SYNTHESIS AND BIOSYNTHESIS OF INDOLE ALKALOIDS

by

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B.Sc. Honours, The University of British Columbia, 1963.

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We accept this thesis as conforming to the

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SYNTHESIS AND BIO-SYNTHESIS OF INDOLE ALKALOIDS

ABSTRACT

In part A, a much sought synthesis of the calycanthaceous alkaloids is described. Oxidative dimerization of N- methyltrytamine afforded dl- chimonanthine and meso-chimonanthine, and dl- calycanthine was produced by subsequent acid-catalyzed rearrangement of the carbon skeleton through a tetraminodialdehyde. As the suggested biosynthesis of these alkaloids is represented as occurring by an oxidative dimerization of N- methyltryptamine (itself a natural product) the very direct synthesis described is a biosynthetic model. The discovery of meso-chimonanthine in an extract of Calycanthus floridus is also reported and is the first natural calycanthaceous alkaloid with this stereochemistry to be discovered. As folicanthine and calycanthidine are N-methyl chimonanthines and this methylation has been reported, a synthesis of chimonanthine also represents a formal synthesis of these alkaloids. A proposal for the structure of hodgkinsine, the remaining calycanthaceous alkaloid, is made. A number of synthetic by-products are also described.

In part B evidence for the mono-terpenoid origin of the nine or ten carbon non-tryptophan derived portion of the indole alkaloids is presented. The monoterpene, geraniol- 2- ^{14}C, was administered to Vinca rosea cuttings and the Aspidosperma- type alkaloid, vindoline, was isolated and shown by Kuhn-Roth degradation to be labelled at C-5 as predicted by theory. Feeding experiments with mevalonic acid- 2- ^{14}C are also described.

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A. I. Scott, F. McCapra, E. S. Hall, Chimonanthine. A One-Step Synthesis and Biosynthetic Model. J. Am. Chem Soc. 86, 302, (1964).

E. S. Hall, F. McCapra, T. Money, K. Fukamoto, J. R. Hanson B. S. Mootoo, G. T. Philips and A. I. Scott, Concerning the Terpenoid Origin of Indole Alkaloids. Chem. Comm. 348, (1966).

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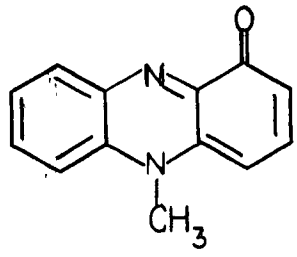
Introduction

The structures of some 1700 naturally occuring alkaloids are known at the present time.[1] Alkaloids are basic nitrogen-containing organic compounds usually with the nitrogen as part of a heterocyclic system. They are in general the metabolic products of higher plants.[2] A few, however, such as pyocyanin (1) from the bacterium Pseudomonas aeruginosa and psilocybine (2) the active principle of Mexican halucinogenic mushrooms of the genus Psilocybe, occur in lower plant forms. One simple indole alkaloid, bufotenine (3), has been isolated from the venom of the toad Bufo vulgaris as well as from plant and fungal sources. Some compounds generally regarded as alkaloids are not basic. Colchicine (4) is an example of an alkaloid with a neutral exocyclic amide nitrogen while some alkaloids occur as quaternary ammonium salts or as tertiary amine oxides.

The taxonomic distribution of alkaloids cannot be fixed with any certainty as the chemistry of the flora of only a few regions of the world have been intensively studied and the greater majority of plants still remain to be examined. It has been estimated that 10-20% of all plants contain alkaloids.[3] This estimate is limited by analytical methods which often fail to detect traces.[3]

Alkaloids can occur in the root, stem, leaf, flower or seeds of plants and accumulation occurs during the active growth of juvenile tissues. The relationship between the age of the plant and alkaloid content and distribution has been reviewed by

Mothes.[3,4] A maximum in alkaloid content often occurs at or about the time of flowering. Alkaloids then often accumulate in the seeds. Although the effect of growing conditions on alkaloid content is a complex matter, generally conditions which promote healthy growth are best for alkaloid formation.



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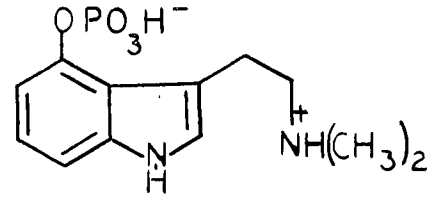

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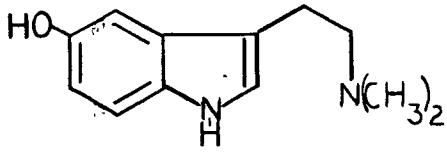

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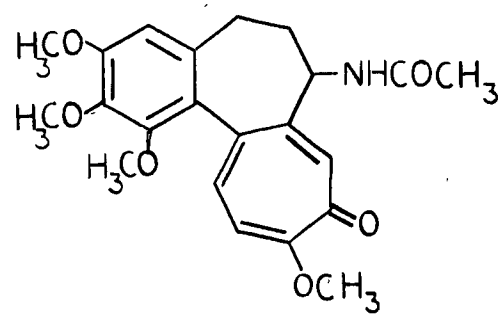

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Figure 1. Atypical Alkaloids.

Very little is known about the function of alkaloids in plants. Of several theories which have been advanced none is entirely

satisfactory. These theories include protection from insects and animals because of the toxic nature of the alkaloids and hence increased chances for survival of the plant, detoxification of potentially dangerous metabolic products by transformations into alkaloids, and regulation of metabolic processes.[3] Objections to the first theory include the often very specific action of alkaloids. The alkaloids of Atropa belladona are extremely toxic to man yet certain insects eat the plant with impunity. Ammonia in high concentrations is known to be toxic to cells but glutamic and aspartic acids have been recognized as being effective in eliminating free ammonia. The toxicity of amino acids known to be converted to alkaloids has not been demonstrated. The argument against a regulatory role for alkaloids is the absence of alkaloids in many plants. A combination of these three general roles may eventually be accepted as the role of alkaloids in plants. In a number of cases proliferation of alkaloid producing plants has resulted from their economic desirability and resultant cultivation by man. Tea, coffee, cocao, tobacco as well as various narcotic species fall into this category.

While alkaloids are generally harmless to plants, much of the commercial interest stems from their diverse physiological effects on animals. These compounds tend to upset the balance of endogenous amines associated with the chemistry of the central nervous system.[5] The range of effects produced by alkaloids is detailed in pharmacological collections[6] and many have been

medically useful.

The processes by which alkaloids are synthesized in plants have long been the subject of study and speculation among organic chemists and biochemists.[7,8,9] A proper understanding of the pathways involved demands a knowledge of the substances which are involved as intermediates and also of the mechanisms by which the various transformations are carried out. Real progress in the study of alkaloid biosynthesis began when organic compounds labelled with carbon-14 and other isotopes became readily available in the early nineteen-fifties. Prior to that time the problems of biosynthesis were only the object of speculation but are now at the very interesting stage of development where hypothesis and experiment can be combined. These problems have been attacked successfully on a broad front and several excellent reviews have been written.[2,10-15] There now seems to be a secure theoretical basis for the details of reaction mechanisms in many instances, and the formal relationship between alkaloids and precursors is becoming more clear.[15]

Notable successes in the synthesis of natural products were made in the first half of this century but there is generally a lack of similarity between the synthetic pathways used by organic chemists in construction of the more intricate systems and the methods and routes presumably employed by nature. During this time a small school developed which was interested in the synthesis of natural products patterned along lines considered also to represent reasonable biosynthetic routes. Many

"biogenetic-type" syntheses have been accomplished and these were reviewed by van Tamelen in 1961.[10] It seems possible that the full potentialities of biogenetically patterned reactions and syntheses are only beginning to be realized. Advantages arise from the realization that even intricate molecules are constructed in nature by a limited number of simple, quite understandable organic reactions and that these reactions can be simulated in the laboratory. Natural products are usually constructed in nature as a consequence of the chemistry of their precursors. Enzyme systems in the cell have an activating effect, as well as serving to hold molecules in a conformation favourable to certain reactions and can determine the stereochemistry of products. The important criterion for a laboratory synthesis is the practical one of whether or not the conceived plan based on suspected natural processes leads to a new or improved laboratory method. A knowledge of biogenetic principles is also invaluable in the structural determination of newly isolated natural products, as many possible structures on the basis of chemical evidence may be unlikely in terms of biogenesis. The term "biogenetic-type" synthesis has been selected to describe an organic reaction designed to follow in at least its major aspects, the biosynthetic pathways proved or presumed to be used in the natural construction of the end product. Little emphasis is placed on reagents or conditions[10] and success may depend upon utilization of reaction types which parallel enzyme-promoted processes by using reagents and conditions not available

to the living system. A few "physiological-type" syntheses have also been accomplished in which not only plausible bio-organic substitutes are used but also specific conditions of temperature, pH, dilution, etc., which supposedly compare to those obtaining in the living cell. Laboratory syntheses which proceed under these conditions are likely to correspond to spontaneous *in vivo* syntheses, that is syntheses not necessarily enzyme-catalyzed.[10]

The first example of a biogenetic-type synthesis was devised and executed by Robinson as early as 1917. Tropinone (5) was obtained in one stage from succinaldehyde, methylamine and acetonedicarboxylic acid which were regarded as reasonable biogenetic precursors[16] (Figure 2). This was accomplished soon after Willstätter's exceedingly lengthy first synthesis of the compound.

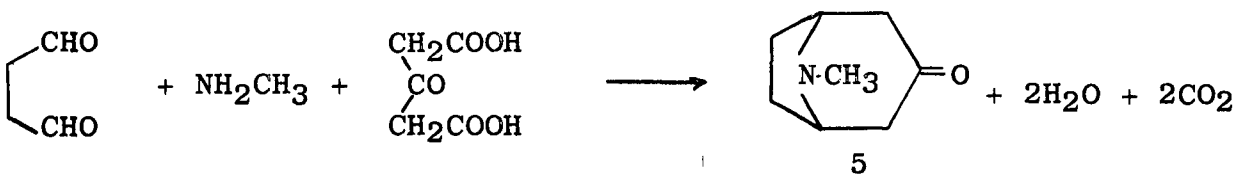

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Figure 2. Biogenetic-type Synthesis of Tropinone.

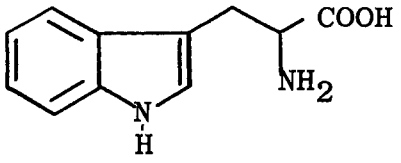
Many postulates have been made concerning biogenetic routes and these arose from two, often closely allied, approaches. The first involved inspection of the structures of alkaloids or other compounds, seeking common structural units and suggesting

possible relationships of these units to simpler natural products. Such deductions have been useful in correlating different groups of alkaloids and for predicting new structures as well as forming a basis for tracer experiments. The recognition of the amino acids, especially lysine (8), ornithine (7), phenylalanine (9), and tryptophan (6) as well as other simple plant bases, as simple units from which alkaloids could arise, led to important information concerning alkaloid synthesis.

The second approach was to correlate alkaloidal structures on the basis of a unifying reaction mechanism. Robinson[9] recognized that if condensation of β-substituted ethylamines with aldehydes could occur in plants then one could account for a wide variety of alkaloidal structures with N-heterocyclic systems. The amino acid phenylalanine (9) can give an amine (10) by decarboxylation and an aldehyde (4) by oxidation. Other synthetic reactions which are important keys to the synthesis of alkaloids are aldol condensations between aldehydes and β-keto acids, and condensations of carbinolamines (-C(OH)N-) with the active methylene of ketones or β -keto acids.[17,13]

The biosynthetic significance of phenol oxidations has long been recognized and is the subject of several excellent reviews[15,18-20] An increasing number of biogenetic-type syntheses make use of phenolic oxidative coupling. This mode of coupling is particularly important in the field of alkaloids as more than 10% of the known alkaloids can be derived by application of the principle of ortho- and para-C-C and C-O coupling,

by coupling of the appropriate phenolic precursors.[15,18,21]



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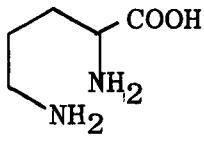

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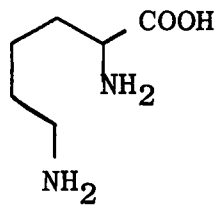

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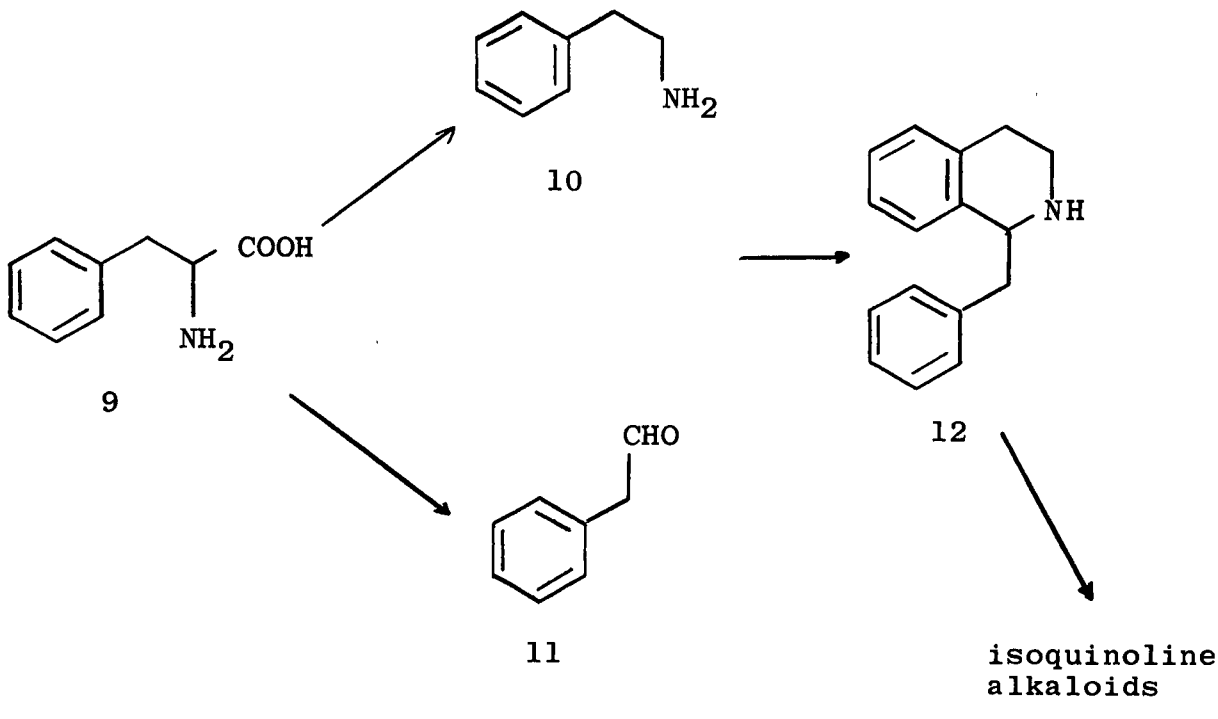
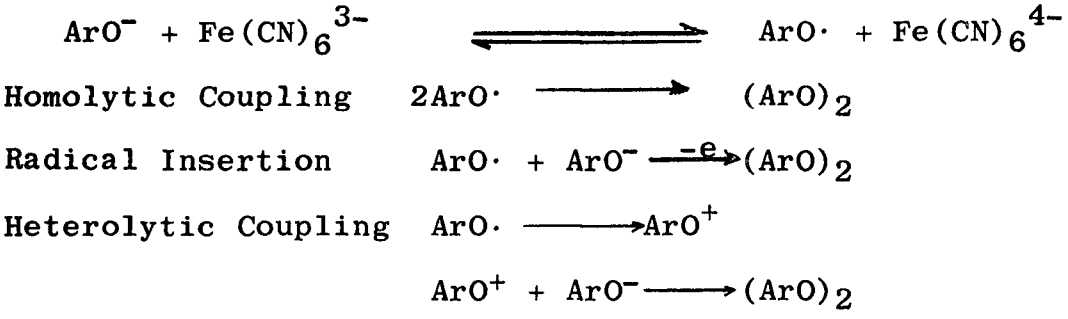
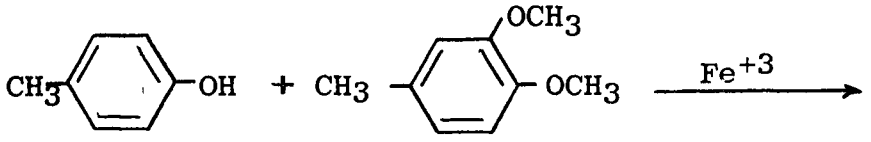


Figure 3. Amino Acids and Alkaloid Biosynthesis.

In considering the mechanism of oxidative dimerization one must distinguish between homolytic coupling (radical coupling), radical insertion, and heterolytic coupling (two electron oxidation to a cationic species). Evidence for the inclusion of a cationic species is lacking and the inability of ArO^+ to capture any nucleophile other than phenol anions offers some circumstantial evidence against this two electron oxidation. Although radical insertion cannot be disregarded it seems unlikely. Evidence includes the absence of cross coupling when p-cresol (13) is oxidized in the presence of a large excess of veratrole (14), and internal coupling of the phenol (15), while its monomethyl ether only dimerizes.[20] Electron spin resonance spectra of phenols undergoing oxidation in alkaline solution give direct evidence of radical intermediates. The oxidation of phenols or of phenol anions by reagents capable of reduction by one-electron affords mesomeric phenol radicals stabilized by spreading of the odd electron by resonance over the ortho and para positions of the aromatic ring. Coupling in the ortho or para position or on the oxygen is then possible. Detailed studies by E.Müller and his colleagues on the analysis of hyperfine splitting of electron spin resonance spectra of these radicals have shown that the free electron density is greater at the para than at the ortho position while the meta position shows a small but non-zero density.[15,22] The most versatile reagents for oxidative coupling are alkaline potassium ferricyanide and ferric chloride. Other oxidants which have been





13 14

no cross coupling (same products as for oxidation of p-cresol) in absence of veratrole

15

when R =H, not when R = CH_3

Figure 4. Alternative Mechanisms and Evidence Against Radical Insertion for Phenolic Oxidative Coupling.

used in C-C, C-O, and C-N formation are manganese and lead dioxides, cerium (IV) and vanadium (V) salts, lead tetra-acetate and Fenton's reagent.

Aminophenols and amines also couple via mesomeric radicals. Simple aromatic amines [23] and o-aminophenols [24] give rise to dimeric phenazines (16) and phenoxazones respectively.

Participation of oxidative coupling in the biosynthesis of the morphine alkaloids has been demonstrated and a biogenetic-type synthesis of thebaine (22) has been achieved. This serves to illustrate the utility of the phenolic coupling

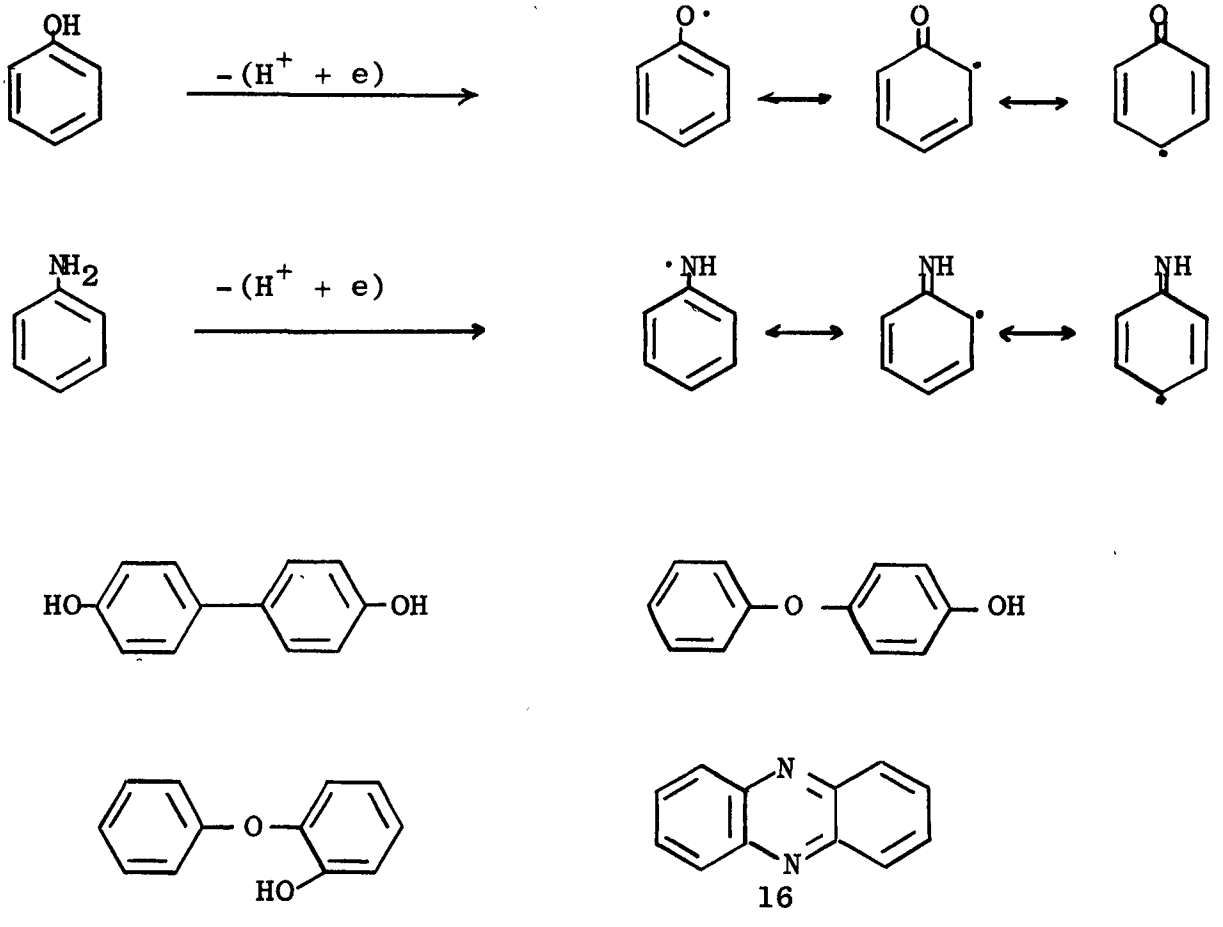


Figure 5. Coupling of Mesomeric Phenol and Arylamine Radicals.

concept. The relationship between benzylisoquinolines (12,17) and morphine (23) was first suggested by Gulland and Robinson[15] and used to deduce the correct structure for the latter. Details of possible mechanisms for the coupling were discussed by Barton and Cohen[18] and are illustrated by conversion of the diphenolic base (17, R = Me) into the dienone (19) followed by ether formation (20) and appropriate reduction. Modifications of the proposed transformation of the dienone (19) into morphine involve reduction to the dienol (21) followed by dehydrative rearrangement. The dienone was found to exist in the "open" form and could be converted into thebaine (22) under very mild laboratory conditions and thence to morphine (23) in realization of the latter scheme. Barton and Battersby and their co-workers have independently demonstrated the participation of oxidative coupling in the biosynthesis of these alkaloids[14, 20,26] The benzylisoquinoline precursor (17, R = Me) labelled with carbon-14 and tritium was converted (0.14% incorporation) into thebaine (22) in Papaver somniferum. The participation of oxidative coupling was inferred from experiments where norlaudanosoline (17, R = H) was incorporated more efficiently than tyrosine but less efficiently than the base (17, R = Me). Labelled tetrahydropapavarine (18) was not incorporated. The labelled precursor (17, R = Me) of totally synthetic origin could be oxidized to the racemic dienone (19) in 0.024% yield using manganese dioxide, as determined by radiochemical dilution.[25]

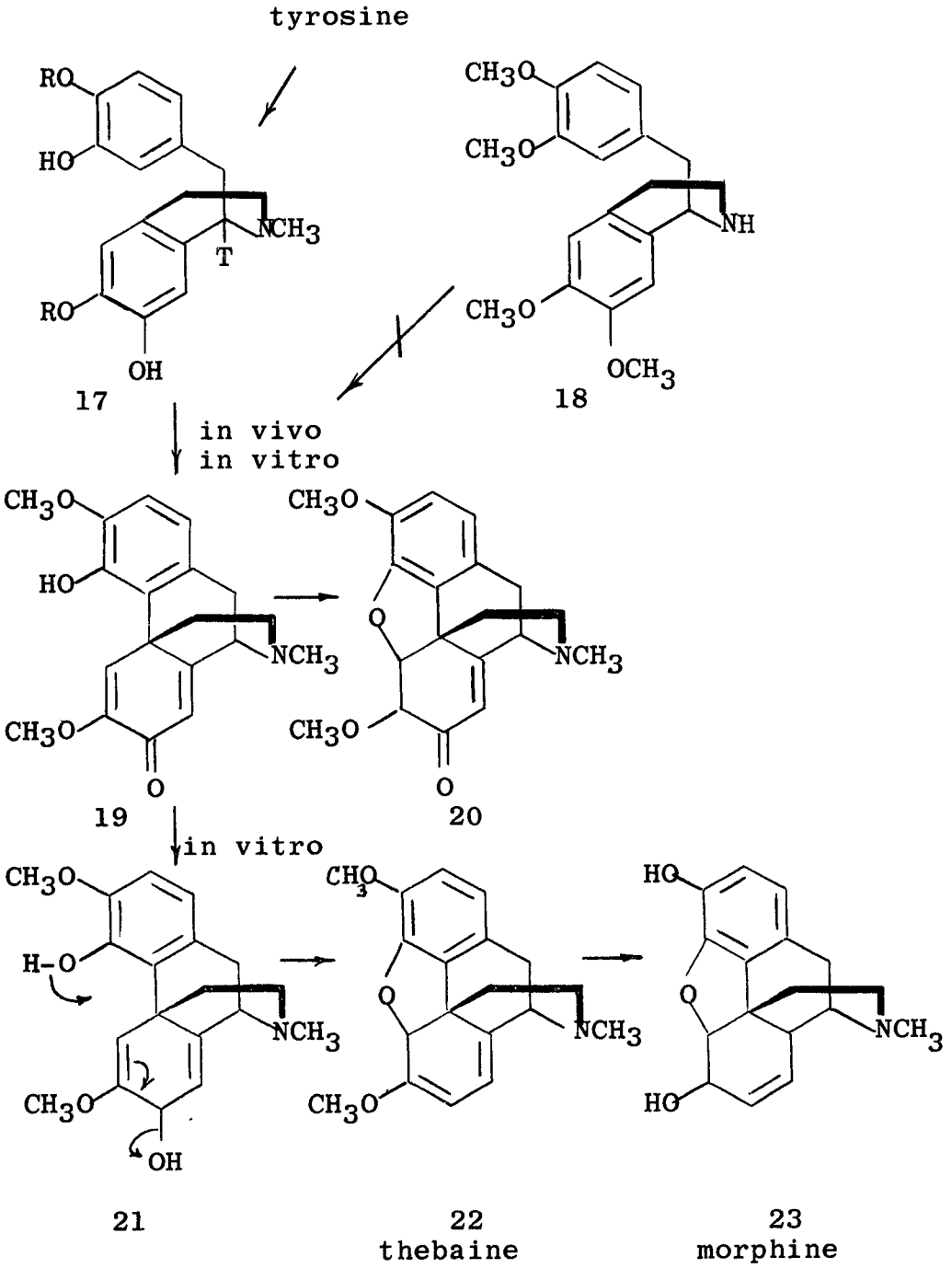
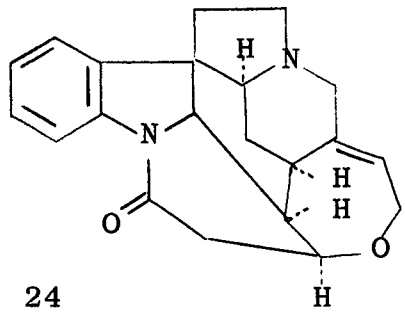


Figure 6. Morphine Alkaloids, Synthesis and Biosynthesis.

Indole Alkaloids

A large number of alkaloids occuring in nature contain aromatic rings and of these a considerable portion contain the indole or dihydroindole nucleus. This large family of indole alkaloids has been isolated from more than twenty-five genera of plants and more than three-hundred structures have been elucidated, as listed by Hesse in a very useful recent publication.[27] No indole alkaloid has acquired the illicit commercial notoriety of the phenylalanine derivative morphine (23) or the availability of its methyl ether, codeine, which is so commonly used as an analgesic. However, some useful and well-known drugs are indole alkaloids. Strychnine (24) has been used as a stimulant for the heart and also as a poison for vermin. Curare is the name given by South American Indians in the Amazon and Orinoco valleys to concentrated aqueous extracts used as arrow poisons which produce cardiac or skeletal muscle paralysis. Over forty alkaloids which bear striking resemblances to strychnine have been isolated in recent years from the particularly potent Calabash curare. Yohimbine (25) was employed as an aphrodisiac in veterinary medicine. Reserpine (26), which is but one of the alkaloids of the Indian Snakeroot, Rauwolfia serpentina, was widely used in native medicine, usually as a sedative. It is useful in treatment of hypertension and of various mental disorders. The alkaloids of Ergot which is a fungus parasitic on cereal grasses, especially rye, have an oxytocic effect useful in childbirth. These



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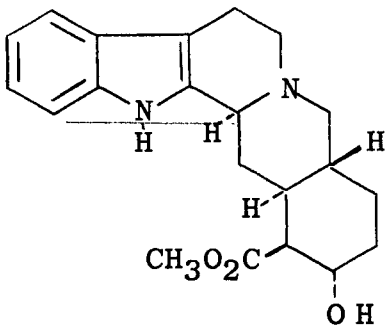
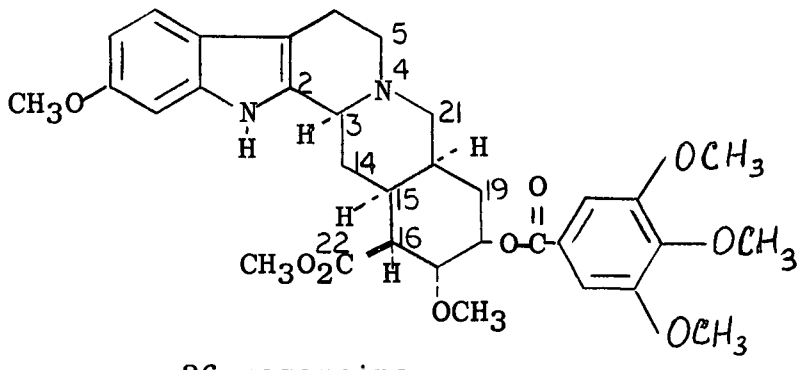
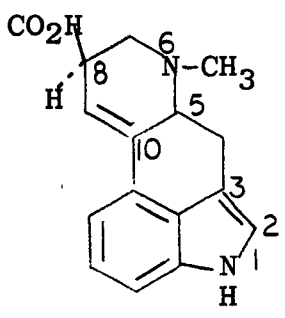
strychnine



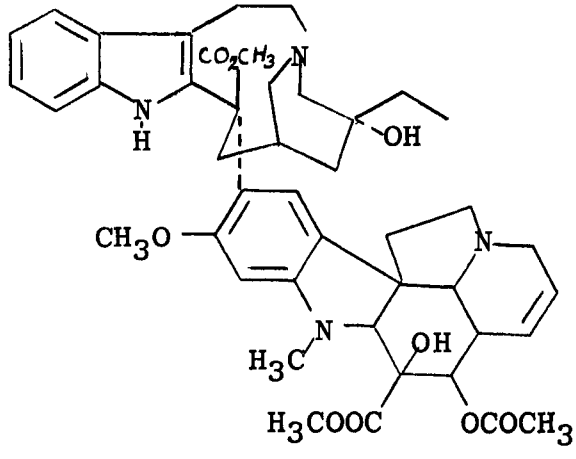
25 yohimbine



26 reserpine



27 lysergic acid



28 vinblastine

Figure 7. Some Indole Alkaloids.

alkaloids are lysergic acid amides. Of the series of synthetic amides of lysergic acid (27) the most interesting is the diethylamide which produces symptoms like those of schizophrenia when administered in extremely small doses.[28] Vinblastine[29] (28) a dimeric alkaloid produced by Vinca rosea Linn is used clinically as a potent anti-leukemic agent. The physiological activity and diversity of interesting and elaborate ring systems will continue to provide interest in indole alkaloid chemistry.

It has long been suspected that the indole alkaloids are derived in part from tryptophan (8) and this has been confirmed in every case where tracer experiments have been carried out.[2, 30-33] These alkaloids are formed in nature from tryptophan in several ways.

Although the indole alkaloids are characterized by elaborate ring systems, a few are simple derivatives of tryptamine (31). Serotonin (5-hydroxytryptamine) is a vascoconstrictive principle of blood, is widely distributed in animal tissue and is involved in the chemistry of the central nervous system.[5] It also occurs in plants. Psilocybine (2) is a related tryptamine derivative and requires no comment other than to note oxidation at the 4-position of the indole nucleus, a position which is important with respect to synthesis and biosynthesis of lysergic acid. Gramine (32) is a degradation product of tryptophan and was the subject of some of the earliest tracer studies.[34] The most significant result of these studies is presented in a recent paper by O'Donovan and Leete[35] in which a mixture of DL-tryptophan-β-^{3}H and DL-tryptophan-β-^{14}C was fed to intact barley

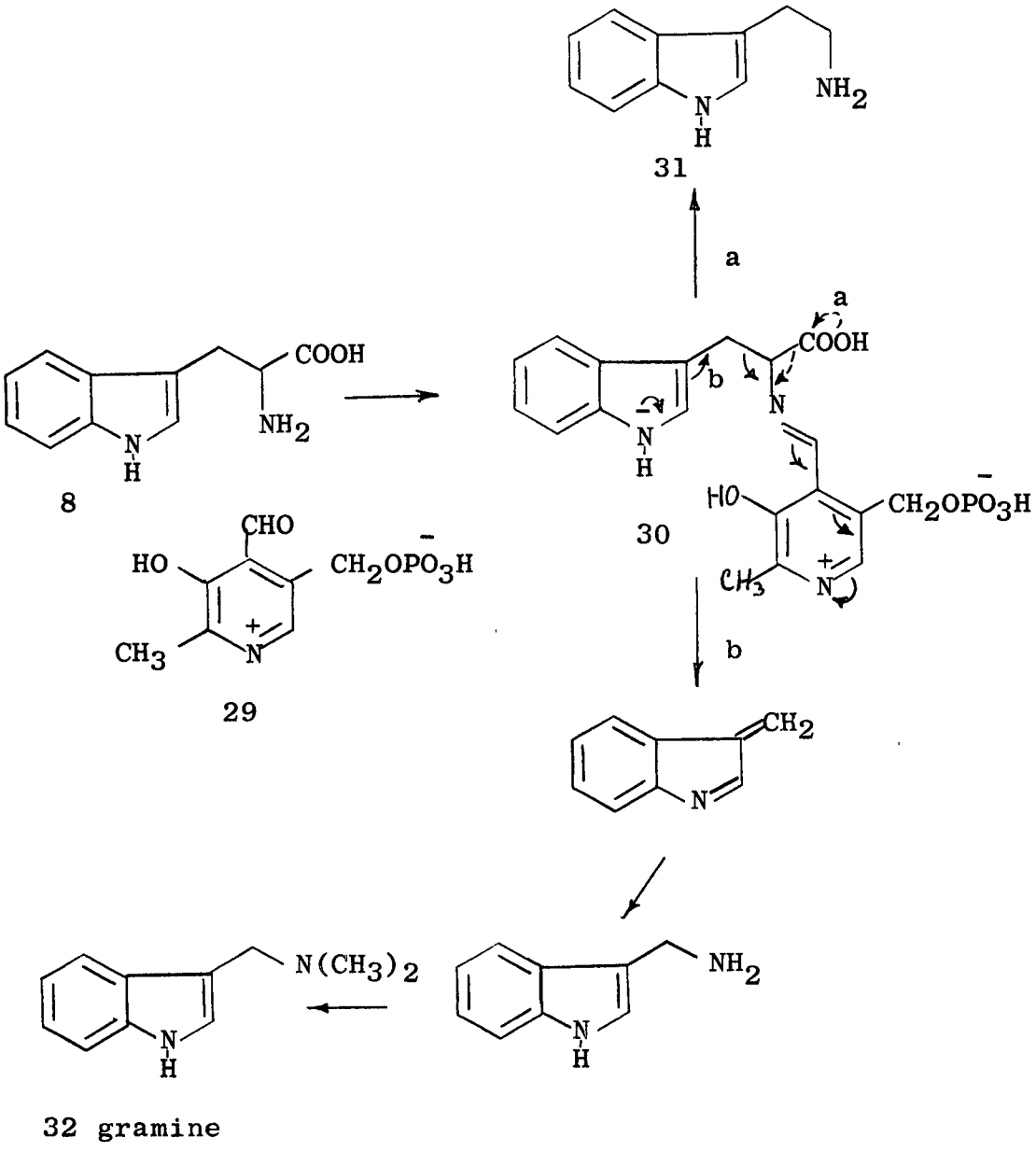
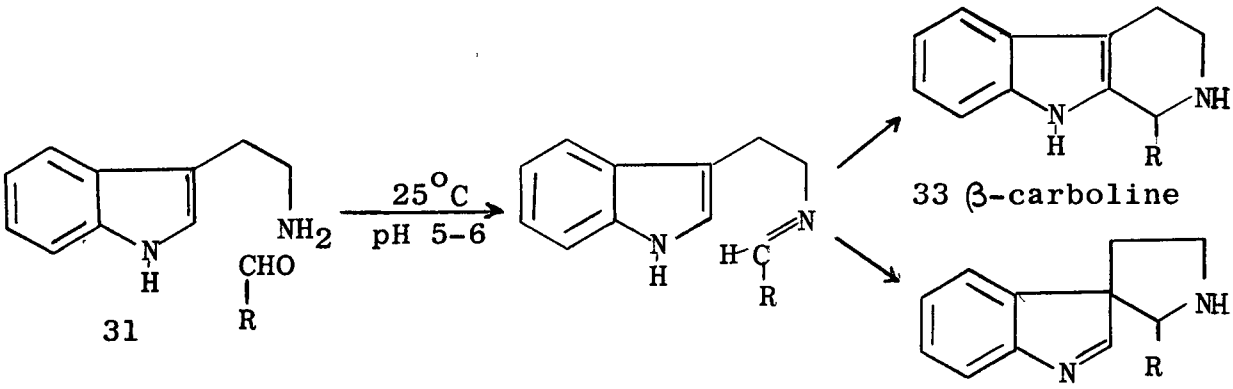


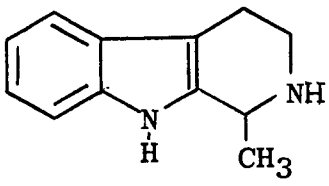
Figure 8. Tryptamine and Gramine from Tryptophan.

seedlings. It was established that all of the activity was located in the methylene group of the gramine side chain. A biosynthetic hypothesis consistent with this result was proposed by Wenkert[36] and is attractive in that it involves pyridoxal phosphate (29) which is also involved in transamination and decarboxylation of amino acids through formation of the Schiff base (30).

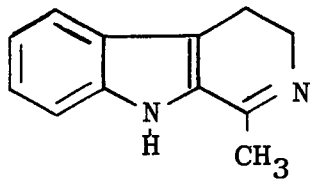
Under physiological conditions (25° C and pH 5-6) tetrahydroharman (35) can be produced *in vitro* in good yield from tryptamine (31) and acetaldehyde.[37] As this is also a reasonable biosynthesis for the β-carboline alkaloids harmaline (36), harmine (37) and harman (38) obtained from *Peganum harmala*, it stands as an early example of a biogenetic-type synthesis. Labelled tryptophan is a proven precursor of the carbolines of *Peganum harmala*.[38] The condensation of tryptamine with aldehydes or other carbonyl compounds, for instance α-keto acids, to yield carboline (33) or indolenine (34) derivatives is recognized as the well-known Mannich reaction of organic chemistry. Yohimbine is a good example of a more complex carboline while strychnine has an indolenine skeleton. (Figures 7 and 9).

The ergot alkaloids are formed from tryptophan and a five-carbon unit. Their biogenesis has been reviewed by Weygand and Floss.[39] Weygand was the first to demonstrate that mevalonic acid is used not only for the synthesis of typical isoprenoid compounds but also for the biosynthesis of alkaloids when he showed a specific rate of incorporation of 16% into some ergot

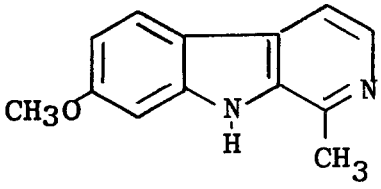




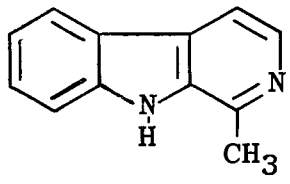
35 tetrahydroharman



36 harmaline



37 harmine

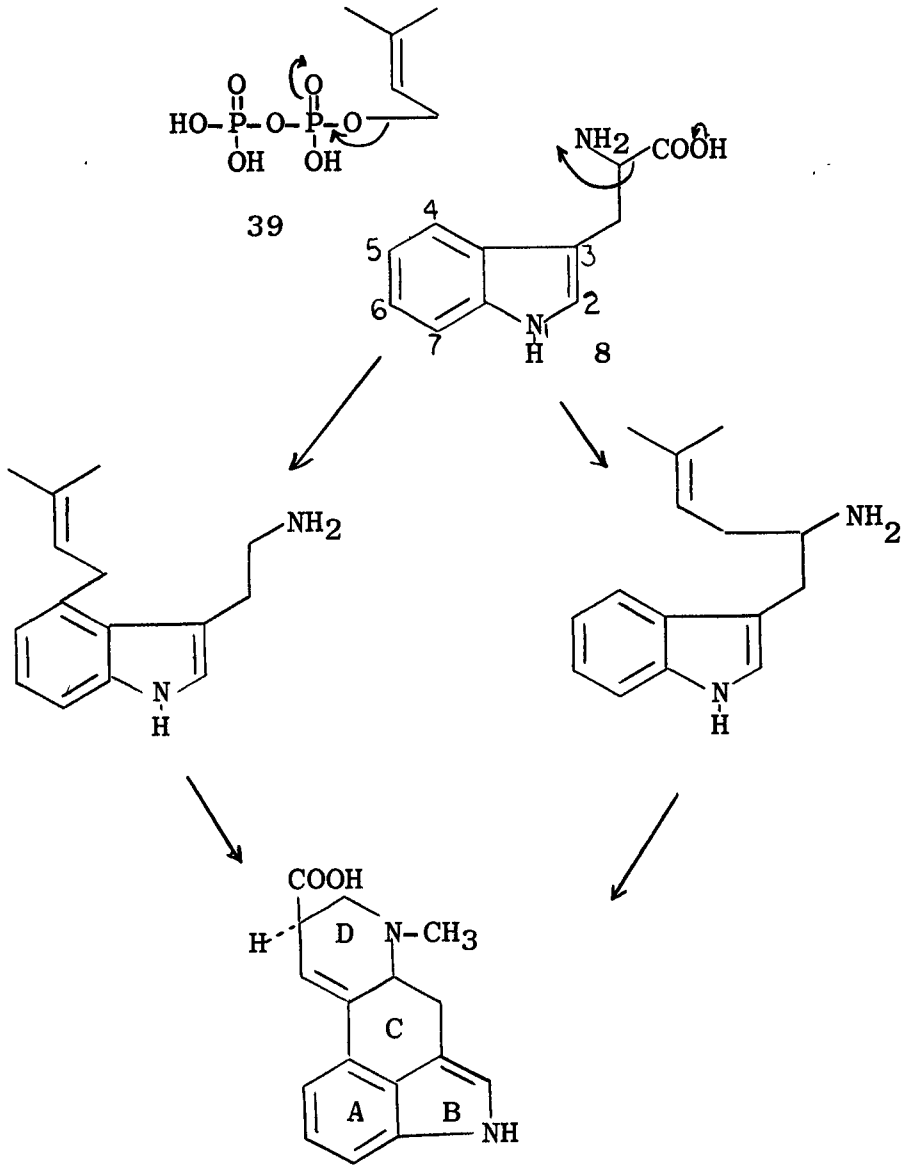


38 harman

Figure 9. Condensation of Tryptamine and Aldehydes.

alkaloids.[40] Furthermore mevalonic acid is incorporated into ergot alkaloids via isopentenyl or dimethylallyl pyrophosphate (39).[41,42] The mechanism of condensation and cyclization is a question which has not been answered completely. Electrophilic substitution of the indole ring is difficult in position four and favoured in position five. The discovery in nature of psilocybine (2.)[43] followed by tracer studies[44] demonstrated that tryptophan can be hydroxylated in nature in the 4-position and such a reaction could play a part in the biogenesis of the ergot alkaloids. The other possibility is direct condensation of tryptophan with dimethylallylpyrophosphate either by attack at the 4-position of the indole ring or by attack with simultaneous decarboxylation at the α-carbon of the tryptophan side-chain followed by cyclization to position four which is favoured for stereochemical reasons even though positions five and seven are electronically favoured.[45]

The majority of indole alkaloids consist of a tryptamine unit plus a nine or ten carbon unit condensed with the nitrogen of the tryptamine side chain and any combination of positions one, two, or three of the indole system. The carbon skeleton of this ten carbon unit is arranged in one of three patterns, each of which can condense with itself and with the tryptamine unit to give rise to the multitude of elaborate ring systems which make the indole alkaloids so interesting. Part B of this thesis is concerned with elaboration of the biogenesis of the non-tryptophan derived portion of these indole alkaloids.



27 lysergic acid

Figure 10. Biogenesis of Ergot Alkaloids.

The Calycanthaceous alkaloids are a small but interesting group of indole alkaloids derived from condensation of two tryptamine units. Part A of this thesis describes the total synthesis of these alkaloids in a simple way which is probably closely related to the way in which they are synthesized in nature.

The biosynthesis of the aromatic amino acids has been worked out using biochemical techniques which include feeding of labelled precursors, growth requirements, and isolation of certain intermediates from mutant strains of E.coli. Shikimic acid-5-phosphate (44) and prephenic acid (45) are important intermediates in this metabolic pathway to aromatic compounds which has become known as the shikimic-prephenic or the carbohydrate route. Shikimic acid (43) is derived from phosphoenolpyruvic acid (41) and erythrose-4-phosphate (42), both being derived from glucose by glycolysis and through the pentose shunt respectively. These metabolic pathways are outlined in figures 11 and 12. The mechanism of aromatization of shikimic acid-5-phosphate (44) to anthranilic acid (46) has not been completely elucidated but the amino group is glutamine-derived. Anthranilic acid is converted to indole-3-glycerol phosphate (47) through its ribonucleotide. The glycerol phosphate side chain is then replaced by serine (48) yielding tryptophan (6). Prephenic acid (45) is derived from shikimic acid-5-phosphate and phosphoenolpyruvate. Aromatization with loss of carbon dioxide followed by amination of the α-keto group yields

tyrosine. Aromatization with loss of carbon dioxide and water gives phenylpyruvic acid and phenylalanine by subsequent amination. Reductive dehydration gives cinnamic acid.[46]

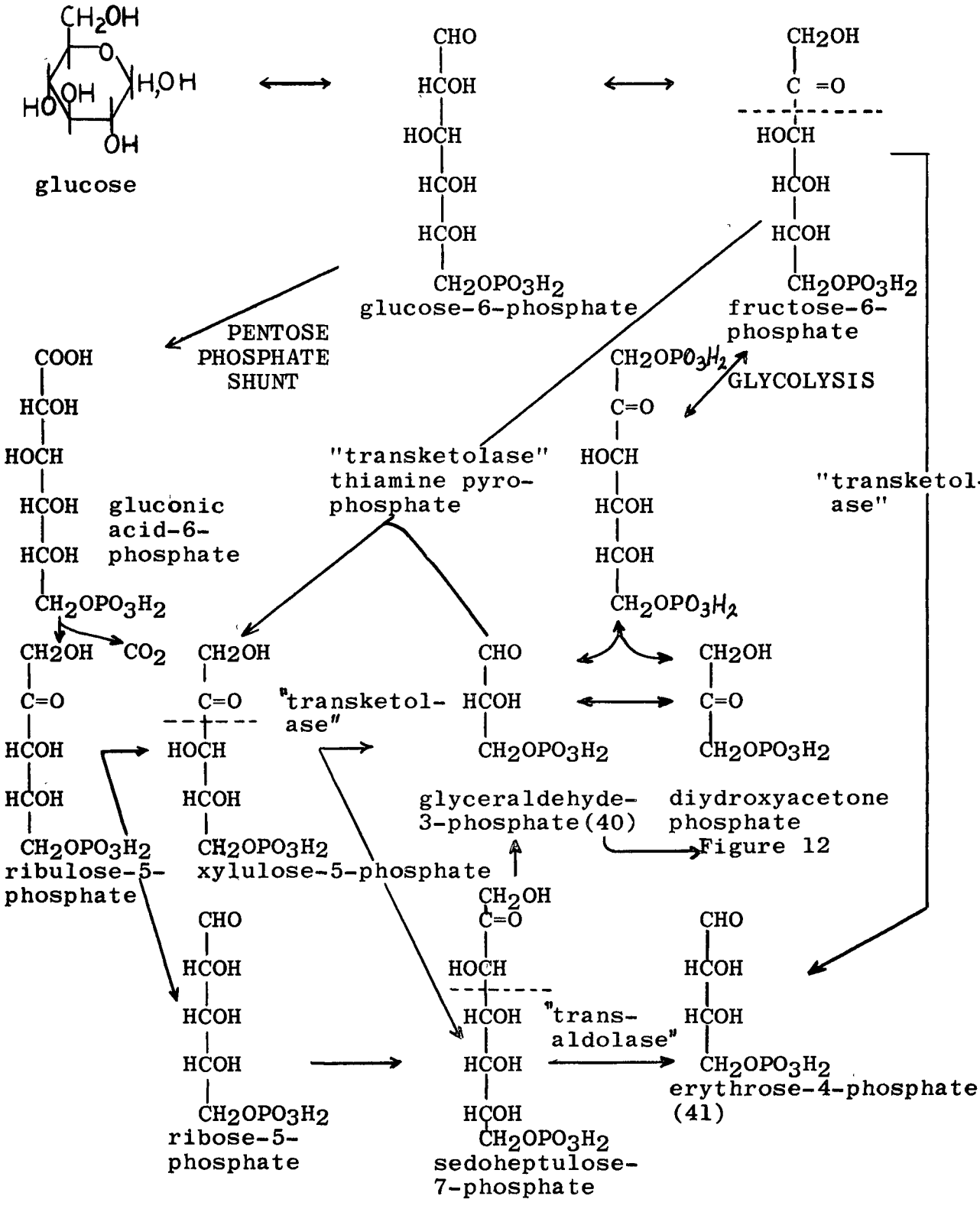


Figure 11. Pentose Shunt and the Glycolytic Pathway.

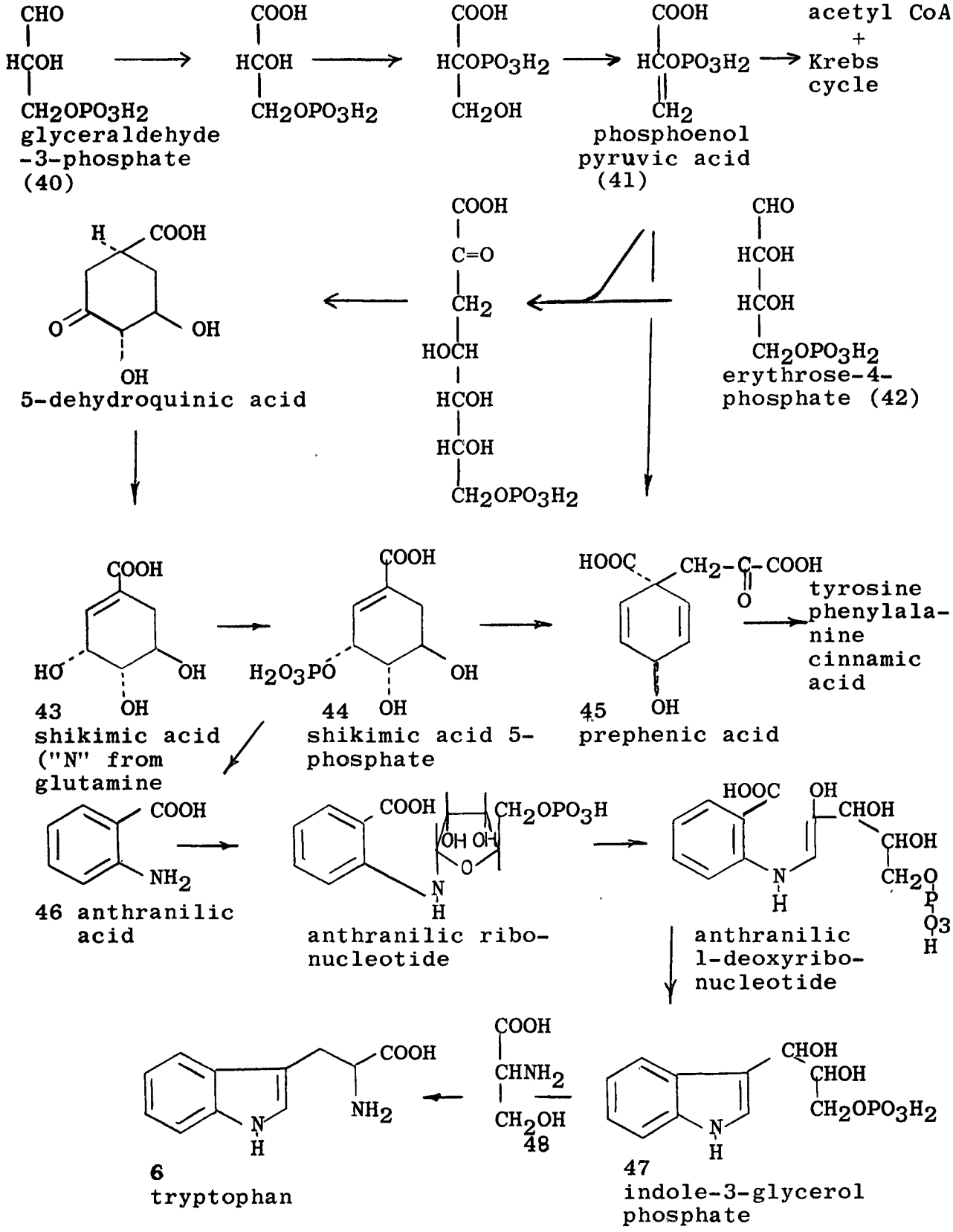


Figure 12. Shikimic-Prephenic Acid Route to Aromatic Amino Acids.

PART A

Calycanthaceous Alkaloids:

A Total Synthesis and Biosynthetic Model

Introduction

The determination of the structure of calycanthine (73α), which is the principal alkaloid of the botanical order Calycanthaceae may be considered one of the classical problems of alkaloid chemistry, while the deduction of the correct structure stands as a tribute to the power of modern biogenetic and mechanistic theory. The seeds of Calycanthus glaucus Willd., which is a shrub native to Georgia, North Carolina and Tennessee, attracted attention because of their poisonous nature. Calycanthine, the active principle, was first isolated from these seeds by G.R.Eccles in 1888.[47] It was not until 1952, however, that a deduction of the correct structure was first made by R.B.Woodward[48] in an advanced course on natural products at Harvard, after synthesis of the degradation product calycanine (69) was accomplished in corroboration and published in 1960.[49] The same suggestion was made by R.Robinson and H.J.Teuber in 1954.[50] The chemical and spectral evidence presented by Woodward in 1960 for the structure and configuration of calycanthine was then confirmed by x-ray analysis of the dihydrobromide dihydrate.[51] The absolute configuration has also been determined.[52]

Before the advent of modern physical tools, structures of organic compounds were based on elemental analysis, molecular weight, chemical tests for functional groups and degradation to produce known compounds or at least simpler compounds, whose structure or structural determination would provide clues to the

initial molecular framework. Structural proposals based on this evidence were then checked by synthesis. The complexity of calycanthine became evident from the remarkable variety of nitrogenous heterocycles produced on degradation. The formation of both indole and quinoline derivatives created substantial difficulties in interpretation and led to some bizarre structural proposals, eg. 59-65. Reviews of this degradative work are found in "The Alkaloids".[53]

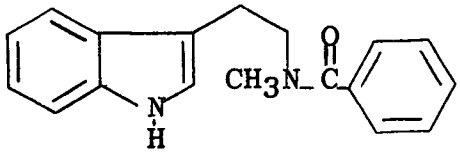
It is interesting to examine some of the evidence which led to the proposal and rejection of these structures. In 1905 Gordin[54] assigned the formula $C_{11}H_{14}N_2$ to calycanthine. This was later doubled[55] then finally revised by Barger *et al.* in 1939 to its present $C_{22}H_{26}N_4$.[56] The first clue to the constitution was afforded by the benzoylation of calycanthine and oxidation of the product with potassium permanganate,[57] yielding a product shown to be identical with synthetic N-benzoyl-N-methyltryptamine (49). It was also known by formation of dinitrosoderivatives and Zerewitinoff determination of two active hydrogens, that two of the four nitrogen atoms were secondary. From this the probable presence in the molecule of the grouping 59 was deduced and supported by the production of β-carboline (51) when the molecule is dehydrogenated with selenium. Isolation of identical substances when either calycanthine or tryptamine (31) is heated with phthalic anhydride gave further support. The action of soda lime on benzoylcalycanthine produced 2-phenylindole (52) and

quinoline (53) while calycanthine yielded N_b-methyltryptamine (50) and a small quantity of a base $C_{12}H_{10}N_2$, at first assumed to be 8-methyl-β-carboline[56] but later disproved by synthesis.[58] Repetition of this degradation yielded mainly norharman (54).[59] The weak base calycanine, $C_{12}H_{10}N_2$ (69) is produced when calycanthine is pyrolyzed, or heated with lead oxide, copper oxide, sulfur,[56] selenium,[60] or zinc dust.[61] β-Carboline (51),[60] skatole (55), 3-ethylindole (56) and lepidine (57)[61] are also formed by the action of selenium (Figure 13).

In 1939 Barger, Madinaveitia and Streuli[56] assumed calycanine was a di-indolylene (60) (containing a quinoline nucleus) which, with a methylamino side chain and a fused piperidine ring (61), was tentatively advanced to represent calycanthine. This structure had several points against it, including the reaction of calycanthine with Ehrlich's reagent only on heating implying that calycanthine must be substituted in the α and β positions of the indole nucleus.

This led to the proposal of 63 as a more likely alternative although the accompanying proposal for calycanine (62) as a lepidyl-β-carboline required an empirical formula $C_{21}H_{15}N_3$.[61] On synthesis 62 proved to be quite different from calycanine. 62 is intensely fluorescent in neutral or acid solutions whereas calycanine is only slightly fluorescent and therefore probably not even a carboline derivative. Structure 64, $C_{15}H_{10}N_2$, was then proposed for calycanine.

Furthermore both of the structural proposals for caly-



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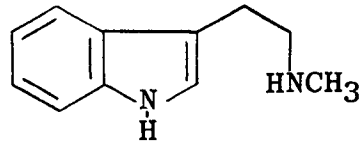
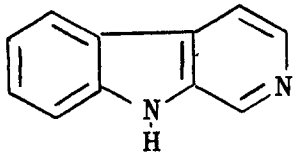
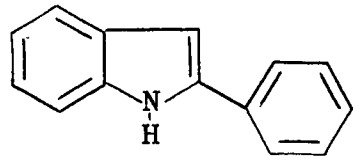


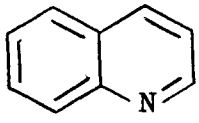
50 N_b-methyltryptamine (dipterin)



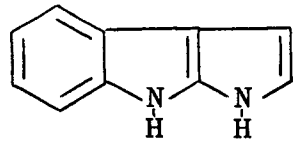
51 β-carboline



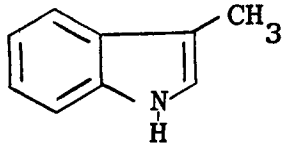
52 2-phenylindole



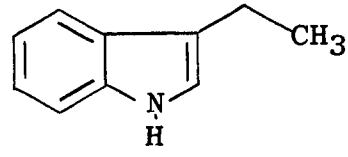
53 quinoline



54 norharman



55 skatole



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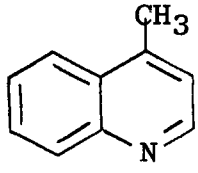

57 lepidine

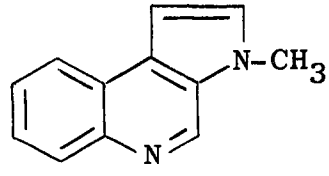

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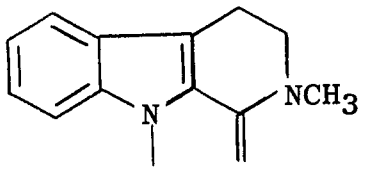
Figure 13. Degradation of Calycanthine.



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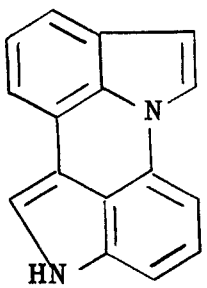

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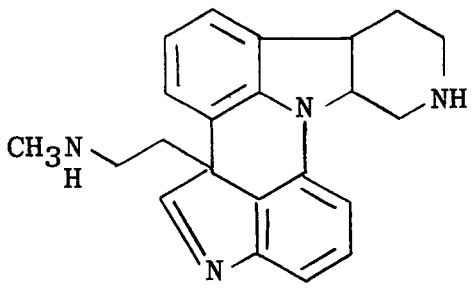

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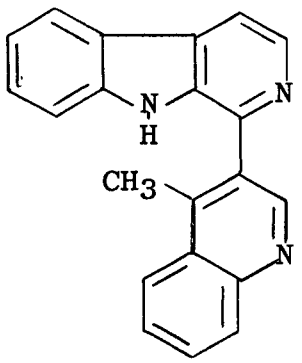

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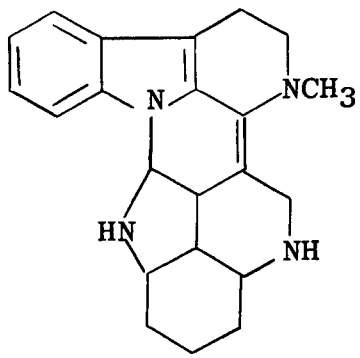

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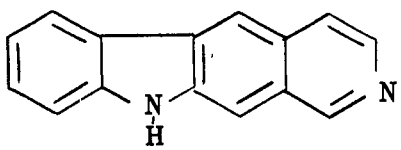

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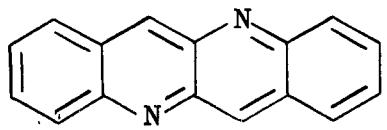

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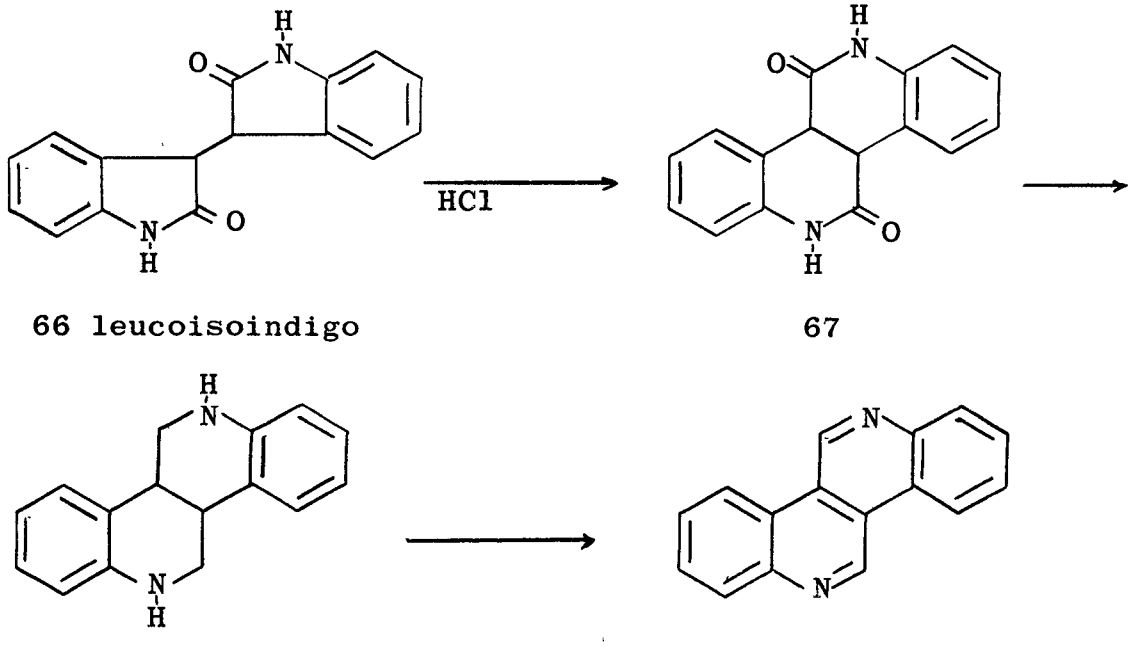
Figure 14. Early Proposals for the Structure of Calycanthine and Calycanine.

canthine (61 and 63) were rendered unlikely when a base, $C_{12}H_{10}N_2$, m.p. 115-6° C [62] was obtained by oxidation of calycanthine by silver acetate in 1% acetic acid solutions. This base which further oxidized by alkaline potassium permanganate to N-oxalyl-anthranilic acid and ammonia[63] has been shown by synthesis[64] to be 3-(N-methyl)-4-pyrroquinoline (58). This degradation product could only be derived from 61 or 63 by a series of highly unusual transformations. More recent spectroscopic properties as well as functional group analysis and colour reactions also exclude these formulations.

The correct structure of calycanthine was deduced on biogenetic and mechanistic principles after the structure of calycanine was finally established. An x-ray examination[65]



66 leucoisoindigo 67

68 69 calycanine

Figure 15. Synthesis of Calycanine.

of calycanine in 1941 showed the molecule to be centro-symmetrical and structure 69 was finally established by synthesis [49] from leuco-isoindigo (60)[66](Figure 15). Leuco-isoindigo is isomerized by heating for five hours in 4 N hydrochloric acid to the δ-lactam 67. Reduction with lithium aluminum hydride in tetrahydrofuran gives the hexahydrodiaza-chrysene (68). Dehydrogenation with palladium chloride in hydrochloric acid [67] or over metallic palladium[68] converted the hexahydroderivative smoothly into 6,12-diazachrysene (69) which was identical in all respects to calycanine derived from caly-canthine. Comparison of the ultraviolet spectrum of caly-canthine; $\lambda_{max.}^{MeCN}$ 252, 310 mμ, $\log\epsilon_{max.}$ 4.26, 3.80 with that of 1,2,3,4-tetrahydroquinoline; $\lambda_{max.}^{EtOH}$ 248, 299 mμ, $\log\epsilon_{max.}$ 3.86, 3.30 suggested that the molecule contained two aromatic rings ortho-substituted with carbon and nitrogen residues. This substitution pattern is amply supported by consideration of the degradation products.(Figure 13).

A simple biogenesis was suggested for calycanthine by its isolation[69] by Manske in 1929 from the seeds of a taxonomically distant species, Meratia praecox, which is a Compositae. The facile formation of N_b-methyltryptamine (50) whose empirical formula, $C_{11}H_{14}N_2$, is one proton more than half that of caly-canthine, as a degradation product of calycanthine, and its occurrence as the natural alkaloid dipterin led to the proposal of oxidative coupling of two molecules of N_b-methyltryptamine as a reasonable biogenesis for the alkaloid.[49,50] This

coupling could be $\alpha\alpha'$, $\alpha\beta$ or $\beta\beta'$. However on mechanistic grounds electrons are primarily available for the oxidative coupling of two indoles at the β-position and the primary product of such a coupling would be 71. This calycanthine isomer may be regarded as equivalent to the tetraaminodialdehyde 72 via hydrolysis of the two imines. This tetraaminodialdehyde is capable of forming five structural isomers (Figure 17) through formation of internal N-acetals with loss of two molecules of water. The stability of calycanthine to acid suggested that it has the configuration which is most favoured on steric grounds. Robinson and Teuber[50] had preferred structure 73δ while Harley-Mason is reported to have preferred 73β.[51] As calycanthine is optically active the forms α, β and δ must contain a cis-fusion of the two six-membered rings A and B. In β and δ the rings A and B must be in the boat form in order to allow the five-membered rings to reach approximate planarity whereas in α a conformation with cis-fused chairs is possible and hence was Woodward's choice for the preferred structure and configuration.[49]

Further chemical evidence for the structure 73α was provided by oxidation of calycanthine by mercuric acetate in aqueous acetic acid, yielding dehydrocalycanthine with the loss of two hydrogen atoms. Because dehydrocalycanthine was smoothly hydrolyzed by alcoholic potassium hydroxide to methylamine and an amide alcohol it was deduced that it was an enamine (74) rather than an amidine. This conclusion was supported by

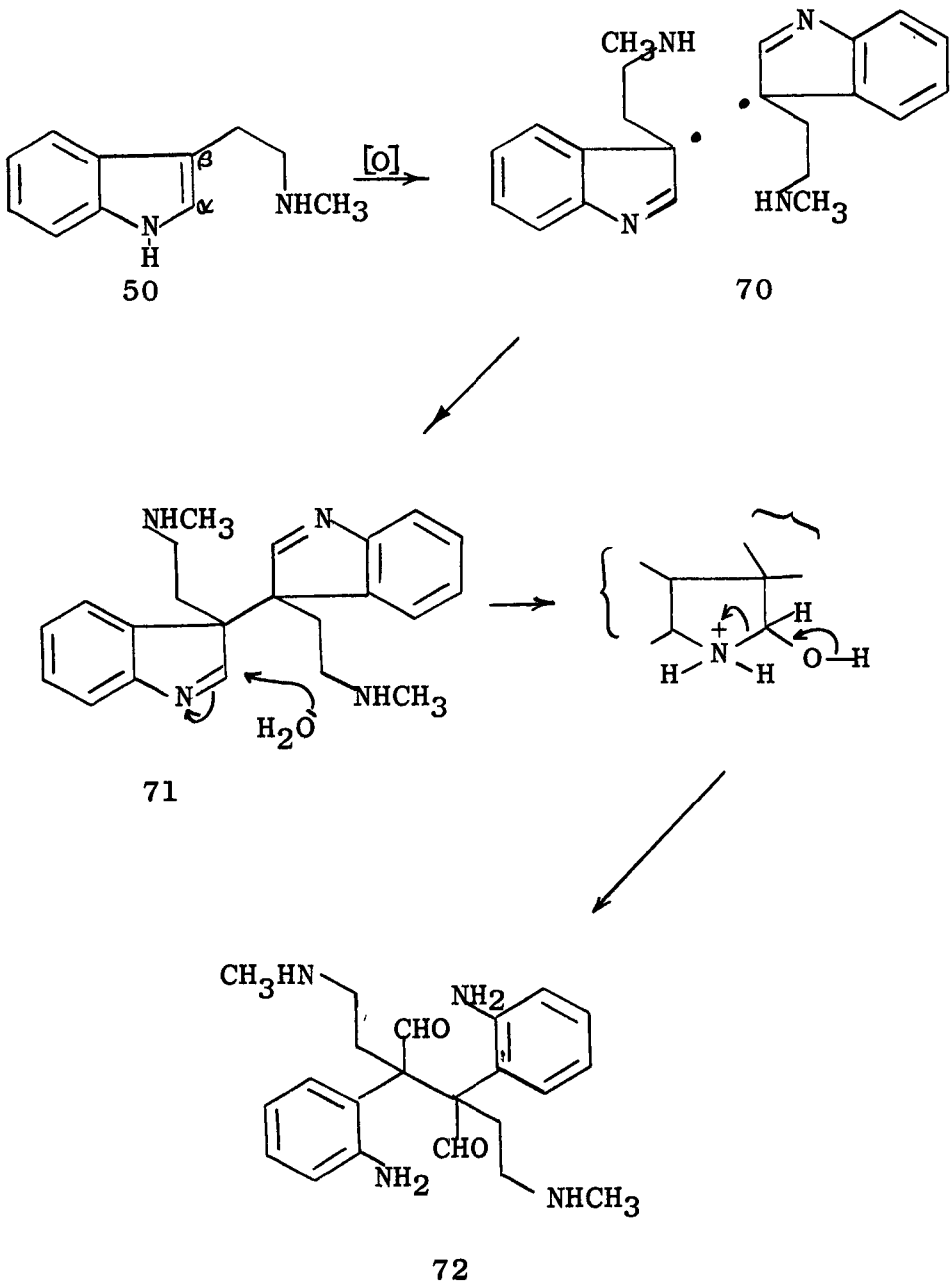


Figure 16. Biogenesis of Calycanthaceous Alkaloids.

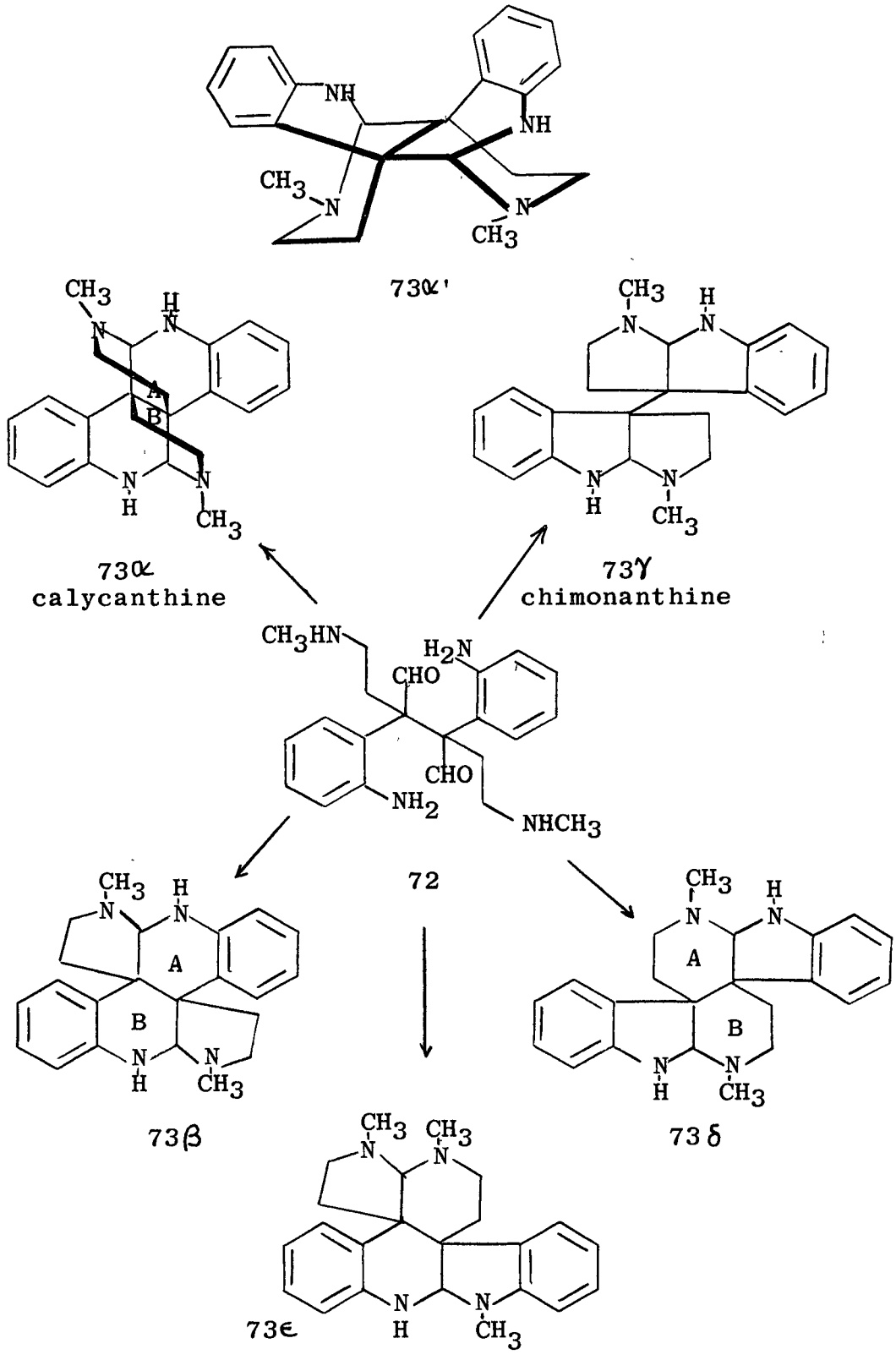
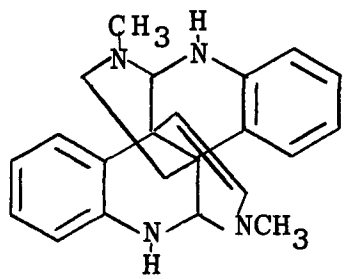


Figure 17. Condensation of Diamino-dialdehyde.

conversion of NN'-dimethylcalycanthine to a closely analogous dehydro-compound. Only in the case of 73α is amidine formation via oxidation precluded by steric considerations.

A comparison of the nuclear magnetic resonance spectrum of calycanthine (40 megacycles) with that of the analogous natural alkaloid physostigmine (75) revealed certain differences in the methylene resonances. In the case of physostigmine the near planarity of the five-membered ring makes geminal methylene hydrogens approximately equivalent with corresponding small



74

coupling constants, and results in a pair of distorted triplet structures one of which (-N-CH_2-) is overlapped by the N-methyl resonance. The complexity of the fine structure associated with the methylene groups of calycanthine is however more typical of methylene groups which are part of a rigid and puckered six-membered ring where the two hydrogen atoms of each methylene are non-equivalent. Structures 73α or δ are hence implicated. The structure and configuration 73α' was confirmed in 1960[51] by x-ray analysis.

The absolute configuration of calycanthine (73α') was

assigned by S.F.Mason in 1962[52] from the shape and signs of the circular dichroism curve associated with the long wavelength absorption band of the aniline chromophore and from a consideration of Moffit's coupled-oscillator theory.[70]

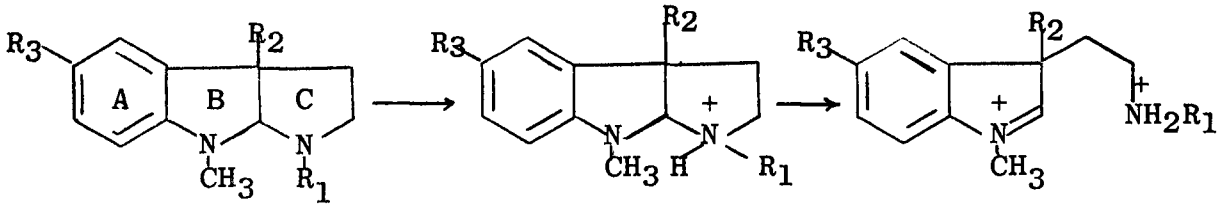
It has been predicted that all five calycanthine isomers will be found in nature. Chimonanthine (73γ) and N-methyl chimonanthines however represent the only other isomer to be identified. It must be noted that since there are two asymmetric centers produced by the oxidative coupling there are two diastereoisomers of the intermediate 72 and this gives rise to two series of isomers (73). With the exception of structure 73ϵ which is structurally disymmetric and hence always potentially optically active one series of isomers must be mesomeric and the other racemic or optically active. One of the striking properties of natural calycanthine is its strongly dextrorotatory nature ($[\alpha]_D = +684^o$).[71] Natural chimonanthine is stongly levorotatory ($[\alpha]_D = -329^o$). It has recently been shown[48] by isomerization of d-calycanthine into l-chimonanthine that these alkaloids belong to the same enantiomorphic series, that is, they have the same absolute configuration.

Chimonanthine

Chimonanthine was isolated in 1960 from the leaves of Chimonanthus fragrans Lindle (Meratia praecox Rehd. and Wils.)[71] This alkaloid, $C_{22}H_{26}N_4$, m.p. 188-189° C was shown by potentiometric titration to be a weak diacidic base of equivalent weight

173. . The ultraviolet spectrum had a maxima at 246 and 304 mμ and underwent a hypsochromic shift of about 7 mμ in dilute acid (0.5 N HCl). This type of spectrum had earlier been noted as characteristic of a so-called Ph-N-C-N type of chromophore[72] and was substantiated by later work. Physostigmine (75)[71] and analogues such as echitinolide,[74] coryminine,[75] as well as calycanthine, calycanthidine[56] and folicanthine[76] containing the tricyclic pyrroloindole system (75) all showed modified indoline type ultraviolet spectra in neutral solution with $\lambda_{max.}$ ca. 250 and 300 mμ. In dilute acidic solution, however, the spectra of physostigmine and its congeners undergo a hypsochromic shift of 8-10 mμ. Hodgson and Smith[72] attributed this shift to protonation of N_b (78), the positive charge on which is then sufficiently close to partially inhibit the delocalization of the lone pair of electrons on N_a over the aromatic nucleus. An excellent study has recently been made on the protonation of tryptamine derivatives in acidic media by Jackson and A.E.Smith[77] which confirms the earlier interpretation by G.F.Smith as to the reasons for the spectral shift. This study is also very important in that it was noted that in more strongly acidic solution (1 M HCl) the spectra of these physostigmine derivatives become very similar to those of tryptamines in stongly acidic solution, and of indolenines. This was clearly consistent with the opening of ring C to give 3H-indolium salts (79). This acid induced ring opening is essential to the biogenetic argument for interconversion of the calycanthaceous alkaloids.

Other spectroscopic studies had also shown that the protonation of indoles in strongly acidic media occurs exclusively at the 3-position with the formation of the corresponding 3H-indolium (indolenine) salt.[78,79]



75 physostigmine $R_1=R_2=CH_3$, $R_3=OCONHMe$ 78 79

76 eserméthole $R_1=R_2=CH_3$, $R_3=OCH_3$

77 deoxynereseroline $R_2=CH_3$, $R_1=R_3=H$

Figure 18. Protonation of Tryptamine Derivatives.

Chimonanthine also was shown[71] to contain two N-methyl groups and had a sharp band at 3440 cm^{-1} in the infrared, interpreted as an aromatic N-H stretching frequency. Reduction with zinc and hydrochloric acid gave a quantitative yield of an indoline identified by comparison of the infrared and ultraviolet spectra as 3-2'-methylaminoethylindoline, indicating that the skeleton of chimonanthine was composed of two tryptamine units.[80] Structures 73α or 73δ were proposed on the basis of

dehydrogenation with zinc dust at 330° C. These conditions gave calycanine from calycanthine with its preformed quinoloquinoline skeleton, but failed to give more than a trace with either chimonanthine or folicanthine. Evidence was also presented that folicanthine was bis-N_a-methylchimonanthine and the Hofmann degradation product of folicanthine dimethiodide was assigned a structure 80, based on indoline rather than quinoline systems by synthesis.[81] This evidence eliminated structures 73 ϵ and 73 β .

A detailed x-ray analysis of chimonanthine dihydrobromide established structure 73 γ as the correct one for chimonanthine.[82]

Folicanthine (85)

Folicanthine with a melting point of 118-119° and a rotation $[\alpha]_D = -365°$ (revised)[83] was first isolated from the leaves of Calycanthus floridus L. in 1951[76] and later from the leaves of Calycanthus occidentalis.[72] The structure proposed after initial studies[76] was based on an incorrect molecular formula and on four degradation products which were not definitely identified. Subsequent investigation [84] identified three of these compounds as N_a,N_b-dimethyltryptamine (81), N_a,N_b,N_b-trimethyltryptamine and N-methylnorharman (54) and this with an indoline type of ultraviolet spectrum led to reformulation of the structure. In later work folicanthine was found to give an 86% yield of N_a,N_b-dimethylindoline (81) on reduction with zinc in hydrochloric acid.[70] The presence of a Ph-N-C-N

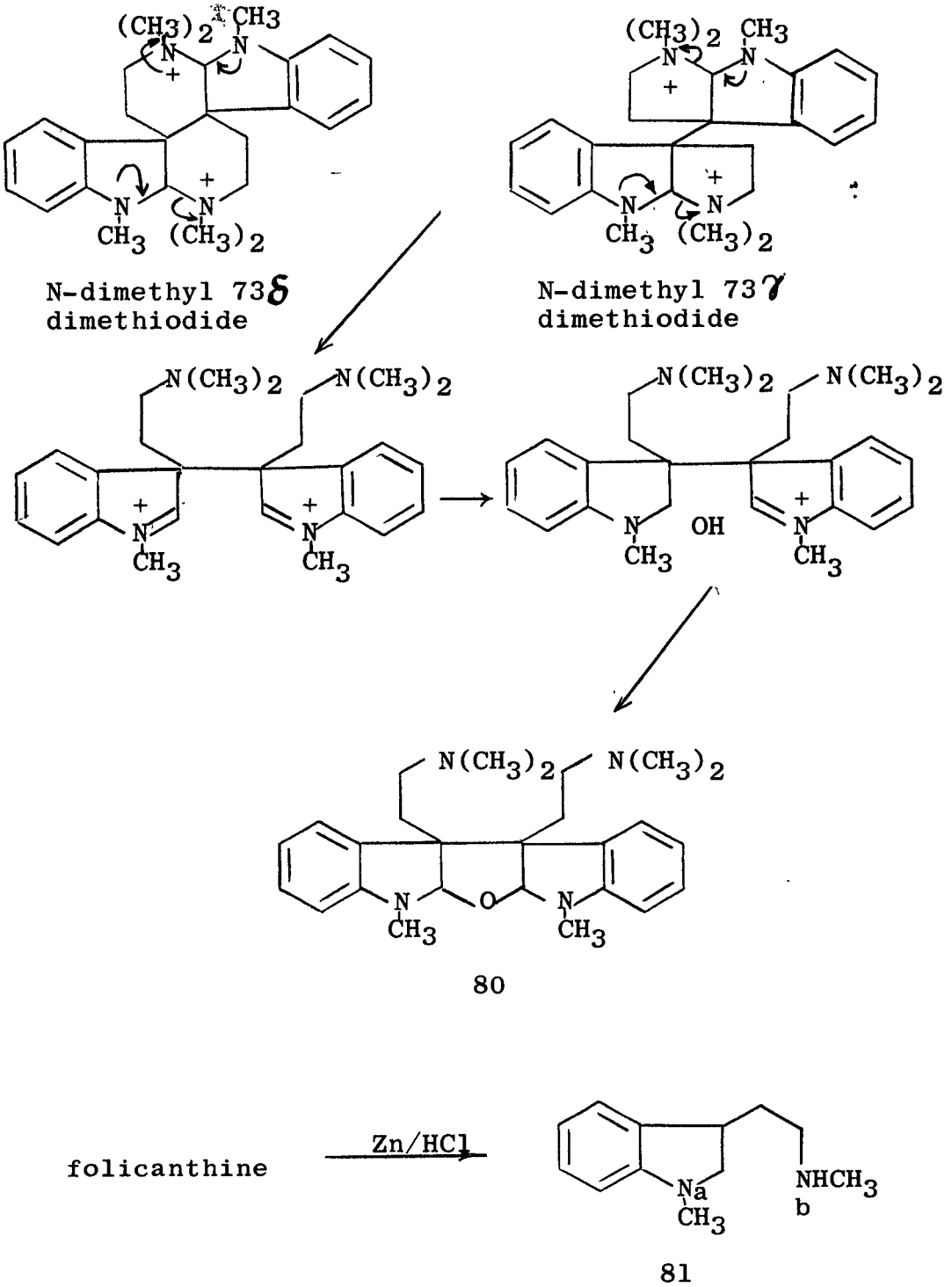


Figure 19. Important Degradation Products of Folicanthine.

system was verified by ultraviolet spectral measurements, and potentiometric titration with a molecular weight determination, supported a dimeric structure but led to another incorrect structural proposal.[70] Identification of the product from basic treatment of folicanthine dimethiodide[80] by synthesis[81] finally led to the proposal of bis-N-methyl calycanthine structures 73 δ or γ for folicanthine. An infrared spectrum for folicanthine differing only in the NH and N-methyl regions from that of chimonanthine but quite different from calycanthine, very similar rotations and easy reduction of both by zinc and acid to indolines while calycanthine is unaffected led to the conviction that folicanthine was bis-N_a-methyl chimonanthine (73γ). The rapid formation of normal dimethiodides in contrast to the complicated reactions of calycanthine [49,85] and the similarity of the mass spectrum to that of chimonanthine[86] showing facile cleavage to a strong fragment at one-half the molecular weight supported this structure. Finally the successful methylation by G.F.Smith[87] of chimonanthine to folicanthine confirmed this structure (73γ,85).

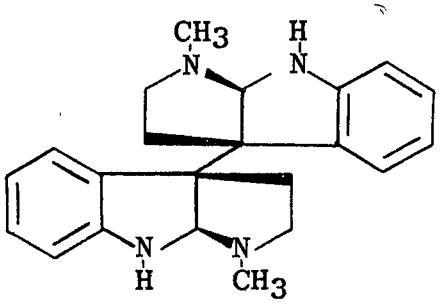
Calycanthidine (84)

Calycanthidine, melting point 142° C, was first isolated by Barger in 1939 as the minor alkaloid from the seeds of Calycanthus floridus L..[56] A hexahydro β-carboline skeleton based on a molecular formula $C_{13}H_{16}N_2$ was deduced and accepted until recently. In work published in 1962[83] calycanthidine

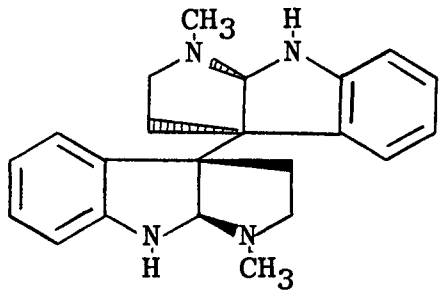
was shown to have the formula $C_{23}H_{28}N_4$, and two Ph-N-C-N chromophores from the ultraviolet spectrum and by potentiometric titration and was thus a fourth member of the dimerized tryptamine group of alkaloids. Comparison of the infrared spectrum with those of the very similar chimonanthine and folicanthine, a similar specific rotation ($[\alpha]_D$ = -317) to chimonanthine and folicanthine, rapid formation of a normal dimethiodide and smooth reduction by zinc and hydrochloric acid to a mixture of indolines, demonstrated that calycanthidine had a chimonanthine-like structure. Isolation of 3-2'-methylaminoethylindoline and 1-methyl-3-2'-methylaminoethylindoline indicated that calycanthidine represented the intermediate stage in the methylation of chimonanthine to folicanthine and this is supported by nuclear magnetic resonance and mass spectral evidence, especially the facile cleavage of the molecular ion. The methylation of chimonanthine to calycanthidine and then to folicanthine has been accomplished.[87]

Hodgkinsine (86)

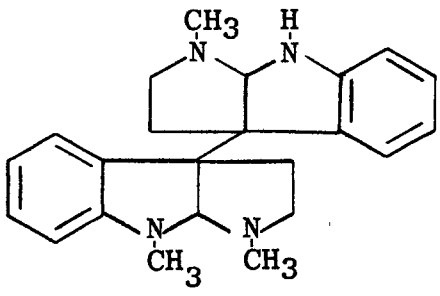
Hodgkinsine with a melting point 128° C and a specific rotation of +60° was isolated in 1960 from the leaves of an Australian shrub, Hodgkinsonia frutescens F.Muell..[88] On the basis of analysis and molecular weight it was assigned a molecular formula $C_{22}H_{26}N_4$[88] and shown to contain the Ph-N-C-N system from its ultraviolet spectrum.[85] It was also reported to be a diacidic base with either one[88] or two[85] NH stretching



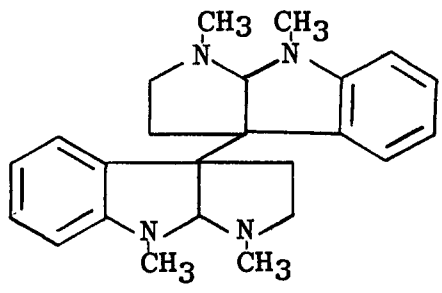
82 l-chimonanthine



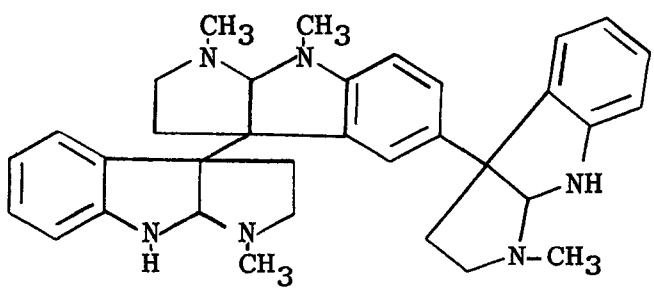
83 meso-chimonanthine



84 calycanthidine



85 folicanthine



86 hodgkinsine (not definitely established)

Figure 20. Calycanthaceous Alkaloids other than Calycanthine.

regions in the infrared and to be isomeric with calycanthine. Failure to produce calycanine on degradation with zinc dust indicated it probably did not have a quinoloquinoline skeleton. This was the extent of knowledge about hodgkinsine when the present studies began. Subsequent studies by G.F. Smith indicated that hodgkinsine could be a dehydrocalycanthine $C_{22}H_{24}N_4$ and eventually a mass spectral determination indicated that hodgkinsine was a tryptamine trimer. This conclusion, supported by nuclear magnetic resonance data, led Professor Smith to propose structure 86 for hodgkinsine.[89] Parallel support is contained in this thesis.

Discussion

The object of this work was to achieve a model synthesis of the calycanthaceous alkaloids along the lines of the proposed biogenetic scheme.[49,50] From the onset the problem was one of reaction conditions since the postulate of $\beta\beta'$-coupling of tryptamine had always seemed reasonable for the biosynthesis of this class of dimeric indole alkaloids. Experimental evidence that tryptamine is the biological precursor of these compounds came with feeding of tryptophan-β-^{14}C to Calycanthus floridus[90] after simulation of the biosynthesis had been achieved in the laboratory.

Many attempts had been made by several workers to achieve coupling of various N-substituted N-methyltryptamines by exposure to such oxidative media as ferric, ceric, and manganous solutions under various conditions known to promote one-electron oxidative coupling of phenolic systems. Starting material was either recovered or degraded to intractable mixtures. The first practical solution to the problem was found by Hendrickson et al.[91,48] who used an oxindole coupling reaction following the precedent of sodiomalonate coupling[92] involving oxidation with iodine under basic conditions. Coupling of the urethan of oxytryptamine (92) gave the urethan protected bisoxindole (94). The urethan grouping was used to prevent oxidation of the secondary amine and was a nice choice as it can be reduced to the requisite N-methyl at the same time that the oxindole is reduced to the required oxidation state with lithium aluminum

hydride. Coupling of the oxindole precluded the possibility of coupling in the α-position.

Oxytryptamine hydrochloride (91) was prepared by a method based on an earlier preparation[93] and developed into a convenient procedure. This preparation involved condensation of isatin (87) and methyl cyanoacetate in piperidine followed by zinc dust reduction in acetic acid to methyl-3-oxindolylcyanoacetate (88). After saponification in 2 N potassium hydroxide the resulting 3-oxindolylcyanoacetic acid (89) was decarboxylated by addition to hot ethylene glycol, yielding 3-oxindolylacetonitrile (90). This nitrile was then reduced with platinum dioxide and hydrogen in ethanolic hydrochloric acid yielding oxytryptamine hydrochloride in thirty per cent overall yield. Shaking an aqueous alkaline solution of oxytryptamine with ethyl chloroformate smoothly afforded the urethan (92).

The oxidative coupling of the urethan (92) was attempted using cupric, ferric and silver ions. Electrolytic and t-butyl hydroperoxide oxidations were also futile yielding only starting material. The oxindole enolate (93), preformed by addition of sodium hydride to the urethan, was successfully coupled to 94 when a solution of iodine in benzene was slowly added. In view of the difficulty of achieving this coupling using normal one electron oxidizing agents a radical coupling process as envisaged for the biosynthesis is probably not involved here. A coupling of one mole of oxindole enolate (93)

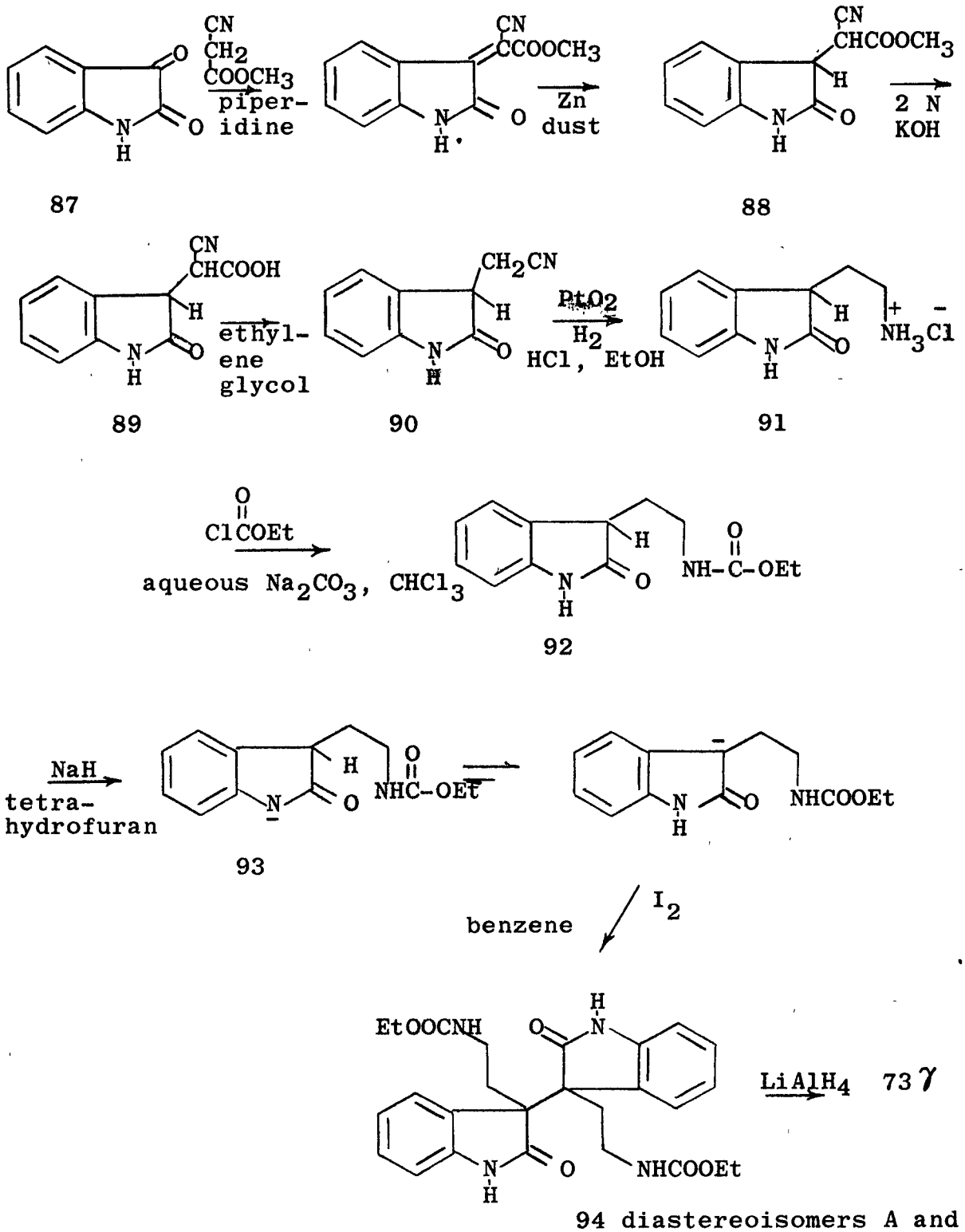


Figure 21. Synthesis and Coupling of Oxytryptamine Urethan(92).

with a mole of 3-iodooxindole could be the mechanism.[92]

Reduction of the two dimeric diastereoisomers (94) with lithium aluminum hydride in tetrahydrofuran yielded mixtures of basic products whose chromophores were not destroyed in acid but showed a hypsochromic shift characteristic of the Ph-N-C-N system which indicated cyclization had occurred. The chromophores of any products which might arise from total reduction of the oxindoles to indolines without cyclization should be destroyed in acid by protonation of the aniline nitrogen. Of the six compounds isolated from the mixture obtained from reduction of one diastereoisomer (94) the major product had a molecular weight of 344 which is two hydrogens less than calycanthine. The ultraviolet spectrum was also significantly different from that of calycanthine. It could be transformed into an isomer also isolable from the mixture by boiling in aqueous acid. These compounds were formulated as monoamidines of 73β and δ respectively. dl-Chimonanthine (82) (3%), dl-calycanthine (73α') (0.2%), N_b-methyltryptamine and a third dehydrocompound were also isolated from the mixture. The crystalline product (6%) isolated from reduction of the second diastereoisomer (94) was a calycanthine isomer by analysis and mass spectrum but the infrared spectrum was significantly different from calycanthine. The preponderant peak in the mass spectrum at m/e 172, which represented one-half of the molecular weight, was forty-five times more intense than the parent peak, an intensity difference which can only be

rationalized in terms of the calycanthine isomer 73 γ with two halves joined together by a single bond. This compound was isolated and characterized before Smith had announced the isolation of natural chimonanthine and showed that it was in fact the γ isomer of calycanthine. Similar but not identical thin-layer chromatographic behaviour to the natural chimonanthine led to the conclusion that this synthetic compound was meso-chimonanthine.

Implicit in the chemistry of the calycanthine isomers is the potential for equilibration of the five isomers in acidic medium via species such as 71 and 72 (Figure 16). dl-Chimonanthine was heated with dilute hydrochloric acid (0.01 N) and the resulting bases, separated on thin-layer plates, produced a pattern of five or six spots. The major ones corresponded to chimonanthine (25%), calycanthine (40%), and N_b-methyltryptamine (5%). When calycanthine was subjected to the same treatment a virtually identical thin-layer pattern was produced which demonstrated the equilibration nature of the interconversion. When natural d-calycanthine was isomerized the generated chimonanthine was found to be levorotatory demonstrating that both alkaloids have the same absolute stereochemistry at the two non-epimerizable centers.[91,48]

Since chimonanthine and calycanthidine have both been methylated to yield folicanthine[89] the synthesis of calycanthine and chimonanthine also serves formally as a synthesis of folicanthine.

A total synthesis of racemic folicanthine has also been achieved by T.Hino in Japan through the 3,3'-disubstituted 3,3'-bisoxindole (95)[81,94] prepared by condensation of N-methylisatin and N-methyloxindole in acid followed by cyanoethylation. Formation of the Schiff base 96 followed by treatment with methyl iodide and acid hydrolysis yielded a bisoxindole(97)which afforded dl-folicanthine on reduction.

The failure of various N-substituted N-methyl tryptamines to couple when exposed to various reagents known to promote one-electron oxidative coupling in phenolic systems (page 9) suggested the absence of the β-radical under these conditions. As autoxidation (i.e. formation of free radicals) is enhanced by anion formation[95] it was decided to make use of the salt-like properties associated with indolyl magnesium halides.

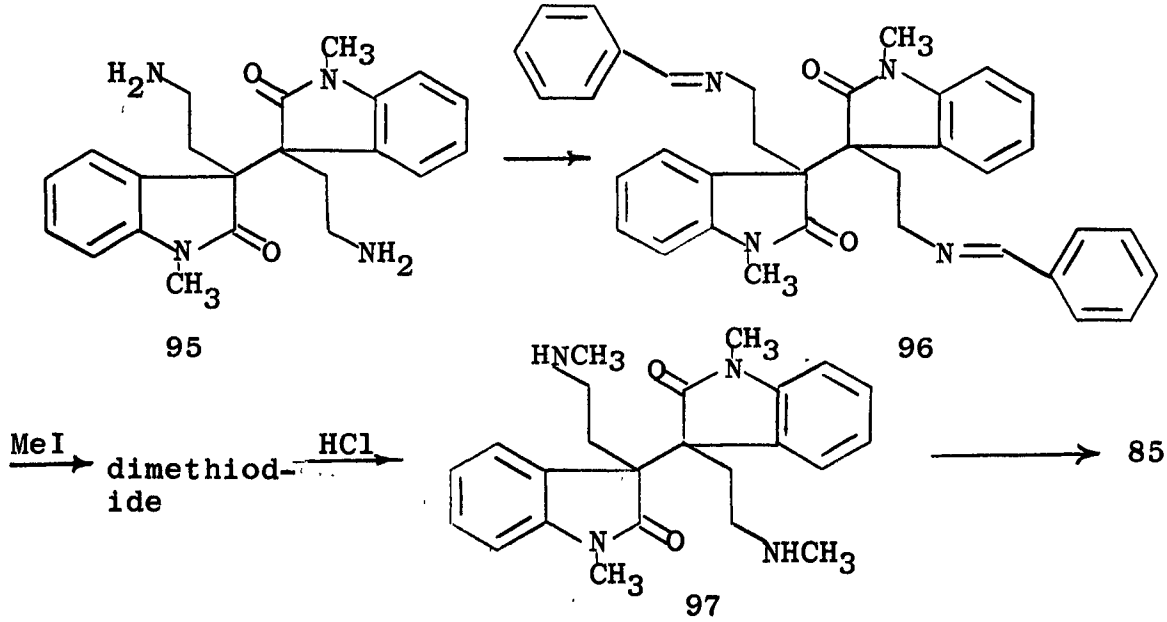


Figure 22. Synthesis of Folicanthine.

As previously mentioned the structural determination of calycanthine was based in part on a mechanistic preference for coupling of β-radicals of N_b-methyltryptamine as opposed to coupling in the α-position. The preference for β-coupling is based on the established availability of electrons in this position and the assumption that an odd electron would also have its highest density in the β-position. The electron densities for indole have been calculated by the method of molecular orbitals,[96] and have established the electronegativity of the β-position. A consideration of Kekulé resonance hybrids leads to the same conclusion.[97] Those structures containing an ordinary benzenoid ring would be expected to be more stable and hence make more important contributions to the hybrid but the only structure contributing a negative charge to the α-position is non-benzenoid while several contributing to negative charge in the β-position are benzenoid. Indoles undergo electrophilic substitution in the β-position with great facility. Nitration, alkylation, acylation, Mannich reaction, halogenation and diazotization all take place in the β-position if sufficiently mild conditions are used to prevent polysubstitution.[98] Recent spectroscopic studies have shown that protonation of indoles in strongly acidic solution occurs exclusively in the β-position even when the β-position is already substituted.[78]

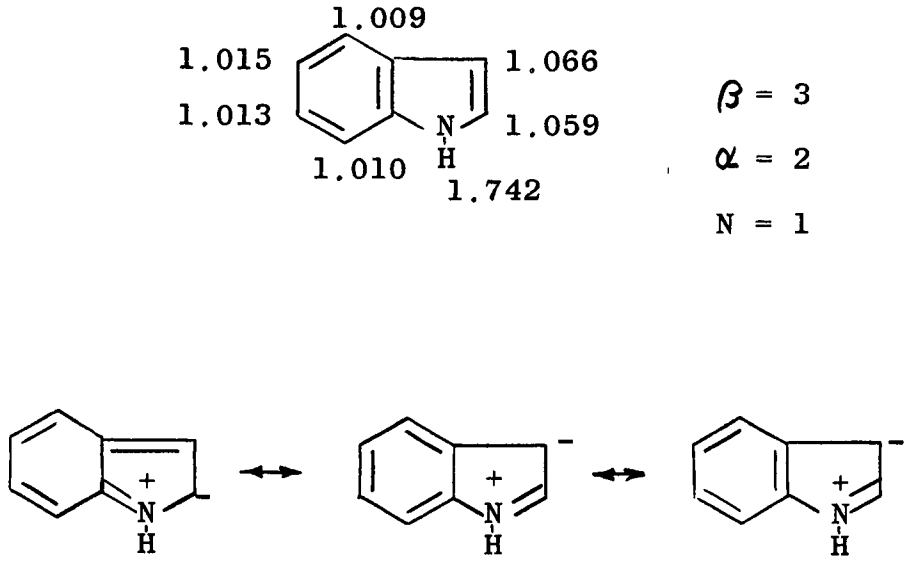


Figure 23. Kekulé Structures and Electron Density of Indole by Molecular Orbital Approximations.

The utility of Grignard reagents derived from indole was first investigated by Oddo in 1911[99] and their nature has since been the object of speculation. Depending on reaction conditions alkylations, acylation, and carbonation of indolylmagnesium halides yields N-, β-, N,β- and rarely α-substituted products.[100,101] These results led to formulation of the indole Grignard reagent as either N-MgX, C-MgX or essentially ionic species. Recent evidence in favour of ionic species has been

obtained from a study of the nuclear magnetic resonance spectra of these reagents in tetrahydrofuran.[102] The failure to detect an N-H in the nuclear magnetic resonance or infrared spectra and the failure of the β-proton resonance to shift to higher field on preparing the Grignard reagent eliminate C-MgX formulations. The striking similarity between the n.m.r. spectra of indolylmagnesium bromide and indolylsodium which presumably contains a largely ionic N-Na bond excludes the possiblity of a covalent N-Mg bond.

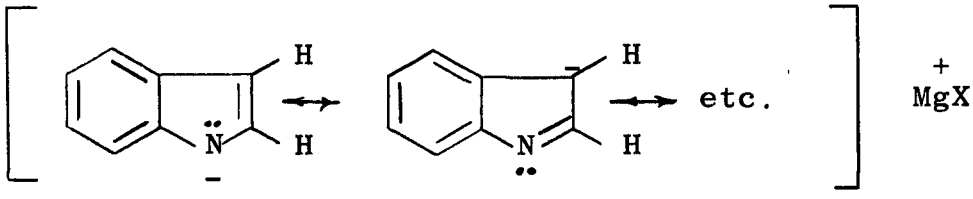


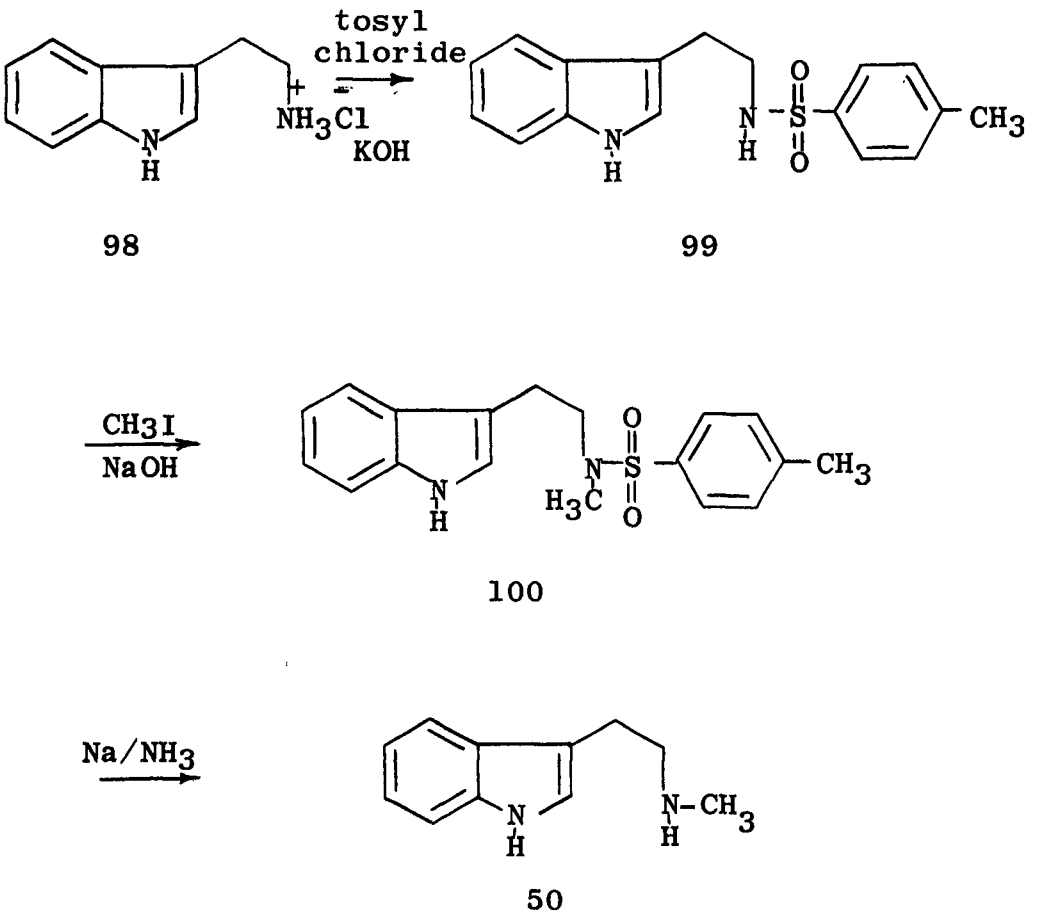
Figure 24. Indole Grignard Reagent.

Kondireff and Fomin in 1914 announced a method for preparation of hydrocarbons based on the coupling action of ferric chloride on organomanganese derivatives.[103] Perhaps the most interesting consequence of this reaction was the discovery of ferrocene in 1951 [104] when ferric chloride was added to cyclopentadienyl magnesium bromide. A systematic study of the coupling of phenylmagnesium bromide with ferric chloride was made in 1930 to define the mechanism.[105] An interesting stoichiometry was observed by titration, metallic iron being one of the products. The coupling could also be initiated with ferrous

chloride.

$$6\ C_6H_5MgBr + 2\ FeCl_3 \longrightarrow 3\ C_6H_5\text{-}C_6H_5 + 2\ Fe + 3\ MgBr_2 + 3\ MgCl_2$$

N_b-methyltryptamine (50) (dipterin), was prepared (Figure 25) using a method devised by Dr. A.C.Day, University of Oxford, England, which is itself a modification of an earlier preparation by Hoshiro and Kobayashi.[106] (An alternative preparation has been described by Witkop which involves formylation of tryptamine hydrochloride followed by reduction with lithium aluminum hydride.[107]) Quaternization of the aliphatic nitrogen was prevented by N_b-tosylation (99) before methylation (100). The protective tosyl group was then conveniently eliminated using the method of Wilkinson[108] involving reductive displacement by sodium in liquid ammonia. The N_b-methyltryptamine was characterized by melting point, its indole chromophore in the ultraviolet, nuclear magnetic resonance and mass spectrum. As the mass spectra of coupling products were very important a discussion of the fragmentation pattern of N_b-methyltryptamine is also important. By far the most abundant fragments were those with m/e 44 and 31. It is well recognized that the most important primary process resulting from electron impact on aliphatic amines is removal of one of the lone pair electrons of the heteroatom followed by simple cleavage of the carbon-carbon bond adjacent to the nitrogen atom.[109] This would result



98 99

100

50

Figure 25. Preparation of N_b-methyltryptamine.

in an ion with m/e 44. Simple cleavage of a C-N bond should give a positive fragment of m/e 30. The strongest peak at higher mass has m/e 131 corresponding to loss of dimethylamine and addition of a proton; m/e 130 is also strong. The other characteristic fragments are m/e 144 corresponding to loss of methylamine and the molecular ion m/e 174 with 8% of the abundance of m/e 131. (Figure 27). Fragmentation patterns will be discussed in terms of the convention introduced by Djerassi[109]

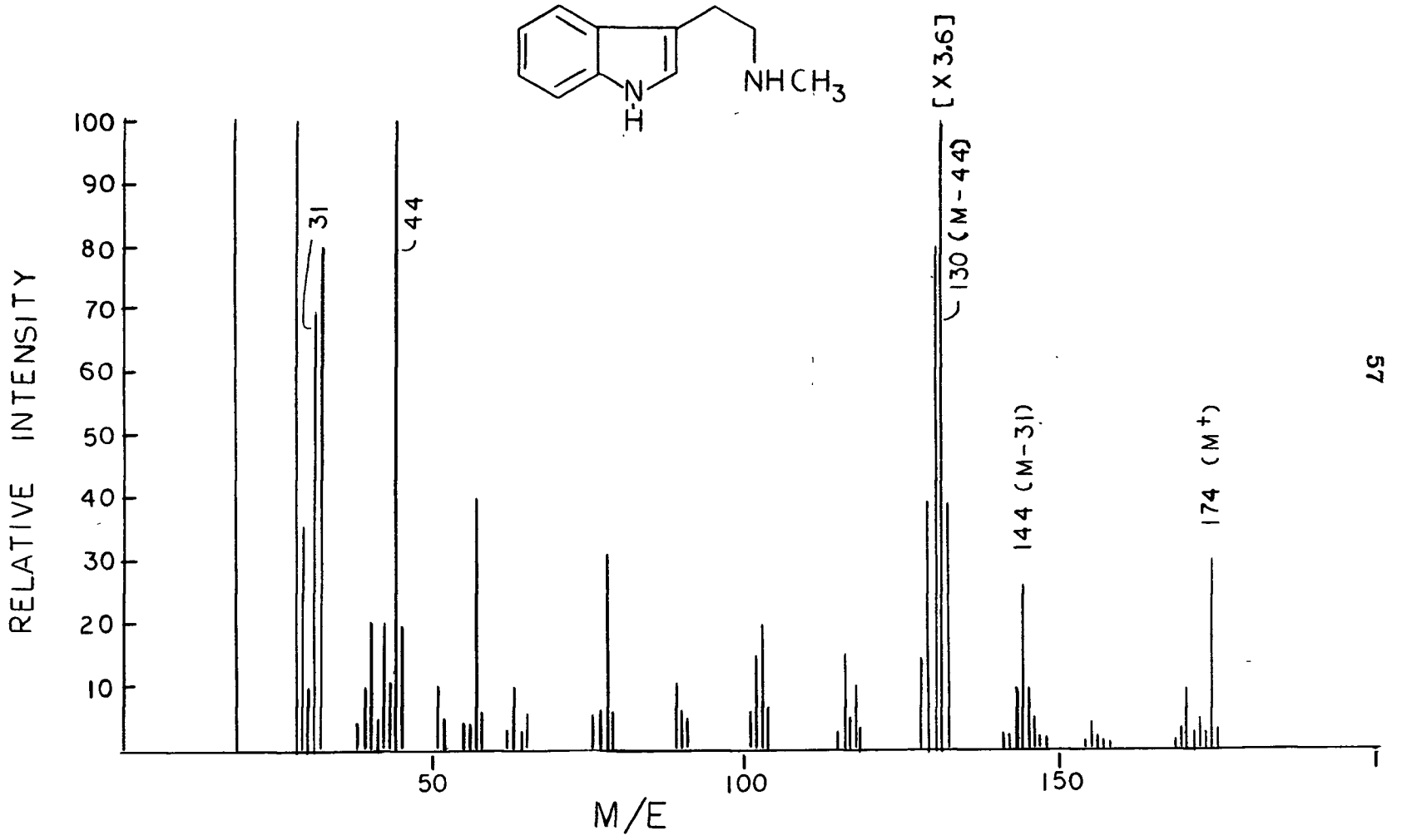


Figure 27. Mass Spectrum of N_b-Methyltryptamine.

where a single headed arrow represents transfer of a single electron (m/e is the ratio of mass to charge).

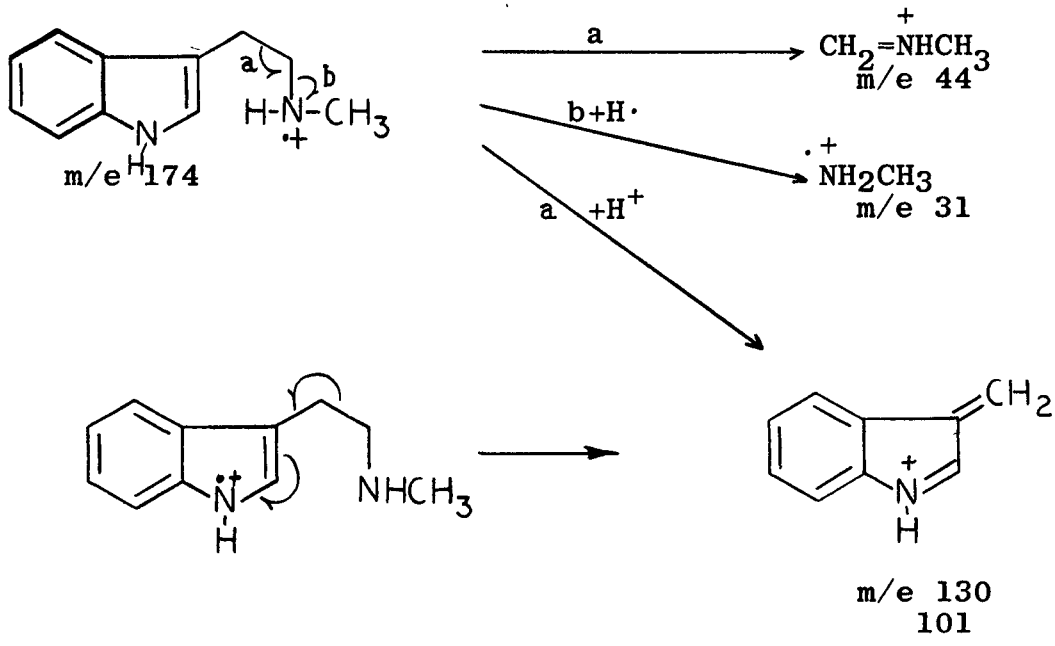


Figure 26. Fragmentation of N_b-Methyltryptamine.

The coupling of N_b-methyltryptamine was successfully achieved by oxidation of its magnesium iodide salt with anhydrous ferric chloride. Preparation of N_b-methyltryptamine-N_a-magnesium iodide was carried out under nitrogen by addition of an ethereal solution of N_b-methyltryptamine to a stirred solution of methyl magnesium iodide prepared in the usual way. Addition of ferric chloride in ether followed by mild hydrolysis with aqueous ammonium chloride gave a mixture of products which were then analyzed by thin-layer chromatography and ultraviolet spectroscopy. Several fractions from preparative thin-layer plates had ultraviolet spectra correspond-

ing to a Ph-N-C-N chromophore which is characteristic of calycanthaceous and physostigmine type alkaloids (an indoline chromophore with a hypsochromic shift in dilute acid).[72,77] This was the first indication that the coupling might have been successful. On separation of the products by chromatography apart from unchanged N_b-methyltryptamine (30%) the principal products were dl-chimonanthine (19%, based on 30% recovery of starting material), meso-chimonanthine (7%), two high melting dimers named A (3.5%) and B (3.5%), and some monomeric oxidation products which were not isolated in crystalline form. The recovery of organic material from the coupling reaction ranged from 60 to 80%. Calycanthine was not detected in the reaction mixture, and was not expected since conditions do not favour equilibration through the tetraminodialdehyde (72). The recovery of starting material was poor unless the reaction was carried out under nitrogen.

The identity of dl-chimonanthine isolated from the reaction mixture was established beyond doubt by correspondence of its infrared, ultraviolet, and mass spectra as well as thin-layer chromatographic behaviour in several solvent systems and its colour reaction with 1% ceric sulphate in 35% sulphuric acid spray, to the natural l-alkaloid. There was no depression of melting point on admixture with a sample of synthetic racemic chimonanthine prepared by Professor Hendrickson.[91,48]

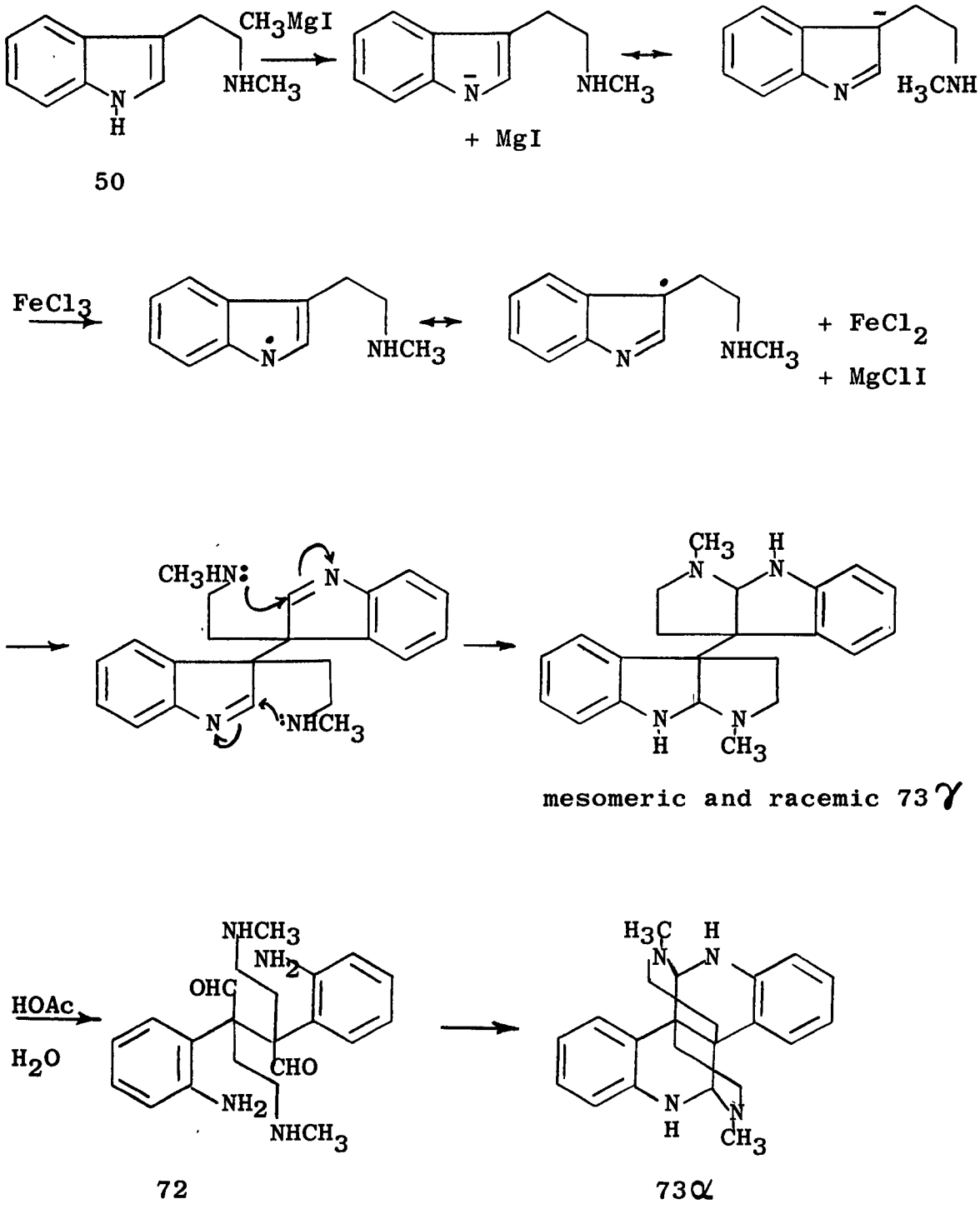


Figure 28. Synthesis of Chimonanthine and Calycanthine.

Previously observed depression of melting point on admixture with a sample of natural chimonanthine received from Professor Smith of Manchester led to the discovery of meso-chimonanthine in nature. This sample of chimonanthine from an extract of Calycanthus floridus was yellow in colour and was received with the instructions that it could be purified by simple chromatography on alumina. After recrystallization from benzene of the first fractions eluted with benzene-ether from an alumina column the white crystals with melting point 199-202° C were used for comparison with the synthetic product. The absence of optical activity, very close resemblance of ultraviolet and solution infrared spectra, a mass spectrum showing a molecular ion at m/e 346 with facile cleavage to a fragment m/e 172 and a less intense fragment at m/e 130 established the identity of this compound as the mesomeric isomer of chimonanthine.[86] This same compound was subsequently isolated from the reaction mixture and was also reported as a product from Hendrickson's synthesis.[48]

As the mass spectrum is so important in characterization of the chimonanthines it should be discussed in detail. The mass spectra of the calycanthaceous alkaloids have been published and discussed.[86,110] Of the five calycanthine isomers (73) only chimonanthine, joined by a single bond would be expected to undergo facile symmetrical cleavage. Symmetrical fragmentation can yield a stabilized benzyl cation (102) which may lose hydrogen to form a fragment of m/e 172 or undergo cyclic

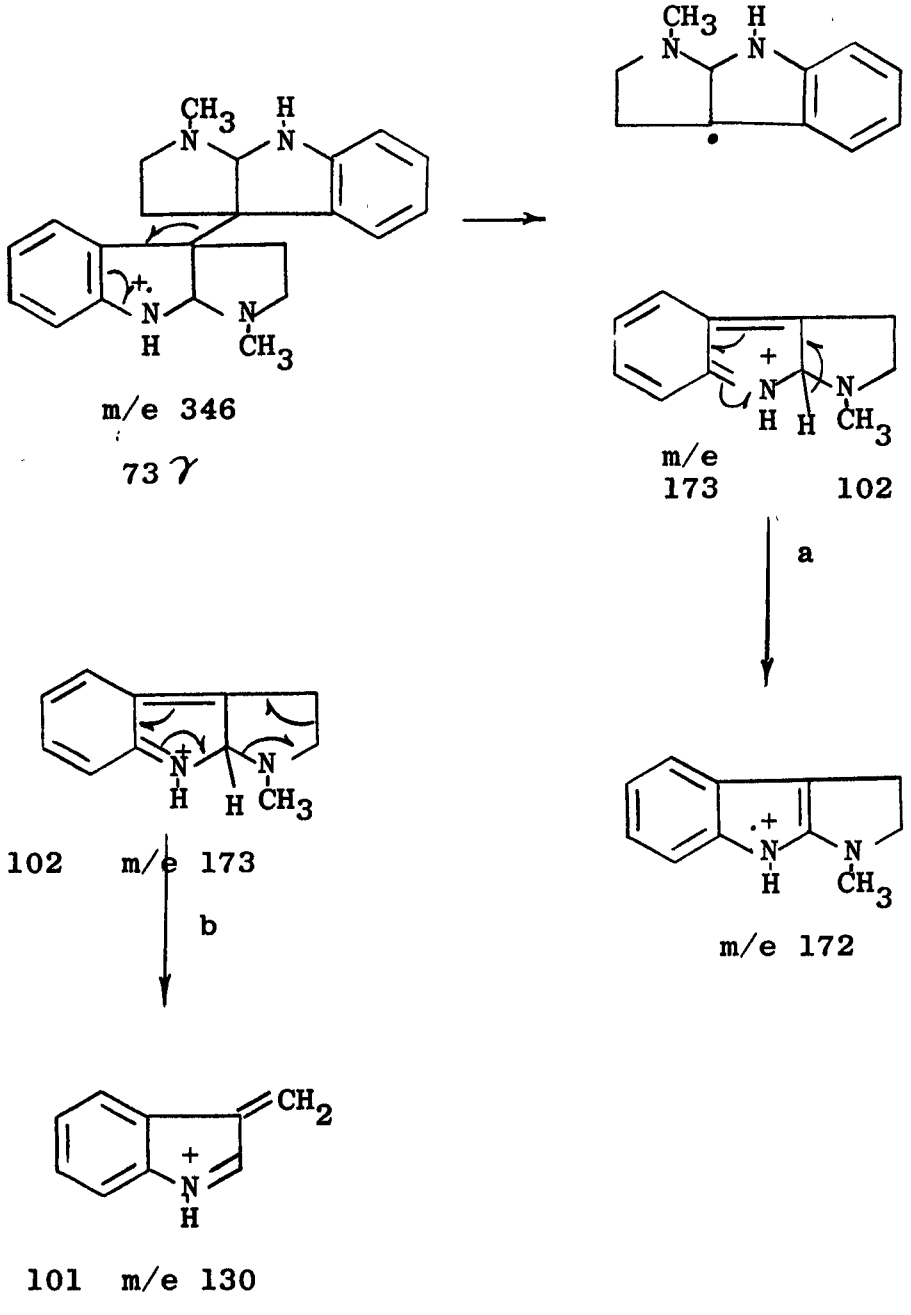


Figure 29. Fragmentation of Chimonanthine.

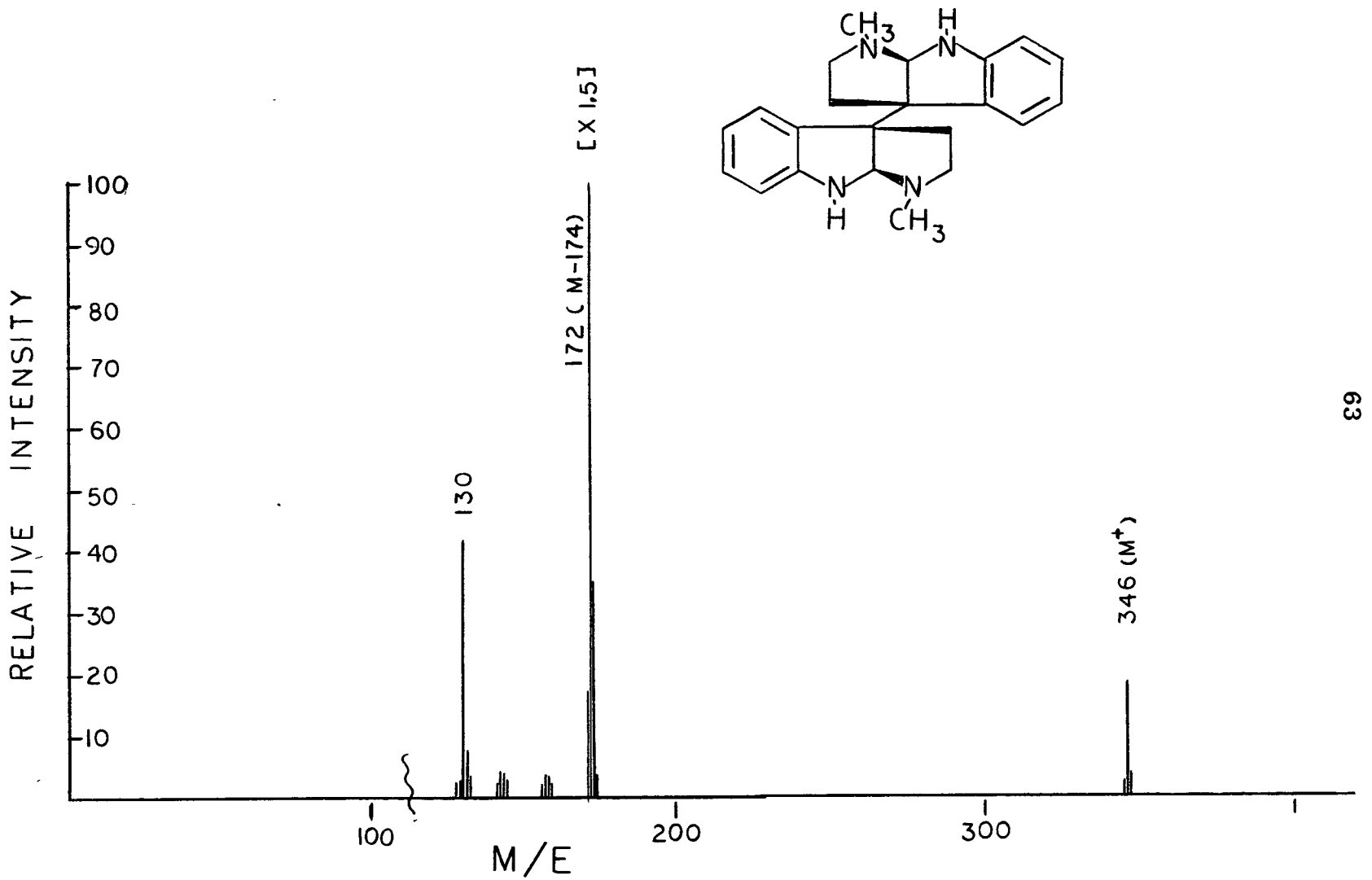


Figure 30. Mass Spectrum of Natural (1) and Synthetic dl-Chimonanthine.

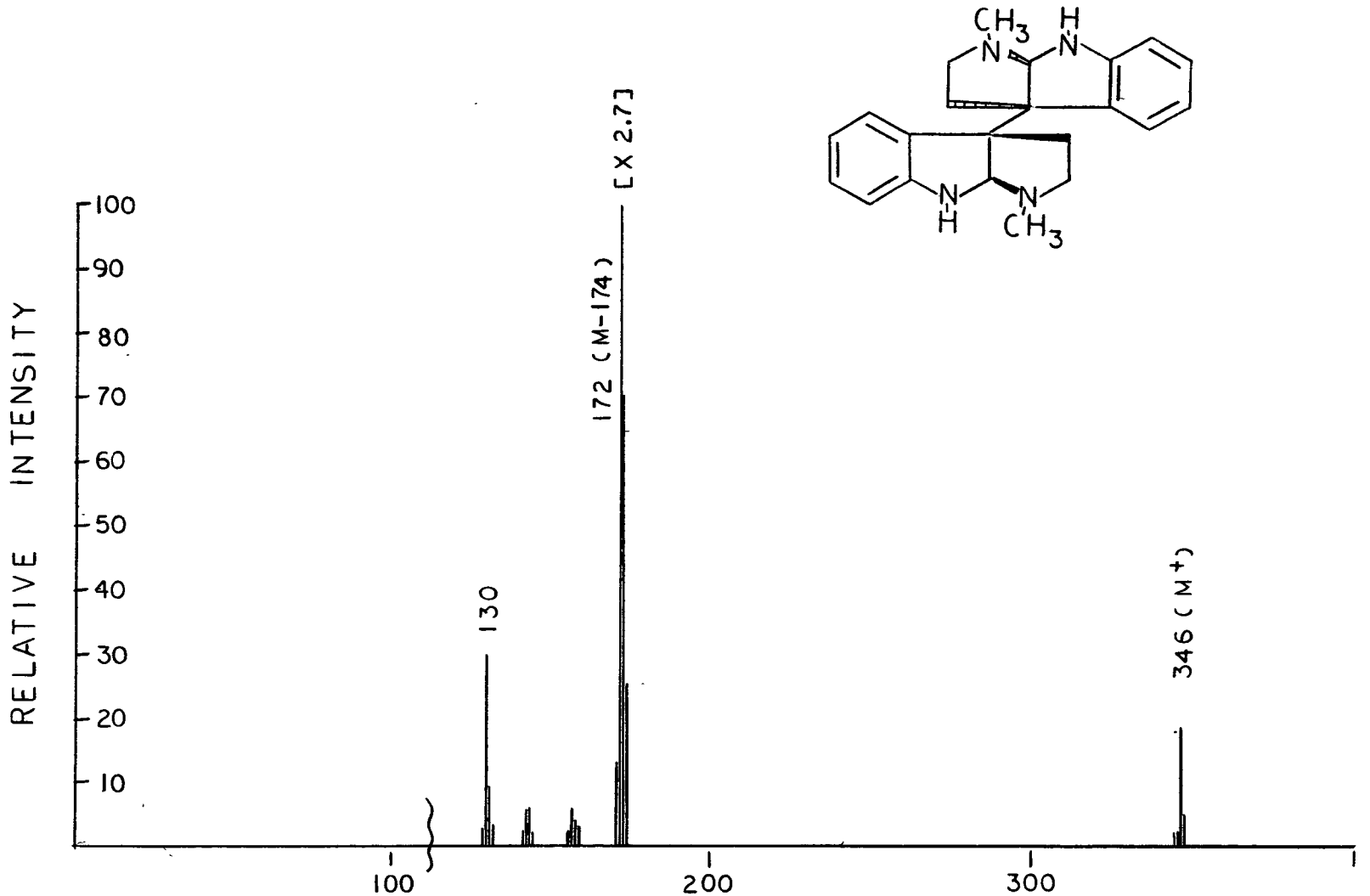


Figure 31. Mass Spectrum of Natural and Synthetic meso-Chimonanthine.

collapse as shown in Figure 29 to a β-methylene indolenine fragment (101) of m/e 130. The molecular ion m/e 346 was found to be less than 12% as intense as the parent fragment at m/e 172 for l- and dl-chimonanthine and 7% for meso-chimonanthine. Previously published spectra[86] indicate 3.5% abundance for the molecular ion, a difference readily attributed to different experimental conditions.

The nuclear magnetic resonance spectra are also of considerable interest. Woodward[49] predicted that the nearly planar five-membered rings of 73γ, which was later discovered in nature as chimonanthine should have more nearly equivalent methylene protons than would be the case for calycanthine 73α or the other isomers with puckered six-membered rings. The methylene resonances of racemic (Figure 32) and mesomeric (Figure 33) chimonanthines are not identical but are similar and contrast with calycanthine (Figure 37). The difference in chemical shift of the N-CH_3 resonances, 7.70τ, for racemic and 7.63τ for mesomeric chimonanthines, is consistent with differences in shielding caused by different positions relative to the aromatic nucleus. An n.m.r. spectrum of the natural mixture from which meso-chimonanthine was isolated had peaks of very similar intensity at 7.63τ and 7.70τ indicating an approximately 1:1 mixture of the diastereoisomers. This analysis is supported by thin-layer chromatography of the mixture in 2% methanol-ether on alumina. The chemical shifts of N<u>H</u> and R_3C<u>H</u> protons are also somewhat different. The splitting of the aromatic protons

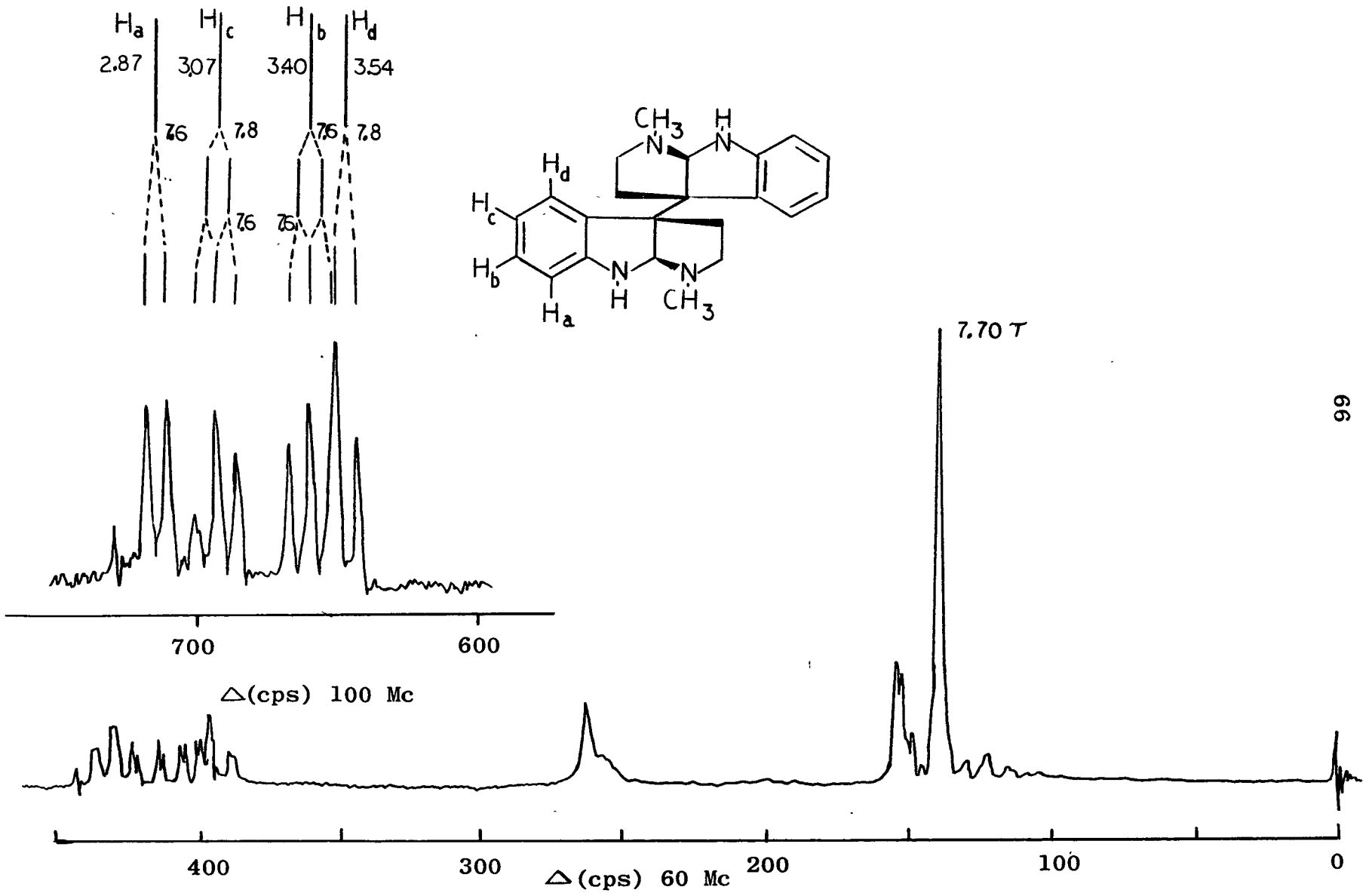


Figure 32. N.M.R. spectrum of dl-chimonanthine.

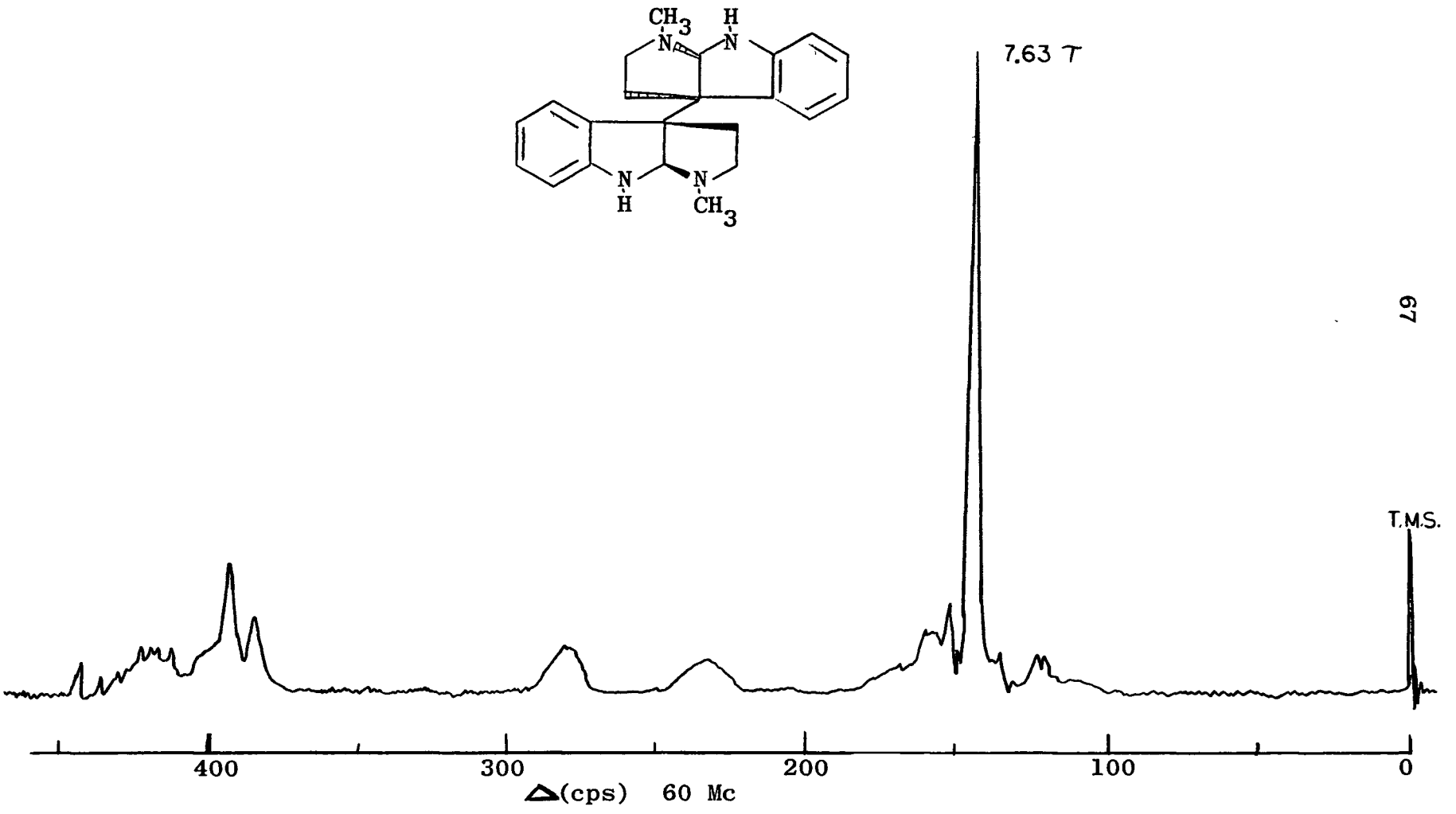


Figure 33. N.M.R. Spectrum of meso-chimonanthine.

could be analyzed in the 100 Mc spectrum of racemic chimonanthine but not of the meso-isomer where several of these protons must be more nearly equivalent. The low field aromatic proton H_a (2.87τ) would be coupled to H_b (3.40τ, J_{ab} = 7.6 c.p.s.). Proton H_b must also be coupled to H_c (3.07τ, J_{bc} = 7.6 c.p.s.) which is para to the nitrogen and expected to be more shielded than H_b. Proton H_c must also be coupled to H_d (3.45τ, J_{cd} = 7.8 c.p.s.). Long range coupling effects which are quite large through π electron systems[111,112] also split protons H_b and H_c (J_{bd} = 1.0 c.p.s., J_{ca} = 1.3).

The isolation of meso-chimonanthine in nature and the isolation of calycanthaceous alkaloids from several orders of plants is indicative of a simple biogenesis with only loose enzyme control.

The synthesis of calycanthine was completed by acid catalyzed isomerization of racemic chimonanthine to calycanthine. Treatment with hot dilute aqueous acetic acid was found to be the best means of accomplishing this isomerization. After thirty hours on the steam bath dl-chimonanthine was transformed into a 1:4 mixture of chimonanthine and calycanthine. In contrast to isomerizations catalyzed by dilute hydrochloric acid there were no minor byproducts or degradation to N_b-methyltryptamine. The presence of other isomers (73) could have been masked by failure to separate them from calycanthine or chimonanthine by thin-layer chromatography. If they were present the quantities involved must have been small. In view of the relative stability

of calycanthine and chimonanthine it seems unlikely that other isomers will be detected in nature.

dl-Calycanthine was identified on the basis of thin-layer chromatographic behaviour and the superimposibility of ultraviolet and solution infrared spectra with those of the natural alkaloid. Melting point depression and differences in the solid state infrared spectra were attributed to different crystalline forms for the resolved and racemic alkaloid. Nuclear magnetic resonance and mass spectra were also identical.

Treatment of meso-chimonanthine with dilute aqueous acetic acid resulted in an essentially two component mixture of starting material and a compound identified as meso-calycanthine (1:2) on the basis of its mass spectrum, an ultraviolet spectrum showing the Ph-N-C-N chromophore, and its melting point of 265-268^{o} C which is higher than d-calycanthine as expected for a more symmetrical molecule.

The mass spectrum of calycanthine is dominated by the molecular ion m/e 346 as the rupture of a single bond cannot result in fragmentation. Other peaks in the spectrum occur by loss of the bridges.[86] Peaks at m/e 288 (17%) and 231 (25%) correspond to loss of one and two bridges respectively. Peaks also occur at m/e 172 and 130. The mass spectrum of the major component after treatment of meso-chimonanthine with acid was strikingly similar to that of natural or racemic calycanthine showing only small differences in abundance of some fragments (notably m/e 172 which is approximately twice as intense

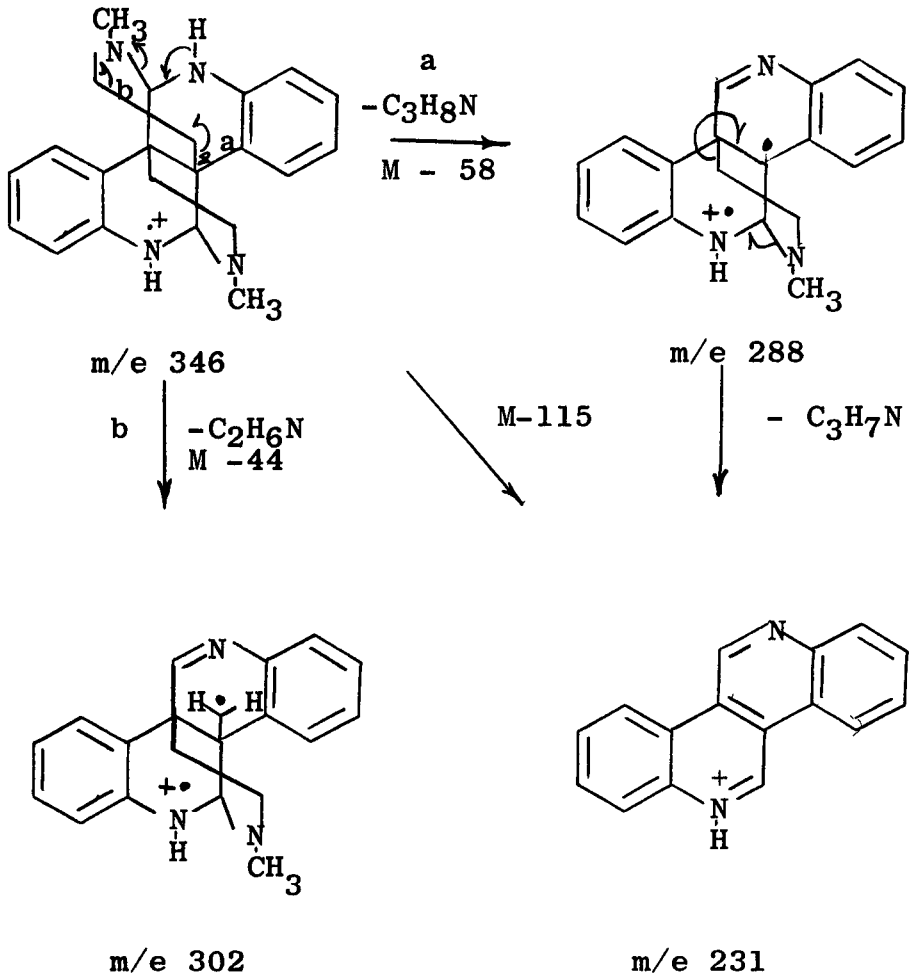


Figure 34. Fragmentation of Calycanthine.

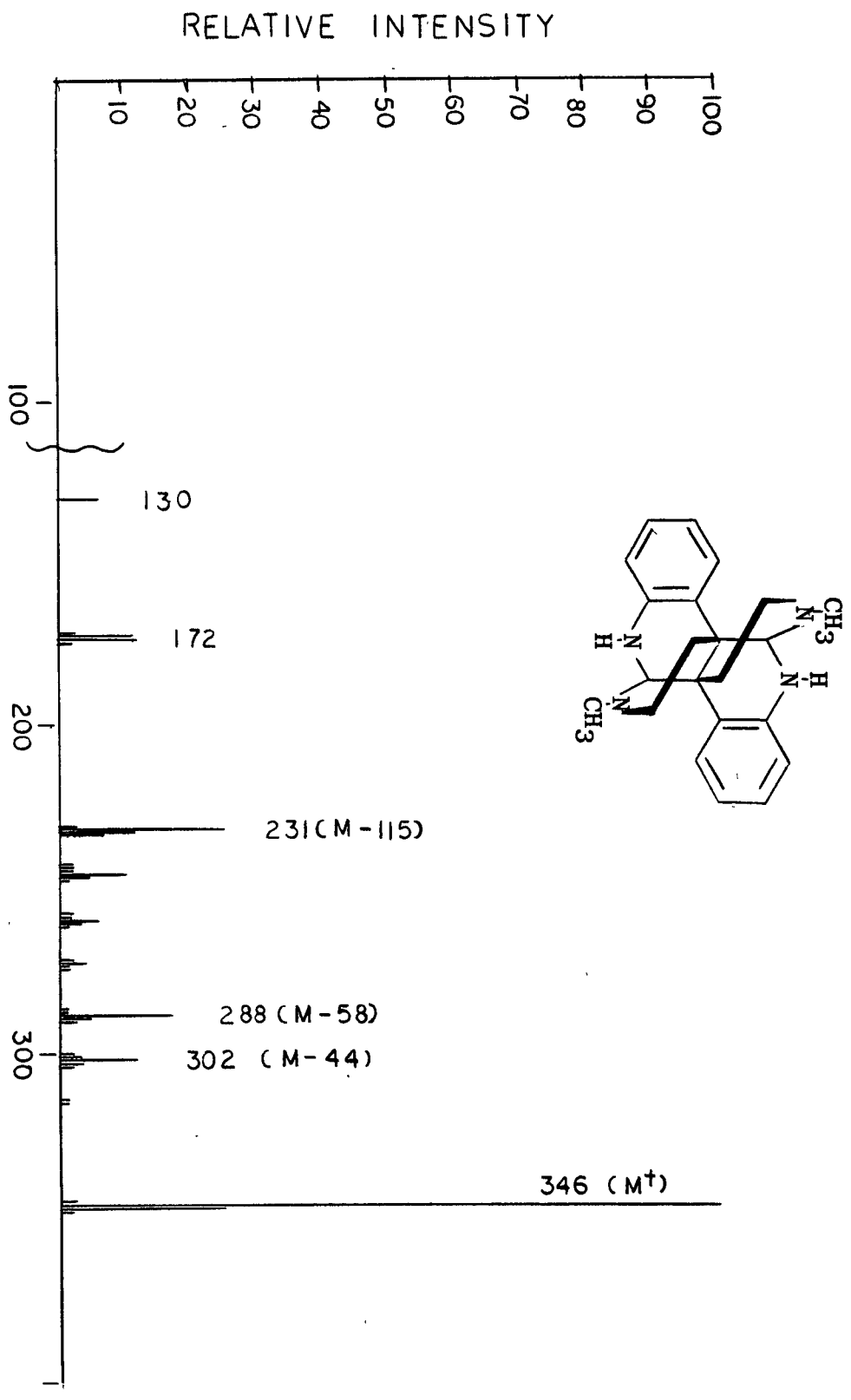


Figure 35. Mass Spectrum of dl-calycanthine.

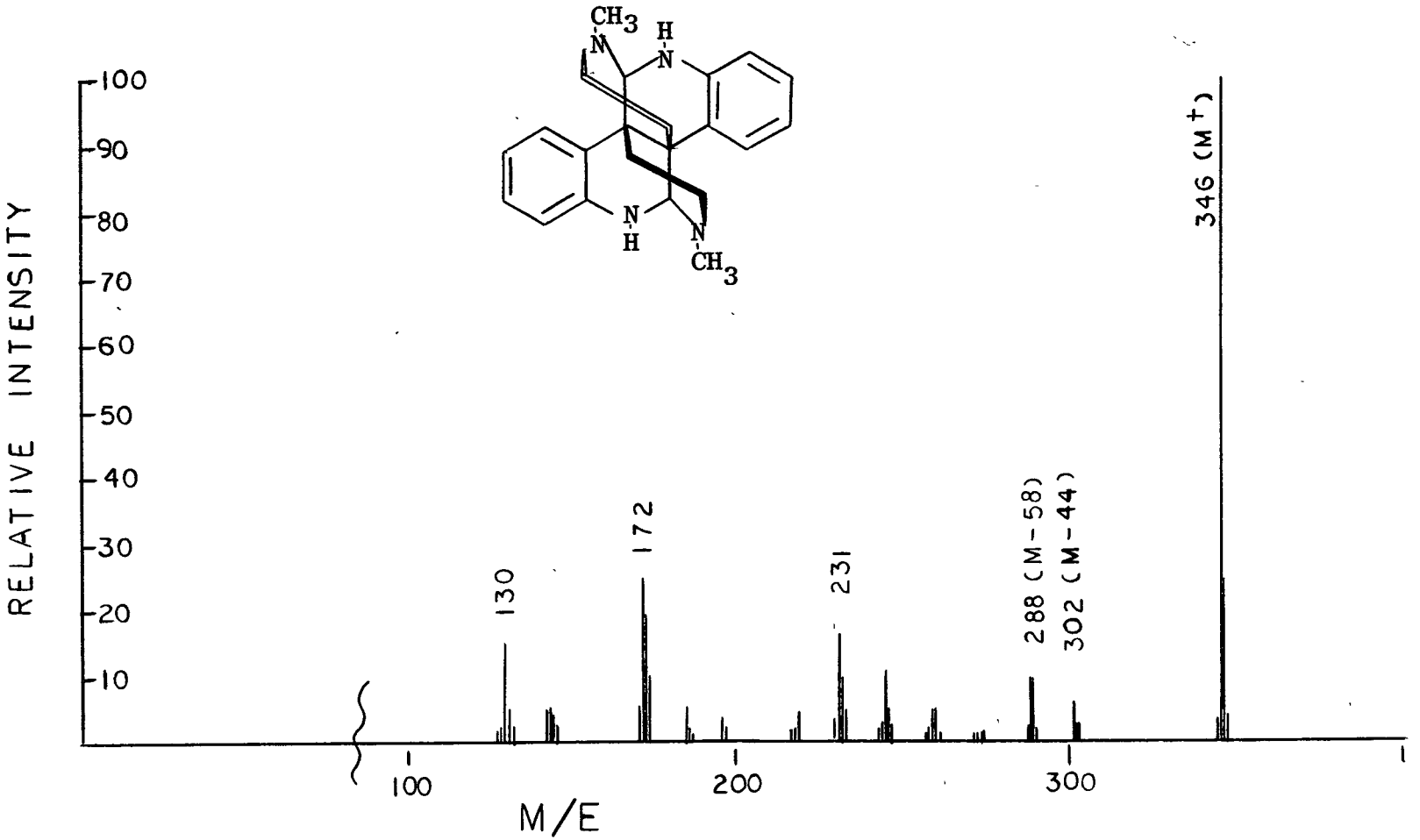


Figure 36. Mass spectrum of meso-calycanthine.

(Figures 35 and 36).).

The nuclear magnetic resonance spectrum of natural d-calycanthine (and the synthetic racemate) has been published before[49] but it is of interest in comparison with that of chimonanthine. Like dl-chimonanthine the aromatic region in the 100 Mc spectrum is readily analyzed and is consistent with the known structure. The chemical shifts and coupling constants are shown in figure 37. The methylene region can be partially analyzed even in the 60 Mc spectrum. The low field proton at 6.85 τ is split into a sextet by coupling with a geminal proton (J = 14 c.p.s.) and two vicinal (J = 14 and 6 c.p.s.) protons. The high field proton is split into a doublet of quartets by geminal coupling (J = 14 c.p.s.) and coupling with two vicinal protons (J = 4 and 2 c.p.s.). From the identical geminal coupling constants it is probable that the high and low field methylene protons are geminal indicating an unusually large chemical shift (1.91τ) between two protons on the same carbon. Such a situation is an interesting example of anisotropic shielding and deshielding by aromatic rings.[111a]

The two synthetic byproducts A and B were originally thought to be either isomeric with calycanthine or to be oxygenation products. The infrared spectra were different from those of the calycanthine isomers, had a sharp NH band at about 3440 cm^{-1} and no carbonyl bands. The ultraviolet spectra (see experimental) were not the same as those of the calycanthine isomers having an extra peak about 280 mμ which showed a

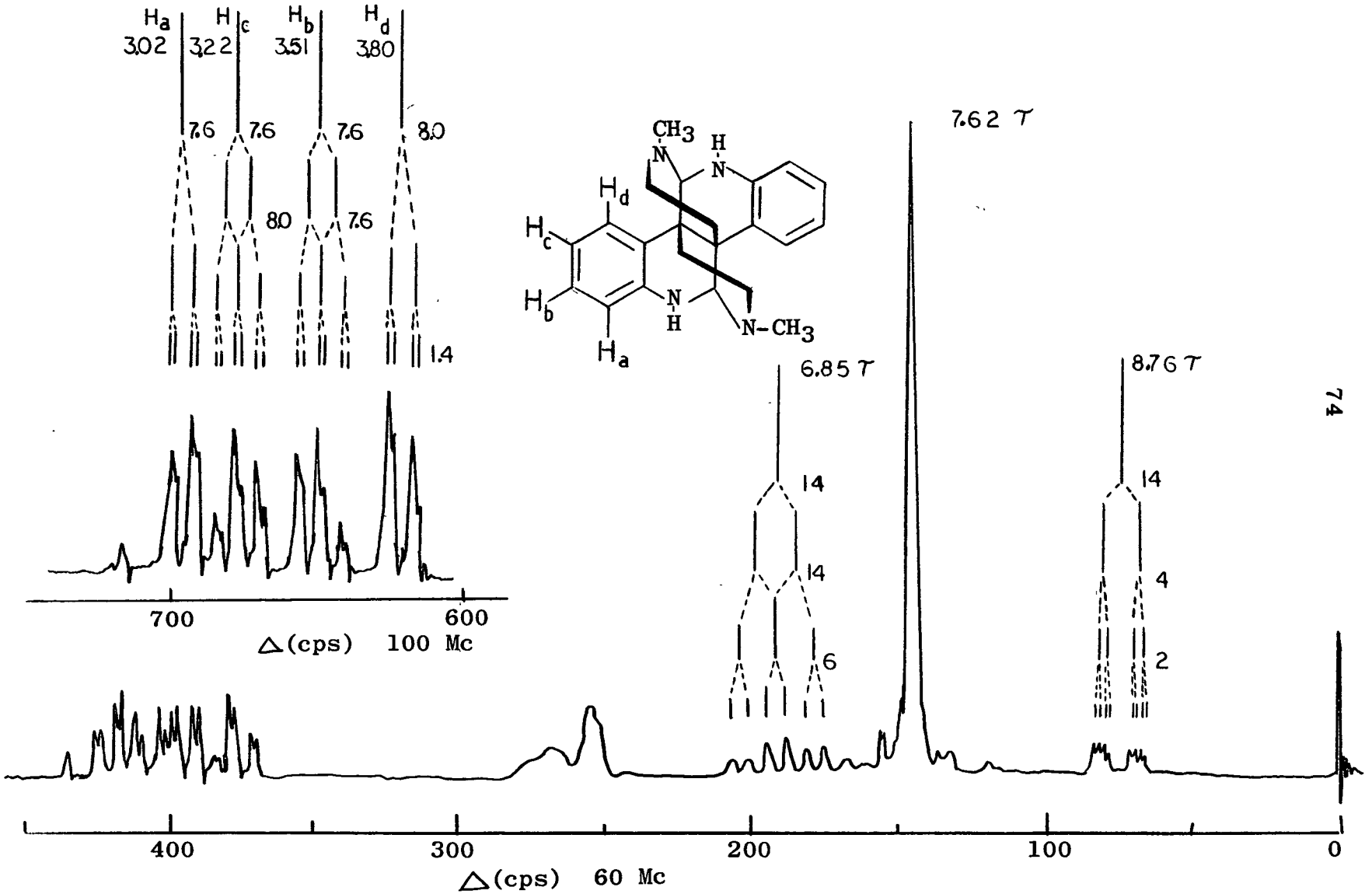


Figure 37. N.M.R. Spectrum of dl-calycanthine.

hypsochromic shift and reduced intensity in dilute acid. Although the spectra obtained by mixing N_b-methyltryptamine and calycanthine (2:1) were quite similar, the indole chromophore was relatively unaffected by dilute acid. The inference was that A and B possessed an indoline chromophore plus a second non-indole chromophore. Treatment of these compounds with aqueous acetic acid failed to yield any calycanthine isomers. On the basis of thin-layer chromatographic evidence compound A was unaffected while compound B was partially transformed into A or a very similar compound. Micro-analysis and mass spectrometry established molecular weights of 344 with formulae $C_{22}H_{24}N_4$ corresponding to dehydrocalycanthines. The mass spectra were very similar to calycanthine with the molecular ion as the most abundant peak. The minor peaks (with differences in intensity) also corresponded to peaks in the calycanthine spectrum (Figures 38, 39). The major difference is the virtual absence of m/e 231 attributed to a protonated calycanine by loss of both ethanamine bridges. This feature was also shared by reduction byproducts A-1 and A-2 from the first synthesis of calycanthaceous alkaloids.[48] The ultraviolet spectra of these compounds were also very similar leading to the suspicion that compound B with melting point 235-240° C might be identical to Hendrickson's compound A-1 with a melting point of 238-242° C. Compound A had a melting point of 274-275° C while A-2 was much lower, melting at 204-205° C. Differences were observed however in the nuclear magnetic resonance spectra. Compounds

A-1, A-2, A and B all had two N-CH_3 peaks in contrast to one for chimonanthine and calycanthine emphasizing the asymmetric nature of the molecules already suspected from the ultraviolet spectra. Compound A-1 has N-CH_3 peaks at 6.73 and 7.35τ while A-2 has peaks at 6.73 and 7.76τ. Compound B has N-CH_3 peaks at 6.78τ and 7.42 τ while A has peaks at 6.72 and 7.61τ. In spite of differences between the byproducts from indole and oxindole coupling it is still attractive to formulate A and B as mono-amidines of isomers 73, which could be formed by over oxidation during the coupling reaction.

Since an amidine with the calycanthine structure (73α) is impossible[49] and the mass spectrum rules out 73γ there are three possibilities. Hendrickson[48] eliminates 73ϵ on the grounds that the difference in chemical shift strongly implies that one N-CH_3 is an amidine methyl but in the case of an ϵ isomer the environments of the two N-CH_3 groups are saturated and similar. Compounds A-1 and A-2[48] were in the racemic series as byproducts from reduction to dl-chimonanthine. Compounds A and B could then be the mesomeric monoamidines of 73 β and δ respectively (Figure 40). Extra oxidation in the meso series would also account for the lower yield of meso than of racemic chimonanthine.

Further evidence for this formulation includes mono-acetylation of A and consideration of the n.m.r. spectrum of B which has one aromatic proton (2.21τ) at lower field than the rest.

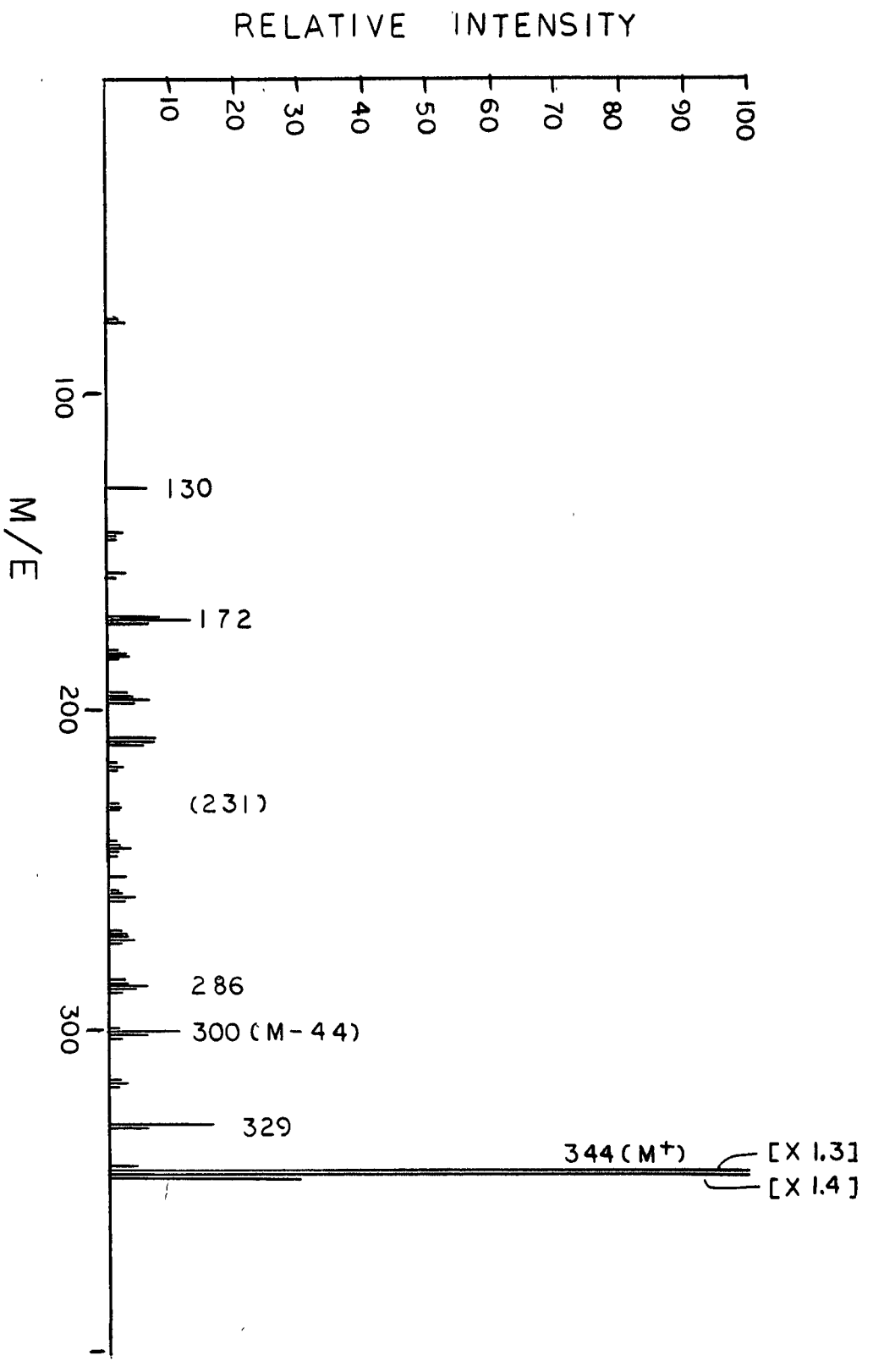


Figure 38. Mass Spectrum of Dimer A.

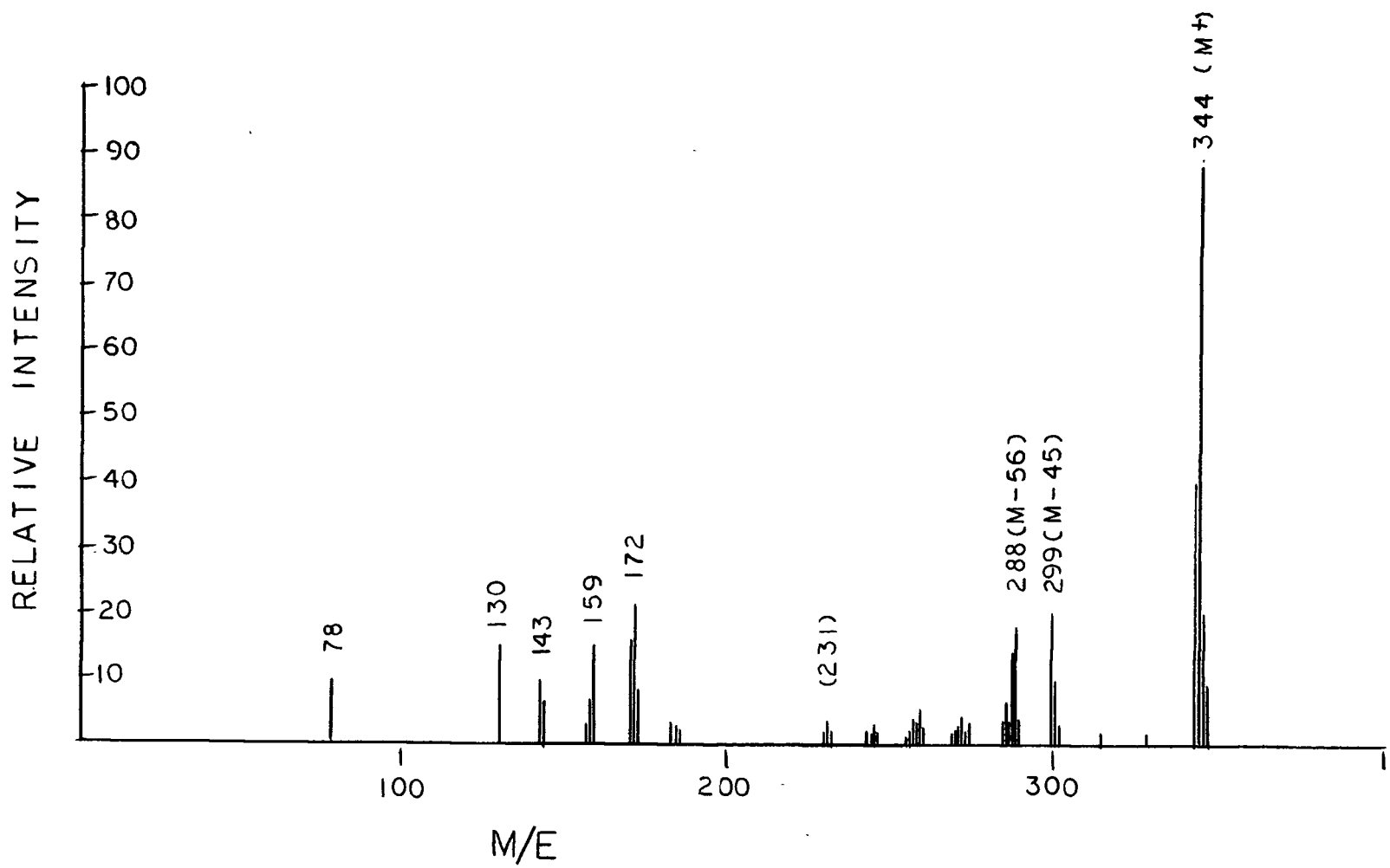
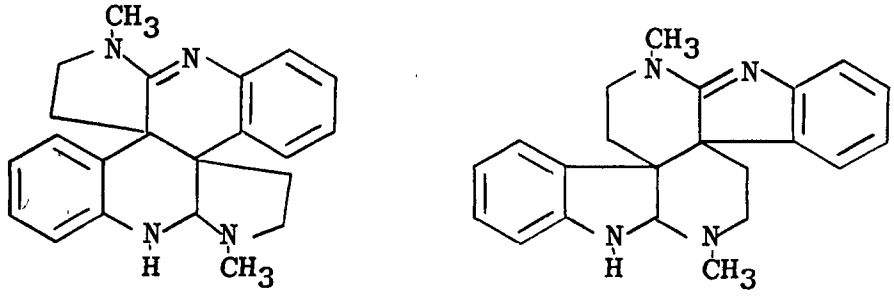


Figure 39. Mass Spectrum of Dimer B.

This is probably the proton ortho to the amidine nitrogen. Compound B has only a single NH and R_3CH by integration and a single methylene proton at high field (8.84τ) which is reminiscent of calycanthine with its six-membered rings. There were eight methylene protons by integration, indicating that the double bond could not be in an ethanamine bridge.



A 73β B 73 δ

Figure 40. Suggested Structures of Compounds A and B.

Compound A was unaffected by attempted hydrogenation and by attempted reduction with lithium aluminum hydride. Oxidation of calycanthine with mercuric acetate and of chimonanthine with mercuric acetate or manganese dioxide failed to yield spots on thin-layer chromatography corresponding to compound A or B. Treatment of these dehydrocompounds with dilute acetic acid did not yield compound A or B. The ultraviolet spectrum of the dehydrocompounds gave no evidence of amidine formation.

An attempt was made to couple N_b-methyltryptamine-N_a-

magnesium iodide in tetrahydrofuran in the hope of raising the yield, as it was much more soluble in this solvent. Only a trace amount of dimeric product was obtained as detected by mass spectrometry in a preparative thin-layer chromatographic fraction with R_f the same as chimonanthine. The major product from this reaction (named C) was shown by analysis and mass spectrometry to have a molecular weight of 188 and a formula of $C_{12}H_{16}N_2$. The infrared had a sharp peak at 3500 cm^{-1} and peaks at 2840 and 2790 cm^{-1} attributable to the NH of a substituted pyrrole and $-N(CH_3)_2$.[113] A typical indole chromophore appeared in the ultraviolet spectrum. The n.m.r. and mass spectra were consistent with formulation of compound C as N_b,N_b-dimethyltryptamine. The melting point, 45-49° C, after recrystallization from ether also is in agreement with this structure.

The second product, D, was shown by its indole type ultraviolet spectrum, absence of an α proton in the n.m.r. spectrum, presence of two NH protons by exchange with D_2O, analysis $C_{15}H_{20}N_2O$, and mass spectrometric molecular weight 244, to be N_b-methyl-α-tetrahydrofuranyltryptamine. This compound probably arises by coupling of the respective radicals, the intermediate α-indolyl radical being less commonly found.

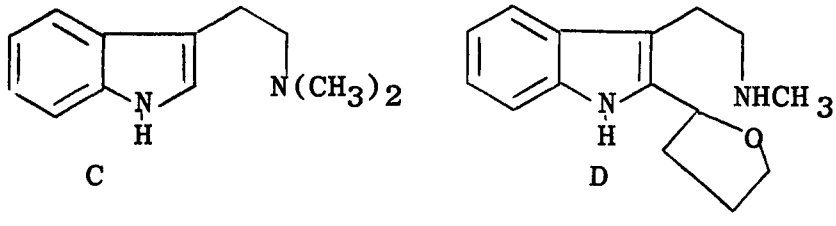


Figure 41. Products from Attempted Coupling in Tetrahydrofuran.

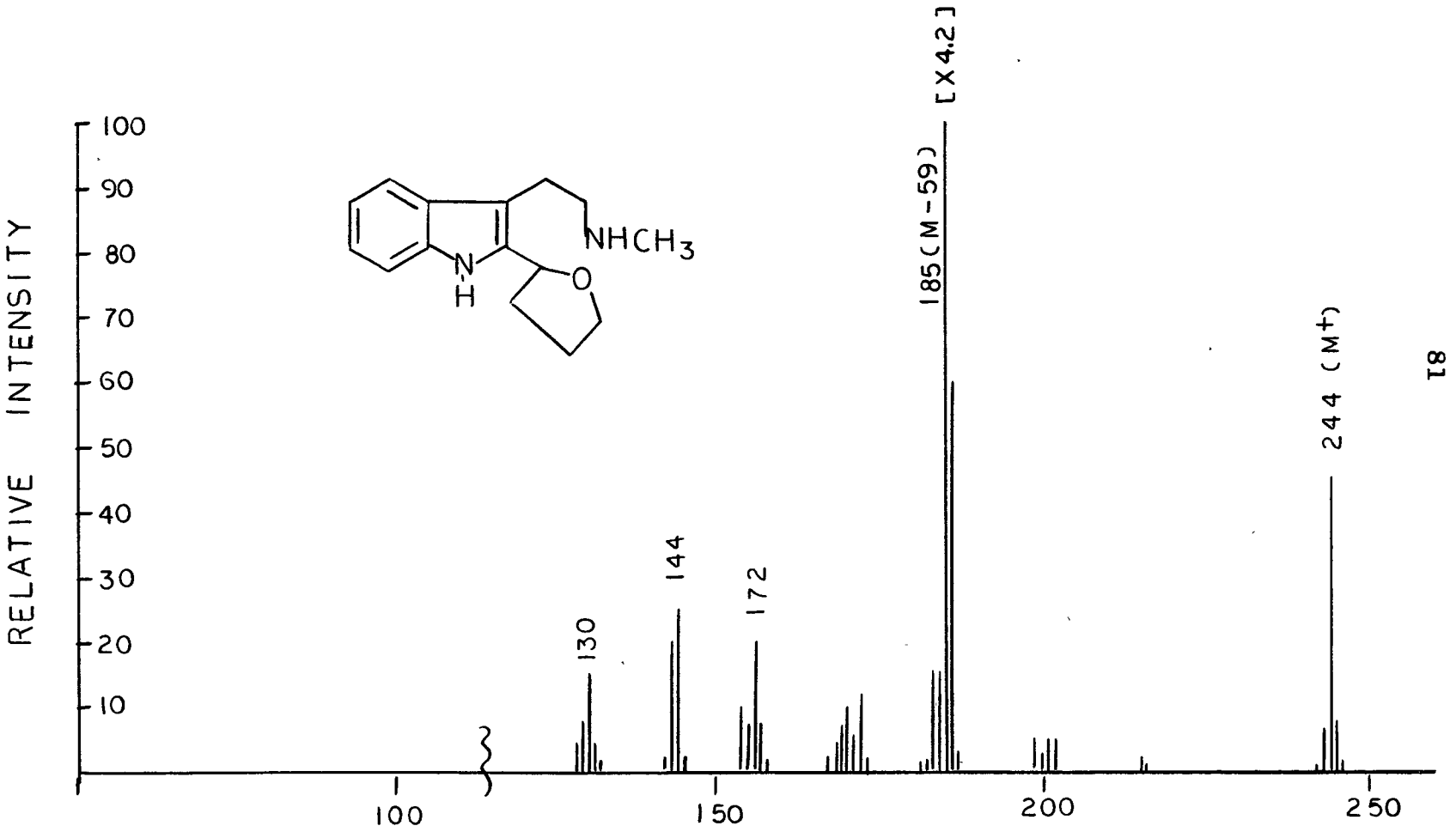


Figure 42. Mass Spectrum of Compound D.

Hodgkinsine

An 80 mg. sample of hodgkinsine was received from Professor Taylor, University of Queensland, when it was thought to be an isomer of calycanthine or a dehydrocalycanthine. Attempted isomerization or hydrogenation followed by isomerization resulted in no evidence for calycanthine isomers. Hodgkinsine during this time was also under study by Professor Smith of Manchester who established that the compound was a trimer of N_b-methyldipterin and suggested structure 86. A mass spectrum and n.m.r. spectrum were determined and are of interest. The base peak is at m/e 172 with secondary peaks at m/e 344 and 518. This pattern indicates facile cleavage of the molecule into three symmetrical fragments. The n.m.r. spectrum has three NCH_3 peaks at 7.59, 7.65 and 7.71τ. The aromatic region integrates for eleven protons with one at slightly higher field than the rest and provides evidence for substitution in an aromatic ring. There are three $\underline{NH}$, three $R_3\underline{CH}$ protons and 21 (12 + 9) methylene plus N-methyl protons. The methylene protons do not extend to as high a field as does the high field proton of calycanthine providing evidence for three five-membered ethanamine rings. A consideration of the biogenesis of hodgkinsine as a probable oxidative coupling of chimonanthine and N_b-methyltryptamine makes substitution on an aromatic ring para to a nitrogen the most probable. Structure 86 for hodgkinsine is a reasonable one.

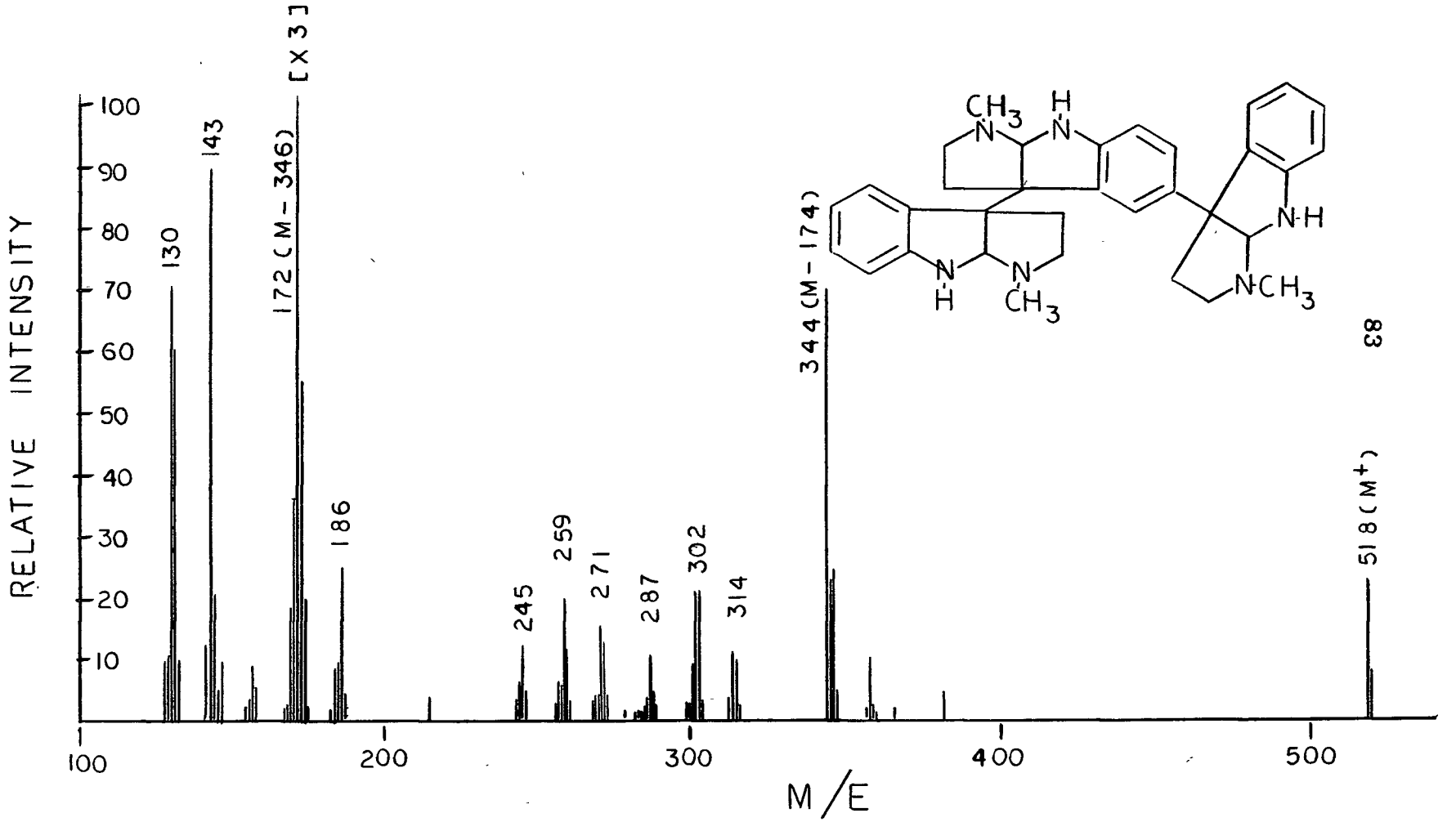


Figure 43. Mass Spectrum of Hodgkinsine.

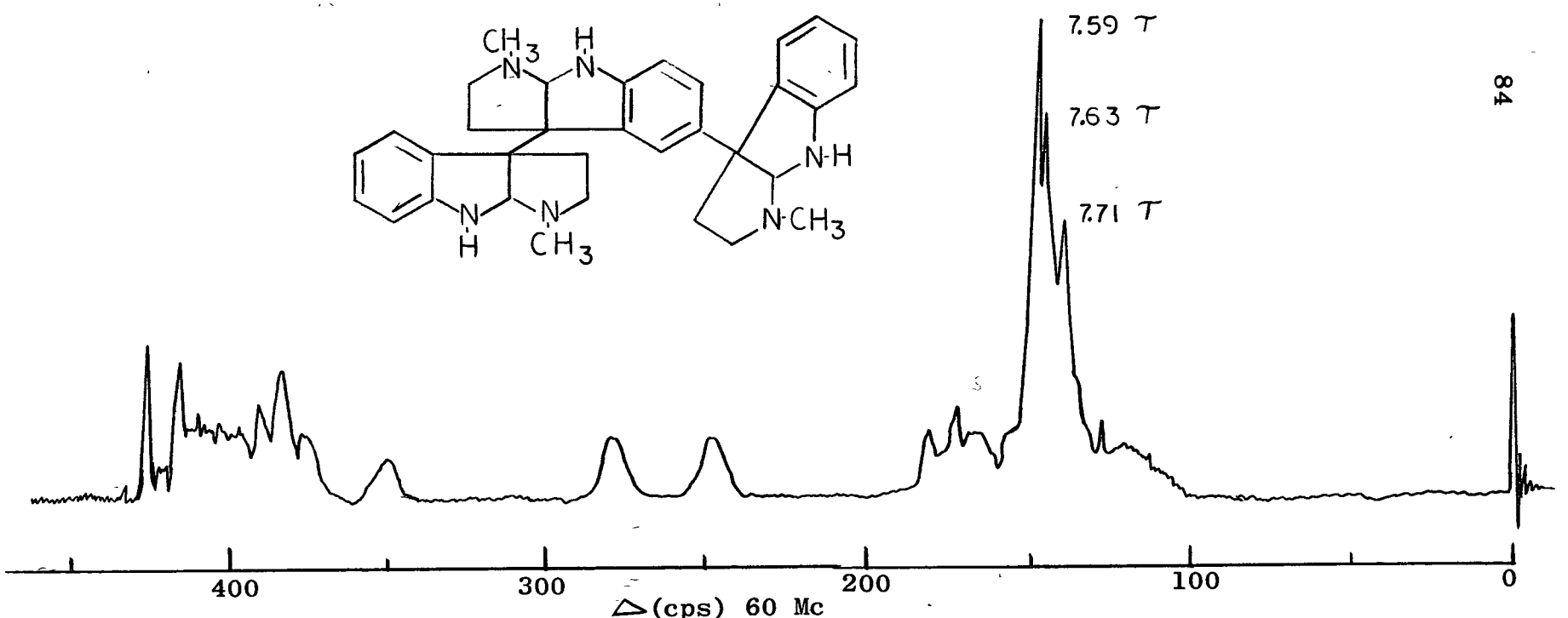


Figure 44. NMR of Hodgkinsine.

Experimental

Melting points were determined on a Kofler hot stage microscope and are uncorrected. Ultraviolet spectra were determined using a Cary 14 spectrophotometer and infrared spectra using a Perkin-Elmer model 137B spectrophotometer unless otherwise stated. Nuclear magnetic resonance spectra (n.m.r.) were recorded at 60 Mc/s on a Varian A60 instrument by Mr.P.Horn or Mrs. A.Brewster and at 100 Mc/s on a Varian HA-100 instrument by Mr.R.Burton of this department. The resonance positions are given in the Tiers τ scale with reference to tetramethylsilane as an internal standard, with types of protons, coupling constants (J) in cycles per second (c.p.s.), and the integrated areas in parentheses. Elemental analyses were performed by Mrs.C.Jenkins of this department.

Mass spectrometric determinations were done by Mr.G.Eigendorf of this department on an Atlas CH4 instrument after this service became available. Thanks are due to Dr.H.Budzikiewicz, Stanford University for the original determination of the mass spectrum of synthetic dl-chimonanthine and to Dr.Taylor, University of Queensland, for his determination of the mass spectrum of synthetic meso-chimonanthine and for his supply of hodgkinsine.

We are grateful to Dr.A.Brossi of Hoffmann-LaRoche, Nutley, New Jersey for a lavish gift of tryptamine hydrochloride. We thank Dr.G.F.Smith, Manchester University, England, for a very generous gift of the crude sample of natural chimonanthine

which proved to be so interesting and for a sample of d-calycanthine. Many thanks are also due to Dr.J.B.Hendrickson for a sample of his synthetic dl-chimonanthine and for a copy of his manuscript before publication.

Alumina G (according to Stahl) was used for thin-layer chromatography. The alumina used for column chromatography was Shawinigan reagent, neutralized by treatment with ethyl acetate.

3,2'-Methylaminoethylindole (N_b-methyltryptamine) (50).

This preparation was devised by Dr.A.C.Day, University of Oxford, England and is a modification of an earlier preparation by Hoshiro and Kobayashi.[106]

3,2'-p-toluenesulphonylaminoethylindole

Tryptamine hydrochloride (3,2'-aminoethylindole) (98) (20 g., 0.102 moles) was suspended in benzene (150 ml.) and treated with p-toluenesulphonyl chloride (21.4 g., 0.112 moles, 10% excess) followed by potassium hydroxide (17 g., 0.3 moles; less when starting with tryptamine) in water (150 ml.). On warming, a clear two phase solution was obtained and allowed to cool with occasional shaking. The white solid, N_b-tosyltryptamine, was filtered off about one-half hour after acidification with dilute hydrochloric acid and chilling in an ice bath. The supernatant liquid was again treated with tosyl chloride after making basic with potassium hydroxide(7 g. excess)

and a few more grams of the tosylate were obtained. The literature method involved recrystallization from benzene-ethanol but this solvent pair is definitely inferior to hot ethanol in which the product is extremely soluble. Yield 30 g., 90%; m.p. 114-115° (literature 115-116°).

3,2'-methyl-p-toluenesulphonylaminoethylindole (100)

N_b-tosyltryptamine (99) (29.8 g., 0.095 moles) was suspended in ethanol (50 ml.) and added to 50% w/w aqeous sodium hydroxide (18 g. NaOH) whereby the compound went into solution on shaking and gentle warming. Methyliodide (16.8 g., 0.118 moles, 24% excess) was added and the solution kept at room temperature overnight. Much crystalline solid had precipitated after two hours. The white crystalline product was filtered off after chilling in an ice bath, washed with a small amount of cold ethanol then recrystallized from a minimum of ethanol. Dilute sodium hydroxide solution was added to the filtrate and the small amount of off-white solid precipitate assumed to be starting material, and treated as before. Yield of N_b-methyl-N_b-tosyltryptamine 20.4 g., 67%; m.p. 118-119° (literature 116-117°). Although the melting point of the unmethylated derivative was very similar, strong depression is shown on mixing.

Detosylation of N_b-methyl-N_b-tosyltryptamine (100)[108]

N_b-methyl-N_b-tosyltryptamine (20 g., 0.064 moles) was dissolved in tetrahydrofuran (350 ml.) and added to liquid

ammonia (1.5 l.) with stirring in a 3 liter 3-necked round bottom flask cooled in a dry-ice acetone bath. Sodium was added in small pieces to the stirred solution until the characteristic deep blue colour of solvated electrons persisted for ten minutes. Ammonium chloride was then added cautiously until the colour disappeared. Ammonia was allowed to evaporate overnight at room temperature. Water and 4 N sodium hydroxide were added and the organic material collected with ether (3 x 100 ml.). The basic product was purified via solution in 1 N hydrochloric acid (3 x 50 ml.), re-extraction with ether (3 x 50 ml.), washing until neutral with water, drying over magnesium sulphate, and evaporation to give a brownish oil which solidified on cooling. The N_b-methyltryptamine (50) could be recrystallized from ether but was very susceptible to oxidation producing a brown ether-insoluble gum presumably through the 3-hydroperoxide and was best crystallized under nitrogen. m.p. 88-89° C. Crude yield; 9.8 g., 93%. Ultraviolet spectrum; (ethanol) $\lambda_{max.}$ 274, 282, 291 mμ, $\epsilon_{max.}$ 7000, 7200, 6000. This is a typical indole chromophore. Nuclear magnetic resonance spectrum; (deuterochloroform) multiplets centered at 2.60 and 2.98τ (aromatic protons plus NH, area 4.8 H), singlet at 3.28τ (α proton, area 1 H), singlet at 7.12τ (methylene protons, area 4 H), singlet at 7.65τ (N-methyl, area 3 H): (benzene) a broad resonance from 3-4τ (aromatic plus NH protons, area 6 H), a broad singlet centered at 7.15τ (methylene protons, 4 H), a singlet at 7.77τ (N-methyl, 3 H). The n.m.r. spectra are

consistent with the structure of N_b-methyltryptamine. The degeneracy of the four methylene protons in deuterochloroform was removed in benzene. The mass spectrum: m/e 174 (M^+, 8%), 144 (M-31, 7%), 131 (M-44+1, 100%), 44 (dimethylamine), 31 (methylamine). Calc. for $C_{11}H_{14}N_2$;Molecular weight 174.

Coupling of Dipterin (N_b-methyltryptamine) (50)

One gram of dipterin was used for several trial oxidations in order to establish reasonable conditions for the oxidation, work-up, and separation of products. A cleaner product is obtained when the coupling is carried out under nitrogen. When one-third of the theoretical amount of ferric chloride is used the yield of chimonanthine is decreased but the amount of recoverable starting material increased. As the indolyl magnesium iodide was found to be quite insoluble in ether the coupling reaction was attempted in tetrahydrofuran without success but with an interesting byproduct. The procedure described is for a scaled up coupling reaction.

Methyl magnesium iodide was prepared in the usual manner from magnesium turnings (1.96 g., 0.805 mmoles) freshly crushed in a mortar and methyl iodide (11.4 g., 0.805 mmoles) in a 3 liter 3-necked flask equipped with a short condenser, a mechanical stirrer with a mercury gland, a 250 ml. dropping funnel, and drying tubes. The apparatus was flame dried and the reaction carried out under a positive pressure of dry nitrogen. A total volume of 500 ml. of commercial anhydrous ether was used.

The dipterin (14 g., 0.805 mmoles) used was the crude material from detosylation of N_b-tosyl-N_b-methyltryptamine dried by azeotroping with benzene (3 x 50 ml.) and dissolved in anhydrous ether (700 ml.). The small amount of ether insoluble impurity was removed by filtration through a pad of glass wool as the dipterin was added dropwise to the stirred solution of methyl magnesium iodide over a period of about one hour. The light yellow solid indolylmagnesium iodide, some in suspension and some on the flask walls, was stirred vigorously for two hours. Anhydrous ferric chloride (15 g., 0.920 mmoles, 15% excess allowing for impurities) was dissolved in anhydrous ether (2 x 200 ml.), decanted from the rust-brown residue, and added dropwise with vigorous stirring to the indolylmagnesium iodide suspension. The blue-black reaction mixture was stirred vigorously fo eighteen hours. The Grignard reagent was decomposed with one and one-half liters of cold saturated ammonium chloride with stirring for two hours leaving light brown aqueous and ether layers and a brown solid. The latter was removed by filtration through a celite pad and washed with saturated ammonium chloride. The ether layer was washed with brine and dried over magnesium sulphate. On evaporation 0.22 g. of a black solid was obtained. The composition of this mixture was not investigated after it was found to contain no chimonanthine by comparative thin-layer chromatography. The aqueous layer was extracted with chloroform (5 x 100 ml.) then enough 6 N sodium hydroxide was added to bring the pH to approx-

imately ten, shaking all the while with chloroform. Powdered cellulose was added to absorb the green gelatinous precipitate of ferrous hydroxide which was then filtered and titurated repeatedly, as was the original brown solid, with boiling chloroform. The aqueous layer was also repeatedly extracted with chloroform. All chloroform extracts which were red-brown in colour were combined, dried over anhydrous magnesium sulphate and evaporated yielding eleven grams (80% recovery) of a product mixture. Analysis by thin-layer chromatography (alumina G, 2% methanol-ether, ceric sulphate spray (1% ceric sulphate: 35% sulphuric acid)) revealed a mixture of five major components including meso-chimonanthine (R_f 0.50, red changing to blue-green changing to yellow), dl-chimonanthine (R_f 0.30, red changing to blue changing to yellow) and N_b-methyltryptamine (R_f 0.10, yellow). Two compounds more mobile in this system than chimonanthine were named compound A (R_f 0.68, purple, fading quickly) and compound B (R_f 0.58, brown after warming). Of the solvent systems tried ether, or ether mixtures, were the only ones found which would separate meso and racemic chimonanthines. 2% Methanol-ether was the most generally useful. 50% Methanol-ether (R_f 0.80, chimonanthine, calycanthine) was useful for analysis of monomeric components (R_f 0.30, N_b-methyltryptamine). Acidic ceric sulphate spray was the most generally useful for detection of compounds as it not only gave brightly coloured spots but spots which changed in colour with time in a characteristic manner. Chloroplatinic acid, Dragendorf reagent[114] and

iodine vapour were also useful. Chloroplatinic acid also gave specific colours and was more sensitive in the case of compounds A and B than was ceric sulphate. Dragendorf reagent was particularly sensitive for all these compounds but showed uniform orange spots. Iodine vapour was very convenient but not very specific.

Chromatographic separation of the products.

The product mixture was dissolved in benzene and subjected to careful chromatography on neutral alumina (grade I, 400 g.). The chromatography was followed by thin-layer chromatography and fractions (250 ml.) combined accordingly. After two liters of benzene had passed through the column compound A was eluted (500 ml., 0.25 g.). Elution with 2% ether-benzene (1000 ml.) produced a mixture of compounds A and B (0.26 g.) then with 10% ether-benzene (500 ml.) a mixture of meso-chimonanthine, compound B and some of compound A (0.30 g.). Elution with 20% ether-benzene (1000 ml.) yielded a mixture of compounds A and B with both racemic and meso-chimonanthines (1.2 g.). dl-Chimonanthine with some of the meso isomer (1.05 g.) was then eluted with 50% ether-benzene (500 ml.) followed by a mixture of chimonanthine, unidentified material (indole chromophore) and N_b-methyltryptamine (1.22 g., 500 ml.). The column was then stripped of organic material using ether (250 ml.), 10% methanol-ether (250 ml.) and finally methanol (1800 ml.). N_b-methyltryptamine was the major component of this dark brown gum (5.2 g.)

and 30% of starting material could be recovered by washing through an alumina column (grade III) using chloroform, chloroform-methanol, and finally methanol.

The other major components were further purified by repeated chromatography then by crystallization and yields calculated on the basis of 30% recovery of starting material.

Compound A (yield 3.5%) was recrystallized from chloroform or benzene and was difficult to redissolve once obtained in the crystalline form. m.p. 274-275° C. Anal. Found: C, 76.26%; H, 7.44%; N, 15.78%. Calc. for $C_{22}H_{26}N_4$: C, 76.3%; H, 7.5%; N, 16.5%. Calc. for $C_{22}H_{24}N_4$: C, 76.7%; H, 7.0%; N, 16.25%; molecular weight 344. Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 249, 276 sh., 283, 304 sh. mμ, $\epsilon_{max.}$ 7100, 7000, 7200, 4500. Ultraviolet spectrum in ethanolic hydrochloric acid: $\lambda_{max.}$ 239 sh., 264, 271 sh., 290 sh. mμ, $\epsilon_{max.}$ 6000, 5300, 5000, 3800. Infrared spectrum in chloroform: $\nu_{max.}$ 3440(w), 2930, 2850, 2790, 1640(s), 1590(s), 1580(s). The n.m.r. spectrum in deuterochloroform: unresolved multiplets centered at 2.95 and 3.45 τ (aromatic protons, area 8 H), broad singlets at 5.34 and 5.80 τ (N<u>H</u> or R_3C<u>H</u>, area each 1 H), unresolved multiplets from 6.4 to 8.1 τ (methylene protons), singlet at 6.72 τ (R_2N-C<u>H</u>$_3$, area 3 H), and singlet at 7.61 τ (R_2N-C<u>H</u>$_3$, area 3 H). The mass spectrum had significant peaks at m/e 344 (M^+), 345 (M + 1, 107%), 346 (M + 2, 23%), 329 (M - 15, 12%), 300 (M - 44, 10%), 286 (M - 58, 4%), 209 (M - 135, 5%), 197 (M - 147, 4%), 172 (M - 172, 10%), 130 (4%).

Compound B (yield 3.5%) was difficult to purify and was shown to be a mixture of two isomers both of which have the same ultraviolet spectrum. The physical data are for the isomer which was slightly more mobile on alumina when developed with ether. m.p. 235-240° C. Anal. Found: C, 76.56%; H, 7.09%; N, 16.10%. Calc. for $C_{22}H_{24}N_4$: C, 76.7%; H, 7.0%; N, 16.25%; molecular weight 344. Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 274, 283, 310 sh. mμ, $\epsilon_{max.}$ 10080, 10100, 6500. Ultraviolet spectrum in ethanolic hydrochloric acid: $\lambda_{max.}$ 267, 276, 293 mμ, $\epsilon_{max.}$ 7600, 7600, 5900. Infrared spectrum in chloroform includes: $\nu_{max.}$ 3440, 3200, 2950, 2830, 1630(s), 1580(s), 1450(s) cm^{-1}. The n.m.r. spectrum in deuterochloroform: a doublet (J = 7.8 c.p.s.) centered at 2.21τ (aromatic proton, area 1 H), a wide multiplet centered at 3.30τ (aromatic protons, area 7 H), broad doublets (J ca. 3 c.p.s.) centered at 5.50 and 5.70τ ($R_3C\underline{H}$ and $R_2N\underline{H}$, each area 1 H), a sextet from 6.1 to 6.65 τ (methylene protons, area 2 H), partially obscured multiplets from 7.0 to 8.1 τ (methylene protons, area 5 H), doublet of multiplets (J = 14 c.p.s.) centered at 8.84τ (methylene proton, area 1 H), singlet at 6.78τ ($N-CH_3$, area 3 H) and a singlet at 7.42τ ($N-CH_3$, area 3 H). The mass spectrum had significant peaks at m/e 344 (M^+), 345 (M + 1, 20%), 346 (M + 2, 10%), 343 (M - 1, 45%), 299 (M - 45, 22%), 288 (M - 56, 20%), 231 (M - 113, 4%), 172 (M - 172, 23%), 159 (16%), 143 (10%), 130 (16%).

Meso-chimonanthine (yield 7%) was recrystallized from

benzene. m.p. 198-202° C. There was no depression of melting point on admixture with natural meso-chimonanthine (see experimental for isolation). Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 248, 305 mμ, $\epsilon_{max.}$ 13000, 4900. Ultraviolet spectrum in ethanolic hydrochloric acid (0.1 N): $\lambda_{max.}$ 239, 294 mμ (typical Ph-N-C-N chromophore). Infrared spectrum in chloroform (0.1 mg./ml.): $\nu_{max.}$ 3440(w), 2920(s), 2850(m), 2800(m), 1615(s), 1480(s), 1460(s), 1395(w), 1340(m), 1310(m), 1240(w), 1150(m), 1120(m), 1022(m), 994(w), 935(w), 908(w) cm^{-1}. The n.m.r. spectrum in deuterochloroform: multiplets from 2.7 to 4.0 τ (aromatic protons, area 8 H), broadened singlet at 5.35 τ (R_2N-H, area 2 H), broadened singlet at 6.15τ (R_3C-H, area 2 H), partially masked multiplets from 7.0 to 8.3 τ (methylene protons, area 8 H), singlet at 7.63 τ (N-CH_3, area 6 H). The mass spectrum had fragments at m/e 346 (M^+, 7%), 172 (M - 174, 100%), 173 (M - 173, 25%), 157 (2%), 144 (2%) and 130 (10%).

dl-Chimonanthine (yield 19%) was recrystallized from benzene. m.p. 183-185° C. There was no depression of melting point on admixture with a sample of synthetic dl-chimonanthine kindly supplied by Professor J.B.Hendrickson and prepared by coupling of oxindoles.[48] Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 247, 303 mμ, $\epsilon_{max.}$ 13600, 5600. Ultraviolet spectrum in ethanolic hydrochloric acid: $\lambda_{max.}$ 239, 294 mμ. Infrared spectrum in chloroform: $\nu_{max.}$ 3440(w), 2920(s), 2850(m), 2800 (m), 1615(s), 1480(s), 1460(s), 1395(m), 1350(m), 1310(m), 1240 (w), 1150(m), 1120(m), 1090(w), 1055(w), 1022(m), 904(w) cm^{-1}.

The n.m.r. spectrum in deuterochloroform: multiplet (can be analyzed in the 100 Mc spectrum as a simple ABCD spectrum) from 2.7 to 3.7 τ (aromatic protons, area 8 H), broad singlet at 5.62τ (R_3C-H, area 2 H), broader singlet at 5.75τ(R_2N-H, area 2 H), multiplets from 7.2 to 8.2 τ (methylene protons, area 8 H), and a singlet at 7.70 τ (N-CH_3, area 6 H). The mass spectrum had fragments at m/e 346(M^+, <12%), 347(M + 1, 1%), 172(M - 174, 100%), 173(M - 173, <33%), 157(2%), 144(2%) and 130(26%).

Attempted coupling of dipterin(50) in tetrahydrofuran

Methyl magnesium iodide was prepared in tetrahydrofuran (dried by refluxing over lithium aluminum hydride) from magnesium (0.28 g.) and methyl iodide (1.63 g.). As not all of the magnesium dissolved even after prolonged heating the reagent was estimated by adding an aliquot (1 ml., 1/60 of reagent) to dilute hydrochloric acid (0.1 N, 3 ml.) and back-titrating with dilute sodium carbonate (0.0173 N, 7.9 ml.) to methyl red, as 75% of the theoretical N_b-methyltryptamine (50) (1.5 g.) dissolved in tetrahydrofuran was added dropwise with stirring and stirring was continued for two hours. The volume was increased (150 ml.) because the indolyl Grignard reagent separated as a brown gum. Anhydrous ferric chloride (1.4 g.) in tetrahydrofuran (50 ml.) was added slowly giving a black apparently homogeneous solution which was stirred under nitrogen overnight. The workup was identical to that using ether as a solvent (see previous experiment) and recovery of organic

material was good (85%).

The absence of either chimonanthine or calycanthine was demonstrated by thin-layer chromatography (alumina G, ether, ceric sulphate). Besides starting material, the two major products which were designated C (R_f 0.5, yellow, 15% of product) and D (R_f 0.3, purple, 5% of product) were isolated by chromatography on alumina.

Compound C was recrystallized from ether and identified from physical data as N_b,N_b-dimethyltryptamine. (Figure 41). m.p. 45-49° C, lit.m.p. 49-50° C.[115] Anal. found: C, 76.74%; H, 8.72%; N, 14.26%. Calc. for $C_{12}H_{16}N_2$: C, 76.54%; H, 8.57%; N, 14.89%; molecular weight 188. The ultraviolet spectrum in ethanol: $\lambda_{max.}$ 277 sh., 283, 292 mμ, $\epsilon_{max.}$ 6600, 7000, 6200, unaffected by dilute acid. This is a typical indole chromophore. The infrared spectrum includes: $\nu_{max.}$ 3500 (NH, substituted pyrrole[113]), 2950, 2870, 2840, 2790 cm^{-1} ($-N(CH_3)_2$).[113] The n.m.r. spectrum in deuterochloroform: multiplets centered at 2.4 and 2.9 τ (aromatic protons, areas 1 and 3 H), a broadened doublet (J = 1.8 c.p.s.) centered at 3.27 τ (α proton, area 1 H), multiplet at 7.21 τ (methylene protons, area 4 H) and a singlet at 7.61 τ ($N(CH_3)_2$), area 6 H). The mass spectrum has m/e 188 (M^+, 100%), 143 (M - 45, 45%), 130 (M - 58, 85%), 115(40%), 58 (M - 130), 44.

Compound D was recrystallized from ether. m.p. 134-147° C. It was identified as a tetrahydrofuranyltryptamine derivative. (Figure 41). Anal. Found(single determination only): C, 73.10%;

H, 8.19%; N, 11.68%; O, 7.1% (by difference). Calc. for $C_{15}H_{20}N_2O$: C, 73.73%; H, 8.25%; N, 11.47%; O, 6.55%: molecular weight 244. The ultraviolet spectrum in ethanol: $\lambda_{max.}$ 276 sh., 283, 290 mμ, $\epsilon_{max.}$ 6700. 6900. 5200. This is a typical indole chromophore and was essentially unchanged in dilute acid. The infrared spectrum includes: $\nu_{max.}$ 3500 (NH, pyrrole), 2910, 2850, 2700 (N-CH_3), 1630, 1670, 1010, 990, 975, 956 cm^{-1}. The n.m.r. spectrum in deuterochloroform: broadened singlet at 0.96 τ (aromatic N-H, exchanges with D_2O, area 0.7 H), multiplet from 2.3 to 3.1 τ (aromatic protons, area 4 H), broadened singlet 3.53 τ (aliphatic N-H, exchanges with D_2O, area 0.9 H), multiplet centered at 6.39 τ (-O-CH_2-, area 3 H), multiplet centered at 7.12 τ (-CH_2-CH_2-N-, area 4 H), singlet at 7.44 τ (N-CH_3, area 3 H) and a broadened doublet (J = 22 c.p.s.) centered at 8.22 τ (-CH-(CH_2)$_2$-CH_2-, area 4 H). The mass spectrum had m/e 244 (M^+, 10%), 198 (M - 45, 1%), 158 (M - 59, 100%), 159 (M - 59, 14%), 172 (M - 72, 3%), 156 (5%), 144 (6%), and 130 (4%).

A mass spectrum of the mother liquors from which compound C (R_f 0.30, ether, alumina, c.f. chimonanthine R_f 0.25) was crystallized showed a fragment at m/e 346 which is the molecular weight of the calycanthine isomers. The corresponding m/e 172 peak was also evident. It was concluded that a small amount of the coupling reaction took place in tetrahydrofuran (>1%).

Discovery of meso-chimonanthine in nature

A sample of crude chimonanthine (201 mg.) from an extract of Calycanthus floridus was received from Dr.G.F.Smith for purposes of comparison with the synthetic product. As this material was yellow in colour part of it (83 mg.) was washed through an alumina column with benzene (50 ml.), 10% ether-benzene (50 ml.), ether, and finally chloroform. The ether-benzene fraction was recrystallized twice from benzene yielding white crystals (20 mg.) m.p. 199-202° C. lit. l-chimonanthine m.p. 188-189° C.[71] This compound showed strong depression of melting point on admixture with the synthetic chimonanthine first isolated (m.p. 183-185° C). A check of rotation, $[\alpha]_D^{23}$ (chloroform) 0.0°, led to its identification as meso-chimonanthine and discovery of the same material in the N_b-methyl-tryptamine coupling product. For physical data see experimental data for the synthesis. There was a complete correspondence of infrared, ultraviolet, n.m.r., and mass spectra. Behaviour on thin-layer chromatography in several solvent systems including the colour reaction with ceric sulphate was also identical. An n.m.r. spectrum in deuterochloroform of the crude material showed singlets of equal intensity at 7.63 τ (-N-C$\underline{H}_3$ of l-chimonanthine) and at 7.72 τ (-N-C$\underline{H}_3$ of meso-chimonanthine). Thin-layer chromatographic analysis (alumina G, 2% methanol-ether, 1% ceric sulphate:35% sulphuric acid) revealed a two component (approximately 1:1) mixture.

Preparation of dl-calycanthine by isomerization of dl-chimonanthine.

Trial experiments were first carried out on samples of synthetic dl-chimonanthine (5 mg.) in dilute hydrochloric acid (4 N, 1 N, 0.1 N and 0.01 N) (5 ml.) under nitrogen at 20° and 100° C for times ranging from two hours to twenty days. After addition of potassium carbonate and extraction with chloroform the alkaloid mixture was examined by thin-layer chromatography (alumina, ether, ceric sulphate). The most drastic conditions led to recovery of only a small amount of alkaloid and this was mainly N_b-methyltryptamine while the mildest conditions produced no observable change. Intermediate conditions produced spots corresponding to N_b-methyltryptamine, dl-chimonanthine and calycanthine (R_f 0.80, red changing to grey-green changing to mauve when sprayed with 1% ceric sulphate: 35% sulphuric acid) as well as some very minor spots.

A second series of experiments using acetic acid, dl-chimonanthine (2 mg.) in preboiled distilled water (5 ml.), under nitrogen was carried out and found to be superior in that essentially no N_b-methyltryptamine or minor products were produced.

dl-Chimonanthine (30 mg.) was dissolved in water (5 ml.) containing acetic acid (5 drops) and heated on a steam bath (30 hours) under nitrogen. The alkaloid recovered (27 mg.) was a mixture of two components (1:4) corresponding to chimonanthine and calycanthine with only a trace of N_b-methyltryptamine.

The major component was isolated by chromatography (20 mg.) on alumina, recrystallized from benzene and compared with natural d-calycanthine. White crystals: m.p.244-247° C, which showed depression of melting point on admixture with natural d-calycanthine. The solid state infrared spectra (KBr discs) were not identical but all other physical data, including the solution infrared, which were run on a Perkin Elmer 21 and found to be superimposable, were identical.

The ultraviolet spectrum in ethanol: $\lambda_{max.}$ 250, 309 mμ, $\epsilon_{max.}$ 19000, 6300; in dilute hydrochloric acid; $\lambda_{max.}$ 240, 298 mμ. The infrared spectrum (chloroform): $\nu_{max.}$ 3480(m), 2960(s), 2820(s), 1607(s), 1585(m), 1490(s), 1450(m), 1378(m), 1315(s), 1303(s), 1287(m), 1268(m), 1240(m), 1190(m), 1167(s), 1157(m), 1118(w), 1107(m), 1067(s), 1040(s), 1025(s), 975(w), 958(w), 886(w), 865(w) cm^{-1}. The n.m.r. spectrum in deuterochloroform: complex of multiplets from 2.90 to 3.90τ, easily analysable in a 100 Mc spectrum (see Figure 37) (aromatic protons, area 8 H), a broad singlet at 5.52τ ($R_2N\underline{H}$, exchanges in D_2O, area 2 H), a broadened singlet at 5.72τ (N-C$\underline{H}$-N, sharp in D_2O, area 2 H), a sextet centered at 6.85τ with coupling constants 14, 14 and 2 c.p.s. (methylene protons, area 2 H), multiplets from 7.2 to 8.1τ (remaining methylene protons, area 4 H) and a singlet at 7.62τ ($NC\underline{H}_3$, area 6 H). The mass spectrum was in sharp contrast to chimonanthine with m/e 346 (M^+, 100%), 347(M + 1, 25%), 302(M - 44, 12%), 288(M - 58, 17%),

259(6%), 245(10%), 231(M - 115, 25%), 172(M - 174, 10%), and 130(6%).

Isomerization of meso-chimonanthine

Meso-chimonanthine (10 mg.) was dissolved in dilute acetic acid (10 drops in 5 ml. water) and heated under nitrogen on the steam bath for 24 hours. Examination by thin-layer chromatography after work up revealed a two component mixture (1:2) contaminated with a small amount of N_b-methyltryptamine. The component (major) other than starting material had a decided tendency to tail and streaked from the origin to R_f 0.8 (alumina, ether, mauve colour fading quickly with ceric sulphate) unless very lightly spotted.

Partial separation was achieved by chromatography on alumina using benzene-ether mixtures and purification by recrystallization from benzene. White crystals: m.p. 265-268° C. Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 245, 307 mμ. The mass spectrum was strikingly similar to that of dl-calycanthine differing only slightly in the abundance of some fragments: m/e 346 (M^+, 100%), 347 (M + 1, 25%), 302 (M - 44, 7%), 288 (M - 58, 10%), 259 (5%), 245 (11%), 231 (M - 115, 15%), 172 (26%) and 130 (16%).

Isomerization of Compounds A and B

Compounds A and B (10 mg.) were subjected to the same isomerization conditions as was meso-chimonanthine and examined

by thin-layer chromatography. A large portion was not recovered from aqueous solution but compound A was apparently unaffected while compound B was partially transformed into A. This was not confirmed by isolation. Neither calycanthine nor chimonanthine was formed.

Acetylation of compound A

Compound A (20 mg.) was treated with acetic anhydride (0.5 ml.) and pyridine (0.5 ml.) for 15 minutes. The product (R_f 0.2, alumina, ether; orange, 1% ceric sulphate:35% sulphuric acid) was shown to be the monoacetate by integration of the acetate methyl (8.01τ) in the n.m.r. spectrum. No starting material was found in the product. The ultraviolet spectrum in ethanol: $\lambda_{max.}$ 250 sh., 273 sh., 280 mμ, was very similar to that of compound A.

Attempted hydrogenation of compound A

Compound A (16 mg.) was shaken in ethylacetate with pre-hydrogenated palladium on charcoal (1:10) for two hours. No hydrogen was absorbed.

Attempted reduction of compound A

Compound A (95 mg.) and lithium aluminum hydride (60 mg.) were refluxed overnight in tetrahydrofuran (5 ml., dried by refluxing over $LiAlH_4$). Excess hydride was destroyed by addition of ethylacetate. After filtration and evaporation

thin-layer chromatography showed virtually unchanged starting material. The ultraviolet spectrum was also unchanged.

Hodgkinsine

A sample of hodgkinsine (80 mg.) was received from Dr. Taylor in Australia. It was believed then to be either isomeric with calycanthine or to be a dehydro isomer.

Isomerization

Hodgkinsine (5 mg.) was dissolved in preboiled distilled water containing acetic acid (3 drops) and heated under nitrogen on the steam bath (15 hours). The sample (4 mg.) was recovered by extraction with chloroform of the solution which had been made basic with sodium bicarbonate. Analysis by thin-layer chromatography in several solvent systems (i.e. alumina, ether, ceric sulphate) revealed hodgkinsine (R_f 0.2, red changing to pink then to yellow), some material at the origin, a major product (R_f 0.54, red changing to red-brown) and a minor product (R_f 0.70, red changing to pink). Neither hodgkinsine nor its acid isomerization products corresponded to products or acid isomerization products from the coupling reaction.

Reduction

Hodgkinsine (9.8 mg.) was shaken with presaturated palladium-charcoal (1:10) in ethanol for three hours. After

90 minutes 0.70 equivalents (M.W. 344) of hydrogen had been absorbed (1.0 equiv., M.W. 520) and absorption of hydrogen proceeded only very slowly after this time. After filtration analysis by thin-layer chromatography showed some unchanged hodgkinsine and a major spot at the origin (R_f 0.0, alumina, ether, red changing to orange changing to dark brown; R_f 0.3, alumina, methanol-chloroform 1:10). The chromatographic behaviour of this reduction product was very similar to N_b-methyltryptamine but the colour reactions with acid-ceric sulphate were quite different. dl-Chimonanthine (19.7 mg.) was treated in the same way but no hydrogenolysis occurred.

Isomerization

The hodgkinsine reduction mixture (3 mg.) was dissolved in distilled water (5 ml.) containing acetic acid (3 drops) and heated under nitrogen on the steam bath (18 hours). Analysis by thin-layer chromatography failed to reveal any calycanthine isomers. The pattern was that of a mixture of hodgkinsine reduction and acid isomerization products.

Attempted reduction with lithium aluminum hydride

Hodgkinsine (5 mg.) was refluxed with lithium aluminum hydride (60 mg.) in dry tetrahydrofuran (dried by distillation from $LiAlH_4$) and worked up in the usual way. Analysis by thin-layer chromatography revealed unchanged hodgkinsine.

Dehydrocalycanthine

Calycanthine (30 mg.) and mercuric acetate (58.1 mg.) were stirred under nitrogen at room temperature for seventeen hours in dilute acetic acid (5%). A few milligrams of calycanthine dissolved in dilute acetic acid (5%) served as a blank and yielded unchanged calycanthine. A slight cloudiness soon appeared (30 min.) accompanied by a yellow colouration but there was no further apparent change (17 hours). The solution was then heated on a steam bath (2 hours) yielding a white precipitate of mercurous acetate and a lime-green solution. The workup[116] involved filtration, bubbling hydrogen sulphide through the solution and removing precipitated mercuric sulphide by filtration through a celite pad, basification with potassium carbonate and extraction into chloroform (16 mg.). The mercurous acetate precipitate was washed with dilute acetic acid (5%), then with acetone, weighed (10 mg.) and found to represent only about 25% oxidation. Thin-layer chromatography (alumina, ether, ceric sulphate) revealed a mixture of calycanthine, several minor components and two major components. The first (R_f 0.65, purple changing to yellow with violet edges) was not dissimilar to the hodgkinsine isomerization product but was proven different by chromatography of the appropriate mixture. The second (R_f 0.60, reddish changing to mauve changing to grey) could not be separated from compound B by thin-layer chromatography. This compound was isolated (25%) by chromatography on alumina (benzene-ether mixtures) and recrystal-

lized from benzene. m.p. 211-214° C. Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 238, 312 mμ, $\epsilon_{max.}$ 4500, 1120: in dilute hydrochloric acid: $\lambda_{max.}$ 238, 295 mμ. This spectrum was quite different from that of compound B.

Dehydrochimonanthine

dl-Chimonanthine (7 mg.) and mercuric acetate (5% excess) were stirred under nitrogen on a steam bath for 2.5 hours in dilute acetic acid (5%) and worked up in the same way as calycanthine (2 mg. recovered). The recovered material was a mixture of calycanthine, dehydrocalycanthine and chimonanthine.

dl-Chimonanthine (16.5 mg.) and manganese dioxide (35 mg.) were stirred under nitrogen for two days in benzene-ether (1:1) giving, besides material at the origin, a compound more mobile than chimonanthine (R_f 0.8, alumina, methanol-ether 1:20). The ultraviolet spectrum in ethanol: $\lambda_{max.}$ 248, 305 mμ indicated no change in the indoline chromophore.

Physical properties of hodgkinsine

Colourless needles, m.p. 126-134° C. lit. 128° C.[88] The ultraviolet spectrum in ethanol: $\lambda_{max.}$ 246, 306 mμ, $\epsilon_{max.}$ 13000, 6400. The n.m.r. spectrum in deuterochloroform: multiplet from 2.8 to 3.8 τ (aromatic protons, area 10 H), broadened singlet centered at 4.12 τ (aromatic proton, area 1 H), broadened singlets centered at 5.33 and 5.88 τ (N-C<u>H</u>-N and R_2N-<u>H</u>, area 2.7 and 2.9), a series of multiplets from 7.0 to

8.3 τ under three singlets at 7.59, 7.63 and 7.71 τ (methylene and R_2-N-$\underline{CH_3}$ protons, total area 21 H (12+9)). The mass spectrum was most instructive with m/e 518 (M^+, 7%), 519 (M + 1, 2%), 358 (M - 160, 2%), 344 (M - 174, 23%), 345 (M - 173, 10%), 346 (M - 172, 10%), 314 (M - 174 - 30, 3%), 302 (M - 174 - 42, 7%), 287(M - 174 - 57, 3%), 271 (M - 174 - 73, 5%), 259 (M - 174 - 85, 7%), 245 (M - 174 - 99, 4%), 186 (M - 174 - 158, 8%), 172 (M - 346, 100%), 143 (30%), 130 (22%). The abundances quoted may not be accurate as the intensity of the parent peak was not accurately known.

Alkaloids from the seeds of Calycanthus floridus

Calycanthus floridus L. seeds (50 g.) purchased from the F.W.Schumacher Co., Sandwich, Mass., U.S.A., were ground in a Waring blender and triturated with light petroleum ether (3 x 200 ml.) yielding a quantity of light yellow oil (13.5 g., 27%). The remaining solid was triturated with chloroform (3 x 200 ml.) then extracted in a soxhlet apparatus. On evaporation of the chloroform extracts a yellow oil was obtained (2.2 g., 4.3%) and examined by thin-layer chromatography (ether, alumina, ceric sulphate). Aside from the oil at the solvent front, and a very minor spot at the origin calycanthine (major alkaloid) and calycanthidine (R_f 0.35) were detected. No chimonanthine or other components corresponding to minor products of the coupling reaction were detected. After standing for several days calycanthine crystallized from the oil.

Attempted resolution of dl-chimonanthine

All attempts to resolve synthetic dl-chimonanthine by crystallization of the mono- or di-salts of d-10-camphor-sulphonic acid were unsuccessful. Resolution attempts as salts of tartaric acid were also unsuccessful. Regeneration of the free bases was conveniently accomplished by washing the salts through an alumina column with ethanol.

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PART B

THE ORIGIN OF THE NON-TRYPTOPHAN DERIVED PORTION OF THE INDOLE ALKALOIDS

Introduction

The relationship of tryptophan to the indole alkaloids as discussed in the general introduction is almost self-evident and has been conclusively demonstrated.

Tryptophan-2-^{14}C (1) has been found to be incorporated by Rauwolfia serpentina into ajmaline (2), serpentine(3), and reserpine(4).[1,2] Systematic degradation of the ajmaline[2] and serpentine[3] showed that the radioactivity was confined to C-5 as expected. The degradation of ajmaline (2) was accomplished by fusion with soda lime[4] yielding N_a-methylharman (5) which was converted to its methiodide and reduced with sodium borohydride to a tertiary base (6). Reduction (sodium in liquid ammonia) of the methiodide of the tertiary base (6) gave a single compound in high yield identified as 2-ethyl-1-methyl-N,N-dimethyltryptamine (7). Hofmann degradation of this compound yielded a 3-vinylindole (8) in excellent yield which was converted to a diol with osmium tetroxide and cleaved with periodate to yield 2-ethyl-3-formyl-1-methylindole (9) and formaldehyde which was isolated as its dimedone derivative. The indole derivative was inactive but formaldehyde dimedone derivative and all other intermediates in the degradative scheme had essentially the same specific activity as the ajmaline which indicated that the ajmaline was labelled solely at C-5.

Dl-Tryptophan-3-^{14}C (10) has been incorporated (6.8%) into ibogaine (11) by five month old Tabernanthe iboga plants.[5] The location of the active carbon atom was established as C-7 by

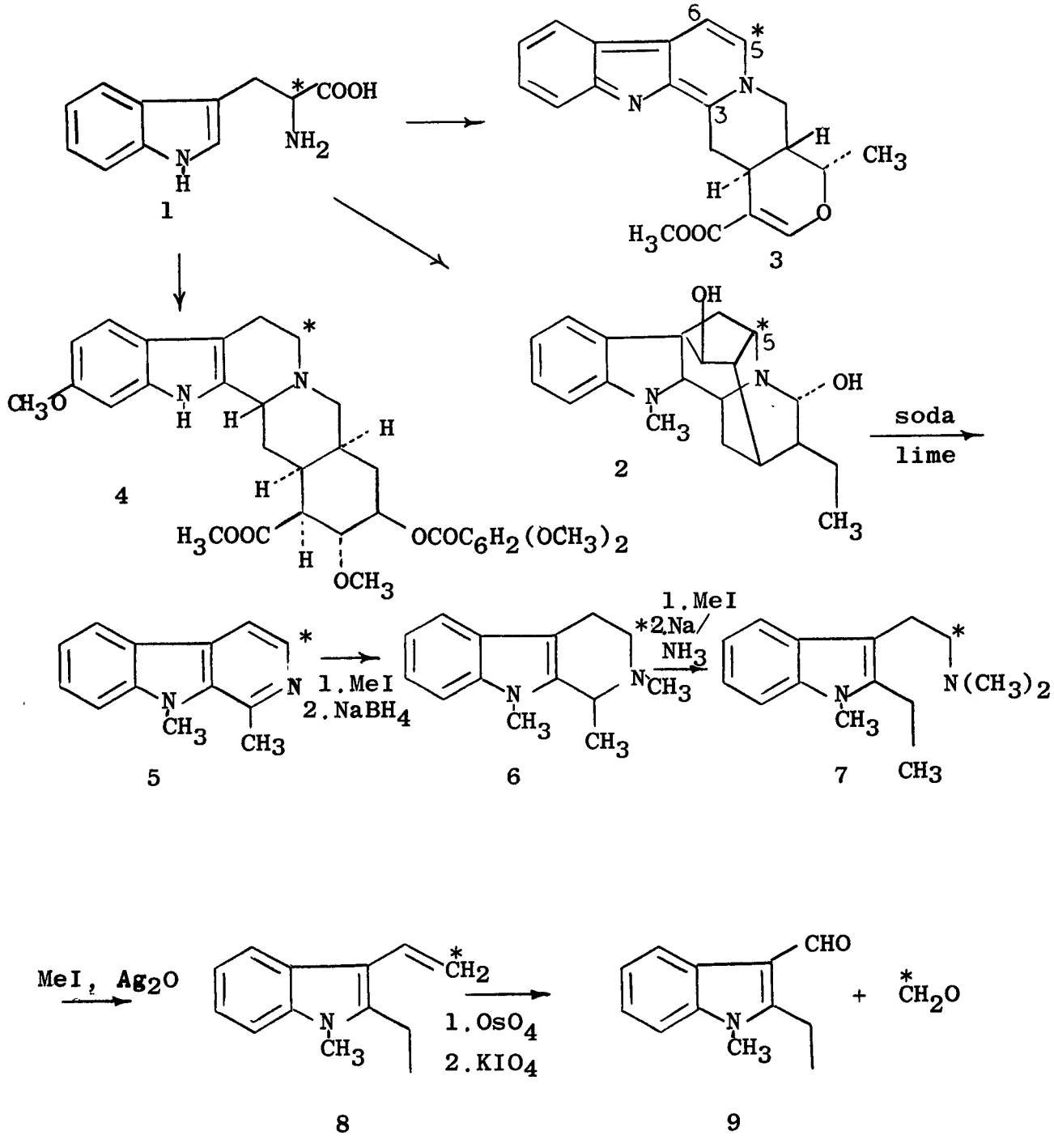


Figure 1. Incorporation of Tryptophan into the Rauwolfia Alkaloids.

degradation. Fusion of the alkaloid with a mixture of sodium and potassium hydroxide yielded 1,2-dimethyl-3-ethyl-5-hydroxy-indole (12) which was oxidized with aqueous 80% chromium trioxide affording a mixture of labelled propionic and un-labelled acetic acids. After separation by chromatography the propionic acid was oxidized with potassium dichromate in 18 N sulphuric acid to acetic acid which was then subjected to a Schmidt reaction affording inactive methylamine and carbon dioxide with 80% of the activity of ibogaine.

Radioactive vindoline has recently been isolated from Vinca rosea plants which had been fed DL-tryptophan-2-^{14}C with 0.32% incorporation.[6,7] A systematic degradation established that essentially all of the activity was located at C-10.[7] The key step of the degradation was the formation of N_a-methylnor-harmine (14) by soda lime distillation of vindoline.[8] The degradation was then identical to that already described for ajmaline[2] (figure 1) and the formaldehyde isolated as its dimedone derivative had essentially the same activity as the active vindoline (13).

The biogenesis of the non-tryptophan, nine or ten carbon, portion of the indole alkaloids was less obvious and has long been the subject of speculation. Several elaborate theories have been promulgated. The initial tracer studies tended to confuse rather than clarify the matter.

The Barger-Hahn hypothesis concerning the biogenesis of the carbon skeleton of yohimbine (19) was advanced concurrently with

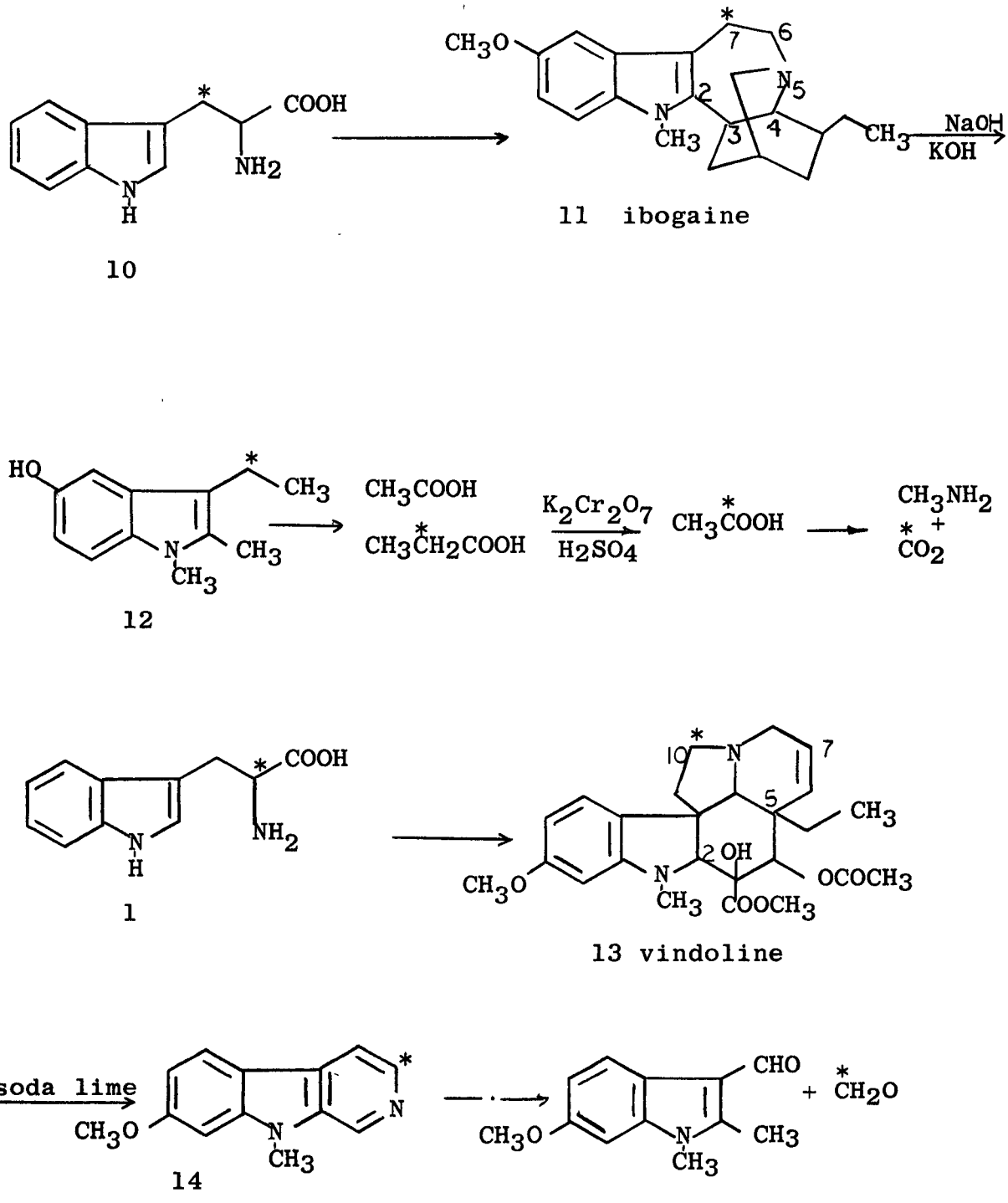
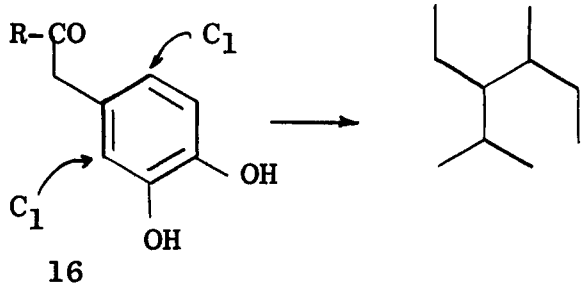


Figure 2. Incorporation of Tryptophan into Ibogaine and Vindoline.

the structural elucidation of that alkaloid.[9-11] They considered yohimbine (19) to be derived from tryptamine (15), tyrosine as m-hydroxyphenylacetaldehyde (16, R = H) or its equivalent and formaldehyde or its one carbon equivalent. This proposal was supported by condensation of tryptamine with aldehydes and enolic α-keto acids to yield carboline derivatives under physiological conditions.[10,12] The aromatic analogue (18) of yohimbine was also synthesized in this way.[11] A difficulty encountered by this suggestion is that ring E would be aromatic whereas it is alicyclic in yohimbine and most related alkaloids. The proposal gained considerable support, however, as a consequence of the attractive suggestion of Woodward.[13] This related pathway involves condensation of 3,4-dihydroxyphenylacetaldehyde (16a, R = H) or its equivalent with the β-position of tryptamine (15), and reaction with a one carbon unit to give 25. Addition of an anionoid C-2 to the α position of the indoline and fission of the carbocyclic ring between the hydroxyl-bearing atom C-3 and C-4 with incorporation of an acetate unit (not necessarily in the order shown in figure 3) could simply explain the origin of strychnine (28). This type of ring cleavage named after Woodward then played a major role in theories dealing with the biogenesis of other alkaloids including cinchonine (23a), quinine (23b), ajmaline (2), etc..[14,15]

Woodward recognized that the carbon skeleton of ajmaline (2) and cinchonine (23a) could be derived from a compound 20 by Woodward cleavage of ring E of the aromatic analogue of

a. Schematic.



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b. Biogenesis of Yohimbine, Ajmaline and Quinine

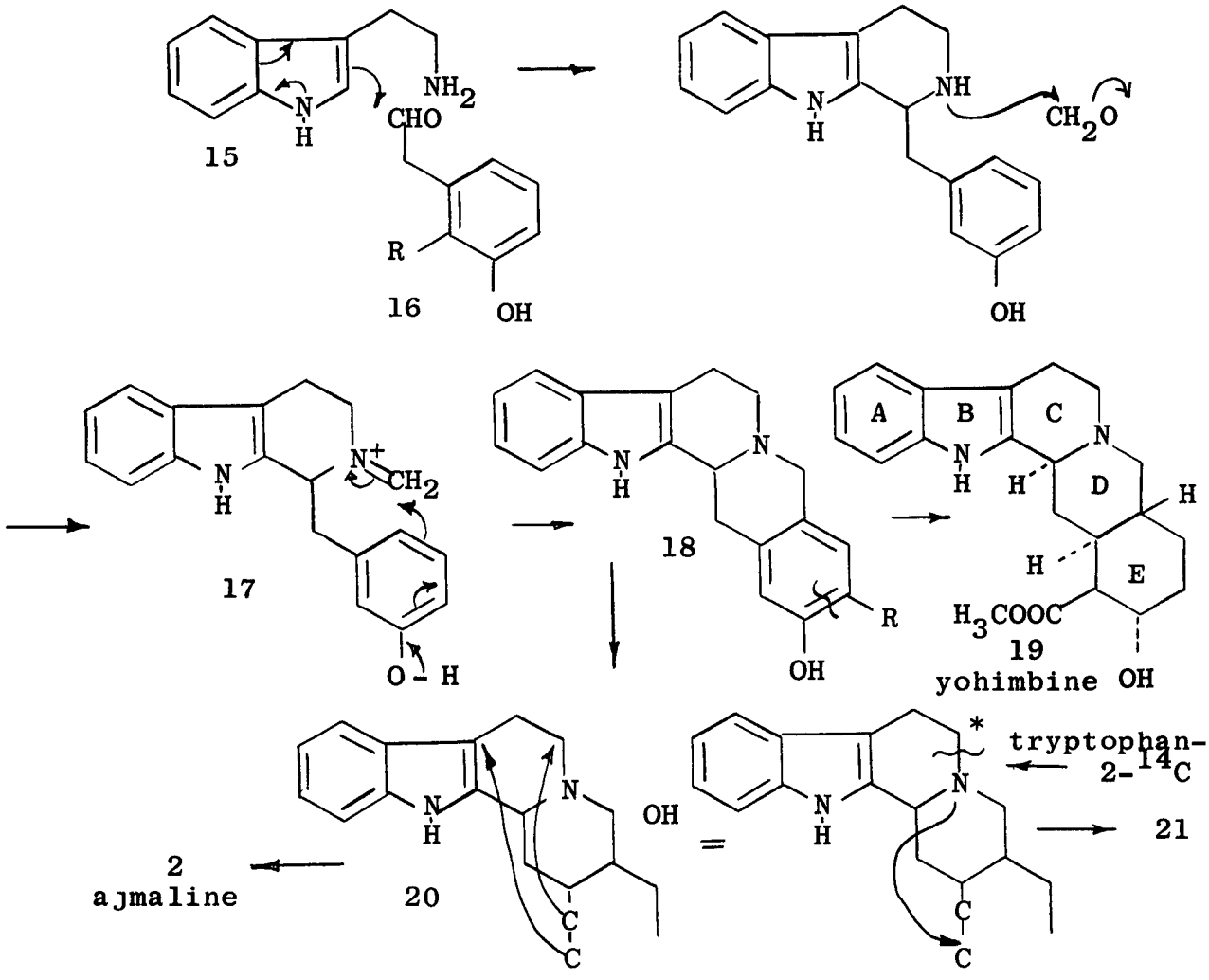
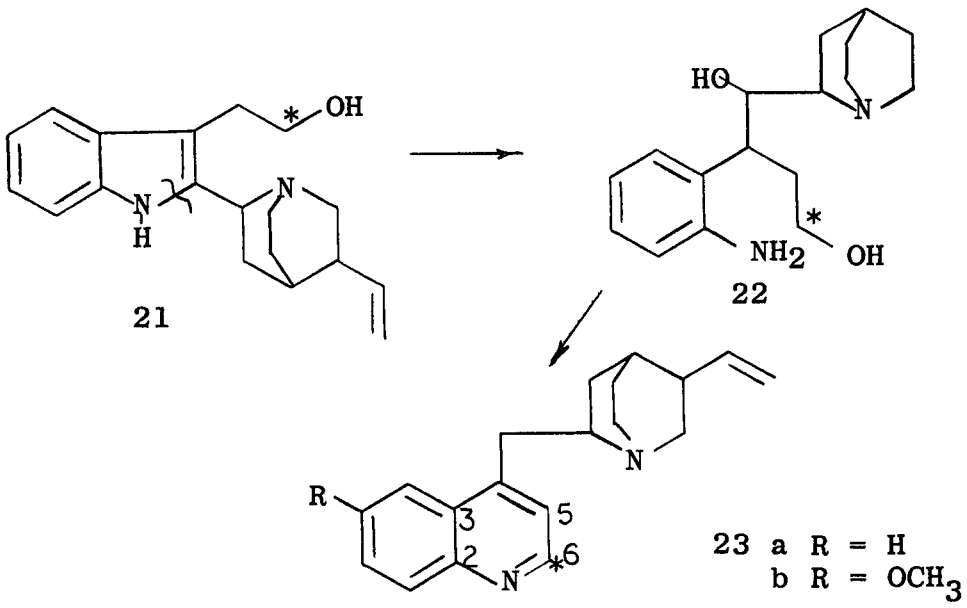
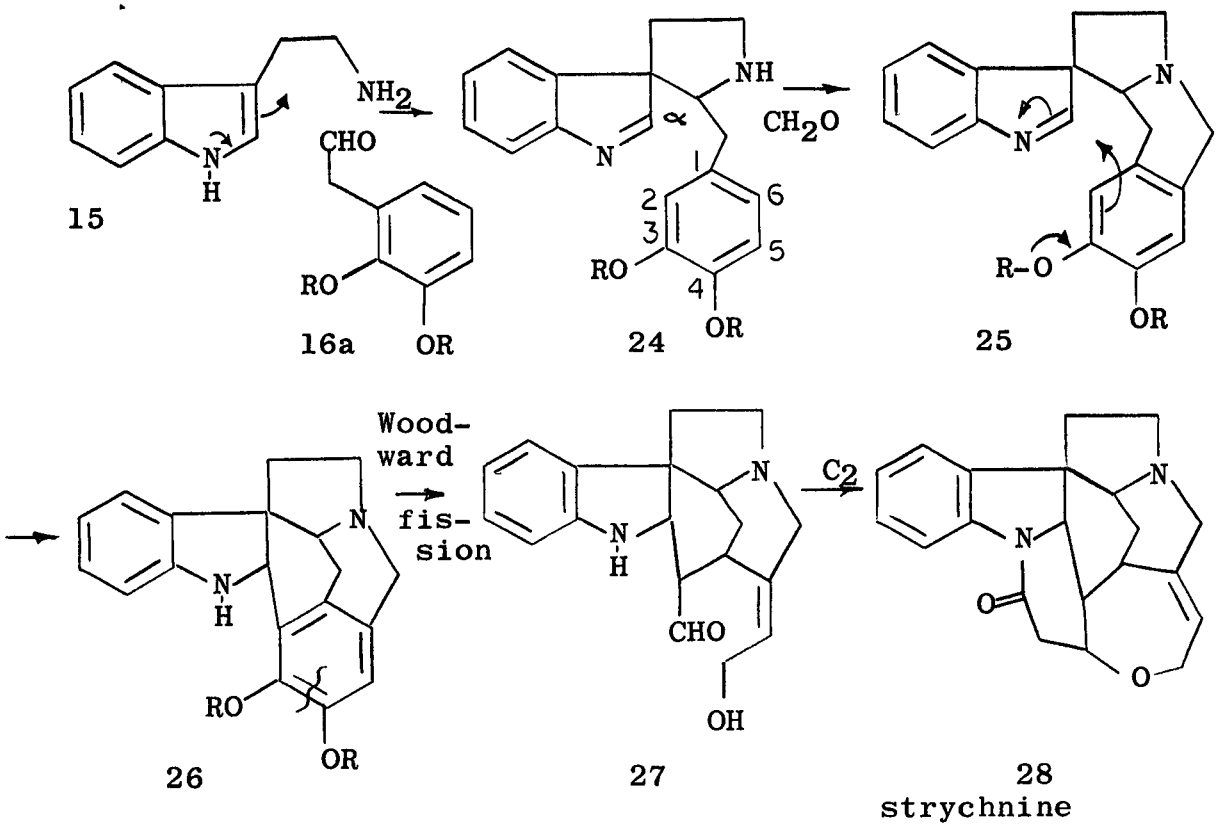


Figure 3. Barger - Hahn - Woodward Theory.

Figure 3. continued.



c. Suggested Biogenesis of Strychnine.



yohimbine (18).[14] Ajmaline is derived by the hydroxylation and condensation of ring E with the carboline ring as shown in figure 3. It was further suggested that compound 20 could also undergo ring cleavage and ring coupling to yield cinchonamine (21), cinchonamine, by a third cleavage and coupling, yielding the cinchonine, quinine (23) skeleton. Although cinchonine (23a) and quinine (23b) are quinoline alkaloids tryptophan-2-^{14}C (1) is in fact incorporated into quinine by Cinchona succirubra and after degradation all of the radioactivity was found at C-6[17] as predicted by theory.[14]

Robinson[15] elaborated the Barger-Hahn scheme to account for the presence of the extraneous carbomethoxy group in ring E of yohimbine. He suggested expansion of aromatic ring E to a tropolone which could then collapse with extrusion of the carbomethoxy group.

A study of the stereochemistry of the indole and phytochemically related alkaloids by Wenkert and Bringi[18] led to the observation that carbon-15 was invariably of one configuration. ψ-Akuammicine[19] was until recently the only known exception. Wenkert and Bringi then drew attention to the deficiencies of the Barger-Hahn biosynthetic model and proposed an elegant alternative. The three interdependent difficulties raised by the older theory are: (a) the state of oxidation of ring E, (b) the absolute configuration of C-15 and (c) the origin of the carbomethoxy group. With the exception of alstonine no yohimbine type compounds possess aromatic ring E, and all have

asymmetric centers at the D/E ring juncture except alstonine and sempervirine. Furthermore, reduction of a phenol by natural systems has never been observed. Finally, the lack of clarity regarding the expansion of phenolic ring E to a tropolone and the mechanistic unlikelihood of the contraction of the latter to a β- rather than an α-conjugated ester make the origin of the ester group very vague.[18]

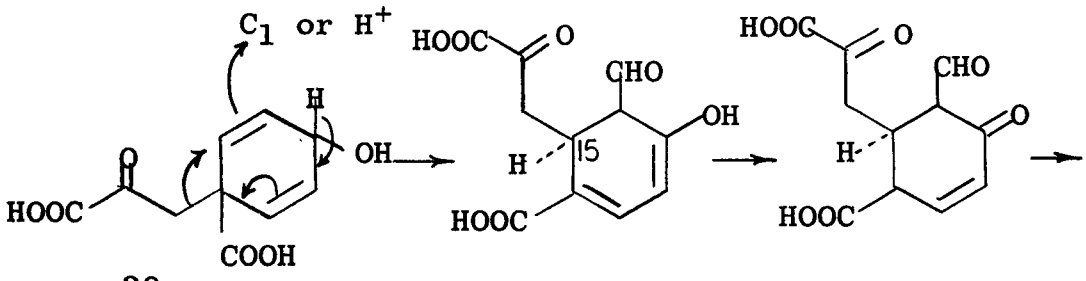
A carboxysubstituted hydroaromatic moiety was then suggested as a substitute for phenylalanine as a precursor of the yohimbine skeleton. Davis had recently demonstrated that shikimic (43, page 25) and prephenic acids (45, page 25) were the progenitors of the aromatic amino acids.[21] A hydrated prephenic acid was then considered as the crucial intermediate from which most indole bases were derived and by which their absolute configuration is determined.[18,22] However the lack of intervention of such hydrated forms in the microbiological conversion of shikimic to prephenic acid[23] suggested that prephenic acid was itself the direct progenitor of these alkaloids.[24]

Rearrangement of prephenic acid (45, page 25) (29) by a 1,2-shift of the pyruvate residue and condensation with a C-1 equivalent followed by hydration affords a unit (30) readily discernible in yohimbine (19). While the rearrangement of prephenic acid might be expected to yield racemic products (i.e. ψ-akuammmine (34)) enzymatic intervention at this point could be responsible for the specific absolute configuration in the natural products. Seco-prephenate-formaldehyde (S.P.F.) (31)

could then arise by a retro-aldol reaction. Condensation with tryptamine through an intermediate (36) could then yield alkaloids such as corynantheine (32), ajmalicine (33), sarpagine (35), and ajmaline (2) as outlined in figure 5. The strychnine skeleton (e.g. 34) could similarly be derived from 36 by attack of the formylacetate group at the α-position of the indole followed by transannular cyclization to the iminium ion (37).

A mechanism by which the Aspidosperma (13, 61) and Iboga (11, 62) alkaloid skeletons could be derived from a tryptamine-S.P.F. unit (36, 37) was also described by Wenkert in the later paper.[24] These structurally more complex systems require rearrangement of the S.P.F. unit. This rearrangement could be visualized as proceeding via a retro-Michael reaction of the intermediate 37 resulting in a formyl acetate moiety attached to the α-position of the indole (38). Michael reactions after appropriate changes in oxidation level could result in compounds 41 and 42 which are very similar to the natural alkaloids quebrachamine (45) and cleavamine (46). Internal Mannich reactions (transannular cyclization) as shown in figure 5 could then yield the closed Aspidosperma and Iboga carbon skeletons (43 and 44).

An interesting aspect of the transannular cyclization reactions 41 to 43 and 42 to 44 as postulated by Wenkert for formulation of Aspidosperma and Iboga carbon skeletons in nature, is that model reactions have been accomplished. Kutney and co-workers[25,26] in this department effected an aspidosperma-



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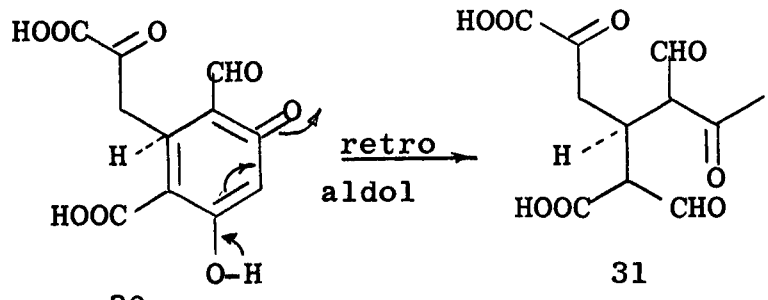

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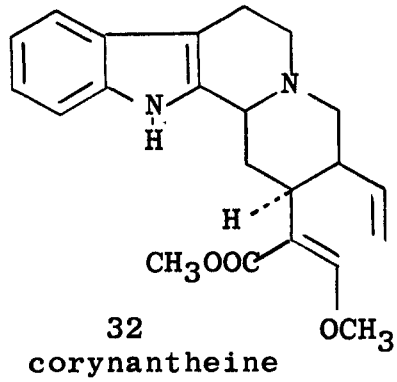
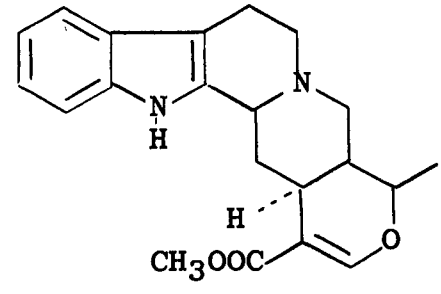
31

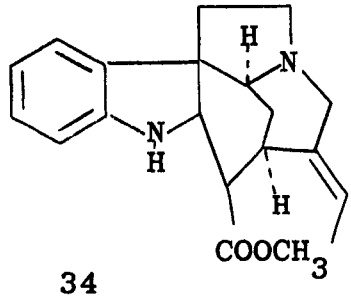
seco-prephenate-formaldehyde
(S.P.F.)



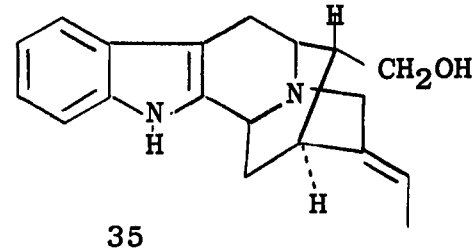
32
corynantheine



33
ajmalicine



34
akuammicine
(ψ = racemic)



35
sarpagine

Figure 4. Wenkert-Bringi Hypothesis-S.P.F. Unit.

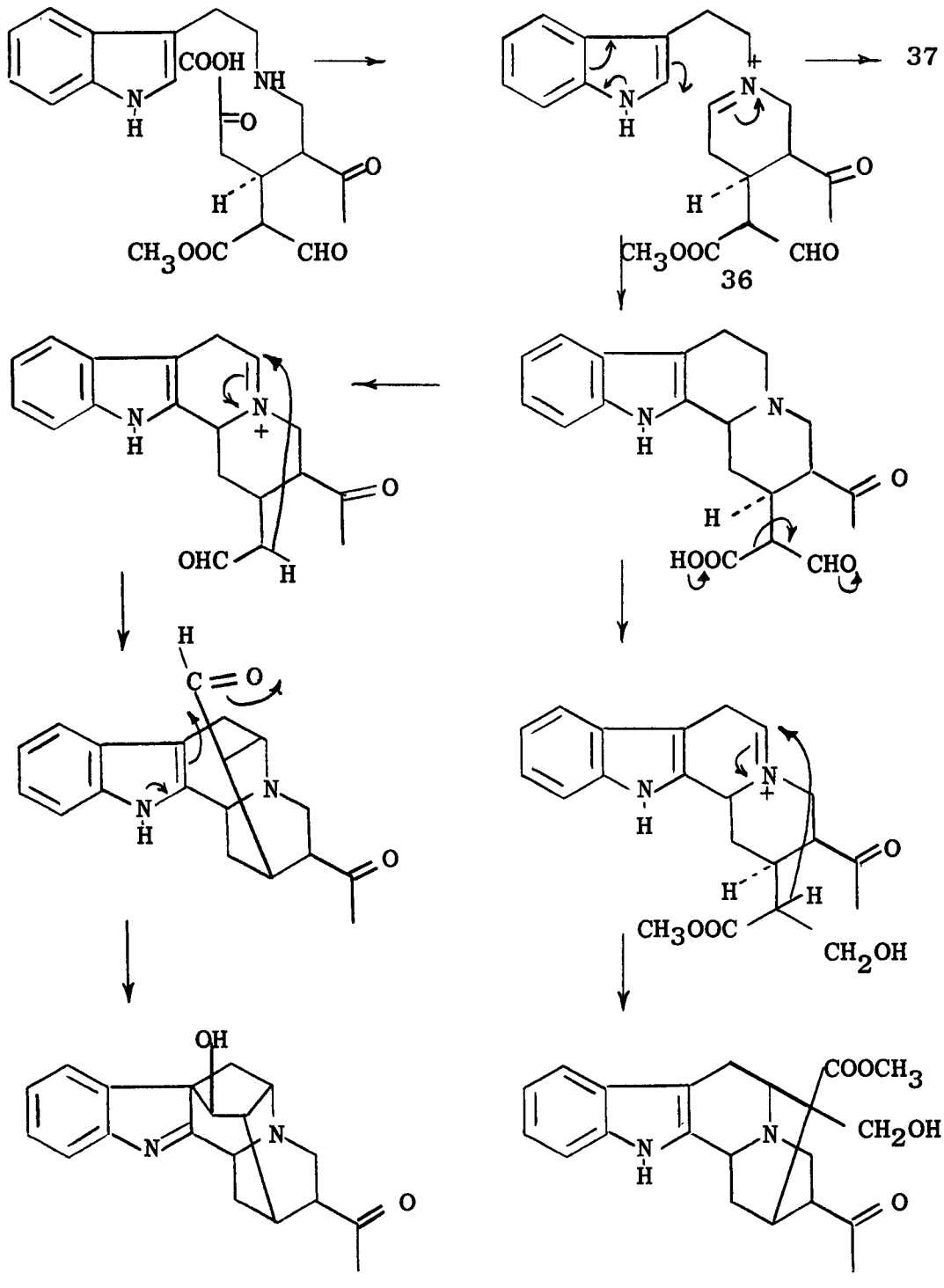
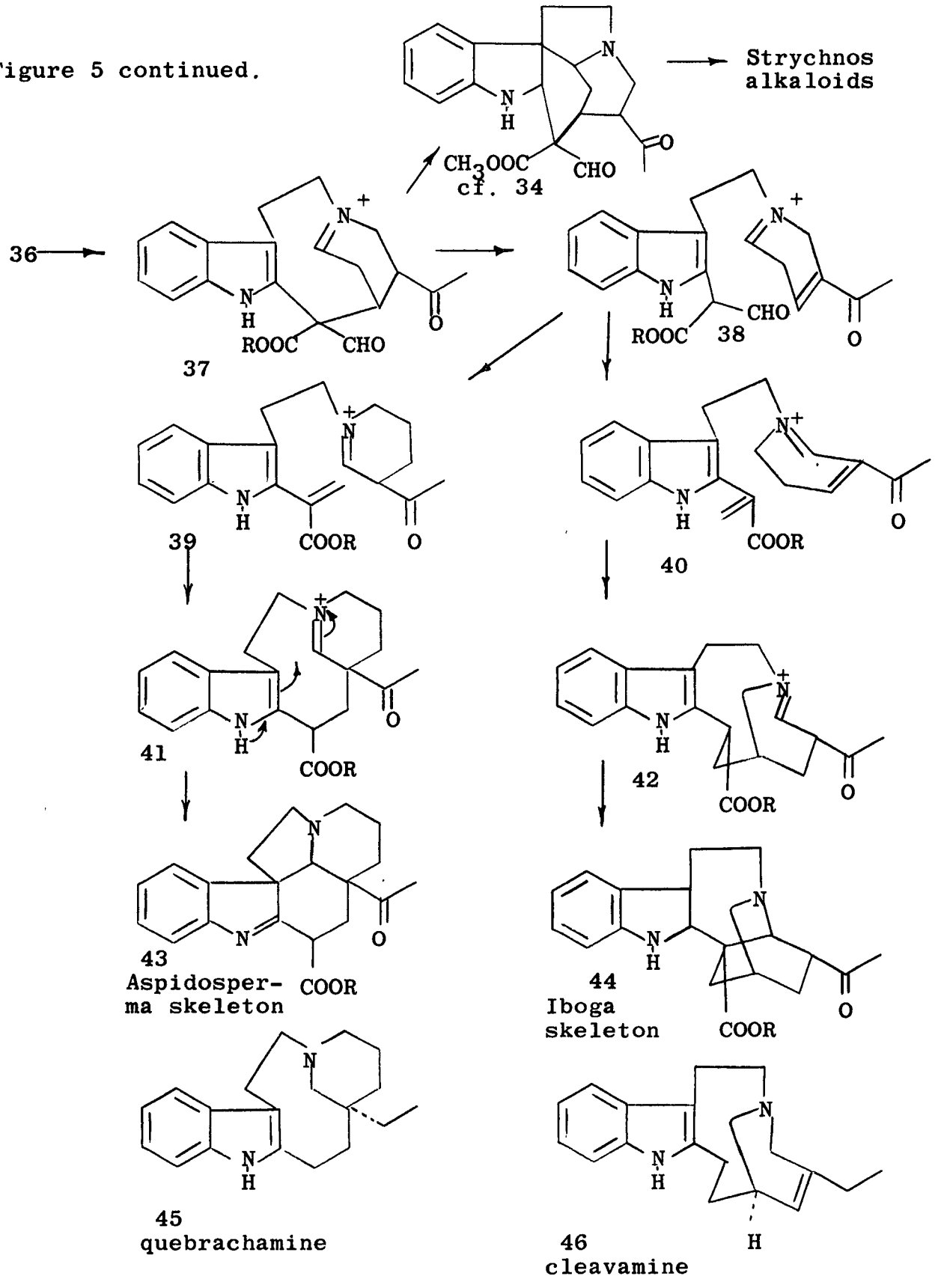


Figure 5. Condensation of S.P.F. and Tryptamine.

Figure 5 continued.



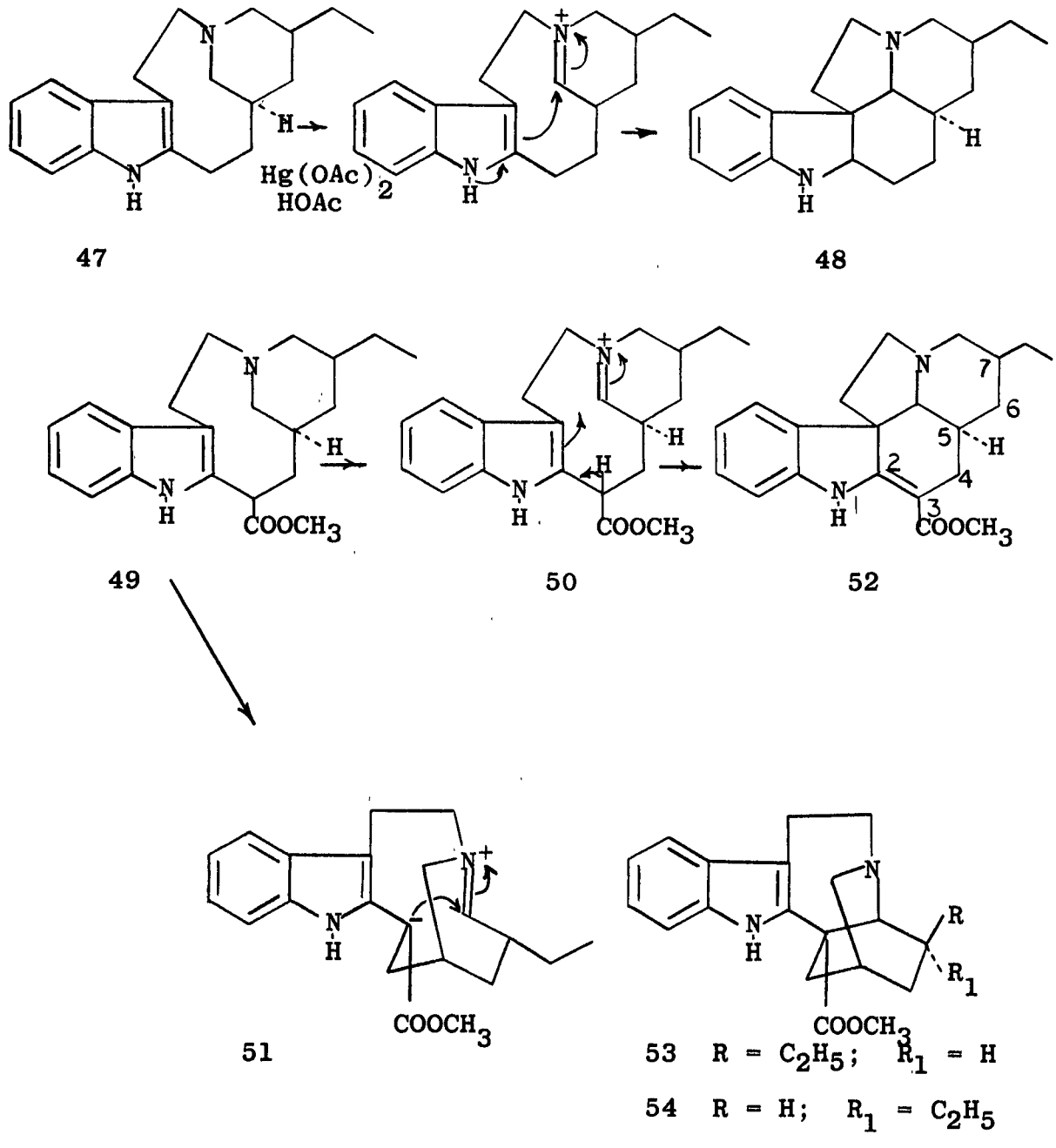


Figure 6. *In vitro* Transannular Cyclization.

like transannular cyclization on dihydrocleavamine (47) with mercuric acetate in acetic acid. Carbomethoxydihydrocleavamine (49) when oxidized with mercuric acetate to alternative iminium salts 50 and 51 cyclizes to 5-desethyl-7-ethylvincadifformine (52) and to the ibogaine-like bases coronaridine (53) and dihydrocatharanthine (54).

A third theory based on structural relationships was independently proposed by Thomas[27] and Wenkert[24] in 1961. It was suggested that the non-tryptophan moiety of the indole alkaloids was monoterpenoid in origin. Wenkert[24] noted the structural identity of the carbon skeleton of the glucosides oleuropeine (77),[28] gentiopicrin (78),[29] bakankosin (79),[30] and swertiamarin (73)[31] with his seco-prephenate-formaldehyde unit (31). He also noted that cleavage of the cyclopentane ring of the monoterpenic glucosides, verbenalin (63),[30] genipin (64), [32] aucubin (65),[33] and asperuloside (66)[34] at a specific bond would give an S.P.F. type of carbon skeleton with the required absolute configuration of the non-tryptophan part of the indole alkaloids. Hendrickson[35] when discussing these monoterpenes at a later date ascribed one reasonable mechanism for this ring opening process. (Figure 8; 71 ⟶ 72). The possibility that these glucosides were prephenate derived was also suggested by Wenkert. The aromatic ring E of yohimbine (19) would be formed by ring closure as opposed to the preformed ring of the Barger-Hahn and prephenic acid theories. The proposed mode of formation of Aspidosperma (61) and Iboga (62)

skeletons would be the same as for the later stages of the prephenic acid theory. (Figure 5).

Thomas[27] also proposed that the biosynthesis of gentianine (75),[36] oleuropeine (77), swertiamarin (73) and bakankosin (79) was related to that of the non-tryptophan moiety of the indole alkaloids and pointed out that this concept (requiring the combination of tryptamine with a monoterpene) would be consistent with the established mode of biosynthesis of the ergot alkaloids. Tryptophan and mevalonic acid were known to be precursors (see page 18) of these mould alkaloids. According to Thomas the ten carbon unit would be derived from two units of mevalonate (55) via a cyclopentanoid monoterpene (59) in such a way that C-21 of yohimbine (19) would correspond to C-5 of the precursor (carbonyl carbon of acetate).[27] The carbomethoxy group (C-22) would be derived from either C-2 or C-3a of mevalonate (methyl carbon of acetate) and neither of these ring E substituents would be derived from one-carbon units as was required by the other hypothesis. (Figure 7). Aromatic ring E would be formed in a manner analogous to that involved in the cyclization of swertiamarin (73) to erythrocentaurin (74) as observed by Kubo and Tomita.[31] Thomas also recognized the cyclopentanoid monoterpene structure (59) in the alkaloids actinidine (70),[37] skytanthine (69),[38] and the ring opened structure 60 in the Ipecacuanha alkaloid emetine (76).[39] Aucubin (65) is an example of a nine carbon monoterpene. It differs from the skeleton of genepin (64) in that a carbomethoxy group is missing. This

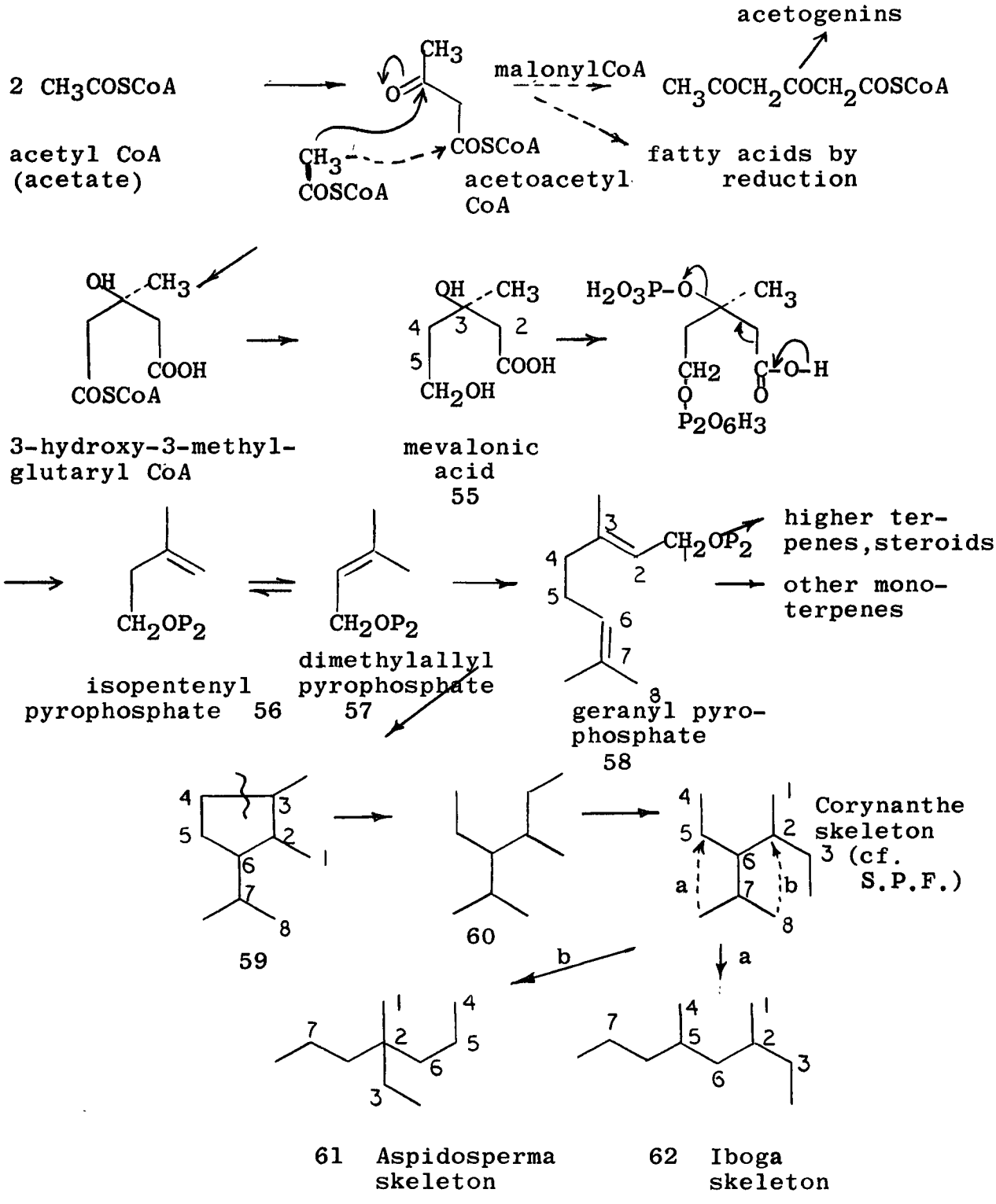
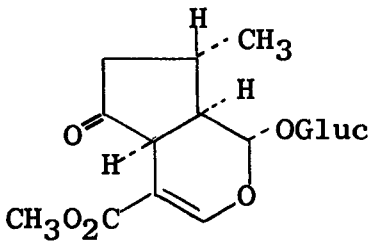
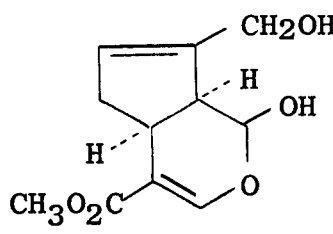


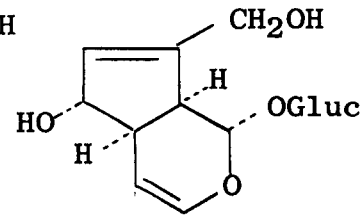
Figure 7. Biogenesis of Geranyl pyrophosphate and the Thomas-Wenkert Theory.



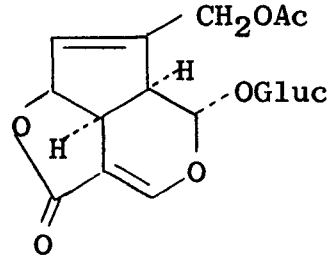
verbenalin
63



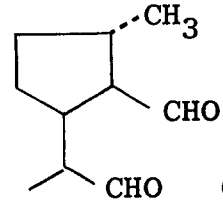
genepin
64



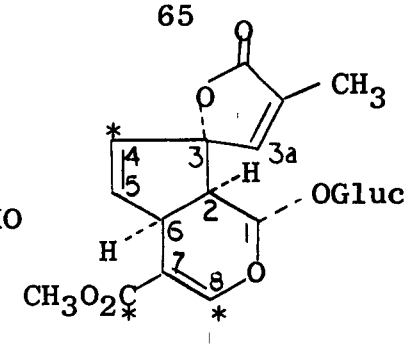
aucubin
65



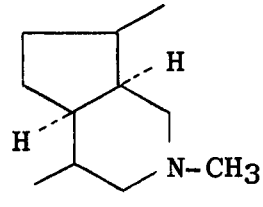
asperuloside
66



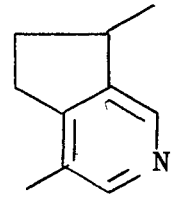
iridodial
67



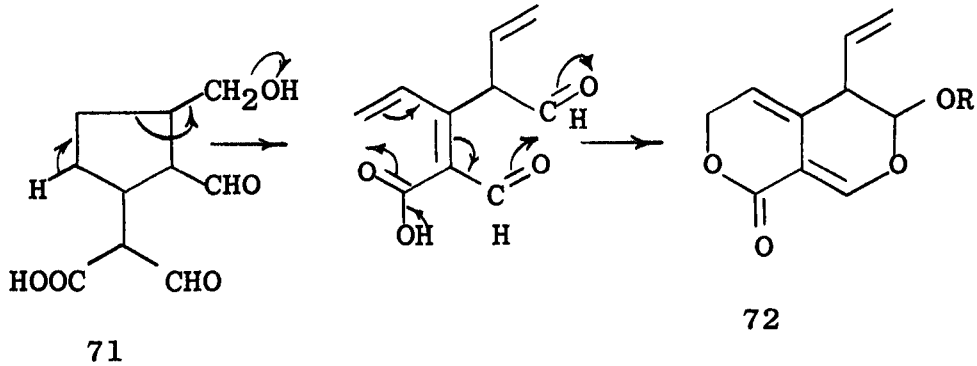
plumieride
68



skytanthine
69



actinidine
70



71 72

Figure 8. Cyclopentanoid Monoterpenes.

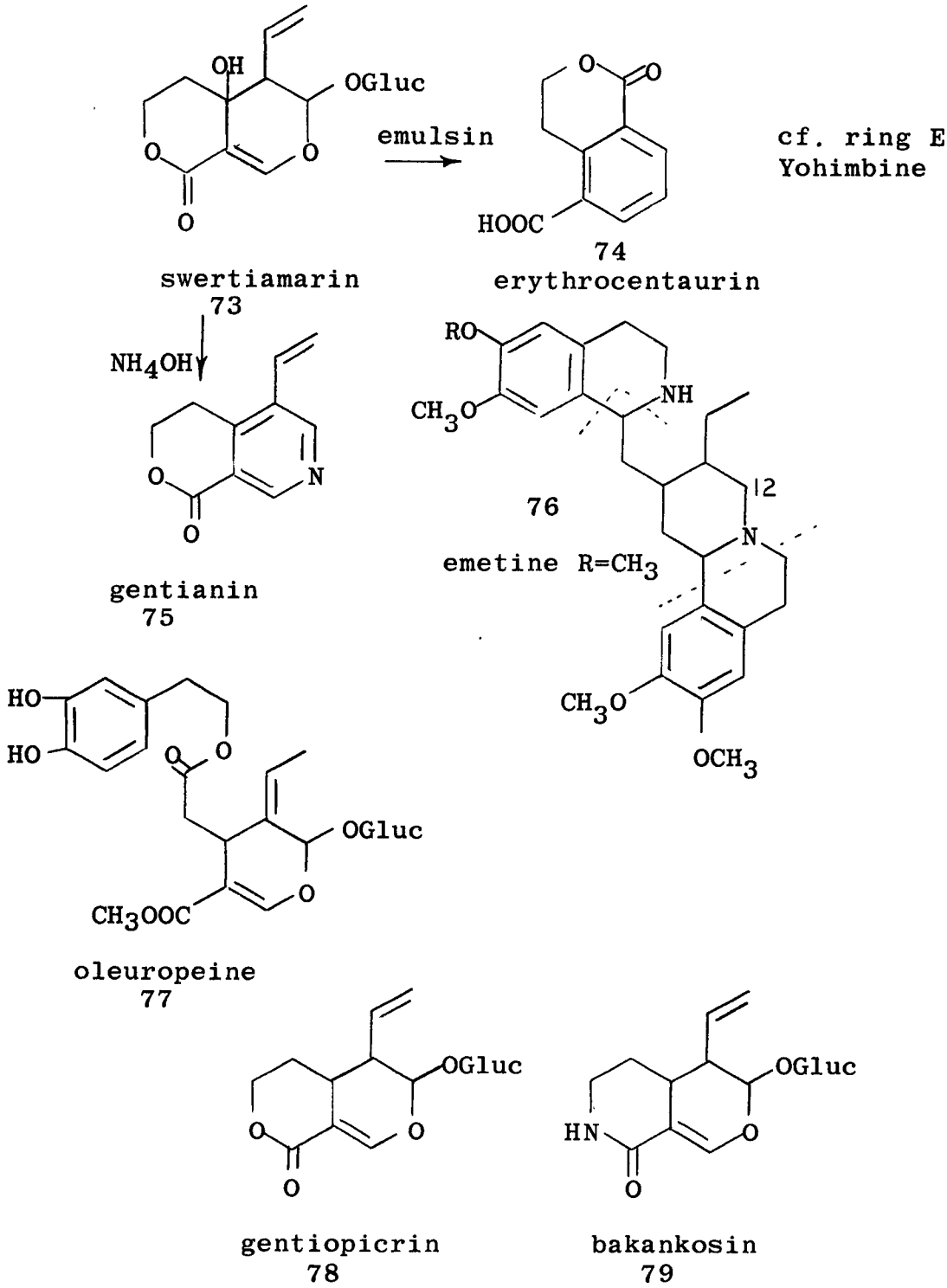


Figure 9. Some Monoterpenes with the "Corynanthe" Carbon Skeleton.

carbomethoxy group is presumably the same as that which is often present in the indole alkaloids.

The biosynthesis proposed for iridodial (67), involving the cyclization of citronellal (cf. 58→59), has been simulated in the laboratory.[40] Yeowell and Schmidt have recently investigated the biogenesis of the cyclopentane-monoterpene glucoside plumieride (68)[41] by feeding of mevalonolactone-2-^{14}C. Radioactivity was found at C-4 and C-7a (Figure 8) as predicted from the cyclization of geranyl pyrophosphate (58).

A fourth possibility for the origin of the non-tryptophan portion of the indole alkaloids was proposed by Schlittler and Taylor in 1960.[42] The suggestion was that the relevant precursor might be formed by condensation of an open chain six carbon acetate unit, a one carbon unit and a three carbon unit. (Figure 10).

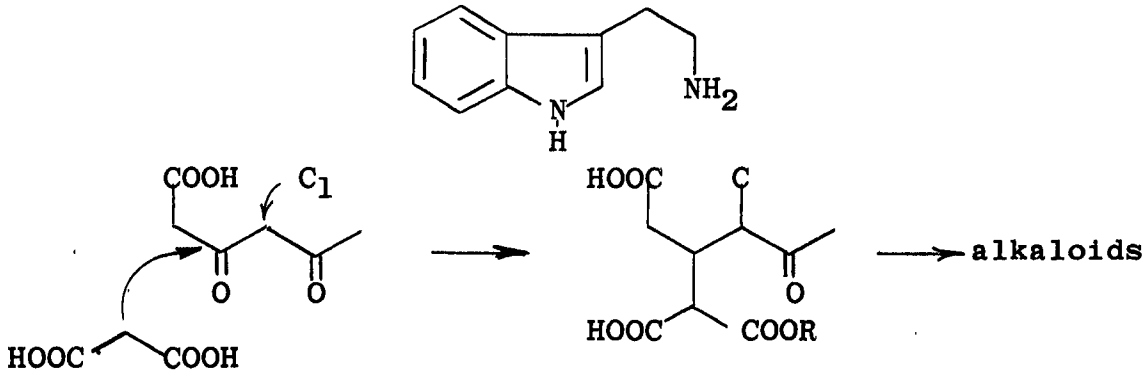


Figure 10. Schlittler-Taylor-Leete Hypothesis.

A series of feeding experiments using Rauwolfia serpentina carried out by Leete seemed to eliminate the Barger-Hahn, the prephenic acid, and the monoterpene theories for the biogenesis of the indole alkaloids. These experiments give results consistent only with the Schlittler-Taylor "acetate" hypothesis.

When sodium formate-^{14}C was fed to R.serpentina it was reported[43] that C-21 of ajmaline (2) became labelled (12% of activity) in agreement with its derivation from the one-carbon pool of the plants as predicted by all theories except the monoterpene theory. In a second paper[44] it was reported that ajmaline (2) isolated from plants which had been fed mevalonic acid-2-^{14}C (55) or phenylalanine-2-^{14}C was completely inactive. This was evidence against the monoterpene and the Barger-Hahn schemes. Alanine-2-^{14}C was fed to test the prephenic acid hypothesis on the assumption that pyruvate formed from alanine by transamination would be incorporated into the side chain of prephenic acid (29). Phosphoenolpyruvate (see page 25) is the actual precursor however and is apparently not readily formed from pyruvic acid[45] hence low incorporation probably doesn't constitute a valid objection. Only 2% of the activity was in the position (C-3 of ajmaline) predicted by theory. Sodium acetate-1-^{14}C was reported[44] as being incorporated into ajmaline (2) with 26% of total activity at positions 3 and 19, with positions 14, 18 and 21 inactive. This is in perfect agreement with the Taylor acetate hypothesis and in contrast to the monoterpene theory which predicts that the label should have been

in positions 21, 19, 16 and 14. Further support was provided by isolation and degradation of serpentine (3). In a third paper[46] malonic acid-1,3-^{14}C was reported as being incorporated into serpentine (3) with 48% of the total activity at position 22 and also into ajmaline with 74% of the activity at C-17. Both results are in agreement with the Taylor hypothesis. (Figure 10).

Later feeding experiments by Battersby[47] not only failed to confirm the Leete results but were in sharp contrast. After the feeding of sodium formate-^{14}C and the isolation of ajmaline from R.serpentina, it was found that the N-methyl group carried not less than 25% of the activity and C-21 had little or no activity. Cephaline (76, R = H) from C.ipecacuanha fed with sodium formate-^{14}C had 67% of its activity in the O-methyl groups whereas C-12 was essentially inactive. Kuhn-Roth oxidation showed a low scatter of activity in the carbon skeleton. This scatter of activity was also observed with ajmaline (2) isolated from R.serpentina plants fed with sodium acetate-1-^{14}C. Acetic and propionic acids isolated after oxidation had low and different levels of activity. These results were strong evidence against the involvement of a one-carbon unit as predicted by several theories. Fresh experimental evidence was urgently required in view of the conflicting results obtained in initial tracer experiments.

Discussion

In spite of much speculation[14,15,18,22,24,27,42] and experimentation[6,43,44,46,47] the origin of the carbon skeleton comprising the non-tryptophan derived portion of the indole alkaloids remained obscure and the results confused. No hypothesis had been unequivocally supported by feeding experiments. The repeated claim that formation of this nine or ten carbon fragment involved one formate, one malonate and three acetate units[43,44,46] had not been substantiated by other workers[47] who observed scattering of the label when acetate was fed to R.serpentina and C.ipecacuanha. This observation was eventually to be confirmed by the original workers.[7] Research was initiated in our laboratory to test what seemed to us the most attractive theory experimentally.

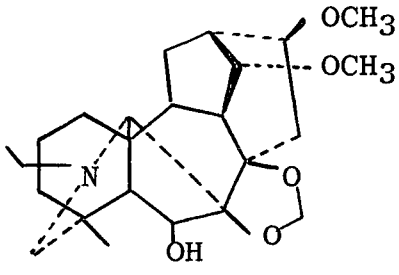
Thomas[27] and Wenkert[24] had proposed that the non-tryptophan portion of the indole alkaloids could be derived from a ten carbon monoterpenoid unit. If this proposal were correct the ubiquitous terpene precursor, mevalonic acid (55), should be utilized in the biosynthesis of indole alkaloids. A radioactive isotopic label in mevalonic acid fed to a plant should be incorporated into the indole alkaloids produced by that plant in a specific manner. Leete, however, had fed active mevalonic acid to R.serpentina[44] and had failed to observe any incorporation of radioactivity into ajmaline (2). On the basis of this negative result and the reported but now discredited specific incorporation of formate, acetate and malonate[43,44,46]

into ajmaline the monoterpene hypothesis appeared untenable.

Kirby,[48] however, found that mevalonic acid was not incorporated by Delphinium elatum plants into the alkaloid delpheline (79) which he recognized as being clearly terpenoid in origin and hence warned that negative experiments should be interpreted with care. The terpenoid origin of delpheline was based on a structural analysis. Postulates concerning biogenetic routes often arise from an inspection of structures seeking common structural units and suggesting possible relationships of these units to simpler natural products (see page 8). The success of this approach has been particularly striking in the large terpene field[35] where many complex and often highly rearranged structures can always be related in terms of reasonable reaction mechanisms to ten, fifteen, twenty or thirty carbon atom uncyclized precursors. These precursors are formed in accordance with the biogenetic isoprene rule by condensation of the five carbon units isopentenyl pyrophosphate (56) and dimethylallyl pyrophosphate (57) which arise by phosphorylation and decarboxylation of mevalonic acid, itself derived from three acetate units. (Figure 7).

The success of feeding experiments depends, among other factors, upon the labelled precursor reaching the site of synthesis at a time when active synthesis is occurring. Success may thus depend on the choice of experimental plant, the age of the plant and the method of feeding. This principle was demonstrated by Benn and May[49] who observed the incorporation

of mevalonic acid-2-^{14}C into lycoctonine (81) and browniine (82) when intact D.brownii plants were fed just before flowering. These workers attributed Kirby's negative results[48] when mevalonate was fed through the cut ends of leaf stalks of young D. elatum plants to the possible confinement of alkaloid formation to the plant roots.



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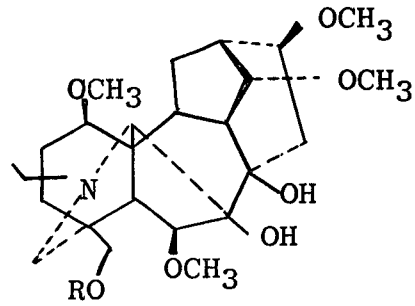
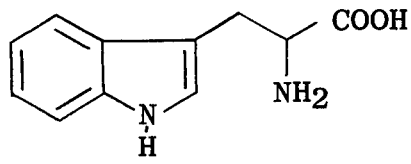
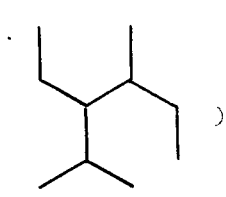


81 R = H
82 R = CH_3

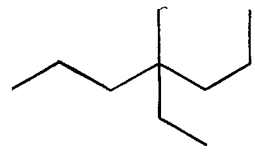
Figure 11. Delphinium Alkaloids.

Structural analysis reveals the astonishing fact that in spite of the many and varied ring systems and the large number of indole alkaloids all of these alkaloids can be classified in terms of three basic patterns for the carbon skeleton of the non-tryptophan portion. These three patterns have been named after botanical families in which representative alkaloids were first discovered. In figure 12 ajmalicine (33) is seen to be representative of the Corynanthe skeleton (60), vindoline (13) of the Aspidosperma skeleton (61) and catharanthine (83) of the Iboga skeleton (62). The structures of vindoline and catharanthine are depicted in such a way as to make this relationship

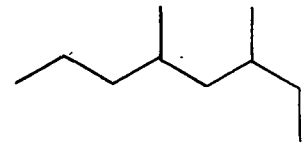




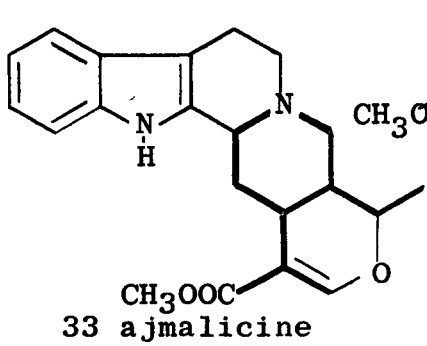
60 Corynanthe



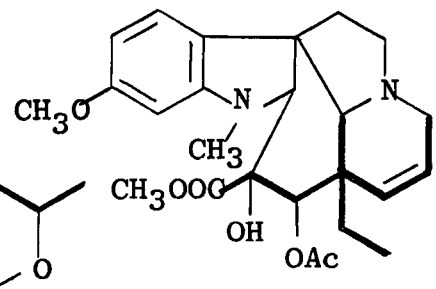
61 Aspidosperma



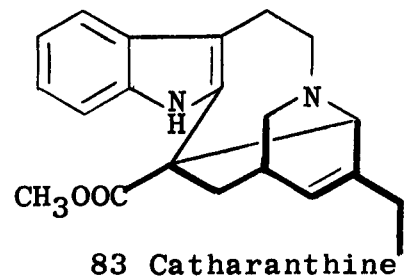
62 Iboga



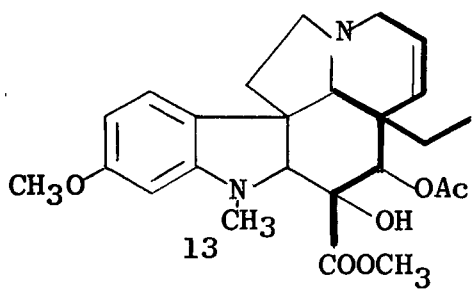
33 ajmalicine
Corynanthe



13 Vindoline
// Aspidosperma



83 Catharanthine
Iboga
//



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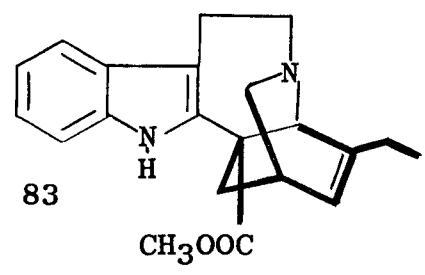
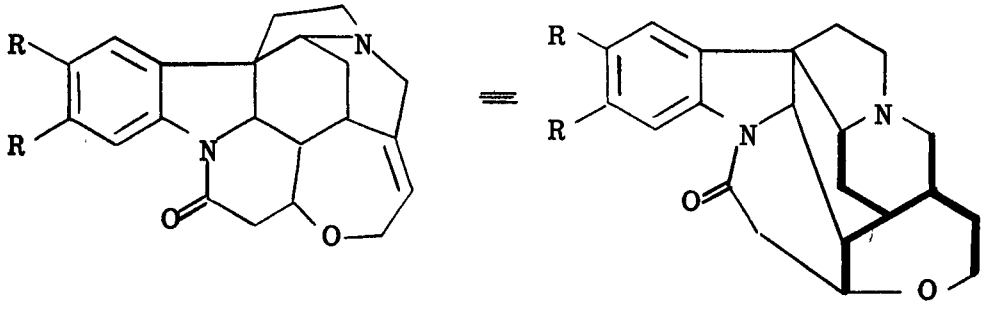
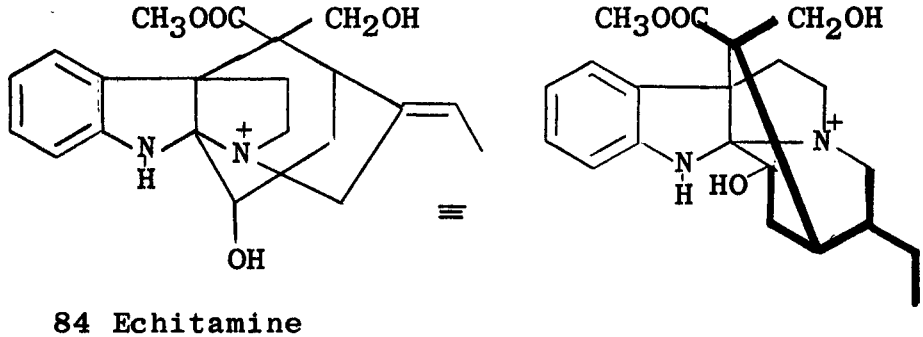


83

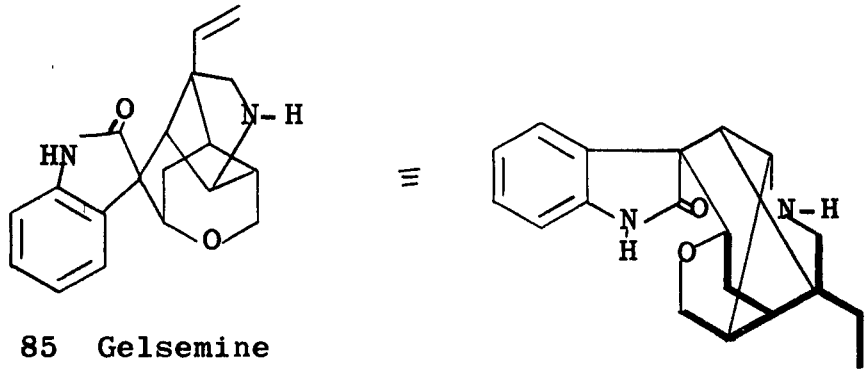
Figure 12. Structural Analysis of Indole Alkaloids.



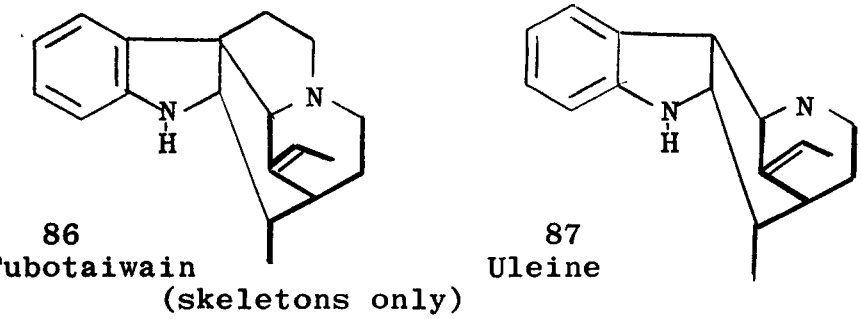
28 Brucine R = OCH_3
Strychnine R = H



84 Echitamine



85 Gelsemine



86 Tubotaiwain

87 Uleine

(skeletons only)

Figure 13. Exotic Corynanthe Ring Systems.

clear. Differences in oxygenation pattern and the absence of the carbomethoxy group account for differences of many indole alkaloids from these three types. Many other alkaloids have quite different ring systems resulting from condensation of the three basic ten-carbon skeletons with tryptophan and with themselves in a variety of ways. The Corynanthe skeleton gives rise to the greatest number of ring systems. A few of these as represented by yohimbine (19), reserpine (4), serpentine (3), ajmaline (2), corynantheine (32), sarpagine (35), and strychnine (28) were encountered in the introduction. The carbon skeletons of some more exotic Corynanthe ring systems are depicted in figure 13. Echitamine (84) and gelsemine (85) are drawn in the usual way and also in such a way as to make the the Corynanthe carbon skeleton obvious. Some of these alkaloids such as apparicine, ellipticine[50] and uleine (87) have apparently lost one or both of the carbon atoms from the tryptophan side chain.

We were fortunate in having close at hand workers experienced in the growth, feeding and extraction of the prolific indole alkaloid producing plant Vinca rosea Linn (Catharanthus roseus G.Don) and are indebted to Dr. Beer[51] for advice and gifts of plant cuttings. Interest in this plant and its relatives (the common periwinkle) has been considerable since the discovery in it of antileukemic alkaloids[51] and has resulted in extensive investigation of the alkaloidal constituents.[52-60] Of some three hundred alkaloids present the structures of more

than fifty are known and represent many structural types. Vindoline (13),[52,54,58] catharanthine (83)[60] and ajmalicine (33) are three of the major alkaloids and possess the Aspidosperma(61), Iboga (62) and Corynanthe (60) skeletons respectively making this plant an excellent choice for biogenetic studies. The structure of vincaleukoblastine (vinblastine) (28)[61] which is one of the antileukemic dimeric alkaloids isolated from V.rosea is on page 15. A discussion of the alkaloids which are found in Vinca species, while a topic of current interest, is beyond the scope of this thesis.

In initial experiments carried out by Dr.I.G.Wright mevalonic acid-2-^{14}C in aqueous solution was administered to freshly cut shoots of Vinca rosea Linn.. By the established biogenesis of monoterpenes from mevalonic acid (55), the monocyclic monoterpene skeleton (59) can be derived and cleavage of a suitably functionalized derivative according to the Wenkert-Thomas theory would yield a fragment (60) labelled as shown. (Figure 15). Utilization of this fragment according to the Wenkert hypothesis[24] would yield vindoline with 50% of the activity at C-8 and 25% of the activity in each of positions 4 and 22. Seven days after administration of 0.30 mc of DL-mevalonic acid-2-^{14}C radioactive vindoline with 0.02% specific incorporation of precursor was isolated and recrystallized to constant activity as its hydrochloride. Desacetylvindoline had essentially the same activity indicating that mevalonic acid was not being degraded to acetate and the label scattered.

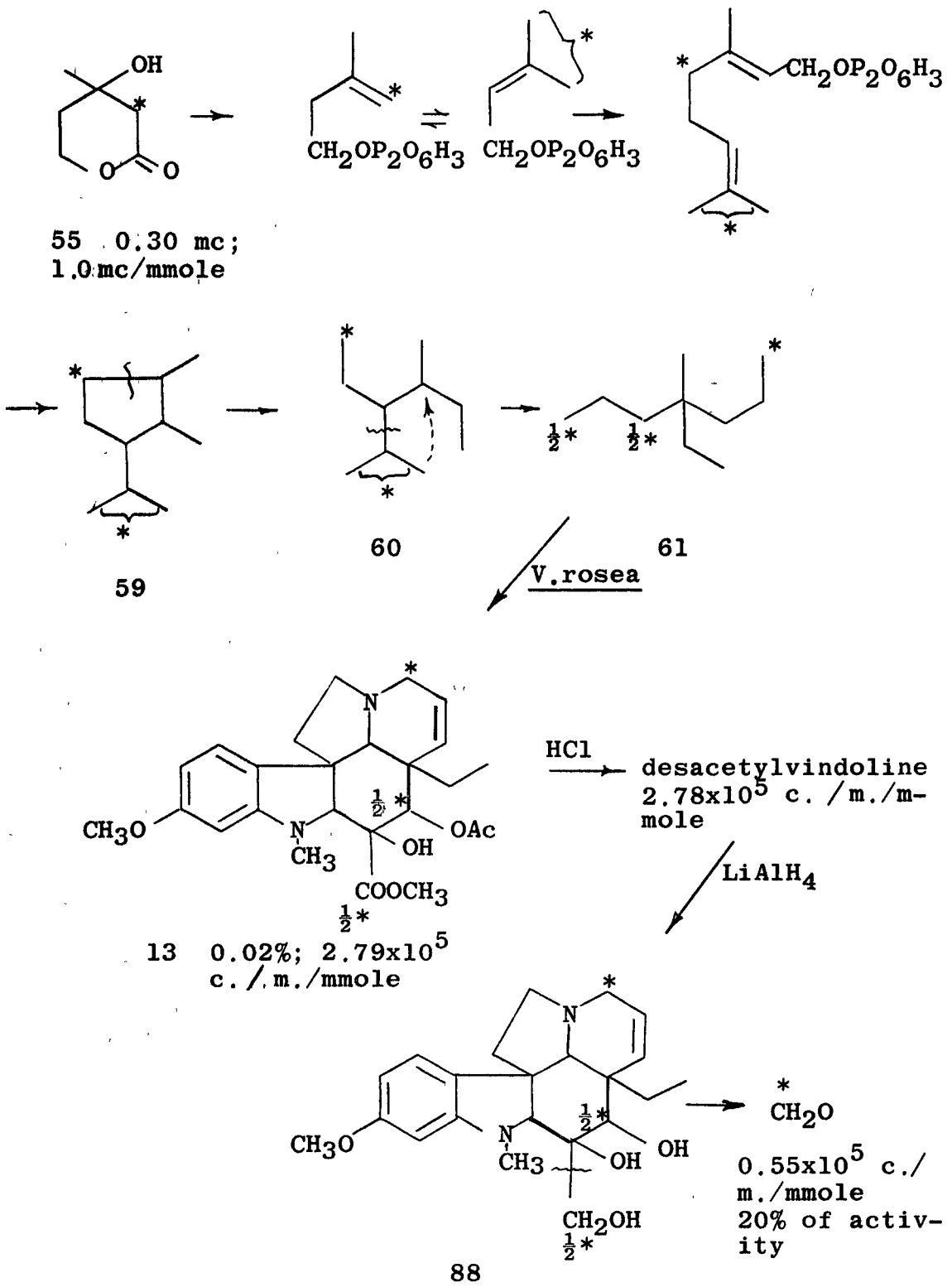


Figure 15. Incorporation of mevalonic acid into Vindoline.

Reduction of desacetylvindoline to vindolinol (88) and cleavage of this vicinal glycol with periodic acid produced formaldehyde which was isolated as its dimedone derivative and shown to have 20% (approximately $\frac{1}{4}$) of the activity of the vindoline, as expected.[62,63] This was excellent evidence for a monoterpenoid origin for the non-tryptophan portion of the indole alkaloids. An alternative mode of cyclization and cleavage of monoterpene precursors[62,63] was also consistent with these results. (Figure 14). No mechanism was suggested.

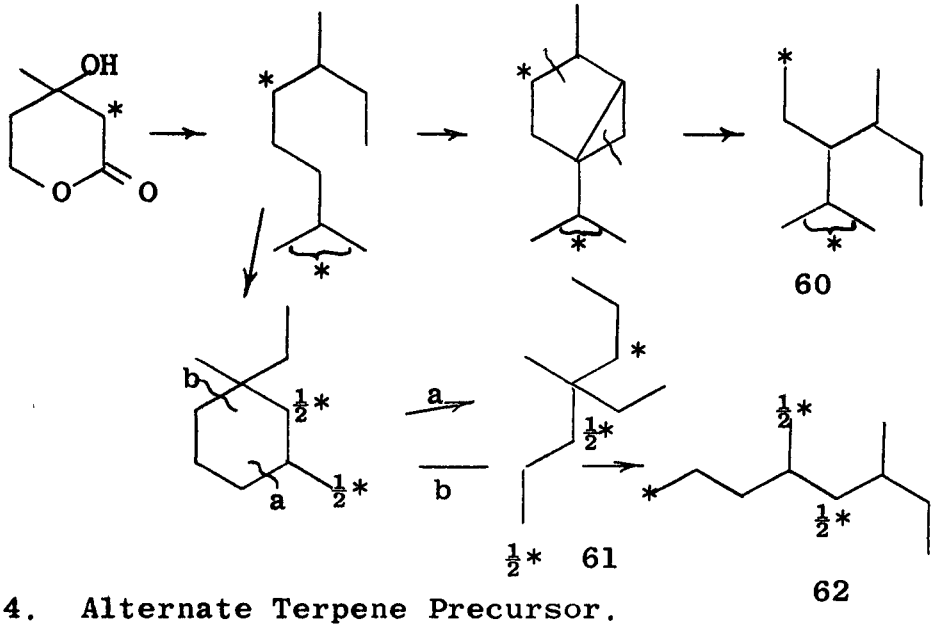


Figure 14. Alternate Terpene Precursor.

Soon after this initial experiment Goeggel and Arigoni[64] reported similar results from feeding of mevalonic acid-2-^{14}C to Vinca rosea and Vinca major L. followed by isolation of vindoline with 0.12% incorporation and reserpinine (89) with 0.01% incorporation. Degradation of vindoline established that the acetate group, the O-methyl, N-methyl and the ethyl side

chain were inactive while C-22 (the carbomethoxy carbon) contained 22.5% of the total activity. Degradation of the reserpinine obtained revealed an appreciable amount of scatter of label as each O-methyl accounted for about 7% of the activity. (Figure 16). These authors had previously established that these methyls were derived from methionine.[65] The carbomethoxy carbon, C-22, accounted for 20% of the total activity while the ethyl side chain had a low level of activity (0-3%).

Battersby also published the results of feeding mevalonic acid-2-^{14}C to Vinca rosea and Rhazia stricta and of feeding mevalonic acid-3-^{14}C to the latter.[66] Radioactive vindoline (13) with 0.05% incorporation, ajmalicine (33) with 0.003% incorporation, serpentine (3) with 0.02% incorporation and catharanthine (87) with 0.04% incorporation were isolated from Vinca rosea. The carbomethoxy groups (C-22) of ajmalicine and catharanthine had 24% and 23% of the total activity. Kuhn-Roth degradation of catharanthine established that C-6, C-20 and C-21 were inactive while ethyl pyridine obtained on reduction had 48% of the activity implying that C-1 and C-18 must by difference carry 29% of the activity. The Thomas-Wenkert theory requires 50% of the label at C-5, 25% at C-1, and 25% at C-22. These results were clearly consistent. The previously mentioned alternative proposal for rearrangement of a terpene precursor however requires 50% of the label at C-22 of the Iboga skeleton. The degradation of catharanthine hence eliminates this suggestion.

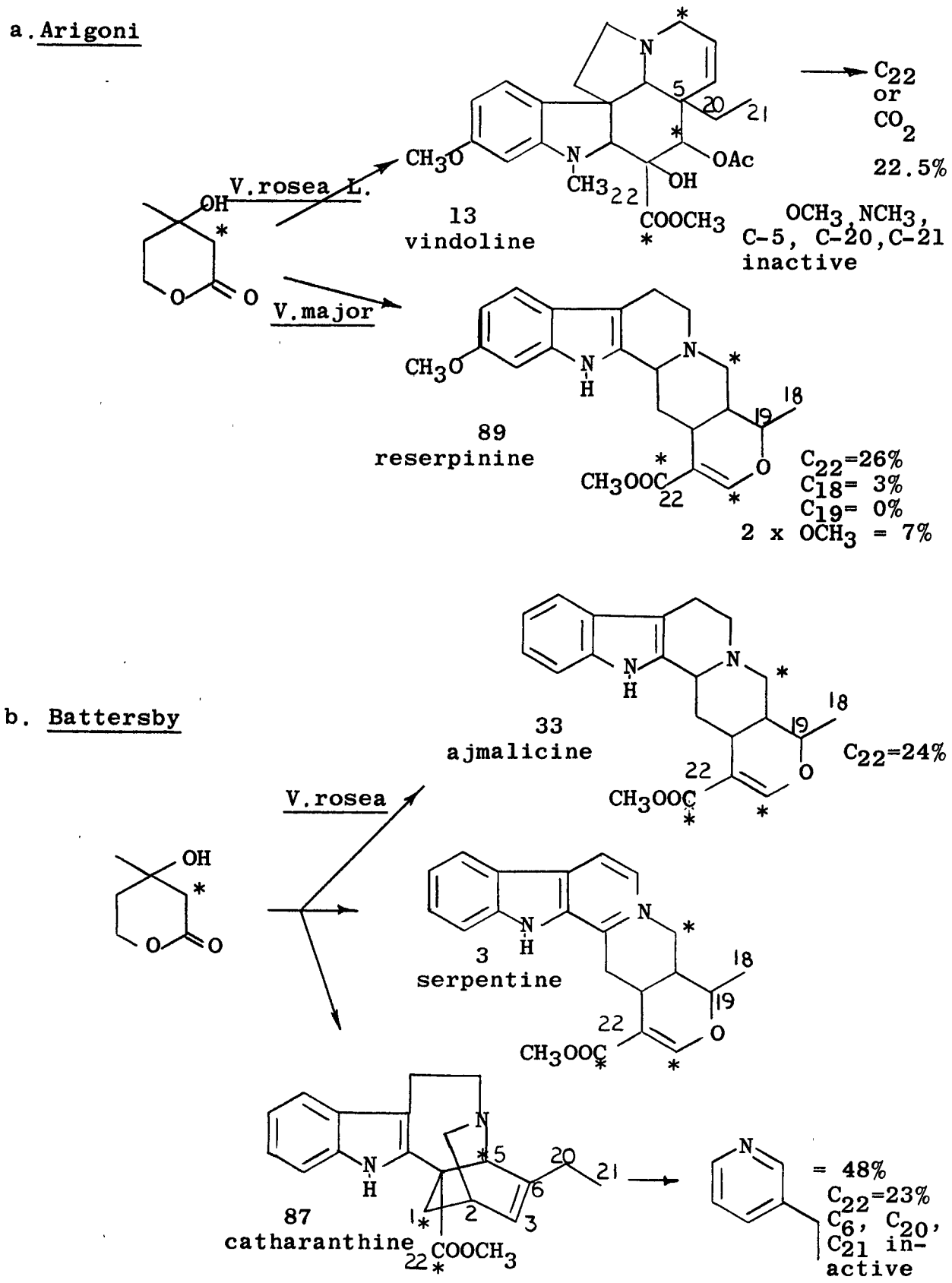
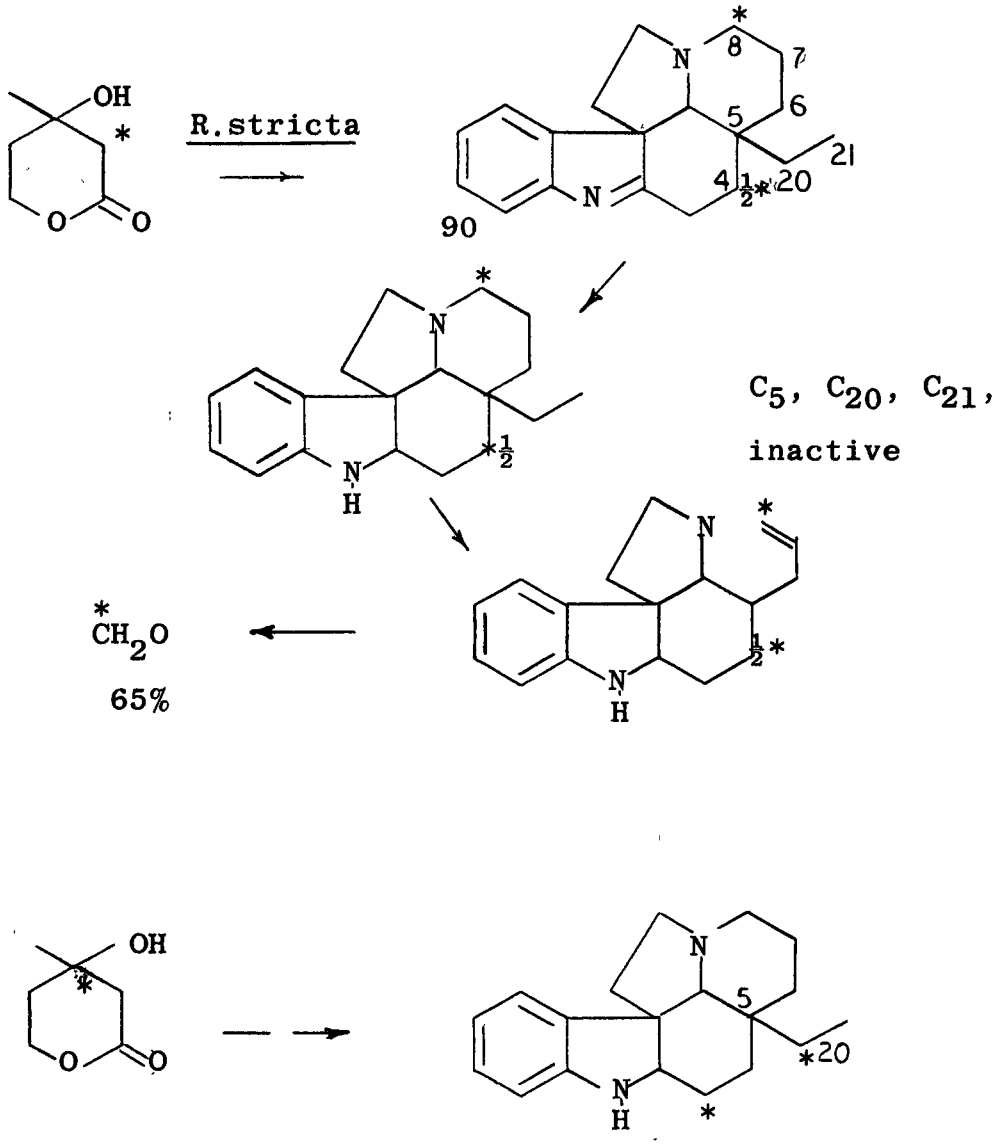


Figure 16. Feeding of Mevalonate to Vinca Species.

Figure 16 continued.

c.Battersby. Feeding of Rhazia stricta.



Active 1,2-dehydroaspidospermine (90) was isolated with 0.15% incorporation of mevalonate-2-^{14}C from Rhazia stricta.[66] C-5, C-20, and C-21 were shown to be inactive while isolation of C-8 as formaldehyde after Emde and Hofmann degradations established that this position carried 65% of the activity. As this alkaloid has no carbomethoxy group 67% is required by theory. Carbon-20 of the same alkaloid labelled by feeding of mevalonate-3-^{14}C was shown to carry 47% of the total activity while C-5 and C-21 were inactive as expected by theory.

Geraniol-2-^{14}C was synthesized for the purpose of feeding to Vinca rosea in the hope of establishing that the terpenoid portion of the indole alkaloids was formed from a ten carbon precursor. This would emphasize the normal terpenoid nature of the indole alkaloids. Successful incorporation of geraniol or geranyl pyrophosphate would also establish this compound as a useful precursor for other studies in the monoterpene field. The successful use of geraniol would also lead to use of labelled farnesol, the fifteen carbon unit from which the sesquiterpenes are derived, and plans to synthesize labelled farnesol were also made. In spite of the vast amount of speculation concerning the biogenesis of terpenes remarkably few tracer studies have been completed. Although geranyl and farnesyl pyrophosphates are proven steroid precursors[67] in no case have they been used for biogenetic studies in the terpene field. As far as is known a study of the biogenesis of gibberellins[68] in which labelled geranylgeraniol was not incorporated is the only

instance where this compound has been used.

There are several reasons why these potentially very useful compounds had not been used for tracer studies. They are extremely insoluble in water and difficulties in administration were envisaged. The phosphate or pyrophosphate is the metabolite utilized by an organic system and while these compounds can be made they would be ionic in nature and were not expected to be able to penetrate cell membranes. A third reason was undoubtedly the fact that labelled geraniol is not commercially available.

Geraniol-2-^{14}C with a specific activity of 0.159 mc/mmole was prepared from one millicurie of ethyl bromoacetate-2-^{14}C. The method employed involved a Reformatsky condensation of ethyl bromoacetate-2-^{14}C (92) with 6-methyl-5-hepten-2-one (91) to yield ethyl 3-hydroxy-3,7-dimethyl-6-octenoate (93) in a very small scale modification of a method described by Ruzicka and Schinz.[69,71] Dehydration was accomplished by pyrolysis of ethyl 3-acetyl-3,7-dimethyl-6-octenoate (94)[69] yielding a 1:2 mixture of <u>cis</u> and <u>trans</u> ethyl 3,7-dimethyl-2,6-octadienoate-2-^{14}C (95) with only a small amount of other double bond isomers. This ester mixture was reduced to a 1:2 mixture of nerol (97) and geraniol-2-^{14}C (96) with lithium aluminum hydride. (Figure 17). All steps in the synthesis were checked on an ordinary scale and compounds characterized by physical data (see experimental). The reactions were then scaled down and repeated until the techniques involved, especially distillation, were

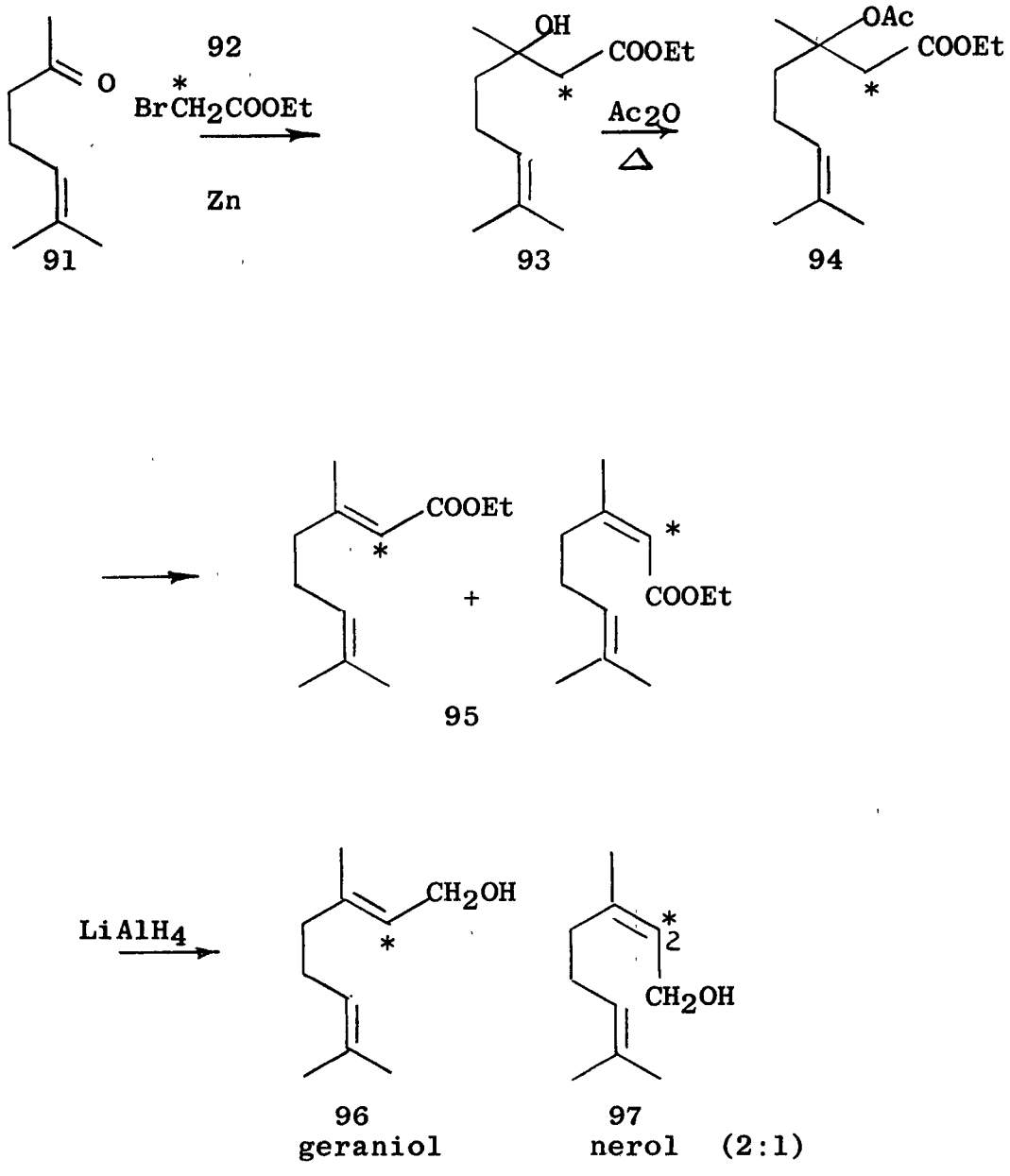


Figure 17. Synthesis of 2-^{14}C Geraniol.

adequate to give reasonable yields in the radioactive synthesis. The infra-red and n.m.r. spectra of products obtained in the radioactive synthesis were compared with those of products in the cold-run. The 1:2 ratio of cis and trans isomers was established by vapor phase chromatography. As nerol and geraniol are both natural products and probably interconvertible in vivo no attempt was made to separate the mixture before feeding. The preparation of a mixture of geranyl phosphate and pyrophosphate by the method of Cramer and Böhm[72] as modified by Popjak and Cornforth[72a] was also tried in anticipation of difficulty in the administration of geraniol to the plant.

Plant tissue is permeable to geraniol but is destroyed by high concentrations. The problem of administration was simply one of adequate dispersion. Several feeding experiments were carried out using cold geraniol until a method was found which did not destroy plant tissue. It was found that when geraniol was made soluble in water with α-lecithin, a natural emulsifying agent from plant sources, or with the non-ionic detergent Tween 20 it could be administered through the cut ends of shoots without obvious damage to the plants.

A small amount of geraniol-2-^{14}C made soluble with Tween 20 was fed to a single Vinca rosea cutting. After one week this cutting was dried between absorbant paper and autoradiographs prepared by exposure of a piece of x-ray film by contact with the leaves and stem for seven days. These autoradiographs showed that radioactivity was distributed throughout the plant.

The stem and leaf veins were apparently the most active but this may be a consequence of the bulk of these tissues. The topmost leaves were apparently as active as the lower ones.

Geraniol-2-^{14}C was fed to ten Vinca rosea cuttings and the alkaloids extracted after seven days: 2.4% of the activity fed was present in the crude alkaloidal fraction. Thin-layer chromatography of the alkaloid mixture revealed the well-known complexity of the mixture. An autoradiograph of a thin-layer plate showed spots corresponding to all of the alkaloidal spots on the plate and was good evidence for incorporation of geraniol into many of the alkaloids of Vinca rosea.

Vindoline (13) was isolated by preparative thin-layer chromatography[54] and crystallized to constant activity after dilution with authentic material. It had a rate of incorporation of about 0.005%. The specific incorporation is not known as the amount of vindoline isolated from the plant is unknown. Vindoline was present in the plant in only small amounts at the time of these feeding experiments but was chosen for study because of experience with this alkaloid and the availability of reasonable amounts of authentic vindoline for purposes of degradation.

Two-hundred 10-14 inch cuttings of one year old Vinca rosea plants were administered 0.282 mc of geraniol-2-^{14}C with a specific activity of 0.159 mc/mmole made soluble in 200 ml. distilled water with 8 drops of Tween 20. After one week the crude alkaloid extract contained 2.1% of the activity administered.

Vindoline was isolated by chromatography and preparative thin-layer chromatography in two solvent systems. 20 mg. of alkaloid showing a single spot on an autoradiograph had an activity of 1350 c.p.m./mg. or 3.11 x 10^{-5} mc corresponding to a specific incorporation of 0.37% and a rate of incorporation of 0.011%. The activity changed very little after several recrystallizations to constant activity as the dihydrochloride. The regenerated alkaloid was then diluted with authentic vindoline and recrystallized from ether to provide a sample suitable for degradation.

If the Thomas-Wenkert proposal for the biogenesis of the non-tryptophan portion of the indole alkaloids was correct the label of geraniol-2-^{14}C should be specifically incorporated at position 5 of vindoline. (Figure 18). Position 5 of vindoline is accessible by careful Kuhn-Roth oxidation as the carbonyl carbon of propionic acid.[75] The other two carbons of propionic acid from the ethyl side chain should also appear as acetic acid. Acetic acid would also be derived from the acetate group by hydrolysis during oxidation. Kuhn-Roth oxidation and separation of propionate and acetate as their p-bromophenacyl esters[76] followed by determination of the specific activity of the two esters would unambiguously locate the activity of vindoline as position 5 if the propionic acid had the same specific activity as vindoline and if the acetic acid was inactive. Any activity in the acetate of vindoline could be independently determined by hydrolysis to desacetyl-

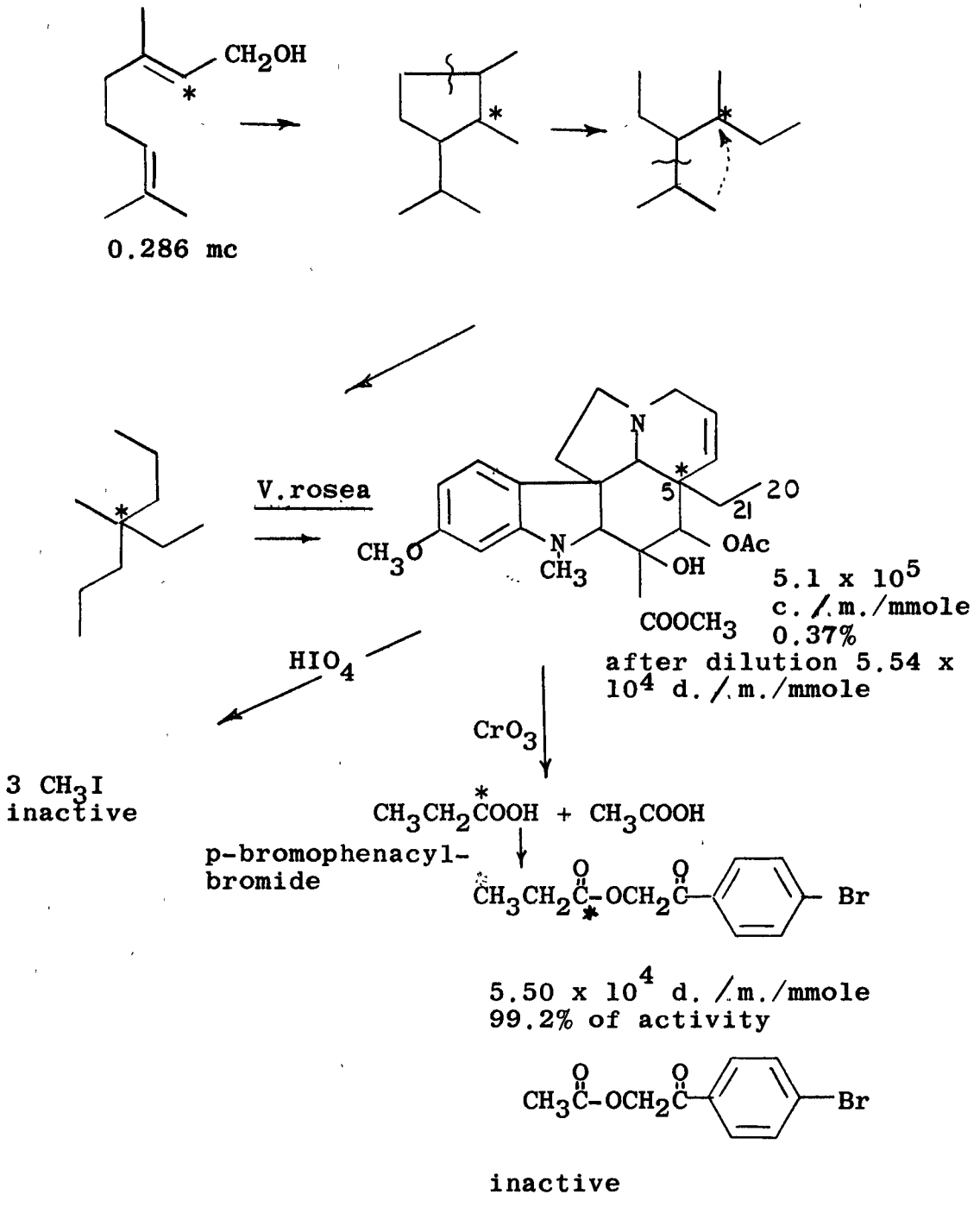


Figure 18. Incorporation of Geraniol into Vindoline.

vindoline. The activity of N-methyl and O-methyl groups is easily determined by volatilization as methyl iodide on reaction with hydriodic acid.[77] Degradation of the precursor and incorporation into the one-carbon pool can thus be checked.

A series of Kuhn-Roth oxidations were carried out in an effort to establish the best conditions[73,74,75] for production of propionic acid. The yield of volatile acids was established by titration and after regeneration of the free acids by passage through an ion exchange column they were separated and estimated as their ethylamine salts by paper chromatography.[74] The best conditions were very similar to conditions published by Lemieux and Purves.[73]

Vindoline-^{14}C (93.6 mg.) with a specific activity of 5.54 x 10^4 d./m./mmole was oxidized with 30% aqueous chromium trioxide and 1.97 equivalents of volatile acids were obtained as estimated by titration with lithium hydroxide. The salts were converted to the p-bromophenacyl esters and separated by preparative thin-layer chromatography on silica gel. When single spot material was obtained it was recrystallized to constant melting point and specific activity determined by liquid scintillation techniques. The p-bromophenacylpropionate had a specific activity of 5.50 x 10^4 d./m./mmole (275 $\pm$ 4.5 c./m. for 2.01 mg. of sample at a counting efficiency of 64%) which is 99 $\pm$ 2% of the activity of vindoline. The p-bromophenacylacetate was inactive within the limits of counting error. The O-methyl and N-methyl groups were also shown to be inactive with-

in the limit of counting error.[77]

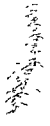
These results constitute unambiguous evidence for specific incorporation of C-2 of geraniol into C-5 of vindoline and establish the monoterpenoid origin of the non-tryptophan portion of the indole alkaloids. Only some questions remain as to the mechanism of condensation and cyclization.

Independent studies by Battersby[78], Arigoni[79], and Scott[80] were published simultaneously with this work and all results are consistent with the Wenkert-Thomas theory. Arigoni fed geraniol-2-^{14}C to Vinca rosea and isolated vindoline with all its activity at position 5. Mevalonate-3-^{14}C was also fed and ajmalicine (33) and vindoline (13) isolated. 47% of the activity of vindoline was at C-20 as expected while C-5, C-21 and the carbomethoxy carbon C-22, as well as the O- and N-methyls were inactive. In the case of ajmalicine C-19 carried 40% of the activity while C-18 was inactive.

Battersby also fed mevalonate-3-^{14}C to Vinca rosea and showed that 42% of the activity was at C-19 as required by theory. Mevalonate-6-^{14}C was incorporated into vindoline (13) and catharanthine (87). The propionic acid residue was inactive in both cases as expected. The mixed phosphate esters of geraniol-2-^{14}C were fed to Vinca rosea and ajmalicine (33), serpentine (3), catharanthine (87) and vindoline (13) isolated. Kuhn-Roth oxidation on desacetylvindoline established that 98% of the activity was located at C-5 as also established by us and Arigoni. Carbon 5 of catharanthine also accounted for all of

the activity in that molecule. In the case of ajmalicine activity was located at C-3, C-14, C-20 or C-21 by degradation to ajmalicol and Kuhn-Roth oxidation. Theory requires that C-20 be labelled.

Scott[80] fed deuterium labelled mevalonate and geraniol to Vinca rosea and was able to observe enhancement of relevant fragments by mass spectrometry of isolated vindoline. This technique could prove to be useful for biogenetic studies provided the incorporation is greater than 0.2%.



Experimental

Melting points were determined on a Kofler block and are uncorrected. Ultraviolet spectra were measured on a Cary 14 spectrophotometer in 95% ethanol. The infrared spectra were taken on a Perkin Elmer Model 137B or a Model 21 spectrophotometer. The nuclear magnetic resonance (n.m.r.) spectra were measured at sixty megacycles per second on a Varian A 60 instrument. The line positions or centers of multiplets are given in Tiers τ scale with reference to tetramethylsilane as the internal standard. Alumina G plates (according to Stahl) or Silica gel G plates (according to Stahl) were used for thin-layer chromatography (T.L.C.). In more recent work the background was made fluorescent to long wave ultraviolet light by admixture of alumina or silica with 2% General Electric Ratma P-1, Type 118-2-7 electronic phosphor. Mass spectra were determined using an Atlas CH-4 mass spectrometer.

The radioactivity was measured with a Nuclear Chicago Model D47 gas flow detector operated as a Geiger counter and mounted in a Model M-5 Semiautomatic Sample Changer, all in conjunction with a Model 181B Decade Scaler. The activities were measured by depositing samples of 0.2 to 0.5 mg. as thin films on standard 1.125 inch diameter aluminum planchettes. The total activities of synthetic precursors and of isolated metabolites are given in millicuries (mc) assuming a counter efficiency of 39%. A Nuclear Chicago Model 180040 liquid

scintillation counter was used for more accurate determinations of radioactivity as required for degradative work. The scintillation mixture consisted of toluene (500 ml.), 2,5-diphenyloxazole (P.P.O.) (2 g.) and 2-p-phenylenebis(5-phenyloxazole) (P.O.P.O.P.) (25 mg.).[81] We are indebted to Dr.P. McGeer of the Faculty of Medicine and Professor G.H.N.Towers of the Department of Botany for the use of their scintillation apparatus. We thank Dr.T.C.Beer of the Cancer Research Institute and also the Eli Lily Company for samples of Vinca alkaloids.

Incorporation of a precursor is reported in two ways. The rate of incorporation is defined as the ratio of the total activity of product to the total activity of precursor times one hundred. The specific incorporation is the ratio of the molar activity of the product to the molar activity of the precursor times one hundred.

The radioactive precursor [DL]-3,5-dihydroxy-3-methylpentanoic lactone-2-^{14}C ([DL]-mevalonolactone-2-^{14}C) was purchased from Merck Sharpe and Dohme of Canada Ltd. in either 0.5 or 1.0 mc quantities. One millicurie of ethyl bromoacetate-2-^{14}C was obtained from the same company and used for the preparation of geraniol-2-^{14}C by standard methods.[69,70]

Synthesis of 3,7-dimethyl-2,6-octadien-1-ol (geraniol-2-^{14}C)

In each step of the synthesis model reactions were carried out to check reaction conditions, yields, and to serve as a

source of authentic intermediates for comparisons in the radioactive series. These reactions were carried out first on an ordinary scale then on a scale close to that expected to be required in the radioactive synthesis.

Normal precautions were observed with respect to handling of radioisotopes. All work was carried out on a metal tray (1 x 19 x 28 inches) in a fume hood lined with polyethylene. A Nuclear Chicago Model 2650 Geiger counter was used to monitor any gross contamination.

Ethyl 3-hydroxy-3,7-dimethyl-6-octenoate-2-^{14}C (93)[69]

The millicurie of ethyl bromoacetate-2-^{14}C with a specific activity of 1.0 mc/mmole was purchased in a break-seal sample tube. The tube was modified by addition of a B_{10} Quickfit socket through which the break-seal could be broken by means of a glass rod and inactive ethyl bromoacetate added after distillation for purposes of scavenging and of dilution. A sidearm set at forty-five degrees to the tube and equipped with a B_{14} Quickfit cone and vacuum takeoff was also added. After cooling the brown liquid the seal was broken and ethyl bromoacetate-2-^{14}C distilled under reduced pressure (12 tor) into a tared, ice-cooled, round bottom flask. After cooling the sample tube inactive ethyl bromoacetate (0.83 g.) was added and distilled as before yielding a sample of sufficient volume (1.050 g., 6.28 mmoles) for synthesis with a calculated specific activity of 0.159 mc/mmole. This was mixed in a dry 5 ml. dropping funnel

with freshly distilled 6-methyl-5-hepten-2-one (0.794 g., 6.28 mmoles) purchased from Aldrich Chemical Co.. The flask was rinsed with one milliliter (2 x 0.5 ml.) of benzene which had been dried by azeotropic distillation to one half its volume and the rinsings combined with the reagents in the dropping funnel. The reaction commenced after brief warming with an oil bath when about 10% of the reagent mixture had been added under anhydrous conditions in a nitrogen atmosphere to a 10% excess of activated granular zinc (0.452 g.) in a 10 ml. round bottom two-necked flask equipped with a condenser and magnetic stirring flea. The zinc had been activated by washing with aqueous hydrochloric acid (5%), distilled water, ethanol, acetone and finally dry benzene, traces of which were removed in vacuo before weighing. Dropwise addition was then continued at such a rate (20 min.) as to maintain refluxing of the gently stirred mixture. The reaction mixture was then refluxed for an hour with vigorous stirring, cooled in an ice-bath and treated with ice-cold 10% sulphuric acid (5 ml.). After transfer to a separatory funnel and rinsing the reaction flask with benzene (2 x 5 ml.) the aqueous and benzene layers were separated. The benzene solution was extracted with cold 5% sulphuric acid (2 x 10 ml.), cold 10% sodium carbonate (5 ml.) and finally washed with water (2 x 5 ml.). The combined solutions were back extracted with ether (2 x 5 ml.) which was washed with 10% sodium carbonate (5 ml.) and with water (2 x 5 ml.). The combined organic layers were dried over anhydrous magnesium

sulphate and filtered into the sublimation apparatus which had been modified for small scale distillations by addition of a cup (2 ml.) to the end of the cold finger. The bulk of solvent was removed under a stream of warm, dry nitrogen. The residual solvent and any low boiling impurities were removed by distillation of the magnetically stirred liquid under reduced pressure (12 tor) using a boiling water bath. Ethyl 3,7-dimethyl-3-hydroxy-6-octenoate-2-^{14}C was then distilled (11 tor) into the cleaned dried cup while the temperature of a magnetically stirred glycerin bath was slowly raised from 130 to 150° C leaving a light brown residue. The product was carefully transferred by pipette and cold finger cup and the distillation jacket rinsed with ether. After removal of solvent and distillation as before a further 28 mg. of the precious product was collected (0.9784 g., 4.56 mmoles, 72.5%). Boiling point, lit.[69] 134°, 15 tor. Infrared: (liquid film) 3540(s), 3000(s), 2940(s), 1640(s), 1460(m), 1380(m), 1330(m), 1205(s), 1040(m), 935(w), 840(w) cm^{-1}. This spectrum is identical to the one obtained for the compound in cold runs as is the n.m.r. spectrum which is consistent with the expected structure. The n.m.r. spectrum: (deuterochloroform) broad triplet centered at 4.89 τ (olefinic proton, area 1.0 H), quartet centered at 5.89 τ (methylene of $-OCH_2CH_3$, area 2 H), singlet at 6.64 τ (hydroxyl proton, area 1 H), singlet at 7.56 τ (C-2 methylene, area 2 H), multiplet centered at 7.99 τ (allylic methylene, area 2 H), singlets at 8.33 and 8.39 τ (terminal methyl protons, area 6 H), singlet

at 8.77 τ (tertiary methyl protons) overlapping a triplet also centered at 8.77 τ (methyl of $-OCH_2CH_3$, total area 6 H). The remaining methylene protons are obscured by methyl resonances.

Ethyl 3-acetyl-3,7-dimethyl-6-octenoate-2-^{14}C [70]

Ethyl 3,7-dimethyl-3-hydroxy-6-octenoate-2-^{14}C (69) (0.978 g., 4.56 mmoles) was refluxed for 6.5 hours with a two molar excess of acetic anhydride in a 10 ml. round bottom flask. Excess acetic anhydride in the yellow reaction mixture was carefully decomposed with water (5 ml.). After separation in a funnel the aqueous layer was extracted with ether (2 x 5 ml.) which had just been used to rinse the reaction flask and condenser. The ether washings were combined with the organic layer and carefully washed free of acetic acid with 10% sodium carbonate (1 x 5, 3 x 2.5 ml.). The carbonate extracts were then back extracted with ether (1 x 5 ml.). The ethereal solution was washed with ether (1 x 5, 1 x 2.5 ml.), then with brine (1 x 4 ml.) and dried over magnesium sulphate in a Craig[82] filtration apparatus. Removal of solvent under a stream of dry nitrogen after filtration yielded a crude acetate (1.11 g., 4.34 mmoles, 95%) whose infrared spectrum was identical to that obtained for the same compound in the inactive synthesis. The compound is characterized by the absence of hydroxyl absorption (3540 cm^{-1}) and by typical bands at 1740 and 1245 cm^{-1} in the infrared.

Ethyl 3,7-dimethyl-2,6-octadienoate-2-^{14}C[70]

Pyrolysis of the acetate of ethyl 3-hydroxy-3,7-dimethyl-6-octenoate was shown by vapor phase chromatography to yield two major products and was therefore a superior method to dehydration with phosphorus oxychloride which yielded four. A carbowax 20M (5 feet x 0.25 inches) column at 140° C with collector, detector and injector temperatures of 150, 250 and 235° C respectively and a helium flow rate of 59 ml./min. was used. Injecting 2.0 μl. of sample retention times were determined for 2-methyl-2-hepten-6-one (5 min.), 3,7-dimethyl-2-trans-6-octadien-1-ol (geraniol) (44 min.), ethyl 3-hydroxy-3,7-dimethyl-6-octenoate (67 min.) and ethyl 3-acetyl-3,7-dimethyl-6-octenoate (93 min.). The products of pyrolysis of the acetate, integrating the areas under the peaks, had retention times of 22 min. (33%) and 29.5 min. (67%). The two components were separated using a carbowax 20M column (10 feet x 0.318 inches) at an operating temperature of 180° C and a helium flow rate of 100 ml./min.. The collector, detector, and injector temperatures were 155, 260, and 240° C respectively. Up to 100 μl. of liquid could be separated under these conditions.

The more mobile component is ethyl 3,7-dimethyl-2-cis-6-octadienoate as deduced from its physical properties and by reduction to nerol with lithium aluminum hydride. Refractive index: $[n]_D^{23}$ = 1.4672. Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 215 mμ, $\epsilon_{max.}$ 11,400 (typical of an α,β-unsaturated ester). Infrared spectrum (liquid film): $\nu_{max.}$ 3002(s), 2925(s), 1723,

1715(s), 1650(s), 1450(m), 1245, 1215(m), 1160(s), 1099(w), 1065(w), 1039(w), 852(w), 821(w), 790(w) cm^{-1}. The n.m.r. spectrum in deuterochloroform which implies that the fraction is a mixture and is thereby difficult to analyze completely includes: broad singlet at 4.38 τ (newly olefinic proton 0.80 H), a broad triplet at 4.87 τ (olefinic proton, area 1.0 H) and a quartet centered at 5.94 τ (methylene of $-OCH_2CH_3$, area 2 H). The mass spectrum showed significant peaks at m/e = 196 (M^+), 151 (M - 45), 128 (M - 69), 124 (M - 72), 101 (M - 95), 86, 83, 69. 43, 41, and 28. The reduction product showed two peaks on vapour phase chromatography using a carbowax 20M column (5 feet x 0.25 inches) operated at 180° C with injector, detector and collector temperatures of 230, 245, and 150° C with a helium flow rate of 91 ml./min.. One peak corresponded to the unreduced ester (retention time 6.0 min.) and the other to 3,7-dimethyl-2-cis-6-octadien-1-ol (nerol) which was purchased from Aldrich Chemical Company as a mixture of the cis (nerol) and trans (geraniol) isomers. A sample of the trans isomer (geraniol) was also purchased from the same company.

The less mobile and major component of pyrolysis is ethyl 3,7-dimethyl-2-trans-6-octadienoate. Refractive index: $[n]_D^{23}$ = 1.4680. Ultraviolet spectrum in ethanol: $\lambda_{max.}$ 215 mμ, $\epsilon_{max.}$ 11,200 (typical of an α,β-unsaturated ester).[87] Infrared spectrum (liquid film): $\nu_{max.}$ 3001 (s), 2920(s), 1723, 1712(s), 1650(s), 1450(m), 1225(s), 1143(s), 1108, 1098, 1061, 1038. 865, 815, 780 cm^{-1}. The n.m.r. spectrum in deuterochloroform:

broad singlet 4.37 τ (olefinic proton on newly formed double bond, area 1.0 H), broad triplet centered at 4.90 τ (original olefinic proton, area 1.0 H), quartet centered at 5.94 τ (methylene protons of $-OCH_2CH_3$, area 2 H), poorly resolved peak at 7.87 τ (methylene and C-3 methyl protons, area 7 H), pair of singlets at 8.33 and 8.39 τ (terminal methyl protons, area 6 H), and finally a triplet centered at 8.80 τ (methyl protons of $-OCH_2CH_3$). The mass spectrum showed significant peaks at m/e = 196 (M^+), 151 (M - 45), 129 (M - 67), 124 (M - 72), 100 (M - 96), 82, 69, 41, and 28.

Ethyl 3-acetyl-3,7-dimethyl-6-octenoate-2-^{14}C (1.11 g., 4.35 mmoles) was very slowly distilled at atmospheric pressure into the collection cup of the micro-distillation apparatus. It had been found that refluxing for ten minutes did not effect complete pyrolysis. Both distillate and residue were transferred to a separatory funnel and washed free of acetic acid with a 10% sodium carbonate solution (3 x 3 ml.). After washing with water (4 x 3 ml.), brine (2 ml.), drying over anhydrous magnesium sulphate, filtration and removal of the ether under a stream of nitrogen the pyrolysate was distilled under reduced pressure (12 tor, 116-123° C) yielding a mixture consisting of at least 90% of cis and trans (1:3) isomers of ethyl 3,7-dimethyl-2,6-octadienoate-2-^{14}C (0.70 g., 3.58 mmoles, 83% yield). The composition is based on a comparison of the infrared spectrum with that from a sample of the same compound which was analyzed by vapour phase chromatography and by interpretation of the

n.m.r. spectrum. The pyrolysis of the acetate is readily checked by disappearance of the acetate absorption maxima at 1735 and 1250 cm^{-1} and appearance of a peak at 1650 cm^{-1} attributed to an asymmetrical double bond. The n.m.r. spectrum (no solvent) with a singlet at 4.38 τ (olefinic proton on newly formed double bond, area 0.90 H) is also identical to that from the cold run.

3,7-dimethyl-2,6-octadien-1-ol-2-^{14}C (geraniol) (97)[70a]

Ethyl 3,7-dimethyl-2,6-octadienoate-2-^{14}C (0.70 g., 3.58 mmoles) in ether (3 ml.) was added at such a rate as to maintain refluxing to a magnetically stirred ethereal solution (5 ml.) of lithium aluminum hydride (100% excess) in a 12 ml. two-necked round bottom flask. The mixture was then refluxed for forty minutes and excess hydride destroyed by cautious addition of wet ether. Dilute hydrochloric acid (2 N, 3 ml.) was added to dissolve aluminates and organic material extracted into ether (3 x 3 ml.). The combined organic layers were washed with water until neutral to pH paper (5 x 3 ml.), with brine (2 ml.), dried over magnesium sulphate, filtered, the solvent removed under a stream of dry nitrogen and the 3,7-dimethyl-2,6-octadien-1-ol-2-^{14}C distilled under reduced pressure. The yield (0.3736 g., 2.46 mmoles, 68.6% from the ester) of the synthetic mixture of geraniol and nerol based on ethyl bromoacetate-2-^{14}C is 39.2%. The infrared spectrum of this mixture is identical to that of previous synthetic mixtures and differs somewhat

from the spectrum of the trans isomer (geraniol) but only in the fingerprint region.

3,7-dimethyl-2,6-octadien-1-phosphate and pyrophosphate[72a]

Geraniol (96) (250 mg., 1.62 mmoles) was mixed with trichloroacetonitrile (1.40 g., 9.75 mmoles, 6 x) in a 100 ml. three-necked round bottom flask with small magnetic stirring bar fitted with a condenser and dropping funnel. Ditriethylamine phosphate (1.17 g., 3.9 mmoles, 2.4 x) dissolved in acetonitrile (34 ml.) was introduced through the dropping funnel over a 3.5 hour period. The reaction mixture was kept at room temperature and stirred constantly during the addition then was allowed to stand 2.5 hours. (Ditriethylamine phosphate had been prepared by dissolving 85% phosphoric acid (11.75 g.) in acetonitrile (50 ml.) then adding triethylamine (20.6 g.) from a dropping funnel to the stirred solution. Considerable heat was evolved. After the first mole of triethylamine had been added the milky suspension became a clear solution. The product failed to crystallize out overnight but crystallized beautifully after addition of a few milliliters of acetone. The phosphate is highly deliquescent and was kept over a desiccant after removal of solvent in vacuo). Dilute ammonia (0.1 N, 50 ml.) and ether (150 ml.) were added to the reaction mixture. After separation the ether layer was extracted with dilute ammonia (0.1 N, 2 x 25 ml.) and the combined aqueous phase washed with ether (3 x 100 ml.). The

aqueous phase was then concentrated on a rotary evaporator (60° C) to 20 ml.. After addition of cyclohexylamine (0.55 ml.) the concentration was continued until crystals appeared then the solution was allowed to stand overnight at 0° C and the monophosphate salt filtered and dried (123 mg.).

The mother liquors were treated with concentrated ammonia (1 ml.) and extracted with ether to remove cyclohexylamine (2 x 15 ml.). A solution of lithium chloride (1 N, 5 ml.) was added to the mother liquors which were then concentrated until crystals appeared, allowed to stand overnight (0° C) and filtered. The pyrophosphate (85 mg.) was dried overnight in vacuo at 60° C.

The homogeneity of the phosphate and pyrophosphate salts were tested by paper chromatography.[83] Whatman #3 MM paper for chromatography was washed with dilute acetic acid (2 N, 250 ml.). After draining for five minutes the paper was washed until neutral with distilled water. Repeated rinsing with a total of five liters per sheet of paper was necessary. The paper was allowed to dry at room temperature overnight then was spotted with the lithium salt of geranyl pyrophosphate and the cyclohexylamine salt of geranyl phosphate. The residue from the mother liquors, ditriethylamine phosphate and dipotassium phosphate were also spotted on the paper which was then rolled into a cylinder, stapled and developed (25 hours) in an isopropanol, ammonia, water (6:3:1) mixture using the ascending technique in a chromatography tank which had been allowed two

days to equilibrate. The paper was dried by standing in a current of warm air from a hair dryer for half and hour then in an oven for five minutes (80° C). The paper was sprayed, first with a mixture of 60% w/w perchloric acid (5 ml.), normal hydrochloric acid (10 ml.) and 4% w/v ammonium molybdate (25 ml.) which had been diluted to 100 ml. with water. It was then dried in a current of warm air (5 min.) then momentarily in an oven (80° C). The paper was then sprayed with freshly prepared 1% stannous chloride in 10% hydrochloric acid yielding blue spots of a phosphomolybdate complex. Geranyl phosphate (R_f 0.6) showed up as a single blue-green spot with a very small amount of material near the origin. The sample of geranyl pyrophosphate (R_f 0.5) contained a large amount of phosphate (blue spot) and a significant amount of impurity near the origin. Orthophosphate (R_f 1.3) was visible as a yellow spot before reduction with stannous chloride when it turned blue-green. The residue from the mother liquors consisted mainly of orthophosphate with a small amount of geranyl phosphate.

Administration of labelled geraniol to *Vinca rosea* Linn. (*Catharanthus roseus* G.don)

The plants (1.0 year old) were grown in a bed in an unheated, shaded greenhouse and about 5% were flowering. These plants had flowered the previous summer and had had no flowers for three months prior to feeding. The crude alkaloid content based on dried weight of stem and leaf material was 0.37% and

vindoline was shown to be present by thin-layer chromatography and by its characteristic crimson colour with ceric sulphate spray(1% ceric sulphate, 35% sulphuric acid).

Because trouble had been anticipated in administering water insoluble geraniol to plants experimental details had been worked out for preparation of the pyrophosphate. The glucoside was also considered as a suitable water soluble derivative. Before preparing a derivative attempts were made to solubilize geraniol using ethanol, dimethylsulfoxide, soap, α-lecithin, Span 20 (sorbitanlaurate) and Tween 20 (polyoxyethylenesorbitan-laurate). Ethanol and dimethylsulfoxide killed the plants in concentrations necessary for solubilization. Ordinary potassium soap also killed the plant. Injection of neat geraniol with a syringe into the stem resulted in collapse of the stem. Painting the chemical or a dilute solution in petroleum ether on the leaves resulted in absorption and desiccation of cells in the painted area with collapse of the veins and eventual stem collapse as the material was transported. Tween 20 and to a lesser extent Span 20 and α-lecithin were capable of solubilizing geraniol so that it could be administered through cut ends of shoots in sufficient concentrations for tracer studies without any obvious damage to the plants.

A six inch cutting (21 leaves) was placed in a test tube containing an aqueous suspension (1 ml.) of 3,7-dimethyl-2,6-octadien-1-ol-2-^{14}C (96,97) (1.67 mg., specific activity 0.159 mc/mmole). The suspension was prepared by shaking the labelled

precursor with one milliliter from a mixture of one drop of Tween 20 in 250 ml. of distilled water. The liquid was absorbed within four hours and water was added as required to keep the cut end submerged. Within three days activity was detectable with the radiation monitor in even the topmost leaves. The cutting which was kept alive under continuous illumination for seven days, lost 5 leaves in this time. Leaves were removed from opposite sides of the stem and pressed between tissue paper between two books until dry. The stem with remaining leaves was also pressed. Leaves and stem with leaves were then left for seven days in contact with Ilford x-ray film and the resulting autoradiograph confirmed that activity was distributed throughout the plant.

Toxicity of geraniol as a function of concentration was checked in a simple experiment by hydrophonic administration to each of two cuttings of one milliliter of solution containing the following concentrations of geraniol and Tween 20 respectively: I. 1 mg., 1/2500 drop; II. 1 mg., 1/10 drop; III. 2 mg., 1/2500 drop; IV. 2 mg., 1/10 drop; V. 5 mg., 1/10 drop; VI. a blank. The ends of the cuttings were kept immersed in water and a record made of the water absorbed. After three days the lower leaves started yellowing in all cases. After five days the lower leaves were particularly dry and falling, stem colouring had disappeared, and the cutting stopped absorbing water under condition V. It was concluded that healthy cuttings of Vinca rosea will tolerate one tenth drop of Tween 20 and two milli-

grams of geraniol but that five milligrams results in observable damage.

Pilot Run

Feeding of geraniol-2-^{14}C to Vinca rosea

When it became obvious that a large number of cuttings would have to be fed in order to obtain enough active vindoline for degradation an illumination chamber was constructed. This consisted of a bank of four twenty-seven watt, eighteen inch fluorescent tubes supported on a metal frame sixteen inches above a nine square foot area covered with polyethylene sheeting. Eight twenty-five inch test tube blocks were made each with two staggered rows of holes to fit 12 x 75 mm. test tubes. Rows, and holes in each row were spaced two inches apart so each block contained space for twenty-four test tubes and a total of one hundred and ninety-two cuttings could be fed and illuminated at once.

Pilot Run

Ten shoots of year old greenhouse grown Catharanthus roseus plants varying from five to eleven inches in height were cut diagonally across the stems. Several cuttings were taken from single plants. Leaves were carefully removed so that the individual cuttings would stand in 12 x 75 mm. test tubes without interference from the leaves. The cut ends were immersed immediately in water in order not to break the capil-

larity of the liquid transport system. Geraniol-2-^{14}C (20.1 mg., 0.021 mc) emulsified in ten milliliters of distilled water with Tween 20 (8 drops/liter) was absorbed through the cut ends of the ten shoots which were then kept alive under constant illumination taking care to keep the cut ends immersed in water for seven days.

The fresh plant material (51 g.) was cut into smaller pieces and macerated with methanol-acetic acid (10:1, 3 x 200 ml.) in a Waring blender, filtering and washing each time on a Buchner funnel until no more green colour remained. The solvent was removed on a rotary evaporator and the residue parititioned between benzene (1 x 200 ml., 1 x 50 ml.) and dilute hydrochloric acid (2 N, 2 x 50 ml., 3 x 20 ml.). The combined aqueous layer was mixed with an equal volume of chloroform, shaken, and the organic layers combined . The dark brown aqueous layer was then washed with chloroform (5 x 40 ml.) which removed a small amount of yellow pigment. Emulsions had not caused any great amount of trouble to this point but the aqueous layer was filtered through a large Whatman #1 paper to help prevent future difficulties. The aqueous layer was made basic with ammonia (pH 10) and the alkaloids extracted into chloroform (10 x 25 ml.) yielding a light yellow extract which after washing with water (4 x 20 ml.) then brine (1 x 30 ml.), filtering, drying over anhydrous magnesium sulphate and evaporation yielded the crude alkaloid (106 mg., 0.2% of fresh plant material), which was counted using the gas flow counter (specific activity

4070 c./m./mg.) and shown to represent 2.4% of the activity fed.

The crude alkaloid was examined by thin-layer chromatography[54] which revealed the now familiar complexity of the mixture and suggested a very low vindoline content. An alumina G plate (5 x 20 cm.) with tungsten phosphor was spotted with the crude alkaloid, alkaloid from band V, and with authentic vindoline and was developed in chloroform-ethylacetate (1:1). This plate after examination under ultraviolet light and exposure to iodine vapour was sprayed with a thin film of Craftint Spray-Art Fixative to preserve the iodine colouration and to fix the alumina. An autoradiograph obtained by exposure of Ilford x-ray film by contact with the plate for seventeen days showed a series of spots corresponding to alkaloids but the band V material in this system showed only a single radioactive spot.

A preparative thin-layer chromatoplate was prepared using Desaga apparatus (blade setting 0.8 mm.) by spreading fifty grams of alumina G and one gram phosphor slurried in ninety milliliters of water on a twenty by sixty centimeter plate. The plate was dried at room temperature in a ventilated area for twenty-four hours. The crude alkaloid in a concentrated chloroform solution was streaked across one end. The plate was developed using a mixture of chloroform-ethylacetate (1:1). Alkaloid bands on the plate were located by fluorescence and by quenching of the fluorescent background. Eight bands were

scraped from the plate and the alumina slurried in chloroform and packed in glass columns as one would normally pack a chromatography column. The alkaloid was then eluted by repeated washing with chloroform, weighed and its activity determined by counting on aluminum planchettes. Band one (R_f 0-2.5) was a complex of fluorescent and quenching bands near to the origin (36 mg., total activity 1.1 x 10^5 counts per minute). Band II (R_f 2.5-2.9) was brightly blue fluorescent (1.7 mg., total activity 3.8 x 10^3 c./m.). Band III (R_f 2.9-3.7) was strongly quenching and by thin layer comparison with authentic material was the vindoline containing band (1.7 mg., total activity 4.9 x 10^3 c./m.). Bands IV, V and VI were very strongly quenching (R_f 3.7-4.1, 4.1-4.4, 4.4-4.9 respectively) and were coloured quickly and intensely by iodine vapour (2.8, 2.9, 2.7 mg., total activity 16.2, 5.6, and 5.4 x 10^3 c./m. respectively). Band IV had a higher specific activity than the vindoline containing band. Band VII was neither fluorescent nor very strongly quenching (R_f 4.9-5.4, 0.9 mg., total activity 1.8 x 10^3 c./m.) while band VIII was again stongly quenching (R_f 5.4-6.0, 1.6 mg., total activity 1.6 x 10^3 c./m.). There was no activity between band VIII and the solvent front. Only about fifty percent of the alkaloid loaded onto the preparative plate was recovered. The origin was not extracted. Speed is required for successful recovery of alkaloids from alumina as they tend to oxidize. It was best not to let developing solvent evaporate entirely from the

plate before bands were scraped off and slurried with chloroform.

Band III was diluted with five milligrams of authentic vindoline as an aid to purification and crystallized from a very small amount of ether at low temperatures (0^{o} C) after seeding. Vindoline is not an easy compound to crystallize in small quantities. Two large colourless crystals (1.2 mg.) were obtained and counted as thin films deposited on three tared planchettes from ethyl acetate until the statistical counting error was less than four percent (I. 0.286 mg., 149 $\pm$ 6 counts per minute per milligram (c./m./mg.), II. 0.310 mg., 165 $\pm$ 6 c./m./mg., III. 0.279 mg., 147 $\pm$ 6 c./m./mg.). The vindoline was recovered from the planchettes, crystallized one more time from ether and counted as before showing no detectable loss of activity. The specific activity was 150 c./m./mg. or 6.85×10^{4} c./m./mmole after dilution with 5 mg. vindoline. Incorporation, 9×10^{-7} millicuries. Rate of incorporation, 0.005%.

Preparative feeding of geraniol-2-^{14}C to Vinca rosea Linn.

Geraniol-2-^{14}C (0.272 mg., specific activity 0.159 mc/mmole, 0.282 mc) was administered hydrophonically to one hundred and ninety-two cuttings of Vinca rosea solubilized with Tween 20 (8 drops/200 ml.) as previously described. After seven days the fresh plant material was extracted for alkaloid. The plants were macerated in fifty gram lots (wet weight 835 g.), extracts combined, and volumes scaled appropriately yielding 2.14 g. of crude alkaloid (0.26%). Rate of incorporation: 2.1%. This

material was shown to contain some vindoline by thin-layer chromatography (Alumina G, chloroform-ethylacetate, 1:1, or ethyl acetate-ether, 1:50, with iodine vapour or ceric sulphate spray). The alkaloid was triturated with chloroform-benzene (3:100, 100 ml.), the dark brown residue shown to contain no vindoline detectable by thin-layer chromatography, and was loaded onto a grade I neutral alumina column (Woelm alumina) and chromatographed. No material was eluted from the column while gradually increasing the eluent polarity through chloroform-benzene (3:100, 400 ml.; 1:20, 200 ml.; 1:10, 200 ml; 1:4, 400 ml.; 1:1, 200 ml.) until a brown band started to come off in chloroform-benzene (3:1). Elution was continued with this solvent collecting aliquots (1 x 100, 1 x 40, 2 x 200 ml.) which were all shown by T.L.C. to contain vindoline. Elution was continued with chloroform (1 x 200, 1 x 500ml.) until very little material was being eluted but this alkaloidal mixture also contained some vindoline. The vindoline containing fractions which represented 0.59 g. of alkaloid were not combined but further purified by preparative thick-layer chromatography. The column was washed with methanol-chloroform (1:20), methanol, then acetic acid-methanol(1:10) giving essentially quantitative recovery of alkaloid. The post chloroform fractions were shown by T.L.C. to contain no vindoline. Six thick-layer alumina plates with fluoresecent background were prepared as described in the previous section.

After streaking approximately 100 mg. of alkaloids in a thin band on each plate they were developed in chloroform-

ethyl acetate 1:1 (8 hours) until the solvent had reached the end of the plate (60 cm.). The vindoline containing bands (R_f 0.3-0.4) were located by reference to thin-layer plates by fluorescence and by quenching of the fluorescent background. These bands were scraped from the plates as soon after developing as possible and the alkaloid eluted with chloroform followed by a bit of methanol yielding 73.6 mg. of light brown solid foam. This material was counted on a planchette (4, 270 c./m./mg.) and found to be more active than the total alkaloid (3,500 c./m./mg.). An alumina chromatogram spotted with the total alkaloid, the semi-purified vindoline, and authentic vindoline was developed in chloroform-ethylacetate (1:1), spots revealed with iodine vapour and the plate sprayed with Craftint Spray-Art Fixative to hold the alumina in place while an autoradiograph was exposed. After 30 days the autoradiograph was developed and showed a series of spots corresponding to the alkaloid spots made visible with iodine and a single spot corresponding to the vindoline fraction. In spite of this the vindoline fraction contained less than fifty percent vindoline as revealed by analysis of the ultraviolet spectrum: $\lambda_{max.}$ 293, 247 sh. mμ, $\epsilon_{max.}$ 8000, 6000. Vindoline: $\lambda_{max.}$ 252, 304 mμ, $\epsilon_{max.}$ 7660, 5540 (typical indole chromophore). Thin-layer chromatography in ethyl acetate-ether (1:50) demonstrated that the material was a mixture of three major components (R_f 0.5, 0.25 (vindoline), and 0.1) and several fluorescent impurities. Vindoline is not fluorescent. The mixture was separated by

preparative thick-layer (0.5 mm.) chromatography on an alumina G (20 x 20 cm.) plate. The vindoline fraction located by quenching of background fluorescence (20 mg.) was now, except for two fluorescent impurities not detectable by other means, a single spot material in two good solvent systems as well as several others tried which did not give as impressive separations with the original mixtures. The characteristic crimson colour with 1% ceric sulphate-35% sulphuric acid spray now matched perfectly that of authentic vindoline.[55] The ultraviolet spectrum also matched (ethanol): $\lambda_{max.}$ 252, 303 mμ ; $\epsilon_{max.}$ 7600, 5500 (typical indolinene). The specific activity (1,350 c./m./mg., 20 mg.) at a counting efficiency of 39% corresponded to an incorporation of 3.11×10^{-5} mc or a rate of incorporation of 0.011%.

As vindoline is difficult to crystallize as the free alkaloid it was crystallized to constant activity as its dihydrochloride. The vindoline was dissolved in ether, filtered, and hydrogen chloride blown over the top of the solution. After centrifugation the solution was again treated with hydrogen chloride until no more precipitate was obtained. The mother liquors were then decanted and the white precipitate washed with a small amount of cold ether. The dihydrochloride was dissolved in methanol (2 drops) and warm ethyl acetate added until the solution became turbid. Crystallization occurred on scratching with a seed crystal. The dihydrochloride was counted after deposition on two aluminum planchettes from chloroform. Average specific

activity: 940 c./m./mg., 5.0 x 10^5 c./m./mmole. The dihydrochloride was crystallized twice more, m.p. 150-152, from ethyl acetate-methanol and again counted. Average specific activity: 960 c./m./mg., 5.1 x 10^5 c./m./mmole. The free alkaloid was regenerated by shaking a chloroform solution (4 ml.) with aqueous ammonia (1 N). The aqueous layer was then washed with chloroform (1 ml.) and the organic layer dried over anhydrous magnesium sulphate, filtered and taken to dryness under a stream of dry nitrogen, removing residual solvent *in vacuo*. The recovered vindoline (6.27 mg.) now showed no fluorescent impurities on thin-layer chromatography and had a specific activity of 5.1 x 10^5 c./m./mmole., 1120 c./m./mg.. Constant activity had been achieved within the limits of counting error which can be large if great care is not taken weighing samples and depositing samples as a uniform film. With care the count can be reproduced within three percent (500 c./m.). A specific activity of 5.1 x 10^5 c./m./mmole corresponds at a 39% counting efficiency to 1.3 x 10^6 disintegrations per minute. As one millicurie corresponds to 3.700 x 10^7 disintegrations per second (2.22 x 10^9 d./m.) the specific activity of vindoline can be expressed as 5.9 x 10^{-4} mc./mmole. As the specific activity of geraniol-2-^{14}C fed to *Vinca rosea* was 0.159 mc/mmole the specific incorporation of the geraniol-nerol mixture into vindoline is 0.37%.

Degradation of Vindoline

The active vindoline (6.27 mg.) obtained by extraction of Vinca rosea was diluted by recrystallization from ether in two crops (first crop 89.4 mg., second crop 19.8 mg., total 109.2 mg) with authentic vindoline (111.6 mg.) obtained from the Eli Lily Company. This material and its degradation products were counted in the toluene scintillation mixture[81] using a Nuclear Chicago Model 180040 Liquid Scintillation Counter. The counting efficiency was established by the channel ratio method. A quenching curve (a plot of counting efficiency versus the ratio of counting rate in two energy channels(B/A) was determined using a series of six acetone quenched samples with activity of 210,200 disintegrations per minute. The counting rates in channel A were respectively 164,784(78.5%), 147,893(70.3%), 115,112(54.8%), 85,926(40.9%), 66,010 (31.4%), and 33,742(16.0%) counts per minute. The counting rates in channel B were at the same time 55,787(B/A = 0.339), 74,595 (B/A = 0.506), 84,244(B/A = 0.731), 74,218(B/A = 0.86), 60,840(B/A = 0.922), 32,902(B/A = 0.975) counts per minute. Calculation of the standard deviation in the counting rates then in the ratios (Appendix I) established that the standard deviations in the ratios were less than 1%. One should be able to use the curve plotted from the above data to establish counting efficiency in a radioactive sample within 1%, subject to the statistical error in counting and in the ratio B/A for that sample.

Oxidation Conditions[73,74,75]

A series of thirteen experiments were required to establish the oxidation conditions under which a suitable ratio of propionic and acetic acids could be obtained so that the p-bromophenacyl esters of these acids could be purified and their specific activity determined. In these experiments three different designs of apparatus were used. One of these, a modified Kjeldahl apparatus[84] involved bubbling steam through a solution of vindoline in the oxidizing mixture. The second consisted of an oxidation chamber with a vapour trap then a splash trap to prevent non-volatile acids being carried from the oxidizing solution during distillation. A side arm was provided for addition of water to compensate for water removed as steam. The third apparatus was used in the event. It was constructed from a fifty milliliter round bottom flask with B_{14} Quickfit neck, a Claisen head modified so that the distillation flask could be inclined 30^{o} from the normal vertical position, a dropping funnel, a Liebig condenser and finally a graduated cylinder for collecting distillate. Carborundum boiling stones were used to prevent bumping and it was found that the bent Claisen head provided remarkable protection against splash. The first seven oxidations were carried out on 5 mg. of vindoline, the next five on 10 mg. and the thirteenth with 100 mg.. The changes in oxidation conditions involved changes in concentration of chromium trioxide, concentration of sulphuric acid, and rate of distillation.

A typical oxidation procedure is as follows: Vindoline (10 mg.) was washed into the 50 ml. round bottom flask with distilled water (5 ml.). The 3 ml. and 5 ml. levels were marked with a grease pencil on the flask. One milliliter of the oxidizing mixture (4 N chromic acid-sulphuric acid-water 4:1:25) was added and the resulting mixture heated immediately to boiling with a micro burner. The chromic acid solution (4 N) was prepared by dissolving chromium trioxide (67 g.) in distilled water (500 ml.), allowing it to stand overnight, filtering through a sintered glass filter, slowly adding concentrated sulphuric acid (125 ml.), then distilling to remove steam volatile impurities. Distillation was continued adding water when required from the dropping funnel so that there was always 3-5 ml. of liquid in the flask. Twenty-five milliliters of distillate was collected in 20 minutes and then a further 25 ml. aliquot collected. Each aliquot was brought to the boiling point to degas it, a drop of phenolphthalein solution added and titrated with dilute sodium hydroxide (0.01015 N). A few crystals of barium chloride added to a one ml. aliquot before titration established the absence of splashing. The volume of the first aliquot was reduced in vacuo at a bath temperature of 55° C to 0.5 ml.. This solution was passed through a column of Dowex 50 (acid form) (0.7 x 5 cm.) to regenerate the free acid.[74] A 25% ethylamine solution (0.2 ml.) was added to the eluant and then the volume reduced in vacuo to about 1 drop. The volatile acid mixture was then analyzed by paper chromatog-

raphy for acetic and propionic acids. Whatman #3 acid washed paper for chromatography was cut in strips (6 inches wide) and one end serrated so solvent could drip from the paper. The ethylamine salts of the oxidation mixture and acetic, propionic and formic salts were spotted on the paper 6.5 inches from the end. The paper was then folded for descending chromatography. Water saturated butanol served as the mobile phase while the chromatography tank was equilibrated with water saturated butanol, 0.025 N in ethylamine. Papers were placed in the tank for one half hour before addition of the mobile phase, then developed for 8 hours. These salts have a reproducible R_f (propionate 0.4, acetate 0.2, formate 0.15) when R_f is measured from the center of gravity of the spot. The front of the spot is concentration dependent to the extent that it was possible to estimate concentration from position of the front. A one to one ratio of acetate and propionate was resolved but overlap became serious as the ratio of propionate to acetate dropped. As there were two sources of acetate in the molecule, the acetyl group and the ethyl side chain, a ratio of 1:3 for the ethyl side chain results in a 1:7 practical ratio when hydrolysis is considered. Propionate was detectable only with great difficulty when the ratio became more than 1:20. In the experiment described no propionate was detectable. The detection of propionate and acetate on paper in the presence of ethylamine was not too easy but a satisfactory method[85] involved drying the paper overnight or in a stream of warm air from a hair

dryer then spraying with bromophenolblue solution followed by aqueous copper sulphate (2%) solution after drying. This spray gave blue spots on a mauve background. The bromophenol blue solution was prepared by dissolving bromophenolblue (300 mg.) in ethanol (300 ml.) then adding 30% aqueous sodium hydroxide (0.25 ml.) to change the colour of the solution from red to blue.

Oxidation of vindoline-^{14}C (13)

Vindoline-^{14}C (93.6 mg., 0.205 mmoles) with a specific activity of 5.54 x 10^4 d./m./mmole was added to 30% aqueous chromium trioxide (5 ml.) in the 50 ml. round bottom flask with modified Claisen head at room temperature.

The oxidation mixture (Lemieux)[73] had just previously been boiled and 30 ml. of water distilled from the reagent to remove any volatile acid impurities (blank titer 0.25 ml.). The reagent was very carefully heated as rapid evolution of carbon dioxide gave rise to considerable frothing in the first few minutes. The mixture was then heated strongly and four 60 ml. fractions of steam volatile distillate collected being careful to keep the volume of the reagent mixture at about 5 ml.. Each fraction was distilled in about fifteen minutes. After bringing to the boiling point and cooling each fraction was titrated with carbonate free lithium hydroxide (0.0128 N). The carbonate free base was prepared by dissolving lithium hydroxide (7 g.) in boiled out distilled water (40 ml.) and allowed to stand undisturbed under a sodalime guard tube for two days while the

lithium carbonate settled out. A portion (1.4 ml.) of this solution was diluted with distilled water (500 ml.) and the solution standardized against 0.01 N hydrochloric acid (5.000 ml.) using a pH meter to determine the end-point. Reagent blank 0.25 ml.; fraction I 26.42 ml., 0.338 mmoles; fraction II 2.45 ml., 0.0313 mmoles; fraction III 1.75 ml., 0.0224 mmoles; fraction IV 0.93 ml., 0.0121 mmoles; total steam volatile acid 0.404 mmoles. 1.97 equivalents of volatile acid were obtained from the radioactive vindoline. The aqueous solutions were evaporated in vacuo (55° C) to about 2 ml. then transferred to a 25 ml. round bottom flask. A few drops of solution were transferred to a scintillation counting vial and solvent removed under a stream of nitrogen. This sample of lithium salt (1.67 mg.) was to serve as insurance in case of unsuccessful isolation of the esters. It was never counted.

Preparation of p-bromophenacyl esters

The volume of solution was further reduced (0.3 ml.) and the original flask rinsed with a few milliliters of ethanol bringing the volume up to 10 ml.. p-Bromophenacylbromide (125 mg., 0.48 mmoles, 10% excess) was added and the solution refluxed for 40 minutes. The residue was separated by preparative thin layer chromatography on a silica gel G plate (0.5 mm. x 20 x 20 cm.) with fluorescent background developing with chloroform. Six bands (A-H) were scraped from the plate and eluted with ether in a Craig filtration apparatus.[82] The

eluted material was then analyzed by thin-layer chromatography. Band A (R_f 0.0 - 0.12) contained a small amount of an unidentified impurity which could have been p-bromophenacylalcohol. Band B (R_f 0.12 - 0.25, 40.33 mg) consisted of p-bromophenacylacetate (R_f 0.37) with two minor impurities at R_f 0.3 and 0.53 (propionate). Band C (R_f 0.25 - 0.26) was a narrow yellow band. Band D at R_f 0.26 - 0.30 (4.01 mg.) was p-bromophenacylpropionate (R_f 0.53 on a thin plate) with a small amount of the acetate (R_f 0.37 on a thin plate). Band E was blank. Band F (R_f 0.35 - 0.47) was p-bromophenacyl bromide. Band G (R_f 0.47 - 0.54) was a small amount of yellow unidentified impurity while band H (R_f 0.62) was a fluorescent impurity. The propionate band was rechromatographed on a standard silica gel thin layer plate (0.1 mm. x 5 cm. x 20 cm.) and after extraction with ether proved to be single spot material on thin-layer chromatography. This sample (0.4 mg.) was recrystallized three times from light petroleum ether to constant melting point, 59 - 60° C; lit. m.p. 61-62° C[86] and counted in the toluene scintillation mixture (2.01 mg., 275 $\pm$ 4.5 c./m.). A channel ratio (B/A) of 0.607 corresponds to a counting efficiency of 64%. This implies a specific activity of 5.50 x 10^4 d./m./mmole which implies that 99.3 $\pm$ 2% of the activity of the vindoline was located in the propionic acid residue. Similarly the p-bromophenacylacetate fraction was rechromatographed and shown by thin-layer chromatography to contain no propionate. It did still retain a small amount of the slow running impurity. An attempt to remove this

impurity by crystallization was not completely successful so the acetate was chromatographed one more time then crystallized three times from light petroleum ether (30-60°) m.p. 83-84° C (lit. 84-85° C).[86] This compound was counted as before (5 mg.) but no activity was detectable: background 24 ± 1 c./m., p-bromophenacylacetate 24 ± 0.5 c./m.. This indicated that none of the activity of vindoline was located in either the ethyl side chain or the acetyl function of vindoline(13).

Counting of N-methyl and O-methyl groups

The determination of alkoxyl groups in the form of ethers or esters is classically accomplished by treatment of the organic compound with boiling hydriodic acid.[77] The liberated iodides are flushed through a condenser and trap into a solution of bromine in acetic acid then estimated iodometrically. For determination of the N-alkyl group pyrolysis of the quaternary ammonium compound is usually required after liquid has been distilled from the residue. An alkoxyl determination was kindly carried out by Mr. P.Borda, the analyst for this department. In two determinations vindoline (10.871 mg.) required 42.24 ml. of sodium thiosulphate (0.009678 N) and 6.146 mg. required 12.24 ml. of sodium thiosulphate (0.01967 N). These figures implied 2.87 and 2.86 equivalents of methyl iodide for one equivalent of vindoline which indicated that the N-methyl, the methoxyl and the carbomethoxyl methyls were all being volatilized as methyl iodide. Active vindoline (4.197 mg, specific activity

5.54 x 10^4 d./m./mmole) was treated with boiling hydriodic acid and the methyl iodide produced bubbled, using the analytical apparatus, directly into the scintillation mixture (5 ml.). This mixture was then counted and no activity was detectable in the sample within the limits of counting error.

Appendix I

Statistical Error

Standard deviation (68% probability of being within)

$$\sigma = \pm \frac{\sqrt{N}}{t}$$

N count
t time

i.e. total count 10,000, one can say 10,000 $\pm$ 100 i.e.1%

$$\sigma = \frac{100}{t}$$

If t = 10 minutes the count is 1,000 $\pm$ 10 c./m.

Addition(background) of Counts

$$\sigma = (\sigma_1)^2 + (\sigma_2)^2$$

multiplying or dividing,

$$Q = R_1/R_2 \qquad \sigma_Q = Q\sqrt{\left(\frac{\sigma_1}{R_1}\right)^2 + \left(\frac{\sigma_2}{R_2}\right)^2}$$

similarly $P = R_1 \cdot R_2$

$$\sigma_p = P\sqrt{\left(\frac{\sigma_1}{R_1}\right)^2 + \left(\frac{\sigma_2}{R_2}\right)^2 + \cdots}$$

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