shown by infrared and nmr spectra, melting point, and mixture melting point.

Rate Studies. The rates of methiodide formation were determined by the method used previously,[6] except for a few minor changes. Instead of using 10 mg of sample, rates were determined on 3 mg of sample in the present study. The acetonitrile that was used was carefully distilled from CaH_2 and had an observed initial resistance of greater than 1,000,000 ohms.

Biosynthesis of Methylcyclopentane Monoterpenoids. I. *Skytanthus* Alkaloids[1,2]

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Abstract: The biosynthesis of the steam-volatile *Skytanthus* alkaloids has been investigated by administering DL-mevalonate-2-^{14}C and L-methionine-methyl-^{14}C into the green stems of mature flowing *Skytanthus acutus* M. plants. Radioactivity from mevalonate-2-^{14}C was incorporated into the alkaloid. Radioactivity from DL-lysine-2-^{14}C was not incorporated, which indicates that lysine is not a precursor. Gas–liquid chromatographic analysis of the alkaloid fraction indicated that the natural oil contains a mixture of which about 90% is α-, β-, δ-, and dehydroskytanthines. The amounts of these alkaloids vary with the parts of the plant, with roots containing most. Specific activities of the alkaloids derived from mevalonate-2-^{14}C and methionine-methyl-^{14}C also vary in different parts of the plant. At least four alkaloids of unknown structure which comprise about 10% of natural *Skytanthus* oil were detected. Chemical degradations on micro quantities of alkaloid to eliminate nitrogen and to determine the amount of radioactivity located in carbons 3, 4, 7, 9, and 10 were devised. It was found that L-methionine-methyl-^{14}C was the precursor of the N-methyl group of β-skytanthine The results of biosynthesis experiments using mevalonate-2-^{14}C as a precursor provide evidence for the formation of skytanthine isomers *via:* (a) an isoprenoid pathway which involves randomization of the label between the terminal methyl carbon atoms of the monoterpene or monoterpenoid (*i.e.*, geranyl pyrophosphate) intermediate in 1.3-year-old plants, and (b) an isoprenoid pathway which does not involve randomization of the label between the monoterpene terminal methyl carbon atoms in 3-year-old plants.

Natural oil of *Skytanthus acutus* M. contains a mixture of alkaloids of the rare monoterpenoid class. At least three skytanthine isomers (Figure 1: Iα, Iβ, Iδ) and a dehydroskytanthine (II) are produced by *Skytanthus acutus* M.[4,5] *Skytanthus acutus* M. is native to the Atacama desert of Chile and owing to the characteristic shape of its seed pods is commonly referred to as "Goats-horn" by natives of the area. *Skytanthus* alkaloids have no known physiological activity, in contrast to most other methylcyclopentane monoterpenoids, most of which possess biological activity. The biosynthesis of the *Skytanthus* alkaloids is of interest because they are terpenoid alkaloids[4–9] and because they may serve as a link to higher alkaloids.[10–13]

The structures of the *Skytanthus* alkaloids suggest that the piperidine nucleus of these molecules could arise from an isoprenoid precursor. The present evidence for the route of formation of the piperidine ring in plants is based largely on studies of the alkaloid biosynthesis of anabasine[14,15] produced by tobacco, homostachydrine[16] by alfalfa, and pipecolic acid[17–20] by various plants and microorganisms. These molecules are derived from lysine. In contrast, the piperidine ring of coniine and conhydrine, which are alkaloids of hemlock, appears to be formed from a poly-β-keto acid derived from four acetate units.[21,22] Thus, if the piperidine ring of skytanthine is isoprenoid in origin, a third

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(2) A preliminary account of this work was presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965, Abstracts, p 115C.

(3) (a) To whom correspondence should be sent. (b) Universidad Tecnica Federico Santa Maria.

(4) E. J. Eisenbraun, A. Bright, and H. H. Appel, *Chem. Ind.* (London), 1242 (1962).

(5) G. C. Casinovi, F. Delle Monache, G. B. Marini-Bettolo, E. Bianchi, and J. A. Garbarino, *Gazz. Chim. Ital.*, **92**, 479 (1962).

(6) We thank Dr. Kurt L. Loening, Director of Nomenclature, Chemical Abstracts, for advice in selecting the numbering system for the methylcyclopentane monoterpenoids. See the Ring Index, p 179, item 1377.

(7) C. Djerassi, J. P. Kutney, and M. Shamma, *Tetrahedron*, **18**, 183 (1962); see also *Chem. Ind.* (London), 210 (1961).

(8) G. C. Casinovi, J. A. Garbarino, and G. B. Marini-Bettolo, *ibid.*, 253 (1961).

(9) G. C. Casinovi, Monache F. Delle, G. Grandolini, G. B. Marini-Bettolo, and H. H. Appel, *ibid.*, 984 (1963).

(10) R. Thomas, *Tetrahedron Letters*, 544 (1961).

(11) E. Wenkert, *J. Am. Chem. Soc.*, **84**, 98 (1962).

(12) W. I. Taylor, *Science*, **153**, 954 (1966).

(13) A. R. Battersby, Plenary Lecture, 4th International Symposium on the Chemistry of Natural Products, Stockholm, Sweden, June 28, 1966.

(14) E. Leete, *J. Am. Chem. Soc.*, **78**, 3520 (1956).

(15) E. Leete, E. G. Gros, and T. J. Gilbertson, *ibid.*, **86**, 3907 (1964).

(16) A. V. Robertson and L. Marion, *Can. J. Chem.*, **37**, 1043 (1959).

(17) N. Grobbelaar and F. C. Steward, *J. Am. Chem. Soc.*, **75**, 4341 (1953).

(18) P. H. Lowry, *Arch. Biochem. Biophys.*, **47**, 228 (1953).

(19) A. Meister and S. D. Buckley, *Biochim. Biophys. Acta*, **23**, 202 (1957).

(20) L. Fowden, *J. Exptl. Botany*, 11, 302 (1960).

(21) E. Leete, *J. Am. Chem. Soc.*, **85**, 3523 (1963).

(22) E. Leete, *ibid.*, **86**, 2509 (1964).

pathway for the formation of piperidine rings by plants exists. Leete[23] has recently reviewed the biosynthesis of the piperidine ring.

The results reported in this paper conclusively establish that the skytanthine isomers are of isoprenoid origin and that lysine is not involved in the biosynthesis. Methionine was shown to be a precursor of the N-methyl group.

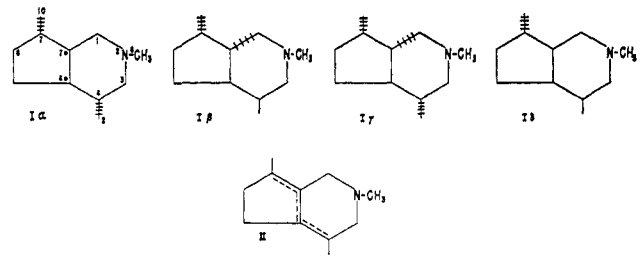


Figure 1. Structures of *Skytanthus* alkaloids. The Greek letters refer to isomers of skytanthine.[4,6] II is dehydroskytanthine.

Experimental Section

Administration of Labeled Compounds. Mature, flowering *Skytanthus acutus* M. plants 3 years old were used except for the experiment reported on 1.3-year-old plants. These plants were grown in a greenhouse.

An aqueous solution of the labeled compound was injected into the stems of a plant. After 4 days the plants were harvested and divided into leaves, green stems, woody stems, and roots for analysis. These parts were stored in plastic bags at −15° until used.

Labeled Compounds Used. Chromatographically pure DL-mevalonic acid-2-^{14}C (N,N′-dibenzylethylenediamine salt obtained from Nuclear Research Chemicals, Orlando, Fla., or New England Nuclear Corp., Boston, Mass., was used.[24] Chromatography was performed using Whatman No. 1 filter paper and 2-propanol–ammonium hydroxide–water (80:5:15) as the solvent. DL-Lysine-2-^{14}C with a specific activity of 0.6 mcurie/mmole was purchased from Tracerlab-Keleket, Waltham, Mass., and L-methionine-methyl-^{14}C with a specific activity of 2.25 mcurie/mmole was obtained from Nuclear Research Chemicals, Orlando, Fla. The amino acids were radiochemically pure as determined by paper chromatography on Whatman No. 1 filter paper and development with phenol saturated with water and 1-butanol–acetic acid–water (60:25:15).

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

Isolation of Steam-Volatile Alkaloids. The frozen plant segments were cut into 0.5-in. pieces and ground with a mortar and pestle, and the pH was adjusted to 10 with 4 *N* potassium carbonate. This homogenate was steam distilled until 300 ml of distillate was collected. The distillate was acidified to pH 2 with concentrated HCl and steam distilled. This latter steam-volatile fraction contained the neutral material. The pH of the material in the steam distillation flask was adjusted to 10 with 4 *N* NaOH and the volatile alkaloids were steam distilled and collected. The neutral and basic fractions were saturated with sodium chloride and extracted with ethyl ether. The ether layers were dried over anhydrous magnesium sulfate, filtered, and concentrated by distillation. The concentrates which remained were each dissolved in a small amount of ether, placed in 1-ml volumetric flasks, and stored at −15° until used.

Gas Chromatography. Analyses of the alkaloids by gas–liquid chromatography were made on 0.125 in. × 10 ft stainless steel columns packed with 10% Carbowax 20M on 100–120 mesh base-treated[25] Firebrick. The injector temperature was kept at 160° and the thermal conductivity detector cell at 230°. Peak areas were measured with a disc integrator[26] and the amounts represented were estimated by comparison with standard curves obtained with pure α- and β-skytanthine and dehydroskytanthine. The pure alkaloids were obtained by preparative gas chromatography (0.375 in. × 10 ft column with the same packing as described above).

Isotope Analyses. Carbon-14 activity of the compounds administered to the plants, the isolated alkaloids, and the products formed during chemical degradation of Iβ was determined using gas radiochromatography and/or liquid scintillation counting.[27,28] The gas radiochromatography measurements were performed with a Perkin-Elmer Model 801 gas–liquid chromatograph equipped with a thermal conductivity detector and a Nuclear-Chicago Model 8200 proportional gas flow counter. The total effluent from the detector was fed into the counting chamber through a heated inlet line. The inlet line and the gas counting chamber were held at 250°. Peak areas were measured using a Nuclear-Chicago Model 8350 automatic integrator. The efficiency was 32% as calculated from a reference standard of *n*-heptane-1-^{14}C, specific activity 0.25 μcurie/μmole.

Chemical Degradation of β-Skytanthine (Iβ). Hofmann Degradation of Skytanthine (Iβ) to the Amino Olefin IV. A 2.5-g sample of β-skytanthine (Iβ), purified by preparative gas chromatography on a 0.375 in. × 10 ft column of alkali-treated Chromosorb P coated with 15% Carbowax 20M, was dissolved in 15 ml of absolute ethanol and 5 ml of methyl iodide was added. The mixture was heated at reflux temperature for 6 hr. The reaction mixture was cooled, ether was added to precipitate the methiodide, and the precipitate was filtered out to give 3.5 g of β-skytanthine methiodide, mp 293–295° (lit.[7] 296–298°). The methiodide, 3.5 g, was dissolved in 200 ml of water and then stirred with freshly prepared silver oxide.[29] The suspension was filtered, and the filtrate was concentrated at 40–50° under vacuum to give 3 g of oil. A 10-μl sample of the N-methyl-β-skytanthine hydroxide (III) was injected into the alkaline 20M Carbowax column. The injection port, column, and detector temperatures were 200, 123, and 250°, respectively. Two peaks were observed, one corresponding to recovered β-skytanthine (Iβ) and the other to the amino olefin IV. The retention times were 16.5 and 12.5 min, respectively. The amino olefin IV was purified by preparative gas chromatography on the alkaline Carbowax 20M column with a yield of about 40%. The infrared spectrum of IV showed bands at 3.26, 6.07, and 11.3 μ.

Amine Oxide Degradation of Amino Olefin IV. A 0.2-g sample of IV was stirred with 1.5 g of 30% hydrogen peroxide for 16 hr until a positive test for base using phenolphthalein was no longer observed. The excess hydrogen peroxide was destroyed by stirring with 24 mg of platinum oxide catalyst until lead sulfide treated paper showed no reaction. The reaction mixture was filtered and concentrated at 30–40° under vacuum and then pyrolyzed. The pyrolysis products were analyzed on the 0.375 in. × 10 ft alkaline Carbowax 20M column. Peaks were observed for N,N-dimethylhydroxylamine VII (1.8 min), the diene VI (2.3 min), and unreacted IV and Iβ.

Ozonolysis of Amino Olefin IV. The amino olefin IV (0.6 g) in ether was dissolved in 10 ml of acetic acid and treated at room temperature for 15 min with oxygen containing ozone. The ozonized solution was transferred to a flask containing a solution of 1 g of ferrous sulfate in 10 ml of water, and the reaction mixture was stirred at room temperature for 30 min, heated for 30 min, and immediately steam distilled into a solution of 0.4 g of dimedon in 100 ml of water. The dimedon derivative was filtered out and crystallized from hot aqueous methanol to give 0.022 g, mp 192–193°. The purity of the dimedon derivative of formaldehyde was tested by thin layer chromatography,[30] using benzene–ethanol–acetate (1:1:4) as the solvent system for separation and 2,4-dinitrophenylhydrazine spray solution for detection.

The steam distillation pot residue was cooled, basified with sodium hydroxide solution, and extracted several times with ether. The ether extract was dried over anhydrous sodium sulfate and filtered, and the ether was removed through a fractionating column to give a concentrate containing the amino ketone VIII.

The amino ketone VIII (80 mg) was obtained by preparative gas chromatography, using a 0.375 in. × 10 ft aluminum column packed with 15% Carbowax 20M loaded on base-washed 60–80 mesh

(23) E. Leete, *Science*, 147, 1000 (1965).

(24) Preliminary experiments indicated that no difference in incorporation of carbon-14 was found when either the free acid or the salt was used as the precursor.

(25) Washed three times with saturated methanolic KOH and once with CH_3OH.

(26) Disc Instruments, Inc., Santa Anna, Calif.

(27) Tri-Carb, Model 314, Packard Instrument Co., La Grange, Ill.

(28) K. S. Yang, R. K. Gholson, and G. R. Waller, *J. Am. Chem. Soc.*, 87, 4184 (1965).

(29) The silver oxide was prepared from 7.0 g of silver nitrate in 70 ml of water containing 1.7 g of sodium hydroxide by heating the resulting suspension at 85° and washing free of alkali with hot water.

(30) Eastman Kodak Type K301 R Chromatogram sheet.

Chromosorb P. The temperatures of the column, injection port, and detector were 140, 200, and 250°, respectively. A gas pressure of 80 psi at a flow rate of 160 cc/min was used. The retention time of the amino ketone VIII was 31 min. VIII was further purified by thin layer chromatography, using silica gel G in 0.2 *N* sodium hydroxide solution for coating the glass plates and hexane–acetone–ethanol (4:1:1) as the solvent system with 1% iodine solution in methanol as a detecting spray. The infrared spectrum of VIII showed an absorption peak at 5.85 μ.

Peroxytrifluoroacetic Acid Oxidation of Amino Ketone VIII.[17] Peroxytrifluoroacetic acid was prepared by adding 1.5 ml of trifluoroacetic anhydride to a stirred mixture of 0.4 ml of 90% H_2O_2 and 7.5 ml of methylene chloride and then stirring at room temperature for 30 min. This product was added dropwise over a period of 5 min to a well-stirred mixture containing 0.6 g of amino ketone VIII in 1 ml of ether and 0.4 g of sodium dihydrogen phosphate in 15 ml of methylene chloride. After addition of the peracid, the reaction mixture was stirred at room temperature for 30 min and heated at reflux temperature for 1.5 hr. The reaction mixture was cooled and a concentrated solution of sodium sulfite added dropwise with stirring until no more gas was released. Water (10 ml) was then added and stirring continued for 30 min to ensure complete hydrolysis of any excess anhydride. The reaction mixture was basified with sodium hydroxide solution and extracted several times with methylene chloride. The methylene chloride extract was dried over anhydrous magnesium sulfate and filtered and the methylene chloride removed by fractionation to give 0.1 g of the amino acetate IX containing a trace of methylene chloride. The infrared spectrum of the amino acetate IX showed absorption peaks at 5.76 and 8.00 μ.

Saponification of the Amino Acetate IX. The amino ester IX (0.1 g) was added to 1 ml of 20% aqueous sodium hydroxide containing a few drops of methanol. The mixture was heated at reflux temperature for 30 min. The alkaline reaction mixture was cooled and extracted exhaustively with ether. This ether extract was dried over anhydrous sodium sulfate and then distilled to give 50 mg of the amino alcohol X which was purified by thin layer chromatography, using silica gel G in 2% sodium hydroxide solution for coating the glass plates, hexane–acetone–ethanol (2:1:1) as the solvent system, and 1% iodine solution in methanol as a detecting spray. The infrared spectrum of the amino alcohol X showed an absorption peak at 2.95 μ.

The alkaline residue remaining from the extraction of the amino alcohol X was acidified with 0.5 *N* sulfuric acid and steam distilled until 50 ml were collected. The steam distillate was neutralized using 0.07 *N* sodium hydroxide. The 10 mg of sodium acetate obtained on evaporation under reduced pressure was purified by chromatography on a Celite column.

Kuhn–Roth Oxidation of β-Skytanthine.[31] β-Skytanthine (Iβ) (20 mg) was oxidized with Kuhn–Roth reagent by heating at reflux temperature for 90 min. The reaction mixture was steam distilled until 50 ml was collected, and the steam distillate was neutralized with 0.07 *N* sodium hydroxide. The 10 mg of sodium acetate obtained on evaporation under reduced pressure was purified by chromatography on a Celite column.

Preparation of Skytanthine-^{14}C Used for Chemical Degradation. The alkaloids from the methionine-methyl-^{14}C biosynthesis experiment were diluted 30-fold with pure β-skytanthine (Iβ) and degraded to give amino olefin IV, the diene VI, and dimethylhydroxylamine VII.

β-Skytanthine-^{14}C formed from mevalonate-2-^{14}C biosynthesis experiments was purified by preparative gas chromatography as previously described, diluted two- to fivefold with pure Iβ, and degraded as described above. A portion of diluted Iβ was degraded using the Kuhn–Roth oxidation procedure.

The specific activity of the diluted alkaloid was redetermined using gas radiochromatography and the liquid scintillation procedures described above.

Results[32]

Distribution of Alkaloids. The distribution of α-, β-, and dehydroskytanthine in mature *Skytanthus acutus* M. plants is shown in Table I. The roots contained about 5% of the total alkaloids. The β isomer predominated in all tissues. These three alkaloids constituted about 90% of the total alkaloids isolated. The remaining 10% of the alkaloid fraction was composed of at least four unknown alkaloids and δ-skytanthine; the structure of these unknown alkaloids is being investigated. It has been previously reported that natural skytanthine alkaloids are a mixture of three and possibly four diastereoisomers (α, β, γ, and δ).[4] No γ isomer was found in this study; however, dehydroskytanthine was found in all parts of the plant. Most of the δ isomer, which constitutes about 1% of the natural skytanthine alkaloids, was located in the roots.

Table I. Distribution of α-Skytanthine, β-Skytanthine, and Dehydroskytanthine in Mature *Skytanthus acutus* M. Plants[a,b]

Plant parts	Skytanthus alkaloids, % α	β	Dehydro
Leaves	0.007	0.06	0.006
Green stems	0.007	0.03	0.009
Woody stems	0.006	0.03	0.02
Roots	0.018	0.13	0.07
Whole plant	0.033	0.25	0.105

[a] Fresh-weight basis. [b] Three-year-old plants.

Alkaloid Biosynthesis. The results obtained using DL-lysine-2-^{14}C, DL-mevalonate-2-^{14}C, and L-methionine-methyl-^{14}C are shown in Table II. The α-, β-, and dehydroskytanthine alkaloids contained 0.56% of the administered carbon-14 from mevalonate-2-^{14}C, none from lysine-2-^{14}C, and 1.04% from methionine-methyl-^{14}C. The incorporation of radioactivity from mevalonate-2-^{14}C along with lack of incorporation from lysine-2-^{14}C provides evidence that the carbon skeleton of skytanthine arises *via* an isoprenoid precursor rather than a pathway which involves lysine. These results confirm and extend the preliminary report of Casinovi and Marini-Bettolo[33] on the incorporation of radioactivity from mevalonate-2-^{14}C into skytanthine produced by sterile excised roots of *Skytanthus acutus* M. A comparison of the specific activities of the skytanthine isomers produced in the various parts of the plant from mevalonate-2-^{14}C and methionine-methyl-^{14}C shows that the highest values were observed in the alkaloids isolated from the green stems. Most of the carbon-14 in the alkaloids is in the β isomer, which might be expected since it is present in the largest amount. The isotope dilution values, which are readily calculated from the specific activities, indicate that the least dilution occurs in the green stems for all three skytanthine isomers. The distribution of carbon-14 in α-, β-, and dehydroskytanthine formed from mevalonate-2-^{14}C was 13, 53, and 34%, respectively. Corresponding values from the methionine-methyl-^{14}C experiment were 9.5, 74.0, and 12.1%. In the methionine-methyl-^{14}C experiment 4.4% of radioactivity was found in an unknown alkaloid.[34]

Chemical Degradation of β-Skytanthine (Iβ). A. Elimination of Nitrogen. The structure of the skytan-

(31) E. J. Eisenbraun, S. M. McElvain, and B. F. Aycock, *J. Am. Chem. Soc.*, 76, 607 (1954).

(32) Unless otherwise stated results and discussion are based on results obtained from 3-year-old plants.

(33) (a) C. G. Casinovi and G. B. Marini-Bettolo, Abstract Ab-3, IUPAC Meeting, London, 1963, p 285; (b) C. G. Casinovi, G. Giovannozzi-Sermanni, and G. B. Marini-Bettolo, *Gazz. Chim. Ital.*, 94, 1356 (1964).

(34) Mass spectral data indicate that this unknown alkaloid has a molecular weight of 165, which corresponds to a dehydroskytanthine

Table II. Incorporation of Carbon-14 from Labeled Compounds into α-, β-, and Dehydroskytanthine Alkaloids Produced by Mature *Skytanthus acutus* M. Plants[a]

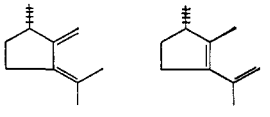
Compd admin (μmoles injected; sp act., μcuries/μmole)	Plant part	*Skytanthus* alkaloids: α isomer Yield, μmoles	α isomer Specific activity, mμcuries/μmole	β isomer Yield, μmoles	β isomer Specific activity, mμcuries/μmole	Dehydro Yield, μmoles	Dehydro Specific activity, mμcuries/μmole
DL-Lysine-2-^{14}C (85; 0.6)	Leaves	14	0	47	0	4	0
	Green stems	2	0	13	0	3	0
	Woody stems	8	0	117	0	20	0
	Roots	38	0	296	0	110	0
DL-Mevalonate-2-^{14}C (21.4; 4.18)	Leaves	144	0.07	1250	0.04	123	0.07
	Green stems	128	0.17	562	0.23	179	0.29
	Woody stems	120	0.18	730	0.05	480	0.12
	Roots	358	0.04	2500	0.02	1410	0.04
L-Methionine-methyl-^{14}C (60; 2.25)	Leaves	21	3.4	69	5.3	7	7.4
	Green stems	3	4.7	4	16.4	1	7.4
	Woody stems	9	0.9	41	5.7	14	1.0
	Roots	38	1.0	152	2.4	75	1.3

[a] Three-year-old plants.

thine skeleton I was initially established by dehydrogenation to actinidine[7,8] and by partial degradation to the amino olefin IV and the amino ketone VIII.[7]

The existence of three isomers of skytanthine (Iα, β, and δ) in *Skytanthus acutus* M., their structures, and their absolute configurations and stereochemistry were established by comparison with isomers of skytanthine obtained by partial synthesis from the nepetalinic acids of known absolute configuration and stereochemistry.[4,5]

Despite these extensive studies, a nitrogen-free degradation product of skytanthine has not been reported. The reported degradation routes were extended to provide a series of molecules which would reveal location of carbon-14 incorporated into the skytanthine skeleton. The results show that when pure β-skytanthine (Iβ) is subjected to the Hofmann elimination reaction it is cleanly converted to the previously reported amino olefin IVa.[7,35,36] The choice of the route to be used for complete removal of nitrogen from IVa was then between a second-stage Hofmann or an amine oxide elimination reaction. These methods were compared and both were found to give nitrogen-free diene. The amine oxide elimination from IVa *via* V was preferred because the resulting diene VI appeared to be a single product, whereas a conventional Hofmann elimination reaction of IVa proceeded to a mixture of dienes possibly having the structures XI and XII. This mixture is to be expected in view of the well-known course of the Hofmann elimination of nitrogen from piperidine to give piperylene (1,3-pentadiene) rather than 1,4-pentadiene.



XI XII

The paucity of starting material and lack of knowledge of the structure of skytanthine possibly prevented earlier workers from fully eliminating nitrogen from the skytanthine skeleton. In our hands, this was accomplished without difficulty and procedures (Figure 2) were developed so that complete nitrogen elimination could be carried out on a milligram scale.

The results presented in Table III show the distribution of label in the skytanthine alkaloids formed from L-methionine-methyl-^{14}C. When the products of the degradation were analyzed by gas radiochromatography it was found that 47% of the radioactivity was in VI and 26 and 27% of the radioactivity was present as unreacted I and IV, respectively. The diene VI was completely devoid of radioactivity. These results provide conclusive evidence that the N-methyl group of the skytanthine alkaloids originated from the methyl group of methionine.

Table III. Distribution of Radioactivity in β-Skytanthine Biosynthetically Formed from L-Methionine-methyl-^{14}C[a]

Compd	Sp act., μcurie/mmole	%
β-Skytanthine (Iβ)	0.10	100
Methine (IV)	0.105	105
Diolefin (VI)	0	0
N,N-Dimethylhydroxylamine (VII)	...	100

[a] Three-year-old plants.

B. Chemical Degradation of β-Skytanthine (Iβ) to Remove Carbons 3, 4, and 9. The previously reported degradation[7,8] of the amino olefin IV to the amino ketone VIII and the amino alcohol X was utilized in obtaining techniques to provide suitable derivatives and degradation products for carbon-14 assay to locate the positions of radioactive labeling in Iβ.[36] The ozonolysis of amino olefin IV readily provided the amino ketone VIII and formaldehyde. The latter was isolated as the dimedon derivative, purified by recrystallization and thin layer chromatography to constant specific activity, and then counted to determine the carbon-14

(35) Analysis of the methines produced in the reaction was made on the combination mass spectrometer–gas chromatograph (prototype of the LKB-9000) using a 0.25 in. × 16 ft glass column packed with 20% Carbowax 20M on 100–120 mesh base-washed[25] Firebrick. The column conditions were 200° for injection port, 125° for the column, and a flow rate of 45 cc of He/min. The retention time of IVb was 21 min and that of IVa was 23 min. Quanitative estimation of the relative amounts of each methine was performed by planimetry. IVa comprised over 99% of the mixture.

(36) We are investigating the Hofmann elimination reaction as applied to δ-skytanthine (Iδ) to determine whether stereochemical alteration in the carbon skeleton of the skytanthine isomers affects the direction of elimination and whether more than one olefin is formed.

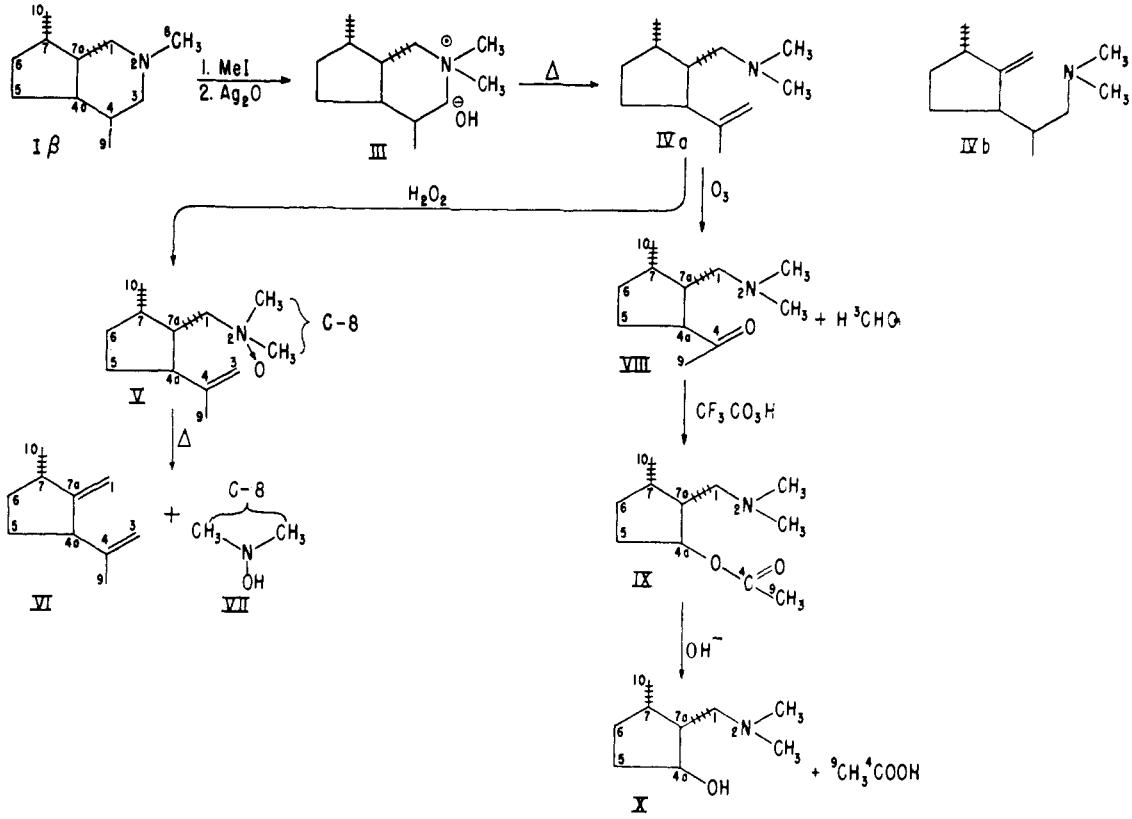


Figure 2. Chemical degradation of β-skytanthine to remove carbons 8, 3, 4, and 9.

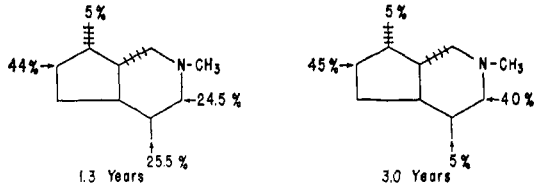


Figure 3. Labeling patterns of β-skytanthine biosynthesized by *Skytanthus acutus* plants from mevalonate-2-^{14}C.

radioactivity at position 3 of Iβ. A conventional Baeyer–Villiger oxidation of the amino ketone VIII with peroxytrifluoroacetic acid[37] yielded the expected amino acetate IX which was hydrolyzed to the amino alcohol X and acetic acid. The acetic acid was isolated and used to determine the carbon-14 radioactivity of carbons 4 and 9 of Iβ.

The results presented in Table IV show the distribution of the label in β-skytanthine (Iβ) formed from mevalonate-2-^{14}C using plants of two ages. The data reported in this table are the results of specific activity determinations made using the liquid scintillation method on weighed, purified samples of Iβ and its derivatives. Gas radiochromatography experiments (3-year-old plants) on Iβ, IV, VIII, and X agree with the results obtained using liquid scintillation counting, but in general these were less reliable because of the low specific activities encountered. The dimedon derivative of formaldehyde and acetate were counted only by the liquid scintillation technique.

A Kuhn–Roth oxidation on the carbon-14-labeled amino alcohol X would provide information on the amount of radioactivity residing in carbons 7 and 10. Because insufficient carbon-14-labeled X was available, this degradation was applied directly to β-skytanthine-^{14}C which yields acetate representing carbons 7 and 10 and 4 and 9.

Three-Year-Old Plants. Results from the chemical degradation indicated that carbon 3 contained 40% of the radioactivity present in the original β-skytanthine-^{14}C (Iβ). The amino alcohol X and the sodium acetate formed from the amino ketone VIII had 49 and 5% of the radioactivity, respectively (Table IV). Results from the Kuhn–Roth oxidation on the original Iβ are shown on the last line of Table IV and indicate that 5% of the carbon-14 resides in carbons 4 and 9 and 7 and 10. These data are interpreted to mean that about 10% of the original radioactivity present in β-skytanthine is present in carbons 4 and 9 and 7 and 10 (Figure 3).

Of the original activity present in β-skytanthine-^{14}C, 94% (carbons 3, 4, 9, and the amino alcohol X) was recovered in these experiments (Table IV). These results combined with the results of the Kuhn–Roth oxidation of Iβ provide convincing evidence for the nonrandomization of the carbon-14 label in β-skytanthine-^{14}C formed from mevalonate-2-^{14}C. In addition, a Kuhn–Roth oxidation was performed on α-skytanthine possessing a specific activity of 9.25 mμcuries/mmole. The sodium acetate obtained was found to possess a specific activity of 0.70 mμcurie/mmole, which indicates that 7.6% of the radioactivity was located in carbons 4 and 9 and 7 and 10.

16-Month-(1.3 years)-Old Plants. The degradation of β-skytanthine-^{14}C from 1.3-year-old plants yielded entirely different results than were obtained with 3-year-

(37) W. F. Sager and A. Duckworth, *J. Am. Chem. Soc.*, 77, 188 (1955).

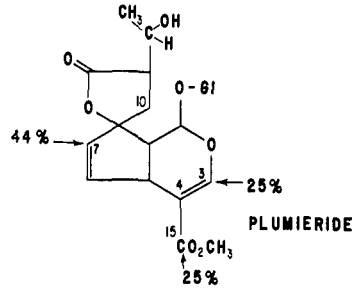
Table IV. Distribution of Radioactivity in β-Skytanthine Biosynthetically Formed by *Skytanthus acutus* Plants from Mevalonate-2-^{14}C

Compound	β-Skytanthine carbon atom	3 years old: Sp act., mμcuries/mmole	3 years old: %	1.3 years old: Sp act., mμcuries/mmole	1.3 years old: %
β-Skytanthine (Iβ)	All	8.87	100	5.63	100
Methine (IV)	All	8.7	98	5.70	101
Ketone (VIII)	All but 3	5.1	57	4.25	75.5
HCHO (dimedon deriv)	3	3.5	40	1.37	24.4
Amino alcohol (X)	All but 3, 4, and 9	4.3	49	...	...
Sodium acetate (from VIII)	4 and 9	0.41	5	...	...
Sodium acetate (from Iβ)	4 and 9, also 7 and 10	0.41	5	0.77	13.7

old plants (Table IV). Owing to the limited quantity of labeled β-skytanthine-^{14}C, chemical degradation could not be carried beyond the formation of the amino ketone VIII and the dimedon derivative of formaldehyde (carbon 3). The results indicate that 24% of the radioactivity is located in carbon 3. Sodium acetate recovered from the Kuhn–Roth oxidation of the β-skytanthine contained about 14% of the radioactivity present in the alkaloid. A theoretical value of 15% of the radioactivity would be expected to be found in the sodium acetate if, in the original β-skytanthine-^{14}C, 25% of the radioactivity was located in carbon 9 and 5% in carbon 10 (this is assuming that the acetate obtained from the Kuhn–Roth oxidation of Iβ is formed in equal amounts from carbons 4 and 9 and 7 and 10). On the other hand, if carbons 9 and 10 each contained 25% of the radioactivity, then the acetate obtained from Kuhn–Roth oxidation would be expected to contain 25% of the radioactivity. If these carbons contained 5% of the radioactivity present originally, then the acetate obtained would be expected to contain 5% of the radioactivity also. The fact that the acetate contained 14% of the radioactivity provides evidence that one of carbons 9 and 10 contained about 25% and the other contained about 5% of the original radioactivity present in β-skytanthine-^{14}C. To further support this view, a Kuhn–Roth oxidation of dehydroskytanthine-^{14}C (obtained from the same biosynthesis experiment) possessing a specific activity of 41.0 mμcuries/mmole was carried out and sodium acetate possessing a specific activity of 6.10 mμcuries/mmole corresponding to 16.5% of the radioactivity originally found in II, was isolated. These data and the data shown in Table IV lead to the conclusion that about 25% of the radioactivity originally present in β-skytanthine-^{14}C (Iβ) is located in carbon 9 and that 5% is located in carbon 10 (Figure 3). It would then appear that in the young, flowering *Skytanthus acutus* M. plants, randomization of the label from mevalonate-2-^{14}C at the monoterpene level occurs when the two terminal methyl groups were equivalent (*i.e.*, geraniol, geranyl pyrophosphate, or some methylcyclopentane monoterpenoid intermediate). A comparison of the different labeling patterns of β-skytanthine-^{14}C (Iβ) biosynthesized from mevalonate-2-^{14}C using 1.3-year-old and 3-year-old *Skytanthus acutus* M. plants is shown in Figure 3.

Discussion

It is of interest to compare these results with those obtained by Yeowell and Schmid.[38] Results from chemical degradation of plumieride formed biosynthetically from mevalonate-2-^{14}C showed that randomization occurred between carbon atoms 3 and 15 (25% of the radioactivity residing in each), and that carbon 7 contained 44%. It can be assumed that



carbon atom 10 would contain about 6% of the radioactivity, which is in agreement with our finding on β-skytanthine. Yeowell and Schmid[38] suggested that randomization might occur after ring closure of the cyclopentanoid ring and that carbon atoms 3 and 15 might be equivalent aldehyde groups. Randomization of the terminal methyl label in the isoprenoid portion of certain indole alkaloids has also been reported,[39-41] and the proposed mechanisms are similar to that suggested for plumieride. These data are also supported by labeling experiments using geranyl pyrophosphate-2-^{14}C[13,40-43] and geraniol-2-^{14}C.[42-44] For a review on methylcyclopentane monoterpenoids as intermediates in the biosynthesis of indole alkaloids see Taylor.[12] It seems logical that randomization might occur at the monoterpenoid level in these compounds since carbon 15 of plumieride is further oxidized to a carboxylic acid group and this biological oxidation might be expected to proceed in a stepwise direction involving alcohol, aldehyde, and finally acid. In skytanthine carbon atom 9 (equivalent to carbon 15 of plumieride) remains as a methyl carbon, and the chance for randomization of label *via* a similar type of intermediate involving methyl groups as carbon atoms 3 and 9 exists at the geraniol or geranyl pyrophosphate state in β-skytanthine biosynthesis. In contrast to the experiments showing the occurrence of randomization, the

(38) D. A. Yeowell and H. Schmid, *Experentia*, 20, 250 (1964).

(39) F. McCapra, T. Money, A. I. Scott, and I. G. Wright, *Chem. Commun.*, 1, 537 (1965).

(40) H. Goeggel and D. Arigoni, *ibid.*, 1, 538 (1965).

(41) A. R. Battersby, R. T. Brown, R. S. Kapil, A. O. Plunkett, and J. B. Taylor, *ibid.*, 2, 47 (1966).

(42) A. R. Battersby, R. T. Brown, J. A. Knight, J. A. Martin, and A. O. Plunkett, *ibid.*, 2, 346 (1966).

(43) P. Loew, H. Goeggel, and D. Argoni, *ibid.*, 2, 347 (1966).

(44) E. S. Hall, F. McCapra, T. Money, K. Fukumoto, J. R. Hanson, B. S. Mootoo, G. T. Phillips, and A. I. Socti, *ibid.*, 2, 348 (1966).

results obtained by Birch, *et al.*,[45] from the incorporation of mevalonate-2-^{14}C into the terpenoid side chain of mycelianamide indicated that 80% of the radioactivity was not randomized, and it was suggested that the degree of randomization observed was more likely a result of the chemical degradative procedure than of a lack of specificity during biosynthesis. It is possible that the extent of randomization which can occur on the appropriate intermediate (*i.e.*, geranyl pyrophosphate) is subject to control at the enzyme level and by the pool size of the substrate. Insufficient knowledge exists on both of these points to permit a definite conclusion.

There may be several possible types of enzymatic control. For example, different enzyme inhibitors or different levels of enzyme may be present in the old and young plants. Also, it may be possible that the particular enzyme responsible for randomization of the monoterpenoid intermediate does not exist in the old plant. An analogous situation is known to occur in the rapid appearance and disappearance of diamine oxidase in pea seedlings.[46] Control by the substrate pool size can best be visualized by considering that the young plant has a rather large pool of monoterpenoid intermediate and that the two terminal methyls become equivalent. In the old plant perhaps only a small pool of monoterpenoid intermediate exists, and this substrate is immediately utilized in the biosynthesis and does not remain long for randomization of the methyl carbons to occur.

An alternative explanation, although remote, to the finding of randomization of label in the 1.3-year-old plant is the possibility of incorporation of mevalonate-U-^{14}C which has been formed by degradation of the administered mevalonate-2-^{14}C to $^{14}CO_2$ which, in turn, is reincorporated *via* CO_2 fixation. The results recently reported by Battu and Youngken[47] suggest that degradation of mevalonate to CO_2 and reincorporation of the latter into monoterpenes of *Mentha piperita* can occur.

Our data also support the mechanism of isomerization of isopentenyl pyrophosphate proposed first by Agranoff, *et al.*,[48] and later established by Shah, *et al.*[49] In this mechanism, the methylene carbon atom of isopentyl pyrophosphate is protonated, the proton from carbon atom 2 is discharged into the medium, and dimethylallyl pyrophosphate is formed. In the reverse reaction, a proton is added stereospecifically at carbon atom 2 of dimethylallyl pyrophosphate. Hence this isomerization does not result in a randomization of label originally present in the methylene group of isopentenyl pyrophosphate.

The metabolic relationship of mevalonate-2-^{14}C and β-skytanthine-^{14}C (Iβ) in 1.3-year-old plants and 3.0-year-old plants has been examined and this compound has been implicated in the biosynthesis of skytanthine by *Skytanthus acutus* M. The pathway for the biosynthesis of skytanthine isomers is not well understood. The contrast in our finding of randomization of label in β-skytanthine formed from mevalonate-2-^{14}C in 1.3-year-old plants with that of nonrandomization of label in 3.0-year-old plants requires additional study before its significance can be determined. Variation in an enzymatic reaction mechanism due to age appears to to be a new phenomenon.

(45) A. J. Birch, M. Kocor, N. Sheppard, and J. Winter, *J. Chem. Soc.*, 1502 (1962).
(46) R. H. Kenton and P. J. G. Mann, *Biochem. J.*, 50, 360 (1952).
(47) R. G. Battu and H. W. Youngken, *Lloydia*, 29, 360 (1966).
(48) B. W. Agranoff, H. Eggerer, U. Henning, and F. Lynen, *J. Biol. Chem.*, 235, 326 (1960).
(49) D. H. Shah, W. W. Cleland, and J. W. Porter, *ibid.*, 240, 1946 (1965).

Communications to the Editor

Reactions of Vapor-Produced *t*-Butyl Carbonium Ion Injected into Liquid Isobutylene

Sir:

The reactions of specific, highly reactive hydrocarbon ions in the liquid phase are difficult to study directly. Ions may be produced in the liquid by direct liquid radiolysis, but the situation is complicated because many other reactive species such as electrons, radicals, and excited molecules are produced simultaneously. This complex situation may be simplified by producing specific ions in the vapor phase and injecting them by means of an electric field into a liquid or solid matrix. Under such conditions the positive ion is separated from its concomitant electron and is accelerated into the liquid or solid alone. To achieve this we describe a successful experimental method which is based on previous experiments by Schlag and Sparapany.[1,2] By this method we have studied the liquid phase reaction of *t*-butyl carbonium ion with isobutylene to form C_8 carbonium ions and their subsequent neutralization reactions.

The apparatus has been described.[3] A krypton resonance lamp[4,5] with intensity of greater than 10^{15} quanta/sec ionizes research grade isobutylene in the vapor phase. An electric field at right angles to the photon beam drives the positive ions into a detachable arm containing liquid isobutylene. The liquid arm of the cell extends into a dewar and is thermostated with a cold nitrogen gas stream; the temperature may be varied by changing the flow rate. A voltage of 400 v was applied to the external electrodes during photolysis. At $-128°$ the per cent conversion of isobutylene was linear beyond 120 min of photolysis time; the conversion rate was 0.01%/min. Gas chromatography showed

(1) E. W. Schlag and J. J. Sparapany, *J. Am. Chem. Soc.*, 86, 1875 (1964).
(2) J. J. Sparapany, *ibid.*, 88, 1357 (1966).
(3) L. Kevan, J. Zimbrick, and N. S. Viswanathan, Annual Summary Report AFRPL-TR-66-359, Nov 1966, p. 76.
(4) H. Okabe, *J. Opt. Soc. Am.*, 54, 478 (1964).
(5) L. J. Stief and R. J. Mataloni, *Appl. Opt.*, 4, 1674 (1965).