXR) and 0.33% cyclohexyl dimethylsuccinate (CHDMS) coated on Chromosorb P was used for the gas chromatographic separations on the combination instrument. The column temperature was regulated by temperature programming from 70° to 180°.

Gas Chromatography Materials. Apiezon L, JXR, CHDMS, Chromosorb W, and Chromosorb P were purchased from Applied Science Laboratories, State

College, Pennsylvania.

Oil of catnip was purchased from Fritzche Brothers, Inc., New York City, New York.

Nepetalactone and caryophyllene were supplied by Dr. E. J. Eisenbraun, Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma.

PROCEDURES

Preparative gas chromatographic fractionation was achieved on the 20% Apiezon L preparative column with operational parameters as follows: injection port temperature 250°, oven temperature 220°, detector temperature 250°, helium flow 120 ml./min., and injection volumes of 100 µl. The mixture was divided into fractions as indicated on the tracing in Figure 4.

Fraction A was analyzed on the JXR-CHDMS column at a column temperature of 85° with an injection port temperature of approximately 100° and a helium flow rate of 20 ml./min. The injection port temperature was not rigidly controlled on the combination instrument.

Fraction B was subjected to 3°/min. programmed temperature analysis on the JXR-CHDMS column at oven temperatures ranging from 85° to 130° with a flow rate of 20 ml./min. and injection port temperature of approximately 100°.

Fraction C was resolved into its components by chromatography on the JXR-CHDMS column with temperature programming from 140° to 190° at 3°/min. with a helium flow rate of 20 ml./min. and injection port temperature of approximately 160°.

The mass spectrometer was operated at an electron energy of 20 eV and an ion source temperature of 250°. The pressure in the mass spectrometer analyzer tube near the ion source was 10^{-5} mm. Hg, and the pressure in the ionization chamber was approximately 10^{-4} mm. of Hg.

The results reported in this chapter have been corrected for background spectra. Background spectra were taken during the analysis of each of the essential oil fractions. These spectra were subtracted from all sample spectra.

All mass spectra reported here are in terms of relative abundance, with the most intense ion being taken as 100%.

RESULTS AND DISCUSSION

Gas chromatographic analysis of catnip oil indicates that it is a complex mixture of compounds. The crude oil was fractionated by gas liquid chromatography and divided into fractions A, B, and C as is indicated in Figure 4. Approximately 0.1, 0.15 and 3 ml. of these respective fractions was collected.

Previous workers (51, 59-62) have studied the composition of Nepeta oils by the use of large scale extractions, distillations and chromatography on alumina columns. Since only small amounts of material were available, it was considered desirable to use the combination mass spectrometer-gas chromatograph for composition studies. Further reference to mass spectrometric-gas chromatographic analysis will be abbreviated to MS-GC analysis.

MS-GC Analysis of Fraction A. Resolution of most of the components of this mixture (Figure 5) on the combination instrument was achieved.

Satisfactory spectra of compounds of low concentration such as A1, A2, and A3 were not obtained. The spectra of samples A5, A7 and A8 were not reported because they were mixtures of two compounds.

It is seen in Figure 5 that four of the five reported spectra for Fraction A have parent ions of m/e = 136. There is considerable similarity in the spectra of samples A4, A6, A10 and A11 in that they all show significant ions at m/e = 136, 121, 105, 93, 91, 80, 79, and 69 (Table I). Their similar cracking patterns tends to indicate that all of these compounds are structurally related. Variations in the fragmentation of these samples seems to be mainly in intensity. Since the amount of sample introduced into the mass spectrometer overloaded the instrument in the cases of samples A4, A6, and A11, intensities were not accurately measured on fragments of high abundance.

A comparison of the spectra of monoterpene hydrocarbons reported by Ryhage (65) with the spectra of the compounds in Fraction A makes it seem probable that compounds A4, A6, A10 and A11 are of the ocimene-myrcene type (Figure 6).

Ryhage reported allo-ocimene (XXXVI) to have an intense molecule ion (m/e = 136) with the most intense ion in the spectrum occuring at m/e = 121 = M-15. These published spectra show a strong tendency of the parent ion to give the M-15 ion for all the members of this series. The high intensities of ions at m/e = 136 and 121 in the spectrum of sample A6 probably indicates that these ions are stabilized by a conjugated system.

Since β-ocimene-X (XXXVII) and β-ocimene-Y (XXXVIII) are geometric isomers, the spectra of these two compounds are very similar (65). The

most abundant ion in the spectra of these compounds is m/e = 93 = M-43. This fragment was shown to arise from both the molecule ion (m/e = 136) and the ion of m/e = 121.

The high intensities of the ions at m/e = 93 = M-43 in samples A4, A10 and A11 is probably the result of a smaller degree of stabilization in the parent molecule. It is probable that M-43 is formed by the loss of an isopropyl radical (C_3H_5).

Myrcene (XXXIX) was found (65) to be the most easily decomposed member of this group with a base peak at m/e = 41. A metastable ion at m/e = 24.3 indicated the formation of m/e = 41 from m/e = 69. These findings suggest that the chief point of fragmentation in this compound is at the bond connecting the two isoprene units.

Variations in fragment intensities obtained with different combination instruments makes it inadvisable to assign structures to A4, A6, A10 and A11 based on published spectra. However, the close similarity of fragmentation patterns makes it probable that these four compounds are composed of allo-ocimene (XXXVI), β-ocimene-X (XXXVII), β-ocimene-Y (XXXVIII), and myrcene (XXXIX). No attempt was made to assign a structure to sample A9.

It is doubtful that all of these monoterpene isomers were present in the original oil of catnip. Juneja (66) has shown that allylic alcohols readily dehydrate to a mixture of trienes during gas chromatographic analysis if the injection port temperature is above 150° C. If alcohols such as geraniol (XL), nerol (XLI) and linalool (XLII) were present in the crude oil, it is probable that the 250° C. inlet temperature used in the preparative GLC fractionation would have dehydrated

these alcohols to a mixture of trienes such as those observed.

MS-GC Analysis of Fraction B. Temperature programmed gas chromatographic analysis of Fraction B (Figure 7) on the combination instrument partially resolved this mixture into its components. Due to low concentration and poor separation of components, satisfactory spectra were not obtained for several compounds. It will be seen in Figure 7 that there are large variations in the masses of the components of this fraction ranging from m/e = 138 to 220. Gupta et al. (61) reported that Nepeta leucophylla oil contains phenylethyl acetate (XXIX) (MW = 150), ethyl benzoate (XXXII) (MW = 150), and phenylethyl isovalerate (XXX) (MW = 192). Compounds which possess these apparent molecular weights are also present in the oil of Nepeta cataria (Table II), but interpretation of the mass spectral data failed to confirm the presence of the characteristic fragments for the aromatic nucleus at m/e = 65, 77, 91, and 108. The complexity of the spectral data made it inexpedient to attempt to give structural assignments to the various compounds of Fraction B on the basis of their mass spectra.

MS-GC Analysis of Fraction C. Resolution of this mixture into four major components was achieved by temperature programmed gas chromatography on a JXR-CHDMS column in the combination instrument (Figure 8). Satisfactory spectra of samples C4, C7, C8, and C12 were obtained. The masses of all samples in which there was mixing of components or low concentration of sample introduced into the mass spectrometer were deleted. Samples C4 and C7 (Tables III and IV) have been identified by gas chromatography retention times and molecular weights as caryophyllene (II) and nepetalactone (I) respectively. Little work has been done on the

interpretation of mass spectra of sesquiterpene hydrocarbons. Without the aid of published work or isotopic labeling, an adequate interpretation of these spectra is virtually impossible. For this reason the interpretations presented here are of a theoretical nature based on current knowledge of fragmentations of related compounds. The only justification for these interpretations is that they may serve as a point of reference for future studies.

The base peak in the spectrum of caryophyllene is m/e = 93. It is not possible to tell if this ion is formed by direct decomposition of the molecule ion or in a two step reaction. The ion at m/e = 93 ($C_7H_9^+$) is prevalent in all of the spectra of bicyclic monoterpenes (65). Friedman (67) has proposed that this is a cyclic ion having some of the character of the tropylium ion rather than a simple open chain. This would partially explain the apparent stability of this fragment. A route for the formation of the fragment ion with m/e = 93 is provided for by the bond cleavages indicated in Figure 9 with subsequent loss of a proton. Direct decomposition of caryophyllene to ion m/e = 109 may be initiated by cleavage of the bond allylic to the two double bonds in the nine membered ring with subsequent cleavages at the bonds indicated in the four membered ring. The production of the ion of mass m/e = 121 is most easily rationalized by fragmentation as indicated in the figure. Expulsion of a gem-dimethyl group along with one hydrogen is characteristic of many terpenes (65,67). A pathway for the formation of the fragment m/e = 161 = M-43 by the expulsion of an isopropyl radical ($C_3H^{\cdot}$) from caryophyllene is shown. The formation of this radical from a gem-dimethyl compound must involve hydrogen migration. Ions of mass m/e = 189 = M-15

and m/e = 190 = M-14 are also evident in the spectrum of caryophyllene. It is possible for both M-15 and M-14 to be formed in several different ways. Differentiation between these pathways is impossible at the present.

Nepetalactone (Table IV) is considerably different from the other compounds investigated in that the parent ion (m/e = 166) is the most abundant ion in the spectrum. It is generally accepted that the most facile ionization in carbonyl compounds is at the carbonyl oxygen (68). This subsequently leads to cleavage of the bonds adjacent to the carbonyl group. Alpha-cleavage in lactones is more predominant at the carbon-carbon bond than the carbon-oxygen bond (69). Initial cleavage of the carbon-carbon bond adjacent to the carbonyl group followed by cleavages at other positions in the molecule could theoretically account for some of the fragments observed experimentally. The expulsion of CO and CO_2 from lactones (69) and esters (67) is well documented. Ions at m/e = 138 = M-28 and m/e = 122 = M-44 in the spectrum of nepetalactone are probably the result of such eliminations. In the breakdown of a molecule following electron impact, it is possible that either of the product fragments may retain a positive charge. This may explain the presence of both ions of m/e = 122 = M-44 and m/e = 44. Other fragmentation products which are probably the result of an α-cleavage are the ions at m/e = 109, 82, 69, and 55 (Figure 10). The small number of ions in the spectrum which may be due to alpha carbon-oxygen cleavage is in agreement with the findings of others (70). The ions of m/e 110 and 69 may be rationalized by cleavages such as those indicated in the figure.

Sample C12 in Table VI shows prominent ions at m/e = 65, 77, 91, 103 and 131 with an apparent molecular ion of m/e = 208. The cinnamic acid esters exhibit fragmentation patterns (70) in close agreement with those of sample C12. Parent molecular ion intensities have a tendency to decrease with increasing molecular weight in these esters. In the cinnamate esters an ion is observed at m/e = 131 due to the loss of the alkoxyl radical. The other two most characteristic ions in the spectrum of cinnamates are those of m/e = 103 and 77. These are accounted for as shown in Figure 11. The ion of m/e = 91 is probably the tropylium ion. This ion may decompose further (71) to give the cyclopentadienyl cation (m/e = 65). Since no cinnamates of parent mass 208 are known, it is likely that the ion of m/e = 208 is not the true molecular ion.

SUMMARY

Mass spectral-gas chromatographic analysis of catnip oil indicates the presence of a large number of different compounds. The complexity of this essential oil and the diversity of the compounds present introduced several problems. In some cases gas chromatographic separation was not sufficient to provide the high purity samples necessary for mass spectrometric analysis. Interpretation of the mass spectra is particularly difficult when none of the structural features of the molecule are known. Future tabulation of data from a large series of compounds will facilitate the interpretation of these spectra.

The occurrence of caryophyllene and nepetalactone in the essential oil of Nepeta cataria L. has been established by MS-GC analysis using authentic samples of these compounds as a basis of comparison.

The probable occurrence of an ester of cinnamic acid and a family of monoterpene trienes in the oil samples analyzed is suggested by the mass spectral data.

TABLE I

TABULATION OF THE INTENSE IONS IN THE SPECTRA OF THE COMPOUNDS IN FRACTION A

m/e	A4 %	A5 %	A9 %	A10 %	A11 %
41	10.0				100.0
43	17.6				
44	6.0				
53	4.7				
55	11.3	33.3			
56	2.0				
57	1.5				
65	2.2				
66	1.4				
67	15.3	61.3	100.0	19.3	10.6
68	12.0		100.0		
69	4.0	100.0	24.6	6.0	4.3
70	1.0		7.3		
71	0.8		16.6		
76	43.3	100.0	15.3		
77	6.9	21.3		45.3	16.0
78	48.6	100.0	45.3	70.0	
79	25.3	100.0	22.6	50.6	54.6
80	8.7	28.0	32.6	76.7	17.3
81	1.7		9.3	25.3	100.0
82					12.6

TABLE I (continued)

m/e	A4 %	A5 %	A9 %	A10 %	A11 %
83			14.0		
91	80.6	100.0	56.7	56.0	18.0
92	100.0	100.0	45.3	44.6	14.6
93	100.0	100.0	61.3	100.0	60.0
94	34.0	100.0	76.0	39.3	18.6
95	4.6	24.0	23.3	13.3	12.0
96			14.0		
103	4.3		6.1		
104	5.6		5.1		
105	23.3	28.0	12.7	46.0	12.6
106				24.7	
107	24.0	52.0	35.3	15.3	6.7
108	4.2	17.2	22.0		
109	1.8		3.5		68.0
110			2.6		10.7
111			9.3		
117			27.3		
118			7.4		
119	5.5	11.3	100.0	12.7	8.0
120	1.4		36.7		
121	36.6	100.0	42.7	28.0	15.3
122	8.6	14.0			

TABLE I (continued)

m/e	A4 %	A5 %	A9 %	A10 %	A11 %
124					82.6
125					8.0
132			14.0		
133			3.1		
134			100.0	4.2	4.1
135			14.0		
136	20.0	11.3	36.7	13.3	19.3
137	2.3	11.3	4.2		
139			6.3		
154			7.7		
155			0.7		

TABLE II

TABULATION OF THE INTENSE IONS IN THE SPECTRA OF THE COMPOUNDS IN FRACTION B

m/e	B13 %	B14 %	B15 %	B16 %	B19 %	B20 %	B21 %	B23 %	B24 %	B25 %
43	58.7		100.0							
53			16.0							
54			8.6							
55	23.5	78.7	57.3							
57			22.0							
59			72.0							
65				9.6			50.0			
67	17.0		23.6					31.3		
68	23.5		30.7							
69	35.3		16.0					64.7	56.1	
70	6.5									
71	100.0		8.7							
72	9.4									
76			18.0							
77							21.3			
78			53.0				14.0			
79		18.2	100.0				11.3		14.6	
80			100.0				82.5		38.2	61.5
81			23.3		100.0		48.9	100.0	100.0	100.0
82					17.0			34.0	29.2	34.4
83	17.6	100.0			6.9			9.3		18.9

TABLE II (continued)

m/e	B13 %	B14 %	B15 %	B16 %	B19 %	B20 %	B21 %	B23 %	B24 %	B25 %
84										14.8
85										27.0
86	43.5									
87	2.6				15.9				12.4	
88								18.0		
91	14.1			12.7		10.9	17.3			
92	20.0			40.6						
93	75.8			13.9			16.6		24.7	16.4
94			100.0						16.9	22.1
95							34.6	31.3	32.6	71.3
96			100.0					73.3	61.8	24.6
97					10.4			14.0	16.8	
98		44.2			13.4					
100					15.9					
101								70.0	61.8	
103						5.3	24.0			
104							40.6			
105		8.8	47.3			15.0	15.3			
106		6.4	65.3							
107		18.5	40.6							18.4
108		10.8								

TABLE II (continued)

m/e	B13 %	B14 %	B15 %	B16 %	B19 %	B20 %	B21 %	B23 %	B24 %	B25 %
109			58.0		30.0			40.6	100.0	51.6
110	12.9							26.6	30.3	22.5
111	76.5									13.1
112	6.5									
114					12.8			6.1	7.86	
115						6.5		5.1		
117						23.7				
119				17.6						
120				100.0						
121		6.8	51.3	28.5		9.9		29.3	19.1	14.8
122									7.8	
123			100.0					11.3	22.4	100.0
124			27.3		19.4					
125					12.4					
127					17.0			20.0		
128			10.0					20.0	2.2	
129					28.2					
131							46.0			
132						18.6	28.0			
133									13.5	
134				6.4						

TABLE II (continued)

m/e	B13 %	B14 %	B15 %	B16 %	B19 %	B20 %	B21 %	B23 %	B24 %	B25 %
135							31.3			
136	22.4		48.0							8.2
137	0.3		26.0							12.3
138			100.0					34.6	37.0	27.8
139	3.7	24.2	24.0				13.3	6.2		
140		2.1								
141					14.7			9.0	14.6	4.9
142					11.7					
147						44.4				
148						100.0				
149						2.8				
150		2.6								7.4
151										8.2
152	2.9			54.5	26.5					
153				5.2	13.5					
154	1.8									
155	0.2									
161						7.0	41.3	4.0	10.1	
162						2.4	100.0			
163							9.0			
166								22.5	40.4	8.2
167								10.0	11.2	9.8

TABLE II (continued)

m/e	B13 %	B14 %	B15 %	B16 %	B19 %	B20 %	B21 %	B23 %	B24 %	B25 %
169					4.2				5.6	
170								6.6	4.7	
175								24.0	3.1	
178										10.6
179										1.6
184					3.3					
187					0.5					
189						2.6			1.2	
190								11.3	1.8	
192						2.6				
220										4.8

TABLE III

TABULATION OF THE INTENSE IONS IN THE SPECTRUM OF SAMPLE C4

m/e	%	m/e	%	m/e	%
205	0.9	135	33.6	95	15.7
204	7.2	134	30.6	94	22.8
190	5.2	133	81.7	93	100.0
189	19.7	122	15.7	92	22.9
176	4.7	121	36.9	91	49.7
175	9.6	120	33.6	81	13.5
162	9.0	119	43.0	80	34.7
161	48.2	109	68.7	79	14.3
149	3.3	108	11.8	78	42.6
148	15.3	107	48.2	69	71.3
147	21.0	106	30.6	67	25.2
136	6.43	105	53.2		

TABLE IV

TABULATION OF THE INTENSE IONS IN THE SPECTRUM OF SAMPLE C7

m/e	%	m/e	%	m/e	%
168	0.7	120	6.9	78	3.1
167	9.8	111	15.2	77	4.3
166	100.0	110	16.4	75	9.0
165	1.6	109	50.0	73	20.7
153	5.8	108	4.1	70	3.6
152	1.8	107	11.4	69	61.9
151	10.0	105	11.4	68	11.4
150	2.6	97	5.4	67	47.6
149	24.3	96	17.9	66	19.8
148	2.9	95	65.5	65	20.5
147	4.8	94	18.6	57	7.9
139	2.9	93	11.9	56	7.1
138	32.0	91	7.1	55	20.5
137	14.9	85	19.0	54	3.6
135	9.0	84	11.7	53	5.5
133	3.1	83	12.6	45	5.0
124	11.0	82	25.2	44	16.4
123	84.6	81	76.2	43	2.9
122	56.0	80	38.1	42	19.3
121	13.3	79	12.4	41	2.9

TABLE V

TABULATION OF THE INTENSE IONS IN THE SPECTRUM OF SAMPLE C8

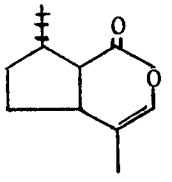
m/e	%	m/e	%	m/e	%
180	0.8	125	18.1	81	30.3
179	6.0	124	8.1	80	96.9
169	3.0	123	25.6	79	8.9
168	6.3	121	3.9	78	8.1
167	4.1	114	23.6	69	46.6
166	1.8	113	100.0	68	20.4
164	5.1	111	55.4	67	72.4
154	7.2	110	10.6	57	13.3
153	93.8	109	16.9	56	15.6
151	5.0	108	15.8	55	12.7
150	7.0	107	13.6	54	6.8
146	3.9	100	10.0	44	0.7
139	5.9	96	100.0	43	15.0
135	9.0	95	48.8	42	1.6
127	22.5	93	10.0	41	15.9
126	34.4	82	7.5		

TABLE VI

TABULATION OF THE INTENSE IONS IN THE SPECTRUM OF SAMPLE C12

m/e	%	m/e	%	m/e	%
208	2.8	163	3.4	131	21.6
194	2.1	162	5.3	119	17.3
193	19.0	161	26.8	104	2.8
192	100.0	149	1.7	103	7.8
191	14.1	147	14.0	91	17.2
179	1.3	134	6.3	77	6.3
178	7.9	133	16.6	65	8.4
165	22.4	132	4.9		

Figure 3. Constituents of the essential oils of different Nepeta species



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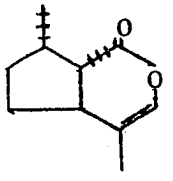

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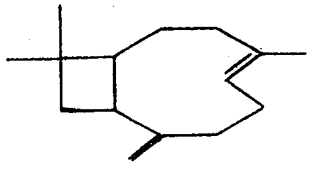

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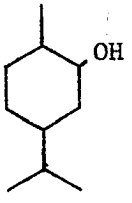

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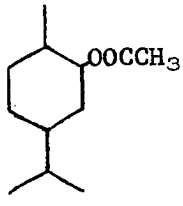

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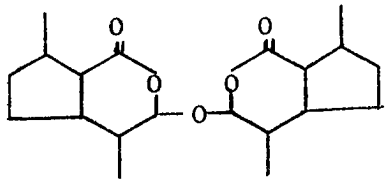

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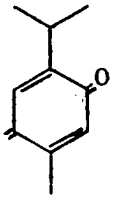

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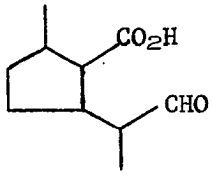

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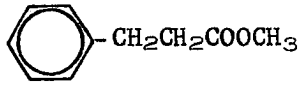

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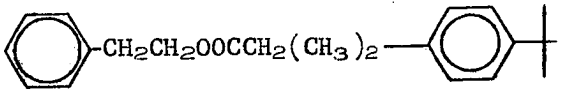

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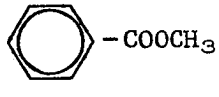

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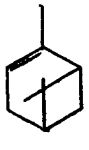

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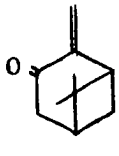

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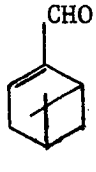


XXV

Figure 4. Gas liquid chromatographic analysis of crude oil of catnip

The conditions were as follows:

Column - 10' x 3/8" aluminum

Column Packing - 20% Apeizon L coated on Chromosorb W

Column Temperature - 220°

Inlet Temperature - 250°

Carrier Gas - Helium

Flow Rate - 120 ml./min.

Detector - Thermal conductivity

Detector Temperature - 250°

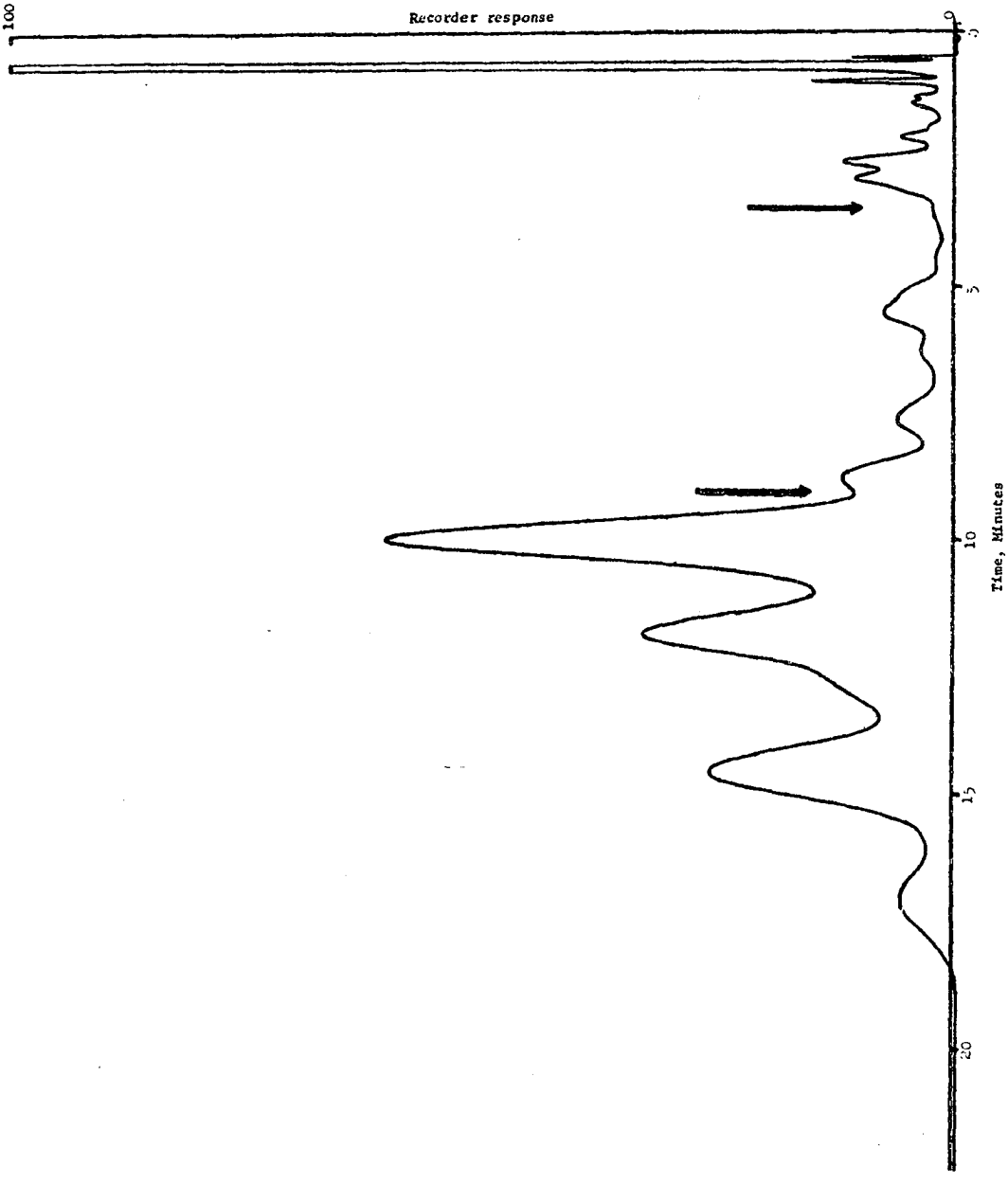


Figure 5. Gas liquid chromatographic analysis of Fraction A

The conditions were as follows:

Column - 20' x 5/32" coiled glass

Column Packing - 2% JXR and 0.33% CHDMS coated on Chromosorb P

Column Temperature - 85°

Inlet Temperature - 100°

Carrier Gas - Helium

Flow Rate - 20 ml./min.

Detector - Mass spectrometer

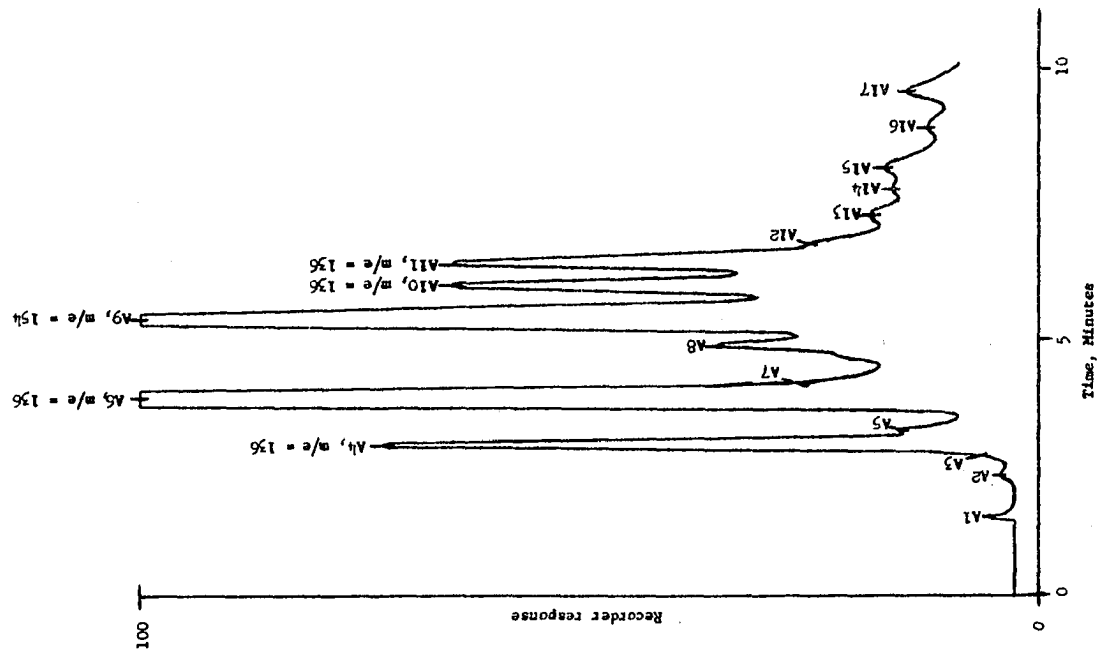
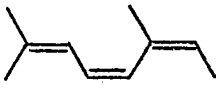


Figure 6. Some compounds composing Fraction A and the terpenols from which they may have arisen



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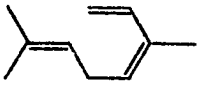

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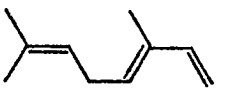

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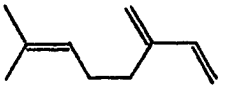

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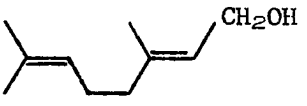

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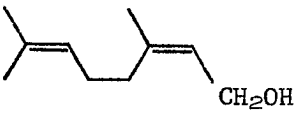

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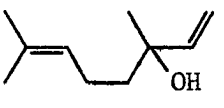


XLII

Figure 7. Gas liquid chromatographic analysis of Fraction B

The conditions were as follows:

Column - 20' x 5/32" colied glass

Column Packing - 2% JXR and 0.33% CHDMS coated on Chromosorb P

Column Temperature - programmed from 85° to 130°

Inlet Temperature - 100°

Carrier Gas - Helium

Flow Rate - 20 ml./min.

Detector - Mass spectrometer

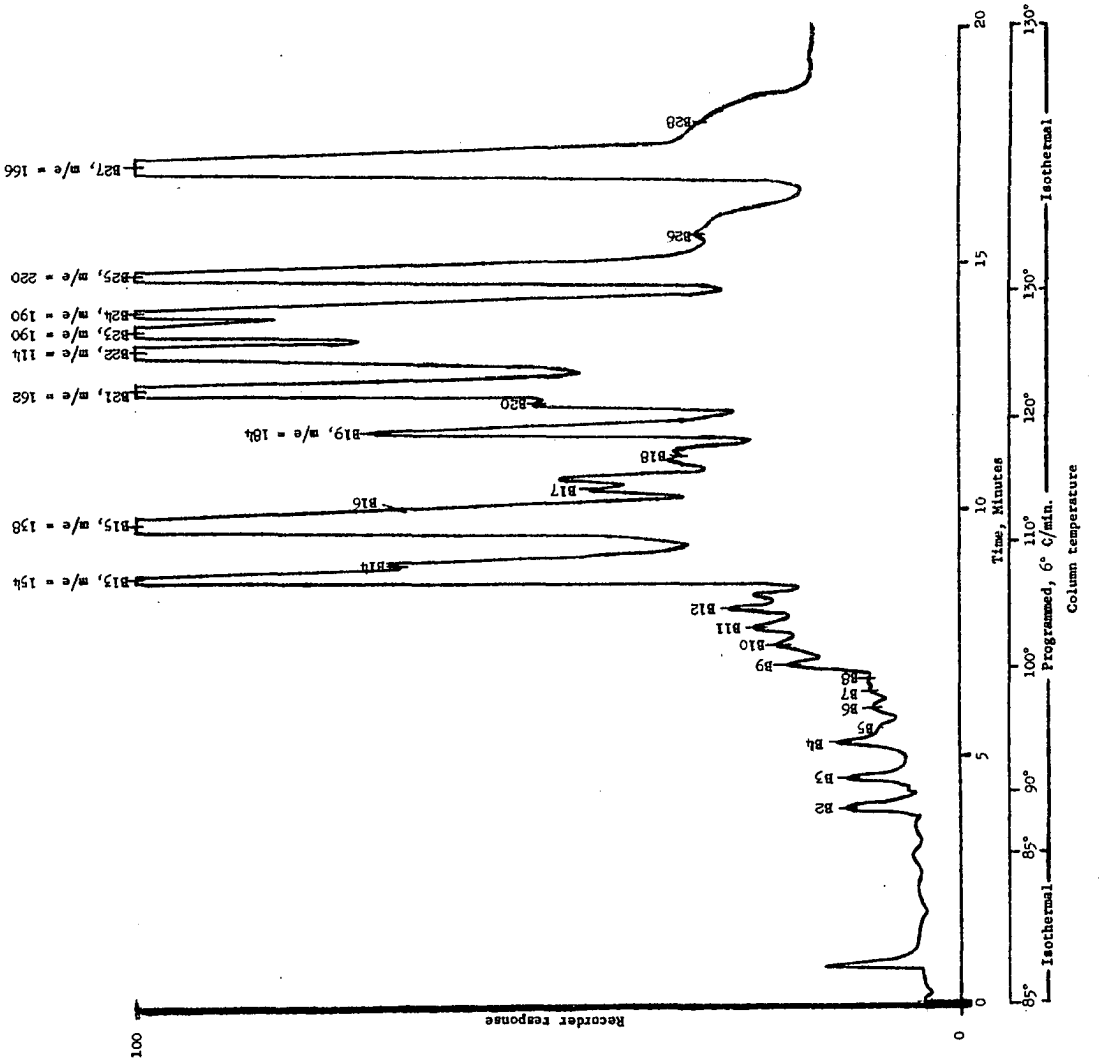


Figure 8. Gas liquid chromatographic analysis of Fraction C

The conditions were as follows:

Column - 20' x 5/32" coiled glass

Column Packing - 2% JXR and 0.33% CHDMS coated on Chromosorb P

Column Temperature - Programmed from 140° to 190°

Inlet Temperature - 160°

Carrier Gas - Helium

Flow Rate - 20 ml./min.

Detector - Mass spectrometer

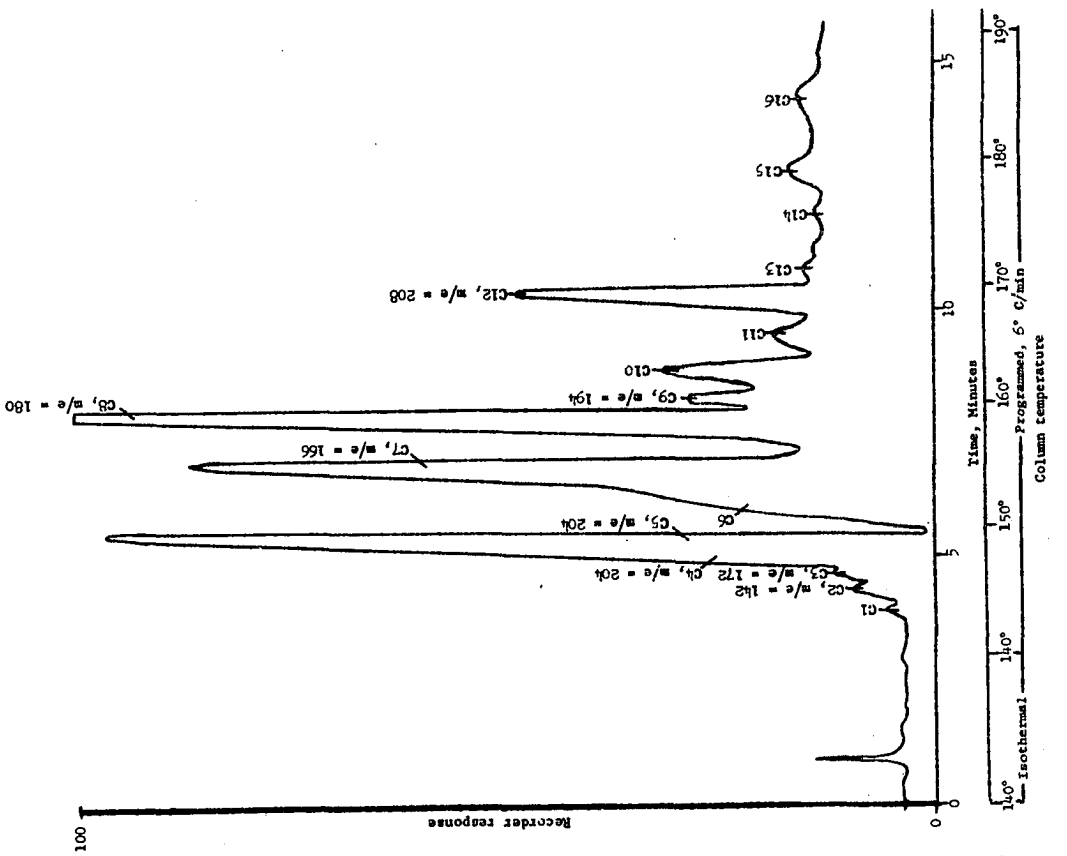
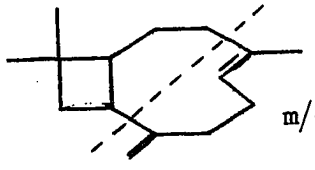


Figure 9. Proposed fragmentation of caryophyllene



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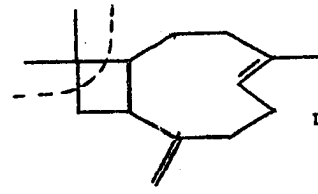

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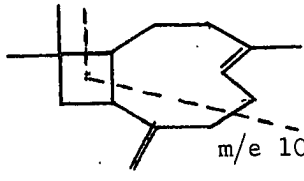
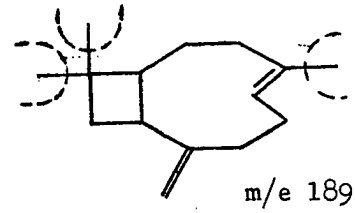


m/e 109



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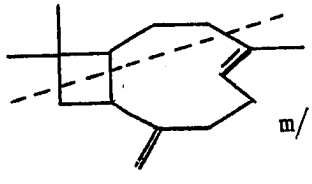


m/e 121

m/e 190

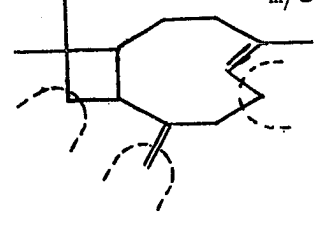
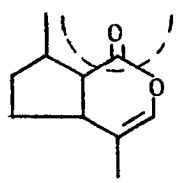


Figure 10. Proposed fragmentation of nepetalactone



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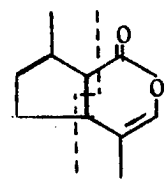
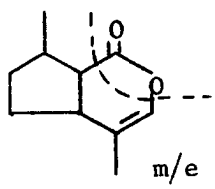
m/e 69





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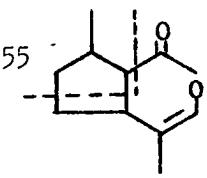

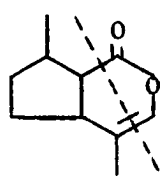

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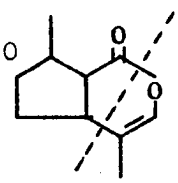
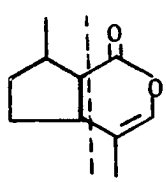
m/e 110





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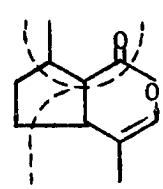
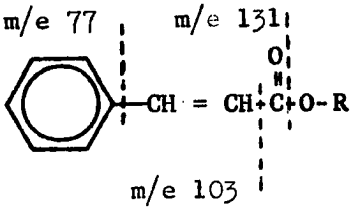


m/e 69

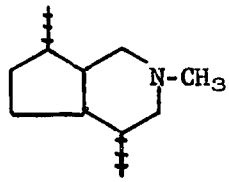
Figure 11. Proposed fragmentation for cinnamate ester



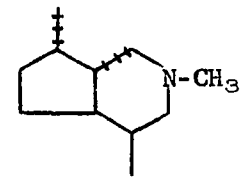
CHAPTER IV

THE DISTRIBUTION AND ANALYSIS OF NEPETALACTONE ISOMERS FROM DIFFERENT *NEPETA* SPECIES

The extensive investigations of the methylcyclopentane monoterpenoids of plant and animal origin have shown that most stereochemical variations occur at the 4a and 7a positions (Figure 12). Skytanthine (37, 72) and nepetalactone (I) (53) have been found to exist in both the



α-skytanthine



β-skytanthine

cis-trans and *trans-cis* configurations.

Bates and Sigel (73) estimated by gas liquid chromatography that nepetalactone (I) obtained from commercial oil of catnip was a mixture containing approximately 75% of the *cis-trans* isomer and 25% of the *trans-cis* isomer. During our studies on the biosynthesis of nepetalactone (I) it was observed that *Nepeta cataria* L. plants from greenhouse stock produced 99.9% of the *cis-trans* isomer (I) and 0.1% of the *trans-cis* isomer (Ia) which will be referred to as epinepetalactone.

The recent report by Eisner (2) that nepetalactone can serve as an insect repellant focuses attention on the role which this methylcyclopentane

monoterpenoid might have in insect control. It would be of considerable interest to know if nepetalactone (I) and epinepetalactone (Ia) are equally effective as insect repellants.

This chapter reports the development of an analytical technique for analysis of mixtures of nepetalactone (I) and epinepetalactone (Ia). Variations of isomer ratios among different oil samples of Nepeta is also reported and discussed.

EXPERIMENTAL PROCEDURE

Plant Material. The various Nepeta species were grown in the greenhouse either from seeds or cuttings. Additional oil of catnip was obtained from catnip plants grown near Fairbury, Nebraska and East Lansing, Michigan.

Isolation of the Essential Oils. The plants were subjected to steam distillation for one-half hour, using an all glass system. The distillate was saturated with sodium chloride and extracted with ethyl ether. The ether solution was dried over anhydrous magnesium sulfate, filtered and concentrated at atmospheric pressure. The essential oil was stored at -15° C. until used.

Gas Liquid Chromatography. Gas liquid chromatography was performed on a Beckman Model GC-2A gas chromatograph equipped with a Bristol, 1 millivolt recorder, a thermal conductivity cell and a Disc Instruments, Incorporated, disc integrator. Stainless steel columns, 250' x 0.02" and 400' x 0.02" coated with Apeizon L and LAC 886 respectively, were used. Nepetalactone (I) and epinepetalactone (Ia) were chromatographed on the Apeizon L column at 160°. The dimethyl nepetalinates were

chromatographed on the LAC 886 column at 178°. Peak areas were measured by electromechanical integration and quantitation of amounts made by comparison with standard curves obtained with pure compounds. Nepetalactone, nepetalic acid, and nepetalinic acids with known absolute configuration and stereochemistry were available from earlier studies (52) and were also freshly prepared.

Conversion of Nepetalactone (I) and Epinepetalactone (Ia) to the Dimethyl Nepetalinates. Approximately 0.1 ml. of crude catnip oil was treated with 1 ml. of 10% NaOH and stirred for 1 hr. at room temperature. The neutral components of the oil were removed by three successive extractions with equal volumes of ethyl ether. After adjusting to pH 2 with 2 N HCl, the solution was extracted again with ethyl ether, concentrated, and esterified with diazomethane. Air oxidation for 48 hrs. at room temperature yielded the half ester of nepetalinic acid. The sample was prepared for gas chromatography by esterifying a second time with diazomethane (52). This oxidation scheme is shown in Figure 12.

RESULTS AND DISCUSSION

Epimerization is a reaction characteristic of lactones with asymmetric centers α to the carbonyl group. These compounds lose protons reversibly from the α position and an equilibrium between the diastereoisomers is established. It is conceivable that lactones in the vapor phase could be epimerized by contact with metals or by alkali in solution. Therefore, the previously reported analyses of nepetalactone-epinepetalactone ratios using alkaline hydrolysis and vapor phase chromatography were re-investigated.

The Analytical Method. This problem was approached through simultaneous analysis of oil samples by direct gas liquid chromatography and by chemical degradation of nepetalactone (I) and epinepetalactone (Ia) to the corresponding dimethyl esters of nepetalinic acid (Figure 12, XLV-XLVc). By comparing the quantitative results from these two techniques, it was possible to determine the accuracy of the analytical techniques (see Table VII).

Nepetalactone (I) and epinepetalactone (Ia) were chromatographed on an Apiezon L column while the dimethyl nepetalinates were separated on a LAC 886 column. Complete separation of nepetalactone, epinepetalactone (Figure 13) and the dimethyl nepetalinates (Figure 14) was achieved.

The results shown in Table VII indicate that the quantitative data obtained from chemical degradation and direct GLC analysis are in close agreement. The Nebraska, and Fritzche oils were found to contain large amounts (30%) of epinepetalactone (Ia) while nepetalactone (I) obtained from pyrolyzed nepetalic acid (52) and Nepeta cataria L. contained only small quantities of Ia.

The close agreement of the data from the two analytical techniques makes it possible to draw several conclusions. It is evident that there is not a thermal equilibrium between the diastereoisomers I and Ia in the gas chromatography inlet. Subsequent experiments varying the inlet temperatures from 200° to 250° indicated that no change occurred in the isomer ratio of the Michigan catnip oil. It is also concluded that the strong base (10-20% KOH) used in the hydrolysis of nepetalactone (I) to nepetalic acid (XVIII-XVIIIc) does not epimerize nepetalactone at, prior to, or after ring opening.

Effect of Isolation Conditions on Nepetalactone-Epinepetalactone Ratios.

It was considered that amine rust inhibitors present in the steam lines, prolonged steam distillation, or metal salts might cause epimerization of nepetalactone (I) to epinepetalactone (Ia). A mixture of nepetalactone (I) and epinepetalactone (Ia), isomer ratio 99:1, was subjected to simulated steam distillation conditions by heating with 10 volumes of water at the reflux temperature for several hours. Extended exposure to any or all of these three conditions was ineffective in changing the isomer ratio (Table VIII).

Nepetalactone-Epinepetalactone Ratio Variations of Different Nepeta Species. Environmental conditions, such as climate and season, appeared to have no discernible effect on isomer ratios. The Michigan oil and samples obtained from the majority of Nepeta cataria L. plants collected throughout the growing season in Oklahoma yielded more than 99% nepetalactone (I).

Plants were occasionally found in the Nepeta cataria greenhouse stock which showed up to 32% epinepetalactone (Ia). Since no distinct morphological differences were observed in these plants, it appeared that the differences were due to biological variation of the specie. This point is still under investigation.

From the results shown in Table IX, it is evident that a constant nepetalactone-epinepetalactone ratio exists in different parts of Nepeta cataria L. plants. This is in contrast to the methylcyclopentane alkaloid produced by Skytanthus acutus Meyen, where it is found that the α- to β-skytanthine ratios in roots, leaves, and stems vary considerably.

Large variations in isomer ratios were found in different species of Nepeta. In Table IX it is seen that both Nepeta cataria and Nepeta mussini produce over 99% nepetalactone (I), while Nepeta nuda produces predominantly epinepetalactone (Ia). Nepeta citredoria produces both nepetalactone (I) and epinepetalactone (Ia). It is likely that the wide variations in isomer ratios in these species is of genetic origin. The lower ratio of nepetalactone (I) to epinepetalactone (Ia) in the commercial catnip oil and the Nebraska oils may be due to one of three things: (1) the mixing of species which contain large concentrations of epinepetalactone (Ia) with Nepeta cataria L. during harvest; (2) hybridization resulting from different species growing together; or (3) variability within the species. The variation in the greenhouse stock which had been classified as Nepeta cataria L. suggests that variation within the species is possible.

The existence of both nepetalactone (I) and epinepetalactone (Ia) in the same plant may have some importance in biosynthetic studies of nepetalactone. Studies on the biosynthesis of α- and β-skytanthine provide an interesting analogy to those of I and Ia. Auda et al. (74) observed a considerable variation in the ratio of α- to β-skytanthine in the different parts of Skytanthus acutus Meyen. Comparison of the specific activities of the skytanthine isomers, biosynthetically labeled from mevalonic acid-2-^{14}C showed that the specific activity of β-skytanthine was always 2 to 3 times higher than that of α-skytanthine. This suggests that β-skytanthine (trans-cis configuration) may be the precursor of α-skytanthine (cis-trans configuration).

SUMMARY

A new method for the analysis of nepetalactone (I) and epinepetalactone (Ia) mixtures has been developed. The nepetalactone-epinepetalactone ratios in samples of different biological origin have been studied with this technique.

From these studies the following conclusions were reached: (1) mixtures of nepetalactone (I) and epinepetalactone (Ia) may be analyzed by direct gas chromatography or chemical degradation without epimerization; (2) the 99.9:0.1 ratio of nepetalactone (I) to epinepetalactone (Ia) is constant for all parts of most Nepeta cataria L. plants; however, occasional plants were analyzed which contained over 30% of epinepetalactone (Ia); (3) there is a wide variation in the nepetalactone-epinepetalactone ratio in various Nepeta species.

TABLE VII

RELATIVE CONCENTRATIONS OF NEPETALACTONE ISOMERS IN *NEPETA cataria* OILS

Sample	Nepetalactone Isomers		Methyl Nepetalinates			
	cis-trans %	trans-cis %	α %	δ %	β %	γ %
Wisconsin Oil	29	71	13	12	38	36
Nebraska Oil	69	31	23	35	22	20
Michigan Oil	99	1	64	36	trace	trace
Fritzche Oil	60	40	-	-	-	-
Nepetalactone from Nepetalic Acid	99	1	68	32	trace	trace
Nepetalactone from extraction of *Nepeta cataria**	99.6	0.4	73	27	trace	trace

*The oil was extracted with ethyl ether.

TABLE VIII

EFFECT OF DIFFERENT ISOLATION TECHNIQUES ON THE NEPETALACTONE-EPINEPETALACTONE RATIO*

Sample Number	Isomerization Conditions		Nepetalactone Isomers	
	Reflux time hours	Catalyst Added	cis-trans %	trans-cis %
1	2	none	98.9	1.1
2	24	none	98.9	1.1
3	2	steam condensate	98.9	1.1
4	2	Zn	98.9	1.1
5	2.5	ZnO	98.9	1.1
6	2	Al	98.9	1.1
7	2	Al_2O_3	98.9	1.1
8	4	Fe	98.9	1.1
9	4	FeO	98.9	1.1

*The reflux temperature was 98.7° C.

TABLE IX

RELATIVE CONCENTRATION OF NEPETALACTONE ISOMERS FROM DIFFERENT SOURCES

Plant Species	Plant Age	Part of Plant	Nepetalactone Isomers cis-trans %	Nepetalactone Isomers trans-cis %
Nepeta cataria	Mature Flowering	Whole Plant	99.9	0.1
Nepeta cataria	Mature Flowering	Flowers	99.9	0.1
Nepeta cataria	Mature Flowering	Leaves	99.9	0.1
Nepeta cataria	Mature Flowering	Stems	99.9	0.1
Nepeta mussini	Young	Whole Plant	99.9+	trace
Nepeta citredoria	Young	Whole Plant	64.0	36.0
Nepeta nuda	Young	Whole Plant	24.0	76.0

Figure 12. Conversion of nepetalactone and epinepetalactone to dimethyl nepetalinates

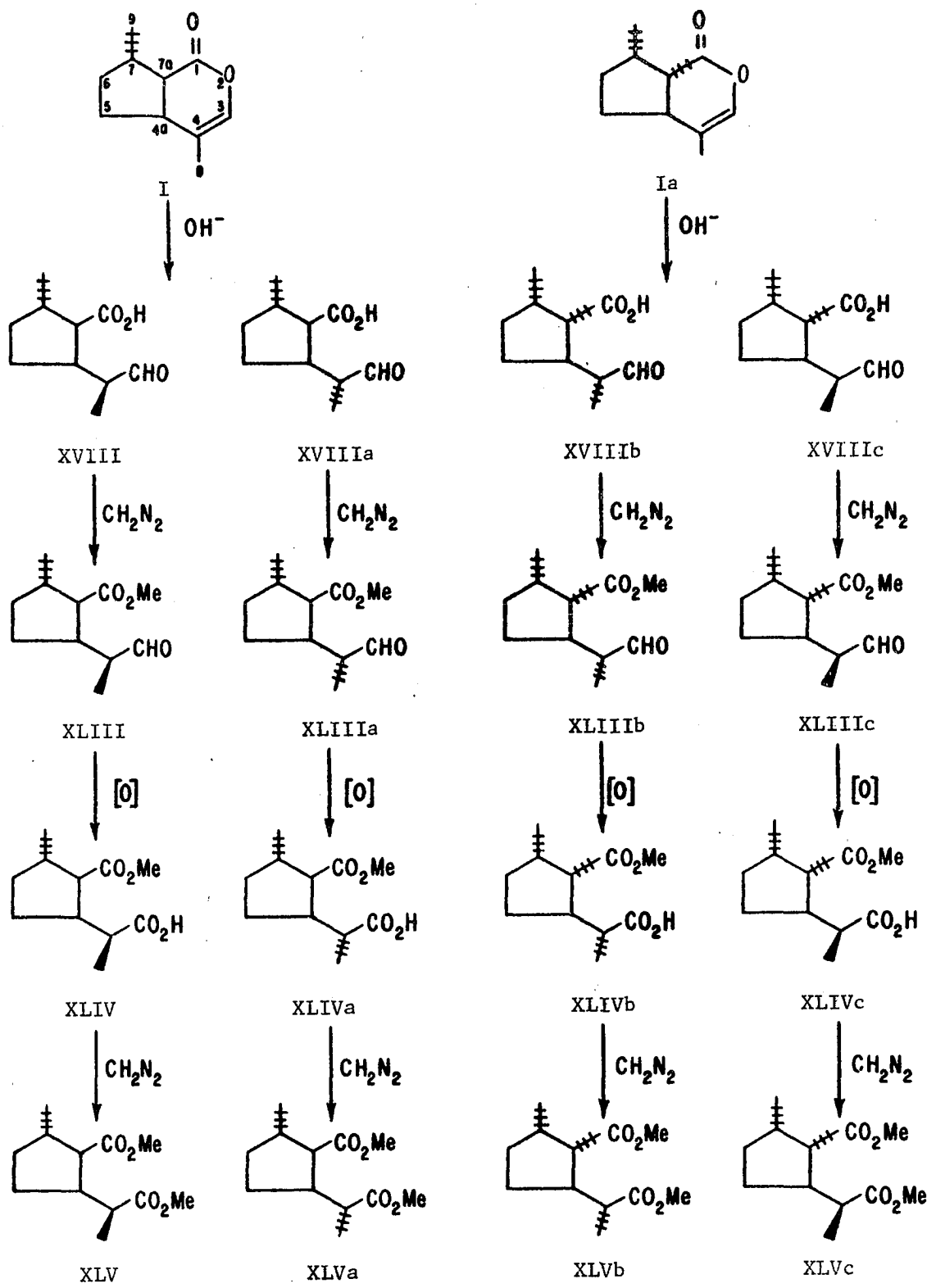


Figure 13. Gas liquid chromatographic analysis of the dimethyl-nepetalinates

0.05 μl of the mixture was used. The column was 400' x 0.02", LAC 886 and was kept at 162° C. A hydrogen flame detector was used (Perkin-Elmer Model 801 Gas Chromatograph).

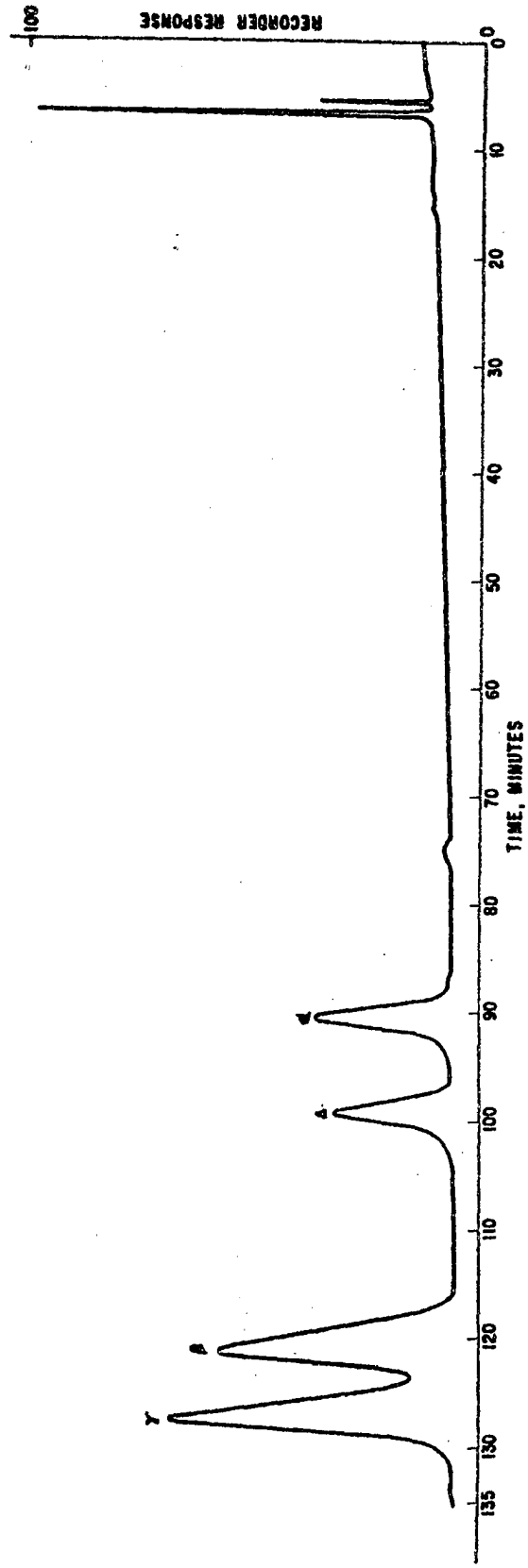


Figure 14. Gas liquid chromatographic analysis of various Nepeta species

Conditions were as follows:

Column - 250' x 0.02" capillary

Coating - Apiezon-L

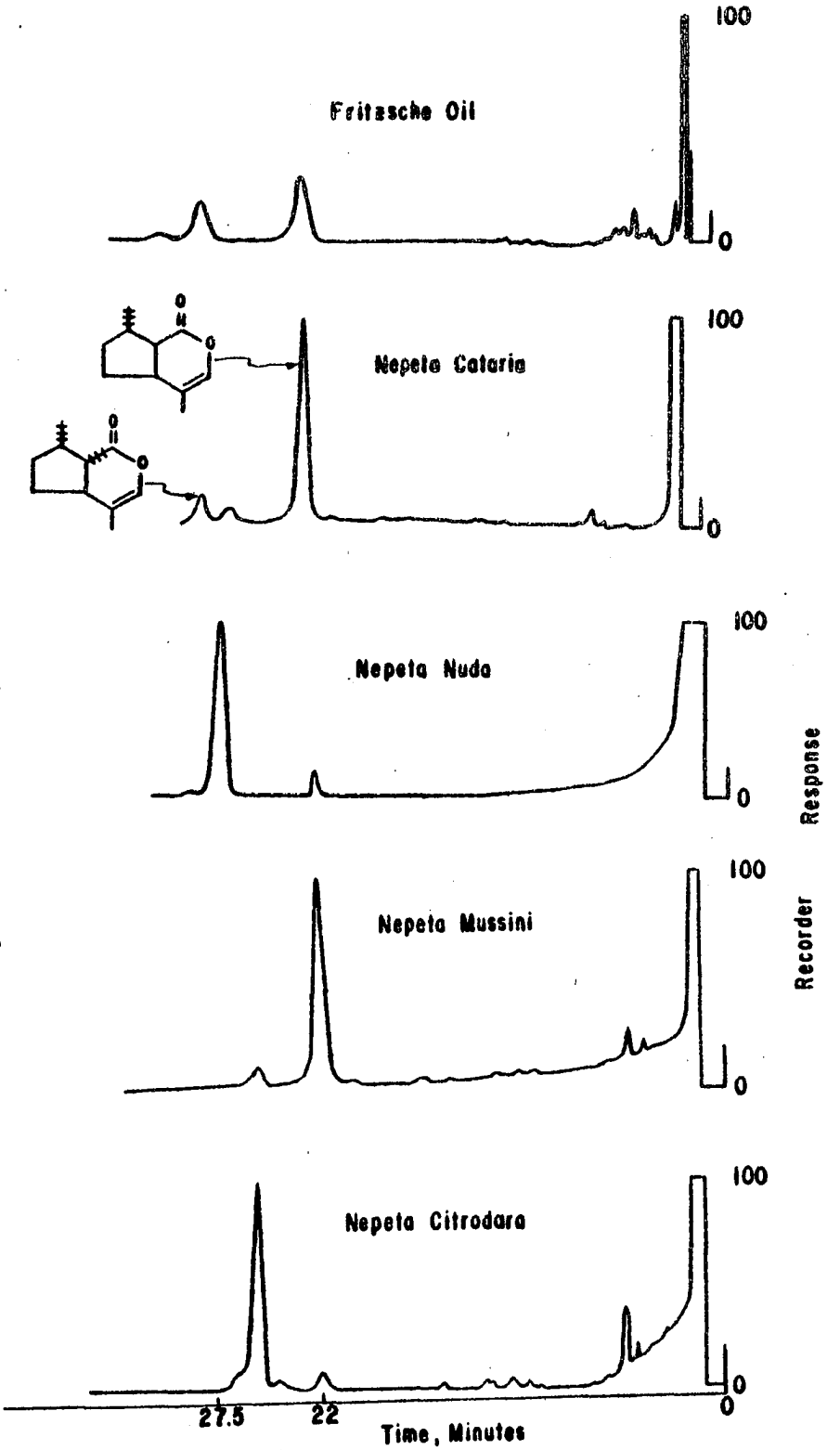
Column Temperature - 160° C

Injection Block - 190° C

Carrier Gas - Helium

Flow Rate - 2 cc/min.

Detector - Thermal conductivity



CHAPTER V

THE BIOSYNTHESIS OF NEPETALACTONE

The structure and stereochemistry of a molecule often provides some documentation of the chain of events that occurred in its biosynthesis. In the case of the methyl cyclopentane ring system found in nature, it is suggested that the carbon skeleton is derived from known isoprenoid precursors. The isoprene rule as formulated by Ruzicka (75) provides as powerful a tool for studies of the biosynthesis of terpenes as it did in their structure elucidation. Most theoretical discussions of terpene biosynthesis have their origin in what is currently known about the biological generation of the isoprene unit.

The generation of isopentenyl pyrophosphate (XLVI)— the biological isoprene unit — from acetyl coenzyme A (XLVII) and malonyl coenzyme A (XLVIII) probably occurs by the route outlined in Figure 14. Recent findings (75, 76) indicate that XLVII and XLVIII are converted directly to mevalonic acid (XLIX) without passing through the free coenzyme A esters of hydroxymethylglutaric acid (L) and acetoacetic acid (LI). The intermediates are bound to the enzyme at all times until the final release of mevalonic acid (XLIX) (77, 78). It is seen in the figure that acetoacetyl-enzyme is a pivotal compound in the biosynthesis of fatty acids and isoprenoid compounds. The conversion of mevalonic acid (XLIX) to isopentenyl pyrophosphate (XLVI) is catalyzed by three enzymes; mevalonate

kinase, mevalonate-5-phosphate kinase, and mevalonate-5-pyrophosphate kinase.

The formation of mevalonate-5-phosphate (LII) from mevalonic acid (XLIX) and ATP through the action of mevalonate kinase was first recognized by Tchen (79) in yeast enzyme preparations. Seventy-fold purification of this enzyme was achieved by treatment of a high-speed supernatant with protamine sulfate followed by ammonium sulfate and calcium phosphate gel fractionations (80). Leevy and Popják (81) have reported the partial purification of mevalonate kinase from pig liver. Both of these enzymes were reversibly inhibited by p-chloromercuribenzoate, indicating the presence of an SH group(s) necessary for enzyme activity.

Utilization of mevalonate-5-phosphate (LII) and ATP in the formation of mevalonate-5-pyrophosphate (LIII) by mevalonate phosphate kinase has been observed in yeast (82) and liver (83) systems.

The conversion of LIII to isopentenyl pyrophosphate (XLVI) has been reported (84) to be a bimolecular reaction between LIII and ATP to form ADP, Pi, CO_2, and isopentenyl pyrophosphate (XLVI). Evidence has been presented (85) that the terminal phosphate of ATP reacts with the tertiary hydroxyl group of mevalonate-5-pyrophosphate (LIII) during its decarboxylation to isopentenyl pyrophosphate (XLVI). Attempts to isolate a phosphorylated intermediate were unsuccessful.

It is found that part of the isopentenyl pyrophosphate (XLVI) must be converted to dimethylallyl pyrophosphate (LIV) before polymerization to higher isoprenoid analoges may occur. Lynen (86) showed this conversion by the enzyme isopentenyl pyrophosphate isomerase to be inhibited by iodoacetamide. At equilibrium the ratio of XLVI to LIV is 87:13 (87).

The addition and removal of the proton at the 2 position is found to be stereospecific in this reversible reaction. An enzyme substrate complex has been isolated in which the intermediate is proposed to be bound covalently to a sulfhydryl group of the enzyme protein.

The condensation of dimethylallyl pyrophosphate (LIV) and isopentenyl pyrophosphate (XLVI) occurs in the formation of geranyl pyrophosphate (LV) with the elimination of pyrophosphate ion. In this reaction there may be a stereospecific elimination of a proton from the 2 position of isopentenyl pyrophosphate (XLVI). Experimental verification of this point however, awaits further purification of the enzyme involved in the catalysis.

The work of Yeowell and Schmid (31) clearly establishes that the cyclopentanoid ring system is derived from isoprenoid precursors. Mevalonate was shown to be incorporated exclusively into the aglycone portion of the cyclopentanoid glycoside plumieride (VIII). Roughly 50% of the total radioactivity of the glycoside was located at carbon 7, while the other 50% was evenly distributed between carbons 3 and 15 (Figure 1).

After the formation of geranyl pyrophosphate (LV), pathways for the genesis of monoterpenes must necessarily diverge from those for the formation of other isoprenoid compounds. All further discussions which are presented in the literature are of a theoretical origin based on related structures and stereochemistry and have no foundation in nature. These theoretical discussions are of value only insofar as they provide the worker with a model upon which he may orient his thinking.

In 1955, Sir Robert Robinson (88) proposed a scheme for the biogenesis of methylcyclopentane monoterpenoids on the basis of the novel structural features of some of the monoterpenes found in nature. Geraniol (XL) and citral (LVI) were considered to be the first intermediates. A stereospecific enzymatic reduction of citral (LVI) would yield L-citronellal (LVII), after which terminal oxidation would yield 2,6-dimethyloct-2-en-1,8-dial (LVIII). Terminal oxidations of this type have been reported by Williams (89) to occur in the rabbit. Enzymatic cyclization of the acyclic dialdehyde LVIII would then yield iridodial (XIV) as outlined in Figure 15.

Robinson and his colleagues (90) were able to cyclize the aldehydo-acetal of LVIII to L-iridodial (XIV) by 50% acqueous acetic acid treatment. Cavill (88) feels that his reaction constitutes a simulation of the biogenetic process used in the synthesis of L-iridodial (XIV). In vitro biogenetic simulation is not widely accepted and the significance of this reaction in relation to what might occur naturally is therefore questionable.

Iridodial (XIV) is formulated to be an equilibrium mixture of the 1,5-dialdehyde and its cis-trans (LIX) and trans-cis (LIXa) lactol tautomers (91). These detailed investigations of the reactions of iridodial (XIV) indicate that the cis-trans configuration is preponderant. Oxidation of the appropriate lactol tautomer would yield nepetalactone (I) and epinepetalactone (Ia).

Cookson (92) has demonstrated that citral (LVI) may be cyclized by UV irradiation to photocitral-A (LX) containing the methylcyclopentane ring system. Photocitral-B (LXI) was also produced in small amounts.

The stereochemistry of the ring substituents in photocitral-A was not determined by these workers. Since this reaction also provided a route to the cyclopentane ring system it deserves equal consideration in the biogenetic scheme for nepetalactone (I).

This chapter reports some results which indicate that an isoprenoid biosynthesis pathway is utilized in the formation of nepetalactone by Nepeta cataria L. plants. Preliminary findings concerning the biosynthesis of caryophyllene (II) by this plant are also presented.

EXPERIMENTAL PROCEDURES

Plant Material. Nepeta cataria plants were grown in the greenhouse either from seeds or cuttings. Additional oil of catnip was obtained from catnip plants grown in fields near Fairbury, Nebraska and East Lansing, Michigan.

Isolation of the Essential Oils. The plants were subjected to steam distillation for one-half hour, using an all glass system. The distillate was saturated with sodium chloride and extracted with ethyl ether. The ether solution was dried over anhydrous magnesium sulfate, filtered and concentrated at atmospheric pressure. The essential oil was stored at -15° C. until used.

An alternate procedure for the isolation of the essential oil without the use of steam employed a hexane extraction. The hexane extract was filtered through a 1:1 super cel-magnesium oxide column after drying over anhydrous magnesium sulfate. The quality of the essential oil obtained is fully as good as that obtained by steam distillation; however, more lipid material is obtained in the extracts than from steam distillation.

Chromatographic Analysis of Nepetalactone and Caryophyllene. The separation of nepetalactone and caryophyllene from the other components of catnip oil was accomplished by gas chromatography and thin layer chromatography. Since many compounds are present in the oil it is possible that radiochemically pure nepetalactone and caryophyllene were not obtained with this thin layer chromatographic technique; however, this technique was used when it was necessary to recover small amounts of the labeled substrates so that their specific activity could be determined.

Gas liquid chromatography 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 millivolt recorder and a Disc Instruments, Incorporated disc integrator. Analysis of nepetalactone (I) and caryophyllene (II) was performed: (a) on 20' x 1/8" stainless steel columns packed with 20% Apeizon L on 60-80 mesh Chromosorb W (acid washed) and a column temperature of 225° or (b) on a 250' x 0.02" stainless steel column coated with Apeizon L and a column temperature of 160°.

Analysis of the degradation products of nepetalactone (I) and nepetonic acid (XVIII) was performed on a 250' x 0.02" stainless steel column coated with Apeizon L. The temperature was held at 160° C. and the helium flow rate was 2 ml./min. Nepetalactone and caryophyllene were identified by comparison of their retention times with standard compounds and by using combination mass spectrometry-gas chromatography (63). Nepetalic acid (XVIII) and nepetalinic acids (XXI-XXIa) with known absolute configurations and stereochemistry were available from earlier studies (54) and were also freshly prepared. Peak areas were measured

by electro-mechanical integration and quantitation of amounts made by comparison with standard curves obtained with pure compounds.

Commercially available thin layer chromatography plates (20 x 5 x 0.4 cm.) were coated with a 0.5 mm. layer of silica-gel G applied as a slurry consisting of 60 g. of silica-gel G and 115 ml. of water. The coated plates were activated in the oven 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 available standards were placed beside the unknowns for a direct comparison of R_f values. The plate was developed with a hexane:acetone:ethanol (40:10:4) mixture. The compounds were stained by exposing the plates to iodine vapor.

Labeled Compound Used. DL-mevalonic acid-2-^{14}C (N,N' dibenzylethylene-diamine salt) obtained from Nuclear Research Chemicals, Orlando, Florida, was converted to the free acid which had a specific activity of 1.7 mc./mmole. To regenerate the free acid, a weighed amount of the salt was dissolved in a minimal amount of water and the pH adjusted to 10 with 1 N NaOH. Dibenzylethlenediamine was removed by two extractions with an equal volume of ethyl ether. The ether extracts were combined and extracted with 0.5 volume of NaOH solution (pH 10). This washing was added to the original aqueous solution. The mevalonate-2-^{14}C was purified by descending preparative paper chromatography using Whatman No. 1 paper and 85% isopropyl alcohol as the developing solvent. An R_f of 0.48 was observed for the mevalonate (XLVIII). Its purity was checked by paper chromatography with n-butanol:acetic acid:water (4:1:1) and only one spot was observed.

Isotope Analysis. The counting technique used was dependent on the specific activity of the isolated material and the method used in the isolation. Samples of low specific activity were usually subjected to thin-layer chromatography and the radio assay was accomplished by scraping the plates clean at the appropriate areas followed by direct counting of absorbent and compound in a liquid scintillation spectrometer. 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 has an efficiency of 44% in the Tri-Carb liquid scintillation spectrometer. The iodine vapor used to detect the compounds had no measurable effect on the liquid scintillation counting efficiency. Quenching and absorption of labeled compounds by silica-gel G were measured by the internal standard method using benzoic acid-^{14}C and the results showed no measurable effect on counting efficiency at the concentrations used, consequently, no correction factors were used.

The radioactive components separated by gas chromatography were measured using a Nuclear-Chicago Model 4998 continuous gas flow monitoring system. This instrument has a counting efficiency of 18% at a detector temperature of 225° using methane at a rate of 100 ml./min. as the counting gas.

Nepetalic Acid (XVIII) From Oil of Catnip. The essential oil was extracted with an excess of 10% sodium hydroxide. The alkaline solution was acidified and extracted twice with equal volumes of ether. After drying with anhydrous magnesium sulfate and concentration under diminished pressure, the residue was taken up in a minimal quantity of warm petroleum

ether (b.p. 40-60° C.). Nepetalic acid (XVII) slowly crystallized from the solution after dry-ice cooling. The acid was filtered in several crops.

Alkaline Hydrogen Peroxide Degradation of Nepetalic Acid (XVIII). A solution containing 1 g. of nepetalic acid (XVIII) in 10 ml. of 10% potassium hydroxide was treated with 5 ml. of 30% hydrogen peroxide. The temperature was usually maintained at 27°, with stirring, for 1 hour. The solution was then treated with several mg. of platinum oxide and the stirring continued for an additional 15 min. After acidification and extraction with ether, the solution was dried with anhydrous magnesium sulfate and concentrated. The samples were prepared for gas chromatography by esterification with diazomethane.

Alkaline Hydrogen Peroxide Degradation of Nepetonic Acid (XIX). A solution of 0.15 g. of nepetonic acid (XIX), 1 ml. of 10% sodium hydroxide, and 0.5 ml. of 30% hydrogen peroxide was stirred at room temperature for 12 hrs. At the end of this period the excess hydrogen peroxide was decomposed by adding several mg. of platinum oxide. After acidification and extraction with ether, the solution was dried over magnesium sulfate and concentrated. The samples were analyzed by gas chromatography after esterification with diazomethane.

Separation of Nepetolactone From the Alkaline Hydrogen Peroxide Degradation Products. The ether extract from the alkaline hydrogen peroxide degradation of nepetalic acid was extracted with saturated sodium bicarbonate to remove acidic material. The neutral ether layer yields nepetolactone.

Nepetic Acid. A solution of 10 mg. of nepetonic acid or nepetolactone in 0.5 ml. of 10% sodium hydroxide was treated with 0.5 ml. of 10%

bromine-20% potassium bromide solution by dropwise addition over a 1 hr. period. After acidification the solution was evaporated to dryness at 0.1 mm. pressure. Distillation at 200° C. (0.1 mm.) yielded nepetic acid.

Preparation of the Methyl Ester of 2-Acetoxy-5-Methylcyclopentane Carboxylic Acid. Nepetonic acid (XIX) was oxidized according to the method of Emmons (93). The peroxytrifluoroacetic acid solution was prepared by dropwise addition of 0.5 ml. of trifluoroacetic anhydride to a suspension of 0.8 ml. of 90% hydrogen peroxide in 0.5 ml. of cold methylene chloride. This solution was added over 20 min. to a suspension of 1.3 g. of dry disodium hydrogen phosphate in a mixture of 1.5 ml. of methylene chloride and 0.2 g. of methyl nepetonate. After addition was complete, the solution was heated under reflux 30 min. and the insoluble salts were then collected by filtration. The salts were washed with 1.0 ml. of methylene chloride. The combined filtrates were washed with 10% sodium carbonate and dried over magnesium sulfate. Concentration and vacuum distillation yielded 64% of methyl-3-acetoxy-5-methylcyclopentane carboxylate and 36% of dimethyl nepetate by gas chromatographic analysis.

RESULTS AND DISCUSSION

Histological Examination of Nepeta cataria L. Leaves. Green leaves of Nepeta cataria L. in various stages of development were fixed in FAA (formalin-acetic acid-alcohol), dehydrated in a tertiary butyl alcohol series and embedded in paraffin. Transverse serial sections were cut at 10 microns and stained in standard safranin-fast green. Microscopic examinations were made with high dry and oil immersion objectives.

Black and white microphotographs were made at magnifications of 562.5X and 1125X.

Microscopic examination of leaf-cross section prepared from leaves at three different stages of development show (Figure 17) several types of glandular structures occurring 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 (Figure 17A) no stalk is visible; in the intermediate stage (Figure 17B) formation of a stalk with a globular head is seen. In the late stage (Figure 17C) 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 (94). The essential oil of Nepeta cataria L. is probably accumulated in these glands.

Occurrence of Nepetalactone and Caryophyllene in Nepeta cataria L. Plants. The essential oil from the leaves, stem, and flowers of mature flowering Nepeta cataria L. plants contains about 85% nepetalactone, 10% caryophyllene and 5% of a mixture of other compounds (Figure 4). These parts of the plant yield roughly 0.3% of this steam volatile oil on a wet weight basis. Plants 1 month old were found to contain 0.15% and 0.01% of nepetalactone and caryophyllene respectively (ratio 15:1). It appears that there is a continuous increase of these compounds which reaches a maximum at senescence.

There is a considerable variation in the absolute content of nepetalactone and caryophyllene between the upper parts of the plant and the

root system. Fresh roots (693 g.) yielded 5 x 10^{-7}% (4 mg.) of nepetalactone and 4 x 10^{-6}% (0.03 mg.) of caryophyllene. The yield of nepetalactone is 5 x 10^{-5} of that to be expected from an equivalent weight of leaves and stems. The ratio of nepetalactone to caryophyllene (125:1) differs widely from that found in the upper parts of the plant (8:1).

Mevalonic Acid-2-^{14}C As A Terpenoid Precursor. When mevalonic acid-2-^{14}C was tested as a precursor for nepetalactone and caryophyllene biosynthesis by Nepeta cataria L. plants, radioactivity was incorporated into these compounds in experiments using plants of different ages. The extent of incorporation varied with time and with the parts of the plant. The incorporation of radioactivity from mevalonic acid-2-^{14}C produced by young plants (approx. 4 cm. tall) into nepetalactone and caryophyllene as a function of time is shown in Figure 18. A rapid incorporation of carbon-14 into caryophyllene was observed but this leveled off at about 50 hours and remained constant for the duration of the experiment. In contrast there is an initial rapid period of incorporation of carbon-14 into nepetalactone which levels off at about 12 hours, remains constant for nearly 125 hours and then begins to rise again. The leveling off in the latter curve is indicative of mevalonic acid being converted to another precursor which initially is further away from nepetalactone in the biosynthetic pathway, but which after 125 hours, has been converted to a more direct precursor. Such a metabolic system might operate via the "metabolic grid" system proposed by Birch (95). It should be pointed out that the specific activities of nepetalactone (2.5 μμc./μmole) and caryophyllene (111 μμc./μmole) and the extent of incorporation of carbon-14 into these molecules was very low, being 0.011 ± 0.004% and

0.014 ± 0.002% respectively.

The specific activity of nepetalactone isolated from new leaves is 6.3 times greater than that isolated from old leaves of the same plant (Table X). Possible explanations for the higher specific activity of the material from new leaves are: (1) there may be greater metabolic activity and, therefore, an increased rate of synthesis in the new leaves would be expected to occur 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.

To establish that mevalonic acid-2-^{14}C was being metabolized to $^{14}CO_2$, the CO_2 released by plants that had been injected with radioactive mevalonic acid was collected and analyzed. The increase of respiratory $^{14}CO_2$ was linear with respect to time up to 30 hours, after which time no further increase in evolution of $^{14}CO_2$ was observed (Figure 19). A total of 0.14% of the administered mevalonic acid-2-^{14}C was evolved as respiratory CO_2 during the 50 hour duration of the experiment.

A preliminary investigation of the site of synthesis of nepetalactone indicates that the roots are not required. Plants with excised roots were dipped into an aqueous solution containing 1.9 μc. of mevalonic acid-2-^{14}C (1.0 mc./mmole). Nepetalactone and caryophyllene were isolated by TLC and their radioactivity measured. The results indicated that 0.01% of the administered radioactivity was recovered in nepetalactone and 0.03% was recovered in caryophyllene. These results are similar to those obtained using whole plants (Figure 18), consequently, it may be concluded that roots are not required for the biosynthesis of

these terpenoids.

The method used in administering mevalonic acid-2-^{14}C to Nepeta cataria L. can influence the rate of uptake of the compound. Uptake by plants with excised roots is the most rapid with detectable radioactivity appearing in the growing tips within 15 minutes. From stem injections, about 2 hours is required for translocation to the upper growing tips and from the crown (the base from which the stems protrude) injections, about 12 hours is required.

A preliminary $^{14}CO_2$ feeding experiment was done on a whole plant in a gas tight chamber equipped with an end-window counter for continuous monitoring of the atmosphere in the vessel. Incandescent lamps mounted around the chamber provided continuous illumination during the duration of the experiment. The plant incorporated 96% of the administered $^{14}CO_2$ (0.645 mc.) in 48 hours. Nepetalactone separated from the other components of the crude oil by GLC and counted with the continuous gas flow monitor was found to have a specific activity of 11.7 mμc./μmole.

Degradation of Nepetalactone (I). Based on the structure of nepetalactone (I) it would be predicted that radioactivity from mevalonic acid-2-^{14}C would be incorporated into carbons 3 and/or 8 and 6 and/or 9 if an isoprenoid biosynthetic pathway exists. The primary objective of the oxidative degradation of nepetalactone (I) was to determine the location of labeled carbon atoms. A combination of the haloform reaction and alkaline hydrogen peroxide oxidation was used in an attempt to accomplish this objective.

It is necessary to convert the nepetalactone (I) in crude catnip oil to nepetalic acid (XVIII) by alkaline hydrolysis prior to alkaline

hydrogen peroxide degradation. The contaminating neutral components of the essential oil were still present after alkaline hydrolysis and were removed by extraction with ether. When the volume of the solution was small (0.1 ml.) and the surface area of the vessel large, as much as 50% of the nepetalic acid (XVIII) was converted to the nepetalinic acids (XXI-XXIa). The ratio of α-nepetalinic acid (XXI) to Δ-nepetalinic acid (XXIa) was usually 2:1. The presence of these nepetalinic acids was confirmed by comparison with retention times of authentic standards on Apeizon L and LAC 886 capillary columns. By bubbling molecular oxygen through a 10% sodium hydroxide solution of nepetalic acid a 78% yield of the nepetalinic acids was obtained. Purging the solution with nitrogen during hydrolysis greatly reduced the formation of nepetalinic acids. This undesirable side reaction is probably caused by the dissolved oxygen in the solution. When small scale reactions were run, the isolation of crystalline nepetalic acid (XVIII) was not attempted.

The alkaline hydrogen peroxide degradation of nepetalic acid (XVIII) and nepetonic acid (XIX) to nepetolactone (XXIV) (Figure 20) was achieved by adding 30% aqueous hydrogen peroxide to the nepetalactone hydrolysate after the extraction of the neutral components. The reaction rate was influenced by both temperature and base concentration. Time course studies using GLC analysis showed that the oxidation of nepetalic acid (XVIII) was complete in 10 minutes at 27°, while 50-60 minutes were required at 0°. As the temperature was increased to 54°, degradation of nepetonic acid (XVIII) became pronounced. The degradation of the nepetonic acid (XVIII) formed in the initial reaction also increases as the concentration of base in the reaction is increased from 10% to 30%.

Hydrogen peroxide concentration does not seem to be as critical in these oxidative reactions. Increasing the hydrogen peroxide content from 10% to 30% produces little change in reaction products.

The mechanism of formation of nepetolactone (XXIV) and the nepetalinic acids (XXI-XXIa) is best explained by attack of the hydroperoxide anion (OOH^-) at the carbonyl carbon with formation of a hydroxy hydroperoxide (Figure 20). On the basis of other work it is probable that this compound would rearrange to either a carboxylic acid (96) or to an ester (97). The formation of nepetonic acid (XVIII) is rationalized by a different mechanism. Elkik (96) and Doering (98) have found that there is a strong tendency for the hydroperoxide anion and other forms of active oxygen to react at the position α to the carbonyl group in strong base (Figure 20). The peroxide formed in the initial reaction would subsequently rearrange to nepetonic acid (XIX).

The degradation of nepetonic acid (XIX) would be expected to proceed by the same mechanism (Figure 21) as the degradation of nepetalic acid (XVIII) with the formation of trans-cis nepetic acid (XXII), 3-methylcyclopentanone (LXII), and 2-hydroxy-5-methylcyclopentane carboxylic acid (LXIII). Treatment of nepetonic acid (XIX) with an alkaline hydrogen peroxide solution resulted in the formation of a number of products. Trans-cis nepetic acid (XXII), 3-methylcyclopentanone(LXII), and 2-hydroxy-5-methylcyclopentane carboxylic (LXIII) were identified as degradation products by GLC after treatment with diazomethane. Identification of these compounds is based on comparison of retention times and sample mixing on Apeizon L and SE-30 columns. The methyl ester of LXIII was prepared from methyl 3-acetoxy-5-methylcyclopentane carboxylate by

hydrolysis with 10% NaOH and esterification with diazomethane.

Nepetolactone (XXIV) was separated from the acidic nepetalic acid (XVIII) degradation products by extraction of ether solutions of this mixture with saturated sodium bicarbonate. Both nepetolactone (XXIV) and nepetonic acid (XIX) were converted to nepetic acid (XXII) in approximately 80% yield by treatment with alkaline solutions of sodium hypobromite.

Mevalonic acid-2-^{14}C (0.4 μmole, sp. act. 3.14 μc./μmole) was injected into the stems of young Nepeta cataria L. plants. The difference in the specific activities of the nepetalactone (sp. act. 4.25 μμc./μmole) isolated from the plant and the nepetic acid (sp. act. 3.2 μμc./μmole) indicate that 25% of the radioactivity of the nepetalactone was located in carbon atoms 3 and 8.

SUMMARY

Radioactivity from mevalonic acid-2-^{14}C is incorporated into nepetalactone and caryophyllene by Nepeta cataria L. plants. The extent of incorporation into caryophyllene is greater than that of nepetalactone, however both were low. A chemical degradation for nepetalactone using alkaline hydrogen peroxide and haloform reactions was developed to permit the determination of the amount of carbon-14 labeling in carbon atoms 3 and 8. Twenty-five per cent of the radioactivity in nepetalactone was found to be located in carbon atoms 3 and 8.

TABLE X

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

Precursor	Nepetalactone		
	Plant Part	Radioactivity Recovered cpm/mg	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 μmoles per day for 7 days)	Young Leaves	257	6.3:1
	Old Leaves	41	

Figure 14. Pathway for the biosynthesis of geranyl pyrophosphate

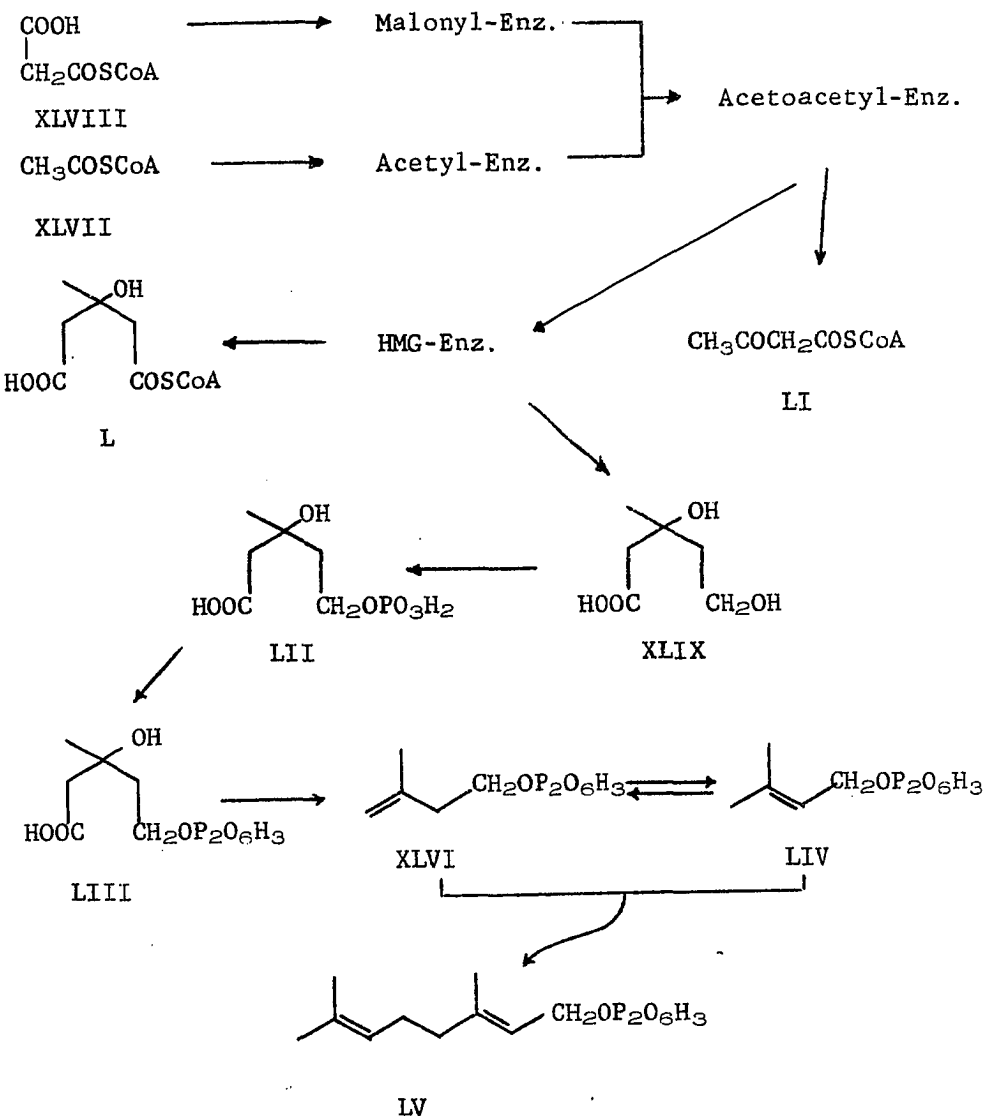


Figure 15. Proposed scheme for the biogenesis of nepetalactone

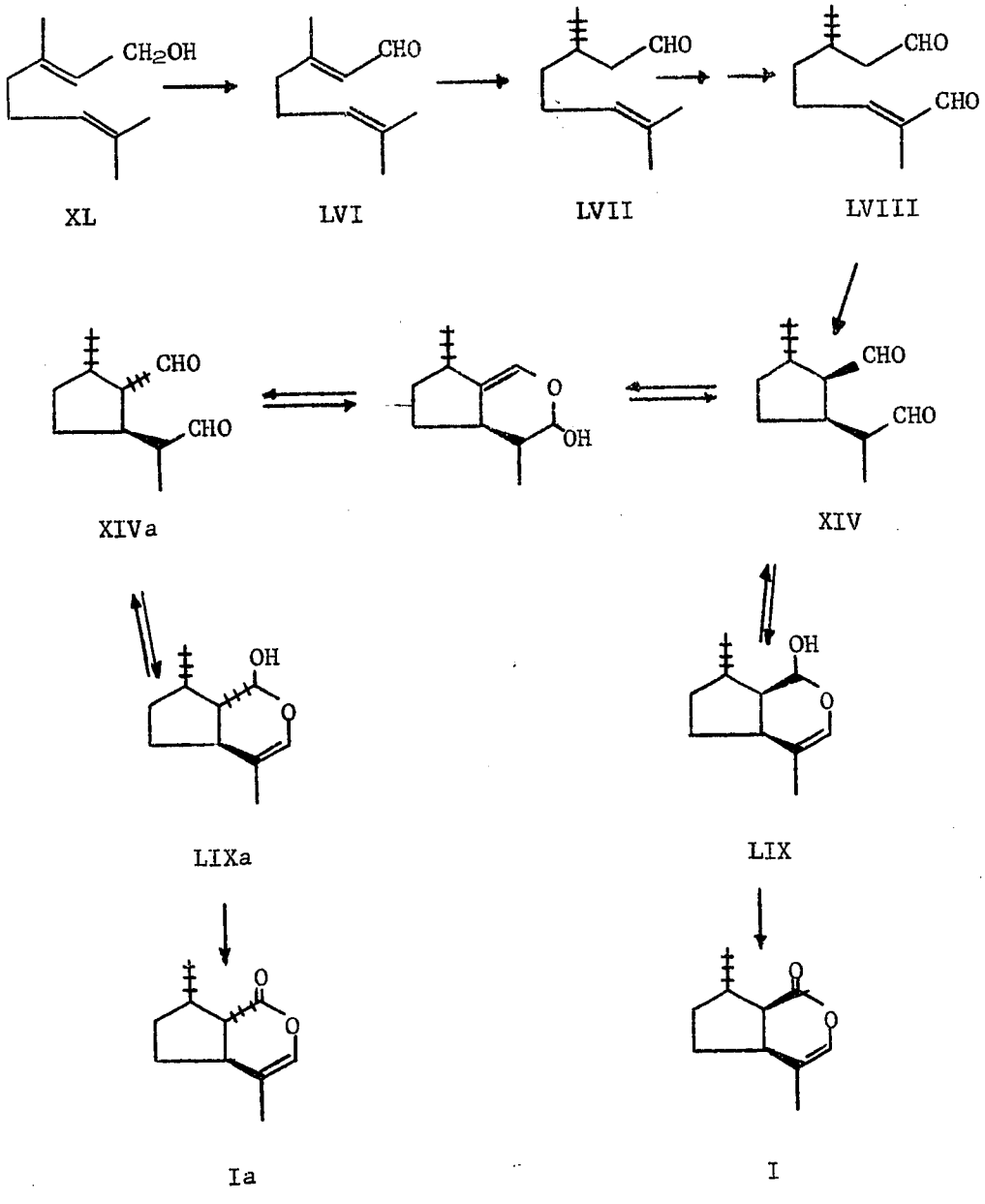
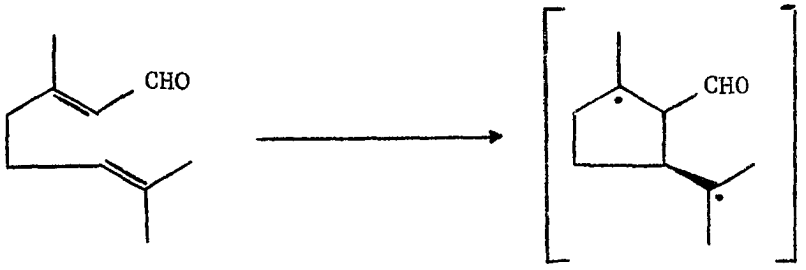
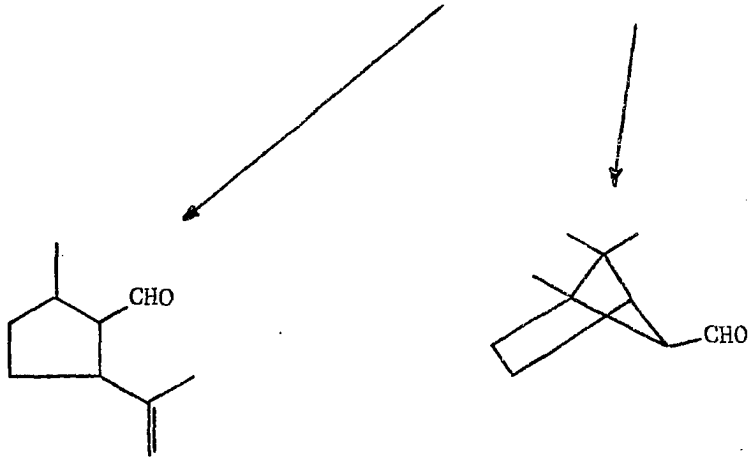


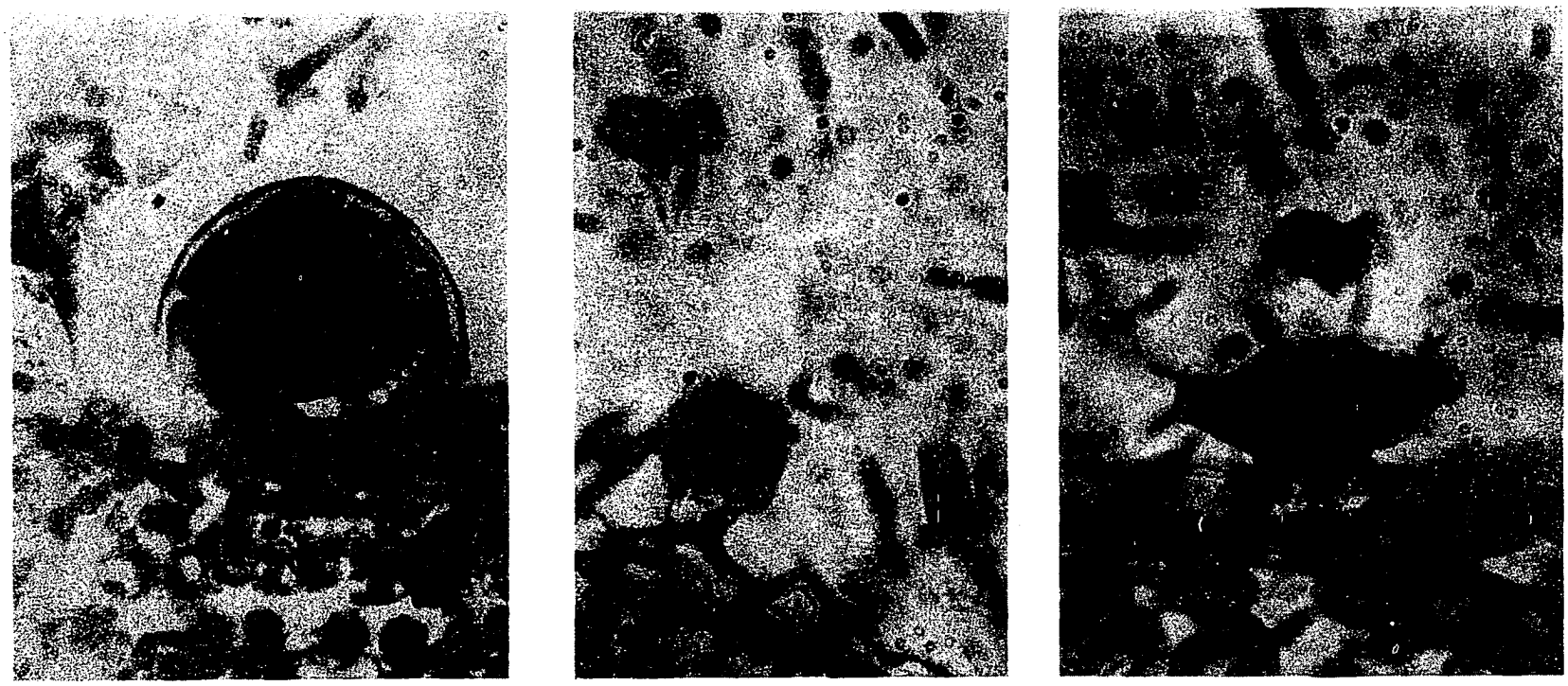
Figure 16. The photochemical cyclization of citral



LVI



LX LXI



A B C

Figure 17. Nepeta Cataria L. Transverse (Cross) Sections of Leaf Tissue

A. Early stage x1125. B. Intermediate stage x562.5. C. Later stage x562.5.

Figure 18. Incorporation of radioactivity from mevalonic acid-2-^{14}C into nepetalactone and caryophyllene produced by Nepeta cataria L. plants 0.32 μc. of mevalonic acid-2-^{14}C was injected into the stem of plants weighing 1.1-2.6 gms. At appropriate intervals 4 plants were harvested and the nepetalactone and caryophyllene isolated and their radioactivity measured by TLC as described under methods.

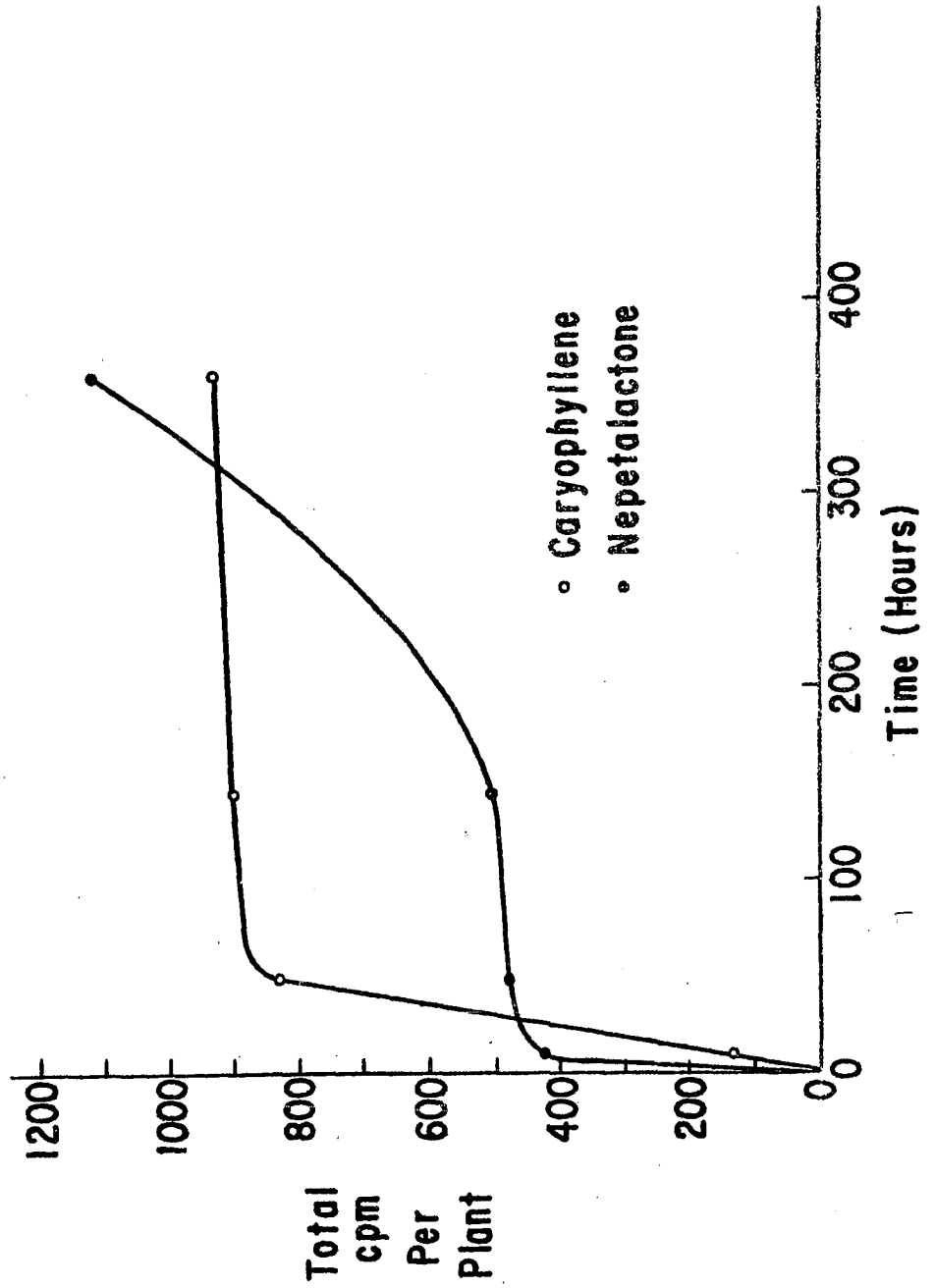


Figure 19. $^{14}CO_2$ released by young Nepeta cataria plant after administration of mevalonic acid-2-^{14}C

1.9 μc. of mevalonic acid-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.

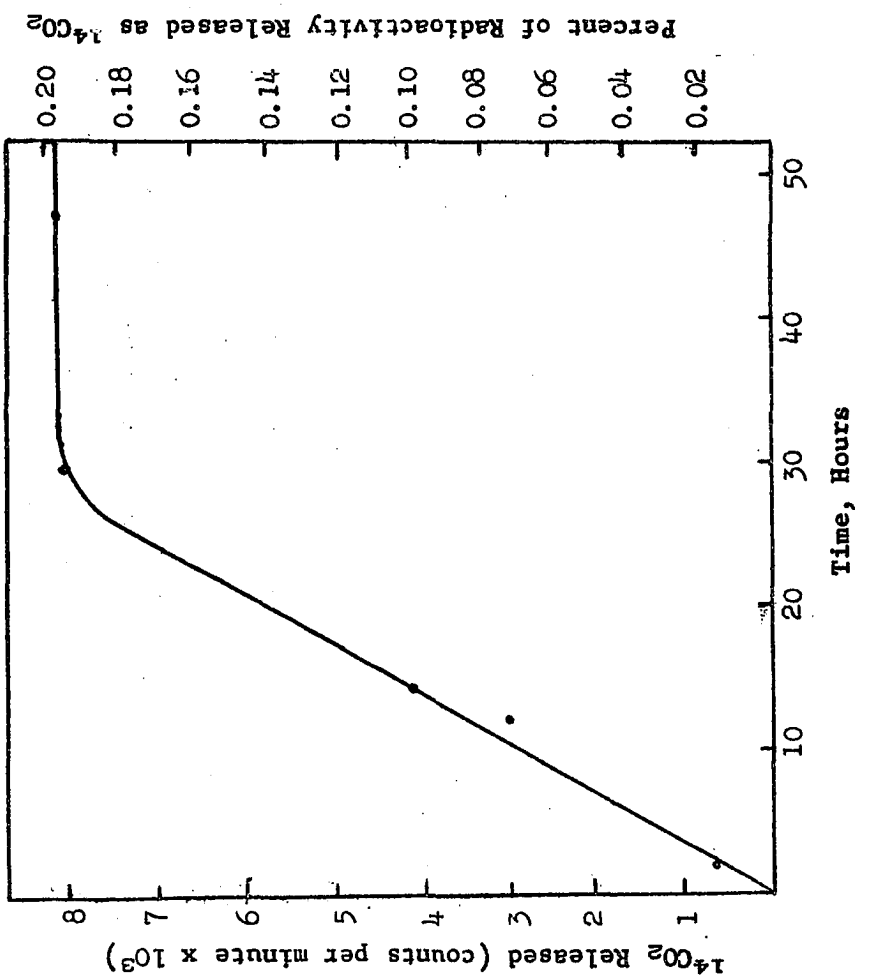


Figure 20. Alkaline hydrogen peroxide degradation of nepetalactone (I)

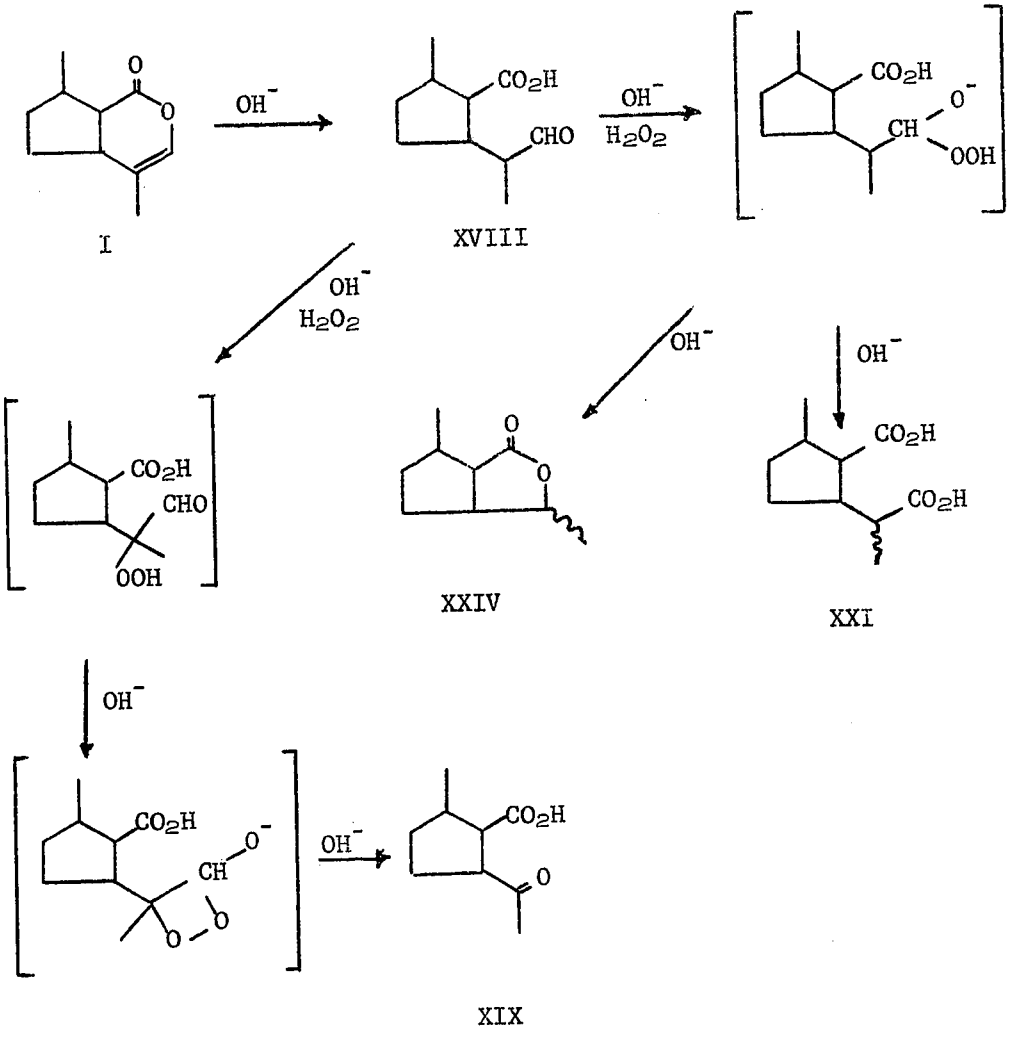
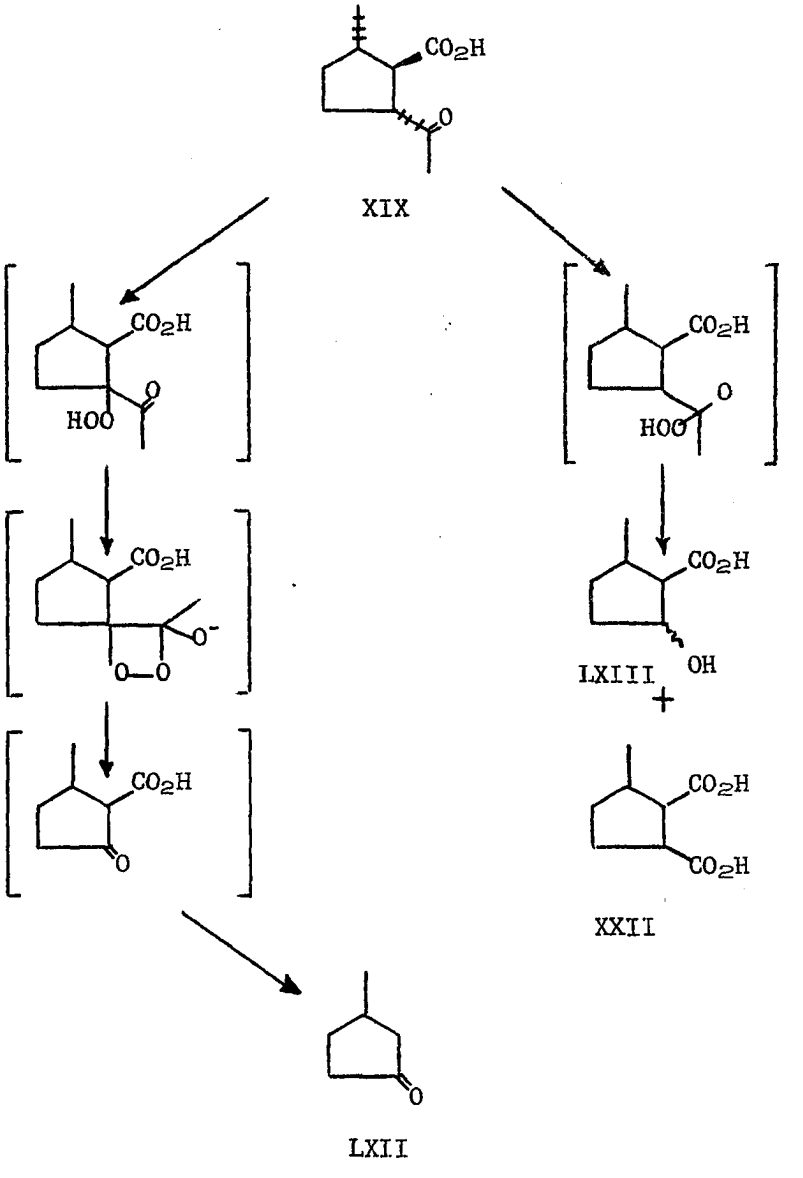


Figure 21. Alkaline hydrogen peroxide degradation of nepetonic acid (XVIII)



CHAPTER VI

SUMMARY

The occurrence of caryophyllene, nepetalactone, and an ester of cinnamic acid in the essential oil of Nepeta cataria L. has been established by combination mass spectral-gas chromatographic analysis. A tabulation of the important spectral ions and their partial interpretation has been presented for these compounds. The presence of a family of monoterpene trienes in the preparative GLC eluant has been established on the basis of their mass spectrum.

A rapid, simple method has been described for determination of nepetalactone and epinepetalactone by gas chromatography. This method was used to obtain information showing that neither steam distillation nor treatment of these compounds with strong base will result in their interconversion during isolation. Nepeta cataria L. was found to produce over 99% nepetalactone while the various species of Nepeta produce widely varying amounts of nepetalactone and epinepetalactone.

Radioactivity from mevalonic acid-2-^{14}C was incorporated into nepetalactone and caryophyllene by Nepeta cataria L. plants. The incorporation into caryophyllene was found to be a direct function of time up to 50 hours, whereas incorporation into nepetalactone occurred in two stages. It is concluded that roots are not required for the biosynthesis of these terpenoid compounds. A chemical degradation for

nepetalactone using alkaline hydrogen peroxide and haloform reactions was developed to permit the determination of carbon-14 labeling in carbon atoms 3 and 8. Twenty-five per cent of the radioactivity in nepetalactone was found to be located in carbon atoms 3 and 8.

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