BIOSYNTHESIS OF *LYTHRACEAE* ALKALOIDS

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SCOPE AND CONTENTS:

The biosynthesis of the alkaloids of Decodon verticillatus, (L.)Ell, a member of the Lythraceae family, was studied by tracer methods. These alkaloids contain a phenylquinolizidine system, whose biosynthetic origin has not previously been investigated.

Labelled samples of lysine, Δ^1-piperideine and phenylalanine yielded radioactive decodine and decinine, the two major alkaloids of D. verticillatus. Systematic degradations of these labelled alkaloids show that lysine, Δ^1-piperideine and phenylalanine serve as specific precursors. Whereas lysine and Δ^1-piperideine entered the quinolizidine ring, the biphenyl ring system of these alkaloids was derived from two units of phenylalanine. In a further experiment, labelled pelletierine was administered to the plants. But this experiment yielded an inconclusive result.

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I. INTRODUCTION

Alkaloids are a large group of naturally-occurring nitrogeneous organic compounds. Most of them contain their nitrogen as part of a heterocyclic system, and are basic in character, even though there are others such as colchicine and capsaicin, which are not heterocyclic and non-basic. Many alkaloids are physiologically active. The overwhelming majority of alkaloids are found in the plant kingdom but a few are isolated from animals and microorganisms. Since 1806 when morphine was first isolated by Sertürner, alkaloids have been extensively investigated primarily by organic chemists. There are a few thousand alkaloids reported and most of their structures are known.[1,2]

Over the years, the chemical interest in alkaloids has centered around the following three aspects: the isolation and structure determinations, synthesis and biosynthesis. Compared to the advanced stage of study of isolation and structure elucidation, the study of alkaloid biosynthesis is in its infancy.

Ever since the structure of a large number of alkaloids had been elucidated around the turn of this century, organic chemists have speculated about the biogenetic origin of these substances. It was the pioneering ideas of Trier[3] and Robinson[4] which provided most of the original hypotheses of the biogenesis of alkaloids. These biogenetic speculations were advanced on the basis of visual dissection of the complex alkaloid skeletons into simpler structural units, which were plausibly derivable from common primary metabolites, and on the basis of analogy with known reactions of organic

chemistry. While most of the classical hypotheses were later proved to be correct, some were shown to be wrong and thus had to be either discarded entirely or modified.

Little progress in the study of alkaloid biosynthesis was achieved before 1950, although a number of techniques were used for these investigations which sometimes yielded controversial results.[5] Since around 1951, when radioisotopes became available, we began to see real progress in the study of alkaloid biosynthesis. Marion and coworkers[6,7] are the pioneers in the use of tracers in these studies. During the last two decades, precursor-product relationships of practically all the major alkaloid skeletons have been investigated. Numerous reviews on alkaloid biosynthesis have appeared in the last few years.[8-15]

As a result of extensive tracer experiments, it can be concluded that most alkaloids are formed from a relatively small number of organic compounds such as amino acids, acetic acid and mevalonic acid. Moreover, there are relatively few "in vitro" reaction-types which could account for the formation of these alkaloids. The most important organic chemical reactions involved are: Schiff base formation; Mannich reaction; aldol condensation; oxidations and reductions; and rearrangements.[16]

The usual procedure used in biosynthetic work is to choose a putative precursor, labelled with an isotope such as ^{3}H, ^{14}C or ^{15}N, and administer this labelled compound to the intact living plant or to part of the plant, such as excised shoots or root tissue, by a variety of techniques. After an arbitrary period of growth of the plant in contact with the tracer, ranging from a few days to a few months, the desired alkaloid is isolated and rigorously purified to constant radioactivity.

The labelling pattern of the radioactive product is then established by chemical degradation. If the results of degradation show that the incorporation of the tracer is non-random, the administered compound is regarded as a specific precursor of the alkaloid. Sometimes it is useful to use doubly or even triply labelled substrates, since such experiments can establish intact incorporation of the precursors. It is from studies of this type that most of the knowledge of precursor-product relationships in alkaloid biosynthesis has emerged.

The chemical degradation of a labelled product can be time-consuming and often a difficult process. Recently, mass spectrometry has been introduced to the study of alkaloid biosynthesis.[17-20] Although NMR techniques have been used in biosynthetic work,[21-23] they have not yet been applied in the field of alkaloid biosynthesis. So far these spectroscopic methods have only been successful for the study of the distribution of label in products which contain a relatively high percentage of the tracers. Increasing application of physical methods in the future will be expected since the non-degradative spectroscopic method eliminates the necessity of chemical reactions required to isolate specific labelled carbon atoms.

It is to be pointed out that experimental findings with tracers have to be interpreted with caution. The time course and the sites of biosynthesis of plant products, and problems of permeability of the added precursors through the cell membranes will have to be taken into consideration. Sometimes, even the modes of feeding techniques could be important. Thus, in some cases an alkaloid is biosynthesized in young plants but not in the older ones or vice versa showing that the formation of alkaloids may be seasonal. Nicotiana glauca is capable of synthesizing nicotine

and anabasine in the roots and so the intact plant growing hydroponically has been used for tracer studies.

Over the last twenty years or so, the initial phase of alkaloid biosynthesis from labelled precursor studies has been achieved; not only have the important primary metabolites which build up the complex molecule been established, even some of the intermediates along the proposed biosynthetic pathway have been isolated, characterized and tested.[10,11] Most of the studies so far are nonenzymatic and do not necessarily establish the normal and obligatory metabolic pathway. Since the majority of alkaloids found in nature are optically active, it seems that enzymes must be involved in alkaloid formation, although some steps along the metabolic pathway may be nonenzymatic. It is apparent then that the final and hardest phase of alkaloid biosynthesis is to isolate and characterize the enzymes which catalyze the individual steps of a postulated biosynthetic sequence. Until then, even such a question as the timing of O- and N-methylation cannot be answered. Although a few enzymes involved in alkaloid biosynthesis have been isolated and characterized, relatively little is known about the detailed mechanism of alkaloid formation at the enzymatic level.[24] This indeed is a challenge to the future investigators of alkaloid biosynthesis.

II. BIOGENESIS OF LYTHRACEAE ALKALOIDS

A. Structural Studies of Lythraceae Alkaloids

The family Lythraceae consists of 22 genera with approximately 500 species.[25] Although some Lythraceae species had been investigated long ago for alkaloidal content, it was not until 1962 that structural studies on the alkaloids of Lythraceae plants were first reported.[26] So far the group of Lythraceae alkaloids has been shown to contain more than a dozen members and most of their structures are known.

Ferris[26] reported the isolation and partial structure determination of seven alkaloids from Decodon verticillatus (L.) Ell. ("water oleander" or "swamp loosestrife"), a plant which occurs in swampy areas. These alkaloids were named decodine, verticillatine, decinine, decamine, vertine, decaline and vertaline, respectively. Subsequently, lythrine, vertine, cryogenine, lythridine, sinicuichine, heimine, sinine, nesodine and lyfoline were isolated from Heimia salicifolia and myrtifolia, and were interrelated with those isolated from Decodon.[25,27,28] It was found later that cryogenine is in fact identical with vertine[29] and lythridine is identical with sinine in all respects.[30,31]

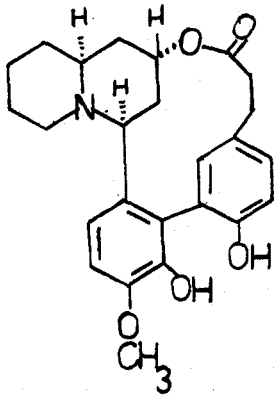
The structure of the first of the Lythraceae alkaloid, lythrine, was elucidated on the basis of X-ray crystallographic and chemical studies.[29] The complete structures of other Lythraceae alkaloids were subsequently reported on the basis of chemical and spectroscopic studies.[30-34] Up to this point it was apparent that all the known Lythraceae alkaloids could be divided into two classes (I and II). The class I alkaloids possess

the biphenyl linkage. These include decodine (1), decinine (2), verticillatine (3), lythrine (4, R = CH_3), lyfoline (4, R = H), vertine (5), decamine (6), nesodine (7) and lythridine (sinine) (8). These structurally related alkaloids have the same basic skeleton but differ in the oxygenation pattern of the aromatic nuclei and/or in configuration at one asymmetric center and/or in the double bond of the lactone ring. An asymmetric center is at C-10 which is the bridgehead of the quinolizidine ring system and thus forms either a trans- or cis-fused quinolizidine ring system.

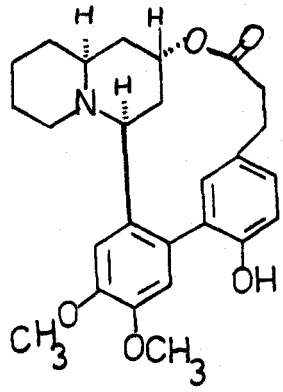
The class II alkaloids possess the biphenyl ether linkage. There are at present five alkaloids isolated from Lythraceae plants which belong to this class. However, only the structures of two of these alkaloids have been reported. The complete structure and stereochemistry of vertaline (9) and decaline (10) have been elucidated by X-ray analysis,[33] chemical and spectroscopic studies.[34] Lagerine, a biphenyl ether alkaloid, was isolated from another species of Lythraceae plants, Lagerstroemia indica L.[34]

Recently, a new class of Lythraceae alkaloids with a very different structural skeleton has been reported.[35, 36] The complete structures of lythranine (11, R_1 = H, R_2 = CH_3 or R_1 = CH_3, R_2 = H), lythranidine (12) and lythramine (13, R_1 = H, R_2 = CH_3 or R_1 = CH_3, R_2 = H), isolated from Lythrum anceps Makina, have been elucidated by chemical and spectroscopic studies. Although they still contain a biphenyl linkage, they do not possess a quinolizidine ring system. Instead a piperidine ring system is present in these three Lythrum alkaloids.

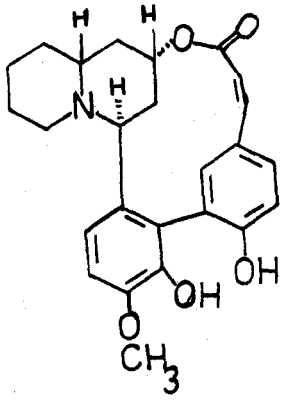
Some of the representative Lythraceae alkaloids are shown in Fig. 1 and Fig. 2.



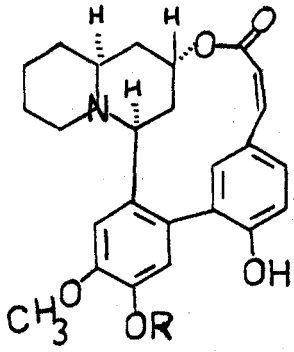
1 decodine



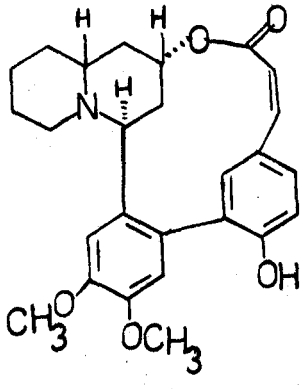
2 decinine



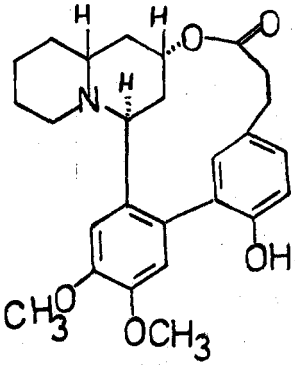
3 verticillatine



4 lythrine (R = CH_3)
lyfoline (R = H)

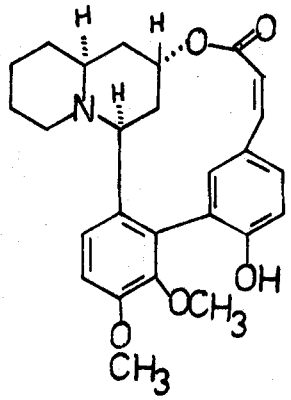


5 vertine (cryogenine)

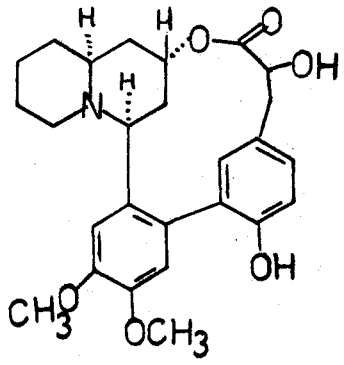


6 decamine

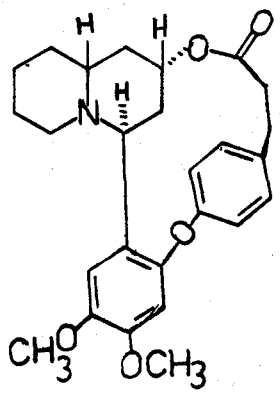
Fig. 1 Lythraceae alkaloids



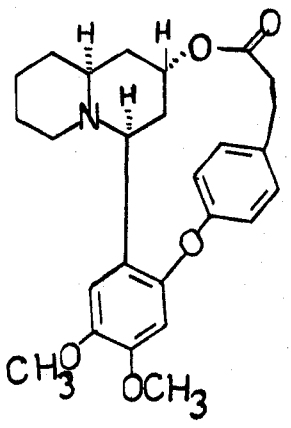
7 nesodine



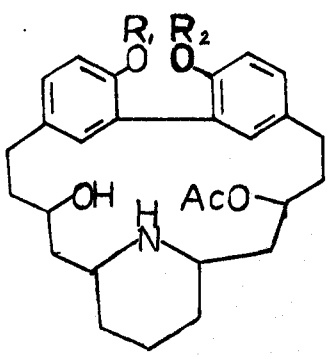
8 lythridine (sinine)



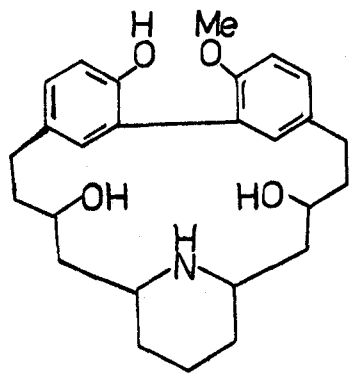
9 vertaline



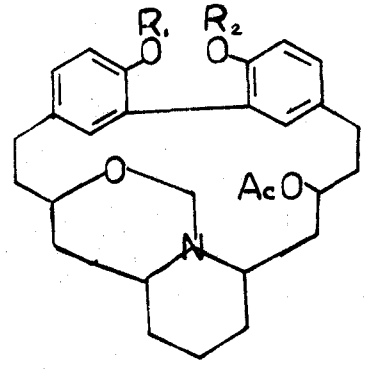
10 decaline



11 lythranine



12 lythranidine



13 lythramine

Fig. 2 Lythraceae alkaloids

B. Biogenetic Schemes for Lythraceae Alkaloids

Clearly, the structural patterns of Decodon and Heimia alkaloids differ in the following ways: the stereochemistry of the quinolizidine ring, the nature of the linkage between the phenyl rings and the oxygenation pattern of the aromatic nuclei. Thus, it seems very probable that they all follow the same biosynthetic pathway, especially in the early stages of their biosynthesis. Any biogenetic scheme postulated should accommodate the features mentioned above.

Several biogenetic schemes have been postulated for the origin of the structural variants of these alkaloids. The first proposed biogenetic scheme[37] is shown in Fig. 3 and designated as Scheme A. Lysine has been shown to be the precursor of many piperidine and quinolizidine alkaloids.[8,9,12] Since the Lythraceae alkaloids also contain a quinolizidine ring, they are likely to be derived from lysine too. The conversion of lysine into Δ^1-piperideine has also been demonstrated in alkaloid biosynthesis.[46,73] Although the conversion of lysine via Δ^1-piperideine to pelletierine (14) has not been shown by an "in vivo" experiment its analog, N-methylpelletierine has been investigated[42,43] and has been shown to be derived from lysine (C_5N) and acetate (side-chain). Condensation of pelletierine with an appropriately oxygenated benzaldehyde derivative (or benzoic acid derivative) such as protocatechuic aldehyde (15) would yield 16. Reduction of the keto group and esterification of the resulting alcohol (17) with p-coumaric acid (18) gives 19. The phenol oxidation would furnish phenolate radicals which can undergo phenol coupling to give the required oxygenated aromatic nuclei (cf. ref. 51). Ortho-ortho C-C phenolate coupling would form the necessary skeleton

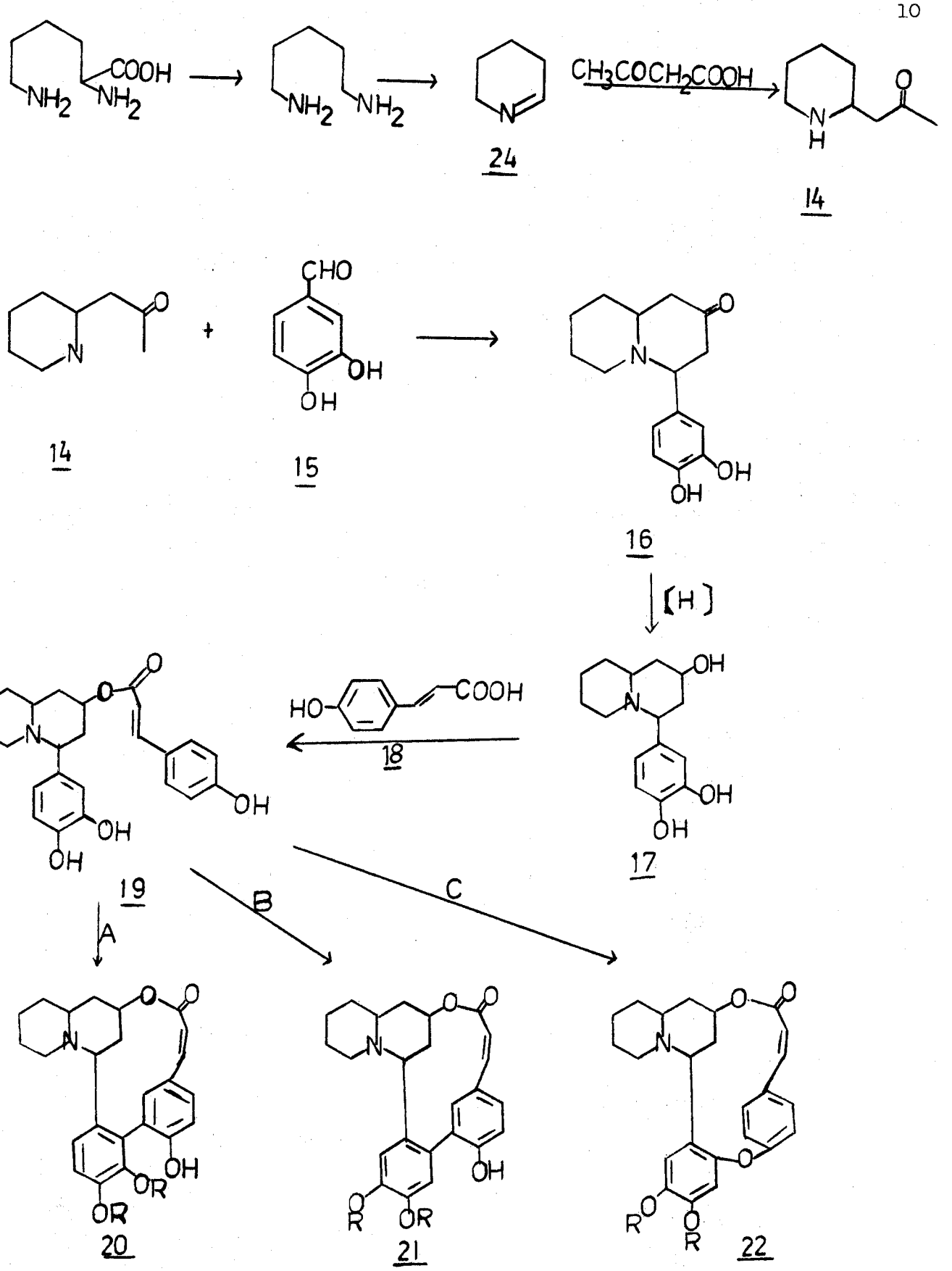


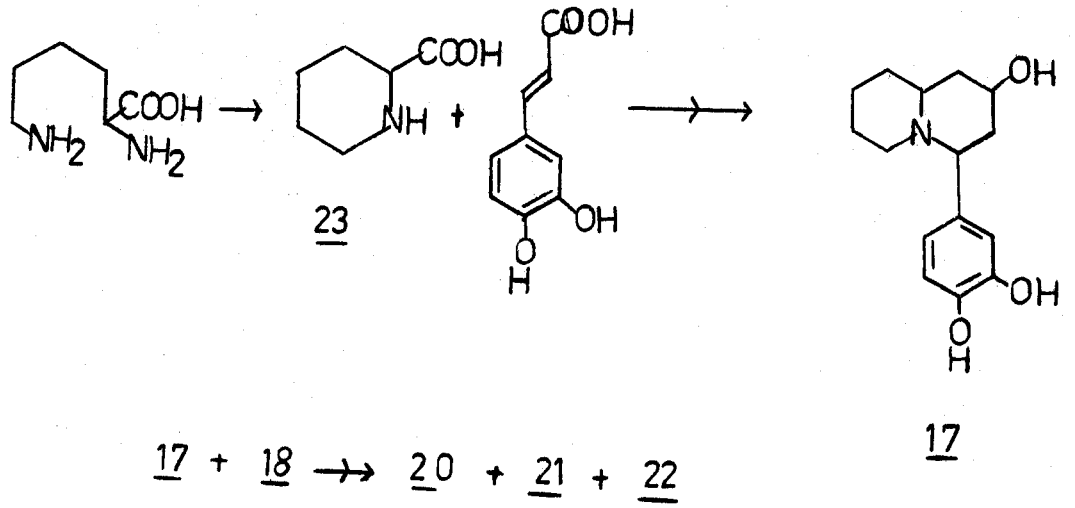
Fig. 3 Biogenesis of the phenylquinolizidine alkaloids of the family Lythraceae (Scheme A)

of compounds of series A (20), such as decodine; ortho-para C-C phenolate coupling gives rise to series B compounds (21), such as decinine; and finally, para-para C-O phenolate radical coupling would result in series C compounds (22), such as vertaline and decaline. The appropriate O-methylation, hydrogenation and hydration of the lactone double bond would account for all the known Lythraceae alkaloid structures. This indeed is a very attractive hypothesis.

It is to be pointed out that the phenolate coupling step may well precede the esterification of alcohol 17. The exact sequence of O-methylation of the phenolic group cannot be ascertained. The required stereochemistry of the quinolizidine ring of all the alkaloids might have resulted during the formation of 16.

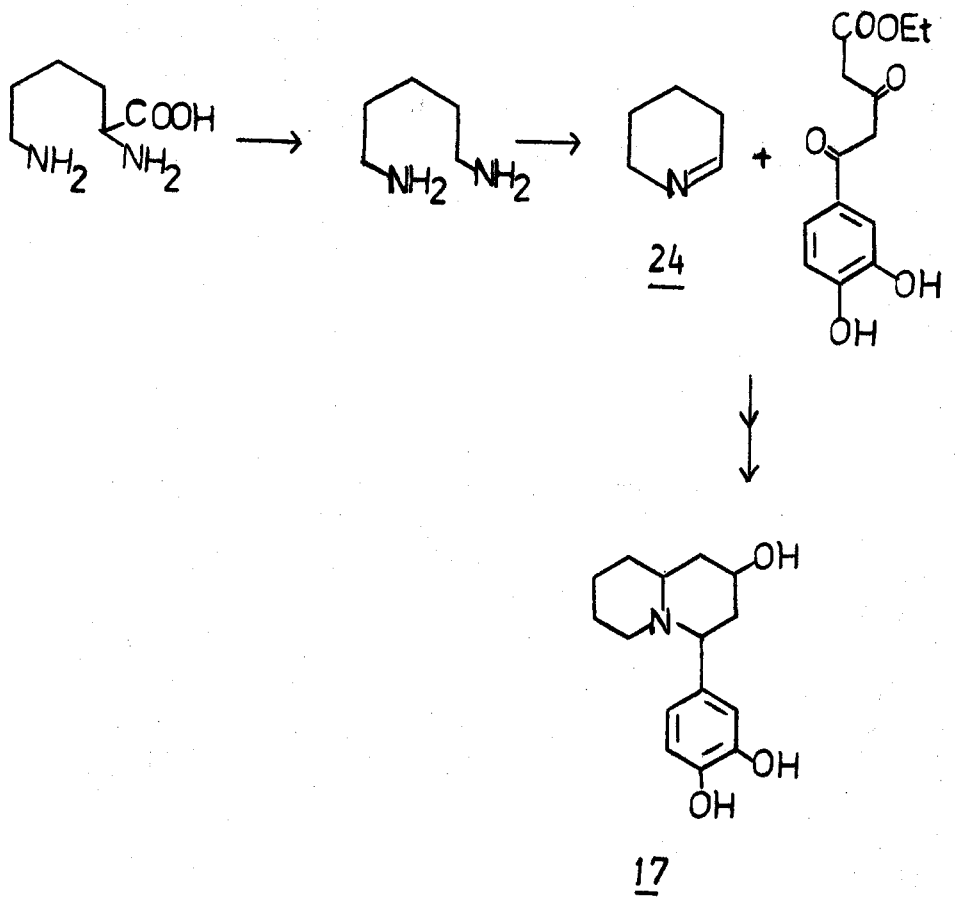
Apart from the hypothesis just discussed, there are three other reported biogenetic schemes[37-39] for Lythraceae alkaloids. They are summarized in Fig. (4), (5), and (6), and are designated as Schemes B, C, and D respectively. In all these schemes, the late step of the biosynthesis is the same as in Scheme A. It is the introduction, by ester formation, followed by a phenol coupling process, of a C_6-C_3 unit, derived from phenylalanine via cinnamic acid. The schemes differ only with respect to the origin of phenylquinolizidine moiety.

Scheme B shows that lysine is converted into pipecolic acid (23) which reacts with cinnamic acid (or its derivative) to form the quinolizidine ring. It should be noted, however, that the coupling of two carboxyl groups is hard to reconcile from the knowledge of organic chemistry, unless the carboxyl group of pipecolic acid is fully reduced to a methyl group before the coupling takes place. The biosynthesis of



17 + 18 ⟶ 20 + 21 + 22

Fig. 4 Biogenesis of the phenylquinolizidine alkaloids of the family Lythraceae (Scheme B)



17 + 18 ⟶ 20 + 21 + 22

Fig. 5 Biogenesis of the phenylquinolizidine alkaloids of the family Lythraceae (Scheme C)

pipecolic acid will be discussed in the following section.

Scheme C shows the formation of quinolizidine ring by condensing Δ^1-piperideine (24) and phenylpropane derivative which, in turn, is derivable from either phenylalanine and acetate or benzoic acid and acetoacetate. This step has been successfully carried out "in vitro."[38]

Scheme D looks especially attractive since a very different structural skeleton of Lythraceae alkaloids has been reported in Lythrum species. It is postulated that the quinolizidine ring system of the Lythraceae alkaloids is derived from a polyketoacid, benzoic acid and ammonia. The biogenetic scheme for Lythrum alkaloids can be outlined as in Fig. 8. The Lythrum alkaloids contain a piperidine nucleus. In fact, some of the piperidine alkaloids, such as coniine and pinidine, have been demonstrated to be of polyketide origin. This will be further discussed in the next section.

At least one more biogenetic scheme which can be added is shown in Scheme E (Fig. 7). Again, this scheme differs from all the others only in the origin of quinolizidine ring. Scheme E suggests that a polyketoacid condenses with cinnamic acid and ammonia to form the quinolizidine ring.

The five equally plausible schemes proposed above can be briefly summarized as follows:

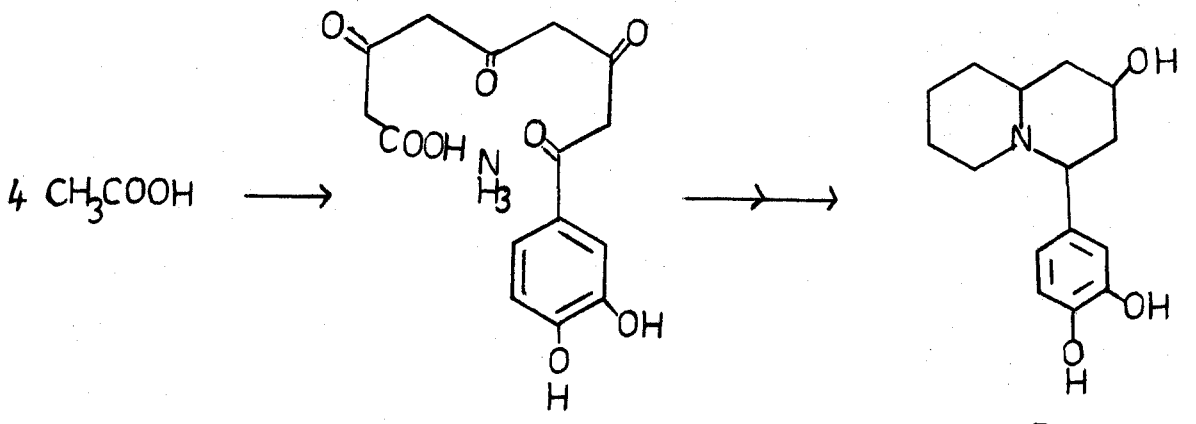
Scheme A: pelletierine + C_6-C_1 + C_6-C_3

Scheme B: pipecolic acid + C_6-C_3 + C_6-C_3

Scheme C: piperideine + C_6-C_3 + C_6-C_3

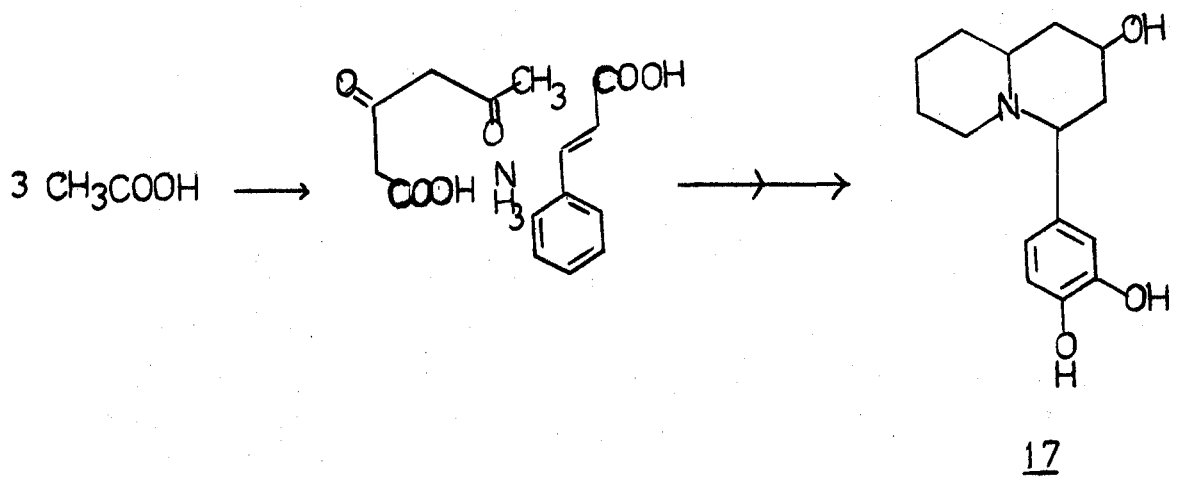
Scheme D: polyketide + C_6-C_1 + C_6-C_3

Scheme E: polyketide + C_6-C_3 + C_6-C_3



17 + 18 ⟶ 20 + 21 + 22

Fig. 6 Biogenesis of the phenylquinolizidine alkaloids of the family Lythraceae (Scheme D)



17 + 18 ⟶ 20 + 21 + 22

Fig. 7 Biogenesis of the phenylquinolizidine alkaloids of the family Lythraceae (Scheme E)

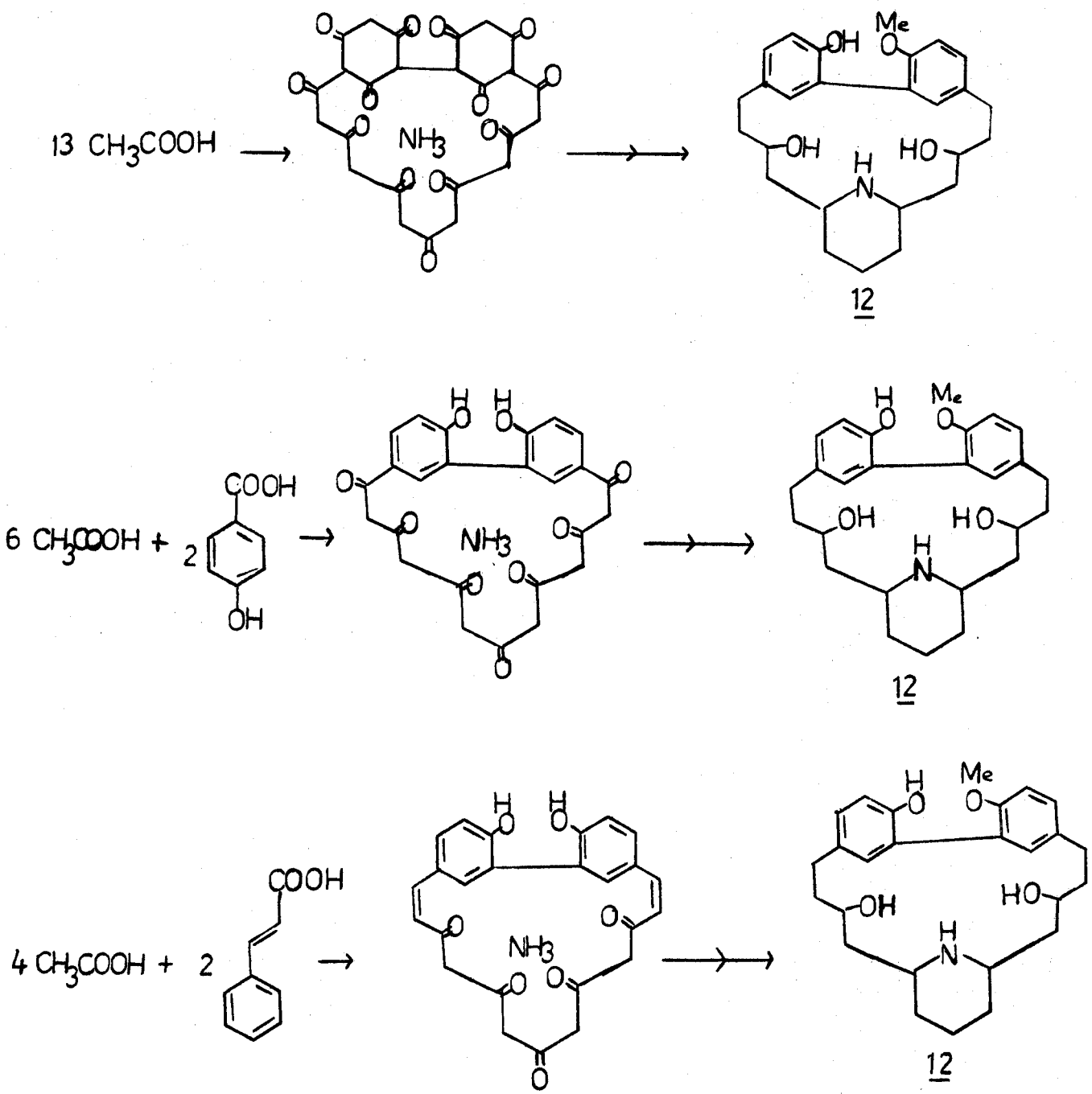


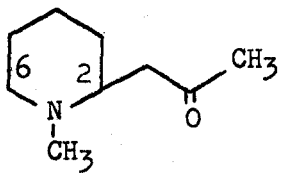
Fig. 8 Biogenesis of the alkaloids of Lythrum anceps Makina

C. Biosynthesis of the Precursors Postulated in the Biogenetic Schemes

As seen in the last section, several precursors have been postulated to be involved in Lythraceae alkaloids biosynthesis. In order to understand the detailed biosynthetic pathway of these alkaloids, it is necessary to discuss briefly the biosynthesis of these precursors.

(i) Pelletierine (14) (Scheme A)

Although the biosynthesis of pelletierine has not yet been investigated "in vivo", the biosynthesis of its analog, N-methylpelletierine (25) has been studied. Moreover, the biogenetically modelled



25

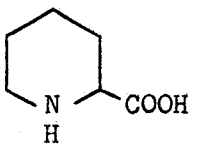
synthesis of pelletierine, following the reactions outlined in Scheme A, has been well established.[40] Pelletierine occurs naturally in Punica granatum. The piperidine nucleus of N-methylpelletierine, a base formed in P. granatum and Sedum sarmentosum, has been shown by tracer studies to be derived from lysine via a nonsymmetrical intermediate.[41,42] All activity in the radioactive N-methylpelletierine, isolated from S. sarmentosum to which 6-^{14}C-lysine had been administered, was found at carbon-6 of its piperidine nucleus.[42] The three-carbon side-chain of N-methylpelletierine has also been found to originate from two units of acetic acid via acetoacetate.[42,43]

The biosynthesis of pelletierine would probably follow the similar pathway. The only difference in their biosynthesis which might be expected

is that the conversion of lysine to pelletierine might proceed either via a nonsymmetrical or a symmetrical intermediate. In other words, when 6-^{14}C-lysine is used as a substrate, the label distribution in the isolated pelletierine from the plants can be either 100% at C-6 or 50% at each of C-6 and C-2 of the piperidine nucleus.

(ii) Pipecolic acid (23) (Scheme B)

Pipecolic acid (23), the cyclic amino acid, is a product of lysine metabolism in animals, microorganisms and higher plants. It has

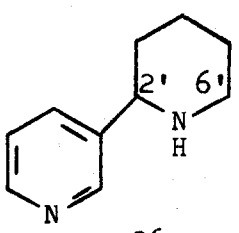


23

been shown by tracer studies[44,45] that pipecolic acid is specifically derived from lysine by way of Δ^1-piperideine-2-carboxylic acid (see Fig. 9).

(iii) Δ^1-Piperideine (24) (Schemes A and C)

Δ^1-Piperideine itself has never been demonstrated to occur naturally in plants but it has been shown to be an effective precursor of anabasine (26), **matrine and lycopodium alkaloids** (see Section III). When 6-^{14}C-Δ^1-piperideine was administered to N. glauca



26

the isolated anabasine had all its activity located at C-6' of the piperidine ring.[46] This result indicates that the double bond in Δ^1-piperideine does not isomerize to C-2 position during the biosynthesis

of anabasine. Anabasine has also been shown to be derived from lysine in a nonsymmetrical manner, since when 2-^{14}C-lysine was administered to N. glauca plants, radioactive anabasine was obtained and shown to contain C-2' of the piperidine ring as the only labelled carbon.[47]

A scheme consistent with these results which has been proposed for the biosynthesis of anabasine, pipecolic acid and N-methylpelletierine is outlined in Fig. 9.

Δ^1-Piperideine has also been shown to be incorporated into matrine and Lycopodium alkaloids. This will be discussed in Section III.

(iv) Polyketide (Schemes D and E)

Structural analogy could be misleading as in the case of piperidine alkaloid biosynthesis. Coniine (27) and pinidine (28) have been shown to be of polyketide origin even though both of them contain a piperidine nucleus. When 1-^{14}C-acetate was administered to hemlock plants, radioactive coniine was obtained. A complete and unambiguous degradation of the resulting coniine showed that the activity was equally distributed between the four starred carbon atoms in the coniine molecule.[48] Pinidine, obtained after administration of 1-^{14}C-acetate to Pinus jeffreyi plants, was systematically degraded. All the activity was located on carbons 2, 4, 6 and 9, and was equally distributed among these positions.[49] Although labelled lysine was also incorporated into pinidine, a partial degradation of the product indicated that the activity was not confined to the piperidine ring. Clearly, acetate can serve as precursors of some piperidine alkaloids. The biosynthesis of coniine (27) and pinidine (28) is shown in Fig. 10.

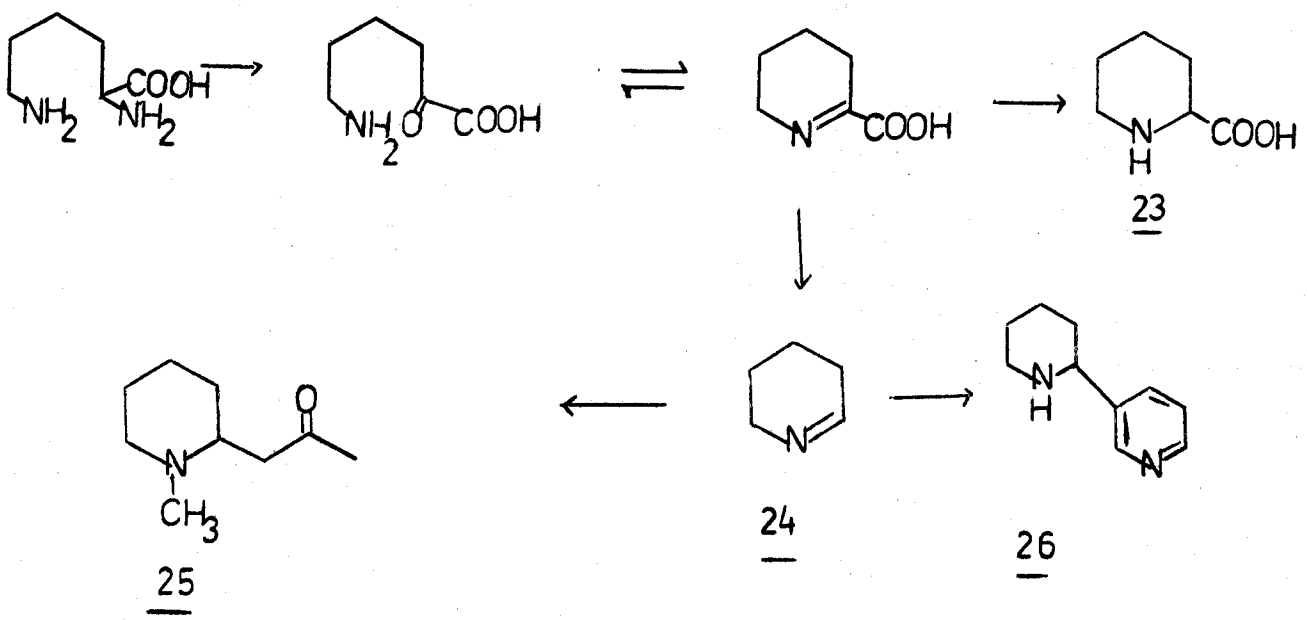


Fig. 9 Biosynthesis of some piperidine alkaloids

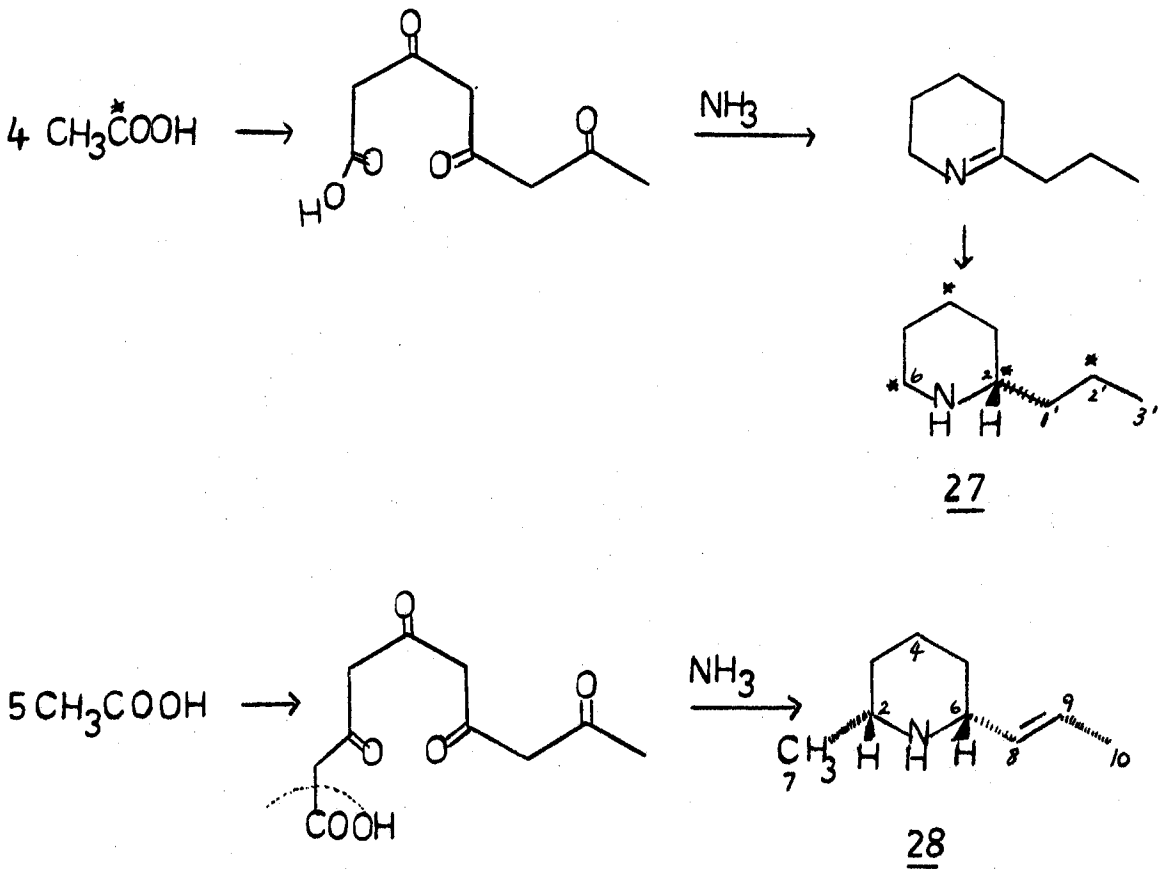


Fig. 10 Biosynthesis of coniine and pinidine

(v) C_6-C_1 and C_6-C_3 units

As shown in all the biogenetic schemes proposed for Lythraceae alkaloids, a late step is the introduction of a C_6-C_3 unit. Furthermore, a C_6-C_3 unit is suggested to be a precursor of the quinolizidine nucleus in Schemes B, C, and E, while a C_6-C_1 unit is suggested as the source of part of the quinolizidine ring system in Schemes A and D. It would be necessary then to discuss some features of the biosynthesis of the C_6-C_1 and C_6-C_3 units and their interrelationship, if any.

It is well established that phenylalanine can serve as a precursor of both C_6-C_1 and C_6-C_3 units of a large number of non-nitrogeneous aromatic compounds, widely distributed in plants, as well as many alkaloids. In plants, trans-cinnamic acid is formed irreversibly by direct deamination of phenylalanine catalyzed by phenylalanine ammonia lyase.[50] The transformation of trans-cinnamic acid into its phenolic derivatives and many C_6-C_3 compounds (phenylpropanoids) as well as into phenolic benzoic acids has been demonstrated.[51] The interrelations of acids of the cinnamic and benzoic species in higher plants is shown in Fig. 11 (cf. ref. 51). It is to be pointed out that while the conversion of cinnamic acid to its hydroxylated compounds seems to be a well established metabolic pathway, the transformation of benzoic acid to its hydroxylated derivatives does not occur in some plants. In Catalpa species, evidence seems to indicate that p-hydroxybenzoic acid is not formed by hydroxylation of benzoic acid.[52]

Some of the alkaloids which are derived from the C_6-C_1 and C_6-C_3 units will be described in the following.

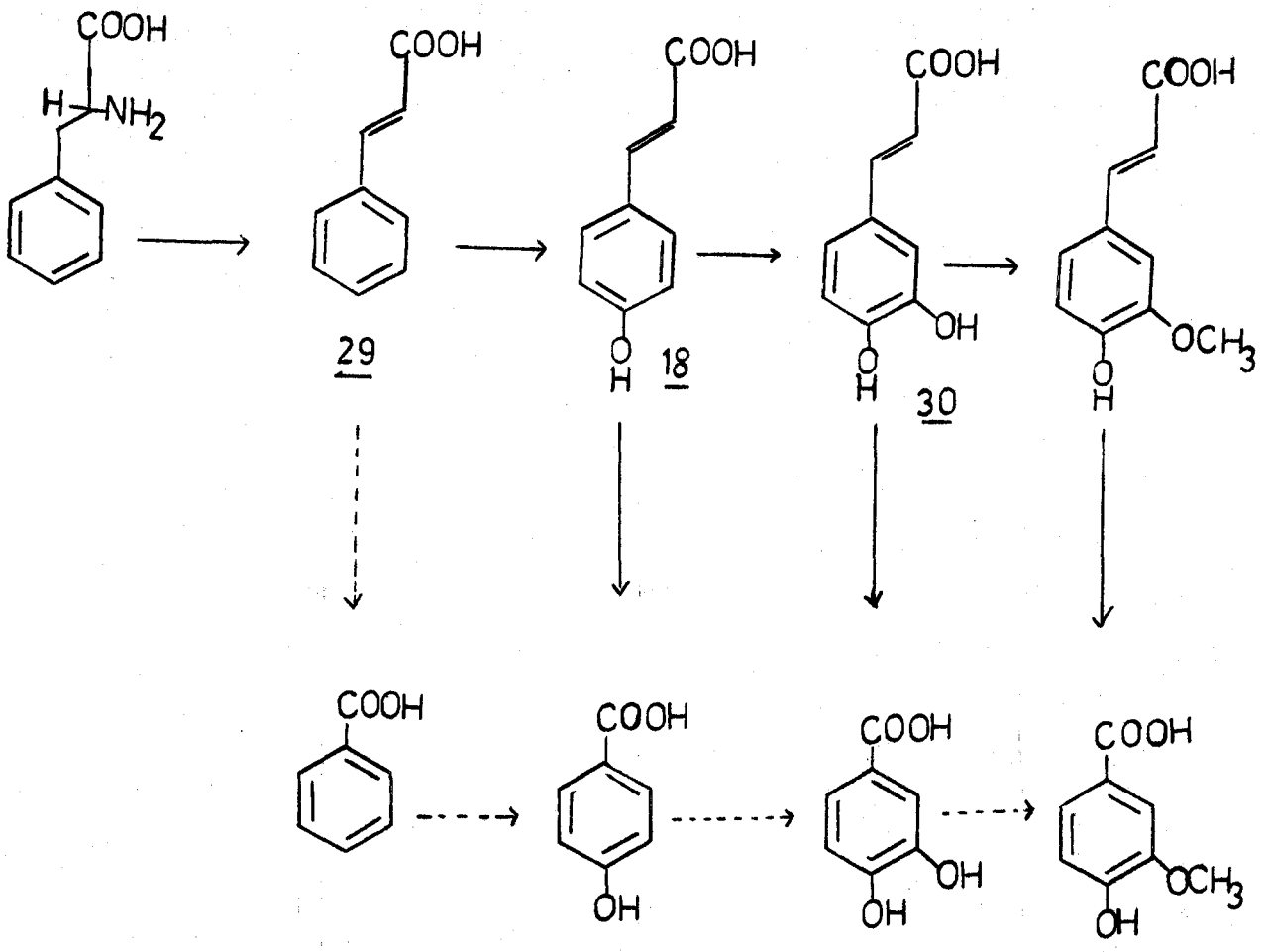


Fig. 11 Origin of aromatic acids in plants (Dotted lines signify possible routes)

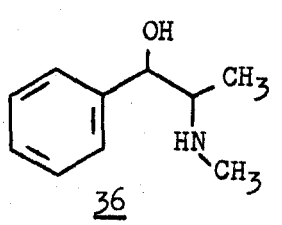
(a) C_6-C_1 unit

The most well-studied alkaloids which contain a fragment derived from a C_6-C_1 unit are those of the Amaryllidaceae. Evidence is accumulating that norbelladine (35) is the progenitor of all the structural variant of the Amaryllidaceae alkaloids. Appropriate ring closures of norbelladine by ortho- or para-coupling of phenolate radicals as proposed by Barton and Cohen[53] lead to the skeletons of all the major Amaryllidaceae alkaloids as shown in Fig. 12. Norbelladine can be dissected into a C_6-C_2-N and a C_6-C_1 unit. These have been shown to be derived from tyrosine and phenylalanine, respectively. No label from radioactive tyrosine was found in the C_6-C_1 unit of the alkaloids while the label from phenylalanine did not enter the other half of the molecule (i.e. C_6-C_2-N).

Since labelled cinnamic acid (29), p-coumaric acid (18) and caffeic acid (30) serve as specific precursors of the C_6-C_1 unit, it is evident that the required hydroxylation of the C_6-C_1 precursor takes place at the level of cinnamic acid. Labelled protocatechuic aldehyde (15), but not p-hydroxybenzaldehyde, is incorporated. Dihydroxylation of cinnamic acid thus precedes side-chain cleavage.

The biosyntheses of belladine (31),[54] lycorine (32),[54,55-57] haemanthamine (33),[55-57] and galanthamine (34)[58] deduced from the available data on the incorporation of these precursors is summarized in Fig. 12.

Phenylalanine has been shown to be a specific precursor of ephedrine (36) and pseudonorephedrine (37). β-^{14}C-Phenylalanine entered



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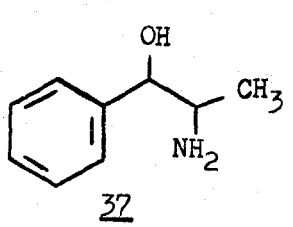

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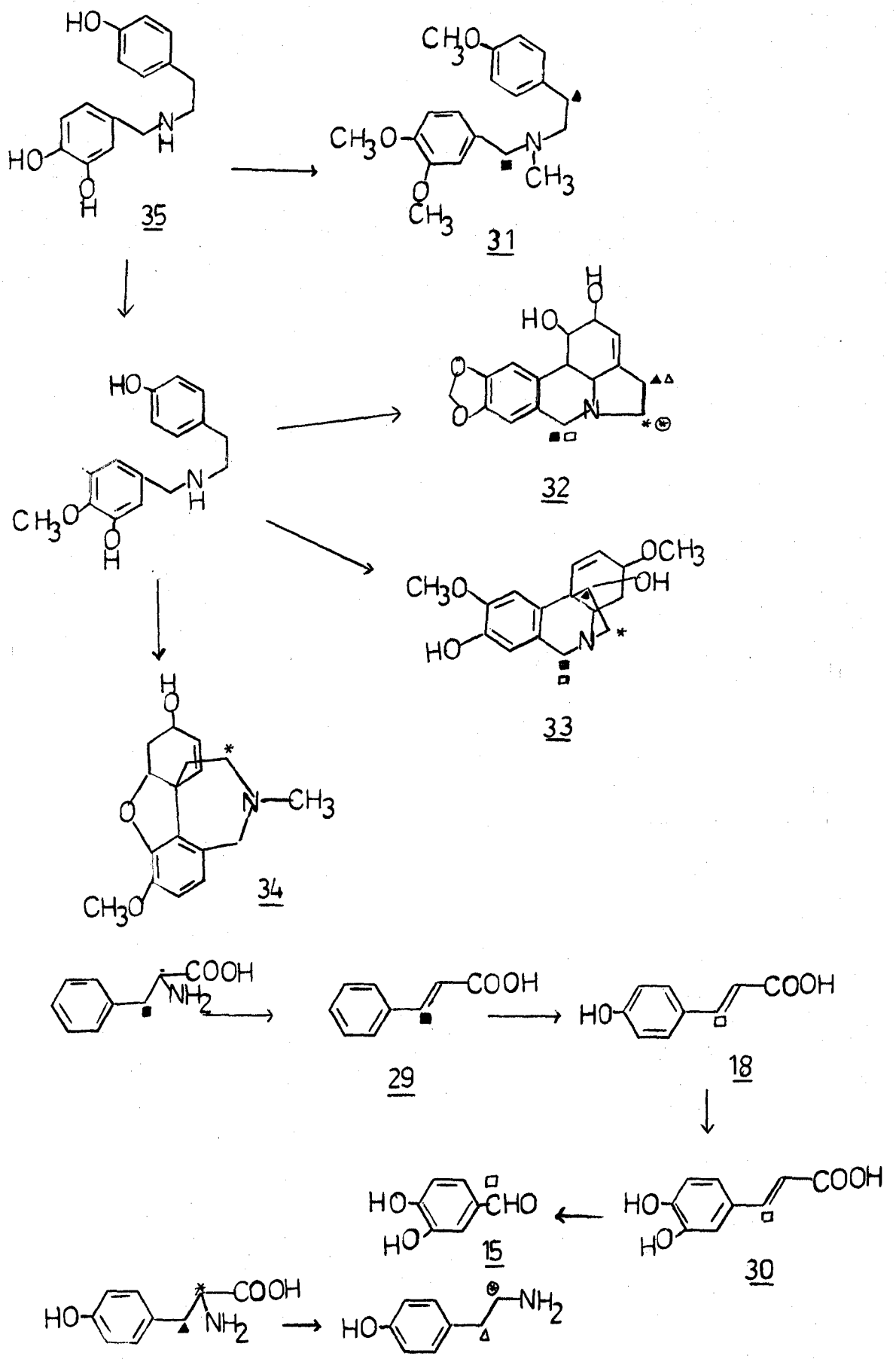
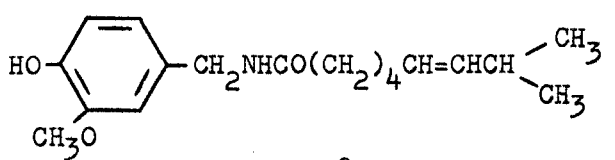


Fig. 12 Biosynthesis of Amaryllidaceae alkaloids

the benzylic carbon of pseudonorephedrine.[59] Whereas α-^{14}C-phenylalanine was not incorporated into ephedrine, label from β-^{14}C-phenylalanine entered the alkaloid.[60] β-^{14}C-Cinnamic acid, [carboxyl-^{14}C-] benzoic acid, [carbonyl-^{14}C-] benzaldehyde were also incorporated into ephedrine and the activity was shown to be localized at the benzylic carbon only.[60] Thus, it appears that ephedrine and pseudonorephedrine are derived from a C_6-C_1 unit.

Another example of alkaloid biosynthesis which involves a C_6-C_1 unit is that of capsaicin biosynthesis. Capsaicin (38), an alkaloid of Capsaicin species, was labelled solely on the methylene group of its vanillylamine residue after the administration of 3-^{14}C-phenylalanine. Labelled tyrosine afforded this alkaloid with very low activity.[61, 62] Again, it seems that phenylalanine was cleaved to the C_6-C_1 unit presumably through cinnamic acid before it was incorporated.



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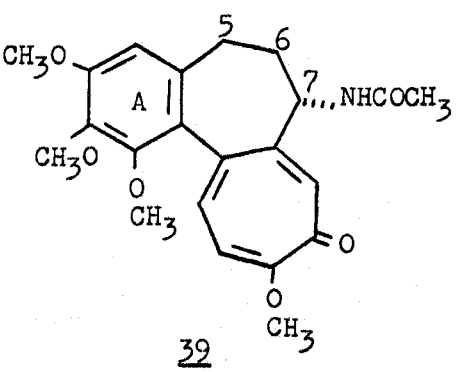

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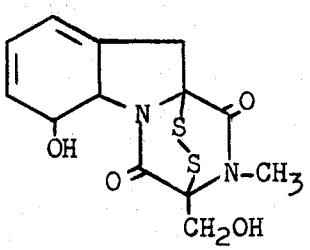
(b) C_6-C_3 unit

Colchicine (39), an alkaloid of Colchicum plants, has been shown to be originated from one unit of tyrosine and one unit of phenylalanine or cinnamic acid by tracer studies.[10] Labelled phenylalanine and cinnamic acid serve as specific precursors of the aromatic ring A and C-5, C-6, C-7 of colchicine molecule.[63-65] The correspondence of the β-, the α-

and the carboxyl - carbon of the phenylalanine side-chain to C-5, C-6 and C-7 of colchicine was demonstrated by tracer studies. Similarly, C-3 and C-2 of cinnamic acid became C-5 and C-6 of colchicine. Clearly, the C_6-C_3 unit of colchicine is derived from phenylalanine by way of cinnamic acid.

Gliotoxin (40) has been established to be derived from the intact molecule of phenylalanine.[20,66] Labelled phenylalanine, 1-^{14}C- and β-^{14}C-phenylalanine were incorporated into gliotoxin. From the result of ^{15}N-phenylalanine studies, it appears that the C_6-C_3 unit is in the form of phenylpyruvic acid or cinnamic acid.[20]

From the above discussion, it is clear that phenylalanine and tyrosine cannot be regarded as equivalent and one cannot replace the other in plant metabolism. In early biogenetic discussions, phenylalanine and tyrosine were regarded as equivalent because of the finding that in mammalian liver phenylalanine could be transformed into tyrosine, a reaction



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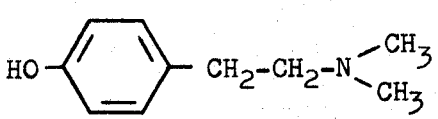

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catalyzed by phenylalanine hydroxylase. So far, only one biosynthetic example in plants is known where phenylalanine and tyrosine appear to be equivalent. Phenylalanine and tyrosine have been reported to be efficient as precursors of hordenine (41).[67] Clearly, there are differences in the pathways of metabolism of phenylalanine and tyrosine in various

organisms (cf. ref. 8, p. 291). It seems that in plant systems the phenolic C_6-C_1 and C_6-C_3 units are derived from phenylalanine while the phenolic C_6-C_2 unit originates from tyrosine.

III. BIOSYNTHESIS OF QUINOLIZIDINE AND RELATED ALKALOIDS

Since the Lythraceae alkaloids contain a quinolizidine ring system, the biosynthesis of quinolizidine and related alkaloids will be briefly reviewed.

A. Quinolizidine Alkaloids

Although there are many alkaloids whose structures contain a quinolizidine ring system, the alkaloids of lupin family are the most extensively studied biosynthetically. In the original biogenetic scheme,[4] it was postulated that lysine and cadaverine were plausible precursors for lupin alkaloids. It has now been shown by tracer studies that lysine and cadaverine are indeed the efficient precursors of lupin alkaloids. 2-^{14}C-Lysine and 1,5-^{14}C-cadaverine were administered to various plant species containing alkaloids of this group and a number of lupin alkaloids were isolated and partially degraded. Approximately one quarter of the total specific activity of lupinine (42),[68] and one sixth of that of sparteine (43),[69] lupanine (44),[70] hydroxylupanine (45),[71] and matrine (46)[73] was found to be at the starred carbon atoms (see Fig. 13), when either 2-^{14}C-lysine or 1,5-^{14}C-cadaverine had served as the substrate. Moreover, 6-^{14}C-Δ^1-piperideine was shown to be incorporated into matrine[73] and the degradation showed that the activity was located at C-2, C-10 and C-15 of the matrine molecule. These results suggested that lysine was incorporated into lupin alkaloids via cadaverine and possibly Δ^1-piperideine. A scheme consistent with these incorporation results is outlined in Fig. 13.

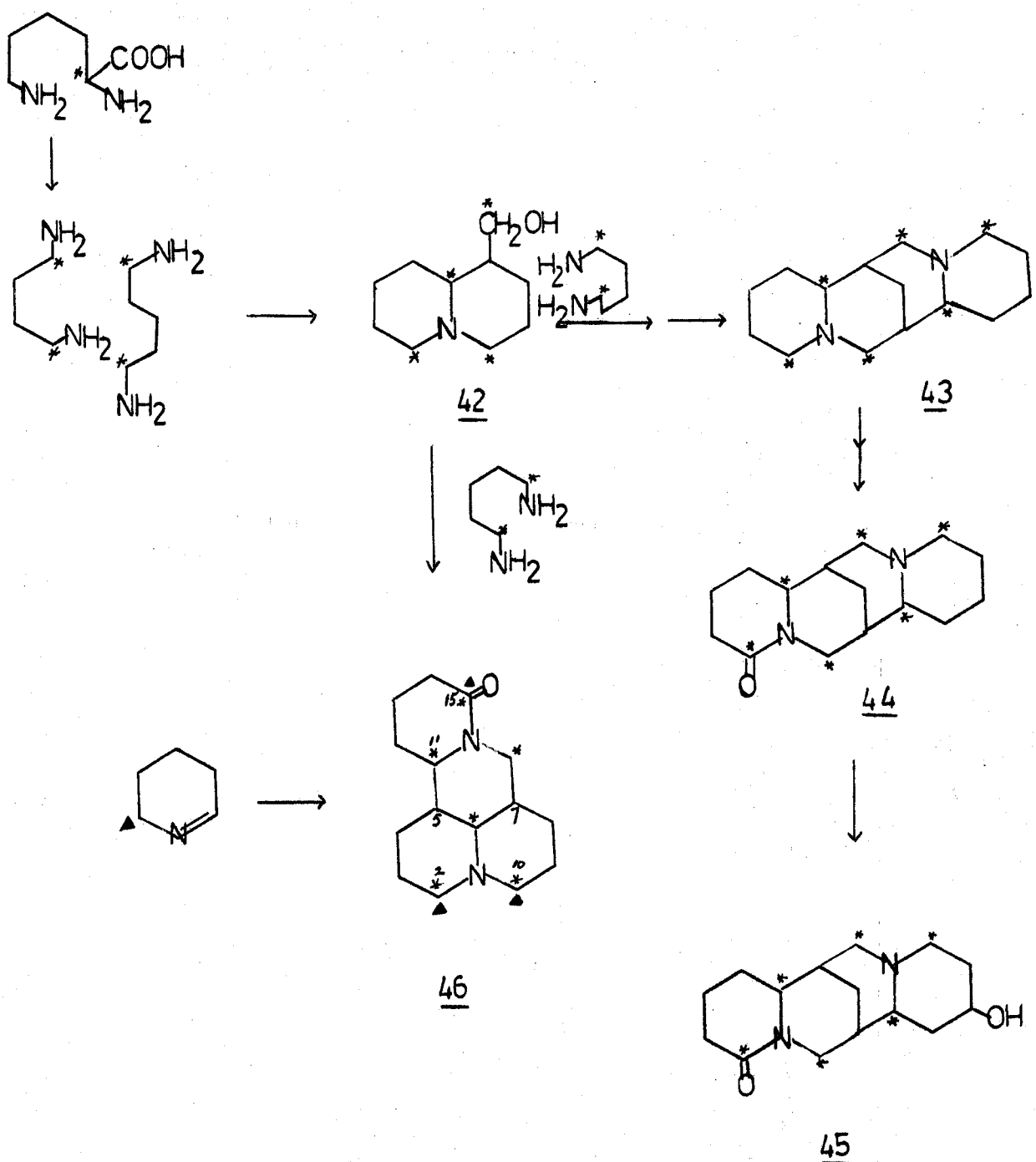
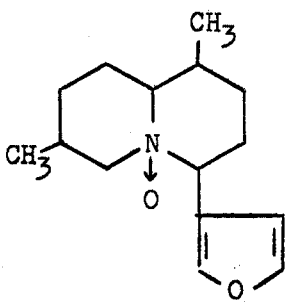


Fig. 13 Biosynthesis of lupin alkaloids

Nupharidine (47), an alkaloid from Nuphur japonicum, appears to be derived from mevalonic acid although it also contains a quinolizidine ring system. The major alkaloid of N. luteum, thiobinupharidine, is a sulfur-containing dimer of deoxynupharidine, which appears to be also of terpenoid origin.[74]



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B. Lycopodium Alkaloids

The hypothesis, that lycopodium alkaloids are modified dimers of pelletierine which in turn is derived from lysine via cadaverine, has received experimental confirmation from tracer studies.[75-78] When 2-^{14}C- and 6-^{14}C-lysine were administered to Lycopodium tristachyum and Lycopodium cernuum, radioactive lycopodine (48)[75] and cernuine (49)[76,77] respectively were isolated. Partial degradations indicated that one-fourth of the activity of lycopodine derived from 6-^{14}C-lysine as well as 2-^{14}C-lysine was localized at each of C-5 and C-9. Likewise, one fourth of the activity of cernuine derived from both radiomers of lysine was localized at C-1. Similar results were obtained when 1,5-^{14}C-cadaverine was administered to L. tristachyum and L. cernuum. However, when 6-^{14}C-Δ^1-piperideine was used as the substrate, half of the activity was localized at C-9 of labelled lycopodine and C-5 was essentially inactive. Conversely, half of the radioactivity of lycopodine, derived from 2-^{14}C-Δ^1-piperideine,

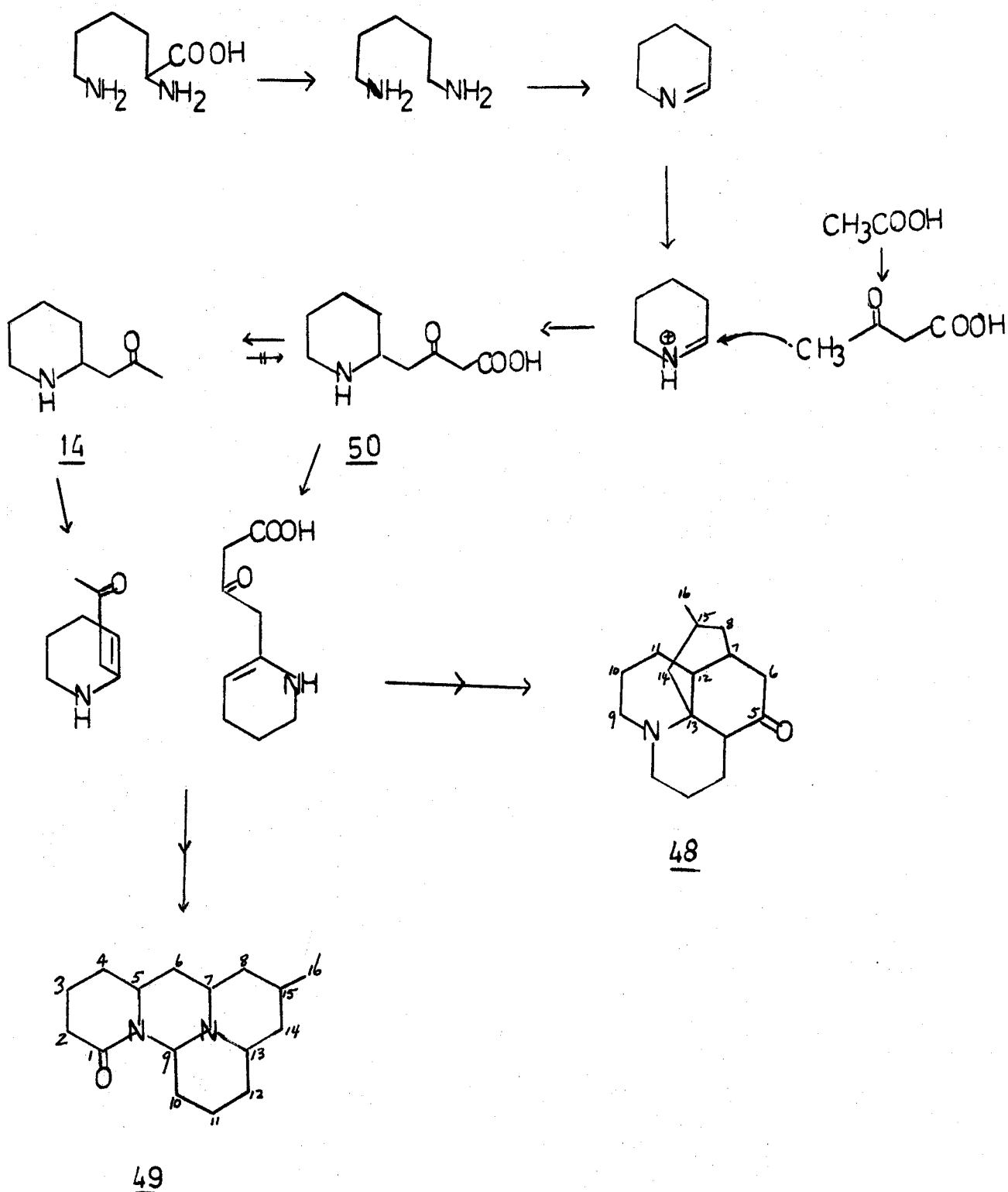


Fig. 14 Biosynthesis of lycopodium alkaloids

was recovered from C-5, whereas C-9 was devoid of activity. Similarly, when 6-^{14}C-Δ^1-piperideine was used as the substrate, half of the radioactivity of cernuine was recovered from C-1. The evidence is clearly compatible with the view that the sequence lysine ⟶ cadaverine ⟶ Δ^1-piperideine constitutes part of the biosynthetic route to lycopodium alkaloids.

Acetate has also been shown to be a precursor of lycopodine. The mode of incorporation of activity from 1-^{14}C-acetate and 2-^{14}C-acetate, supports the inference that two identical C_3 units, generated by decarboxylation of acetoacetate, supply the C_3 units C-6 to -8 and C-14 to -16 of lycopodine.

Administration of labelled pelletierine to L. tristachyum and L. cernuum afforded radioactive lycopodine and cernuine, respectively. Partial degradations showed that an intact pelletierine unit served as a specific precursor of the C_8 unit, C-9 to -16, of lycopodine and of cernuine. It is apparent then that the C_8 unit, C-1 to -8 of these alkaloids, is not derived from this precursor.

The model, shown in Fig. 14, which has been proposed can, in principle, account for equal incorporation of early precursors into the pelletierine-derived as well as the non-pelletierine-derived "half" of lycopodine and cernuine, provided the steady-state concentration of 14 is small compared to that of 50, and the conversion of 50 to 14 is irreversible.

The tracer experiments with labelled cadaverine seem to indicate that cadaverine is a specific precursor of lupin and lycopodium alkaloids. Thus, it has been assumed that cadaverine is a normal precursor of

the lupin and lycopodium alkaloids. Moreover, diamine oxidase activity, capable of converting cadaverine to 5-aminopentanal, has been demonstrated in a preparation from lupin seedlings.[79] Despite these findings, the role of cadaverine in alkaloid biosynthesis is, in general, uncertain. Decarboxylation of lysine has not yet been demonstrated nor has cadaverine been shown to be present in plants. Furthermore, the incorporation of cadaverine into anabasine in Nicotiana glauca plant has been regarded as involving an "aberrant reaction."[46] Cadaverine was also reported to have been incorporated into N-methylpelletierine in Punica granatum[80] although N-methylpelletierine in Sedum sarmentosum had been shown to be derived from lysine in a non-symmetrical fashion.[42] In order to clarify the role of cadaverine in alkaloid biosynthesis, more experiments are needed.

IV. BIOSYNTHESIS OF LYTHRACEAE ALKALOIDS

A. Introduction

The primary objective of this study of the biosynthesis of the Lythraceae alkaloids was the recognition of the origin of the phenylquinolizidine system, which occurs in most of the members of this group (cf. Section II A). When this work was begun several of the biogenetic hypotheses, outlined in Section IIB had been proposed but no experimental work bearing on the biosynthesis of the ring system had been reported.

Biogenetic schemes are useful as working hypotheses in the design of biosynthetic experiments. (That is the only use such hypotheses have. On their own, they are quite irrelevant to actual biosynthetic events). Substrates whose incorporation into the product under investigation is to be examined, can be selected in such a way that one or more biogenetic models can be critically tested.

The three substrates whose implication in the biogenesis of the Lythraceae alkaloids is predicted by several of the hypotheses reviewed in Section IIB, are lysine, acetate and phenylalanine. If incorporation of lysine could be demonstrated, Schemes D and E would be invalidated. If phenylalanine were shown to serve as a precursor, and its mode of incorporation could be determined, one or two of the remaining hypotheses might be eliminated from contention.

In an attempt to study the biosynthesis of the phenylquinolizidine type alkaloids of the family Lythraceae, incorporation of labelled samples of lysine, phenylalanine, Δ^1-piperideine and pelletierine, into decodine

and decinine, the major alkaloids of Decodon verticillatus (L.) Ell, was investigated.

B. Materials and Methods

(i) Cultivation of plants and administration of tracers

A stand of Decodon verticillatus plants was located in a swampy area of Hendrie Valley, Royal Botanical Gardens, Hamilton, Ontario. The isolation of the alkaloid fraction showed that decodine and decinine were the two major alkaloids, as reported.[26]

Preliminary tracer experiments[81] carried out in August and September, when the plants were in full bloom, showed that, whereas radioactivity from ^{14}C-lysine and ^{14}C-phenylalanine was present in the basic fraction extracted from the plants, which contained several alkaloids, the major alkaloids of D. verticillatus, decodine and decinine, were not radioactive. It was concluded that these alkaloids might be formed earlier in the season and that further experiments with D. verticillatus plants should be carried out at an early stage of development. The plants began to break in early June. New shoots had reached a height of about 12 to 18 inches by mid-June. At this stage plants were removed from the swamp and transferred to the greenhouse, where they were propagated either in water or in soil.

Two methods of feeding were used in the first experiment. One was the usual cotton wick method, while in the other, the cut end of the fresh cuttings of the green stems of the plants was immersed into the aqueous solution of tracer placed in a 800-ml beaker. The latter method was not used again, since the alkaloids isolated from the cuttings after four days did not contain radioactivity. The cotton wick method resulted

a good incorporation of the tracer into the alkaloids.

6-^{14}C-DL-Lysine and 2-^{14}C-DL-lysine were each administered to two large Decodon plants in June, 1969 using the cotton wick method. The plants were harvested after they had been kept in contact with tracer for three days.

A sample of doubly labelled phenylalanine was prepared by mixing a known activity of each of 1-^{14}C-phenylalanine and 3-^{14}C-phenylalanine. This gave a sample of 1,3-$^{14}C_2$-phenylalanine with a known specific activity at carbon-1 and carbon-3 of the phenylalanine side-chain (see Table 4). The doubly labelled sample was administered (July, 1969) to two large Decodon plants, which were then harvested and worked up as usual.

Since Decodon plants could be maintained in the greenhouse, it was felt that in order to do further tracer experiments without having to wait until the coming June, plants could be removed from the swamp in late Fall and stored in a cold room for some time before propagating them in the greenhouse. Plants were treated in this manner and Experiments 4-6 (see Table 1) were carried out with three batches of plants which were removed from cold storage in January and February and allowed to break in the greenhouse.

6-^{14}C-Δ^1-Piperideine prepared from 6-^{14}C-lysine (see Experimental) and containing approximately 10% of unchanged starting material, was administered to three Decodon plants when the plants were at an early stage of growth and shoots had grown to a height of 12-18 inches. Another experiment was carried out with similar plants, using a mixture of 6-^{14}C-Δ^1-piperideine (55%) and 6-^{14}C-lysine (45%). Finally, doubly labelled 6,2'-$^{14}C_2$-pelletierine was administered to another batch of Decodon plants.

The harvesting process and work-up of the plant material was done in the usual manner. The details of all the experiments which were carried out are summarized in Table 1.

(ii) Isolation and purification of decodine and decinine

The green stems, shoots and leaves of the plants to which tracers had been administered were detached from the stocks, cut and dried. Alkaloids were extracted from the dried plant material by the procedure described by Ferris.[26] The method of separation of the basic fraction into individual components and the final purification procedure was adapted to work on a small scale.

Extraction with chloroform gave a crude alkaloid mixture. Methanol had been found to give a slightly better yield of crude alkaloids in a pilot extraction, but the basic fraction was always accompanied by a large quantity of non-basic substances. Chloroform was thus chosen for extracting the radioactive plant materials. A preliminary investigation for alkaloidal content of the roots showed that they did not contain any appreciable amount of the alkaloids. The roots were thus discarded in all the tracer experiments.

The alkaloid mixture was analyzed by thin layer chromatography. The solvent system, chloroform/methanol/diethylamine (10:1:1) proved useful in achieving separation of the major components. When the crude alkaloid extracts were chromatographed on silica gel plates in this solvent system, decodine and decinine were clearly separated. At least six other minor alkaloids were detected on t.l.c. using this solvent system. A t.l.c. system with good resolving power is very helpful in tracer experiments, since the thin layer chromatogram can be checked for distribution of activity by using a radioscanner, and this gives

a preliminary indication of the relative amount of activity in individual components of a mixture.

The crude extract containing radioactive alkaloids was further fractionated on a column of neutral alumina (activity IV). Each fraction which was collected, was concentrated to dryness and chromatographed on t.l.c. The fractions containing decodine or decinine were combined. Inactive carrier was added to each of the combined solutions and the product was isolated and crystallized to constant radioactivity. The radioactive samples of decodine and decinine were rigorously purified by recrystallization and sublimation.

The purified samples of decodine and decinine, obtained from individual feeding experiments, were partially degraded to determine the distribution of radioactivity. The reactions which were employed for this purpose are discussed in Section IV.

C. Results

When the crude alkaloid mixture, isolated from the experiments with two radiomers of lysine, was chromatographed on thin layer plates and the t.l.c. was scanned in a radioscanner, two distinctive major radioactive peaks were detected which corresponded in R_f value to decinine and decodine, respectively. However, the radioscan of t.l.c. of the crude alkaloid mixture isolated from the doubly labelled phenylalanine experiment showed, in addition to the two peaks corresponding to decinine and decodine, a third major peak. This "unknown" radioactive compound was not further investigated at this time. It is apparent that the biosynthesis of decinine and decodine in Decodon plants is seasonal. The experiments

carried out in June resulted in good incorporation of tracers into the two major alkaloids, while in July a third alkaloid became labelled. Labelled precursors were not incorporated into decinine and decodine when feeding was carried out in August and September.

When 2-^{14}C-lysine, 6-^{14}C-lysine, 1,3-$^{14}C_2$-phenylalanine, 6-^{14}C-Δ^1-piperideine and 6-^{14}C-Δ^1-piperideine/6-^{14}C-lysine (55:45) (Experiments 1 - 5) were used as substrates, the rigorously purified samples of decodine and decinine which were isolated in each case, were radioactive. In Expt. 4, incorporation of radioactivity from Δ^1-piperideine was less efficient than in the other experiments, and only radioactive decodine was isolated. The samples of decodine and decinine, obtained from the experiment with doubly labelled 6,2'-$^{14}C_2$-pelletierine contained, after rigorous purification, only a little radioactivity, amounting to fewer than five counts/min/mg above background. The results of the incorporation of these labelled compounds are shown in Table 1.

Degradation of radioactive decodine, obtained from the experiments with 2-^{14}C- and with 6-^{14}C-lysine showed that the degradation fragments, β-alanine and γ-aminobutyric acid contained one half of the activity of the intact alkaloid, whereas the fragment 2-piperidineacetic acid contained all the activity (Table 2).

The radioactive samples of decinine, obtained from the two lysine experiments, were degraded as in the case of decodine to afford β-alanine, γ-aminobutyric acid and 2-piperidineacetic acid. The results were analogous to those obtained in the case of decodine and are shown in Table 3.

The samples of radioactive decodine obtained from 6-^{14}C-Δ^1-piperideine (Expt. 4) and from the mixture of 6-^{14}C-Δ^1-piperideine and

TABLE 1

Incorporation of Precursors

Expt. No.	Precursors	Nominal Total Activity mCi	Nominal Specific Activity mCi/mmole	Weight of dry plant material (g)	Products: Decodine[d] Specific Activity (counts $min^{-1} mmole^{-1}$) $\times 10^{-4}$ after dilution with inactive carrier (mg)	Products: Decinine Specific Activity (counts $min^{-1} mmole^{-1}$) $\times 10^{-4}$ after dilution with inactive carrier (mg)
1	6-^{14}C-DL-Lysine[a]	0.1	48	22	2.51 ± 0.04 (600)	2.51 ± 0.03 (600)
2	2-^{14}C-DL-Lysine[b]	0.1	1.6	32	5.65 ± 0.04 (650)	9.60 ± 0.14 (400)
3	1,3-$^{14}C_2$-DL-Phenylalanine	0.2	4.0	22	2.69 ± 0.03 (600)	3.26 ± 0.05 (400)
	from 1-^{14}C-DL-Phenylalanine[b]	0.1	2.1			
	3-^{14}C-DL-Phenylalanine[a]	0.1	48			
4	6-^{14}C-Δ^1-Piperideine[c] (containing 10% 6-^{14}C-DL-lysine)			17	0.88 ± 0.01 (350)	not isolated
	from 6-^{14}C-DL-Lysine[a]	0.1	48			
5	6-^{14}C-Δ^1-Piperideine[c]			18	4.10 ± 0.04 (400)	3.80 ± 0.03 (400)
	from 6-^{14}C-DL-Lysine[a]	0.05	48			
	plus 6-^{14}C-DL-Lysine[a]	0.05	48			
6	6,2'-$^{14}C_2$-Pelletierine			26	residual activity (65)	residual activity (50)
	from 6-^{14}C-Pelletierine[c]	0.03	1			
	2'-^{14}C-Pelletierine[c]	0.02	0.25			

[a] Commissariat a l'Energie Atomique, France

[b] New England Nuclear

[c] See Experimental

[d] Assayed as dimethyldecodine

Table 2

Incorporation of lysine and Δ^1-piperideine into decodine

Precursor	6-^{14}C-lysine		2-^{14}C-lysine		6-^{14}C-Δ^1-piperideine		6-^{14}C-Δ^1-piperideine 6-^{14}C-DL-Lysine	
Expt. No.	1		2		4		5	
Product	SA[a]	RSA[b]	SA	RSA	SA	RSA	SA	RSA
Dimethyldecodine	2.51 ± 0.04	100 ± 1	5.65 ± 0.04	100 ± 1	0.88 ± 0.01	100 ± 1	4.10 ± 0.04	100 ± 1
DNP-β-alanine	1.28 ± 0.02	51 ± 1	[c]	49 ± 1	0.66 ± 0.01	75 ± 1	2.32 ± 0.03	57 ± 1
DNP-Y-aminobutyric acid	1.28 ± 0.02	51 ± 1	2.80 ± 0.6	50 ± 1	—		—	
DNP-2-piperidine-acetic acid	2.48 ± 0.05	99 ± 3	5.68 ± 0.06	100 ± 1	—		—	

[a] Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}

[b] Relative specific activity percent (dimethyldecodine = 100)

[c] The DNP-β-alanine (specific activity (1.46 ± 0.02) x 10^4 counts min^{-1} $mmole^{-1}$ was obtained by oxidation of a sample of dimethyldecodine, (specific activity (2.98 ± 0.05) x 10^4 counts min^{-1} $mmole^{-1}$) which was prepared by carrier dilution of the sample, specific activity (5.65 ± 0.04) x 10^4 counts min^{-1} $mmole^{-1}$.

Table 3

Incorporation of lysine into decinine

	6-^{14}C-lysine		2-^{14}C-lysine	
Expt. No.	1		2	
Product	SA[a]	RSA[b]	SA	RSA
Decinine	2.51 ± 0.03	100 ± 1	9.60 ± 0.14	100 ± 2
DNP-β-alanine	1.38 ± 0.03	55 ± 1	5.13 ± 0.11	53 ± 1
DNP-γ-aminobutyric acid	1.31 ± 0.02	52 ± 1	5.51 ± 0.06	57 ± 1
DNP-2-piperidine-acetic acid	2.66 ± 0.04	106 ± 2	9.70 ± 0.13	101 ± 2

[a]Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}

[b]Relative specific activity percent (decinine = 100)

Table 4

Incorporation of 1,3-$^{14}C_2$-DL-phenylalanine into decodine and decinine (Expt. 3)

PRECURSOR	SA[a]	RSA[b]
1,3-$^{14}C_2$-DL-phenylalanine	4.68 ± 0.07	100 ± 2
β-^{14}C	2.56 ± 0.05	54.6 ± 1.3
carboxyl-^{14}C	2.13 ± 0.06	45.4 ± 1.4

$$\frac{^{14}C \text{ at } \beta\text{-carbon}}{^{14}C \text{ at carboxyl carbon}} = \frac{54.6 \pm 1.3}{45.4 \pm 1.4} = 1.20 \pm 0.05$$

PRODUCTS	SA	RSA
Dimethyldecodine	2.69 ± 0.03	100.0 ± 1.3
Hemipinic Anhydride	1.25 ± 0.03	46.4 ± 1.2
4-Methoxyisophthalic Acid	0.71 ± 0.01	26.6 ± 0.6
Kuhn-Roth Acetate[c]	0.59 ± 0.01	21.8 ± 0.6

$$\frac{^{14}C \text{ in 4-Methoxyisophthalic acid (C-1'')}}{^{14}C \text{ in Kuhn-Roth acetate (C-3'')}} = \frac{26.6 \pm 0.6}{21.8 \pm 0.6} = 1.22 \pm 0.04$$

	SA	SA	RSA
Methyldecinine	3.26 ± 0.05	2.71 ± 0.03	100 ± 1
4-Methoxyisophthalic Acid	0.71 ± 0.02		21.9 ± 0.6
Kuhn-Roth Acetate[c]		0.46 ± 0.01	17.0 ± 0.4

$$\frac{^{14}C \text{ in 4-Methoxyisophthalic acid (C-1'')}}{^{14}C \text{ in Kuhn-Roth acetate (C-3'')}} = \frac{21.9 \pm 0.6}{17.0 \pm 0.4} = 1.29 \pm 0.05$$

[a]Specific activity (counts min^{-1} $mmole^{-1}$) x 10^{-4}

[b]Relative specific activity (percent) (starting material = 100)

[c]Assayed as acetyl-α-naphthylamide

6-^{14}C-lysine (Expt. 5) were also degraded. The results (Table 2) show that 75% activity of the intact alkaloid was recovered as β-alanine in Expt. 4 while β-alanine isolated from the alkaloid obtained in Expt. 5 contained only 57% of the label activity.

Degradation of radioactive decodine obtained from the experiment with 1,3-$^{14}C_2$-phenylalanine showed that hemipinic anhydride (46%), 4-methoxyisophthalic acid (27%) and acetic acid (22%) (Table 4) account for almost all the activity of the intact alkaloid.

Similarly, 4-methoxyisophthalic acid (22%) and acetic acid (17%), obtained by degradation of the radioactive decinine, derived from 1,3-$^{14}C_2$-phenylalanine account for 39% of the total activity (Table 4). The remaining activity was not accounted for, since the yield of dimethoxyphthalic acid formed in the degradation was too low to permit isolation and radioassay.

Finally, the basic fraction isolated from the plants to which 6,2'-$^{14}C_2$-pelletierine had been administered was highly radioactive. But when decodine and decinine were separated from the crude alkaloid extract, and rigorously purified, their activities were less than five counts/min/mg above background. The alkaloids were not degraded.

D. Discussion

(i) Lysine as a precursor

The results of the partial degradation of the samples of decodine and decinine obtained from the feeding experiments with 2-^{14}C- and with 6-^{14}C-lysine show that lysine serves as a specific precursor of ring A of the alkaloids, and that incorporation takes place via a symmetrical intermediate.

Since 2-piperidineacetic acid (53) contains all the activity of decodine and decinine derived from either 2-^{14}C- or 6-^{14}C-lysine, incorporation of lysine into the alkaloids is non-random and thus lysine serves as a specific precursor. Moreover, when 1-^{14}C-lysine was administered to D. verticillatus, the alkaloid fraction obtained was totally inactive.[81] This means that the carboxyl group of lysine does not enter the alkaloids. It is likely that an intact C_5N unit, derived from lysine, is incorporated and that lysine was not degraded to smaller fragments which then entered the alkaloid. If this had happened the specific activity of 2-piperidineacetic acid would not have corresponded to that of the intact alkaloid because other carbon atoms would presumably have contained some of the activity (cf. ref. 8).

One of the remaining problems is the mode of incorporation of lysine into the alkaloids. It is well established that lysine can be incorporated into alkaloids by way of either a symmetrical or a non-symmetrical intermediate (see Sections IIC and III). Since β-alanine (51) and γ-aminobutyric acid (52) obtained by the degradation of either decodine and of decinine contained one half of the activity of the intact alkaloids, regardless of whether 2-^{14}C- or 6-^{14}C-lysine had been the precursor, the lysine-derived C_5N unit enters the alkaloids by way of a symmetrical intermediate. If lysine had entered the alkaloids by way of a non-symmetrical intermediate, the β-alanine and γ-aminobutyric acid isolated would have contained no activity when 2-^{14}C-lysine had been used as a substrate while they would have contained all the activity in the case of the 6-^{14}C-lysine experiments. It is to be noted also that both the isolated β-alanine and γ-aminobutyric acid contain practically

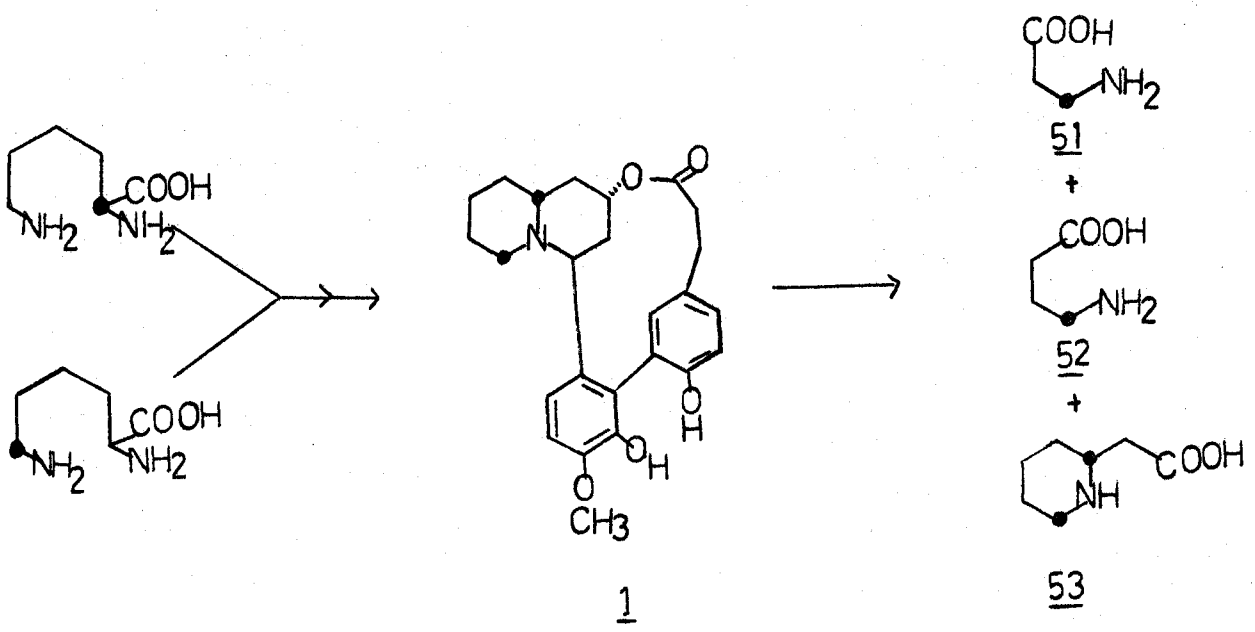


Fig. 15 Incorporation of 2-^{14}C-lysine and 6-^{14}C-lysine into decodine

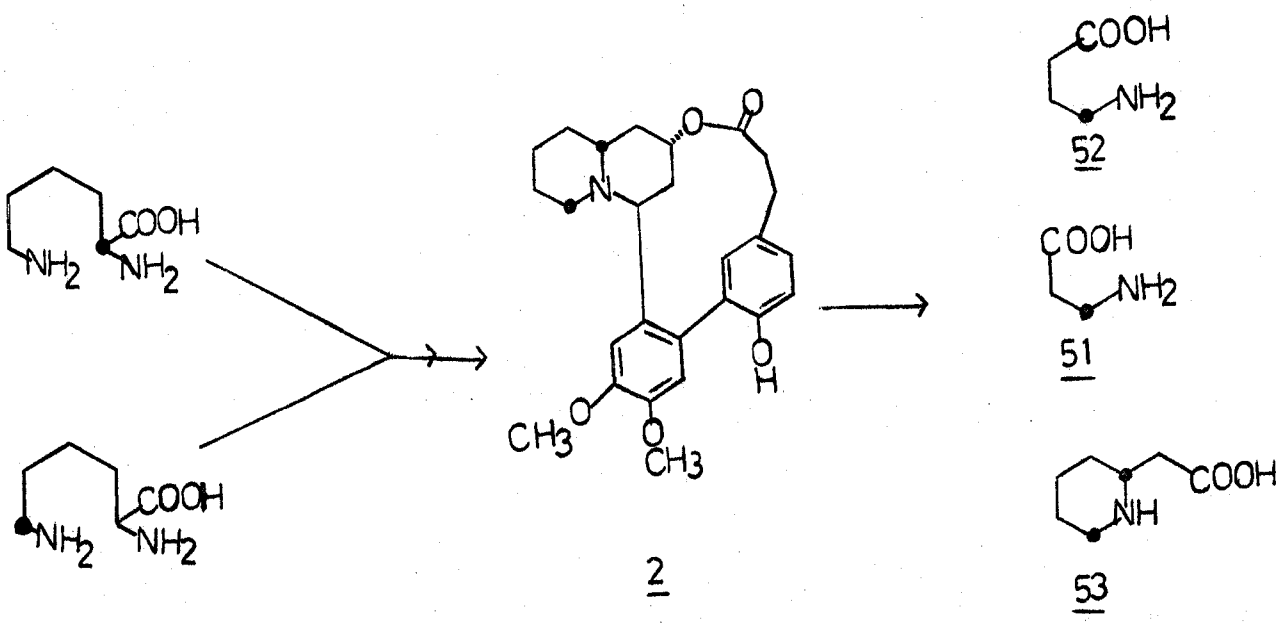


Fig. 16 Incorporation of 2-^{14}C-lysine and 6-^{14}C-lysine into decinine

the same amount of activity. This further strengthens the inference that the activity distribution in the radioactive samples of decodine and decinine is non-random and the label in the alkaloids is localized at C-6 and C-10 of the quinolizidine ring of the alkaloids.

It can be concluded on the basis of the present results that ring A of the two major Lythraceae alkaloids, decodine and decinine, is derived specifically from lysine via a symmetrical intermediate. In this respect, the biosynthesis of decodine and decinine parallels that of the lupin and the lycopodium alkaloids, and is different from that of anabasine and N-methylpelletierine. But, whereas two molecules of lysine are incorporated into the quinolizidine system of the lupin alkaloids and of lycopodine and cernuine, only one C_5N unit of lysine enters the quinolizidine ring of decodine and decinine.

On the basis of the results of the studies of the incorporation of the radiomers of lysine into decodine and decinine, (Fig. 15, 16) Schemes B, D and E can be eliminated. Only two hypotheses, as shown in Schemes A and C, remain to be tested further.

(ii) Δ^1-Piperideine as a precursor

Δ^1-Piperideine has been shown to be incorporated efficiently and specifically into anabasine,[46] into two lycopodium alkaloids[77] and into matrine.[73] The derivation of all of these bases from lysine has been demonstrated. When the incorporation of lysine into decodine and decinine had been demonstrated, the intermediacy of Δ^1-piperideine was tested.

Incorporation of Δ^1-piperideine into anabasine, as well as into lycopodine and cernuine takes place without isomerization of the two carbon atoms adjacent to nitrogen, even though lysine is incorporated

symmetrically into the lycopodium alkaloids but non-symmetrically into anabasine. If Δ^1-piperideine served as a precursor of the quinolizidine system, non-symmetrical incorporation was to be expected.

It follows that all activity from 6-^{14}C-Δ^1-piperideine should be contained in β-alanine, obtained on degradation. However, the β-alanine (51) isolated by degradation of the decodine obtained in Expt. 4, contained only about 75% of the total activity (Fig. 17).

Two possibilities arise from this result: (i) either two different modes of entry of Δ^1-piperideine, one symmetrical and the other non-symmetrical, are operative simultaneously, or (ii) the 6-^{14}C-Δ^1-piperideine used as precursor was contaminated with 6-^{14}C-lysine. It was found, on radioscanning, that the sample of precursor did, indeed, contain 10% of its radioactivity in lysine.

To test these alternative possibilities further, an experiment (Expt. 5) with a mixture of 6-^{14}C-Δ^1-piperideine and 6-^{14}C-lysine was carried out. The activity recovered in the β-alanine obtained by degradation of the decodine, obtained from this experiment corresponded to 57% of the activity of the intact alkaloid. The results obtained in Experiments 4 and 5 can be rationalized as follows:

Suppose that the site of biosynthesis of decodine is highly localized. Suppose further that the entry of lysine and Δ^1-piperideine into the cell compartment, where biosynthesis takes place, is the rate limiting step of the biosynthetic process, and that this transport follows simple first order kinetics.

Then, the rates of transport of labelled lysine and Δ^1-piperideine into the site of biosynthesis will be given by

$$R_L = K_L[L] \quad \text{and} \qquad (1)$$

$$R_P = K_P[P] \qquad (2)$$

where R_L and R_P are the rates of transport of lysine and Δ^1-piperideine, respectively.

K_L and K_P are the rate constants of the respective processes and [L] and [P] are the initial concentrations of labelled lysine and Δ^1-piperideine, respectively.

Since it is assumed for purposes of this discussion that transport of substrate into the site of synthesis is the rate limiting step, it follows that the rates of entry of label into the product must be proportional to the rates of entry of labelled substrates into the site of biosynthesis, and that the distribution of label within the final product is a function of these rates.

It is proposed to evaluate the ratio of rate constants K_L/K_P from the experimental data of Expt. 5, and to use the value of the ratio so obtained to predict the incorporation pattern of Expt. 4, and to compare this prediction with the pattern which was actually observed.

In Expt. 5:

$$\text{Given:} \quad \frac{[L]}{[P]} = \frac{45}{55} \qquad (3)$$

Found: β-alanine contains 56.5% of the activity of the intact decodine.

Since activity from 6-^{14}C-lysine enters decodine symmetrically (Expt. 1), the observed distribution of activity may be interpreted as indicating that 43.5% of the total activity of the alkaloid is located at C-10 and is derived from lysine, and that of the activity at C-6, 43.5% is derived from lysine, and 13% from 6-^{14}C-Δ^1-piperideine.

Since it is assumed that this distribution pattern reflects the rates of entry of substrates into the site of synthesis, it follows that

$$\frac{R_L}{R_P} = \frac{2 \times 43.5}{13} \qquad (4)$$

Then, from eq. (1) and (2)

$$\frac{K_L}{K_P} = \frac{R_L}{R_P}\frac{[P]}{[L]} \qquad (5)$$

and substituting the values obtained in (3) and (4)

$$\frac{K_L}{K_P} = \frac{2 \times 43.5}{13} \times \frac{55}{45} = 8.18 \qquad (6)$$

In Expt. 4,

$$\text{Given: } \frac{[L]}{[P]} = \frac{10}{90} \qquad (7)$$

Also, from (1) and (2)

$$\frac{R_L}{R_P} = \frac{K_L}{K_P}\frac{[L]}{[P]} \qquad (8)$$

Then, substituting the values from (6) and (7) into (8), it is predicted that

$$\frac{R_L}{R_P} = 8.18 \times \frac{10}{90} = 0.91 \qquad (9)$$

From this value it can be calculated (as shown below) that the predicted distribution of activity in the decodine from Expt. 4 should be such that 76.2% of the total activity should be located at C-6.

$$\frac{2 \times \text{Lys-derived}}{\text{Pip-derived}} = 0.91$$

$$2 \times \text{Lys-derived} + \text{Pip-derived} = 100$$

$$\therefore 0.91\ \text{Pip-derived} = 100 - \text{Pip-derived}$$

$$\text{Pip-derived} = \frac{100}{1.91} = 52.35$$

$$\therefore \text{Lys-derived} = \frac{100 - 52.35}{2} = 23.85$$

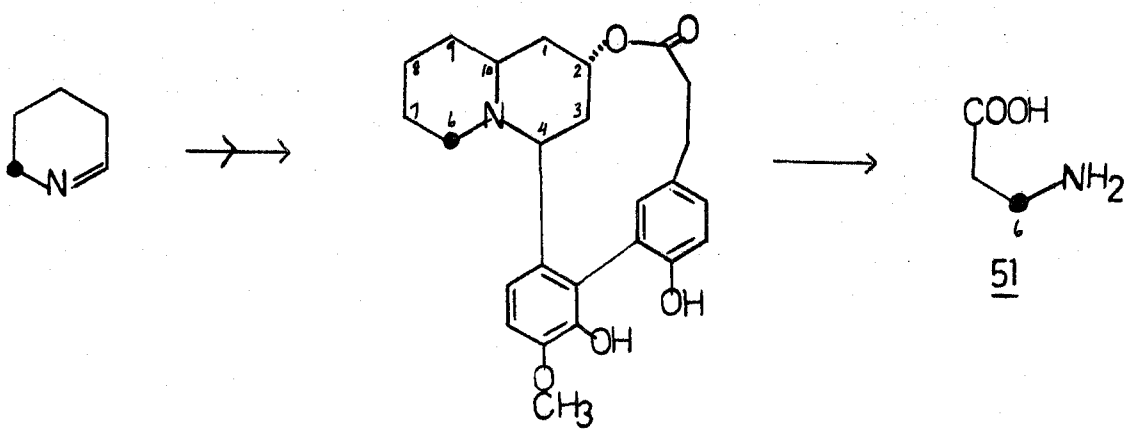
$$\therefore \text{At C-6, the activity} = 52.35 + 23.85 = 76.2$$

$$\text{At C-10, the activity} = 23.85$$

The observed value, 75.3% recovered in β-alanine (i.e. C-6), is in remarkably good agreement with this prediction.

If the assumptions made in this simple treatment are valid, the argument supports the view that Δ^1-piperideine serves as a specific precursor of decodine and that in the course of its incorporation, the identity of the two carbon atoms adjacent to nitrogen is maintained (Fig. 17).

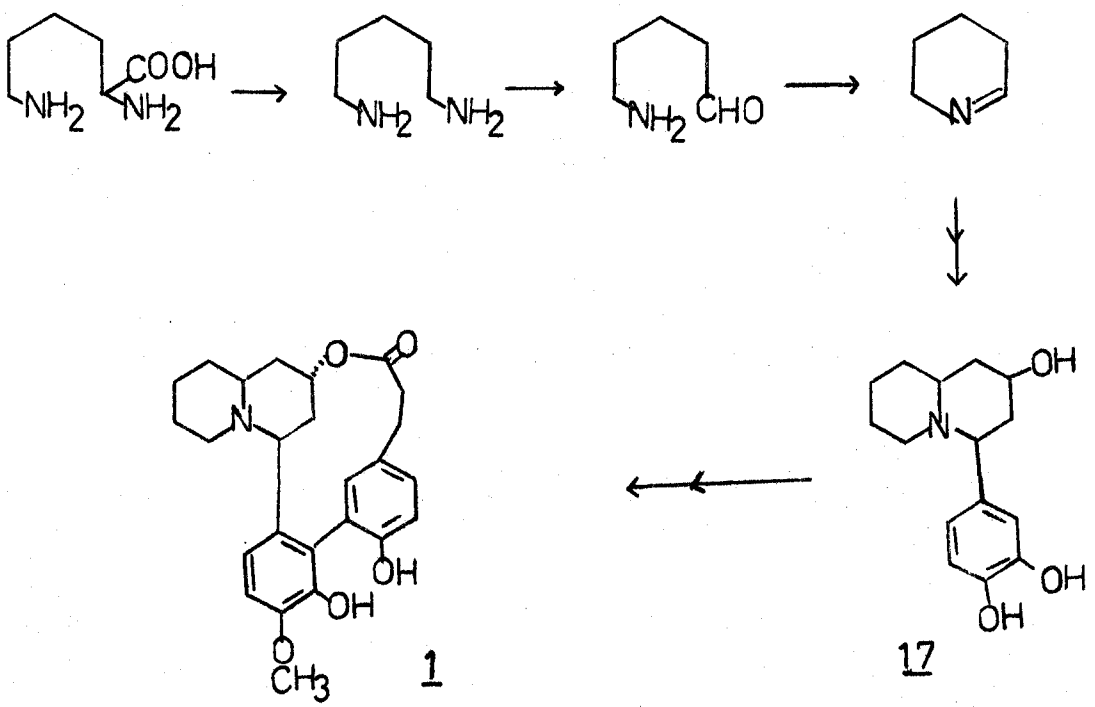
One further question arises. Since in the proposed biosynthetic pathway (Fig. 18) Δ^1-piperideine is regarded as an intermediate between lysine and the alkaloid, the presence of an increased concentration of Δ^1-piperideine should suppress the incorporation of lysine into the alkaloid. The prediction is not borne out by the experimental results.



51

1

Fig. 17 Incorporation of 6-^{14}C-Δ^1-piperideine into decodine



1 17

Fig. 18 Biosynthesis of the quinolizidine ring system of decodine (cf. Scheme A)

This anomaly can be rationalized on the basis of the assumption that the rate of the actual biosynthetic processes is fast relative to the rate of transport of substrates into the biosynthetic site.

The argument based on the distribution of activity observed in Experiments 4 and 5 leads to the inference, that, had the 6-^{14}C-Δ^1-piperideine been free from lysine, all the activity from the intact alkaloid would have been recovered as β-alanine. It follows that Δ^1-piperideine is incorporated non-symmetrically, and that it serves as a specific precursor of decodine. It is a likely intermediate on the biosynthetic pathway. These conclusions are consistent with earlier findings, that the double bond of Δ^1-piperideine does not migrate to the alternative C-N position, in analogy to the observed behaviour during the biosynthesis of other alkaloids, viz. anabasine, lycopodine, cernuine and matrine.

Several reasons can be advanced to account for the inefficient incorporation of Δ^1-piperideine into decodine. Firstly, Δ^1-piperideine might penetrate cell membranes less readily than lysine (as discussed above). Secondly, due to its unstable nature, part of the Δ^1-piperideine might be destroyed. It has been shown that Δ^1-piperideine can be converted chemically to tetrahydroanabasine.[82] Incubation of cadaverine with a diamine oxidase preparation from pea seedlings yielded tetrahydroanabasine, by spontaneous dimerization of the primary product of the reaction, Δ^1-piperideine.[83,84] Δ^1-Piperideine has also been reported to be a less efficient precursor than lysine or cadaverine in matrine biosynthesis,[73] whereas anabasine[46] and lycopodine[77] incorporate Δ^1-piperideine much more efficiently than lysine.

It is clear that when Δ^1-piperideine is being tested as a possible intermediate in alkaloid biosynthesis, it should be free from labelled lysine, unless the incorporation of Δ^1-piperideine into the alkaloid is very much faster than that of lysine as in the case of lycopodine biosynthesis. Only then can reasonable and clear-cut results be expected. Unfortunately we were unable to achieve complete purification of the samples of labelled Δ^1-piperideine. The labelled Δ^1-piperideine was always contaminated by 5-10% lysine, the starting material, from which it is prepared.

Lysine can be converted to Δ^1-piperideine by two pathways: while lysine appears to be converted non-symmetrically into Δ^1-piperideine in anabasine biosynthesis,[46] it appears to be converted symmetrically into Δ^1-piperideine in the course of the biosynthesis of the lycopodine alkaloids.[77] It is now shown that the Decodon alkaloids are derived from lysine via a symmetrical intermediate. This symmetrical intermediate must lie between lysine and Δ^1-piperideine since Δ^1-piperideine is shown to enter the alkaloid non-symmetrically.

Since cadaverine is a symmetrical molecule, it can be postulated to be an intermediate between lysine and Δ^1-piperideine. Cadaverine can be formed chemically by the decarboxylation of lysine. It has been demonstrated to be an efficient intermediate in the biosynthesis of the lupin and lycopodium alkaloids, both of which have also been shown to be derived from lysine via a symmetrical intermediate. When 1,5-^{14}C-cadaverine was fed to lupin and lycopodium plants, the same labelling pattern of the resulting alkaloids was observed as in the case of either 2-^{14}C-lysine or 6-^{14}C-lysine (see Section III). Although labelled cadaverine

has not been fed to Decodon plants, and evidence has not been obtained, it is likely that cadaverine is indeed a precursor of the Decodon alkaloids and that its mode of incorporation will be similar to that of lysine.

(iii) Phenylalanine as a precursor

In the foregoing sections the origin of the quinolizidine ring of the Lythraceae alkaloids has been examined. In order to learn more about the origin of the remaining carbon atoms, incorporation of potential precursors of the biphenyl ring system must be investigated. In all the biogenetic hypotheses, outlined in Section IIB, a C_6-C_3 unit is postulated to be involved at a late stage of biosynthesis. Furthermore, a C_6-C_3 unit has been suggested to supply part of the quinolizidine ring (Schemes B, C and E). A C_6-C_1 unit is proposed as the source of a portion of the quinolizidine ring in Schemes A and D. Phenylalanine is known to be a precursor of a wide variety of alkaloids containing C_6-C_3 or C_6-C_1 units (see Section IIC). Thus, in order to demonstrate the origin of the biphenyl ring system, incorporation of labelled phenylalanine was tested.

The alternatives demand that activity from 3-^{14}C-phenylalanine should be located solely, but not necessarily equally, at C-4 and C-1‴. The alternatives differ in their predicted distribution of activity from 1-^{14}C-phenylalanine. In one case this activity should be located at C-2 and at C-3‴, in the other, solely at C-3‴. Distribution of activity from 2-^{14}C-phenylalanine would be predicted to be located either at C-3 and C-2‴ or solely at C-2‴.

Degradation reactions were devised which extracted four of these six carbon atoms, viz., those expected to carry label from 1-^{14}C- and from 3-^{14}C-phenylalanine (Section V). The alternative possibilities

envisaged by the hypotheses were tested by means of an experiment with multiply labelled 1,3-$^{14}C_2$-phenylalanine. The expected sites of label in decodine derived from 1,3-$^{14}C_2$-phenylalanine would be C-4, C-1‴ and C-3‴, if one C_6-C_3 unit and one C_6-C_1 unit derived from phenylalanine were incorporated. However, C-2, C-4, C-3‴ and C-1‴ would be labelled if two intact C_6-C_3 units had entered the alkaloids.

It should be mentioned that after the results of the experiment with labelled phenylalanine had been obtained, a report on the incorporation of 3-^{14}C-phenylalanine into a closely related Lythraceae alkaloid, cryogenine (5), obtained from **Heimia** salicifolia L. seedlings, was published.[39] It was shown that 3-^{14}C-phenylalanine was incorporated specifically into C-4 and C-1‴ of cryogenine. This result was consistent with but did not discriminate among the alternative biogenetic hypotheses.

As seen in Table 4, the three products, hemipinic anhydride (54a), 4-methoxyisophthalic acid (55) and acetic acid account for almost the entire activity of the intact alkaloid. This indicates that the incorporation of phenylalanine into decodine is non-random and that this amino acid is a specific precursor of the alkaloid. Of the total activity accounted, 49% is located within the C_6-C_3 unit, ring D, C-1‴, -2‴, -3‴. Since the distribution of label within this unit (C-1‴/ C-3‴ = 1.22 ± 0.04) is identical, within experimental error, with the distribution of ^{14}C within the precursor (β-/carboxyl = 1.20 ± 0.05), and since as mentioned above label at C-1‴ of the closely related alkaloid, cryogenine (5) has now been shown to be derived from the β-carbon of the precursor,[39] intact incorporation of the carbon chain of phenylalanine into the phenylpropanoid unit of the alkaloid is demonstrated.

The rest of the activity of the alkaloid (46%) is confined to C-4 of the quinolizidine nucleus, a site known[39] to be derived from the β-carbon of phenylalanine in cryogenine. It is evident that whereas the β-carbon of the precursor enters two carbon atoms, C-4 and C-1‴ of the alkaloid, the carboxyl carbon enters only one site of the product, the carbonyl carbon of the phenylpropanoid moiety. It follows that the phenylalanine-derived moiety which enters the phenylquinolizidine system of the alkaloid cannot be an intact C_6-C_3 unit. The problem still remains whether it is a C_6-C_1 unit or a C_6-C_2 unit derived from phenylalanine, which enters the phenylquinolizidine ring. In the latter case, the C_6-C_2 unit may arise by the intact incorporation of a C_6-C_3 unit derivable from phenylalanine followed by decarboxylation at a later stage.

It has been shown (Section IIC) that phenolic C_6-C_1 and C_6-C_3 units are generally derived from phenylalanine whereas phenolic C_6-C_2 units are derivable from tyrosine. Since, furthermore, tyrosine cannot replace phenylalanine in plant metabolism, it is likely that ring C and C-4 of decodine are derived from phenylalanine via a C_6-C_1 unit. The transformation of phenylalanine to the corresponding C_6-C_3 and C_6-C_1 units in Decodon plants may follow the sequence found to occur in other plant species and outlined in Fig. 11.

Decinine, isolated from the 1,3-$^{14}C_2$-phenylalanine experiment, was also radioactive. The results of degradation shown in Table 4 indicate that the phenylpropanoid unit, ring D, C-1‴, -2‴, -3‴ of the alkaloid has accounted for 39% activity of the intact alkaloid (i.e. 4-methoxyisophthalic acid (55) (22%) and acetic acid (17%)). Since the distribution of label within this C_6-C_3 unit (C-1‴/C-3‴ = 1.29 ± 0.05) is identical, within

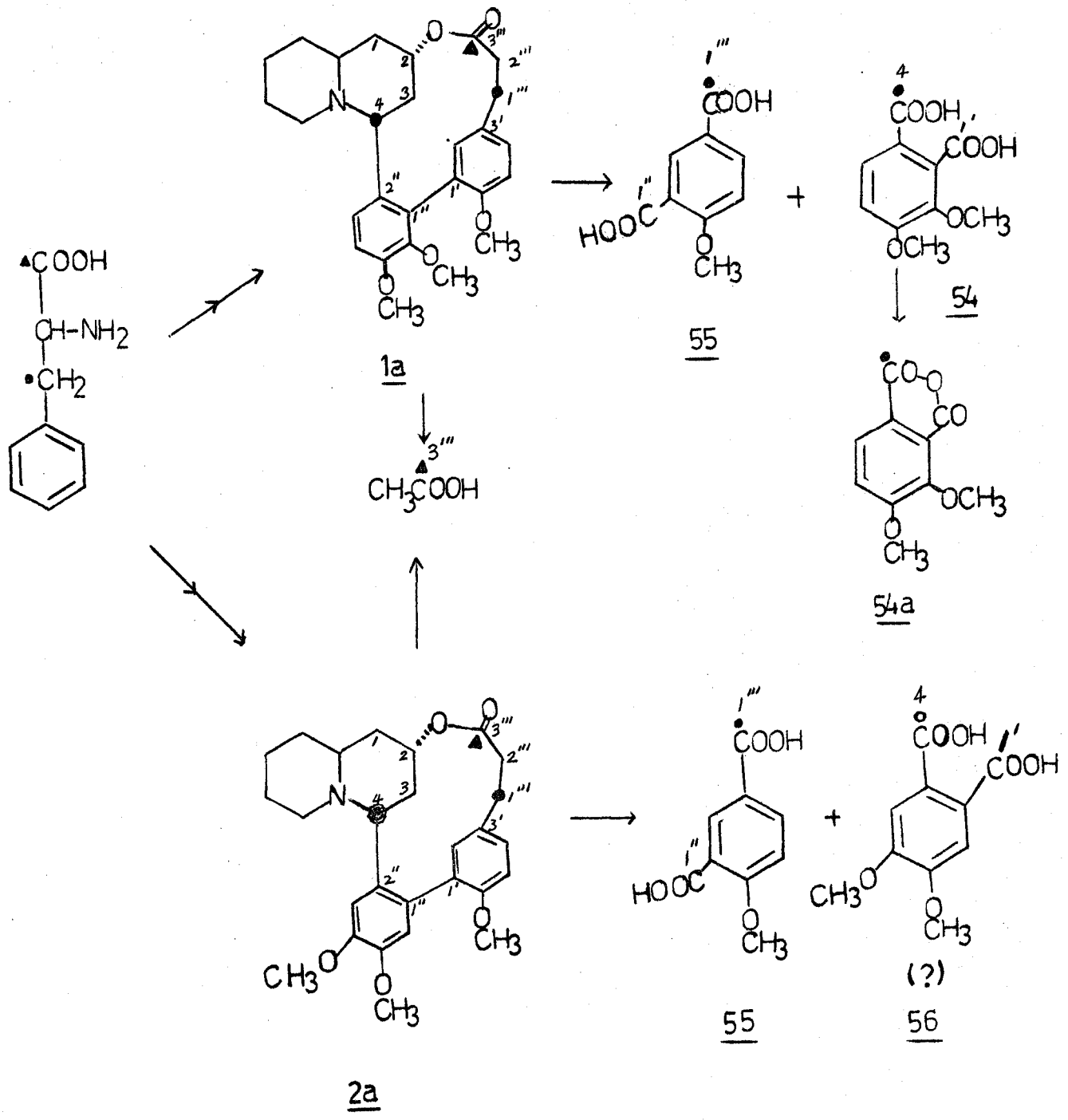


Fig. 19 Incorporation of 1,3-$^{14}C_2$-phenylalanine into decodine and decinine

experimental error, with the distribution of ^{14}C within the precursor (8-/carboxyl = 1.20 ± 0.05), it is evident that, as in the case of decodine, an intact carbon chain of phenylalanine has entered the alkaloid. The remaining activity should have been recovered in dimethoxyphthalic acid. It is unfortunate that the yield of this degradation product was too low for isolation, and that the rest of the activity is not accounted for experimentally. Nevertheless, on the basis of the other analogies found in the distribution of activity in decinine and decodine, it would be surprising indeed if the biosynthesis of decinine did not follow the same pathway as that of decodine. The results of the incorporation of phenylalanine into decodine and decinine are summarized in Fig. 19.

The mode of incorporation of phenylalanine, together with earlier evidence on the derivation of ring A of decodine and decinine from lysine and Δ^1-piperideine, makes it likely that the hypothesis illustrated in Scheme A is the most probable biosynthetic pathway of the Lythraceae alkaloids. On the basis of the results obtained from labelled lysine, Δ^1-piperideine and phenylalanine, the biosynthesis of the Lythraceae alkaloids can be outlined as shown in Fig. 20.

(iv) Pelletierine as a precursor

Since lysine, Δ^1-piperideine and phenylalanine serve as specific precursors of the major alkaloids of Decodon verticillatus, and are incorporated in a quite characteristic manner, the hypothesis illustrated in Scheme A seems to be the most likely model for the biosynthesis of the Lythraceae alkaloids. Scheme A suggests that pelletierine is an intermediate on the route from lysine to the phenylquinolizidine system of decodine. This view is consistent with the evidence which has been

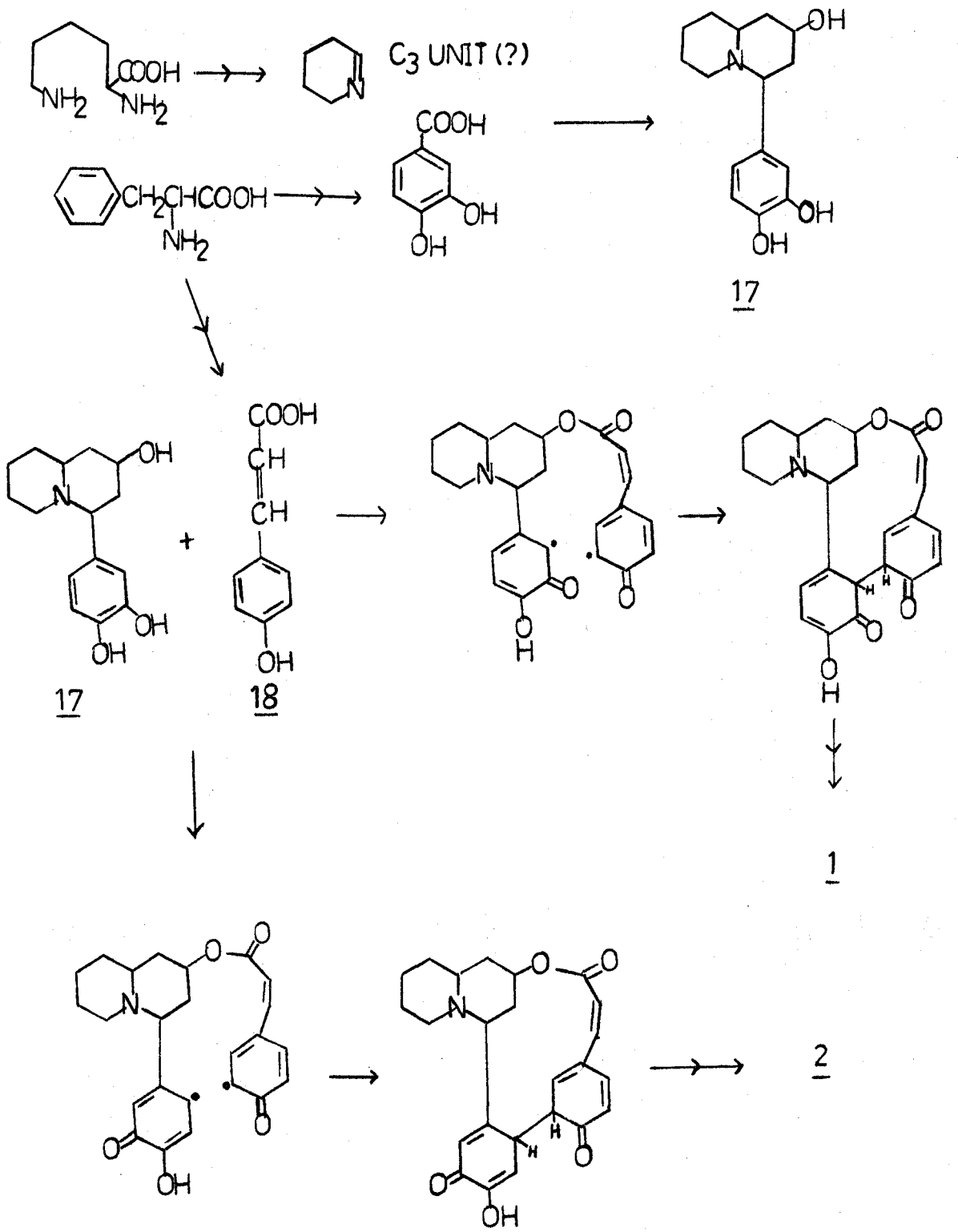


Fig. 20 Biosynthesis of decodine (1) and decinine (2)

presented, and which accounts for the origin of all the carbon atoms in decodine except C-1, -2, -3 of the quinolizidine ring.

Pelletierine has been shown to occur naturally in Punica granatum and has been demonstrated to be incorporated efficiently into lycopodine[78] and cernuine,[76] two of the lycopodium alkaloids. The portion of lycopodine and of cernuine which is derived from pelletierine, has in turn been shown to be derived from lysine and acetate. If pelletierine could be demonstrated to be incorporated specifically into the major alkaloids of Lythraceae, this would not only show it to serve as an intermediate in the biosynthesis of these alkaloids, but would also provide evidence for the origin of the remaining three carbon atoms, C-1, -2, -3 of the quinolizidine ring.

However, when doubly labelled 6,2-$^{14}C_2$-pelletierine was administered to Decodon plants, decodine and decinine were isolated with an activity of less than five counts/min/mg above background, a result which can only be regarded as **inconclusive.**

Negative results in alkaloid biosynthesis must be interpreted with caution. Several suggestions can be advanced to rationalize this negative experiment with labelled pelletierine.

(a) It is conceivable that pelletierine is not a "direct" intermediate in the biosynthetic sequence of Decodon alkaloids even though lysine and Δ^1-piperideine are precursors of the C_5N unit and acetate of the C_3 unit, C-1, -2, -3, respectively (cf. Scheme A). A possible scheme which excludes pelletierine is illustrated in Fig. 21. In place of pelletierine, a derivative (57 or 58), containing a carboxyl group may be required for the condensation with the C_6-C_1 unit.

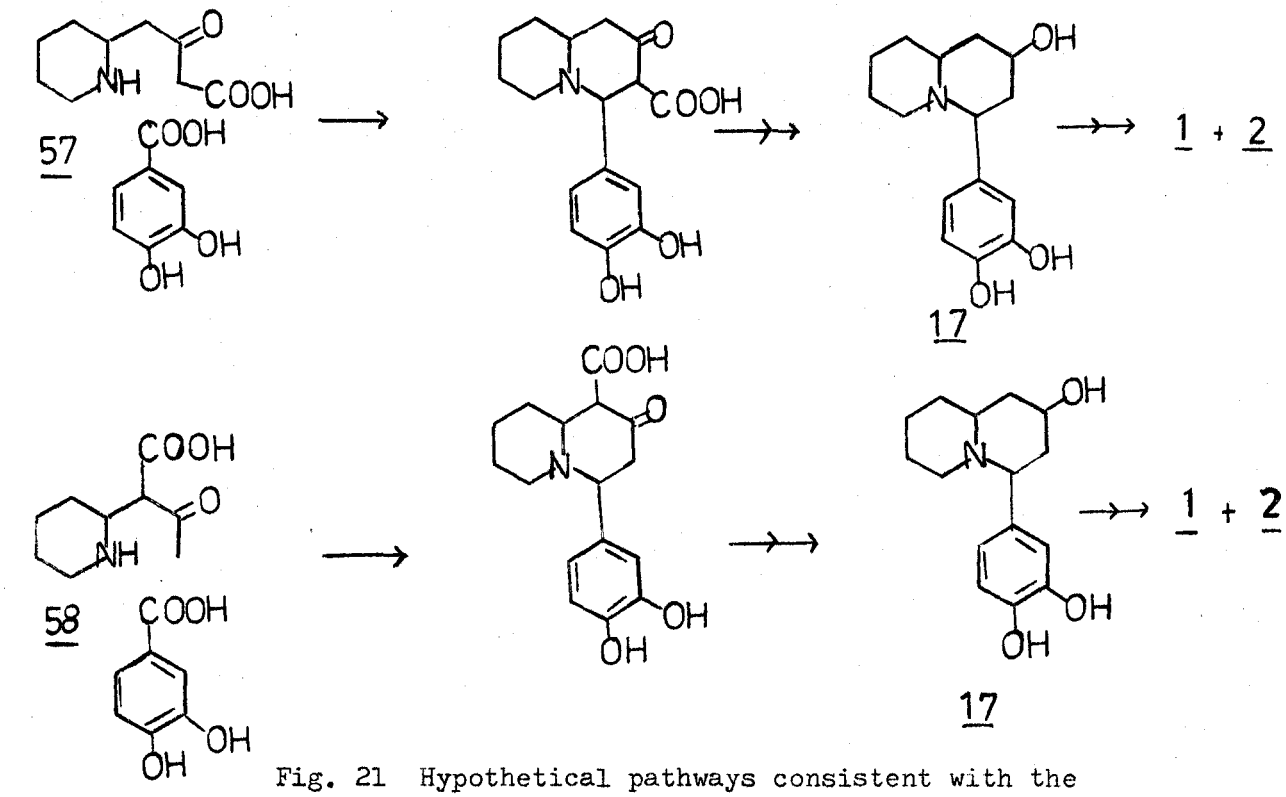


Fig. 21 Hypothetical pathways consistent with the incorporation data

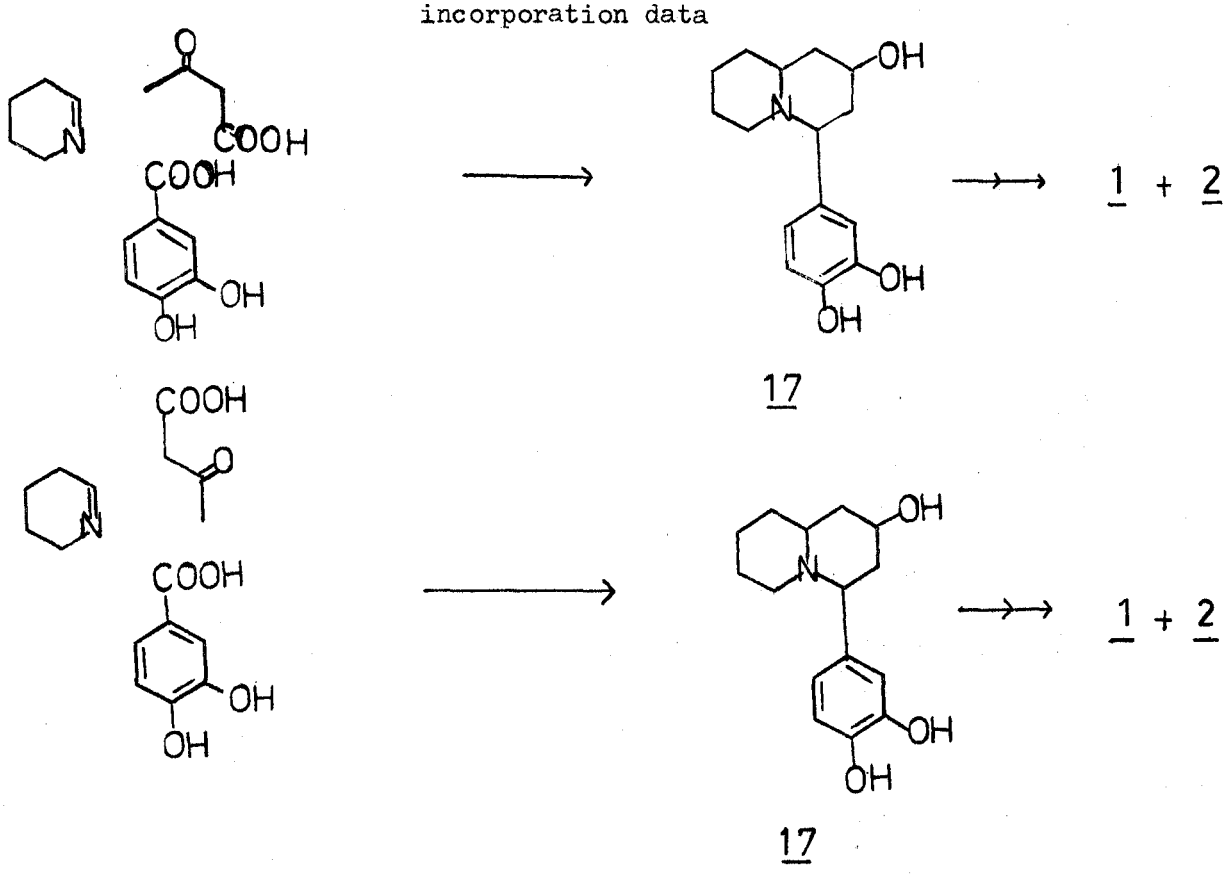


Fig. 22 Hypothetical pathways consistent with the incorporation data

Alternatively, the condensation of Δ^1-piperideine, acetoacetate and a benzoic acid derivative may take place on an enzyme surface without release or uptake of intermediates (Fig. 22).

(b) The administered pelletierine did not reach the site of alkaloid synthesis because the compound did not penetrate the cell membranes of the Decodon plants. It may be significant in this context that Δ^1-piperideine, another base, was incorporated into the alkaloids of Decodon plants less efficiently than the amino acid lysine, even though closer to the end product.

(c) The biosynthesis of decodine and decinine in Decodon plants is seasonal. Whereas incorporation of labelled precursors into decodine and decinine was observed to be good in June, decodine and decinine were not radioactive when the substrates were administered in August and September. The feeding experiments with pelletierine was carried out in April, on plants which had been forced. It might well be that under these conditions, the plants were not actively synthesizing the alkaloids even though the experiments in April were performed on plants whose developmental age was comparable to that of the plants used in the lysine and phenylalanine experiments.

(d) With this particular plant system, it might be that pelletierine had been degraded to smaller fragments before it reached the site of alkaloid synthesis.

The outcome of the tracer experiment with labelled pelletierine must be regarded as inconclusive. Further experiments with labelled pelletierine must be conducted at a time when decodine and decinine are being actively synthesized in Decodon plants.

(v) Conclusion

The results of the tracer experiments with Decodon verticillatus, here described, favor the biosynthetic Scheme A outlined in Fig. 3 . Labelled lysine and Δ^1-piperideine are incorporated into the C_5N unit, C-6 to C-10, N, of the quinolizidine ring. Although no experiment with lysine labelled with ^{15}N, has been carried out, it might be inferred by analogy with the origin of the piperidine nitrogen of anabasine,[85] that the nitrogen atom in decodine and decinine is derived from the ε-nitrogen of lysine.

Further, it has been demonstrated that the biphenyl ring system of the Decodon alkaloids originates from two phenylalanine-derived fragments. Whereas one phenylalanine unit is incorporated as an intact C_6-C_3 entity, to afford the phenylpropanoid unit, the other unit is derived from phenylalanine, by loss of carbon. Although conclusive evidence has not yet been obtained, from the accumulated knowledge of the precursors of phenolic C_6-C_2 and C_6-C_1 units, it is likely that a C_6-C_1 unit, probably a benzoic acid derivative, has entered the phenylquinolizidine ring.

These results account for the origin of the carbon skeleton of the alkaloids, except for that of carbon-1, -2, -3 of the quinolizidine ring. Although an experiment with labelled pelletierine did not give any definitive evidence regarding the origin of the remaining carbon atoms (i.e. C-1, C-2, -3), it is likely that they are derived from a unit of acetoacetate.

V. THE CHEMISTRY OF DEGRADATIONS OF DECODINE AND DECININE

When radioactive substrates are used in a study of alkaloid biosynthesis, a precursor-product relationship is not necessarily established even if the isolated alkaloids are shown to be radioactive. The degradation of radioactive alkaloids to isolate individual labelled carbon atoms or group of atoms is a necessary component of any biosynthetic study. As far as possible, the total activity of the intact alkaloid must be accounted for in terms of the activity at individual sites. This will then show that the incorporation of tracer into the alkaloid is non-random and the administered substance has thus served as a specific precursor of the alkaloid. To achieve the isolation of individual labelled atoms or groups of atoms, whose radioactivity can be determined, systematic and unambiguous degradation methods must be devised. These methods are, in the first place, selected in such a way that carbon atoms are extracted, which are the predicted centers of labelling, on the basis of the working hypothesis which is being tested.

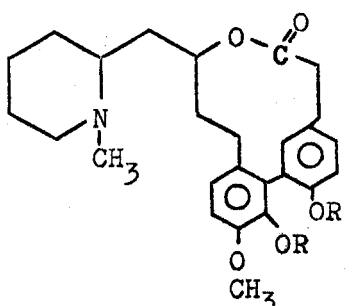
A. The Lysine-derived Carbon Atoms

On the basis of the hypotheses under test, one or both of C-6 and C-10 of the quinolizidine nucleus of the alkaloids would be the expected positions of label, when 2-^{14}C-lysine and 6-^{14}C-lysine are used as substrates.

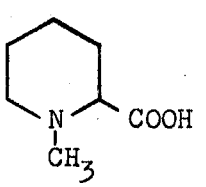
The Lythraceae alkaloids do not lend themselves very readily to the reactions commonly employed to degrade the carbon skeleton of a molecule and the isolation of individual carbon atoms. The attempted

oxidation of dimethyldecodine by either potassium permanganate or chromic anhydride in pyridine has been reported[26] to be unsuccessful. Hofmann degradation of several decodine derivatives was also unsuccessful[86] and the attempted Hofmann degradation on cryogenine, a Lythraceae alkaloid closely related to decodine or decinine, yielded a complex mixture of products.[87]

A fragment containing C-6 and C-10 might have been accessible by Emde degradation. The Emde reduction[32] of the methiodide of diacetyldecodine resulted in the cleavage of the quinolizidine ring to give 59. Oxidation of 59, resulted in N-methylpipecolic acid (60),[88] which is suitable for further degradation. However, the yield obtained in this oxidation was extremely low. This sequence of reactions thus did not appear promising and was not pursued further.



59 (R = -COCH$_3$)



60

An alternative approach to these carbon atoms was based on a method which had been used in the degradation of another quinolizidine alkaloid, sparteine. Drastic chromic acid oxidation of this alkaloid has been reported to yield a mixture of ω-amino acids which was separated into its components.[69] Similar products were obtained when dimethyldecodine and decinine were oxidized under analogous conditions. Since decodine

does not sublime readily and is therefore difficult to purify, a derivative, O,O-dimethyldecodine, was used for degradations. Dimethyldecodine is readily obtained when decodine is treated with diazomethane.

Dimethyldecodine was oxidized by chromic acid to give a complex mixture of products from which ω-amino acids were separated. In order to facilitate the isolation of individual components, the amino acid mixture was treated with 2,4-dinitrofluorobenzene and converted into the respective dinitrophenyl (DNP) derivatives. The resulting DNP-derivatives of the amino acids were readily separated on preparative t.l.c. when the solvent system benzene/pyridine/acetic acid (80:20:2) was used. The bands corresponding to DNP-β-alanine (51a), DNP-γ-aminobutyric acid (52a) and N-dinitrophenyl-2-piperidineacetic acid (53a) were isolated individually. Authentic samples of DNP-β-alanine and DNP-γ-aminobutyric acid were purchased commercially while DNP-2-piperidine-acetic acid was obtained by converting an authentic sample* of 2-piperidineacetic acid to its N-dinitrophenyl derivative by a standard procedure. The disadvantage of this oxidation process is that it gives a very low yield of the individual amino acids, (300 mg of starting alkaloids giving 3-8 mg of each of the products). Among them, DNP-β-alanine is the major product formed in most cases.

B. The Phenylalanine-derived Carbon Atoms

On the basis of the hypotheses under examination, phenylalanine would be expected to serve as the precursor of one or two C_6-C_1 units, and one or two C_6-C_3 units forming part of the ring system of the Lythraceae alkaloids. Thus label from 1-^{14}C-phenylalanine would be expected to enter one or both of C-2 and C-3''' and label from 3-^{14}C-phenylalanine one or both

*Dr. A. I. Meyers is thanked for sending us an authentic sample of 2-piperidineacetic acid.

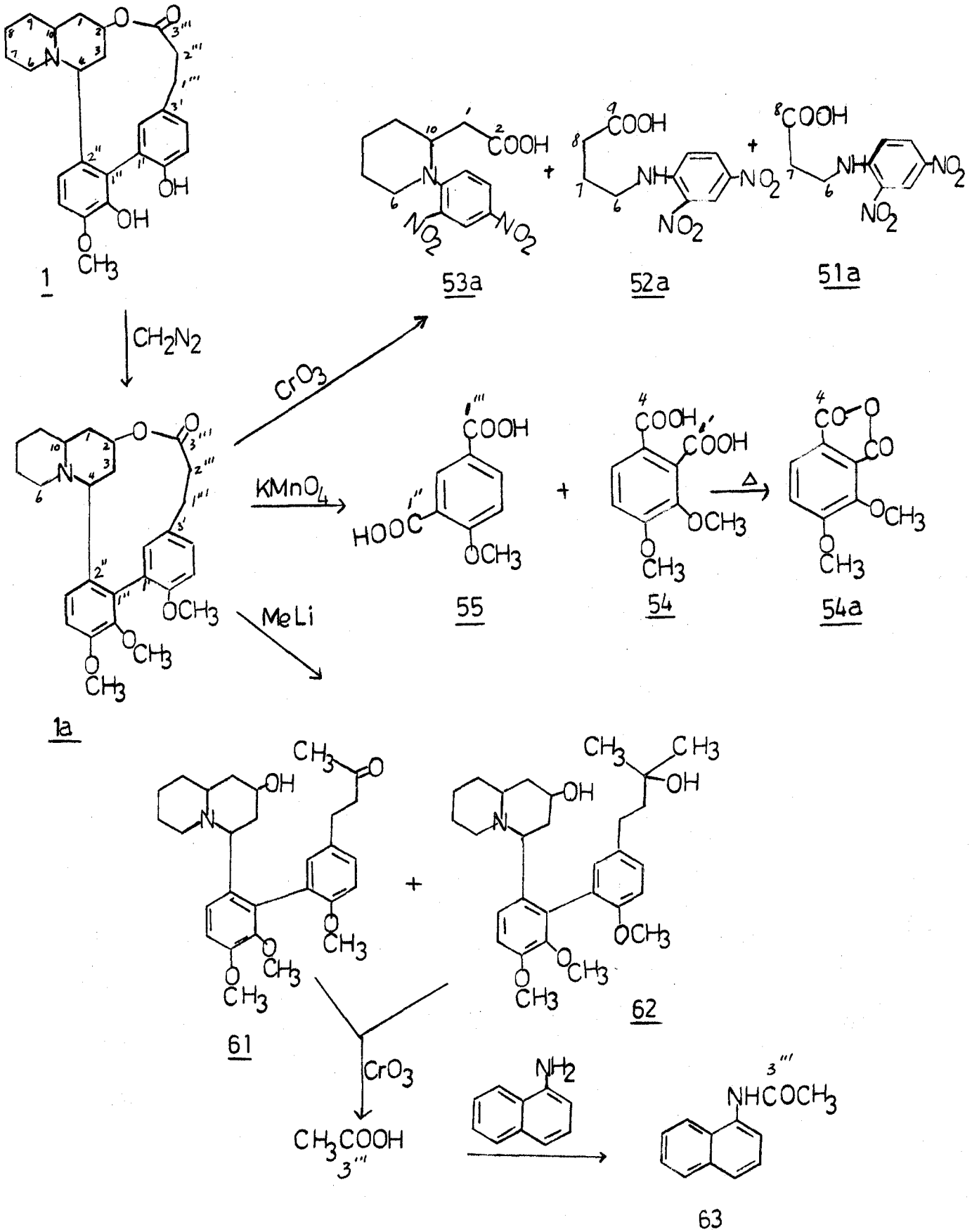


Fig. 23 Chemical degradations of decodine

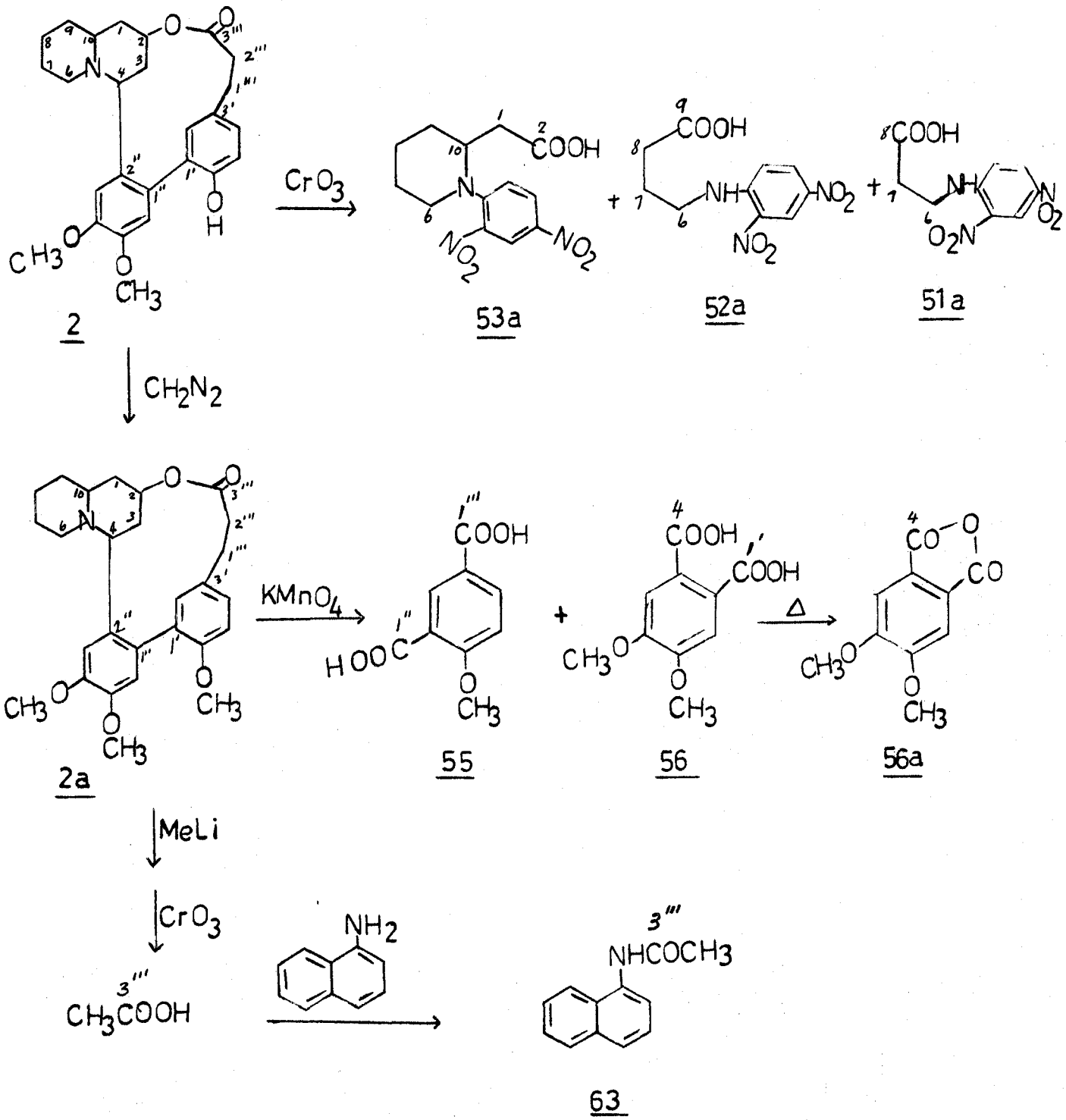


Fig. 24 Chemical degradations of decinine

of C-4 and C-1'''. Degradation attempts were directed at these carbon atoms.

Potassium permanganate oxidation of dimethyldecodine has been reported[26] to give hemipinic acid (54) and 4-methoxyisophthalic acid (55), while oxidation of methyldecinine would afford 4,5-dimethoxyphthalic acid (56) and 4-methoxyisophthalic acid (55). Hemipinic acid contains the carbon atoms of ring C, C-4, C-1' of the alkaloids. All these carbons except one are derived from carbon atoms of the aromatic rings of the alkaloids. The sole remaining carbon represents C-4, a site of expected activity derived from 3-^{14}C-phenylalanine. Similarly, 4-methoxyisophthalic acid contains carbon atoms of ring D, C-1''', C-1'' of the alkaloids, of which only one is not derived from an aromatic nucleus and represents C-1''', corresponding to the second expected site of activity derived from 3-^{14}C-phenylalanine. In other words, if the hypothesis were correct, the activity found in hemipinic acid and 4-methoxyisophthalic acid would represent activity originally present at C-4 and C-1''', of the alkaloids, respectively. Further degradation of 4-methoxyisophthalic and hemipinic acid would separate the individual carbons in question. However, due to the very low yield of these compounds obtained in the oxidation such further degradation could not be pursued.

Permanganate oxidation of dimethyldecodine gave a mixture of products from which 4-methoxyisophthalic acid and hemipinic acid were isolated. The former was obtained in higher yield than the latter, presumably because ring C, containing two methoxy groups, is more susceptible to oxidation than ring D, which contains only one.

Analogously, oxidation of methyldecinine gave 4-methoxyisophthalic acid (55) and 4,5-dimethoxyphthalic acid (56). 4-Methoxyisophthalic acid

again predominates. The yield of 4,5-dimethoxyphthalic acid is variable. In several runs it was not possible to isolate weighable amounts of this product.

The carbonyl carbon, C-3''', of the alkaloids was also isolated. One method of isolating a carbonyl carbon atom of a lactone ring is by phenylation to form a diphenyl derivative, from which benzophenone, whose carbonyl carbon represents the desired carbon atom, is then obtained by oxidation. Attempts to carry out this sequence with dimethyldecodine gave the degradation product in very poor yield. However, when the dimethyldecodine was treated with methyllithium, reaction took place readily. The resulting product, a mixture of two compounds, presumably the mono (61) and dimethylated (62) derivatives (mass spectrum, molecular ion m/e 483 and 467, respectively) was oxidized under Kuhn-Roth conditions to afford acetic acid, whose carboxyl group originated from the required carbon atom. The acetic acid, isolated as the α-naphthylamide, was obtained in less than five milligrams from two hundred milligrams of dimethyldecodine or methyldecinine.

The remaining carbon atom of importance in this context, C-2, is obtainable as the carboxyl carbon of 2-piperidine-acetic acid by chromic acid oxidation as described in the last section.

VI. EXPERIMENTAL

A. Administration of Labelled Compounds

Decodon verticillatus plants were found in a swampy area of Hendrie Valley, Royal Botanical Gardens, Hamilton, Ontario. The plants used in Expt. 1 - 3 were transported from the swamp to a greenhouse in June, 1969 when they were fresh growth and were about 1 and 1½ feet tall.

Two methods of feeding were used in the first experiment. 6-^{14}C-DL-lysine (75μCi) (Expt. 1) was administered to intact plants by the cotton wick method after they had been kept in the greenhouse for three days. Cotton thread was inserted by means of a thin sewing needle into approximately 20 green stems. The end of each thread was placed in a 1-ml beaker. The labelled compound, dissolved in glass-distilled water (10 ml) was placed evenly into the beakers and was absorbed into the plants through the cotton wicks. After the original tracer solution had been absorbed, the beakers were repeatedly (at least three times) refilled with glass-distilled water. The plants were kept in contact with the tracer for three days and then harvested. All the green stems were cut and dried in an oven for two days before the plant material was extracted. Since a preliminary extraction of the roots had shown that they did not contain any appreciable amount of alkaloids, the roots were discarded in all the experiments.

In another feeding method, green shoots were cut from the plants. The cut ends were placed in a 800 ml beaker. The aqueous solution containing 6-^{14}C-DL-lysine (25μCi) was added to the beaker so that the

volume of the solution in the beaker was 700 ml. The activity of the aqueous solution was determined immediately after the tracer was added. The radioactivity of the solution was assayed daily and on the fourth day, only 5% of the radioactivity remained in the solution. The green stems in the beaker were then harvested and worked up. However, this method of feeding was later found to yield no radioactive alkaloids and was not used after the first experiment.

2-^{14}C-DL-Lysine (100μCi) (Expt. 2) was also fed to two large Decodon plants using the cotton-wick method as described above.

A sample of doubly labelled 1,3-$^{14}C_2$-DL-phenylalanine (200μCi) (Expt. 3) was administered to the plants by the cotton-wick method as described. It was prepared in the following manner: solutions of 1-^{14}C-DL-phenylalanine (100μCi in 10 ml glass distilled water) and 3-^{14}C-DL-phenylalanine (100μCi in 10 ml glass distilled water) were mixed in a volumetric flask (25 ml), and the solution was diluted to 25 ml. The total activity of 1-^{14}C-phenylalanine and of 3-^{14}C-phenylalanine before mixing and of 1,3-$^{14}C_2$-phenylalanine after mixing, was determined by liquid scintillation counting. Triplicate samples (10μl) were used in each case.

The plants used in Experiments 4, 5 and 6 were collected from the swamp in late November, 1969, stored in a cold room and propagated in the greenhouse after January, 1970 as they were needed for the experiments. The plants were grown in soil.

The samples of labelled compounds used in Expt. 4 and Expt. 5 were prepared* as follows: N-bromosuccimide (45 mg, 0.26 mmole) was

*The synthesis of these labelled compounds was carried out by Dr. R. N. Gupta.

added to 6-^{14}C-DL-lysine monohydrochloride (0.1 mCi) mixed with inactive lysine monohydrochloride (23 mg, 0.125 mmole) in water (3 ml) in a small flask. The flask was rotated by means of a rotatory evaporator, under mild suction, while immersed in a water bath of 40°. When the solution had become colorless (20 min), five drops of concentrated hydrochloric acid and distilled water (7 ml) were added. The solution was chromatographed on a silica gel plate developing with solvents: 1-butanol:acetic acid:water (2:1:1). The chromatogram was then scanned in a radioscanner (Radiochromatogram Scanner, Model 7201, Packard Instrument Company). The radioscan showed that the sample contained 6-^{14}C-Δ^1-piperideine (R_f 0.52) with 10% 6-^{14}C-lysine (R_f 0.24) as its starting material. This sample was used as substrate in Expt. 4.

For the preparation of the sample used in Expt. 5, the above procedure was followed except that lesser amount (0.15 mmole) of N-bromosuccimide was used. The resulting mixture was chromatographed on a thin layer plate (silica gel) and the t.l.c. was radioscanned. The sample was a mixture of 55% 6-^{14}C-Δ^1-piperideine and 45% 6-^{14}C-lysine.

A doubly labelled sample of 6,2'-$^{14}C_2$-pelletierine was prepared* as described below. 6-^{14}C-Pelletierine was synthesized by an established method[40] from 6-^{14}C-DL-lysine and inactive acetoacetic acid while 2'-^{14}C-pelletierine was prepared from inactive lysine and 3-^{14}C-acetoacetic acid generated by hydrolysis of ethyl 3-^{14}C-acetoacetate. The two labelled samples of pelletierine hydrochloride were mixed and recrystallized from methanol/ether to give a sample of intermolecularly doubly labelled 6,2'-$^{14}C_2$-pelletierine hydrochloride. The specific activity of the labelled carbons, C-6 and C-2', was determined in this way: 6,2'-$^{14}C_2$-pelletierine

(150 mg), which was obtained by mixing a small sample (0.2 μCi) of the original 6,2'-$^{14}C_2$-pelletierine hydrochloride used in Experiment 6 with inactive pelletierine hydrochloride (350 mg), and recrystallizing the mixture to constant activity, was subjected to Kuhn-Roth oxidation by an established method[42] to afford acetic acid. The acetic acid so obtained was converted into acetyl-α-naphthylamide (see below). Pipecolic acid was isolated from the remaining aqueous reaction mixture by a method already described in the literature.[42]

A summary of the feeding experiments which were carried out is shown in Table 1.

B. Isolation and Purification of Decodine and Decinine

The aerial parts of D. verticillatus plants to which 6-^{14}C-lysine had been fed, were dried and ground to a fine powder in a blender (Osterizer Galaxie 10). The powder (22 g) was moistened with aqueous ammonia (10%, 10 ml) and was continuously extracted with chloroform for two days in a Soxhlet extractor. The chloroform extract was evaporated in vacuo until a slurry was formed. Sulfuric acid (20% w/v, 5 ml) was added to the slurry and the mixture was heated on a steam bath for 25 minutes before it was diluted with water (30 ml). The final portion of chloroform was evaporated in vacuo, and the aqueous layer was decanted from the solid residue. A new portion of chloroform (4 ml) was now added to the residue to make a slurry, sulfuric acid (20%, w/v, 5 ml) was added, the mixture was heated and diluted with water (30 ml). Chloroform was again evaporated and the aqueous layer again decanted. This procedure was repeated three times. The combined aqueous solution ($\sim$ 100 ml) so obtained was extracted with ether (2 x 30 ml). The ether layer was discarded while the aqueous

layer was basified with solid sodium carbonate. The resulting basic aqueous solution was extracted with chloroform (4 x 30 ml). The chloroform extract was dried (Na_2SO_4) and evaporated to dryness in vacuo to give a basic fraction containing a mixture of crude alkaloids. Yield: 180 mg.

The plant material obtained from Experiments 2 - 6 was worked up similarly, and a fraction of crude alkaloid obtained in each case. The crude alkaloid extract was chromatographed on thin layer plates (silica gel GF_{254} containing 4% KOH). Many solvent systems for thin layer chromatography were tried for separating the complex mixture of alkaloids. The solvent system consisting of diethylamine/methanol/chloroform (1:1:10) was found to be the best in separating the two major alkaloids, decodine (R_f 0.40) and decinine (R_f 0.73). The chromatogram was developed with iodine vapor or with Draggendroff's reagent.

The thin layer chromatogram was then scanned for radioactivity in a radioscanner. The radiochromatograms obtained from the crude extracts of 6-^{14}C-lysine and 2-^{14}C-lysine experiments showed two well-separated major peaks, corresponding to decodine (R_f 0.40) and decinine (R_f 0.73) while the radiochromatogram obtained from the experiment with 1,3-$^{14}C_2$-phenylalanine indicated that in addition to the two major alkaloids, decodine and decinine, another alkaloid (R_f 0.60) was also radioactive. This "unknown" alkaloid was not further examined at this time.

In Expt. 4, the incorporation of labelled precursor was so low that the radiochromatogram of the crude alkaloid extract did not show much activity.

In Expt. 5, the two major alkaloids were again labelled but were accompanied by an "unknown" compound (R_f 0.65) whose R_f value was very close to that of decinine (R_f 0.73).

In the final experiment (Expt. 6) interpretation of the radiochromatogram obtained from the crude basic extract was complicated by the fact that some unchanged administered labelled pelletierine which itself is a base, had been recovered.

The crude alkaloid extract (180 mg) was then fractionated in a column (7-10 mm diameter) using neutral alumina (activity IV, 10 g) as an absorbant. The solvents used for eluting the alkaloids were in the following sequence: petroleum ether (30-60°); petroleum ether/benzene (1:4); benzene; benzene/chloroform (1:4); chloroform; 5% methanol in chloroform; and methanol. At least 24 fractions, each containing 20 ml solvent were collected. Each fraction was checked for radioactivity in a liquid scintillation counter and then concentrated, chromatographed on t.l.c. (silica gel containing 4% KOH, developed with chloroform/methanol/diethylamine (10:1:1)). The fractions containing the same compounds were combined. In general, decinine was eluted from the column by petroleum ether/benzene (1:4) whereas decodine was found mostly in the benzene/chloroform (1:4) fractions. However, the elution sequence of these alkaloids from the column varied somewhat from experiment to experiment, and depended on the activity and amount of alumina used and the size of the column. In order to conserve radioactive materials, inactive decodine and decinine were added to their respective fractions and the diluted product crystallized from methanol until constant radioactivity had been attained. Radioactive decodine and decinine were obtained in this way

in all the experiments which were carried out. The results of the isolation of labelled decodine and decinine from the different feeding experiments are shown in Table 1.

C. Degradations of the Alkaloids

Conversion of decodine (1) to dimethyldecodine (1a)

Decodine (1) (300 mg) was dissolved in methanol (15 ml) and excess ethereal diazomethane (75 ml) was added. The mixture was kept in a cold room (0 - 4°) for two days. The solvent was then removed. Methanol (10 ml) was added to the residue and the mixture was warmed on a steam bath and filtered. The methanolic filtrate was concentrated and dimethyldecodine (1a) (308 mg) crystallized out. Repeated recrystallization from methanol yielded dimethyldecodine, m.p. 207-208° (lit.[26] 203.5-205°).

Conversion of decinine (2) to methyldecinine (2a)

Decinine (2) (300 mg) was dissolved in methanol (15 ml) and excess diazomethane (75 ml) was added. The mixture was kept in a cold room (0 - 4°) for two days. The solvent was removed. Methanol (10 ml) was added to the residue, and the mixture was warmed on a steam bath and filtered. The filtrate was concentrated and methyldecinine (2a) (305 mg) crystallized out. Repeated recrystallization from methanol yielded methyldecinine, m.p. 169-170° (lit.[26] 171-173°).

Chromic acid oxidation of dimethyldecodine (1a) and decinine (2)

Dimethyldecodine (300 mg) was dissolved in hot sulfuric acid (30% v/v, 3 ml) and chromium trioxide (1.5 g) in water (3 ml) was added in several portions. The resulting mixture was heated under reflux with stirring for 48 hours. The mixture was cooled and extracted with ether (3 x 10 ml). The ether layer was rejected. The aqueous layer was diluted

with water (10 ml) and heated on the steam bath. Hot aqueous barium hydroxide solution was then added until the solution was neutral (pH 7). After one hour the mixture was filtered, the precipitate was washed with hot water (3 x 10 ml) and the combined filtrate and washings were concentrated in vacuo. Sodium carbonate (35 mg) and methanolic 2,4-dinitrofluorobenzene (10%, 1.5 ml) was added and the resultant mixture was allowed to stand for two hours at room temperature with occasional stirring. The pH of the solution was maintained at 8 - 9 with additional sodium carbonate. At the end of two hours, the solution was extracted with ether (3 x 8 ml) and the ether extract was discarded. The solution was then acidified by addition of dilute hydrochloric acid (6N) and extracted with ether (3 x 10 ml). The dried (Na_2SO_4) ether extract was evaporated to dryness. The deep yellow residue, containing the 2,4-dinitrophenyl (DNP) amino acids, was dissolved in methanol (0.5 ml) and applied to silica gel plates (GF_{254}, 0.5 mm thickness). Development with benzene/pyridine/acetic acid (80:20:2) led to the appearance of four well separated bands. The major band (R_f 0.50) was identical with that of an authentic sample of N-2,4-dinitrophenyl-β-alanine. The band with an R_f value of 0.65 was identical with an authentic sample of N-2,4-dinitrophenyl-Y-aminobutyric acid while the top band (R_f 0.76) corresponded to N-2,4-dinitrophenyl-2-piperidineacetic acid. The minor band (R_f = 0.22) was not further examined, but may have been N-2,4-dinitrophenylglycine. The amino acid DNP-derivatives were scraped off the plates separately and eluted from the absorbant with methanol. The methanolic extract (60 ml) of the DNP-β-alanine band (R_f 0.50) which was the most abundant was evaporated to dryness. The residue was dissolved in dilute hydrochloric acid (6N, 5 ml) and water (2 ml) was then added. The mixture

was extracted with ether (3 x 10 ml). The ether layer was washed with water (8 ml), dried (Na_2SO_4) and evaporated to dryness. The yellow residue was sublimed at 160-170° and 1×10^{-3} mm when the DNP-β-alanine (51a) was obtained as a yellow crystalline solid, m.p. 140-142° (lit.[89] 145-146°). Yield: 6 mg. The bands corresponding to DNP-derivatives of γ-aminobutyric acid and 2-piperidineacetic acid were worked up separately in the same way to give pure DNP-γ-aminobutyric acid (52a), m.p. 144-145° (lit.[89] 145-146°), yield: 3.5 mg, and DNP-2-piperidineacetic acid (53a), m.p. 67-70°, yield: 3.5 mg.

Decinine (2) (300 mg) was oxidized in exactly the same process to give the same amino acid derivatives which were separated and purified as described above.

Hemipinic acid (54) and 4-methoxyisophthalic acid (55) from dimethyldecodine (1a)

A suspension of dimethyldecodine (1a) (200 mg) in 2N sulfuric acid (8 ml) was made alkaline by adding 2N sodium carbonate solution (10 ml) to pH 8-8.5. Potassium permanganate (1.1 g) was added with stirring over a period of four hours and the mixture was stirred additionally for another twenty hours at room temperature. The solution was then acidified with 3N sulfuric acid and the excess potassium permanganate and the precipitated manganese dioxide were decomposed by passing sulfur dioxide gas through the solution until it was colorless. The solution was extracted with ethyl acetate (4 x 40 ml) and the organic layer washed, dried (Na_2SO_4), and evaporated, leaving a residue (75 mg), which was shown by thin layer chromatography (on silica gel GF_{254}, developed with benzene/methanol/acetic acid (45:8:4) to contain at least six components, including hemi-

pinic acid (54) and 4-methoxyisophthalic acid (55).

The mixture was then separated by preparative thin layer chromatography (silica gel GF_{254} plates, 0.5 mm thickness, developed with ethanol/water/ammonia (100:12:10)). The major product (R_f 0.48) was eluted with water, the solution was acidified with sulfuric acid (3N) and extracted with ethyl acetate (3 x 25 ml). The organic layer was washed, dried (Na_2SO_4) and evaporated. The residue was sublimed (1×10^{-3} mm, 160°) to give pure 4-methoxyisophthalic acid (55), m.p. 265-270° (lit.[26] 255-260°), identical in all respects with an authentic sample. Yield: 6 mg.

The other product (R_f 0.34) was isolated from the t.l.c. plates and purified as above to give a pure sample of hemipinic acid (54), which was readily converted to its anhydride (54a) on sublimation (1×10^{-3} mm, 135°C). The anhydride (54a), m.p. 157-159° (lit.[26] 158-160°), so obtained was identical with an authentic sample. Yield: 3 mg.

4,5-dimethoxyphthalic acid (56) and 4-methoxyisophthalic acid (55) from methyldecinine (2a)

A suspension of methyldecinine (2a) (200 mg) in 2N sulfuric acid (8 ml) was made alkaline by adding 2N sodium carbonate solution (10 ml) so that the pH of the solution was 8-8.5. Potassium permanganate (1.1 g) was added with stirring over a period of four hours and stirring was continued for twenty hours at room temperature. The solution was then acidified with 3N sulfuric acid and the excess potassium permanganate and the precipitated manganese dioxide were decomposed by adding sodium bisulfite until the solution was colorless. The resulting solution was extracted with ethyl acetate (4 x 40 ml) and the organic layer washed, dried (Na_2SO_4), and evaporated.

The residue (70 mg) was separated by preparative thin layer chromatography (silica gel GF_{254} plates, 0.5 mm thickness, developed with benzene/methanol/acetic acid (45:8:4)). The major product (R_f 0.43) was eluted with water, the solution was acidified with sulfuric acid (3N) and extracted with ethyl acetate (3 x 25 ml). The organic layer was washed, dried (Na_2SO_4) and evaporated and the crude product was sublimed (1×10^{-3} mm, 160°) to give pure 4-methoxyisophthalic acid (55), m.p. 265-260°). Yield: 6 mg.

The other product (R_f 0.25) was isolated from the t.l.c. plates and purified as described above to give a sample of 4,5-dimethoxyphthalic acid (56), which was easily converted to its anhydride (56a) on sublimation (1×10^{-3} mm, 130°C). The anhydride so obtained was identical with an authentic sample, m.p. 176-178° (lit.[87] 178.5-179.5°). Yield: 2 mg.

Reaction of dimethyldecodine (1a) and methyldecinine (2a) with methyllithium and Kuhn-Roth oxidation of the products

Dimethyldecodine (1a) (150 mg) was suspended in anhydrous ether (10 ml) in a dry three-neck flask, flushed with nitrogen gas. Excess methyllithium in ether (5 ml, 2.2 M, Alfa Inorganics, Inc.) was added. The mixture was stirred at room temperature for 5 hours. The flask was cooled in ice and water (10 ml) was added dropwise to decompose the unreacted methyllithium. Dilute sulfuric acid (3N) was then added until the pH of the solution was 8 to 9. The aqueous solution was extracted with ether (3 x 25 ml). The ether layer was washed, dried (Na_2SO_4) and evaporated. The residue (155 mg) was assayed by t.l.c. (silica gel containing 4% KOH, developed in chloroform/methanol (10:1)). No starting material appeared but two new products (R_f 0.40 and 0.57, respectively) were present. The

mass spectrum of the mixture indicated it to be a mixture of two components having molecular ions (m/e) of 483 and 467, respectively.

The above residue without further purification was subjected to Kuhn-Roth oxidation. The residue (155 mg) was dissolved in 3N H_2SO_4 (8 ml) and chromium trioxide (3.0 g) in water (2 ml) was added. The mixture was refluxed for 1.5 hours, and then distilled while water was added to the flask to maintain the volume at about 10 ml. When about 65 ml of distillate had been collected it was titrated with 0.1 N sodium hydroxide (2.3 ml) to pH 9.0 using a pH meter. The solution was then evaporated to dryness to afford crude sodium acetate (8 mg).

Sodium acetate (8 mg) and α-naphthylamine hydrochloride (16 mg) were dissolved in water (1.5 ml). 1-Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (80 mg) was added. After stirring for a few minutes with a glass rod, the dark pink solid which had formed was extracted into ether. The ether extract was washed with dilute hydrochloric acid and then with water, dried (Na_2SO_4) and evaporated. The residue was sublimed *in vacuo* (130°, 3×10^{-3} mm). The white sublimate was dissolved in a little boiling benzene. On addition of petroleum ether, acetyl-α-naphthylamide (63) separated out as colorless needles (6 mg), m.p. 159-160° (lit.[90] 159-160°)

Methyldecinine (2a) (200 mg) was degraded in exactly the same way as described for the dimethyldecodine degradation to give acetic acid which was converted to acetyl-α-naphthylamide (63).

D. Radioactivity Measurements

Radioactivity was assayed on solid samples of finite thickness on aluminum planchettes in a low background gas flow Geiger counting system (Nuclear Chicago Corporation Model 4342). Corrections for background and self-absorption were applied. Samples for counting were prepared as follows: solid materials (0.5-3 mg, weighed to the nearest microgram on a Micro Gramatic balance, Mettler Instrument Corp.) were weighed on aluminum planchettes (diameter 31 mm, area 7.45 cm^2) and dissolved in two drops of dimethylformamide containing collodion (1%). The solution in the planchette was overlaid with a disc of lens tissue (cut to fit the planchette) to ensure even spreading of the sample over the surface of the planchette. The planchettes were dried under an infrared heat lamp for 20 minutes, cooled and counted. Each sample was prepared in either duplicate or triplicate.

Liquid scintillation counting was carried out on a Mark I Liquid Scintillation Computer (Model 6860, Nuclear Chicago Corporation). The efficiency of counting for ^{14}C was determined by external standardization counting with ^{133}Ba. Samples for counting were dissolved in methanol or methanol-water and the diluted Liquifluor (10 ml) was added. The diluted Liquifluor was prepared by diluting a solution of Liquifluor (Nuclear Chicago) with 25 times of toluene, and contains 4 grams of PPO and 50 mg POPOP per liter of solution.

The confidence limits shown in the results (Tables 1 - 4) are standard deviations of the mean. The calculation of the specific activity of a typical compound, from the data obtained from measurements in triplicate samples, will be outlined in the Appendix.

SUMMARY

The biosynthesis of the alkaloids of Decodon verticillatus (L.)Ell, a species of Lythraceae family, has been studied by the tracer technique. Several labelled primary precursors, lysine, Δ^1-piperideine and phenylalanine were administered to Decodon plants and the two major alkaloids, decodine and decinine, were found to be radioactive. Systematic and unambiguous partial degradations of the radioactive samples of these alkaloids were carried out. The results show that lysine, Δ^1-piperideine and phenylalanine serve as specific precursors of these alkaloids.

Radioactive samples of decodine and decinine derived from two radiomers of lysine were degraded to afford β-alanine, γ-aminobutyric acid and 2-piperidineacetic acid. Whereas β-alanine and γ-aminobutyric acid harbour half of the activity of the intact alkaloid, 2-piperidineacetic acid contains all the activity, regardless whether 2-^{14}C- or 6-^{14}C-lysine served as a precursor. This shows that the incorporation of lysine into these alkaloids has occurred through a symmetrical intermediate. The result of the incorporation of Δ^1-piperideine provides further evidence that the quinolizidine ring of these alkaloids is derived from lysine by way of Δ^1-piperideine. Δ^1-Piperideine serves as an intermediate in the biosynthetic pathway of these alkaloids, and the identity of C-6 is preserved in the product. The double bond of the intermediate evidently does not isomerize.

The biphenyl ring of these alkaloids has been shown to be derived from two units of phenylalanine. One intact C_6-C_3 unit derived from phenylalanine has been demonstrated to serve as the precursor of the phenylpropanoid moiety. The other aromatic unit is derived from phenyl-

alanine by loss of carbon. Although conclusive evidence has not yet been obtained, from the accumulated knowledge of the precursors of phenolic C_6-C_2 and C_6-C_1 units, it is likely that a C_6-C_1 unit, probably a benzoic acid derivative, has entered the phenylquinolizidine ring.

On the basis of the experiments with lysine, Δ^1-piperideine and phenylalanine, the origin of all the carbon atoms, except carbon-1, -2, -3, of the two major alkaloids of Decodon plants, has been established.

In an attempt to search for the origin of the remaining carbon atoms, and also to test incorporation of a possible secondary intermediate, a feeding experiment with labelled pelletierine was performed. This experiment yielded an **inconclusive result.**

APPENDIX

Experiment 1: Dimethyldecodine obtained from 6-^{14}C-lysine

Planchette No.	9905	9906	9907
Weight taken (mg)	0.525	0.505	0.440
Molecular weight	451.5	451.5	451.5
Self-absorption factor[91]	0.874	0.874	0.870

Definitions and Symbols:[92]

time (min) for 2000 counts = X

number of time readings = n

mean $\bar{X} = \frac{\Sigma X_i}{n}$

variance = $\Sigma[(X_i-\bar{X})^2]/n$

standard deviation = $\sigma = \sqrt{\Sigma[(X_i - \bar{X})^2]/n}$

best estimate of $\sigma = \sqrt{\Sigma[(X_i - \bar{X})^2]/n-1}$

Planchette 9905	X	$\lvert X-\bar{X}\rvert$	$(X-\bar{X})^2$
1	75.89	4.15	17.223
2	71.11	0.63	0.397
3	72.43	0.69	0.476
4	68.85	2.89	8.352
5	72.08	0.34	0.116
6	69.82	1.92	3.686
7	73.46	1.72	2.958
8	72.06	0.32	0.102
9	72.07	0.33	0.109
10	69.10	2.14	4.580

$\Sigma X = 717.4$

$$\bar{X} = \frac{\Sigma X}{10} = 71.74$$

$\Sigma(X-\bar{X})^2 = 37.999$

$\Sigma(X-\bar{X})^2/n-1 = 4.222$

$$\text{Count rate} = \frac{2000}{71.74} \pm \frac{2000}{71.74} \times \sqrt{\frac{4.222}{(71.74)^2}}$$

$$= 27.89 \pm 0.784 \text{ counts min}^{-1}$$

The background, determined in the same manner, was found to be 2.15 ± 0.16 counts min^{-1}.

The count rate for the radioactive sample was corrected for background:

$$27.89 - 2.15 \pm \sqrt{(0.784)^2 + (0.16)^2}$$

$$= 25.74 \pm 0.801 \text{ counts min}^{-1}$$

Corrections for self-absorption and normalization to specific activity

$$= 25.74 \pm 0.801 \times \frac{451.5 \text{ mg mmole}^{-1}}{0.525 \text{ mg} \times 0.874}$$

$$= (2.5367 \pm 0.079) \times 10^4 \text{ counts min}^{-1} \text{ mmole}^{-1}$$

The corresponding values derived from samples on planchettes 9906 and 9907, were $(2.5195 \pm 0.062) \times 10^4$ and $(2.4815 \pm 0.042) \times 10^4$ counts min^{-1} $mmole^{-1}$, respectively.

$$\text{The mean specific activity} = \left(\frac{2.5367 + 2.5195 + 2.4815}{3} \pm \frac{\sqrt{(0.079)^2 + (0.062)^2 + (0.042)^2}}{3}\right) \times 10^4$$

$$= (2.5126 \pm 0.036) \times 10^4 \text{ counts min}^{-1} \text{ mmole}^{-1}$$

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