BIOSYNTHESIS OF LYCOPODIUM ALKALOIDS

BIOSYNTHESIS OF LYCOPODIUM ALKALOIDS

By

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SCOPE AND CONTENTS:

An investigation of the biosynthesis of lycopodine, the major alkaloid of Lycopodium tristachyum, has revealed that L-lysine, but not D-lysine is incorporated into this alkaloid. D-lysine serves as a precursor to pipecolic acid in this species. Sodium malonate has been demonstrated to be a specific precursor to lycopodine, but attempts to verify the postulated intermediacy of piperidineacetic acid in the elaboration of this alkaloid were unsuccessful.

As a means of studying the postulated conversion of lycopodine to annotinine, the major alkaloid of Lycopodium annotinum, specifically labelled 9-^{14}C-lycopodine was synthesized. However the specific incorporation into annotinine of several precursors known to be incorporated into lycopodine could not be demonstrated.

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INTRODUCTION

1.1 RADIOACTIVE TRACERS IN THE STUDY OF ALKALOID BIOSYNTHESIS

The alkaloids form a structurally diverse family of natural products of plant origin, which are grouped together solely because of the common presence of a nitrogen which is almost always basic. The physiological activity of many of the alkaloids has generated a wide interest in the chemistry of these compounds. However virtually nothing is known about the function of alkaloids within the organisms which produce them.

Early biogenetic studies of these compounds were usually centered on one of two areas. Attempts were made to monitor the sometimes dramatic fluctuation in alkaloid content or to discern the particular portion of the plant in which the alkaloids were being synthesized (1). Many of the conclusions reached in these studies were subject to considerable uncertainty and the realization of results was very slow. There was no evidence which would relate alkaloids to definite biochemical or biophysical functions and thus explain their significance in the metabolism of the plants that produced them. In the absence of anything concrete a number of speculative notions developed. For example the production of alkaloids was thought to parallel some phase of protein metabolism, but since the alkaloids were often found in tissues of low metabolic activity they were usually considered as waste material. They would however be available to the plant for further exploitation and could

thus represent an energy source or a nitrogen source.

Although the chemical literature on alkaloids is rich with biogenetic speculation based purely on chemical analogy, the verification of these ideas had to await the development of an experimental probe. Many of the biogenetic speculations on the alkaloids owe their origin to the insight of Sir R. Robinson, who suggested that alkaloids were synthesized from amino acids and a small number of simple molecules. Many of his proposals, based solely on a visual dissection of the molecules, have been experimentally verified (2). Yet as he pointed out such "chemical speculations" allowed a series of substances to be placed in a possible order of biogenesis but did not allow any conclusions to be drawn about the mechanism of the individual steps.

A chemical probe to test the validity of biogenetic postulates in vivo became possible with the advent of radioisotopes of relatively long lifetimes (3) such as ^{3}H and ^{14}C. Since they first became available in the late nineteen forties the use of radioactive isotopic tracers has rapidly gained popularity and they have been widely used in the study of biosynthetic routes in many biological systems. Their main advantage over rare but stable isotopes such as ^{2}H, ^{13}C, ^{15}N and ^{18}O as probes for biological research is their detection and quantitative measurement in very small quantities.

The usual technique in metabolic studies using radiotracers has been to administer a specifically labelled precursor and after a suitable period of incubation to harvest the specimens, thereby stopping the metabolism of the organisms. The compound under study is isolated,

rigorously purified, then degraded chemically to ascertain the pattern of labelling. The degradation ideally involves chemical transformations which will uniquely isolate a single carbon atom as a suitable derivative. The derivative is then assayed for radioactivity. The suspected intermediate is considered to be a precursor if the labelling in the product follows a predicted pattern (i.e. non-random incorporation). A suspected intermediate is a specific precursor if intact incorporation can be demonstrated, usually by the use of double labelling. The same ratio of one isotope to another in the precursor and the product is considered as evidence of intact incorporation.

Several complementary techniques have been developed to demonstrate a precursor-product relationship. An "isotope sparing" or "metabolite swamping" experiment is sometimes used to demonstrate that a compound under examination is indeed an intermediate on a biosynthetic pathway. The repression of incorporation of label into a product from a general carbon source (or a precursor known to label the product specifically) by a large quantity of inactive compound suggests that the diluent is an intermediate on the biosynthetic pathway between the known precursor and the product.

The presence of a compound which is a suspected intermediate has sometimes been demonstrated by the addition of a large quantity of the inactive compound to the crude extract from a tracer experiment during the isolation sequence. After rigorous purification to constant activity the demonstration of non-random incorporation of activity is evidence that the compound under study is an intermediate. This technique is

called "carrier dilution".

More recently a technique (which will be more fully discussed later) has been described for determining the chirality of a known precursor (4). By studying the incorporation of a doubly labelled precursor in which one isotope is restricted to one optical isomer of the precursor, while the second isotope is present in both antipodes a stereoselective requirement for the incorporation of the precursor may be demonstrated.

The interpretation of results from feedings with labelled compounds must be treated with considerable care. In particular any conclusions drawn from negative results must be carefully scrutinized. The incorporation of label into a product is the cumulative result of several processes. Incorporation is the result of, (i) transport of the precursor to the site of metabolism, and (ii) the transformation of the precursor to the product. The order of intermediates in a particular biosynthetic sequence has sometimes been assessed on the basis of incorporation efficiencies. Yet the differences in efficiencies with which precursors are converted to a product may reflect only differences in the rate at which the different precursors are transported to the site of synthesis. Alternatively the different incorporation efficiencies may reflect only variations in the size of the indigenous metabolic pools. The problems are somewhat compounded when applied to studies of plant biosynthesis as opposed to studies on microorganisms. Whereas in the case of microorganisms one studies the average action of thousands of individuals, the studies on plants are conveniently conducted

on only a few specimens and may not necessarily reflect "normal behaviour". In studies with microorganisms it is comparatively easy to approach "physiological conditions" while a potential precursor is being administered, but it is very difficult to do the same with plants. A possible result is that a plant may alter its metabolic behaviour in response to the experimental conditions. As an example, feedings to plants are often carried out on cuttings. However, it is known that a rapid breakdown of protein takes place in the leaves after excision leading to an accumulation of soluble nitrogen chiefly in the form of amino acids (5). Dilution of the labelled precursor may result. Other techniques which have been used to administer a labelled compound to a plant include injection, root feeding, wick feeding, and application to the leaves.

Attempts have been made to approach "physiological conditions" by exposing the plants to an isotopically labelled environment under "steady state conditions". As defined by Rapoport (6) the requirements for the steady state condition are twofold: (i) that all growth conditions other than the presence of labelled carbon dioxide must be normal, (ii) the specific activity of the carbon dioxide must not vary during the exposure. If these conditions are satisfied then the sequence of intermediates can be inferred on the basis of incorporation efficiencies. However the apparatus required to perform such an experiment is elaborate and the experimental conditions are difficult to achieve. The technique has yet to gain wide popularity.

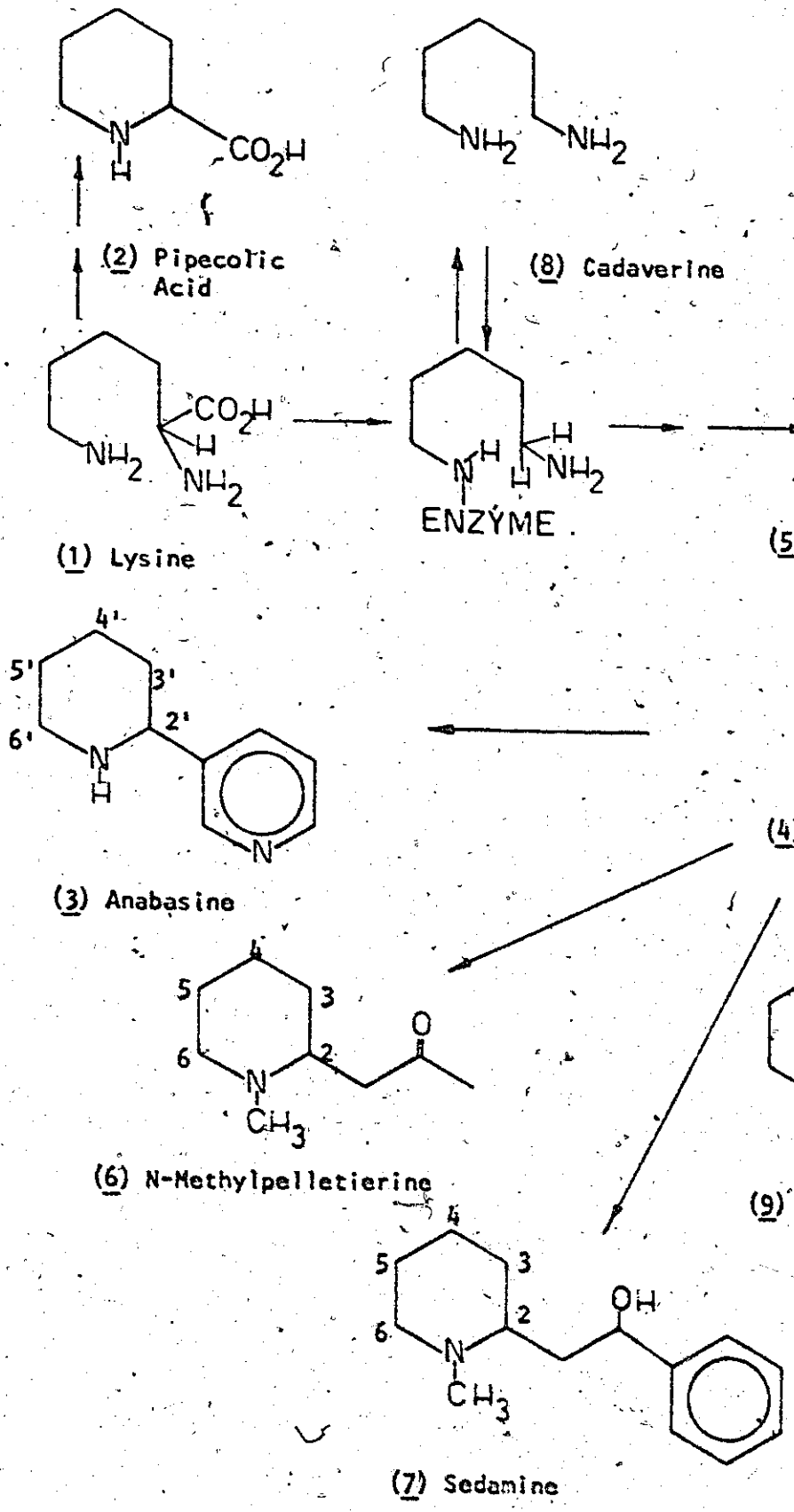
Yet despite what would appear to be a plethora of uncontrollable factors, considerable information has been obtained concerning the biosynthesis of natural products by using isotopic tracers in metabolic studies. Precursor-product relationships have now been described for many families of alkaloids and many structural groups have been related to intermediates of primary metabolism. The next stage of research will involve the testing of biosynthetic sequences, the isolation of the individual intermediates, and a study of the mechanism of the individual transformations. This will involve the isolation of enzymes, defining the required cofactors, and a detailed study of the reaction kinetics. This area of alkaloid research is virtually untouched (7).

1.2 LYSINE METABOLISM - ORIGIN OF THE PIPERIDINE RING

1.2.1 PIPERIDINE ALKALOIDS

Lysine (1) has long been regarded as a precursor of the hetero ring of many naturally occuring piperidine derivatives. This postulate has been verified in the case of pipecolic acid (2) and several alkaloids from diverse sources. The simple piperidine derivatives have been most extensively studied and will be considered first. A biosynthetic scheme which illustrates the origin of these alkaloids is outlined in Scheme 1.

The administering of D,L-2-^{14}C-lysine to Nicotiana glauca R. Grah led to the formation of anabasine (3) labelled exclusively at the 2' position (8). The nitrogen of the piperidine ring is preferentially derived from the ε-amino group of lysine and the immediate precursor of the hetero ring is thought to be Δ'-piperideine (4), the cyclized



Biosynthesis of Piperidine Alkaloids

Scheme 1

form of 5-aminopentanal (5). It has been shown that D,L ε-^{15}N, 2-^{14}C-lysine entered the piperidine ring of anabasine without change in the ^{15}N:^{14}C ratio (9) while when D,L α-^{15}N, 2-^{14}C-lysine was tested as a precursor, the anabasine which was isolated contained no detectable ^{15}N enrichment but was labelled with ^{14}C at the C-2' position of the ring.

Several other piperidine alkaloids have been shown to arise in similar fashion. N-Methylpelletierine (6) a base from Sedum sarmentosum Bunge was found to incorporate lysine without randomizing the activity. All the activity from a feeding of D,L-6-^{14}C-lysine was demonstrated to be at the C-6 position of this alkaloid (10). Analogous results were observed in separate feedings of D,L-2-^{14}C-lysine and D,L-6-^{14}C-lysine to Sedum acre L. The sedamine (7) which was isolated contained all the activity of the intact alkaloid at the C-2 and the C-6 position, respectively (11). Confirmatory evidence that lysine supplies the intact five carbon unit comes from doubly labelled feeding experiments. Sedamine, and N-methylpelletierine isolated from separate feedings using D,L-4,5-$^{3}H_2$, 6-^{14}C-lysine and D,L-6-^{3}H,6-^{14}C-lysine each showed a ^{3}H:^{14}C ratio which was identical with that of the precursor (12,13). More recently (4) it has been demonstrated that sedamine, N-methylpelletierine, and anabasine are synthesized from L-lysine. This selectivity of incorporation has been demonstrated by the use of doubly labelled tracers.

In each of the cases mentioned above lysine serves as a specific precursor and moreover this incorporation does not take place via a

symmetrical intermediate. This in turn suggests that the double bond of Δ^1-piperideine cannot be labile. As predicted, anabasine isolated from a feeding of 6-^{14}C-Δ^1-piperideine to N glauca, was found to contain all the label at C-6' (14). The fact that cadaverine (8) acted as a relatively efficient precursor of this alkaloid (15) was attributed to the non-specificity of the enzymes involved in its oxidation to Δ^1-piperideine. This transformation has been studied in several biological systems (16).

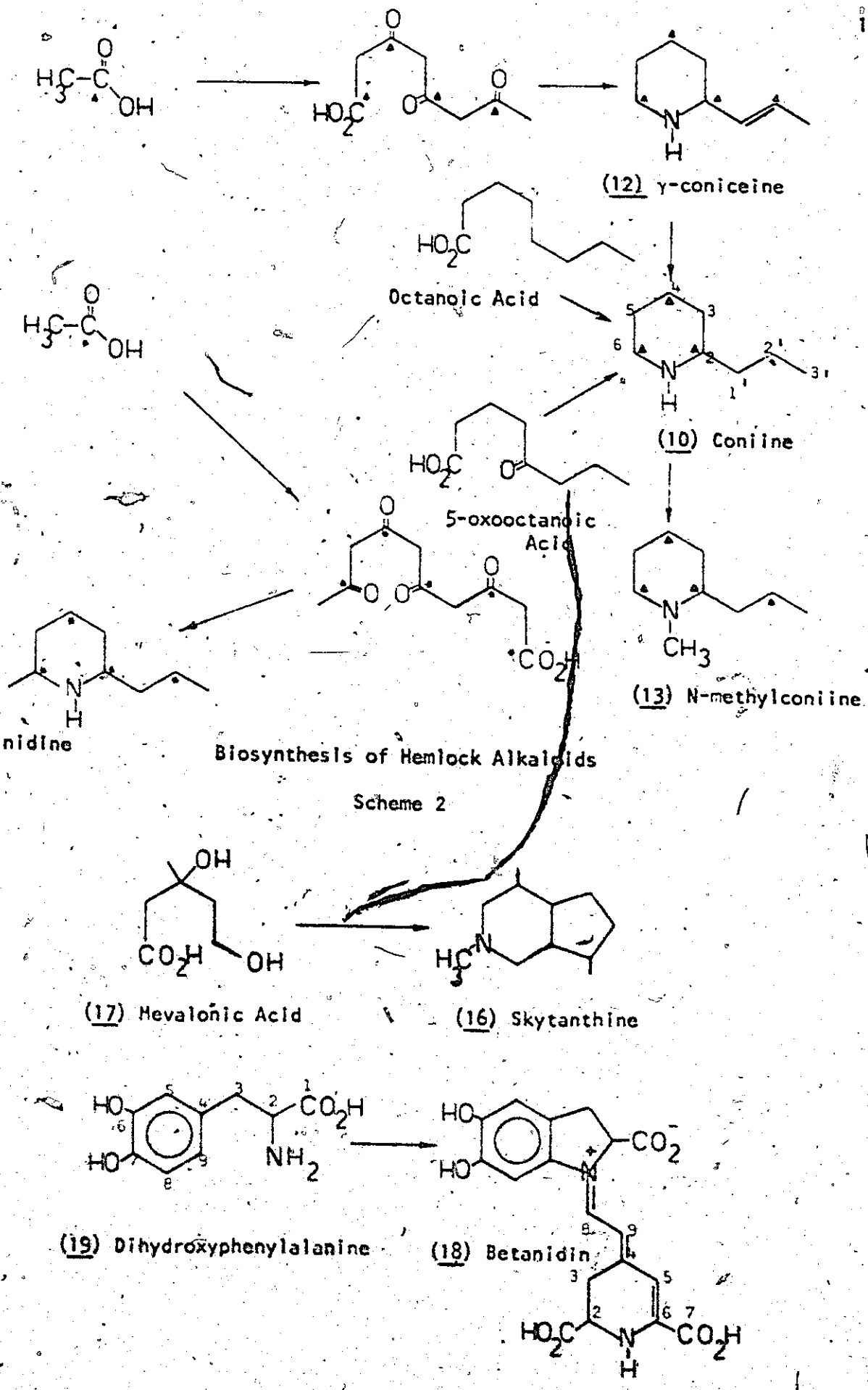
Evidence has been obtained which further describes the role of cadaverine and militates strongly against the incorporation of cadaverine by an "aberrant pathway". Cadaverine has been shown to be a normal constituent of S. acre. The cadaverine which was isolated from a feeding of D,L-4,5-$^3H_2$, 6-^{14}C-lysine to this species was found to have a ^3H:^{14}C ratio which was identical with the initial feeding solution, and with the sedamine which was also isolated (17). This result is consistent with the postulate that cadaverine bound to an enzyme complex serves as a precursor. As long as the equilibrium between the bound and unbound species greatly favours the bound species then the tracer evidence gathered to date is in accord with the intermediacy of cadaverine. The enzyme complex diamine oxidase has been postulated to be involved in the conversion of the bound cadaverine to Δ^1-piperideine. By the use of doubly labelled precursors a hitherto unsuspected stereospecificity has been demonstrated for this interconversion. The fact that the decarboxylation of lysine by L-lysine decarboxylase (ex Bacillus cadaveris) is known to take place with stereochemical specif-

icity was used to prepare two enantiomeric chirally labelled 1-^{3}H cadaverines (17). One sample was prepared by the action of L-lysine decarboxylase on D,L-2-^{3}H-lysine (1B-^{3}H-cadaverine) while the enantiomer was prepared by the decarboxylation of D,L-lysine (using the same enzyme) in the presence of tritiated water (1A-^{3}H-cadaverine). It is not known however, whether the decarboxylation of L-lysine by this enzyme occurs with retention or by inversion. Thus the absolute stereochemistry of the two labelled enantiomers cannot be assigned.

Each of the enantiomers was separately mixed with 1-^{14}C-cadaverine and administered to S. sarmentosum. N-Methylpelletierine and N-methylallosedridine (9) were isolated, the ^{3}H:^{14}C ratio determined and then they were partially degraded to determine the site(s) of tritium labelling. Whereas in the feeding where 1B-^{3}H,1-^{14}C-cadaverine was tested as a precursor the isolated alkaloids contained tritium only at C-6, the alkaloids from the second feeding where 1A-1-^{14}C-cadaverine was tested as a precursor contained tritium at C-2 and C-6. It was found that in the first feeding the ^{3}H:^{14}C ratio of the alkaloids was one half of that of the initial feeding solution while in the latter feeding the ^{3}H:^{14}C ratio of the alkaloids was the same as that of the initial feeding solution. Thus the oxidative deamination of cadaverine in S. sarmentosum is stereospecific.

1.2.2 THE HEMLOCK ALKALOIDS

Not all Piperidine alkaloids are of lysine origin. The Hemlock alkaloids provide a notable exception. The biosynthetic pathway to these alkaloids is outlined in Scheme 2. Based on the efficiencies of



Biosynthesis of Hemlock Alkaloids

Scheme 2

incorporation of L-U-^{14}C lysine (18), of U-^{14}C-Δ^1-piperideine and of U-^{14}C-Δ^1-piperideine-2-carboxylic acid (18,19) a biosynthetic pathway involving the intact incorporation of lysine was postulated for coniine (10). However the isolated alkaloid from these feedings was not of high enough activity to allow the pattern of labelling to be determined by chemical degradation. A pathway involving lysine became suspect when activity from 1-^{14}C sodium acetate was shown to enter the hetero ring of coniine as well as the side chain. Moreover Leete was able to demonstrate that the activity was equally divided among the four even numbered carbon atoms of the molecule (20). Thus coniine was of poly-β-keto acid origin formed from a linear combination of acetate units. The earlier observation that generally labelled lysine can serve as a precursor could be explained by a breakdown of lysine to acetate (scheme 3) prior to its incorporation into coniine.

A similar biosynthetic origin from acetate was demonstrated for pinidine (11). A partial degradation of this alkaloid isolated from a feeding of 1-^{14}C-sodium acetate to Pinus jeffrei demonstrated that this compound was labelled at alternate carbon atoms (21). The fact that 2-^{14}C-lysine when tested as a precursor gave rise to a labelling pattern in the alkaloid which was identical with that from 1-^{14}C-sodium acetate necessitates a breakdown of this precursor prior to incorporation.

The sequence of formation of the Hemlock alkaloids has been studied using carbon-14-dioxide (22) and has been suggested to follow the order: γ-coniceine (12) to coniine (10) to N-methylconiine (13). The oxygenated Hemlock alkaloids appear to be synthesized at a much

slower rate. Octanoic acid (14) and 5-oxooctanoic acid (15) have been shown to be efficient precursors of coniine (23).

Other biosynthetic pathways to the Piperidine alkaloids have also been observed but these pathways are limited in number. Skytanthine (16) a base from Skytanthus acutus has been shown to be derived from two molecules of mevalonic acid (17) (24). Although ultimately derived from acetate the attachment is not linear as in the hemlock alkaloids. Betanidin (18) (the aglycone of betanin) a base from cactus fruits (Opuntia sp.) has been shown to be derived from dihydroxyphenylalanine (19) (25).

1.2.3 PIPECOLIC ACID

Pipecolic acid has been implicated as a lysine metabolite in several biological systems. Soon after its detection and isolation from natural sources (26,27) pipecolic acid was shown to be a lysine metabolite in higher plants (28), Neurospora crassa (No. 4545, G.W. Beadle) (29), and the rat (30). Thus in bean plants (Phaseolus vulgaris) activity from D,L-6-^{14}C-lysine (28), from D,L-2-^{14}C-lysine (31) and from L-1-^{14}C-lysine (32) was found to be incorporated into pipecolic acid. In a lysine less mutant of N. crassa L-1-^{14}C lysine was found to label pipecolic acid (29) and activity from L-6-^{14}C lysine was incorporated into this same compound in the rat (30,33). Although the distribution of activity within the labelled pipecolic acid was not determined in any of the cases mentioned above, and thus intact incorporation has not been conclusively demonstrated, the accumulated tracer results make it likely that pipecolic acid is derived from the intact

carbon chain of lysine.

Rothstein and Miller (34) demonstrated that the α-amino group of lysine is lost in its conversion to pipecolic acid. When D,L-ε-^{15}N lysine was administered to the rat as a potential precursor a significant portion of the label was retained in the pipecolic acid yet when D,L-α-^{15}N-lysine was similarly tested no significant portion of the label was retained. This conclusion is corroborated by recent doubly labelled feedings in which D,L-6-^{3}H,6-^{14}C-lysine was administered to the rat, N. crassa, P. vulgaris, and S. acre. The pipecolic acid isolated from these feedings was found to have, in each case, a ^{3}H:^{14}C ratio which was identical with that of the precursor (12). A second series of experiments in which D,L-2-^{3}H,6-^{14}C lysine was tested as a precursor of pipecolic acid in these systems resulted in pipecolic acid which contained carbon-14 but no tritium (13). These experimental observations suggest the intermediacy of α-keto-ε-aminocaproic acid (20) between lysine and pipecolic acid and negate the possibility that α-aminoadipic acid-ε-semialdehyde (21) might serve as an intermediate.

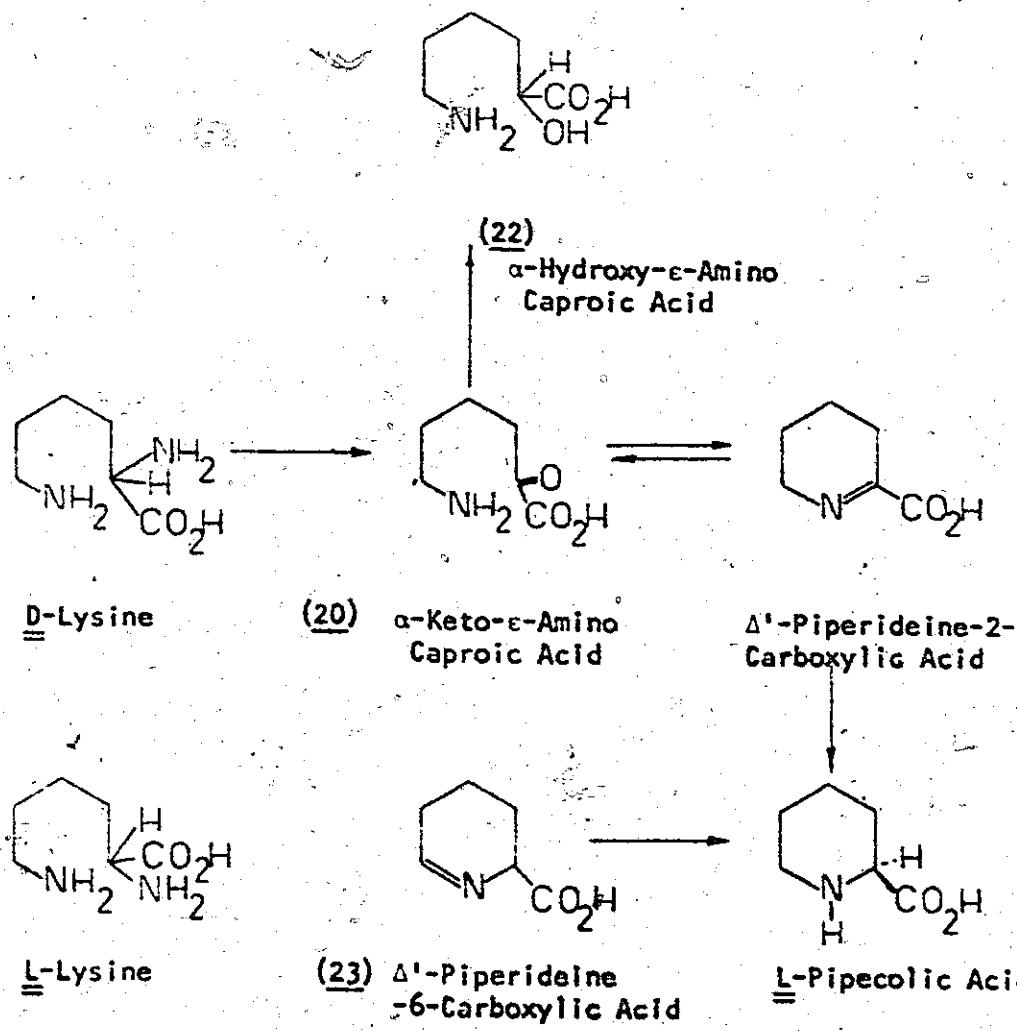
Work with N. crassa suggested that it is the L isomer of pipecolic acid which is lysine derived. When L-1-^{14}C-lysine was administered in the presence of radioinactive D,L-pipecolic acid and the isolated pipecolic acid was resolved into its two enantiomers, only the L isomer contained significant label above background (29). This observation coupled with their isolation of radioactive α-hydroxy-ε-aminocaproic acid (22) led these authors to postulate that in N. crassa Δ'-piperideine-2-carboxylic acid (23) the cyclization product of α-keto-ε-aminocaproic

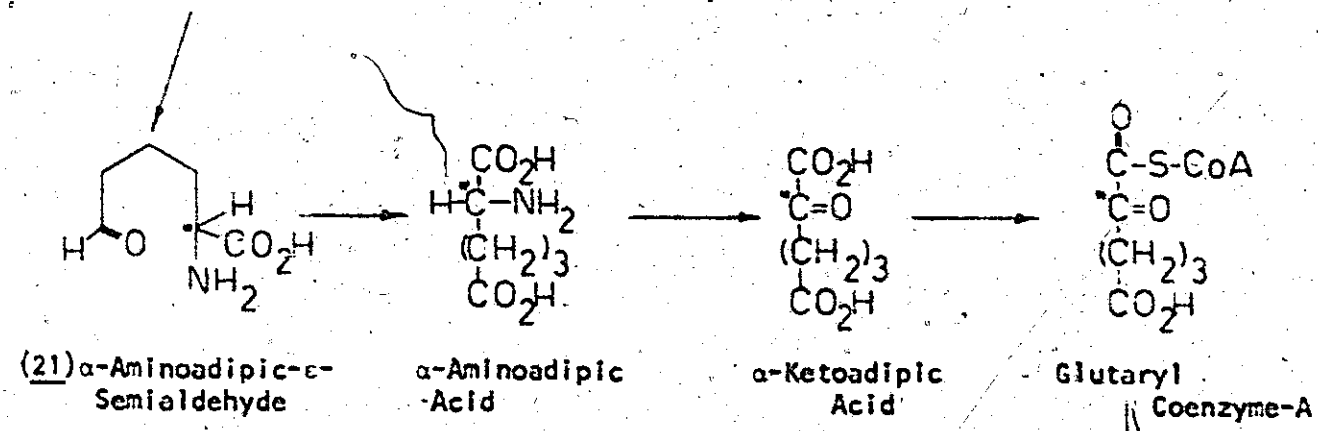
acid is stereospecifically reduced to the L isomer of pipecolic acid. A potential route for the catabolism of lysine in these systems is outlined in scheme 3.

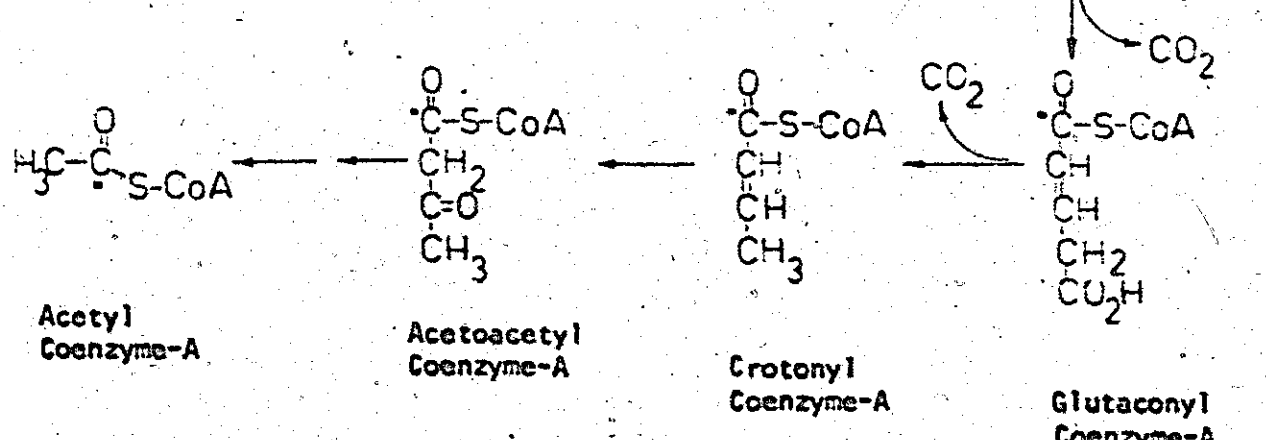
More recently (4) the chirality of the lysine precursor has been re-examined. When a mixture of L-4,5-$^{3}H_2$-lysine and D,L-6-^{14}C-lysine was administered to three plant specimens the isolated pipecolic acid was found to have lost all the tritium relative to carbon-14. This would suggest that D-lysine but not L-lysine was being incorporated into the hetero ring of pipecolic acid. Companion experiments in which a mixture of D,L-4,5-$^{3}H_2$-lysine and D-6-^{14}C-lysine was administered to the same species resulted in pipecolic acid which had a ^{3}H:^{14}C ratio which was one-half of that of the initial feeding solution. If D-lysine were being selectively incorporated into pipecolic acid then one-half of the tritium relative to carbon-14 should have been lost. These observations support other recent evidence that L-pipecolic acid is derived from D-lysine in mammals (35,36) and in microorganisms (37,38) and contradict the earlier conclusions that L-lysine is incorporated into pipecolic acid.

1.2.4 LUPIN ALKALOIDS

The biosynthesis of one other family of alkaloids is also worthy of consideration. A biosynthetic derivation from lysine has been established for several lupin alkaloids but lysine has been shown to be incorporated via a symmetrical intermediate. This contrasts with the mode of incorporation of lysine into anabasine. Lupinine (24) isolated from separate feedings of D,L-2-^{14}C-lysine and D,L-6-^{14}C-lysine


(22)
α-Hydroxy-ε-Amino
Caproic Acid
D-Lysine
(20) α-Keto-ε-Amino
Caproic Acid
Δ'-Piperideine-2-
Carboxylic Acid
L-Lysine
(23) Δ'-Piperideine
-6-Carboxylic Acid
L-Pipecolic Acid

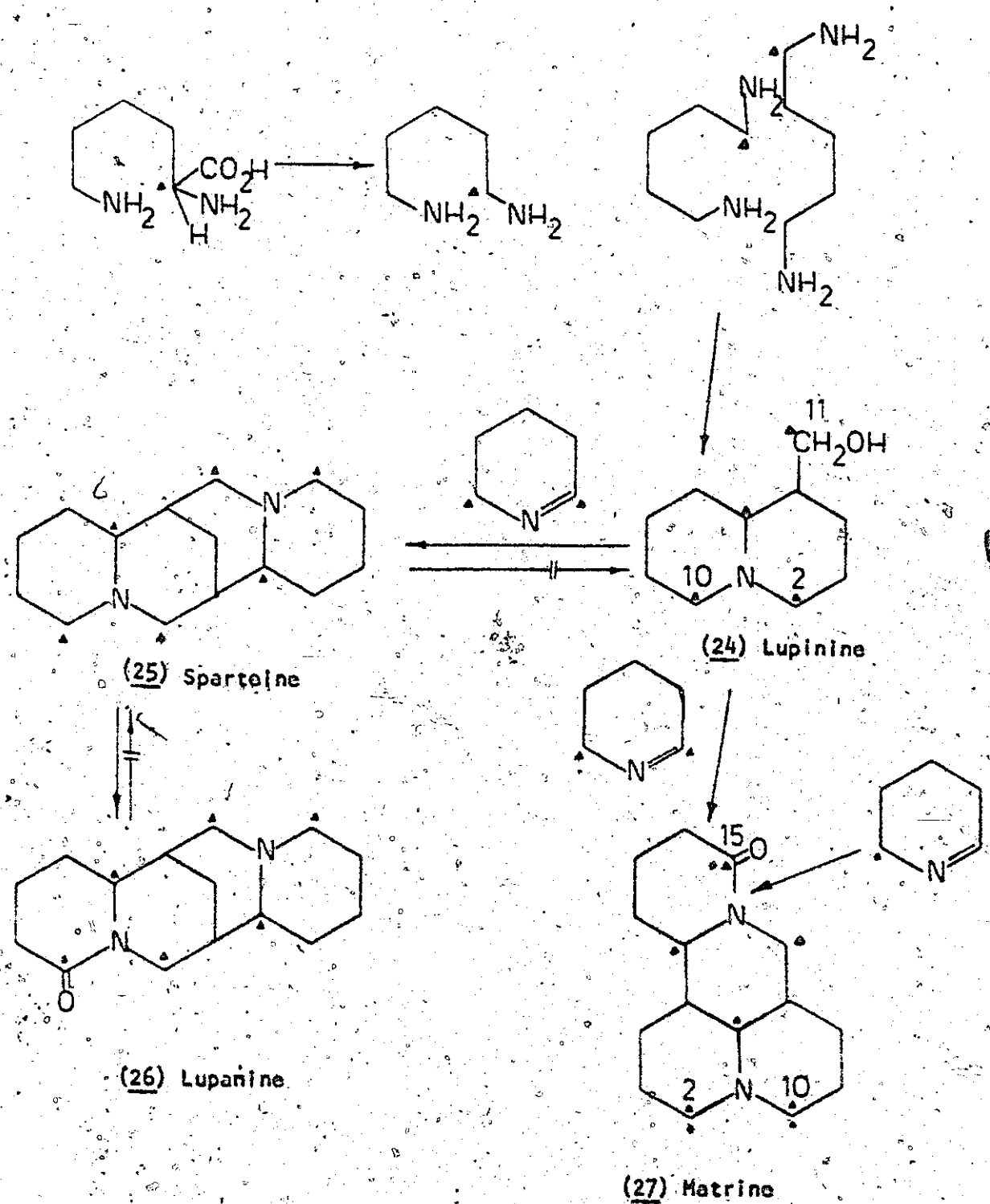

(21) α-Aminoadipic-ε-
Semialdehyde
α-Aminoadipic
-Acid
α-Ketoadipic
Acid
Glutaryl
Coenzyme-A


Acetyl
Coenzyme-A
Acetoacetyl
Coenzyme-A
Crotonyl
Coenzyme-A
Glutaconyl
Coenzyme-A

Scheme 3

to Lupinus luteus was partially degraded to determine the pattern of labelling. In both cases 25% of the activity of the intact alkaloid was found at C-11, while approximately 25% of the activity was found to be at C-2 and at C-10 (39,40). Partial degradation of sparteine (25) isolated from the same plant source demonstrated that one sixth of the activity resided on the marked carbon atoms of figure (25) (41). Similar results were observed in the case of lupanine (26) (42) and matrine (27) (43). A feeding of 6-^{14}C-Δ^1-piperideine was found to label carbons C-2, C-10, and C-15 of matrine (44). The interconversion of these alkaloids has also been studied. It has been demonstrated that activity from lupinine enters sparteine, and that activity from sparteine enters lupanine, however the reverse processes do not occur (45).

Since in all cases labelled cadaverine led to labelling patterns in the alkaloids identical with those from labelled lysine it has been suggested that cadaverine may indeed be a normal intermediate. A sequence which is compatible with these observations is shown in scheme 4. Confirmatory evidence for the intermediacy of cadaverine was sought by studying the incorporation of doubly labelled progenitors. A solution of D,L-α-^{15}N-2-^{14}C-lysine was administered separately to L. luteus and to Sarothamnus scoparius. In each case sparteine was isolated and the ^{14}C:^{15}N ratios were measured. This ratio was found to be three times that of the initial feeding solution (41). When account is taken of the fact that for every three molecules of lysine which are incorporated only two atoms of nitrogen are retained this result is



Biosynthesis of Lupin Alkaloids

Scheme 4

consistent with the postulate that the α-amino group and the ε-amino group are equally effective in supplying the nitrogen atoms of sparteine. The intermediacy of cadaverine is however not mandatory because it is conceivable that the dilution of the ^{15}N may result from exchange with the nitrogen pool prior to its incorporation. It is interesting to note that diamine oxidase activity has been demonstrated in a preparation from lupin seedlings (45).

1.3 LYCOPODIUM ALKALOIDS

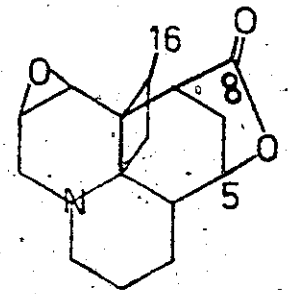
1.3.1 Historical Introduction

Alkaloids of the genus Lycopodium have not been subjected to scientific scrutiny until fairly recently, although they have been known to exist for many years. The first example was observed and recorded by Bödeker in 1881 (46) when he isolated a nitrogenous base from Lycopodium complanatum L. It was however the work of Manske and Marion, whose comprehensive assay of eleven species of Lycopodium for alkaloids, stimulated a wide interest in this family of compounds. Using the techniques of crystallization, selective solubility, and sublimation they were able to isolate and partially characterize some thirty-five new bases; a tribute to their patience and manipulative skills. They demonstrated the general occurrence of alkaloids within the genus and provided the impetus for further research into this group of compounds. Their efforts are documented in a review (47).

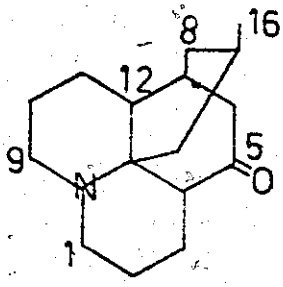
Structural studies, first begun in the nineteen forties, reached fruition in 1957 when the structure of annotinine (28) was realized by

Wiesner and his co-workers (48). This structure was verified by X-ray crystallographic studies of annotinine bromohydrin the following year (49). The second base whose total structure was elucidated was lycopodine (29) (50), the major alkaloid of this series. Since then many new alkaloids of diverse structure have been described and undoubtedly new structural numbers will be isolated as more species of club moss are examined. Only about twenty-two species have been examined to date although it has been estimated that there are over four hundred members of this genus (51).

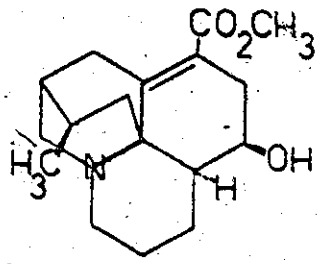
Representative examples of this class are illustrated in figure 1. The skeletal array represented by lycopodine is by far the most common. Alkaloids of this skeletal type are found in many species of wide geographical distribution whereas the other skeletal configurations as represented by annotinine (28), annopodine (30), annotine (31), cernuine (32), luciduline (33), lycopecurine (34), lycodine (35), lyconnotine (36), selagine (37), serratinine (38) and fawcettidine (39) are considerably more restricted in distribution. The majority of these latter alkaloids are elaborated in only one or two species. Lycopodium cernuum L. is the only species studied to date which does not appear to elaborate any alkaloids of the lycopodine structural type (52). This is surprising in view of the fact that of the twenty-two species of Lycopodium which have been studied lycopodine has been found to be a constituent of sixteen of them. Although the majority of this class are tetracyclic and mononitrogenous, pentacyclic and tricyclic structures as well as dinitrogenous bases are also known. A common



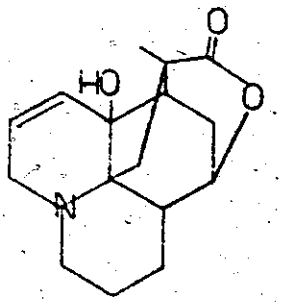
(28) Annotinine



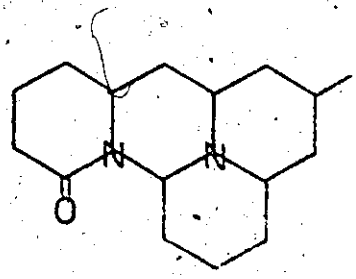
(29) Lycopodine



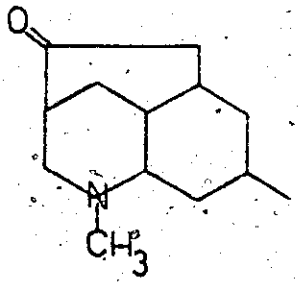
(30) Annopodine



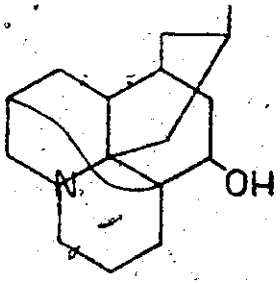
(31) Annotine



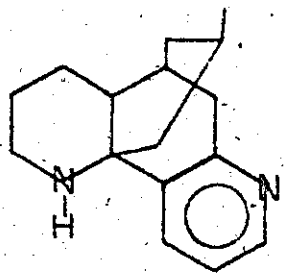
(32) Cernuine



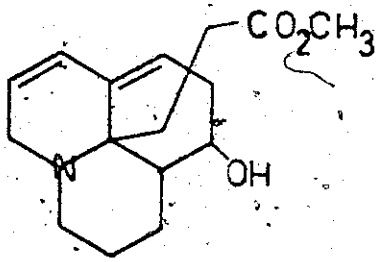
(33) Luciduline



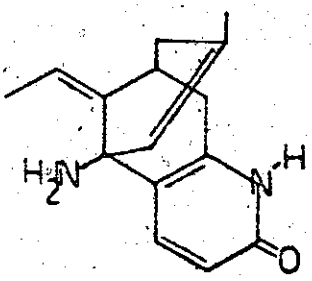
(34) Lycopecurine



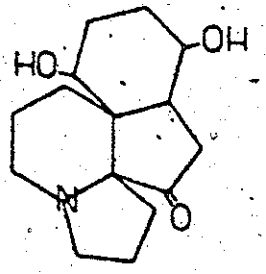
(35) Lycodine



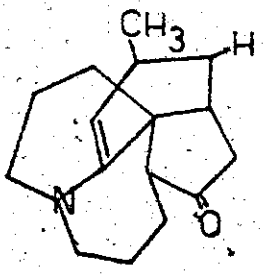
(36) Lyconnotine



(37) Selagine



(38) Serratinine



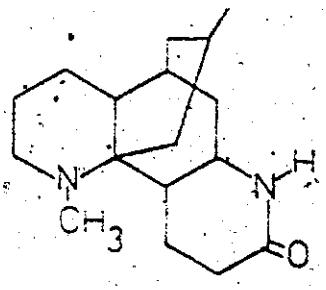
(39) Fawcettidine

Representative Lycopodium Alkaloids

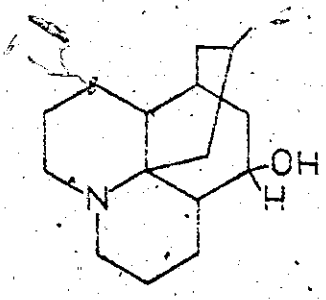
Figure 1

biogenetic origin for many of these alkaloids has been postulated on the basis of similar structural features.

Several of the alkaloids of this series have been interrelated chemically by transformations of one alkaloid to another and several hypothetical schemes interrelating the alkaloids have been proposed. Examples of the former are found in studies designed to determine the relative stereochemistry of an alkaloid of the series. Ayer and Iverach (75) have transformed α-obscurine (40) to dihydrolycopodine (41) and lycopodine (29) has been converted to lycodine (35) by Anet and Rao (54).



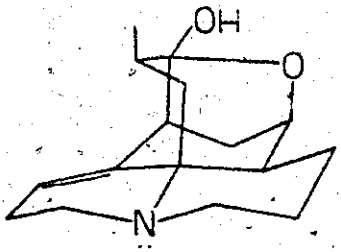
(40)



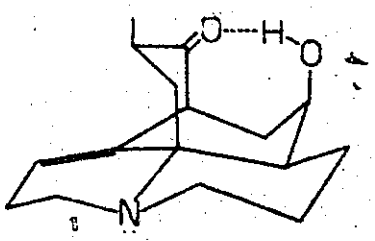
(41)

A relationship between acrifoline, a minor alkaloid of Lycopodium annotinum Linn, and annotinine (28), the major alkaloid of this species, has been suggested and a plausible mechanism for their interconversion has been offered. Acrifoline is known to exist partially as a hemiketal (42) and partially as an internally hydrogen bonded hydroxyketone (43)

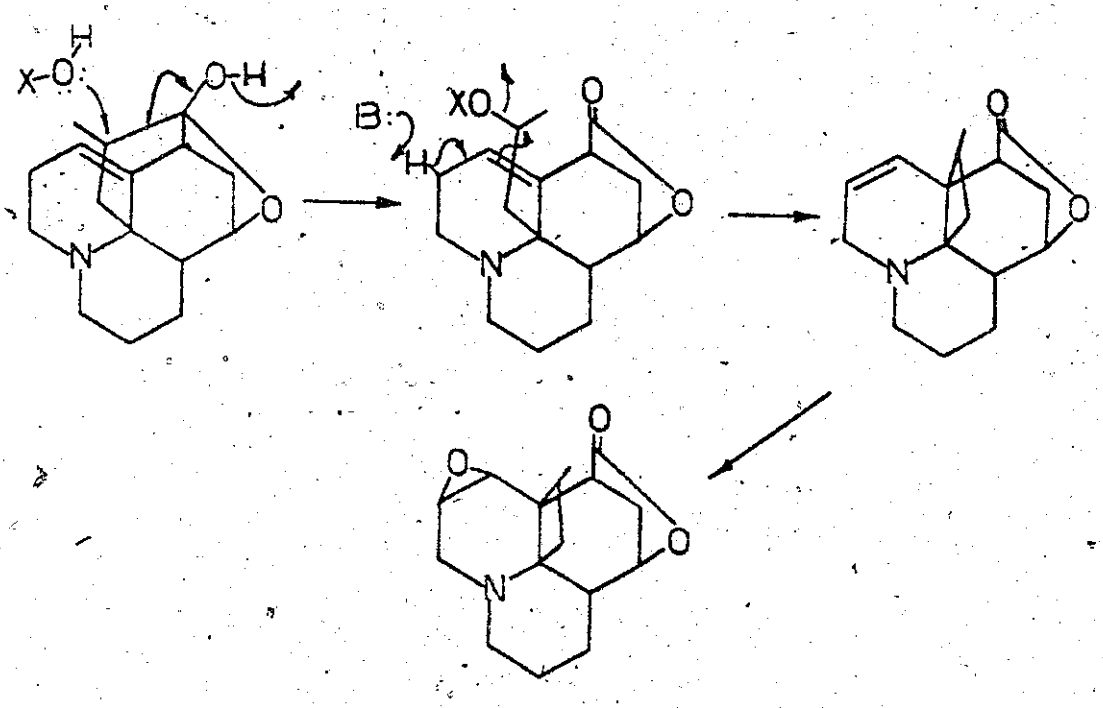
(55). Wiesner has pointed out the structural similarities between annotinine and acrifoline written as a hemiketal and has suggested the following potential mechanism for their interconversion (56).



(42)



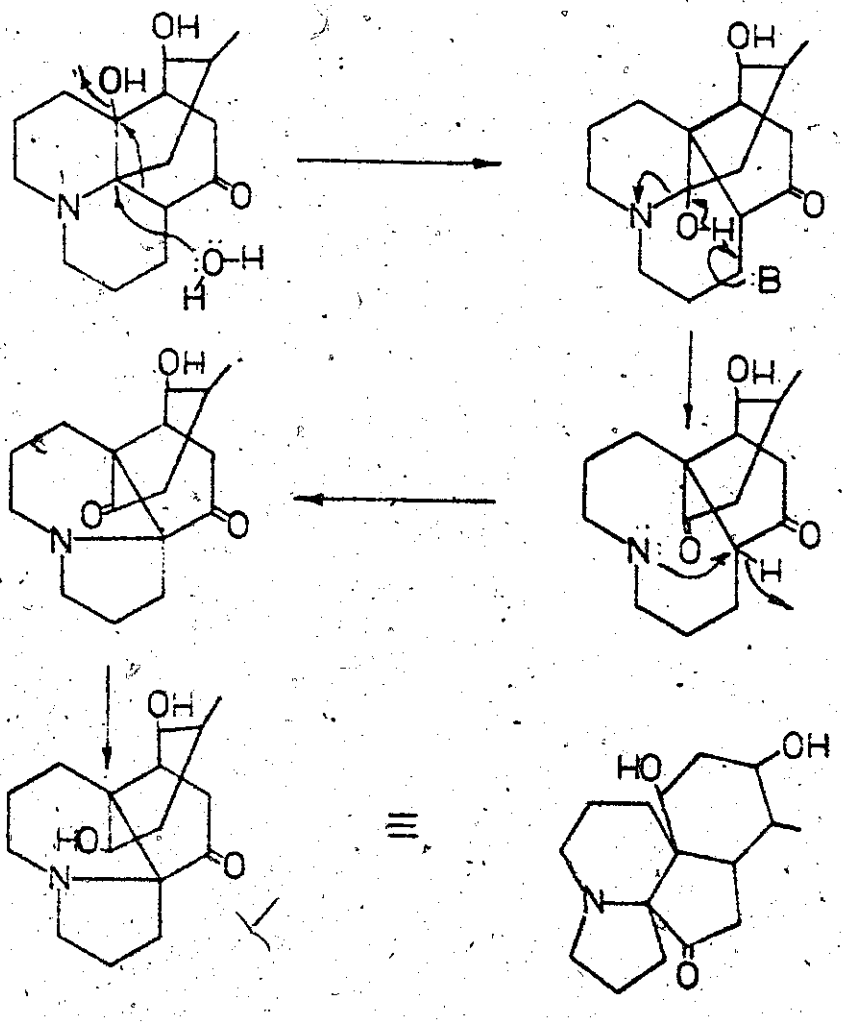
(43)



(28)

It should be noted that lycopodine (29) has been chemically transformed into annofoline (11,12-dihydroacrifoline) by Ayer and co-workers (53).

It has been suggested by Inubushi (57) that serratinine (38) the major alkaloid of L. serratum var. thunbergii, may arise from a suitably oxidized lycopodine derivative. His mechanism is outlined below.

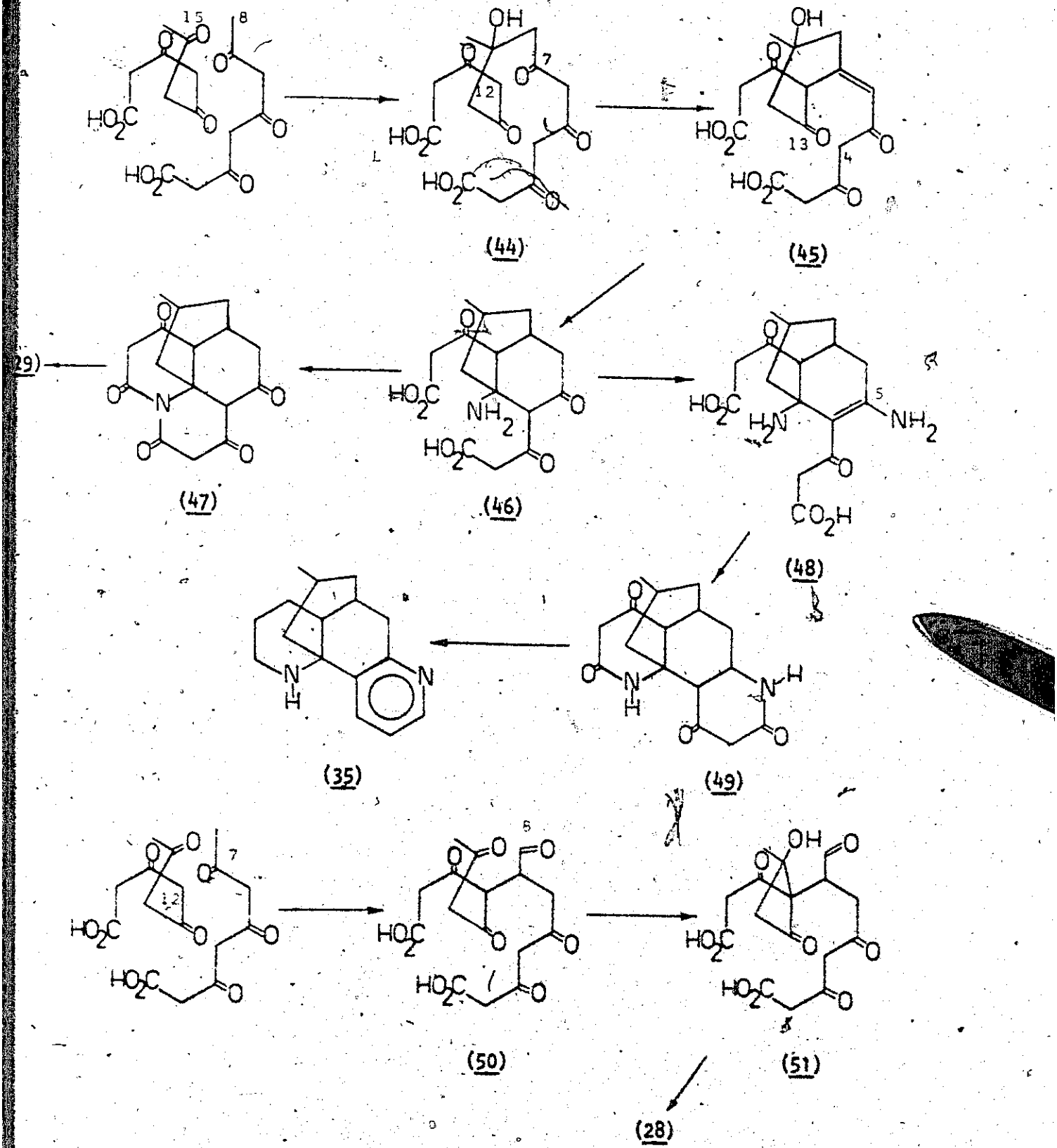


(38)

Although these transformations in no way constitute proof of a biosynthetic link between alkaloids of this class they offer proposals which may be experimentally tested and they illustrate the evolution of the idea of a common intermediate from which the various structural types may be elaborated. They suggest a common pattern within the diversity of structure of this series of compounds.

1.3.2 BIOGENESIS OF LYCOPODIUM ALKALOIDS

The first suggestion for the biogenesis of an alkaloid of this group was advanced by Leete (58). He proposed that annotinine was synthesized from β,γ ketocaproic acid, malonic acid, and two units of aspartic acid. However as the structure of other members of the series became known it was realized that this scheme would not be applicable to them. A simple yet elegant proposal by Conroy (59) was widely applicable. His suggestion is outlined in scheme 5. The $C_{16}N$ structural unit was seen to arise from the condensation or dimerization of two units of triketooctanoic acid (unbranched eight carbon chains) which were in turn derived from acetate. The numbering system in general use with the lycopodium alkaloids (luciduline provides the only exception) follows this proposal. The carbons C-1 to C-8 form one chain while the carbons C-9 to C-16 form the other chain. Ring junctions were envisaged to form via aldol condensations as for example between the C-8 methyl and the C-15 carbonyl to form the intermediate (44). A similar condensation between the C-12 methylene and the C-7 carbonyl followed by the appropriate dehydration results in the intermediate (45). After reduction of the double bond a Mannich reaction involving the



Polyketide Hypothesis

Scheme 5

methylene C-4, carbonyl C-13, and a molecule of ammonia leads to the intermediate (46). The appropriate lactam formation results in the immediate progenitor of the lycopodine type alkaloids (47). This same postulated intermediate (46) can serve as a precursor to the dinitrogenous $C_{16}N_2$ structural unit. Introduction of a second molecule of ammonia at the carbonyl site C-5 (48) and subsequent lactam closure leads to the intermediate (49). Removal of oxygen leads to lycodine (35) and the obscurines.

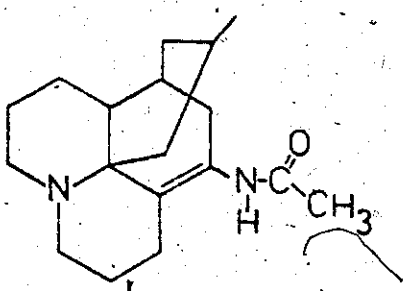
The scheme for annotinine (28) represents only a minor variation in these pathways. Instead of C-8, C-15 aldolization a bond is formed between C-7 and C-12 to give after reduction the intermediate (50). Oxidation at C-8 to the level of a carbonyl now prohibits C-8, C-15 condensation. An alternate aldolization between C-12 and C-15 now takes place to form the cyclobutane ring (51). Mannich condensation, lactamization, adjustment of the oxidation state and subsequent lactonization results in the structural array of annotinine.

Alternatively it has been suggested by Wiesner (56) that the cyclobutane ring is formed by modification of the ring "D" of lycopodine. A C-8, C-15 bond rupture followed by C-12, C-15 aldolization results in the required ring. This proposal is of particular interest as the position at which this takes place along the biogenetic route is not specified. It suggests that lycopodine is a central intermediate and a common precursor to the lycopodium alkaloids.

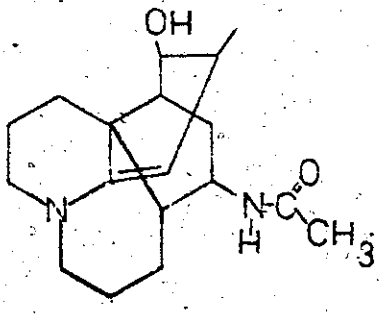
The order of the individual steps of the sequence is arbitrary yet each step is mechanistically plausible. It should be noted that

the acetate hypothesis greatly aided the investigation of this class of compounds. It provided the impetus for and lent direction to experimental research and was effectively used as a tool in structural elucidation. An example in point is the structure of cernuine which was proposed on the basis of degradation studies and its compatibility with the polyacetate hypothesis (52). A similar argument was advanced when the structure of luciduline was proposed. This is possibly one of the reasons for the wide acceptance of this scheme despite the lack of experimental verification.

Another biogenetic scheme which has been postulated for the derivation of the Lycopodium alkaloids views the $C_{16}N_2$ skeleton as providing the clue to the formation of these compounds (60). A visual dissection of this structure (dashed line of structure (52)) yields two C_8N units which by analogy to certain of the piperidine alkaloids could arise from lysine and a three carbon fragment derived from acetate. A suitable combination of two of these "pelletierine residues" results in the lycodine type skeleton. The lycopodine type skeleton is thought to result from a rearrangement subsequent to this dimerization. Oxidative scission of the C-5,N and the C-1,N bonds, loss of nitrogen and re-cyclization results in the lycopodine skeleton. Thus it is considered significant that the majority of tetracyclic mononitrogenous bases are oxygenated at C-5 while in other cases a nitrogen atom is attached to C-5. Examples of the latter case are found in the structures of flabelline (53) and serratinidine (54). The C_8N structural moiety forms integral portions of skeletons of cernuine and luciduline; thus



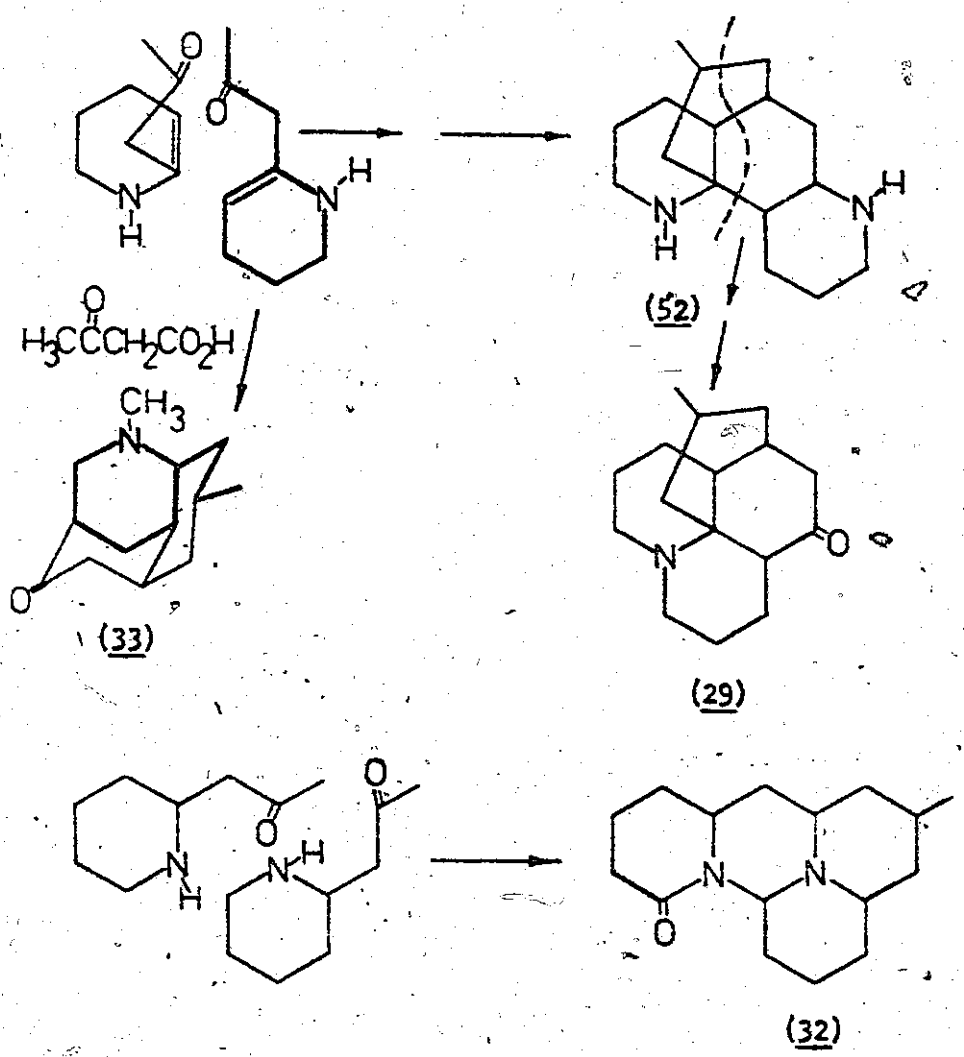
(53)



(54)

the pelletierine hypothesis accounts equally well for their formation. Two "pelletierine units" could dimerize by an alternative sequence to give rise to the structure of cernuine while one of these units in combination with a four carbon unit (possibly acetoacetate) results in the skeleton of luciduline. Alternatively luciduline could be derived from a combination of two C_8N units followed by a loss of a C_4N fragment. Methylation of the nitrogen is seen to take place at a later stage. These postulates are summarized in scheme 6.

The possible involvement of lysine in the biogenesis of these alkaloids had previously been suggested by Wiesner (56) who pointed out that the lupinine skeleton is present in the lycopodine structure. Lysine has been shown to be a specific precursor to lupinine (61). It has been suggested by Iverach (62) that two molecules of lysine could combine with one molecule of isopentenyl pyrophosphate to give the lycopodine skeleton. A modification of the accepted scheme of lupanine

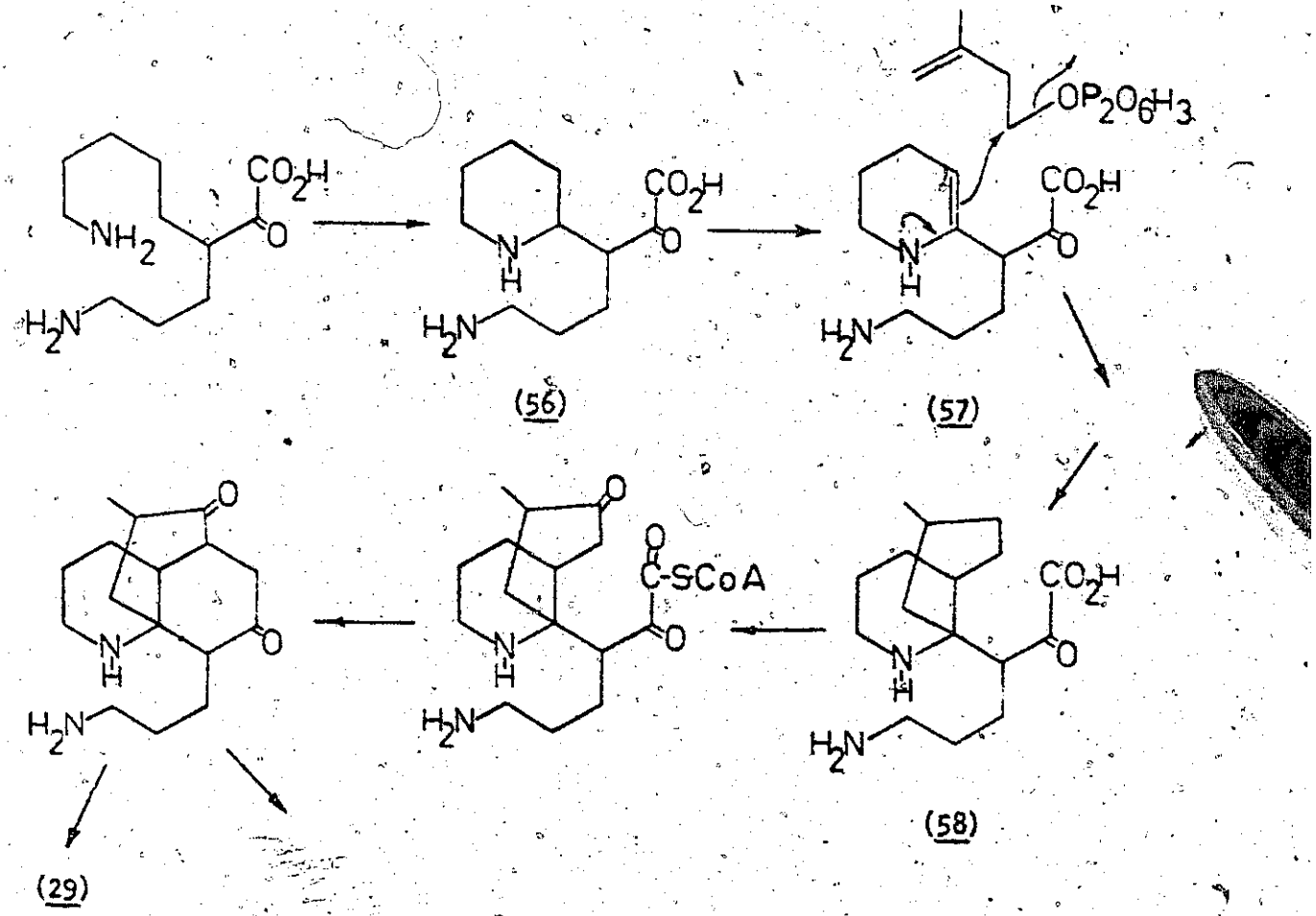


Pelletierine Hypothesis

Scheme 6

elaboration could yield the key intermediate (56), which in turn could be activated by oxidation to the corresponding enamine (57). Reaction with a unit of isopentenyl pyrophosphate would yield an intermediate (58) containing the required sixteen carbons. Appropriate ring closures

would give rise to the lycopodine or to the lycodine type skeletons. This proposal is shown in scheme 7.



Scheme 7.

1.3.3 BIOSYNTHESIS OF LYCOPODIUM ALKALOIDS

Of the alkaloids of this class, the biosynthesis of lycopodine has been most extensively studied, in part because of its apparently wide occurrences throughout the genus and its ready availability as the major alkaloid

of several species. Also several potential degradative sequences were available from earlier structural studies (63). The polyketide scheme has been invalidated, at least in the case of lycopodine and cernuine by the results of acetate feedings to L. tristachyum Pursch, and to L. cernuum, respectively.

Sodium acetate-1-^{14}C and sodium acetate-2-^{14}C were administered to L. tristachyum in separate experiments. The radioactive lycopodine which was isolated was subjected to Kuhn-Roth oxidation to isolate C-15 and C-16 collectively as acetic acid. Not only was the activity of the product other than the 1/8 (12.5%) of the activity of the intact alkaloid, a prediction of the polyketide scheme, but the values differed from each other. Acetic acid was indeed a specific precursor of lycopodine (60) but the incorporation did not occur by the condensation of two tetraacetyl units. The intermediacy of mevalonic acid or its biological equivalent isopentenyl pyrophosphate is also negated by the result from the feeding of sodium acetate-2-^{14}C. The acetic acid isolated in this case was found to contain 25% of the activity of the intact alkaloid and not the 33% demanded if mevalonic acid is to serve as a precursor. The finding of 25% of the activity to be collectively in carbons 15 and 16 is predicted by the pelletierine hypothesis. Also as predicted by this hypothesis 50% of the activity of the intact alkaloid was observed in the acetic acid from a feeding of sodium acetate-1-^{14}C.

Lysine and cadaverine were shown to be precursors of the five carbon units C-9 to C-13, and C-1 to C-5, of lycopodine. However in

contrast to the mode of entry of lysine into the piperidine alkaloids the integrity of the carbons C-2 and C-6 of lysine are lost (60). It was found that 25% of the activity of the intact structure was found at the carbonyl carbon (C-5) and similarly 25% was found at the C-9 position. This was the case whether D,L-2-^{14}C-lysine, or D,L-6-^{14}C-lysine served as a precursor. By inference 25% of the activity would be at C-1 and 25% would be at C-13. The observation that C-2 and C-6 of lysine are equilibrated prior to their incorporation into lycopodine precludes Iverach's suggestion that (58) serves as an intermediate. The intermediacy of (58) would require that the integrity of the two positions be maintained at least in ring "C". The intermediacy of cadaverine in the biosynthesis of lycopodine could account for the equilibration of carbons C-2 and C-6 of lysine. These experimental observations were consistent with the view that lycopodine originates from the dimerization of two "halves" each of which are derived from lysine and acetate.

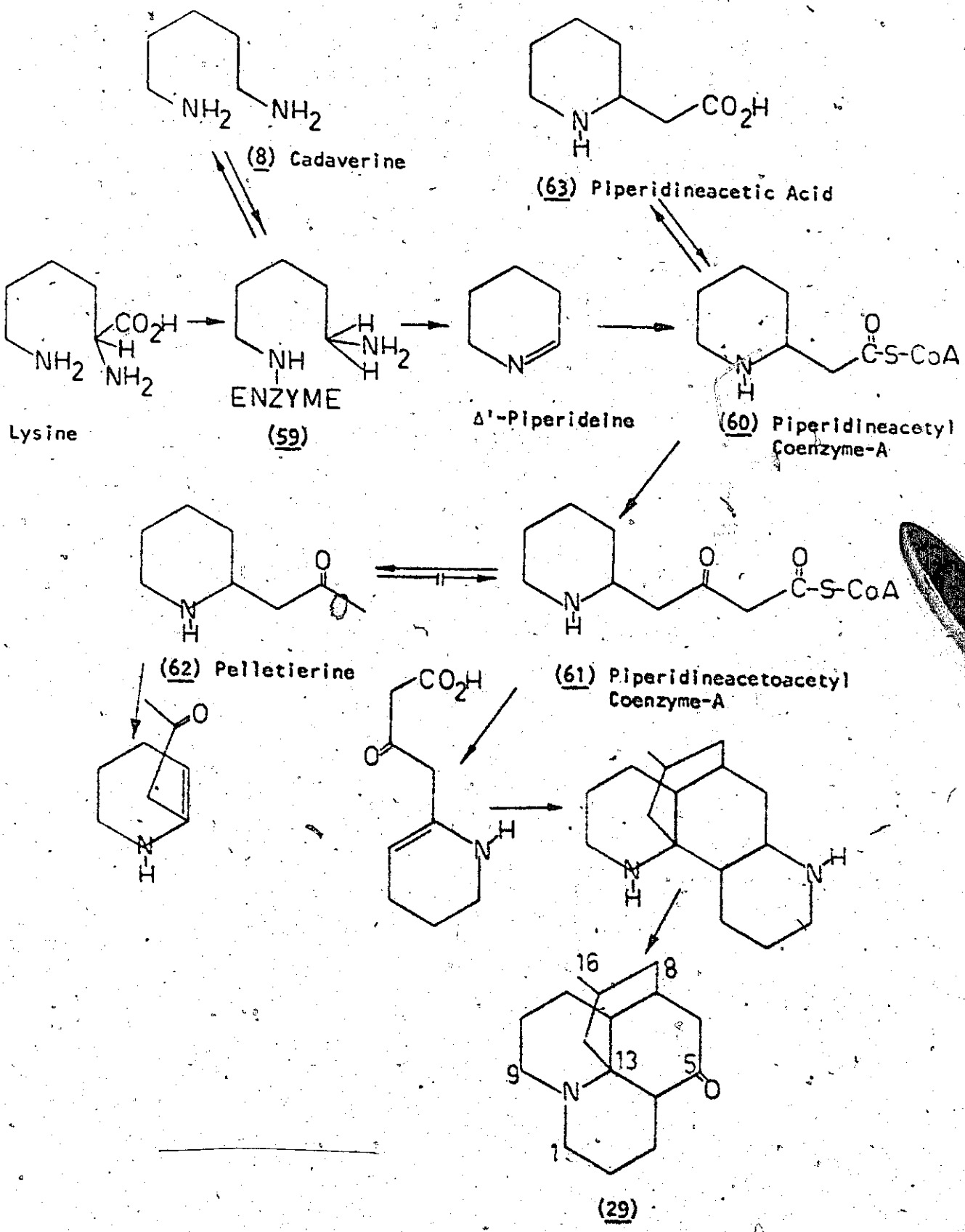
Δ^1-Piperideine and pelletierine have also been shown to be precursors to lycopodine. When 2-^{14}C-Δ^1-piperideine was administered, 50% of the activity was found at the carbonyl carbon (C-5) and by inference the remaining activity was at C-13. When 6-^{14}C-Δ^1-piperideine was tested as a precursor 50% of the activity was demonstrated to be at C-9. Thus the integrity of carbons C-2 and C-6 of Δ^1-piperideine are maintained in its incorporation into lycopodine. Pelletierine, although a specific precursor of this alkaloid, was found to label only that portion of the molecule containing carbons C-9 to C-16 (64). Within

experimental error all the activity of the alkaloid isolated from a feeding of 6-^{14}C-pelletierine was found at position C-9. Thus there existed an apparent paradox; (i) either lycopodine resulted from a dimeric species and only one of the moieties could be replaced by pelletierine (and pelletierine was not a normal precursor), or (ii) if pelletierine were a normal precursor then the alkaloid could not have been generated by a "true dimerization". The latter possibility was apparently contradicted by the results of several previous feedings in which the two halves were equally labelled. 2'-^{14}C-2-Allylpiperidine was also tested as a potential precursor. Isolation of the carbonyl carbon (C-5) of lycopodine as benzoic acid showed that no activity was located at this position and thus 2'-allylpiperidine did not serve as a precursor for the C-1 to C-8 unit.

Efforts were then turned to determine whether pelletierine was a normal intermediate in the route from lysine to lycopodine. Pelletierine was shown to incorporate label from cadaverine and Δ^1-piperideine at the same time as lycopodine was being synthesized within the plant. Using the carrier dilution technique pelletierine was shown to be a normal constituent of this plant. That pelletierine was indeed an obligatory precursor to lycopodine was elegantly demonstrated in two "metabolite swamping" experiments (65). In the presence of a large quantity of cold pelletierine, 1,5-^{14}C-cadaverine was administered to L. tristachyum and the isolated lycopodine was partially degraded to determine the pattern of labelling. In contrast to the 25% of the activity of the intact alkaloid recovered from the carbonyl carbon

(C-5) when 1,5-^{14}C-cadaverine was fed alone, 44% of the activity was found at C-5 in this case. By inference 44% of the activity would be found at C-1 while the remaining 12% would be split equally between C-9 and C-13. This experiment shows that the incorporation of label into the C-9 to C-16 unit of the molecule was considerably suppressed. Similarly when 2-^{14}C-Δ^1-piperideine was fed in the presence of cold carrier pelletierine, suppression of the incorporation of activity into the C-9 to C-16 unit was observed. Thus 90% of the activity of the lycopodine was found at C-5, and by inference 10% of the activity would be at C-13. These experiments demonstrate that pelletierine is an obligatory intermediate and thus lycopodine is not a true dimer of two pelletierine equivalents.

A model for the biosynthesis of lycopodine which is consistent with the above observations is outlined in scheme 8. Cadaverine is considered to be a normal precursor of this alkaloid. Thus, the rate of interconversion between the enzyme bound (59) and unbound cadaverine (8) is considered to be large relative to the rate of conversion of the bound species to Δ^1-piperideine which would account for the equilibration of the carbons C-2 and C-6 of lysine. The source of the carbon fragment which condenses with Δ^1-piperideine to give ultimately piperidineacetoacetyl coenzyme A (61) (the enzyme bound equivalent of piperidineacetoacetic acid) is not specified but could be acetic acid or malonic acid. This intermediate (61) is thought to be irreversibly decarboxylated to give pelletierine (62). Provided the steady state concentration of pelletierine is small relative to its immediate

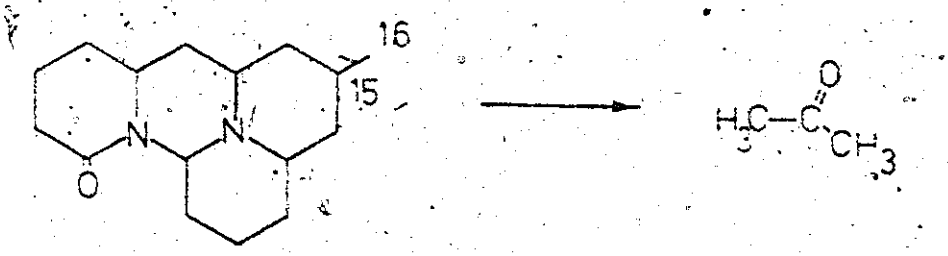

(8) Cadaverine
(63) Piperidineacetic Acid
Lysine
ENZYME
(59)
Δ¹-Piperideine
(60) Piperidineacetyl Coenzyme-A
(62) Pelletierine
(61) Piperidineacetoacetyl Coenzyme-A
(29)

Scheme 8

precursor (61) then this model can account for the equal incorporation of six different substrates into both halves of lycopodine. A condensation of pelletierine and piperideineacetoacetic acid (or its enzyme bound equivalent) both in a suitably oxidized state results in the dinitrogenous skeleton. Subsequent rearrangement leads to lycopodine.

Evidence has been sought for the involvement of piperideineacetic acid (63) in lycopodine biosynthesis. A sample of 3-^{14}C-2-piperideineacetic acid was synthesized and together with L-4-^{3}H-lysine was administered to lycopodine. Although the isolated lycopodine contained tritium it contained very little radioactive carbon (66) thus casting doubt on the intermediacy of this compound.

The biosynthesis of cernuine (32), a Lycopodium alkaloid of very different skeletal structure, has also been studied.



(32) Cernuine

As predicted by the pelletierine hypothesis 50% of the activity of the intact alkaloid was found in the acetic acid (C-15 and C-16) when 1-^{14}C-sodium acetate served as a precursor. When 2-^{14}C-sodium acetate was

administered and cernuine isolated, partial degradation demonstrated that 25% of the activity was at the carbons C-15 and C-16. Labelled lysine, cadaverine, and Δ¹-piperideine were found to be incorporated into each half of the molecule with equal efficiency (67). Pelletierine also served as a specific precursor for cernuine but label was incorporated only into the C-9 to C-16 portion of the molecule but not the C-1 to C-8 unit. These results are exactly analogous to those obtained from studies on lycopodine and they lend support to the postulate that the Lycopodium alkaloids are biosynthesized from common intermediates.

1.4 OBJECTIVES OF THIS RESEARCH

The research reported in this thesis was undertaken with the ultimate objective of experimentally demonstrating a direct biosynthetic relationship between lycopodine and annotinine. Lycopodine has been postulated to be a common precursor to several mononitrogenous alkaloids, a role suggested in part by the wide taxonomic distribution of lycopodine among the species of the genus while the other alkaloids of this class are considerably more restricted in distribution.

The choice of annotinine for investigation as a potential product of lycopodine transformation was suggested for several reasons. A considerable body of chemical information was available from earlier structural studies, and thus, several potential degradation sequences of this compound existed in the literature. Also annotinine was readily available as the major alkaloid of L. annotinum, a species common to

Canada. The postulated transformation would be chemically interesting, if not spectacular. The transformation would of necessity involve several steps for which collectively there existed no analogue in the literature.

One method of experimentally demonstrating the interconversion would be to synthesize specifically labelled lycopodine and then to administer it to L. annotinum. The annotinine could then be isolated and assayed for activity. A direct conversion of the precursor to the product might be demonstrated by chemically degrading the isolated annotinine to determine the site(s) of labelling. Thus a primary objective of this research was to synthesize specifically labelled lycopodine.

It follows that if lycopodine is transformed into annotinine then those molecules which are known to specifically label lycopodine should also be incorporated into annotinine. Another primary objective of the research was to demonstrate the incorporation into annotinine of those compounds known to label lycopodine.

As a supplement to the above it was proposed to study further the biosynthetic pathway from lysine to lycopodine with the view to explaining the apparent anomaly that pelletierine was incorporated into only the C-9 to C-16 half of lycopodine while all other precursors which had been studied were found to label both halves of lycopodine equally. Specifically we proposed to test the postulated intermediacy of piperidineacetic acid in the biosynthesis of lycopodine. We wished also to test malonic acid as a potential precursor to this alkaloid and to define the chirality of the lysine which is incorporated into lycopodine.

DISCUSSION OF RESULTS

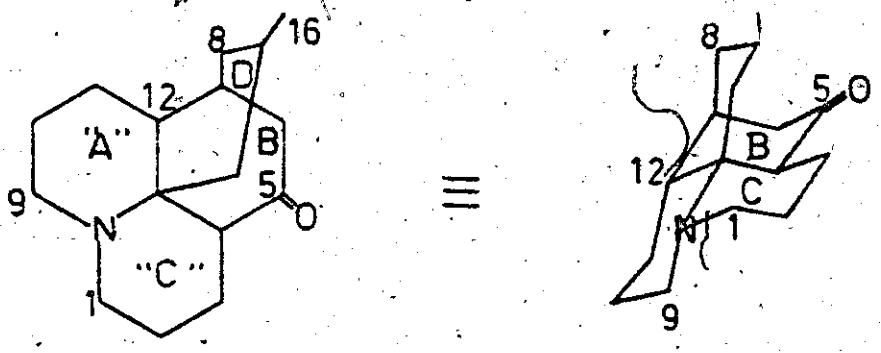
Considering the experimental evidence gathered to date concerning the biosynthesis of lycopodine, cernuine, and by inference of the dinitrogenous Lycopodium alkaloids it becomes increasingly of interest to attempt to establish a direct biosynthetic link between two or more of these alkaloids. Specifically it would be of interest to find experimental basis for the postulated role attributed to lycopodine as a central intermediate. A possible means of attack would be to synthesize specifically labelled lycopodine and to test it as a possible precursor.

2.1 SYNTHESIS OF 9-^{14}C-LYCOPODINE

2.1.1 GENERAL CONSIDERATIONS

Although two total syntheses of lycopodine (29) (68,69) and several synthetic studies on this ring system (70,71,72) have been reported in the literature it was felt that a more direct route to specifically labelled lycopodine would be to modify the existing structure rather than to attempt a total synthesis. Of the carbons of the lycopodine skeleton which can be isolated by chemical degradation procedures C-9 is readily accessible, being α-to nitrogen and thus susceptible to chemical modification. The equivalent carbons of annotinine (28) and acrifoline (42) (two other mononitrogenous alkaloids of this class of compounds which might arise from a biological trans-

formation of lycopodine) are also accessible by chemical degradation. C-9 is also remote from the carbons postulated to undergo modification during the biosynthetic transformation of lycopodine to annotinine or acrifoline.

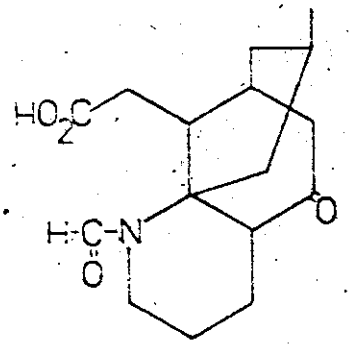


(29)

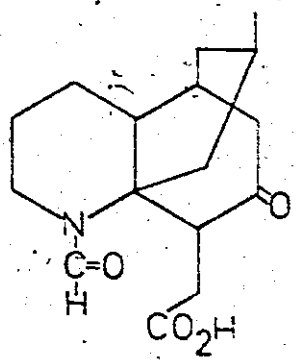
Our approach to this problem was to open ring A, remove carbon C-9, substitute a radioactive carbon atom, and recyclize to the tetracyclic structure of the starting compound. No problems with the stereochemistry of the final product were envisaged as the chiral centres of this compound are remote from the sites of reaction of our proposed scheme.

2.1.2 OPENING OF RING A OF LYCOPODINE

Law (73) has reported that the oxidation of lycopodine with potassium permanganate in aqueous medium yields the N-formyl keto acid (64) in moderate yield. No interfering products from competing side reactions have been isolated and the isomer (65) does not seem to be formed.



(64)



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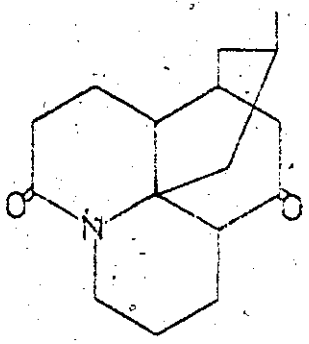
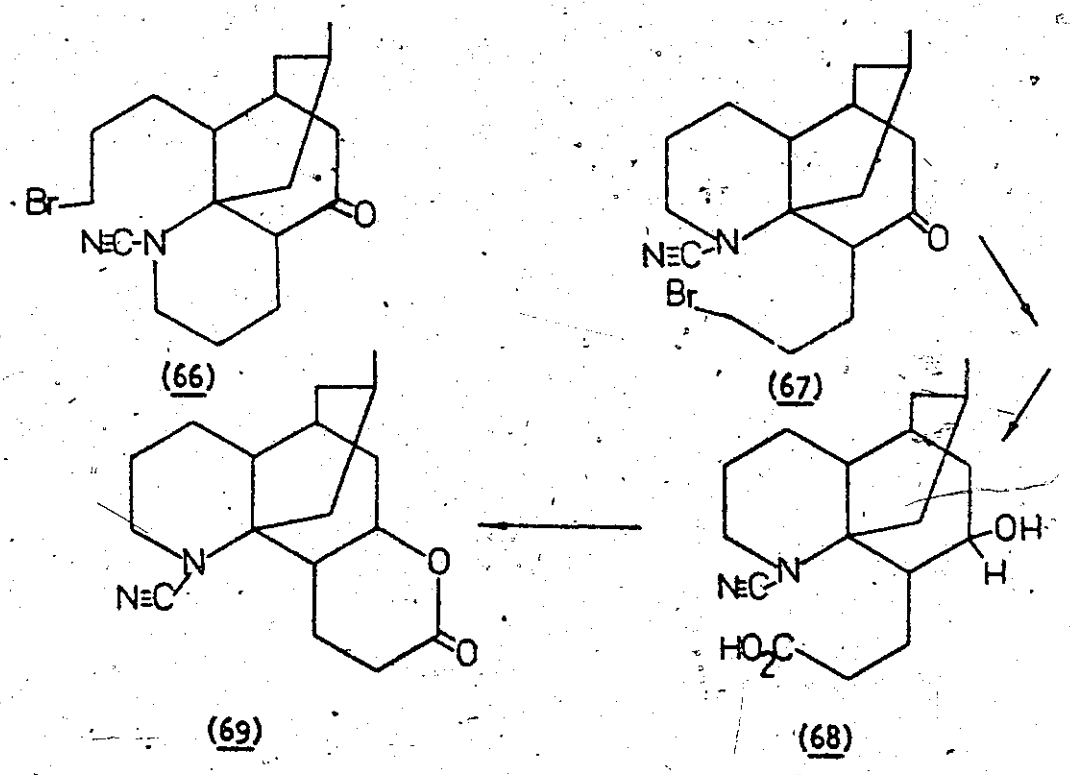
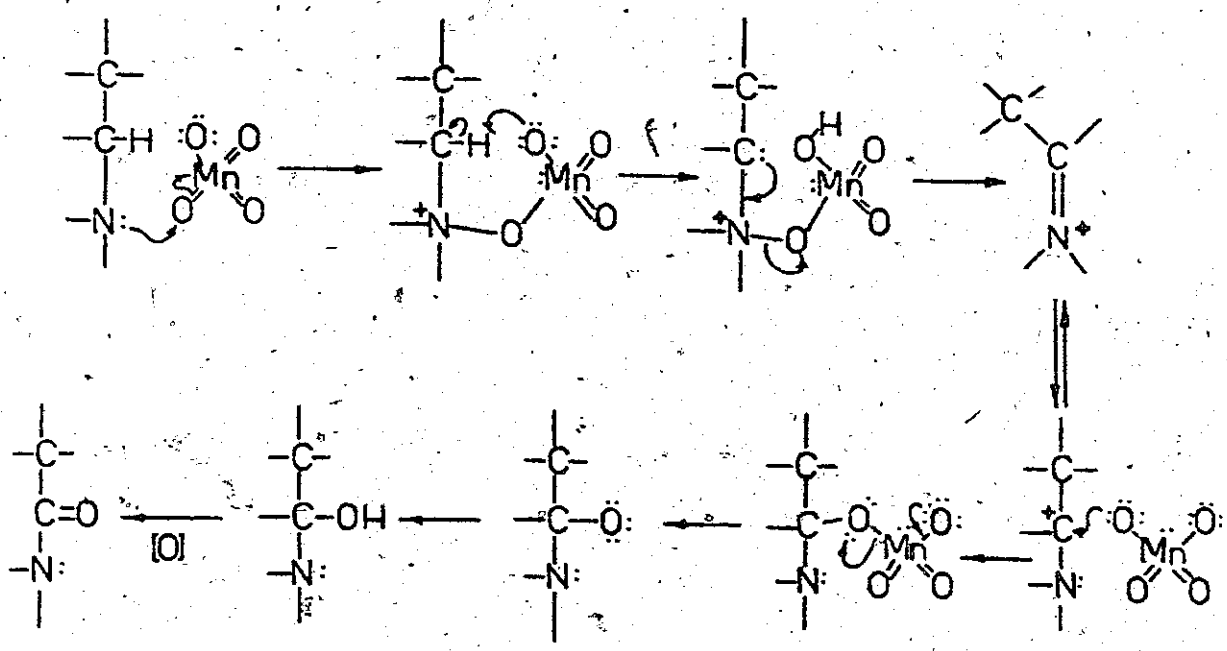


(70)

Evidence that isomer (64) and not (65) is the reaction product comes from a series of related reactions. Lycopodine, when treated with cyanogen bromide under conditions of the Von Braun reaction, yields two additional products which have been formulated as (66) and (67) (74). The β isomer (67), when converted to the corresponding keto acid then reduced to the hydroxy acid (68) cyclized spontaneously to the lactone (69). The α isomer when subjected to the same treatment failed to lactonize. The N-formyl acid, obtained by borohydride reduction of the N-formyl keto acid (64) failed to lactonize in refluxing benzene and p-toluenesulfonic acid (73). It has also been demonstrated that when lycopodine is oxidized with potassium permanganate in acetone only the α-lactam (70) is isolated (75). Conclusive proof of the identity of the product from the aqueous oxidation of lycopodine is provided by this synthesis which relates product (64) with lycopodine α-lactam (70).



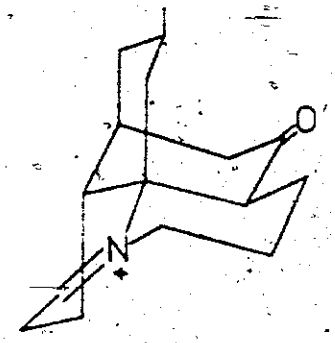
It is interesting to speculate on why this permanganate oxidation is specific to the ring A of lycopodine. Wenkert (76) has postulated a mechanism for the permanganate oxidations of carbons α to nitrogen which relates carbinolamine formation and lactam formation. His proposal, outlined below, involves electrophilic attack upon the amine by permanganate ion, removal of a proton from the carbon α to nitrogen, and rearrangement to the intermediate immonium compound. A second attack upon this intermediate by manganate ion and transfer of oxygen from the ion to the organic substrate leads to the carbinolamine. Subsequent oxidation of the carbinolamine would yield the lactam, or alternatively elimination of water (β,γ to the nitrogen) would yield the corresponding enamine. The enamine would be readily oxidized to



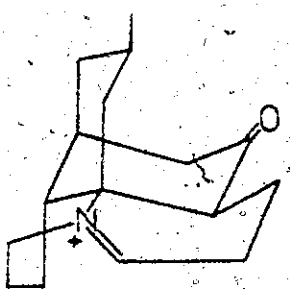
the N-formyl keto acid under the reaction conditions.

It has been demonstrated (77), that β amino ketones, in which the π system of the carbonyl, the α,β sigma bond, and the lone pair of electrons on the nitrogen are parallel, give rise to electron transfer bands in the 220-260 mμ region of the ultraviolet. The ultraviolet spectrum of lycopodine displays an absorption at 217 mμ (ε = 2400) which disappears when the solution is acidified (78). The acid dissociation constant (pK_a) of lycopodine has been found to be lower than the pK_a of dihydrolycopodine (7.44 vs. 9.2) (79). These physical characteristics of lycopodine have been interpreted as evidence for the delocalization of the nitrogen lone pair of electrons.

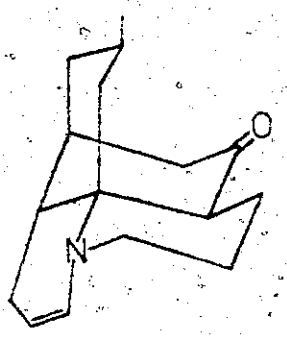
Of the two intermediate immonium compounds (71) and (72) which would result from the oxidation of lycopodine, (71) might be expected to be energetically more favourable than (72) because the resulting π system of the C-9,N double bond could better overlap with the C-4, C-13 sigma bond and the π system of the C-5 carbonyl group. The overlap is not as complete in the case of (72). Once formed the immonium compound (71) would be readily converted to the corresponding enamine (73) in the medium of this reaction.



(71)



(72)

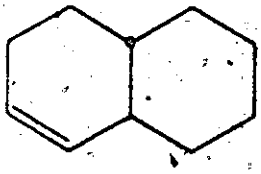


(73)

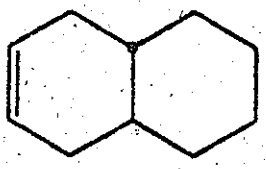
Thus if product yields can be related to predicted thermodynamic stabilities of the postulated immonium intermediates then this argument provides a plausible explanation for reaction only in the ring A of lycopodine.

It has also been pointed out (80) that the intermediate (71) possesses unsaturation which is Δ^1 with respect to the AB cis fused ring while intermediate (72), which would result in oxidation of ring C, possesses unsaturation which is Δ^1 with respect to the BC trans fused ring system. In the case of trans fused octalin the Δ^2 isomer (74) is

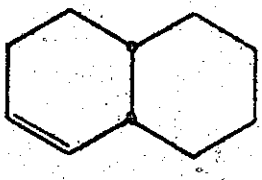
energetically favoured over the Δ^1-isomer (75) while in cis fused octalins the opposite is true; the Δ^1-isomer (76) is favoured over the Δ^2-isomer (77). These differences become appreciable when the unsaturated decalins form part of a polycyclic system. The stability of 9-methyl cis and trans decalin differ only slightly (ΔH_c = 1.39±0.64 kcal/mole (82)). Consequently in some 9-methyldecalin systems the cis isomer is more stable thermodynamically while in others the trans isomer predominates at equilibrium. If the relative stabilities of the cis and trans decalin systems can be considered analogous to the correspond-



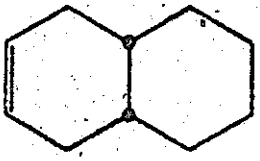
Δ^1 (75)



Δ^2 (74)



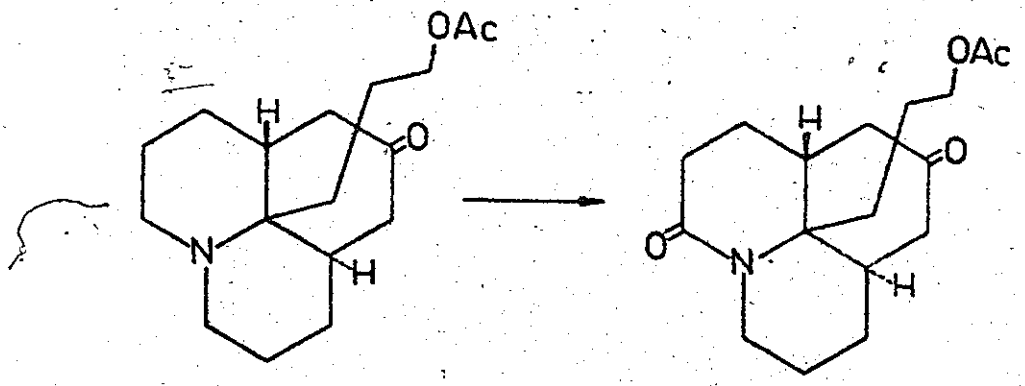
Δ^1 (76)



Δ^2 (77)

ing decahydroquinoline systems then the observed preference for oxidation in the ring A of lycopodine is in keeping with the predicted stabilities of the intermediate immonium compounds (71) and (72).

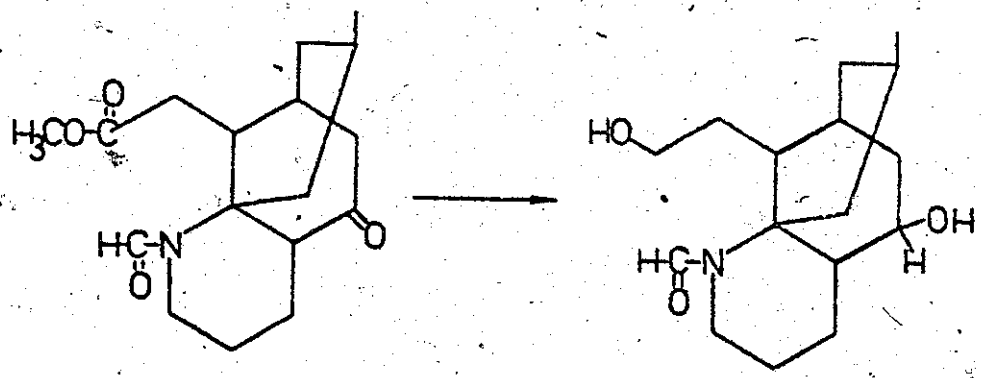
That the ring D can be ignored in the conformational analysis above is suggested by the work of Ayer (69) who found only isomer (78) was isolated from the permanganate oxidation of (79).



(79) (78)

2.1.3 REDUCTION OF N-FORMYL KETO ESTER

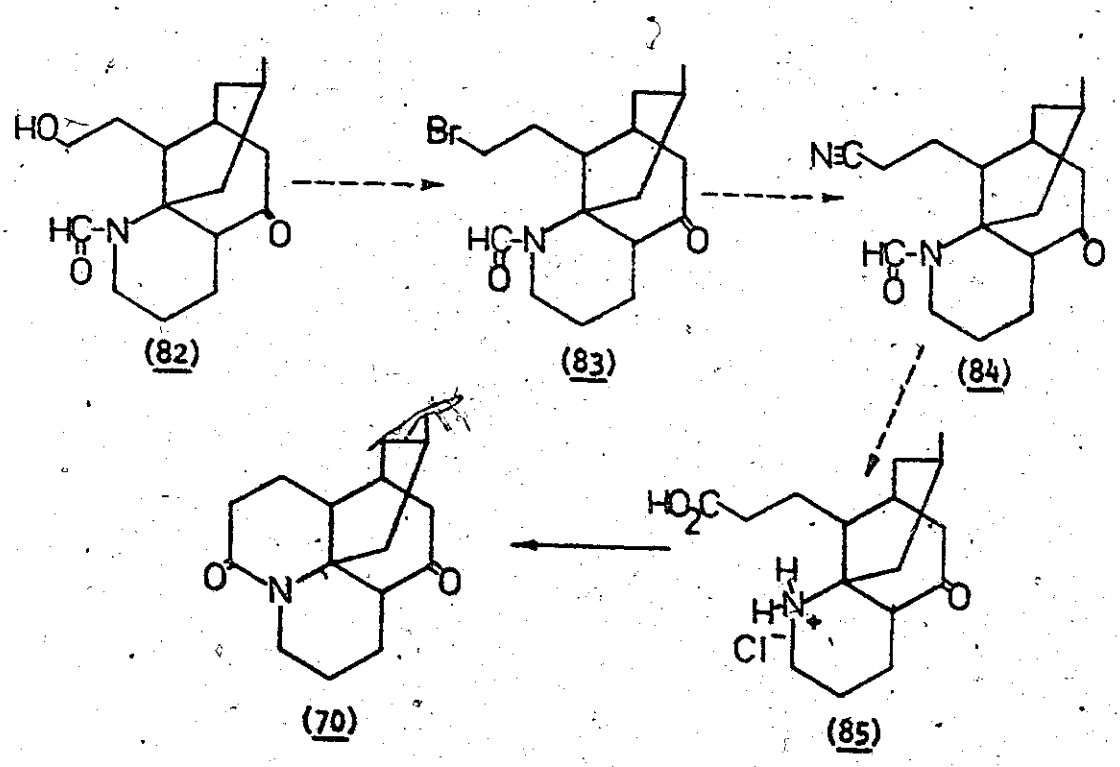
Adduct (64) can be readily esterified with ethereal diazomethane to form the N-formyl keto ester (80). It has been reported that unhindered esters can be reduced to the corresponding alcohols in the presence of an acid function (83) and it was anticipated that in the case of N-formyl keto ester (80), the ester function, and the ketone could be reduced to the corresponding primary and secondary alcohol while the less labile amide function would remain unaffected. Treatment of the ester (80) with sodium trimethoxyborohydride in refluxing 1,2-dimethoxyethane resulted in the isolation of a neutral product ($C_{16}H_{27}NO_3$ m.p. 199-201°) in 29% yield after purification. The product, formulated as (81), was found to absorb at 3450 and 1640 cm^{-1} characteristic of a hydroxyl and N-formyl group respectively. Absorptions at 1740 and 1700 cm^{-1} present in the ester (80) were absent in the spectrum of this product. The yields in this reduction were erratic and separation of



(80) (81)

the totally reduced product from the partially reduced material was difficult.

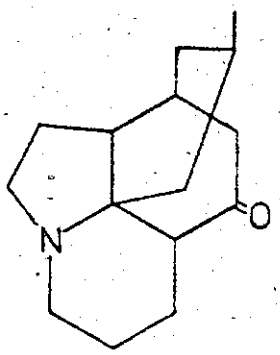
If the dihydroxyformyl compound (81) were obtained in good yield, a selective oxidation of the secondary alcohol at C-5 to the ketone (82) (Oppenauer oxidation) would be expected. Displacement of the hydroxyl (at C-10) first by bromide, then by cyanide would yield the cyanoformyl ketone in the sequence (82) to (84). An acid hydrolysis of the N-formylcyanoketone (84) would be expected to yield the amino acid (85) which had been previously converted first to lycopodine-α-lactam (70) (84), albeit in low yield, and then to lycopodine (29). However because of difficulties in obtaining a good yield of the dihydroxy compound this scheme was abandoned in favour of a more promising route.



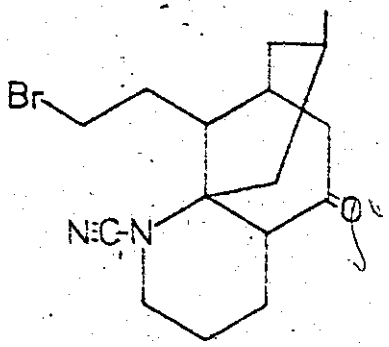
2.1.4 PREPARATION OF A-NORLYCOPODINE

An alternative to the N-formylbromoketone (83), the key intermediate to which a labelled carbon atom could be added, would be the bromocyanamide (86). This product should result from the addition of cyanogen bromide to the tetracylic ketone (87). The Von Braun reaction has been used with success in the structural elucidation of several of the Lycopodium alkaloids. This reagent was known to add preferentially to the "A" ring of lycopodine (29) and acrifoline (42). Our next objective then was to prepare A-norlycopodine (87).

Accordingly, the N-formylketoacid (64) was hydrolyzed in dilute mineral acid. The product, a light brown oil, which foamed under reduced pressure displayed a broad absorption in the region 3100 to



(87)

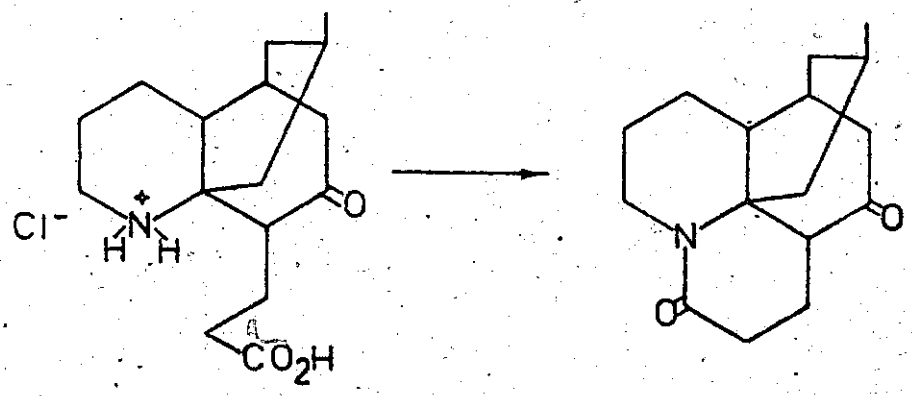


(86)

2700 cm^{-1} and an absorption at 1700 cm^{-1} indicative of an acid and a ketone in a six membered ring. However the amide absorption at 1660 cm^{-1} present in the spectrum of the starting material was absent in the spectrum of the product. Attempts to prepare a crystalline derivative of the product by treating the crude amino acid hydrochloride with alcoholic diazomethane were not successful. This product, a colourless oil, displayed absorptions at 3350, 1740, and 1700 cm^{-1}, indicating the presence of a secondary amine, an ester, and a ketone.

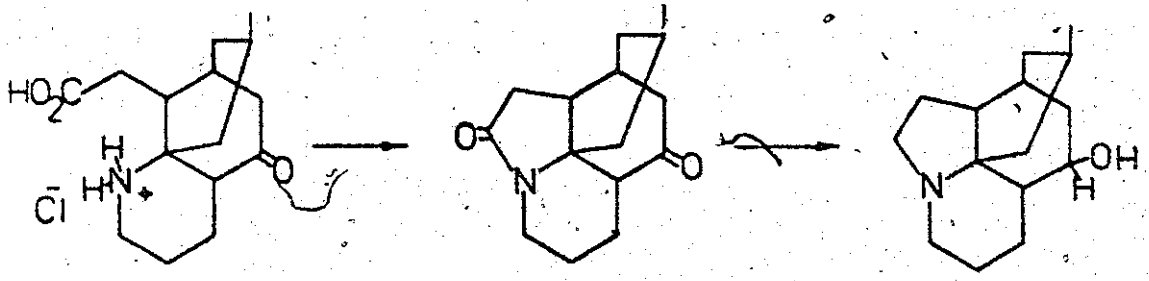
Ayer and Iverach (53) when transforming α-obscurine into dihydrolycopodine cyclized the intermediate (88) to lycopodine-β-lactam (89) using dicyclohexylcarbodiimide in pyridine. The amino acid hydrochloride (90) would be expected to cyclize to A-norlycopodine-α-lactam (91) under similar conditions.

The amino acid hydrochloride, when treated with freshly distilled dicyclohexylcarbodiimide in refluxing pyridine, yielded a neutral crystalline compound ($C_{15}H_{21}O_2N$ m.p. 164-165°) in 27-30% yield after



(88) (89)

chromatography. This product (91) showed absorptions in the infrared at 1680 and 1705 cm^{-1} typical of a γ-lactam and a ketone in a six membered (or larger) ring, respectively.



(90) (91) (92)

Both the α-lactam (70) and the β-lactam (89) of lycopodine have been reduced to dihydrolycopodine using lithium aluminum hydride and when A-norlycopodine-α-lactam (91) was treated with this reagent in refluxing ether a basic crystalline product ($C_{15}H_{25}ON$ m.p. 179-181°) was isolated in 56% yield. This product (92) displayed a strong

absorption at 3410 cm^{-1} but had no other absorptions in the carbonyl region of the infrared. Varying the solvent from ether, to tetrahydrofuran and using diborane as the reducing agent rather than lithium aluminum hydride did not appreciably increase the amount of product. However A-norlycopodine-α-lactam (91) was smoothly reduced with lithium aluminum hydride in refluxing dioxan in 82% yield.

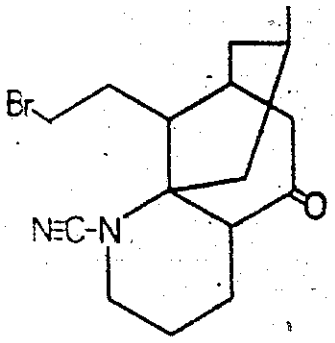
Before attempting to reopen the five membered ring of (91) with cyanogen bromide it was felt that it would be necessary to protect or modify the hydroxyl group at carbon-5 for two reasons. Although acrifoline was successfully treated with cyanogen bromide to give tricyclic addition products (85) dihydrolycopodine gave unsatisfactory results. Secondly, at a later stage it would be necessary to convert the cyano group (bearing the radioactive carbon) to the corresponding carboxylic acid. This is usually effected by hydrolysis in mineral acid. It was possible that under these conditions the hydroxyl group might be eliminated and could not be replaced readily. Oxidation to the corresponding ketone, A-norlycopodine (87) before attempting the Von Braun reaction seemed to be the most promising route. This was accomplished with aluminum isopropoxide and cyclohexanone in refluxing toluene. The product (87) ($C_{15}H_{23}NO$ m.p. 129-130°) displayed a strong absorption at 1700 cm^{-1} but no absorptions in the hydroxyl region of the infrared.

2.1.5 ADDITION OF CYANOGEN BROMIDE TO A-NORLYCOPODINE

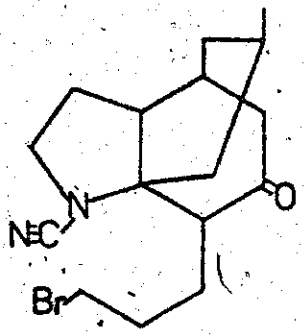
When A-norlycopodine (87) was treated with cyanogen bromide in benzene a colourless crystalline product ($C_{16}H_{25}ON_2Br$ m.p. 179-181°)

was isolated in 56% yield. This product absorbed at 2200 and 1705 cm^{-1} typical of a cyanamide function and a ketone. Only a single component could be detected when this product was chromatographed on silica gel (Rf = 0.42, chloroform; Rf = 0.58, chloroform:methanol (10:1)).

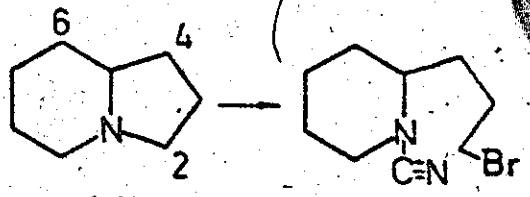
Of the two isomers which could result from the addition of cyanogen bromide to A-norlycopodine, (86) would be expected to predominate over (93). It has been reported (86) that when indolizidine (94), 2-methylindolizidine, and 4-methylindolizidine were treated with this reagent only additions to the pyrollidine ring were observed. This was



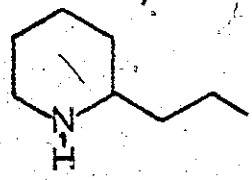
(86)



(93)



(94)



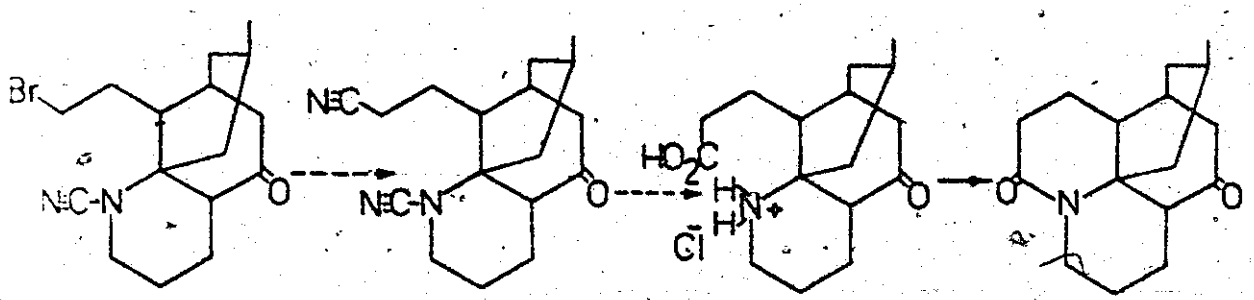
(10)

demonstrated by conversion of the addition products to coniine (10), 2-butylpiperidine, and 1'-methylconiine respectively. Also cyanogen bromide was known to add preferentially to the "A" ring of lycopodine and of acrifoline. Thus the selectivity of the addition was not totally unexpected. The identity of the product was more conclusively

demonstrated in the next step of the synthetic sequence.

2.1.6 INTRODUCTION OF A 1 CARBON UNIT

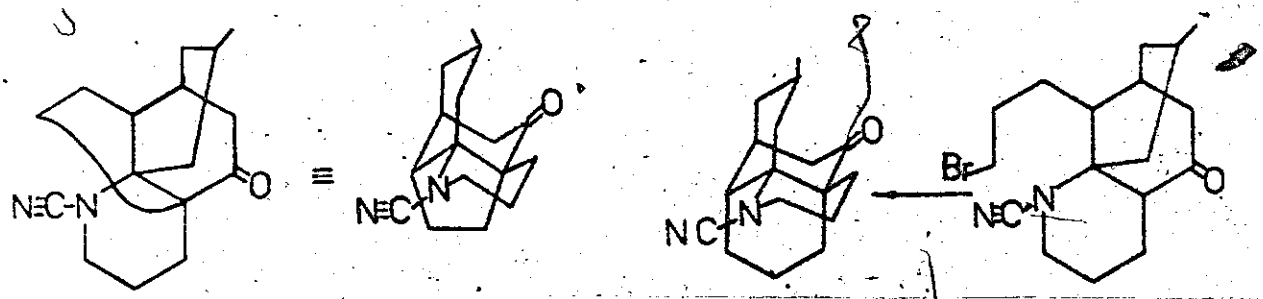
It was our intention to convert the cyanobromo adduct (86) to the corresponding dicyanoketone (95), a step which would allow the introduction of a radioactive carbon into the system. This product (96) would be amenable to conversion to the corresponding amino acid hydrochloride (85) by hydrolysis, the alkyl cyanide being hydrolyzed to the corresponding acid, while the N-cyano group would be lost. Through hydrolysis and decarboxylation the amino acid hydrochloride had been previously cyclized to lycopodine-α-lactam (70).



(86) (95) (85) (70)

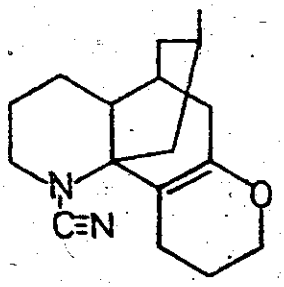
The displacement of halides with metal cyanides is often effected in a boiling aqueous alcoholic solution of the metal cyanide, (87) and sometimes it is possible to promote hydrolysis of the product directly to the acid by prolonged refluxing. Also the bromine of α-cyanobromolycopodine (66) had been displaced previously using alcoholic potassium acetate (74).

When the cyanobromo adduct (86) was refluxed with a slight excess of potassium cyanide, a neutral product ($C_{16}H_{22}N_2O$ m.p. 150-151°) was isolated in 86% yield. The product absorbed at 2200 and 1725 cm^{-1}, demonstrating that the cyanamide function remained intact. The product had lost HBr, but no evidence of olefin formation was observed in the infrared spectrum. The product is formulated as (96) by analogy to the cyclization known to occur when α-cyanobromolycopodine (66) is treated with potassium hydroxide resulting in the formation of α-cyclocyanolycopodine (97). Although the cyclization could occur at either C-4 or at C-6 the presence of a weak but sharp absorption at 1410 cm^{-1}

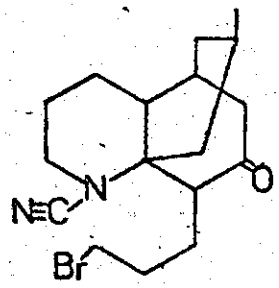


(96) (97) (66)

(indicative of an active methylene group α to a carbonyl function (88,89)) indicates that the cyclization occurred at C-4. β-Cyanobromo lycopodine (67) does not undergo a similar cyclization but rather cyclizes to the enol ether (98) when treated with methanolic potassium hydroxide. Thus the starting material must have been the α-isomer (86) and not the β-isomer (93).

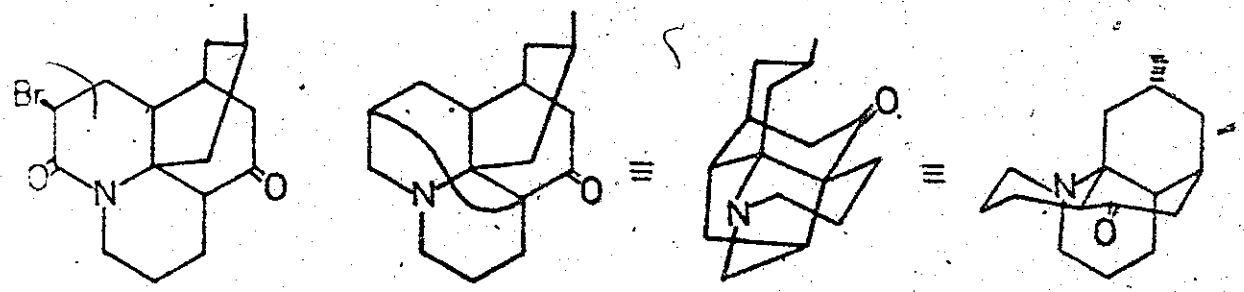


(98)



(67)

Although this product (96) was not useful in this synthesis the unforseen cyclization represents a potential method for converting the lycopodine ring skeleton into the ring system of the pentacyclic Lycopodium alkaloids such as dihydrolycopecurine (100). If a bromine atom can be introduced at C-10 of lycopodine-α-lactam to give (99) it should be possible to generate the enolate anion under similar conditions to those above and displace the bromine to give after reduction dihydrolycopecurine (100). The monobromination of ε-lactams has been effected by treatment with bromine and phosphorous tribromide (90).



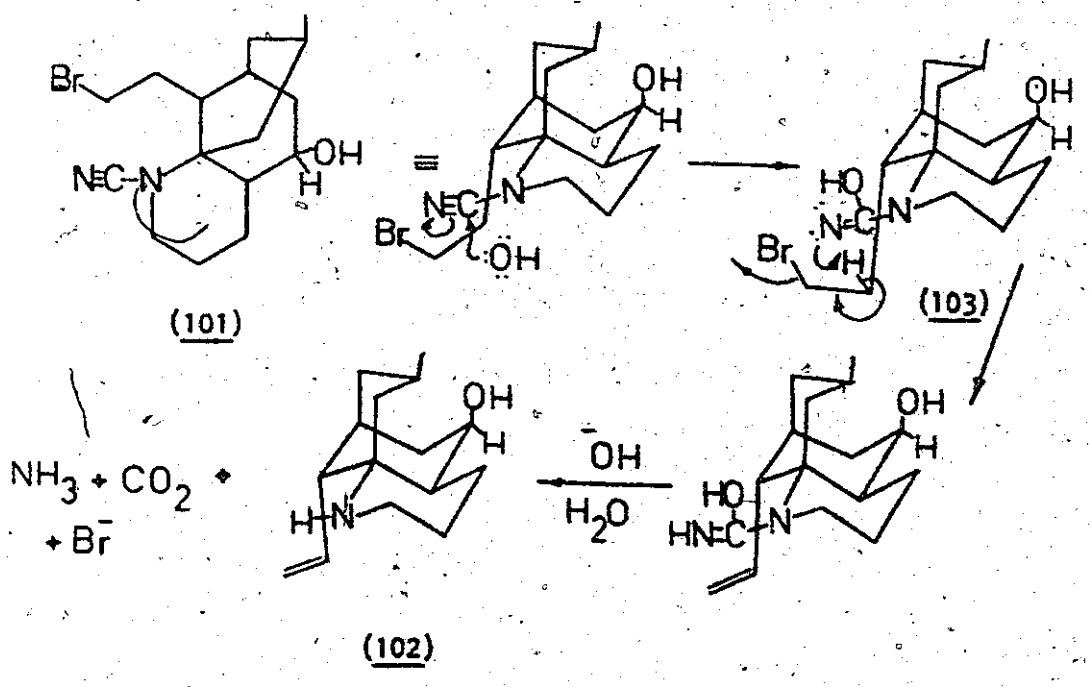
(99) (100)

It was considered that the cyclization of (86) might be avoided if it were treated with sodium cyanide in dimethyl sulfoxide. However, the neutral product (isolated in 72% yield after purification) was indistinguishable from the product (96) by physical methods. A mixed melting point was undepressed.

Efforts were then turned to finding a means of blocking enolization of the C-5 carbonyl. Acetylation of A-nordihydrolycopodine (92) did not produce a good yield of crystalline product and earlier attempts to ketalize the C-5 carbonyl in the lycopodine system had been unsuccessful (presumably because of steric hindrance from the "D" ring which is in close proximity to the carbonyl group). It was decided to attempt the cyanogen bromide addition to A-nordihydrolycopodine (92). When alcohol (92) was treated with cyanogen bromide in benzene a crystalline neutral product (101) ($C_{16}H_{25}ON_2Br$ m.p. 179-181°) was isolated in 55% yield after chromatography. Thin layer chromatography of the product (silica gel, Rf = 0.48, chloroform:methanol (4:1); Rf = 0.66 chloroform:methanol (1:1)) displayed only a single component. This product absorbed at 3450, (-OH) and 2200 cm^{-1} in the infrared region.

When the cyanobromoalcohol (101) was treated with aqueous ethanolic potassium cyanide three major products were isolated. The only basic product had a molecular ion at m/e 235 and displayed prominent absorptions at 3400, 3200, and at 990, and 910 cm^{-1}, suggestive of an alcohol, a secondary amine, and a monosubstituted double bond respectively. There were, however, no cyano or carbonyl absorptions. This product is formulated as (102). This unexpected product might

arise by the following mechanism.

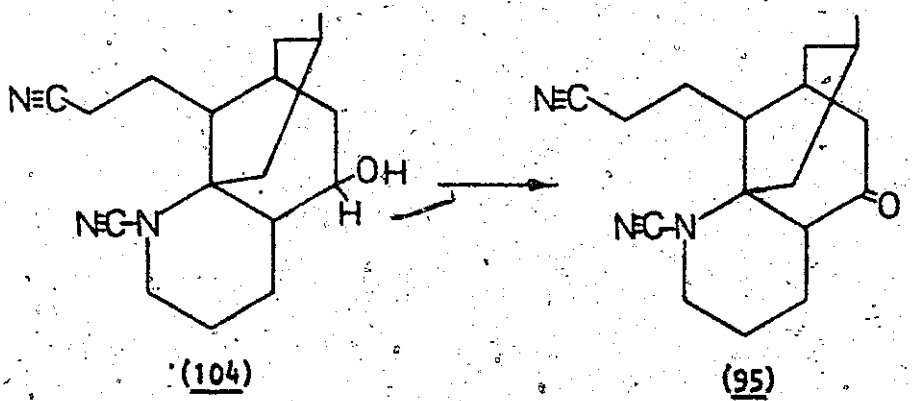


A partial hydrolysis of the cyanamide function would result in the intermediate (103). Molecular models indicate that the nitrogen of the imine (103) can approach a proton of C-11 without appreciable distortion. Removal of a proton from C-11 by this nitrogen generates the urea and results in the elimination of HBr from the alkyl side chain. Subsequent hydrolysis of the urea results in the observed product (102).

The neutral products from this reaction were not investigated in detail. A mass spectrum of this mixture showed no evidence for the

formation of the desired dicyanoalcohol (104).

The cyanobromoalcohol (101) was then treated with sodium cyanide in dimethylsulfoxide and a crystalline neutral product ($C_{17}H_{25}N_3O$ m.p. 214-215°) was isolated in 86% yield. The infrared spectrum of this product (104) displayed absorptions at 3470, 2200 and a weak but sharp absorption at 2245 cm^{-1} indicating the presence of a hydroxyl function and both an alkyl cyanide and a cyanamide function.



(104) (95)

The dicyanoalcohol (104) was converted to the dicyanoketone (95) using Jones reagent. The product ($C_{17}H_{23}N_3O$ m.p. 130-131°) was isolated in 83% yield after purification. It absorbed at 2245, 2200, and 1700 cm^{-1} in the infrared region but the hydroxyl absorption at 3450 cm^{-1} in the spectrum of the reactant was absent.

It has been reported (91) that secondary alcohols can be oxidized to the corresponding ketones using an excess of dicyclohexylcarbodiimide in dimethylsulfoxide. It was felt that it might be possible to perform the cyanide displacement of bromine and then to oxidize the C-5 alcohol of the dicyanoalcohol (104) to the ketone (95)

without having to isolate the product (104) first. This would be advantageous when the radioactive synthesis was being carried out as the number of manipulations with the radioactive intermediates would be reduced. When the bromine displacement was essentially complete (thin layer chromatography) dicyclohexylcarbodiimide and pyridine-trifluoroacetic acid were added to the reaction mixture. After 24 h the reaction was stopped and the products were isolated. However no dicyanoketone (95) could be detected by thin layer chromatography and this procedure was abandoned.

2.1.7 HYDROLYSIS AND RECYCLIZATION

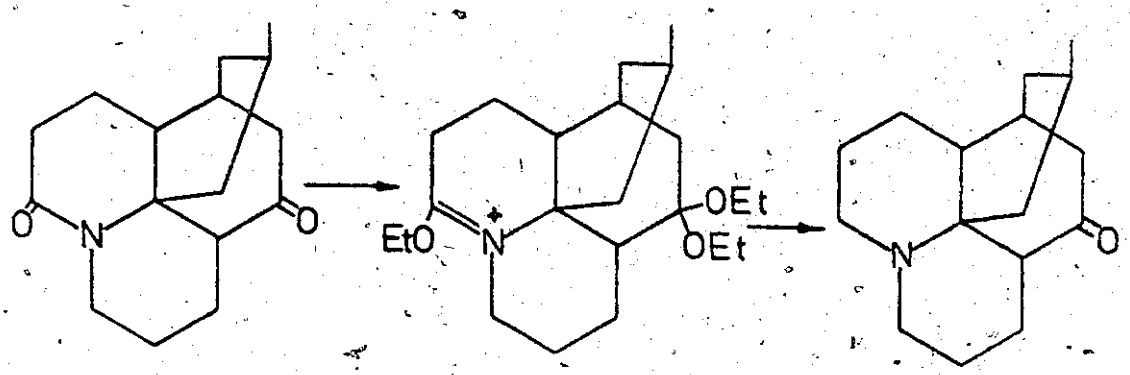
The next step was to convert the dicyanoketone (95) to the corresponding amino acid hydrochloride. It was decided to attempt the hydrolysis in two stages; first to hydrolyze the alkyl cyanide to the carboxylic acid and to esterify the product in situ while at the same time hydrolyzing the cyanamide to the free amine. Subsequently a hydrolysis in aqueous mineral acid would hydrolyze the ester to the amino acid hydrochloride (85). The mass spectrum of the crude reaction mixture after hydrolysis with methanolic hydrogen chloride indicated considerable urethane formation as well as the desired amino ester. However, after prolonged treatment in 30% hydrochloric acid only a mixture of the amino acid (85) and the small amount of the methyl ester remained.

The crude product was treated without further purification, with dicyclohexylcarbodiimide in refluxing pyridine. The crude product was purified on a column of alumina and was indistinguishable by physical

methods from lycopodine-α-lactam (70) (prepared by the oxidation of lycopodine with potassium permanganate). A mixed melting point was undepressed.

A feature of this synthetic sequence not previously considered is that the lycopodine-α-lactam (the critical intermediate in this synthesis of 9-^{14}C-lycopodine) can be prepared readily by oxidation of lycopodine. Thus if intermediate (70) can be prepared with high specific activity it can be diluted at this stage.

The final stage of the synthesis was to convert lycopodine-α-lactam (70) to lycopodine (29). Although this has been accomplished by a two stage sequence (by reduction of the α-lactam to dihydrolycopodine followed by reoxidation to lycopodine) a search for reducing agents capable of reducing amides to amines provided an unexpected benefit. It has been reported that amides have been reduced to amines in high yield by treatment with triethyloxonium fluroborate, followed by reduction of the immonium salt with sodium borohydride (92,93). When this procedure was attempted on lycopodine-α-lactam, lycopodine was isolated in 55% yield after purification. Triethyloxonium fluroborate has been used to ketalize camphor (94) and other hindered ketones. Presumable treatment of lycopodine-α-lactam (70) with this reagent results in the formation of the immonium salt (105) in which the C-5 carbonyl has been ketalized. Diethyl ketals are unaffected by borohydride reduction, and the ketone is apparently regenerated in the subsequent workup.



(70) (105) (29)

2.1.8 RADIOACTIVE SYNTHESIS

9-^{14}C-lycopodine was synthesized following the scheme discussed above. Minor modifications to the procedures and special precautions employed during the actual radioactive synthesis are described in section 3.8 of the experimental. The specific activity of the product was found to be $8.4 \pm 0.1 \times 10^8$ dpm/mmole.

In order to demonstrate that the product was specifically labelled, a small portion of the product was diluted with inactive carrier lycopodine, then degraded to isolate C-9 as formic acid, according to the method of Castillo (60). The product was reacted with 1-naphthylamine hydrochloride and purified to constant activity. The specific activity of the N-formyl-1-naphthylamine ($8.9 \pm 0.1 \times 10^4$ dpm) was found to be 96.7 ±0.2% of the specific activity of the lycopodine ($9.2 \pm 0.2 \times 10^4$ dpm).

2.2 BIOSYNTHESIS OF LYCOPODINE

2.2.1. THE CHIRALITY OF LYSINE INCORPORATED INTO LYCOPODINE

The majority of the intermediates on the biosynthetic route from lysine to lycopodine have now been defined. As some of these intermediates have an optically active center, it became of interest to determine whether a stereochemical requirement exists for their incorporation into lycopodine. The first of these intermediates having a chiral center is lysine itself, and a study was made to determine whether both enantiomers of lysine are incorporated with equal facility or whether only one isomer is incorporated into lycopodine.

In the absence of evidence to the contrary it has often been assumed that it is the "biologically active" L-isomer of a radiolabelled amino acid racemate which is incorporated into alkaloids (95, 96). However attempts to resolve the problem experimentally have been made and are discussed below.

The chirality of a precursor has sometimes been inferred from a comparison of efficiencies of incorporation (observed in parallel experiments) of activity from the racemate and from the labelled D-, and of the labelled L-isomers of the substrate into the product (97,98). Competition experiments (99,100) have also been used in which a labelled racemate is fed alone and in the presence of inactive D- or L-isomer. Suppression of incorporation of label into the product where the active racemate is fed in the presence of unlabelled pure enantiomers indicates that this enantiomer is being incorporated into the product.

A third technique which has been employed has been to compare the efficiencies of incorporation of doubly labelled amino acid race-

mates (labelled with ^{14}C and with ^{3}H or ^{15}N at the chiral center) with similarly labelled pure enantiomers. A comparison of the efficiency of incorporation of the α-^{3}H or $\alpha^{15}N$ relative to ^{14}C will demonstrate whether epimerization of one enantiomer is occurring prior to incorporation (101).

None of these experimental techniques allows an unequivocal conclusion to be drawn about the chirality of the precursor. The use of incorporation efficiencies (based on separate tracer experiments) to discriminate between potential precursors is subject to several uncertainties as outlined in the introduction. Differences in efficiencies of incorporation in a short term experiment may reflect only differences in the rate of transport across cell membranes. A further uncertainty arises when comparing enantiomers. An enantiomer may be converted to its optical antipode prior to incorporation into the biosynthetic product. Such an interconversion could be direct if the required racemase is present (37) or occur via the intermediacy of the corresponding α-keto acid (101). Also when studying plant biosynthesis it is usually practical to study only a small number of specimens, and thus the results are subject to the variability of the individual specimens.

These uncertainties are compounded by methodological difficulties in determining the optical purity of the labelled isomer to be administered. The limits of detection of an unwanted enantiomer in a sample of labelled amino acid are ±1.0% by optical methods and ±0.1% by enzymatic methods (102). The recovery of label in an alkaloid from

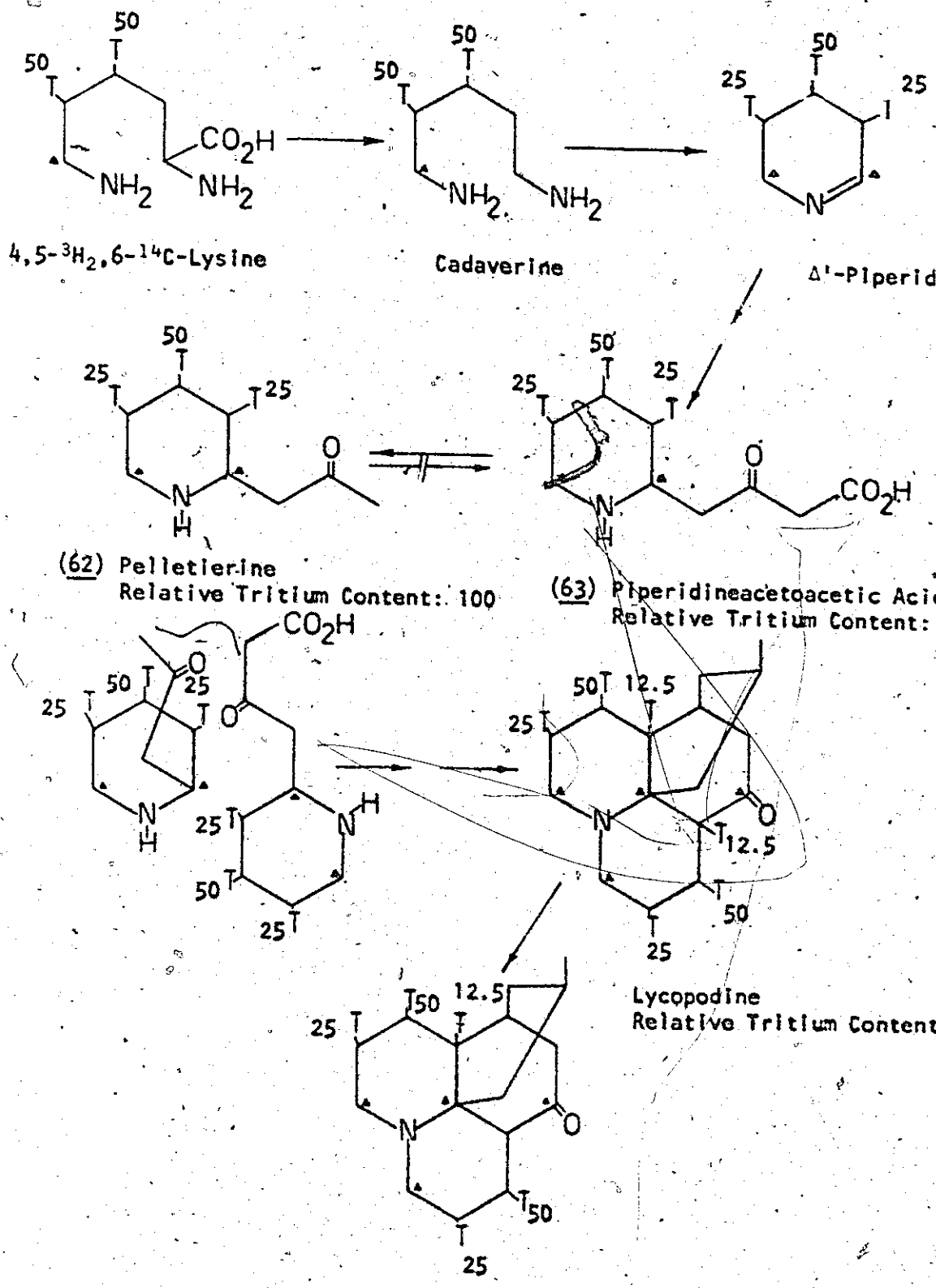
a feeding of a specifically labelled amino acid to an intact plant is often less than 0.1%. Thus it is conceivable that the incorporation of activity into a product from a feeding of a labelled isomer is due only to the preferential incorporation of an enantiomeric impurity in the chiral precursor.

Recently a technique has been described by which the chirality of an optically active precursor may be determined in a single experiment (4). Provided that incorporation of the intact precursor into the product has been previously demonstrated, then the use of a multiply labelled precursor (in which one enantiomer is specifically labelled with one radionuclide while the racemate is specifically labelled with a second nuclide) will demonstrate a specific incorporation of one isomer relative to its optical antipode. The result of such an experiment is self consistent and does not depend on a comparison of parallel experiments.

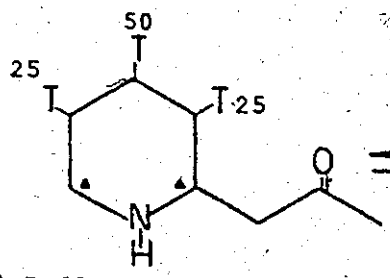
Before discussing the application of this technique to the study of the biosynthesis of lycopodine it is necessary to review some earlier observations which suggest that the carbons of lysine (with the exception of the carboxyl carbon) are incorporated intact into this alkaloid. Castillo (64) observed that doubly labelled lysine was incorporated into lycopodine but that the incorporation was accompanied by a loss of 21.5% of the ^{3}H relative to ^{14}C. Thus the ^{3}H:^{14}C ratio of the product lycopodine was 8.4 ±0.1 from a feeding of D,L-4,5-$^{3}H_2$, 6-^{14}C-lysine having a ^{3}H:^{14}C ratio of 10.7 ±0.2. He suggested that lysine was incorporated into the "pelletierine residue" intact, but

that ^{3}H was lost during the combination of these "two residues" (now thought to be pelletierine and piperidineacetoacetic acid). Two potential schemes for the loss of ^{3}H relative to ^{14}C are outlined in schemes 9 and 10. In scheme 9 a minimum loss of 18.75% of the ^{3}H relative to ^{14}C is predicted. This scheme suggests that no loss of ^{3}H occurs in the incorporation of lysine into pelletierine, and into piperidineacetic acid but ^{3}H is lost during the subsequent combination of these residues. Thus one-half of the ^{3}H at C-3 of pelletierine and at C-3 of piperidineacetic acid should be lost when these two residues are combined to form lycopodine. It is also considered that all the ^{3}H at C-4 of lycopodine is lost prior to or during isolation of this product, by exchange through enolization. Scheme 10 represents a variation of this mechanism. This scheme suggests that prior to their combination, pelletierine and piperidineacetic acid are oxidized to the corresponding immonium compounds, (106) and (107), respectively. A rapid equilibration between the immonium compounds, and the corresponding enamines (108 and 109) results in loss of all the ^{3}H at C-3 of each residue. This scheme predicts a maximum loss of 25% of the ^{3}H relative to ^{14}C. No account of isotope effects has been taken in either scheme. Equilibration at the stage of Δ^{1}-piperideine could result in loss of 25% as well.

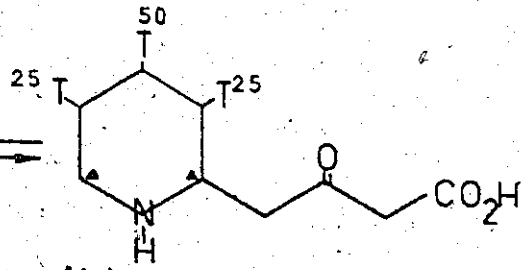
Another observation bears on this problem. It was found that lycopodine isolated from a feeding of D,L-4,5-$^{3}H_2$,2-^{14}C-pelletierine to *L. tristachyum* had a ^{3}H:^{14}C ratio which was identical with that of the initial feeding solution. This confirms that ^{3}H which is attached to


4,5-$^{3}H_2$,6-^{14}C-Lysine
Cadaverine
Δ¹-Piperideine
(62) Pelletierine
Relative Tritium Content: 100
(63) Piperidineacetoacetic Acid
Relative Tritium Content: 100
Lycopodine
Relative Tritium Content 175/200
Lycopodine
Relative Tritium Content 162.5/200 = 81.25%

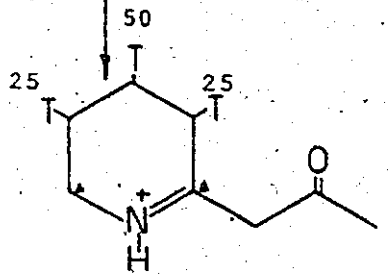
Scheme 9



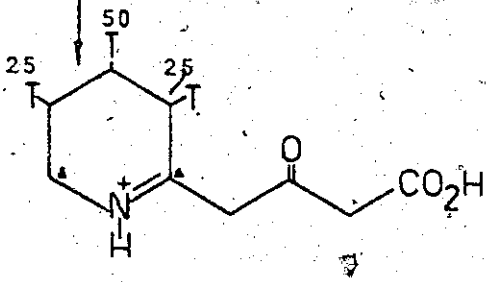
(62) Pelletierine
Relative Tritium Content: 100



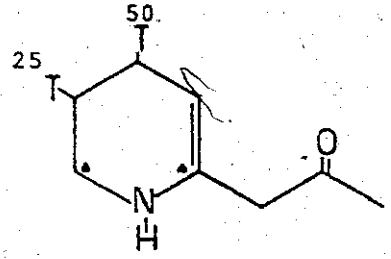
(63) Piperidineacetoacetic Acid
Relative Tritium Content: 100



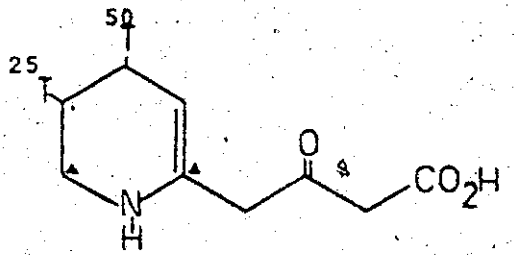
(106) Relative Tritium Content: $\frac{100}{100}$



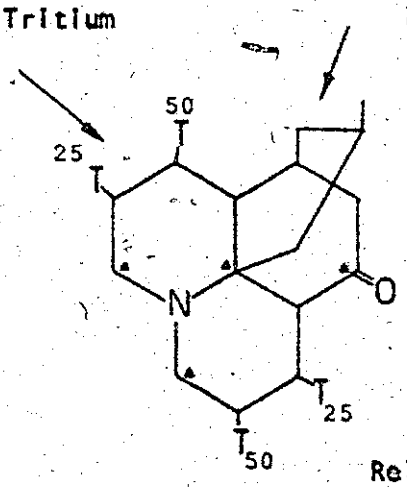
(107) Relative Tritium Content: $\frac{100}{100}$



(108) Relative Tritium Content: $\frac{75}{100}$



(109) Relative Tritium Content: $\frac{75}{100}$



Relative Tritium Content: $\frac{150}{200} = 75\%$

Scheme 10

C-4 or C-5 of either residue (62) or (63) is retained in lycopodine both in accordance with scheme 9 and scheme 10.

To determine the chirality of the lysine incorporated into lycopodine, two feeding solutions were prepared; one contained D,L-4,5-$^{3}H_2$-lysine and D-6-^{14}C-lysine, while the second contained L-4,5-$^{3}H_2$-lysine and D,L-6-^{14}C-lysine. The ^{3}H:^{14}C ratios of these intermolecularly doubly labelled lysine solutions were determined and they were administered, in separate experiments, to L. tristachyum. The ^{3}H:^{14}C ratios predicted for a product which has selectively incorporated either D-lysine or L-lysine from these feeding solutions are tabulated in Table 1.

Feeding Solution	^{3}H:^{14}C	Predicted ^{3}H:^{14}C Ratio of Product Incorporating:		
		D-lysine Selectively	L-lysine Selectively	Both D- and L-Isomers Equally
D,L-4,5-$^{3}H_2$-Lysine / D-6-^{14}C-Lysine	10	5	∞	10
L-4,5-$^{3}H_2$-Lysine / D,L-6-^{14}C-Lysine	10	0	20	10

Table 1

A plot of the $^{3}H:^{14}C$ ratio in the product over the $^{3}H:^{14}C$ ratio in the precursor vs. the percentage of the product derived from L-substrate for; (i) a feeding solution containing L-^{3}H and D,L-^{14}C-precursor, and for (ii) a feeding solution containing D,L-^{3}H and D-^{14}C precursor is included in the appendix.

As mentioned in the introduction the biosynthesis of pipecolic acid (2) has been investigated in several biological systems. This cyclic amino acid is considered to retain the intact carbon skeleton as well as the ε-amino group of lysine. Its genesis from D-lysine has been demonstrated in mammals (36), in higher plants (4), and in microorganisms (37). Although the biosynthesis of this compound had not been studied in L. tristachyum it seemed likely that it was derived from D-lysine here as well.

Lycopodine and pipecolic acid were isolated from each experiment, purified to constant activity, and the $^{3}H:^{14}C$ ratio of each product was determined. The results are tabulated in Table 2. It can be seen that the $^{3}H:^{14}C$ ratio of the lycopodine isolated has increased approximately ten fold in experiment 1 and has increased approximately two fold in experiment 2. These changes in the $^{3}H:^{14}C$ ratio suggest that lycopodine is derived entirely from L-lysine. Alternatively one might argue that the changes in $^{3}H:^{14}C$ ratio reflect only a preferential transport of L-lysine to the site(s) of alkaloid synthesis. If only one isomer were entering the plant, it could be epimerized to the enantiomer prior to incorporation into lycopodine and thus the results would be inconclusive. This possibility is negated by the fact that

Table 2

Chirality of Lysine Incorporated into Lycopodine

Experiment Number		Specific Activity (^{14}C) dpm per mM	$^{3}H/^{14}C$ Ratio[1]	% of Product Derived From L-Lysine
1.	Feeding Solution			
	D,L-4,5-$^{3}H_2$-Lysine[3] / D-6-^{14}C-Lysine[4]		11.1 ±0.1	
	Lycopodine	1.41 ±0.05 x 10^5	89.9 ±0.9, 90.0 ±1.1	
	Lycopodine[2]	1.41 ±0.05 x 10^5	114.2 ±1.0, 114.3 ±1.2	95 ±1%
	Pipecolic Acid	2.2 ±0.02 x 10^6	6.43 ±0.1, 6.42 ±0.1	7 ±1%
2.	Feeding Solution			
	L-4,5-$^{3}H_2$-Lysine[5] / D,L-6-^{14}C-Lysine		8.5 ±0.2	
	Lycopodine	2.46 ±0.06 x 10^5	13.3 ±0.2, 13.3 ±0.1	
	Lycopodine[2]	2.46 ±0.06 x 10^5	16.9 ±0.2, 16.9 ±0.1	100 ±1%
	Pipecolic Acid	1.84 ±0.06 x 10^6	0.48 ±0.03, 0.49 ±0.02	2 ±1%

(1) Each value represents the average of a duplicate determination - when two values are quoted the second value is the average of a duplicate determination after further purification.

(2) A correction factor of (100/78.75 x $^{3}H/^{14}C$) has been applied to the $^{3}H/^{14}C$ ratios of the lycopodine to account for the loss of tritium relative to carbon-14 observed in the incorporation of D,L-4,5-$^{3}H_2$, D,L-6-^{14}C-lysine into lycopodine.

Table 2 (Continued)

Chirality of Lysine Incorporated into Lycopodine

(3) Nominal Specific Activity 34 mCi/mmole, New England Nuclear.

(4) Prepared by the action of L-lysine decarboxylase ("Type II" ex Bacillus cadaveris, Sigma Chemical Co.) on a solution of D,L-6-^{14}C-lysine - nominal specific activity 48 mCi/mmole, Commissariat à l'Energie Atomique, France.

(5) Nominal Specific Activity 0.25 Ci/mmole, Radiochemical Center.

D-lysine also enters this plant as evidenced by the changes in $^{3}H:^{14}C$ ratios observed in pipecolic acid. The pipecolic acid isolated from experiment 1 had a $^{3}H:^{14}C$ ratio which was approximately one half of the $^{3}H:^{14}C$ ratio of the initial feeding solution while the $^{3}H:^{14}C$ ratio of this product from experiment 2 was approximately one-eighteenth of the $^{3}H:^{14}C$ ratio of the initial feeding solution. These results suggest that only 7% and 2% of this product was derived from L-lysine. Thus pipecolic acid is derived almost entirely from D-lysine, and both isomers of lysine are assimilated and metabolized by L. tristachyum.

The results of these two experiments are analogous to the results from similar feedings to N. glauca, S. sarmentosum, and S. acre. In all systems the alkaloids are derived from L-lysine while pipecolic acid is derived almost entirely from D-lysine.

The changes in $^{3}H:^{14}C$ ratio observed in the lycopodine isolated from experiments 1 and 2 have been calculated on the basis of a predicted 21.5% loss of ^{3}H relative to ^{14}C when 4,5-$^{3}H_2$,6-^{14}C-lysine serves as a precursor. This loss of ^{3}H might be avoided if 4-^{3}H-lysine rather than 4,5-$^{3}H_2$-lysine were used as a precursor. Scheme 9 and scheme 10 both predict the loss of ^{3}H, only from the C-5 of lysine. A feeding solution containing L-4-^{3}H-lysine, and D,L-6-^{14}C-lysine was prepared, and after the $^{3}H:^{14}C$ ratio of this solution had been determined, it was administered to L. tristachyum. Lycopodine and pipecolic acid were isolated, purified to constant activity, and the $^{3}H:^{14}C$ ratio of these products was determined. The results of this experiment, recorded in Table 3, suggest that lycopodine and pipecolic acid are each derived from equal

Table 3

The Incorporation of Lysine into Lycopodine

Experiment Number		Specific Activity (^{14}C) dpm per mM	$^{3}H/^{14}C$ Ratio[1]	% of Product Derived From L-Lysine
3.	Feeding Solution[2][4] L-4-^{3}H-Lysine / D,L-6-^{14}C-Lysine[3]		12.5 ±0.2	
	Lycopodine as free base	6.54 ±0.04 x 10^5	13.0 ±0.1	52.0 ±1%
	Lycopodine as perchlorate salt	6.54 ±0.06 x 10^5	13.1 ±0.1, 13.1 ±0.1	52.5 ±1%
	Pipecolic Acid	2.62 ±0.08 x 10^6	12.9 ±0.1	51.5 ±1%
	Purified Lysine (from feeding solution)		8.1 ±0.1	
	Lysine (after enzymatic decarboxylation)		8.1 ±0.1	0 ±1%
	Cadaverine (from enzymatic decarboxylation)		7.7 ±0.1	97.5 ±1%

(1) Each value represents the average of a duplicate determination - when two values are quoted the second value is a duplicate determination after the product has been further purified.

(2) Nominal Specific Activity 29 mCi/mmole - Radiochemical Center, Amersham.

(3) Nominal Specific Activity 48 mCi/mmole - Commissariat a l'Energie Atomique, France.

(4) Shown experimentally to be D,L-4-^{3}H-lysine and not L-4-^{3}H-lysine.

amounts of D- and L-lysine. These results contradict those of experiments 1 and 2 and in the case of pipecolic acid with the results of others (4,35,36,37,38).

Fortunately, a small sample of the initial feeding solution had been set aside for further study and this was chromatographed on paper and the lysine was recovered by eluting with water. The $^{3}H:^{14}C$ ratio of the purified product was determined and it was assayed for chiral purity by subjecting the lysine to enzymatic assay. The enzyme used, L-lysine decarboxylase, is known to decarboxylate only the L-isomer of lysine to give cadaverine. Radioinactive D,L-lysine was added to the reaction mixture to ensure a complete reaction of the radioactive lysine. The reaction was stopped, the product was partially purified, and the mixture was rechromatographed on paper. A radioscan of this chromatogram displayed two peaks of equal area with Rf corresponding to lysine and cadaverine. Thus the decarboxylation reaction was complete. The two products were subsequently recovered and the $^{3}H:^{14}C$ ratio of each product was determined. These results are recorded in Table 3.

Unfortunately, the solvent from the sample of the initial feeding solution had been allowed to evaporate to dryness and considerable autoradiolysis of the sample ensued as evidenced by the generally high level of activity observed throughout the chromatogram of the crude feeding solution. As a result, the $^{3}H:^{14}C$ ratios of the original feeding solution and of the purified lysine from the solution are not comparable.

Had the original feeding solution contained only L-4-^{3}H-lysine and D,L-6-^{14}C-lysine then the cadaverine (the enzymatic decarboxylation product) should have a ^{3}H:^{14}C ratio which is twice that of the purified lysine from the initial feeding solution. The unreacted lysine from the decarboxylation reaction should contain ^{14}C but no ^{3}H. If, on the other hand, the original feeding solution contained D,L-4-^{3}H-lysine and D,L-6-^{14}C-lysine, then the ^{3}H:^{14}C ratio of the products from the decarboxylation reaction (cadaverine and unreacted lysine) should each be the same as the ^{3}H:^{14}C ratio of the purified feeding solution.

As can be seen from Table 3 the latter situation pertains. Within experimental limits the ^{3}H:^{14}C ratio of the unreacted lysine was unchanged, while the ^{3}H:^{14}C ratio of the cadaverine was only slightly lower than the ^{3}H:^{14}C ratio of the purified feeding solution. The plot of the predicted change in ^{3}H:^{14}C ratio between the precursor and the product vs the percentage of product derived from L-lysine, for a feeding solution of L-^{3}H,D,L-^{14}C-tracer (Figure 3, appendix) may also be used to determine the chiral composition of the purified lysine from the initial feeding solution. The observed change in the ^{3}H:^{14}C ratio between the cadaverine and the starting lysine mixture suggests that 48.75 ±1% of the lysine mixture was L-4-^{3}-lysine and 51.25 ±1% of this sample was D-4-^{3}H-lysine. The original solution contained D,L-4-^{3}H-lysine and not L-4-^{3}H-lysine.

The fact that D,L-4-^{3}H,D,L-6-^{14}C-lysine is incorporated into lycopodine essentially without change in the ^{3}H:^{14}C ratio suggests that the carbons of lysine (with the exception of the carbonyl carbon) are

specifically incorporated into this alkaloid. The loss of 21.5% of the ^{3}H relative to ^{14}C when D,L-4,5-$^{3}H_2$,D,L-6-^{14}C-lysine is incorporated into lycopodine must be due only to the loss of ^{3}H from the C-5 position of lysine and cannot be due to a breakdown of this precursor and subsequent recombination of the fragments prior to incorporation into lycopodine. The fact that D,L-4-^{3}H,D,L-6-^{14}C-lysine is incorporated into pipecolic acid essentially without change in ^{3}H:^{14}C ratio suggests that lysine serves as a specific precursor to this compound.

As mentioned earlier in the discussion a prerequisite to determining the chirality of a precursor using this general method is that intact incorporation of the multicarbon fragment of the precursor into the product must be demonstrated. The fact that 25% of the activity of lycopodine isolated from a feeding of D,L-6-^{14}C-lysine was demonstrated to be at carbon C-9 (60) and that 25% of the activity of lycopodine isolated from a feeding of D,L-2-^{14}C-lysine was found at C-5 (60) coupled with the preservation of ^{3}H:^{14}C ratio in lycopodine from the feeding above necessitate that the carbons C-2 to C-6 of lysine are incorporated into this compound intact.

2.2.2 PIPERIDINEACETIC ACID AS A PRECURSOR TO LYCOPODINE

To account for the incorporation of pelletierine (62) only into the C-9 to C-16 portion of lycopodine a model (scheme 8) involving the intermediacy of piperidineacetoacetic acid between lysine and this alkaloid was proposed. Thus piperidineacetoacetic acid could serve as the immediate precursor of the C-1 to C-8 portion of lycopodine while pelletierine (the decarboxylation product of this key intermediate)

would serve as the precursor to the C-9 to C-16 portion of this alkaloid. As proposed by this model a stepwise addition of two, two carbon fragments (from malonyl coenzyme-A) to Δ^1-piperideine (4) would account for the formation of this intermediate. The addition of one, two carbon unit to Δ^1-piperideine would result in the formation of the coenzyme bound ester of piperidineacetic acid (60). Thus piperidineacetic acid might serve as a precursor to lycopodine. Evidence was then sought for the presence of this compound in L. tristachyum.

As the detection of piperidineacetic acid in nature has not been reported in the literature, a generalized method for the separation of amino acids was adapted to the isolation of this product. The procedure makes use of the fact that secondary amines react with nitrous acid to form neutral N-nitrosoamines while primary amines are converted to the corresponding alcohol and tertiary amines are unchanged under the reaction conditions. Thus a separation of secondary amines from tertiary and primary amines can be effected, the free secondary amine being easily regenerated by hydrolysis of the N-nitroso derivative.

As piperidineacetic acid is difficult to crystallize, and as a means of further purifying this product efforts were turned to finding a derivative which would be suitable for counting. The 2,4-dinitrophenyl derivatives of piperidineacetic acid and of pipecolic acid are readily prepared and can be separated by preparative thin layer chromatography (silica gel, benzene:pyridine:acetic acid (6:1:1). However being highly coloured these derivatives are unsuitable for counting by liquid scintillation techniques. Because accurate counting of ^3H in the

presence of ^{14}C can only be effected by this technique another derivative was sought. Attempts to remove the colour of the derivatives by reduction with hydrogen over platinum was unsuccessful. It was found that the phenylthiohydantoins of piperidineacetic acid and pipecolic acid are colourless, easily prepared and are readily differentiated by physical characteristics. This derivative was employed in the experiment described below.

A doubly labelled solution containing D,L-4-^{3}H-lysine, and 1-^{14}C-malonic acid was prepared. The ^{3}H:^{14}C ratio of this solution was determined before it was administered to L. tristachyum. Lycopodine and piperidineacetic acid were isolated (the latter compound by the technique of carrier dilution) and purified to constant activity. The ^{3}H:^{14}C ratios of these products were determined. The results are tabulated in Table 4. Although the piperidineacetic acid appeared to contain a significant quantity of ^{3}H, no ^{14}C above background was detected. Unfortunately the specific activity of the product was too low to allow a chemical degradation to determine the site(s) of labelling within the molecule. Thus the incorporation of label may not be direct.

If the ring of piperidineacetic acid results from the intact incorporation of carbons C-2 to C-6 of lysine it is difficult to see how the side chain arises if not from a two carbon fragment from either acetate, or malonate. The lack of incorporation of malonate into the side chain of piperidineacetic acid is surprising.

Alternatively the activity in the product might be due to the presence of a small quantity of very active impurity. That this might be the case is suggested by a further experiment in which a sample of impure $^{3}H,^{14}C$-lysine was administered to L. tristachyum. Piperidineacetic acid was isolated by the carrier dilution technique. The N-phenylthiohydantoin of this product was prepared and crystallized from hot methanol. Inactive N-phenylthiohydantoin of pipecolic acid (10 mg.) was then added to the crystallization mother liquors and reisolated. The N-phenylthiohydantoin derivative of pipecolic acid and of piperidineacetic acid when assayed for activity, were found to have the same $^{3}H:^{14}C$ ratio even after several crystallizations. The N-phenylthiohydantoin of pipecolic acid was found to be one hundred fold more active than the N-phenylthiohydantoin of piperidineacetic acid. Thus it is likely that the activity associated with the piperidineacetic acid isolated in experiment 4 was due to a small quantity of active pipecolic acid.

As the incorporation of label from malonic acid into lycopodine had not been previously demonstrated, it was decided to degrade partially the alkaloid isolated from this experiment to determine the sites of labelling. The pattern of labelling would be expected to be the same as that resulting from the incorporation of 1-^{14}C sodium acetate, or 3-^{14}C sodium acetoacetate, namely C-15 and C-7, should be labelled and each carbon should contain 50% of the activity of the intact alkaloid.

The lycopodine was oxidized with chromium trioxide and C-15 and C-16 were recovered collectively as acetic acid. A portion of this

Table 4

Piperidineacetic Acid as a Precursor to Lycopodine

Experiment Number		$^{3}H:^{14}C$ Ratio	Specific Activity (^{14}C) dpm per mM x 10^{-4}	Relative Specific Activity
4.	Feeding Solution D,L-4-^{3}H-Lysine (1) 1-^{14}C-Malonate (2)	5.2 ±0.1		
	Piperidineacetic Acid as Phenylthiohydantoin	No ^{14}C above background	3.00 ±0.03*	
	Lycopodine free base	0.74 ±0.01	195.6 ±1.6	
	Lycopodine diluted for degradation		49.2 ±0.1	100 ±0.2
	Acetic Acid as α-naphthylamide		22.9 ±0.1	46.5 ±0.2
	Methylamine as N-methyl-2-naphthoamide		0.03 ±0.06	0 ±0.3

*Specific Activity of ^{3}H (dpm per mM x 10^{-4})

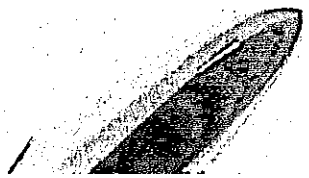
(1) Nominal Specific Activity 34 mCi/mmole - Radiochemical Center Amersham Searle

(2) Nominal Specific Activity 17 mCi/mmole - Radiochemical Center Amersham Searle

Table 4

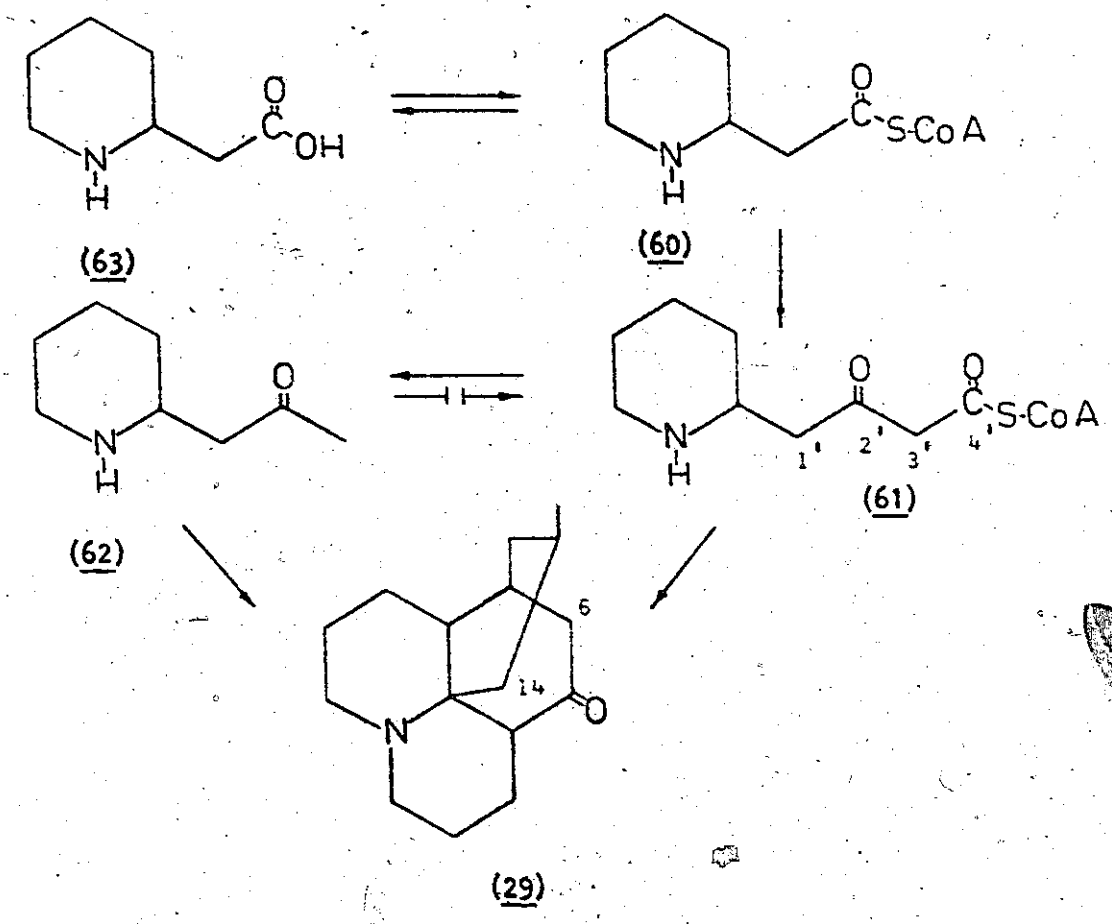
Piperidineacetic Acid as a Precursor to Lycopodine

Experiment Number		Specific Activity (^{14}C) dpm per mM x 10^{-4}	Relative Specific Activity
5.	Lycopodine free base	286	
	Lycopodine as perchlorate salt	287	
	Lycopodine diluted for degradation	104	100 ±0.1
	Acetic Acid as α-naphthylamide	26.2	25.1 ±0.1
	Methylamine as N-methyl-2-naphthoamide	25.4	24.4 ±0.1



product was converted to its α-naphthylacetamide which, when purified to constant activity, was found to have a specific activity which was approximately one-half of the specific activity of the lycopodine. The remainder of the labelled acetic acid was further degraded to recover the methyl group (originally C-16 of lycopodine). Treatment of the acid with sodium azide yielded methylamine which was recovered by distillation. Reaction of this product with 2-naphthoyl chloride gave N-methyl-2-naphthoamide which was purified and assayed for activity. This product was found to be inactive. These results are summarized in Table 4. Thus 47% of the activity of the intact alkaloid was at C-15 which is in close agreement with the 50% postulated by the pelletierine hypothesis. Thus malonate serves as a specific precursor to lycopodine.

A further attempt to gain evidence for the intermediacy of piperidineacetic acid in the biosynthetic pathway between lysine and lycopodine was made. As demonstrated above, malonate serves as an efficient precursor to lycopodine and by inference to the side chain of piperidineacetoacetic acid (61), the postulated crucial intermediate in the elaboration of this alkaloid. If piperidineacetic acid also serves as a precursor to lycopodine then a feeding of a large quantity of inactive piperidineacetic acid in the presence of 2-^{14}C-sodium malonate should suppress incorporation of activity into the C-1' of piperidine-acetoacetic acid and thus into the C-14 and the C-6 of lycopodine. Thus the C-16 and the C-8 of lycopodine should each contain more than the 25% of the activity of the intact alkaloid (predicted when 2-^{14}C



(29)

sodium malonate alone serves as a precursor to lycopodine). This could be demonstrated experimentally by isolating the C-15 and C-16 as acetic acid (Kuhn Roth degradation). The result of this metabolite swamping experiment (experiment 5) is summarized in Table 4. The presence of piperidineacetic acid has had no observable effect on the incorporation of 2-^{14}C-malonate into lycopodine. The C-16 was found to contain 25.1 ±0.1% of the activity of the intact alkaloid and by inference 25% of

the activity would be found at C-6, at C-8, and at C-14.

In summary, the results of earlier feedings of 2'-^{14}C-piperidineacetic acid to L. tristachyum, (66) experiment 4, and experiment 5 it seems very unlikely that piperidineacetic acid serves as a precursor to lycopodine. The working model for the biosynthesis of this product should be modified accordingly. Two interpretations are possible. It is conceivable that even though the coenzyme bound ester of piperidineacetic acid serves as a precursor to lycopodine, no mechanism exists within this species for the conversion of this acid to the ester. Alternatively piperidineacetoacetic acid (or its coenzyme bound ester (61)) arises from the direct combination of a four carbon unit (possibly acetoacetic acid or its biological equivalent) with Δ^1-piperideine. This latter possibility should be investigated experimentally.

2.3 FEEDINGS TO L. ANNOTINUM

It has been suggested that lycopodine may serve as a central intermediate in the biosynthesis of many of the mononitrogenous bases of the Lycopodium alkaloids. If indeed a precursor-product relationship exists between lycopodine and these bases, then those compounds which serve as a precursor to lycopodine must also serve as precursors to the other alkaloids. With this in mind it was decided to attempt to study the biosynthesis of annotinine, the major alkaloid of L. annotinum, and of acrifoline, a minor base from this species, using compounds known to be incorporated into lycopodine. The following compounds were administered to L. annotinum in separate experiments:

1-^{14}C-sodium acetate, 2-^{14}C-sodium acetate, 1,5-^{14}C-cadaverine, D,L-6-^{14}C-lysine, 6-^{14}C-Δ^1-piperideine, and 2-^{14}C-malonate. In each experiment both annotinine and acrifoline were isolated, purified, and assayed for activity. Although in most cases annotinine and acrifoline each contained activity above background, there was not enough activity to allow a chemical degradation to determine the pattern of labelling. The results of the feeding experiments are tabulated in Table 5.

In an attempt to improve the efficiency of incorporation of label into these alkaloids the normal procedure for administration of label was modified. The contact time of the tracer solution with the cuttings was increased from two to four days in experiments 12 and 13. The feedings were varied throughout the growing season of this species and the total activity administered in each feeding was increased from 100 μC to 1 mC in the case of 1-^{14}C-acetate, and 2-^{14}C-acetate (experiments 12 and 13). A feeding was also attempted on only the tips (tissue which developed during the same growing season) where the concentration of preformed alkaloid would be expected to be particularly low. Although activity from this feeding of 1,5-^{14}C-cadaverine was incorporated into annotinine the total activity of the product was again too low to degrade.

It is interesting to note that Leete and Louden (103) isolated annotinine from a feeding of 1-^{14}C-acetate which had a specific activity high enough to allow a chemical degradation. However they report only that the results of their degradation were incompatible with the polyketide hypothesis.

Table 5

Feedings to L. Annotinum

Experiment Number	Date	Precursor	Total Activity Fed	Specific Activity CPM per mM x 10^{-4}	
				Annotinine	Acrifoline
6.	June 1969	1,5-^{14}C-cadaverine[1]	50 µC	0.080 ±0.007	0.255 ±0.002
7.	July 1969	6-^{14}C-Δ'-piperideine[2]	100 µC	0.270 ±0.01	4.83 ±0.10
8.	July 1969	D,L-6-^{14}C-lysine[5]	100 µC	0.055 ±0.003	0.170 ±0.008
9.	June 1969	2-^{14}C-sodium acetate[3]	300 µC	No activity above background	0.0976 ±0.006
10.	June 1970	2-^{14}C-sodium acetate[4]	500 µC	0.182 ±0.001	2.09 ±0.10
11.	August 1971	6-^{14}C-Δ'-piperideine[2]	100 µC	0.185 ±0.02	0.807 ±0.08
12.	August 1971	1-^{14}C-sodium acetate[4]	1 mC	No activity above background	0.493 ±0.02
13.	August 1971	2-^{14}C-sodium acetate[4]	1 mC	0.380 ±0.01	0.619 ±0.03
14.	September 1971	2-^{14}C-sodium malonate[4]	100 µC	0.1930 ±0.01*	0.0981 ±0.006
15.	June 1972	1,5-^{14}C-cadaverine[1]	50 µC	0.120 ±0.02	not isolated

			Piperidineacetic Acid-2,4 DNP	Piperidineacetic Acid-2,4 DNP Methyl Ester
*DPM per mM x 10^{-4}				
13.	August 1971	2-^{14}C-sodium acetate[4]	0.3616 ±0.02	No activity above background
14.	September 1971	2-^{14}C-sodium malonate[4]	0.793 ±0.0004	0.0183 ±0.0003

[1] New England Nuclear Corporation. [2] Prepared from D,L-6-^{14}C-lysine ex C.E.A. France. [3] Volk.
[4] Radiochemical Center, Amersham Searle. [5] C.E.A. France.

One possible explanation for the low levels of incorporation into annotinine of activity from several diverse precursors is that this alkaloid is synthesized and metabolized only very slowly in this species. Thus a high level of preformed alkaloid would dilute any labelled annotinine.

Piperidineacetic acid was also isolated from this species in experiments 12 and 13 and converted to 2,4-dinitrophenyl-2-piperidineacetic acid. Although there appeared to be activity in this product, from both feedings, the activity was not retained when this derivative was converted to the corresponding methyl ester. Thus activity observed in 2,4-dinitrophenylpiperidineacetic acid must have been due to a persistent impurity.

2.4 A SUMMARY OF RESULTS

9-^{14}C-Lycopodine has been synthesized and thus a method for testing the postulated transformation of lycopodine into annotinine is now available. Alternatively, the labelled alkaloid could be used in the search for metabolic products of lycopodine.

Because we were unsuccessful in demonstrating the incorporation into annotinine of those precursors known to label lycopodine it was decided not to administer the labelled lycopodine to L. annotinum until further experiments had been carried out. Specifically, it would be advantageous to determine how rapidly and at what period during the year annotinine is being synthesized and how rapidly it is being metabolized. Indeed one would like to have some indication that our technique

of administering potential precursors to this species is effective in transporting them to the site of alkaloid synthesis.

The biosynthetic route from lysine to lycopodine has been further elucidated. L-Lysine but not D-lysine has been demonstrated to be incorporated into lycopodine. Malonate has also been demonstrated to serve as a specific precursor to this alkaloid.

As far as it is possible to do so experimentally the intermediacy of piperidineacetic acid in the biosynthesis of lycopodine has been discounted. Either no mechanism exists within L. tristachyum for the conversion of the coenzyme bound ester of piperidineacetic acid to the corresponding free acid or piperidineacetoacetic acid is synthesized by the addition of a four carbon unit to Δ^1piperideine.

EXPERIMENTAL

3.1 METHODS AND MATERIALS

Melting points were recorded on a Kofler micro hot stage apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer 337 or on a Beckmann I.R. 5 spectrophotometer in chloroform unless otherwise stated. Mass spectra were routinely run on all samples to verify their molecular weights either on a Hitachi RMU-6A mass spectrometer, or on a C.E.C. 21-110B double focusing instrument. Relative peak heights are quoted in brackets after the m/e value. Mass spectrometry was used to confirm the elemental composition of some compounds. Mass measurements were made using procedures described elsewhere (104).

Microanalyses were performed by A.B. Gygli Microanalyses Laboratory, Toronto, Ontario.

3.2 SYNTHESIS OF 9-^{14}C-LYCOPODINE

3.2.1 N-FORMYL KETO ACID (64)

Lycopodine 1.00 g. (4 mmoles) and oxalic acid 0.510 g. (4 mmoles) were dissolved in 50 ml. of water. Finely ground potassium permanganate 2.14 g. (13.5 mmoles) was added in small portions to the ice cold stirred solution over a period of one hour. The reaction was continued at 0°C for 12 h. then allowed to come to room temperature. When no excess permanganate could be detected the reaction was again

cooled to 0°C, acidified with dilute acid, then decolourised by slowly adding sodium bisulfite.

The resulting pale yellow solution was then extracted six times with chloroform to remove neutral and acidic products. The organic layers were combined and extracted six times with 5% ammonium hydroxide. Finally, acidic material was recovered by acidifying the ammoniacal washes with dilute hydrochloric acid and extracting with chloroform.

N-formyl keto acid (64) (0.32 g.) was recovered from the crude acid product by crystallization using methanol and diethyl ether as cosolvents. The product melted at 244°-245°C. Lit. m.p. 244°-245° (73). The infrared spectrum of this compound showed a broad absorption in the region 3200 cm^{-1} to 2500 cm^{-1}, and strong sharp absorptions at 1660, 1700, and 1720 cm^{-1} (KBr disk). The mass spectrum of this compound had a molecular ion at m/e 293 (100%). Other prominent peaks were observed at M-18 (25), M-28 (45), M-29 (47), and M-57 (91).

Unreacted lycopodine (0.30 g.) was recovered by basifying the original aqueous solutions with dilute ammonium hydroxide and continuously extracting with ether for two days.

3.2.2 N-FORMYL KETO ESTER (80)

The N-formyl keto acid (64) (0.25 g.) was dissolved in dry ether (50 ml.) and treated with an excess of ethereal diazomethane for one hour. The solvent was removed and the residue was added to a short column of alumina. Elution with ether yielded a colourless oil which crystallized on scratching. Recrystallization from ether afforded

colourless crystals which melted at 117°C. Lit. m.p. 116°C (73). A mixed melting point with authentic N-formyl keto ester (80) was undepressed. The infrared spectrum of this compound displayed absorptions at 1740 cm^{-1}, 1705 cm^{-1}, and 1660 cm^{-1} (CCl_4 solution). The mass spectrum of this compound had a molecular ion at m/e 307 (4) and prominent peaks at M-28 (8), M-85 (14) and M-121 (100).

3.2.3 N-FORMYL DIOL (81)

The keto ester (80) (0.114 g.) was dissolved in 20 ml. of 1,2-dimethoxyethane which had been freshly distilled from calcium hydride. The solution was added dropwise to a cooled stirred solution of sodium trimethyoxyborohydride (1.00 g.) in 20 ml. of 1,2-dimethoxyethane (similarly distilled). The whole system was maintained in an atmosphere of dry nitrogen. After the addition was complete the mixture was heated to reflux for seventeen hours, cooled, and added to ice water. The solution was then extracted four times with 20 ml. of chloroform. The combined organic washes were extracted in turn three times with 20 ml. of 5% hydrochloric acid, twice with 20 ml. of water, and finally twice with 20 ml. of 5% sodium bicarbonate solution. The organic phase was then dried over anhydrous sodium sulfate, filtered, and the solvent was removed to yield a pale yellow oil which crystallized on scratching. Yield: 29 mg. The infrared spectrum of the crystalline product displayed broad absorption centered on 3425 cm^{-1} and a single absorption in the carbonyl region at 1645 cm^{-1}. A mass spectrum of the reaction product displayed a parent ion at m/e 281 (12). Other prominent peaks were observed at M-18 (7), M-31 (8), M-45 (19).

M-85 (100), and M-103 (85).

Recrystallization of the product from methanol-ether furnished an analytical sample. The colourless needles melted at 199°-201°C. Anal. Calc. for $C_{16}H_{27}NO_3$: C, 68.29; H, 9.67; N, 4.98%. Found: C, 68.35; H, 9.97; N, 4.65%. The molecular ion was analyzed by high resolution mass spectrometry. Calc. for $C_{16}H_{27}NO_3$: 281.199; Found: 281.203.

3.2.4 A-NORCYCOPODINE-α-LACTAM (91)

The N-formyl keto acid (1.9g.) was suspended in 50 ml. of water and brought into solution by adding methanol. Hydrochloric acid (2 ml.) was added and the solution was refluxed on a steam cone overnight. The solvent was removed at the pump to yield a light brown oil which foamed under reduced pressure. The infrared spectrum of this oil exhibited a broad absorption in the region 3800 - 2500 cm^{-1} and an absorption at 1705 cm^{-1}.

This material was dissolved in dry pyridine (35 ml.) and added dropwise to a vigorously stirred solution of 1.4 g. of freshly distilled dicyclohexylcarbodiimide in 200 ml. of pyridine in an atmosphere of nitrogen. The mixture was refluxed under nitrogen for 6 h. then stirred at room temperature for a further 22 h. The solvent was removed at the pump and the residue was exhaustively extracted with ether. The ether extract was adsorbed on a short column of alumina and the column eluted with ether until no more dicyclohexylurea could be detected in the eluate. The column was then eluted with chloroform yielding a product that readily crystallized as colourless plates from

ether. The mass spectrum of this compound had a molecular ion at m/e 247 (81), and prominent peaks at M-15 (28), M-43 (35), and M-57 (100). The infrared spectrum had strong sharp absorptions at 1705 and 1680 cm^{-1}. Sublimation at 150°C and 5 x 10^{-3} mm. followed by recrystallization from methanol-ether solution furnished an analytical sample. m.p. 164-165°C. Anal. Calc. for $C_{15}H_{21}NO_2$: C, 72.84; H, 8.56; N, 5.66%. Found: C, 72.85; H, 8.44; N, 5.87%.

3.2.5 A-NORDIHYDROLYCOPODINE (92) FROM A-NORLYCOPODINE-α-LACTAM (91) USING LITHIUM ALUMINUM HYDRIDE -

A-norlycopodine-α-lactam (320 mg.) was added to an excess of lithium aluminum hydride in dry dioxan (50 ml.) and the mixture was refluxed for 24 h. The reaction suspension was cooled and the excess reagent was destroyed by the dropwise addition of wet ethyl acetate. The solvent was removed at the pump and the residue was triturated with chloroform. Basic products were removed from the combined organic washes by extracting four times with 20 ml. of 5% hydrochloric acid. The acid washes were basified and re-extracted three times with 20 ml. of chloroform. The chloroform extract was dried over anhydrous sodium sulfate and the solvent was evaporated. The crude basic product was purified on a column using chloroform as eluent. The product (170 mg.) crystallized on removing the solvent. The mass spectrum of the product displayed a molecular ion at m/e 235 (16) and prominent ions at M-15 (9), M-57 (100), and M-85 (24). The infrared spectrum had a strong absorption at 3410 cm^{-1} but had no absorptions in the carbonyl region. An analytical sample was obtained by subliming (120°C and 5 x 10^{-3}mm.) and recrystallizing the product from ether. m.p. 179-181°C. Anal.

Calc. for $C_{15}H_{25}NO$: C, 76.55; H, 10.71; N, 5.95%. Found: C, 76.76; H, 10.76; N, 5.97%.

3.2.6 REDUCTION OF A-NORLYCOPODINE-α-LACTAM (91) TO A-NORDIHYDROLYCOPODINE (92) USING DIBORANE

A-norlycopodine-α-lactam (90) (480 mg.) was dissolved in a minimum of dry tetrahydrofuran and added to an excess of diborane (generated according to the method of Brown (105)) in dry tetrahydrofuran. The reaction mixture was refluxed in an atmosphere of nitrogen for 22 h., cooled, and excess diborane destroyed by the dropwise addition of acidified tetrahydrofuran. The solvent was removed at the pump and the residue dissolved in 50 ml. of 5% hydrochloric acid. The acid solution was extracted twice with 20 ml. of chloroform to remove any starting material. The aqueous phase was basified with dilute ammonium hydroxide and re-extracted three times with 20 ml. of chloroform to recover the product. The combined chloroform extract was dried over sodium sulfate, filtered, and the solvent evaporated to yield 24.6 mg. of crystalline product. m.p. 180°-181°C. The infrared spectrum of this product was identical with that of (92) from the lithium aluminum hydride reduction of (91).

3.2.7 MODIFIED OPPENAUER OXIDATION OF A-NORDIHYDROLYCOPODINE (92) TO A-NORLYCOPODINE (87)

A-nordihydrolycopodine (92) (212 mg.) was treated with aluminum isopropoxide (1.02 g.) and cyclohexanone (2 ml.) in 50 ml. of dry toluene. The mixture was refluxed with stirring for 41 h., cooled, and added to 50 ml. of 5% hydrochloric acid. The toluene layer was drawn off and the acid phase was extracted twice with 20 ml. of ether. The

aqueous phase was made basic and re-extracted four times with 20 ml. of chloroform to recover the product. The chloroform washes were combined, dried over sodium sulfate, filtered, and the solvent evaporated. The crude product was purified on a short column of alumina using ether as eluent. The product (130 mg.) crystallized from ether as short colourless needles. m.p. 129°-130°C. A strong absorption at 1700 cm^{-1} was observed in the infrared spectrum of this product, but there was no hydroxyl absorption. The mass spectrum of this compound displayed a molecular ion at m/e 233 (41) and other prominent peaks were at M-15 (13), M-43 (10), M-57 (100), and M-85 (22). Thin layer chromatography of the product in two solvent systems ($CHCl_3$ and $CHCl_3$: C_6H_6, 2:1 v/v) indicated only a single product. The molecular ion was analyzed by high resolution mass spectrometry. Calc. for $C_{15}H_{23}NO$: 233.177; Found: 233.178.

3.2.8 CYANOBROMO ADDITION PRODUCT (86) FROM A-NORLYCOPODINE (87)

A-norlycopodine (87) (130 mg.) was dissolved in dry benzene and added dropwise to a stirred solution of freshly distilled cyanogen bromide (500 mg.) in 5 ml. of benzene. The system was maintained in an atmosphere of nitrogen and stirred at room temperature for 34 h. Solvent and excess cyanogen bromide were removed at the pump. The residue was dissolved in 50 ml. of chloroform, washed twice with 10 ml. of 5% hydrochloric acid, once with 10 ml. of water, and finally twice with 10 ml. of 5% sodium bicarbonate. The organic phase was dried over anhydrous sodium sulfate, filtered, and the solvent evaporated. The product (~~110~~ mg.) crystallized on removing the solvent. m.p. 149°-151°C.

Thin layer chromatography of the product using chloroform, or chloroform:methanol (10:1 v/v) showed only a single component. The infrared spectrum of this compound displayed absorption maxima at 2220 cm^{-1} and 1705 cm^{-1} characteristic of a cyanamide and a ketone respectively. A mass spectrum of the compound displayed a molecular ion at m/e 340, 338 (19), M-57 (61), M-81, M-79 (100), M-109, M-107 (57). The molecular ion was analyzed by high resolution mass spectrometry. Calc. for $C_{16}H_{23}N_2OBr$: 340.097, 338.099 (isotopic doublet); Found: 340.099, 338.101.

3.2.9 CYANOBROMO ADDITION PRODUCT (101) FROM A-NORDIHYDROLYCOPODINE (92)

A-nordihydrolycopodine (400 mg.) was dissolved in dry benzene (15 ml.) and added dropwise to an excess of freshly distilled cyanogen bromide in benzene (15 ml.). The reaction was stirred in an atmosphere of nitrogen for 48 h. The solvent and excess cyanogen bromide were then removed at the pump. The residue was dissolved in 20 ml. of chloroform and extracted twice with 10 ml. of 5% sodium bicarbonate, once with 10 ml. of water, and twice with 10 ml. of 5% hydrochloric acid. The organic fraction was dried over anhydrous sodium sulfate, filtered, and the solvent removed to obtain the crude reaction product. The product was purified on a short column of alumina using benzene as solvent. The product (320 mg.) readily crystallized from ether. Thin layer chromatography (silica gel) in two solvent systems ($CHCl_3$:MeOH 4:1, and $CHCl_3$:MeOH 1:1) showed the presence of only one component. The infrared spectrum of this compound displayed an absorption at 3450 cm^{-1}, and a strong sharp absorption at 2200 cm^{-1}. The mass spectrum had a molecular

ion at m/e 342, 340 (19) and prominent peaks at m/e 285, 283, M-57 (100), at m/e 261, M-81, M-79 (61), and at m/e 233, M-109, M-107 (35). High resolution mass spectrometry of two of these peaks Calc. for $C_{16}H_{25}N_2O$: 261.197; Found: 261.196, and for $C_{14}H_{22}N_2O$: 233.165; Found: 233.166. Sublimation of the product (145°C and 5×10^{-3} mm.) followed by recrystallization from ether afforded an analytical sample m.p. 179°-181°C. Anal. Calc. for $C_{16}H_{25}N_2OBr$: C, 56.31; H, 7.38; N, 8.21%. Found: C, 56.42; H, 7.44; N, 8.23%.

3.2.10 ATTEMPTED DISPLACEMENT OF BROMINE FROM CYANOBROMO ADDITION PRODUCT (86) USING POTASSIUM CYANIDE-ETHANOL

The cyanobromo addition product (86) (54 mg.) was dissolved in wet ethanol (10 ml. C_2H_5OH:HOH, 3:2) and added dropwise to a stirred solution of 20 mg. of potassium cyanide and 2 mg. of potassium iodide in wet ethanol (20 ml.). The reaction mixture was heated under reflux for 2 h., cooled, and the solvent evaporated. The residue was dissolved in 20 ml. of chloroform and washed three times with 10 ml. of 5% hydrochloric acid, once with 10 ml. of water, and three times with 10 ml. of 5% ammonium hydroxide. The organic phase was dried over anhydrous sodium sulfate, filtered, and evaporated whereupon the product crystallized, yield 35 mg. The infrared spectrum of this compound displayed strong absorptions at 2200 cm^{-1}, 1705 cm^{-1}, and a weak but sharp absorption at 1415 cm^{-1}, but no other absorptions in the region 1500-1700 cm^{-1}. The mass spectrum had a molecular ion at m/e 258 (100) and prominent peaks at M-28 (59), M-43 (79) and M-57 (39). Sublimation at 125°C and 1×10^{-2} mm. followed by two crystallizations from ether furnished an analytical sample m.p. 150°-151°C. Anal. Calc. for

$C_{16}H_{22}N_2O$: C, 74.38; H, 8.58; N, 10.84%. Found: C, 74.28; H, 8.45; N, 10.73%. High resolution mass spectrometry of the molecular ion Calc. for $C_{16}H_{22}N_2O$, 258.174. Found, 258.173.

3.2.11 ATTEMPTED DISPLACEMENT OF BROMINE FROM CYANOBROMO ADDITION PRODUCT (86) USING SODIUM CYANIDE-DIMETHYL SULFOXIDE

The cyanobromo adduct (86) (40 mg.) was added to 6 mg. of sodium cyanide in 15 ml. of dimethyl sulfoxide and the reaction was heated to 100°C for 1 h. The reaction mixture was cooled, added to 100 ml. of water and extracted three times with 20 ml. of chloroform. The combined organic washes were extracted three times with 20 ml. of water. The organic phase was dried over sodium sulfate, filtered, and the solvent evaporated with residual traces of dimethyl sulfoxide being removed on a vacuum line. The product was crystallized from methanol-ether. The mass spectrum of this compound displayed a molecular ion at m/e 258, and a fragmentation pattern which was identical with (96). Thin layer chromatography (silica gel) of this product using chloroform, or chloroform:benzene, 3:1 as solvent showed behaviour analogous to the product (96) derived from the reaction of (86) with alcoholic potassium cyanide.

3.2.12 DICYANOALCOHOL (104) FROM CYANOBROMO ADDITION PRODUCT (101)

The cyanobromo adduct (101) (130 mg.) was dissolved in 5 ml. of dimethyl sulfoxide and added dropwise to a stirred solution of 12 mg. of sodium cyanide in dimethyl sulfoxide (3 ml.) which had been preheated to 95°C in an oil bath. When the addition was complete the flask was returned to the oil bath and maintained at 95°C for 2 h. The reaction

mixture was cooled, then added to 10 ml. of saturated salt solution. The salt solution was extracted three times with 10 ml. of chloroform. The combined extract was washed four times with 8 ml. of saturated salt solution. The organic layer was dried over sodium sulfate, filtered, and the solvent evaporated with residual traces of dimethyl sulfoxide being removed on the vacuum line. The crude product (30 mg.) crystallized as colourless needles from a methanol ether solution. m.p. 214°-215°C. The infrared spectrum of the product exhibited strong absorptions at 3470, and 2200 cm^{-1} and a weak but sharp absorption at 2245 cm^{-1}. The mass spectrum of this compound displayed a molecular ion at m/e 287 (26), and prominent peaks at M-57 (100), and M-152 (30). Sublimation at 120°C and 5×10^{-3} mm. followed by recrystallization from methanol, ether solution furnished an analytical sample. Anal. Calc. for $C_{17}H_{25}N_3O$: C, 71.03; H, 8.77; N, 14.63%. Found: C, 71.26; H, 8.83; N, 14.53%.

3.2.13 ATTEMPTED DISPLACEMENT OF BROMINE FROM CYANOBROMO ADDUCT (101) USING ALCOHOLIC POTASSIUM CYANIDE

The cyanobromo adduct (101) (30 mg.) was dissolved in 10 ml. of ethanol, water solution (3/2 v/v) and added dropwise to a stirred solution of 20 mg. potassium cyanide and 2 mg. of potassium iodide in 10 ml. of ethanol, water. The reaction mixture was heated under reflux for 24 h., cooled, and the solvent was evaporated. The residue was treated four times with 5 ml. of chloroform. The combined organic washes were extracted twice with 10 ml. of 5% hydrochloric acid. The acid washes were combined, made basic, and re-extracted three times with 5 ml. of chloroform to recover basic products. The organic washes

were combined, dried over sodium sulfate, filtered, and the solvent evaporated to yield a colourless oil (8 mg.). The mass spectrum of the product showed a molecular ion at m/e 235 (25), and displayed prominent peaks at M-15 (9), and M-57 (100) but showed no evidence of bromine. The infrared spectrum had absorptions centered on 3400 cm^{-1} and 3200 cm^{-1} and at 990 cm^{-1} and 910 cm^{-1}.

Neutral products were recovered by drying the original chloroform extracts over sodium sulfate, filtering and evaporating the solvent. These were not investigated in detail; however, the infrared spectrum of this mixture displayed absorptions at 3500, 2210, 1715 and 1660 cm^{-1}. The mass spectrum showed no evidence for the presence of the dicyanoalcohol (104).

3.2.14 DICYANOKETONE (95) FROM DICYANOALCOHOL (104)

The dicyanoalcohol (104) (50 mg.) was dissolved in 10 ml. of acetone and cooled in an ice bath. To the stirred reaction mixture was added 5 drops of Jones reagent (106) prepared by dissolving 2.67 g. of chromium trioxide in 10 ml. of water and 0.23 ml. of sulfuric acid. After 15 min. two more drops of oxidant were added and after a further 10 min., an excess of methanol was added to destroy excess oxidant. The reaction mixture was diluted with water and extracted three times with 10 ml. of chloroform. The organic washes were dried over anhydrous sodium sulfate, filtered, and the solvent evaporated. The reaction product crystallized on removing the solvent. Yield 41 mg. The mass spectrum of the product had a molecular ion at m/e 285 (36), and prominent peaks at M-27 (17), M-43 (23), M-57 (100), and at M-135 (95),

The infrared spectrum displayed absorptions at 2245, 2200 and 1700 cm^{-1}.

Two crystallizations from methanol ether solution furnished an analytical sample. m.p. 130°-131°C. Anal. Calc. for $C_{17}H_{23}N_3O$: C, 71.55; H, 8.12; N, 14.72%. Found: C, 71.50, H, 7.91; N, 14.75%.

3.2.15 LYCOPODINE-α-LACTAM (70) FROM DICYANOKETONE (95)

The dicyanoketone (95) (40 mg.) was dissolved in 10 ml. of methanol freshly dried and distilled from magnesium turnings. The reaction vessel was cooled in an ice bath, dry hydrogen chloride was passed through the reaction mixture for 45 min., and the reaction was refluxed for 12 h. The reaction mixture was cooled and the solvent was removed. The residue was redissolved in 5% hydrochloric acid and was extracted three times with 20 ml. of chloroform. The combined extract was washed once with 10 ml. of 5% hydrochloric acid and this was added to the aqueous phase. After basifying the aqueous phase it was extracted three times with 10 ml. of chloroform to recover the crude product. The chloroform washes were combined, dried over sodium sulfate, filtered, and the solvent evaporated to yield a light brown oil. The infrared spectrum of this product had absorptions at 3375 cm^{-1}, at 1735 cm^{-1}, and at 1700 cm^{-1}.

This compound was dissolved in 25 ml. of 10% hydrochloric acid containing 1 ml. of methanol. The mixture was refluxed for 12 h., then cooled and the solvent removed. An infrared spectrum of the crude product displayed broad absorptions in the region 3100 to 2500 cm^{-1} and a single absorption in the carbonyl region at 1700 cm^{-1}. Without further purification the crude product was dissolved in a minimum of dry pyridine

and added dropwise to a stirred solution of dicyclohexylcarbodiimide (50 mg.) in 10 ml. of pyridine. The system was kept under a nitrogen atmosphere throughout the reaction. The mixture was stirred for 12 h. of reflux, then stirred at room temperature for a further 24 h. The reaction mixture was filtered to remove dicyclohexylurea and the solvent evaporated. The residue was extracted with ether and the combined extract was dried over anhydrous sodium sulfate and evaporated. The product was placed on a short column of alumina and eluted with ether until no more dicyclohexylurea could be detected in the eluate. The column was then eluted with chloroform yielding the product which crystallized on removing the solvent. The product was indistinguishable by thin layer chromatography from authentic lycopodine-α-lactam in four separate solvent systems. The infrared spectrum of the product was identical with that of the α-lactam and a mixed melting point was undepressed. The product displayed absorption maxima at 1705 and 1615 cm^{-1}. m.p. 162°-164°C.

3.2.16 LYCOPODINE-α-LACTAM (70) FROM LYCOPODINE (29)

Lycopodine (1 g.) was dissolved in 50 ml. of acetone which had been freshly distilled from potassium permanganate. To this solution was added over a period of 3 h., 0.6 g. of finely powdered potassium permanganate. When no more excess permanganate could be detected the reaction mixture was filtered and the filtrate evaporated to dryness. The acetone soluble material was re-oxidized in the same manner. After 3 h. of reaction time the excess permanganate was destroyed by the addition of methanol. The solution was filtered and evaporated to

dryness. The residue was dissolved in dilute hydrochloric acid, the pH adjusted to 4 with ammonium hydroxide and the solution extracted with methylene chloride. The extract was dried over anhydrous sodium sulfate, filtered and the solvent evaporated to recover the crude product. The product was purified on a column of alumina using chloroform as eluent. The product was recrystallized from ether. m.p. 163°-164°C. Yield 271 mg.

3.2.17 LYCOPODINE FROM LYCOPODINE-α-LACTAM (70)

Lycopodine-α-lactam (70) (47 mg.) was dissolved in a minimum of dry methylene chloride and added dropwise to a stirred solution of 100 mg. of triethyloxoniumfluroborate (prepared according to the method of Meerwein (107)) in 10 ml. of methylene chloride. The reaction was stirred at room temperature for 21 h. in an atmosphere of nitrogen. The solvent was evaporated and the residue was redissolved in ethanol. The ethanol solution was cooled and an excess of sodium borohydride was added in small portions over ½ h. The reaction mixture was allowed to come to room temperature and stirred for a further 18 h. The solution was made acidic with dilute hydrochloric acid and the solvent evaporated. The residue was taken up in 5% hydrochloric acid and extracted twice with methylene chloride and the extract discarded. The acid phase was made basic and re-extracted three times with chloroform. The chloroform extract was dried over sodium sulfate, filtered, and the solvent evaporated. Thin layer chromatography of the product (silica gel using chloroform:methanol, 4:1) showed the product to be mainly lycopodine. Crystallization from hexane afforded 20 mg. of pure lycopodine.

3.3 FEEDINGS TO L. TRISTACHYUM

Lycopodium tristachyum Pursh (ground cedar or running cedar) is found in the vicinity of Huntsville, Ontario, near Algonquin Park. As attempts to propagate this species in the greenhouse were unsuccessful fresh samples were collected for each experiment. Earlier tracer studies using this species indicated (i) that there was little alkaloid in the subterranean stem of this species of club moss and (ii) that no difference in the efficiencies of incorporation were observed when labelled precursors were administered to intact plants (by the wick method) or to excised shoots.

All feedings to this species were conducted on cuttings, 4 to 5 inches in length, which were gathered into two or three bouquets (so as to have 70 to 90 g. of dried plant material) and placed into 30 ml. beakers, cut surfaces downward. Each bouquet and beaker was placed in a 250 ml. beaker. The radiotracers were dissolved in glass distilled water and this solution was divided equally among the bouquets. Sufficient water was added to each beaker to cover the cut tips. The plant cuttings were kept in contact with this solution (water being added as required) for forty-eight hours. Counting of the residual solutions after this time indicated that less than 0.1% of the initial activity remained. The plants were cut into 1 inch lengths and dried.

3.4 ISOLATION OF LABELLED COMPOUNDS FROM L. TRISTACHYUM

3.4.1 ISOLATION OF LYCOPODINE

The dried plants were ground to a fine powder in an Osterizer Blender, transferred to a Soxhlet thimble and extracted for 48 h. with

methanol. To avoid bumping the solvent was changed after 24 h. The combined methanolic extracts were reduced in volume by distillation and water was added. This procedure was repeated until the temperature of the distillate reached 97°C. The extract was acidified with dilute acid, stirred for two hours, then filtered through celite. The filtrate was washed with ether and these washes discarded. The aqueous phase was made basic with dilute ammonium hydroxide then extracted with chloroform to recover the crude basic fraction. The aqueous phase was set aside, to be worked up later to recover amino acids. The residue from this organic extract was extracted in turn with hexane. The combined hexane extracts were dried (Na_2SO_4), filtered, and the solvent was removed. The crystalline residue was purified on a short column of alumina using benzene as solvent. The product readily crystallized from hexane. Lycopodine was purified to constant activity by successive crystallizations and sublimations (110°C and 5×10^{-3} mm.). Melting point 116°, Lit. m.p. 116° (47).

The perchlorate salt was prepared by adding an ethereal solution of perchloric acid to the product dissolved in ether. The crystals which formed were recovered by filtration and were recrystallized from hot methanol. Melting point 276°-278°C Lit. m.p. 278°C (108).

3.4.2 ISOLATION OF PIPECOLIC ACID

The crude aqueous filtrate which was made basic during the lycopodine isolation procedure was worked up to recover amino acids. Radioinactive D,L-pipecolic acid (100 mg.) was added to this extract and the solvent was evaporated on a steam bath. The residue was

redissolved in a minimum of water and a small quantity of charcoal was added. The suspension was filtered through cotton and the filtrate was added to a column of Dowex 50W x 4 (H^+ form -1.5 x 18 inches). The column was eluted with water until the eluate was clear and tests for carbohydrate material in the eluate were no longer positive. The presence of carbohydrate or related material in the eluate was demonstrated by the development of a violet ring at the interface when 18M sulfuric acid (1 ml.) was added to 1 ml. of the eluate to which 2 drops of alcoholic α-naphthol (20%) had been added. Amino acids were recovered by eluting the column with N ammonium hydroxide. Approximately 200 ml. were required. The ammoniacal eluate was evaporated to dryness on a steam bath and the residue was dissolved in 5 ml. of 50% hydrochloric acid. Sodium nitrite (1.0 g.) was added in small portions over ½ h. After a further ½ h. of reaction time the solution was extracted four times with 15 ml. of ether. The combined organic extracts were dried (Na_2SO_4), filtered and evaporated to dryness.

The yellow oil thus obtained was redissolved in concentrated hydrochloric acid, heated on a steam bath for 15 min., then evaporated to dryness. The residue was crystallized from methanol, ether solution. The crystalline product was applied to a second column of Dowex 50W x 4 (H^+ form -1 x 10 cm.), which was eluted with water until no more radioactivity could be detected in the eluate. Pipecolic acid was recovered by eluting the column with 1N ammonium hydroxide. The ammoniacal eluate was evaporated to dryness and the residue was directly sublimed (180° and 5×10^{-3} mm.). The sublimate was then crystallized from methanol

ether solution. Yield 14-23 mg. Melting point 280°-281° (Lit. m.p. 281°) (109).

This product was dissolved in 3 ml. of pyridine, water (1/1) and the pH was adjusted to 8.6 with sodium carbonate. Freshly distilled phenylisothiocyanate, 4 drops, were added and the solution was heated to 40°C for 2 h. The reaction mixture was acidified, then extracted twice with 2 ml. of chloroform. The organic extracts were combined and the solvent was removed. The residual oil was dissolved in methanol containing 1N hydrochloric acid and heated to 80°C for 1 h. The solvent was removed and the product was crystallized from hot methanol. Yield 25-36 mg. Melting point 162°-163°C. The infrared spectrum of this compound displayed absorption maxima at 1740 and 1210 cm^{-1} but showed no absorptions above 3100 cm^{-1}. The mass spectrum of this product had a molecular ion at m/e 246 (100) and prominent peaks at M-29 (12), M-33 (10), and M-111 (22). Anal. Calc. for $C_{13}H_{14}N_2OS$: C, 63.37; H, 5.73; N, 11.38%. Found: C, 63.29; H, 5.91; N, 11.32%.

3.4.3 ISOLATION OF PIPERIDINEACETIC ACID

Radioinactive D,L-piperidineacetic acid (50 mg.) prepared by oxidizing 2-piperidineethanol with chromium trioxide (110), was added to the basic aqueous filtrate (from the crude plant extract) remaining after the isolation of lycopodine. The subsequent reisolation procedure is exactly the same as that used for pipecolic acid. The ammoniacal eluate from the second ion exchange column was evaporated to dryness on a steam bath. The residue was dissolved in 5 ml. of pyridine, water

(1/1) and the pH was adjusted to 8.6. Freshly distilled phenylisothiocyanate, 5 drops, were added and the solution was heated to 40°C for 2 h. The reaction mixture was made acidic and extracted twice with 2 ml. of chloroform. The organic extracts were combined and the solvent was removed. The residual oil was dissolved in 1M hydrochloric acid in aqueous methanol and heated to 80°C for 1 h. The solvent was removed and the product was crystallized from methanol. Yield 7 mg. Melting point 195°-196°C. The infrared spectrum of this product displayed absorption maxima at 1715 and 1210 cm^{-1} but no absorption above 3100 cm^{-1}. The mass spectrum of this compound had a molecular ion at m/e 260 (100) and prominent peaks at M-29 (18), M-43 (20) and M-125 (15). High resolution mass spectrometry of the molecular ion Calc. for $C_{14}H_{16}N_2OS$: 260.098. Found: 260.099. Anal. Calc. for $C_{14}H_{16}N_2OS$: C, 64.57; H, 6.20; N, 10.77; S, 12.32%. Found: C, 64.57; H, 6.33; N, 10.77; S, 12.23%.

3.4.4 DETERMINATION OF ENANTIOMERIC PURITY OF L-A-^{3}H,D,L-6-^{14}C-LYSINE

L-lysine decarboxylase ("Type II" ex *Bacillus cadaveris*. Sigma Chemical Co.) (10 mg.) was suspended in phosphate buffer (0.2M, pH 6.0) and added to the purified lysine dissolved in 1 ml. of the same buffer. The mixture was warmed to 37°C and gently agitated. After 15 min. of incubation 1 mg. of radioinactive D,L-lysine monohydrochloride was added to the reaction mixture and after 40 min. a further 1 mg. was added. After 8 h. of reaction time the incubation mixture was heated on a steam bath for 10 min., the precipitate was removed by centrifugation, the solid was resuspended in water, and the suspension was

recentrifuged. The combined supernatants were evaporated to dryness and the residue was chromatographed on paper (Whatman 3 mm. - isopropanol/0.880 ammonia/water (8:1:1)). The D-lysine had an R_f^6 of 0.06 while the cadaverine had an R_f of 0.4 in this solvent system. The lysine was recovered by eluting with water and the cadaverine was recovered by eluting with methanol.

3.5 DEGRADATION OF LYCOPODINE

3.5.1 CARBONS -15 and -16 AS ACETIC ACID

Chromium trioxide (2.0 g.) was dissolved in 3 ml. of water and added slowly to 100 mg. of lycopodine dissolved in 5 ml. of 40% sulfuric acid. The reaction mixture was refluxed for 1 h. then allowed to cool. The condenser was thoroughly rinsed with water, the washings were added to the reaction flask and the mixture was distilled with the aid of nitrogen until 50 ml. of distillate had been collected. The distillate was neutralized with sodium hydroxide (2.5 ml. of 0.1 N), evaporated to dryness, and the product was converted to the N-acetyl-1-naphthylamine by dissolving 3.5 mg. of this product in 1 ml. of water and adding 8 mg. of 1-naphthylamine hydrochloride and 20 mg. of 1-ethyl-3-(3-dimethylaminoisopropyl)carbodiimide hydrochloride. The flocculent precipitate was recovered by filtration and directly sublimed (130°C, 5×10^{-3} mm.), then crystallized from benzene. m.p. 157°-159°C (Lit. m.p. 159°-160°) (111).

3.5.2 CARBON-16 AS METHYLAMINE

Sodium acetate (14 mg.) was dissolved in sulfuric acid (1 ml.) sodium azide (50 mg.) was added, and the mixture was heated in a steam

bath in a system attached to three gas traps. The carbon dioxide which was evolved was swept through a solution of 10% sodium hydroxide contained in each of the gas traps with the aid of nitrogen. After 1½ h. the reaction mixture was cooled and the traps were replaced by a second system of traps containing 0.1 N hydrochloric acid. The reaction mixture was further cooled in a dry ice, acetone bath and basified with 10% sodium hydroxide (30 ml.). The reaction mixture was again heated for three hours on a steam bath and volatile material swept into the traps with nitrogen. The solutions from the traps were combined and evaporated to dryness. The residue was dissolved in a minimum of dimethylsulfoxide and added to 1 ml. of pyridine containing 40 mg. of 2-naphthoyl chloride. The reaction mixture was stirred for 24 h., diluted with 5 ml. of water, and extracted three times with 10 ml. of ether. The combined organic extracts were washed four times with 10 ml. of 5% sodium bicarbonate, then dried over sodium sulfate, filtered, and evaporated to dryness to yield a colourless oil which was directly sublimed (80°C, 1 x 10^{-2} mm.) to give a colourless crystalline product. m.p. 111°-112°C (sealed capillary), Lit. m.p. 108°-109°C (112).

3.6 FEEDINGS TO L. ANNOTINUM

Lycopodium annotinum Linn. is found throughout much of northern Ontario. As this species was not readily propagated in the greenhouse fresh cuttings were collected for each experiment. The technique used to administer radioactive compounds to this species were the same as the previously described technique used in experiments with L. tristachyum.

3.6.1 ISOLATION OF ANNOTININE AND ACRIFOLINE

The dried plant material was ground to a fine powder in an Osterizer Blender and extracted in a Soxhlet apparatus for 48 h. with methanol. The solvent was changed after 24 h. The combined methanolic extracts were concentrated by distillation and water was added. This procedure was repeated until the temperature of the distillate reached 97°C. The suspension was acidified, stirred for two hours, then filtered through celite. The aqueous filtrate was washed four times with 50 ml. of ether, basified, and re-extracted exhaustively with chloroform. The chloroform extracts were dried (Na_2SO_4), filtered, and evaporated to dryness. Crystallization of the product from methanol, ethyl acetate afforded annotinine. A second crop of crystals was obtained by concentrating the mother liquors. The two crops of crystals were combined, sublimed (130°C, 5×10^{-3} mm.) and recrystallized from ethyl acetate. Yield 22-49 mg. m.p. 231°-232°. Lit. m.p. 232° (113).

The mother liquors were evaporated to dryness and the residue was dissolved in acetone. Hydrobromic acid (48%) (5 drops) was added and the solution was set aside to crystallize. Recrystallization of the product from acetone yielded colourless plates of acrifoline hydrobromide. m.p. 318°d. Lit. m.p. 315+° (111).

3.6.2 ISOLATION OF PIPERIDINEACETIC ACID

Piperidineacetic acid was isolated from this species by the technique of carrier dilution. Radioinactive D,L-piperidineacetic acid (100 mg.) was added to the basic aqueous filtrate (from the crude plant

extract) remaining after the isolation of the alkaloids. The procedure used for the re-isolation of this product was the same as that used to isolate this product from L. tristachyum. The crude product from the second ion exchange column was dissolved in a minimum of water and added to a solution of 130 mg. of 2,4-dinitrofluorobenzene in water (1 ml.). The pH was adjusted to 9.0 with sodium bicarbonate and the mixture was stirred in the dark for 4 h. The pH was periodically adjusted to 9.0 with sodium bicarbonate. The mixture was extracted three times with 10 ml. of ether, made acid, then re-extracted three times with chloroform. The chloroform extracts were combined, dried (Na_2SO_4), filtered and evaporated to dryness. The product was purified by preparative thin layer chromatography (Rf = 0.83, silica gel - benzene, pyridine, acetic acid (6:1:1)). The product was crystallized from hexane, ether solution. m.p. 150°-152°C. Anal. Calc. for $C_{13}H_{15}N_3O_6$: C, 50.49; H, 4.89; N, 13.59%. Found: C, 50.78; H, 5.09; N, 13.44%. The infrared spectrum of this product displayed a broad absorption in the region 3200 to 2600 cm^{-1} as well as absorptions at 1710, 1515, and 1340 cm^{-1}, characteristic of a carboxylic acid and aromatic nitro groups. The mass spectrum of this derivative had a molecular ion at m/e 309 (9), and prominent peaks at M-17 (22), and M-59 (100).

A portion of this product (10 mg.) was dissolved in ether and treated with an excess of ethereal diazomethane for 6 h. The solvent and excess diazomethane were removed, the residue was dissolved in 10 ml. of chloroform, extracted twice with 15 ml. of 5% sodium bicarbonate, dried (Na_2SO_4), filtered, and the solvent removed. The product

readily crystallized as red-brown prisms from ether. Yield 65 mg. m.p. 153°-155°C. The infrared spectrum of this compound displayed absorptions at 1745, 1515 and 1345 cm^{-1}. The mass spectrum of this compound had a molecular ion at m/e 323 (10) and prominent peaks at M-17 (39) and M-73 (100). Anal. Calc. for $C_{14}H_{17}N_3O_6$: C, 51.60; H, 5.30; N, 13.00%. Found: C, 51.43; H, 5.20; N, 13.11%.

3.7 ASSAY OF RADIOACTIVITY

A weighed portion of the sample to be assayed for radioactivity was transferred to an aluminum planchette, dissolved in a minimum of collodion (2%) in dimethylformamide solution, then spread as a thin film on the planchette by adding a circle of lens tissue. The solvent was evaporated with the aid of an infrared lamp, and the activity was determined using a low background Nuclear Chicago Corporation gas flow system (Model 4342). Each sample was assayed in triplicate and the results corrected for self absorption and background. The quoted confidence limits represent the standard deviations from the mean.

Alternatively activity was assayed by liquid scintillation. A weighed portion of the sample was transferred to a scintillation vial, dissolved in a minimum of methanol or methanol-water, then dispersed with 10 ml. of liquifluor (Nuclear Chicago Corporation) which had been diluted twenty-five times with toluene. Samples were assayed in duplicate, under similar quenching conditions on a liquid scintillation computer (Mark I, Model 6860, Nuclear Chicago Corporation) using external standardization with ^{133}Ba.

3.8 SYNTHESIS OF RADIOLABELLED LYCOPODINE

The procedures followed in the elaboration of 9-^{14}C-lycopodine were essentially the same as those used in the pilot synthesis. Modifications of these procedures and special precautions employed during the synthesis of radioactive lycopodine are described below.

3.8.1 DICYANOALCOHOL (104) FROM CYANOBROMO ADDITION PRODUCT (101)

Cyanobromoalcohol (104) (300 mg.) was dissolved in dry dimethylsulfoxide (5 ml.) and added to a solution of ^{14}C-sodium cyanide (3 mC - 2.6 mg. - New England Nuclear Corporation) and sodium cyanide (19.8 mg.) in dimethylsulfoxide (10 ml.). The reaction mixture was heated to 95°C in an oil bath. After 5 min. sodium cyanide (10 mg.) in dimethylsulfoxide (3 ml.) was added and the heating was continued. After a further 10 min. sodium cyanide (13.4 mg.) was added to the reaction mixture. The mixture was heated for a further ½ h. then allowed to come to room temperature. The product was isolated by the procedure described in section 3.2.12.

3.8.2 DICYANOKETONE (95) FROM DICYANOALCOHOL (104)

The crystalline residue (104) was treated (without further purification) with chromium trioxide following the procedure described in section 3.2.14. The product (95) was crystallized twice from methanol, ether. Yield 178 mg.

3.8.3 HYDROLYSIS AND CYCLIZATION

The radiolabelled dicyanoketone was dissolved in hydrochloric acid (50%, 20 ml.) and refluxed for 12 h. The solvent was removed and

replaced with methanol (freshly distilled from magnesium turnings). Dry hydrogen chloride was passed through the solution for ½ h. and the solution was refluxed for 24 h. The reaction mixture was cooled, then the solvent was removed. The residue was again hydrolyzed in hydrochloric acid (25%, 20 ml.). The solvent was removed and the residue was dissolved in pyridine (8 ml. - freshly distilled from barium oxide) and added to a solution of dicyclohexylcarbodiimide (150 mg.) dissolved in pyridine (25 ml.). The whole system was maintained in an atmosphere of nitrogen. The mixture refluxed for 16 h., then stirred at room temperature for a further 12 h.

The solvent was removed and the residue was partitioned between chloroform and dilute hydrochloric acid. The solvent from the acidic fraction was removed and the residue was again subjected to the hydrolysis and cyclization sequence described above. The residue from the second cyclization reaction was partitioned between chloroform and dilute acid. The organic fractions from the two cyclization residues were combined, dried (Na_2SO_4), filtered, and the solvent removed. The residue was applied to a short column of basic alumina which was eluted with ether until no more dicyclohexylurea could be detected in the eluate. The product was then recovered by eluting the column with chloroform.

Radioinactive lycopodine-a-lactam (70) (40 mg.) was added to the crude reaction product and then recrystallized from methanol, ether. The product was then sublimed (110°C and 1×10^{-2} mm.) and assayed for activity. The product (42 mg.) was found to contain 1.5 mC/mM.

3.8.4 DIHYDROLYCOPODINE FROM LYCOPODINE-α-LACTAM (70)

The radiolabelled lycopodine-α-lactam (70) (42 mg.) was dissolved in dry dioxan (5 ml.) and added to a suspension of lithium aluminum hydride (50 mg.) in dioxan (20 ml.). The mixture was refluxed for 24 h. in an atmosphere of nitrogen. When cooled the excess reagent was destroyed by the dropwise addition of ethyl acetate. The solvent was removed and the residue was dissolved in dilute hydrochloric acid. The acidic solution was extracted three times with 20 ml. of methylene chloride, made basic, then re-extracted four times with 20 ml. of chloroform. The chloroform extracts were combined, dried (Na_2SO_4), filtered, and the solvent removed. The residue was applied to a short column of alumina and recovered by eluting with chloroform.

3.8.5 9-^{14}C-LYCOPODINE FROM DIHYDROLYCOPODINE

A slight excess of Jones reagent (106) (8 drops) was added dropwise to the radiolabelled dihydrolycopodine dissolved in acetone (10 ml.). The reaction mixture was stirred at room temperature for 1 h. Excess dichromate ion was destroyed by the dropwise addition of methanol. The solvent was removed at the pump, the oily residue was dissolved in water, and extracted three times with 20 ml. of chloroform. The combined organic extracts were dried (Na_2SO_4), filtered, and the solvent removed. The crude product was purified on a short column of alumina using benzene as eluent. The product (22 mg.) readily crystallized on removing the solvent. A radioscan of a thin layer chromatogram (silica gel - chloroform) showed the presence of only a single component of R_f corresponding to lycopodine. Specifically no dihydrolycopodine was detected.

3.8.6 DEGRADATION OF LYCOPODINE, C-9 AS FORMIC ACID

A small portion of the lycopodine was diluted with inactive carrier lycopodine (250 mg.) then purified by successive crystallization (from hexane) and sublimation (115°C and 1 x 10^{-2} mm.). When assayed for activity the purified lycopodine was found to contain 9.2 ±0.2 x 10^{4} dpm.

Lycopodine (218 mg., diluted for degradation) and oxalic acid (110 mg.) was dissolved in water (25 ml.) and treated with potassium permanganate (500 mg.) following the procedure described in section 3.2.1. Yield 54 mg.

The product N-formyl keto acid (54 mg.) was converted to the corresponding N-formyl keto ester (80) by treatment with an excess of ethereal diazomethane (section 3.2.2). The crude product was added to a short column of alumina and recovered using ether as eluent. The product (50 mg.) was sublimed (140°C and 5 x 10^{-3} mm.) and crystallized from ether. m.p. 115°C. Yield 50 mg.

The methyl ester was dissolved in sulfuric acid (1M, 10 ml.) and the mixture refluxed for 2 h. Steam was then passed through the reaction mixture until 70 ml. of distillate, containing volatile acids, had been collected. The distillate was neutralized with 0.1 M sodium hydroxide (0.97 ml.) and the solution was evaporated to dryness.

The residue was dissolved in water (2 ml.) containing α-naphthylamine hydrochloride (15 mg.) and 1-ethyl-3-(3-dimethylaminoisopropyl) carbodiimide hydrochloride (40 mg.). N-formyl-1-naphthylamine precipitated after three minutes. The product was sublimed (120° and 5 x 10^{-3} mm.) and crystallized from benzene. Yield 7 mg. m.p. 136-137° Lit. m.p. 139 (110).

Figure 2 Incorporation of L-Substrate into Product

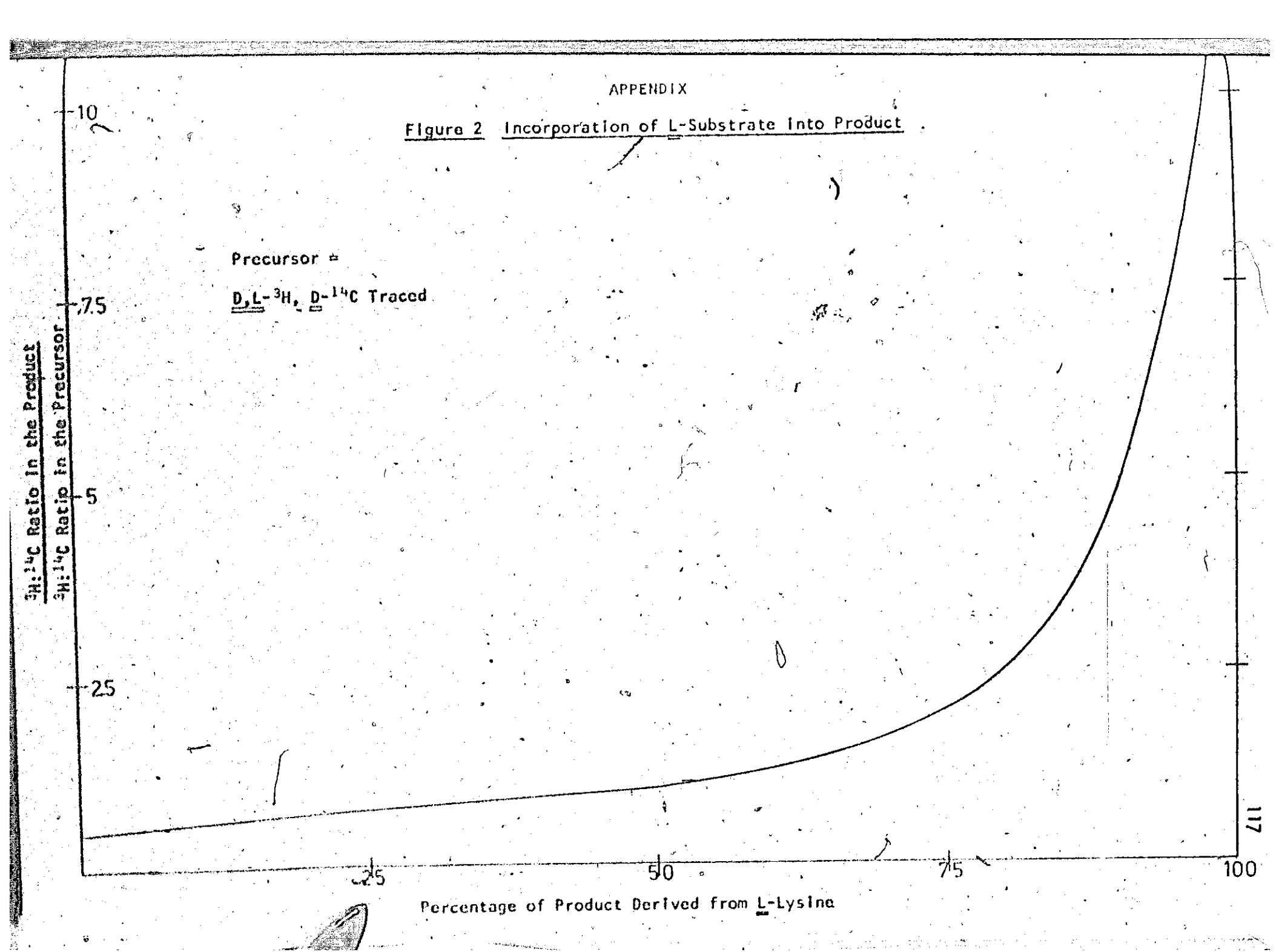
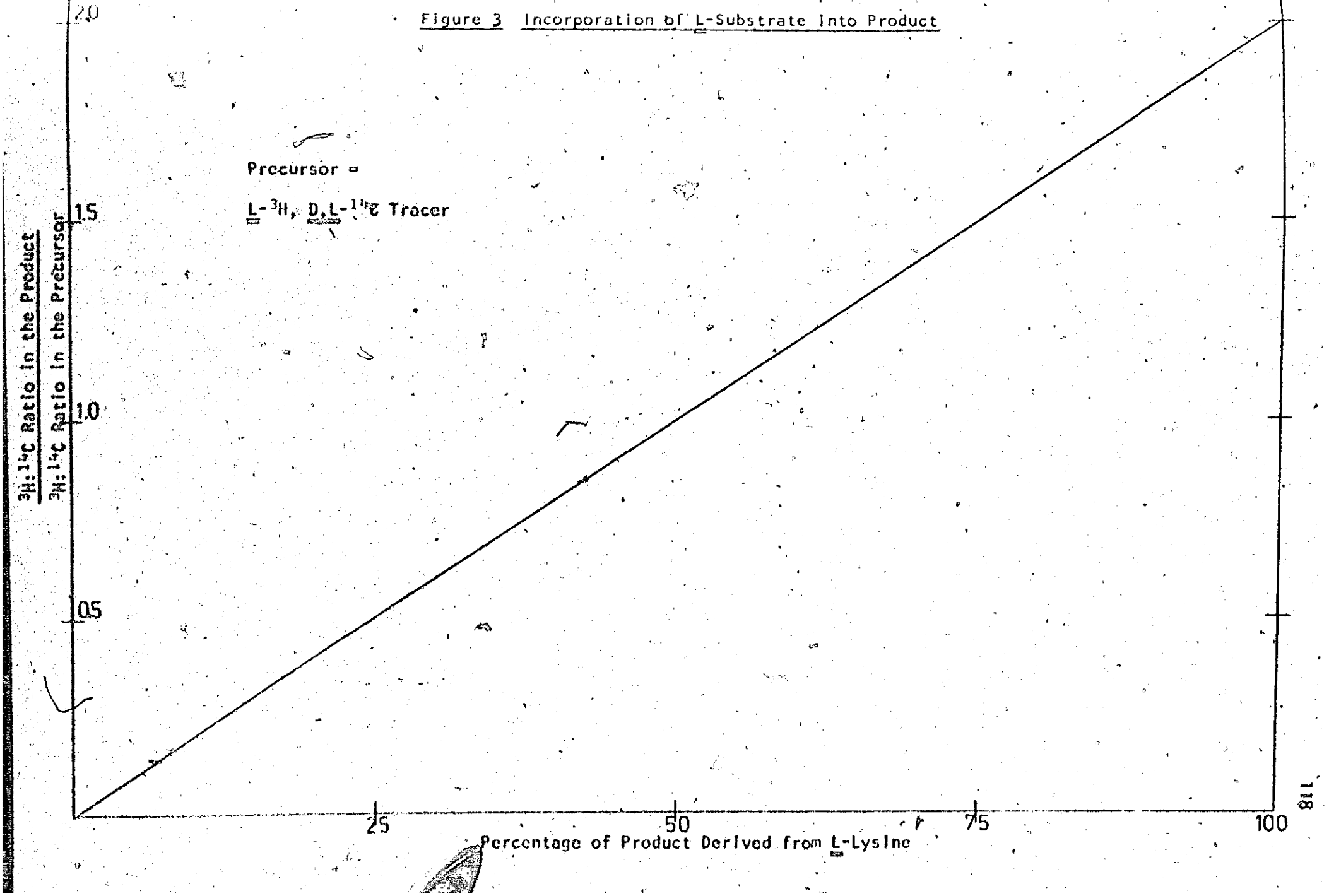


Figure 3 Incorporation of L-Substrate into Product



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