

## Stereochemical aspects of the biosynthetic routes leading to the pyrrolizidine and the quinolizidine alkaloids

Ian D. Spenser

Department of Chemistry, McMaster University, Hamilton, Ontario, Canada, L8S 4M1

Plenary lecture delivered at the 14th International Symposium on the Chemistry of Natural Products of the International Union of Pure and Applied Chemistry, Poznań, Poland, July 9-14 1984.

**ABSTRACT** -- The regiochemistry and the stereochemistry of the biosynthetic sequences leading into retronecine from ornithine and putrescine, and into lupinine, sparteine and lupanine from lysine and cadaverine, is revealed by tracer experiments with intramolecularly doubly  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labelled and chirally  $^2\text{H}$ -labelled substrates. The labelling patterns within the biosynthetic products were established by means of  $^{13}\text{C}$  NMR and  $^2\text{H}$  NMR spectroscopy.

Experiments with  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labelled substrates show that whereas retronecine originates from two putrescine units by way of a  $\text{C}_4\text{-N-C}_4$  intermediate with  $\text{C}_{2v}$  symmetry, the biosynthetic route to lupinine from two cadaverine units excludes the corresponding  $\text{C}_5\text{-N-C}_5$  intermediate. This difference in the biosynthetic construction of the two ring systems, which had hitherto been regarded as biogenetic analogues, is borne out by the labelling patterns observed in the experiments with  $\underline{\text{R}}$ - and  $\underline{\text{S}}$ -(1- $^2\text{H}$ )putrescine and  $\underline{\text{R}}$ - and  $\underline{\text{S}}$ -(1- $^2\text{H}$ )cadaverine, respectively.

Only two of the six C-N bonds within the skeleton of sparteine and of lupanine are derived intact from a C-N bond of cadaverine. This observation, as well as the distribution of  $^2\text{H}$  within samples of sparteine and lupanine, derived from chirally deuterated samples of cadaverine, is explicable in terms of the  $\Delta^1$ -piperidine-trimer model of the biosynthesis of these alkaloids. Other biogenetic hypotheses that have recently been advanced are inconsistent with the experimental data.

Retronecine is derived from  $\underline{\text{L}}$ -ornithine, the lupine alkaloids are derived from the  $\underline{\text{L}}$ -lysine. DeCarboxylation of the amino acids in the course of alkaloid biosynthesis takes place with net retention of configuration.

### 1. GENERAL INTRODUCTION

Three decades have elapsed since the publication of the first chemically rigorous tracer studies, using  $^{14}\text{C}$ , in the field of natural product biosynthesis. These studies, which were carried out in the laboratory of Léo Marion at the National Research Council of Canada in Ottawa, served as models for the many subsequent investigations which have led to the recognition of biosynthetic precursor-product relationships of most groups of natural products. As a result of the success of these investigations the view appears to be gaining ground that little remains to be done and that current investigations of natural product biosynthesis are nothing more than mopping-up operations in a dying field. Nothing could be further from the truth. In fact, only the easy problems have been solved. A much greater effort is now required to advance the state of knowledge in the field of natural product biosynthesis, than was the case in the past.

The primary precursors of most structural groups of natural products have indeed been identified and, in many cases, rigorously established by tracer methods. In most instances the results of these studies have substantiated the clairvoyant speculations of the pioneers of biogenetic analysis whose predictions lent direction to much of the early experimental work. However, the identity of the chemical intermediates which lie on the route from primary precursors to products, and the mechanism and stereochemistry of the

steps in the interconversion of these intermediates, have been established in few instances only. Study of the enzymes catalyzing these interconversions is likewise in its infancy. These are the directions current investigations must take.

There are reasons why, in addition, many of the problems in the field of natural product biosynthesis, that appear to have been solved, bear reexamination: Even though the tracer methodology, whereby speculative predictions based on structural analogy could be tested, has been available for more than thirty years, the tradition of non-experimental biogenetic speculation persists. Furthermore, in this field more than in any other area of bio-organic chemistry, investigators tend to push to the limit -- and beyond -- the interpretation of their results. Also, early radioactive tracer studies depended on simple counters and other unsophisticated equipment and were often carried out with commercially supplied radioactive substances which were not checked for contamination and very often contained unsuspected and unknown radioactive impurities. The products that were obtained were often contaminated with similarly undetected impurities.

Not only have the methods for determining radioactivity increased in sensitivity but new instrumental methods for the detection of stable isotopes have now become available which add new dimensions to the current methodology for biosynthetic studies.

It is my view that many of the old problems must be re-examined not only to substantiate currently accepted concepts of precursor-product relationships, but also to add depth to present knowledge, by probing the pathways by the new methods which were not available when the original investigations took place.

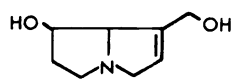
In this lecture I will discuss the results of our re-examination of the biosynthesis of the pyrrolizidine ring system, as represented by retronecine, the heterocyclic component of many Senecio alkaloids, of the quinolizidine ring system, present in lupinine, the simplest of the Lupine alkaloids, and of the bisquinolizidine system present in sparteine and lupanine, two tetracyclic Lupine alkaloids.

It will be my objective to demonstrate that re-examination of these old problems by means of modern methods has led to significant advances in the understanding of the biosynthetic sequences leading to these alkaloids from primary precursors.

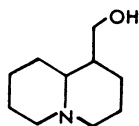
## 2. THE BIOSYNTHESIS OF RETRONECINE AND OF LUPININE

### (i) Symmetry in the biosynthetic routes to retronecine and to lupinine.

The structural homology of the ring skeletons of retronecine and lupinine (Scheme 1) is readily apparent. On the basis of this homology it was suggested by Sir Robert Robinson<sup>1a</sup> that the two ring systems originated in analogous fashion, one from ornithine and putrescine, the other from lysine and cadaverine. In each case two structural units derived from the diamine were thought to condense, yielding a "dimeric" aminodial with  $C_{2v}$  symmetry,  $C_4-N-C_4$  and  $C_5-N-C_5$ , respectively, as an intermediate. Intramolecular Mannich reaction would then yield the pyrrolizidinealdehyde and the quinolizidinealdehyde which were regarded as the progenitors of retronecine and of lupinine, respectively (Scheme 2). Biogenetically modelled syntheses<sup>2,3</sup> supported these speculative proposals which represent the cornerstones of biogenetic thinking: Dissection of the target molecule into subunits related to primary metabolites, intermediates with facile reactivity, and structural analogy as a guide to biogenetic origin.

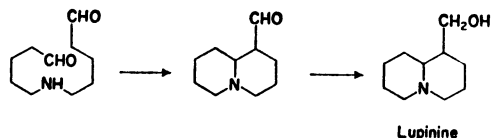
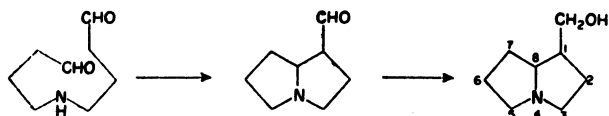


Retronecine



Lupinine

Scheme 1

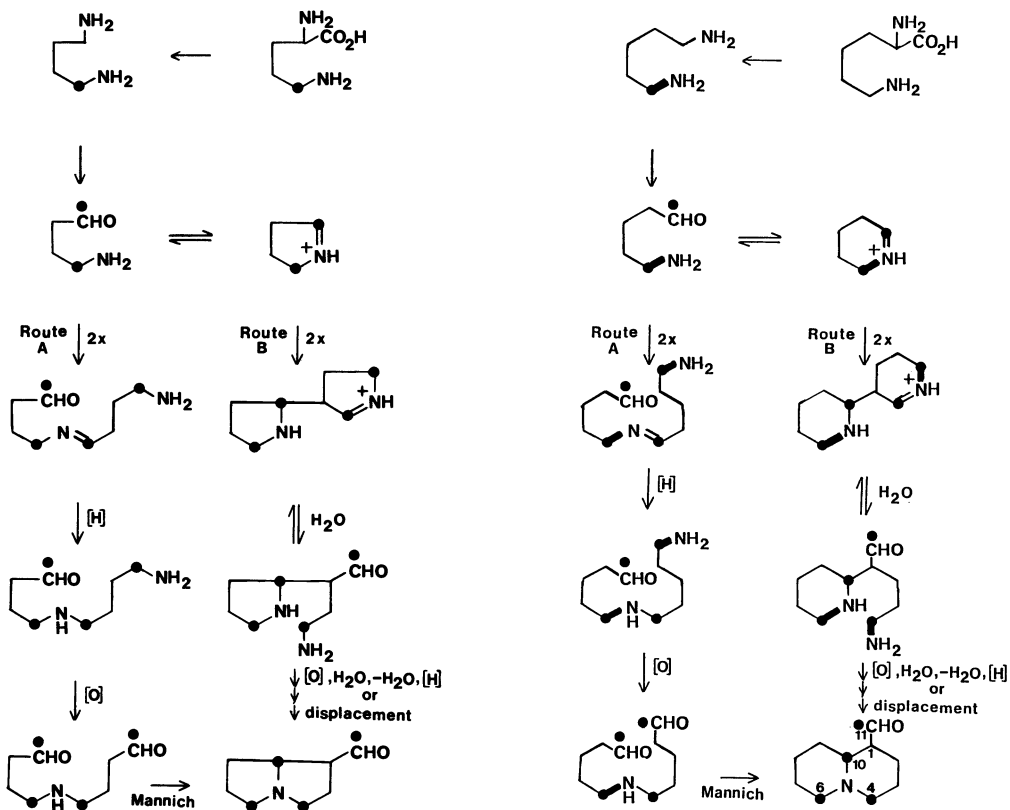


Lupinine

Scheme 2

When these ideas were tested experimentally by means of tracer experiments in which labelled samples of ornithine and putrescine, and of lysine and cadaverine, respectively, were administered to plants of Senecio and of Lupine species, labelled samples of retronecine<sup>4</sup> and of lupinine<sup>5</sup> were indeed obtained. Chemical degradation of the labelled samples of the alkaloids which was carried out in order to determine the sites of labelling led to results which were interpreted as showing that in each case the three carbon atoms adjacent to nitrogen and the carbon atom of the carbinol side chain each bore one quarter of the label derived, in the case of retronecine, either from DL-[2-<sup>14</sup>C]ornithine or DL-[5-<sup>14</sup>C]ornithine or from [1,4-<sup>14</sup>C]putrescine and, in the case of lupinine, either from DL-[2-<sup>14</sup>C]lysine or from [1,5-<sup>14</sup>C]cadaverine. This distribution of label was regarded as confirmation of the biogenetic proposals of Robinson that retronecine was derived from two C<sub>4</sub>N units generated from ornithine via the "symmetrical" (i.e. C<sub>2v</sub>) monomer, putrescine (Scheme 3, Route A), and that lupinine originated from two C<sub>5</sub>N units derived from lysine via the "symmetrical" (i.e. C<sub>2v</sub>) monomer, cadaverine (Scheme 4, Route A), and that in each case a "symmetrical" (i.e. C<sub>2v</sub>) "dimeric" intermediate was a further stage on route to the alkaloids.

It was completely overlooked that the same distribution could arise in several other ways as well. Thus, if putrescine and cadaverine were obligatory monomeric intermediates, then the observed distribution of label from each of the substrates which were tested would have resulted even without a "symmetrical dimeric" intermediate. If, on the other hand, a "symmetrical dimeric" intermediate is implicated, then putrescine and cadaverine, respectively, would not have to lie on the routes from ornithine or lysine, respectively, into the alkaloids in order to obtain the observed distribution.

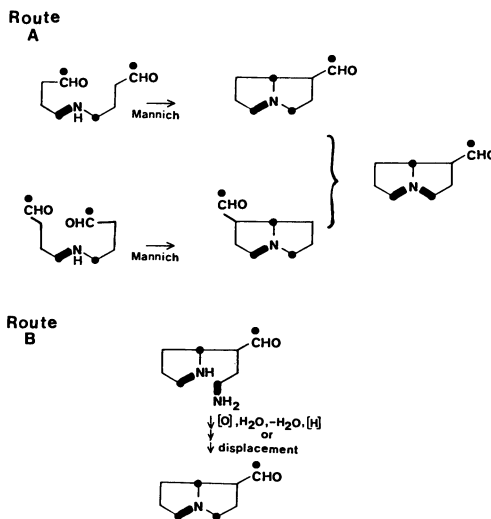


Scheme 3: Two routes from ornithine into retronecine.

Scheme 4: Two routes from lysine into lupinine.

Route A, via a symmetrical (i.e. nondissymmetric, C<sub>2v</sub>), 'dimeric' intermediate; Route B, via dissymmetrical (C<sub>1</sub>) dimeric intermediates.

Two variants of the route from ornithine into retronecine are shown in Scheme 3, and from lysine into lupinine in Scheme 4. Experiments with carbon-labelled substrates cannot discriminate among these variants. What distinguishes the variants from one another is the mode of entry of an intact C-N unit: In each case, Route B proceeds through a set of dimeric intermediates which are dissymmetric ( $C_1$ ) and leads to the product in a structurally unique manner. Route B thus yields a sample of alkaloid in which only one of the three C-N bonds represents an intact C-N unit of the precursor. Route A, on the other hand, proceeds via a "dimeric" intermediate with  $C_{2v}$  symmetry which can yield product in either one of two equivalent ways. Even though the  $C_{2v}$  intermediate contains only one C-N bond derived intact from the monomeric precursor, the product will consist of an equimolar mixture of two species, each of which carries this intact C-N bond at a different site. Scheme 5 illustrates the different modes of entry of an intact C-N unit supplied by putrescine into retronecine.



Scheme 5

The routes via a symmetrical ( $C_{2v}$ ) or a dissymmetric ( $C_1$ ) dimer could be distinguished if it were possible to label a C-N bond. Such "bond-labelling" is in fact possible: Since  $^{15}\text{N}$  has a nuclear spin of  $1/2$ , the  $^{13}\text{C}$  NMR signal due to  $^{13}\text{C}$  adjacent to  $^{15}\text{N}$  appears as a doublet. A tracer experiment with substrate labelled intramolecularly with  $^{15}\text{N}$  and with  $^{13}\text{C}$  at the adjacent carbon atom can serve as a probe for the intact transfer of a C-N unit. If the  $^{13}\text{C}$ - $^{15}\text{N}$  unit has been transferred intact into the biosynthetic product, the signal due to the carbon atom in the  $^{13}\text{C}$  NMR spectrum of the product should appear as a doublet.

Since, in practice, the plant to which the doubly enriched substrate is administered will contain its own endogenous natural abundance supply of product, often in large excess, the signal due to the carbon atom in question will appear as a  $^{13}\text{C}$ - $^{15}\text{N}$  doublet straddling a natural abundance  $^{13}\text{C}$  singlet.

Furthermore, it is customary to administer  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched samples in admixture with natural abundance material, in order to minimize the possibility that C-N bonds, formed *de novo* in the course of metabolism, show  $^{13}\text{C}$ - $^{15}\text{N}$  enrichment of a magnitude similar to that of the C-N units transferred intact from the substrate. It is also desirable to add radioactive tracer as a ready means of determining isotope dilution.

By employing samples of putrescine<sup>4</sup> and of cadaverine<sup>5</sup>, intramolecularly doubly labelled, with  $^{15}\text{N}$  at one of the amino groups and with  $^{13}\text{C}$  at the adjacent carbon atom, the routes from the amines into the alkaloids were examined. If the biosynthetic sequence proceeds by way of the  $C_{2v}$  route (Route A), product must consist of a 50/50 mixture of two species which carry the  $^{13}\text{C}$ - $^{15}\text{N}$  unit, transferred intact from substrate, at two different sites. Two of the carbon atoms adjacent to nitrogen will thus show a  $^{13}\text{C}$ -NMR signal appearing as a doublet, or a doublet straddling a singlet. Since the  $C_{2v}$  symmetry of the intermediate demands that the two species make an equimolar contribution to the product, the two sets of signals must have identical signal areas and, furthermore, must show identical ratios of signal area of doublet/signal area of singlet. If the two multiple signals are not identical, a "dimeric" intermediate with  $C_{2v}$  symmetry cannot lie on the pathway. If the biosynthetic sequence proceeds by way of the  $C_1$  route (Route B) only a single doublet (or doublet straddling a singlet) will appear in the  $^{13}\text{C}$  NMR spectrum of the product.

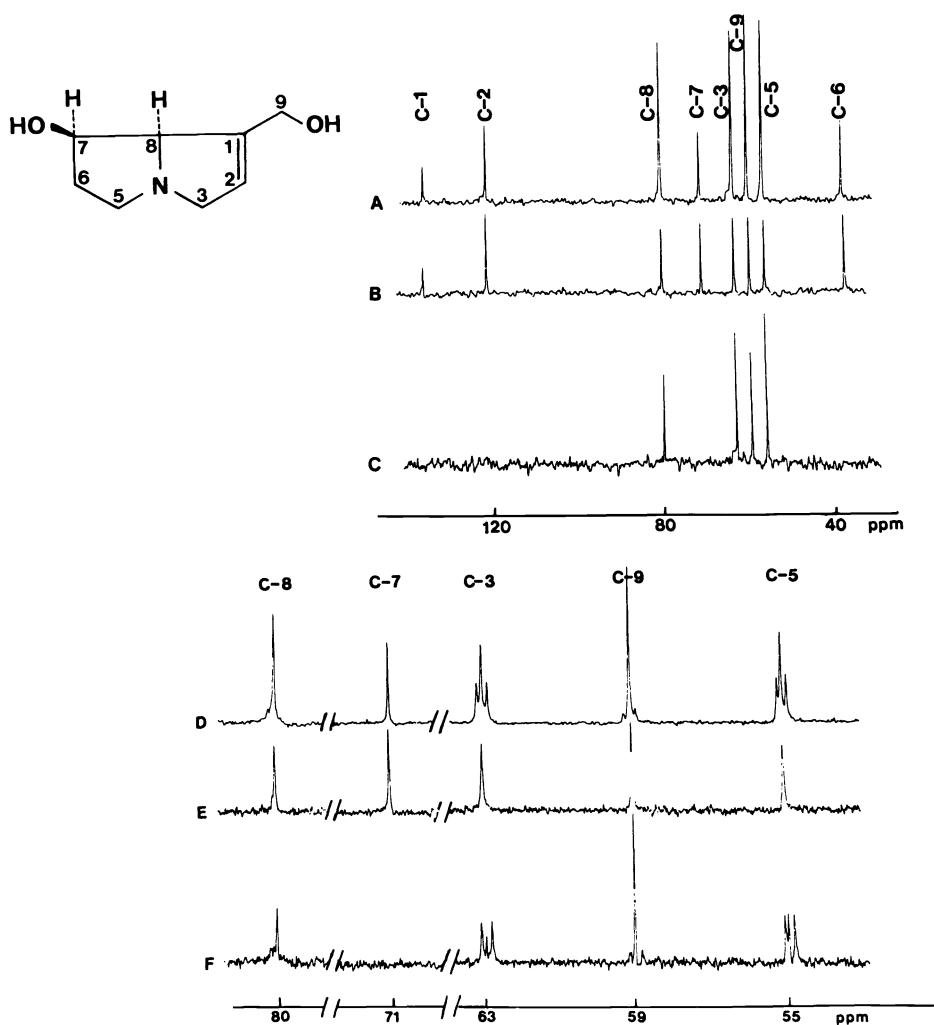


Figure 1: 20.15 MHz  $^{13}\text{C}$  NMR spectra of retronecine hydrochloride. A: proton noise decoupled (PND) spectrum of  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched sample, derived from  $(1-^{13}\text{C}, 1-^{15}\text{N})$ putrescine. B: PND spectrum of natural abundance sample. C: difference spectrum (A minus B). D: PND spectrum (narrow window) of  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched sample. E: PND spectrum of natural abundance sample. F: difference spectrum (D minus E) (Reference 4).

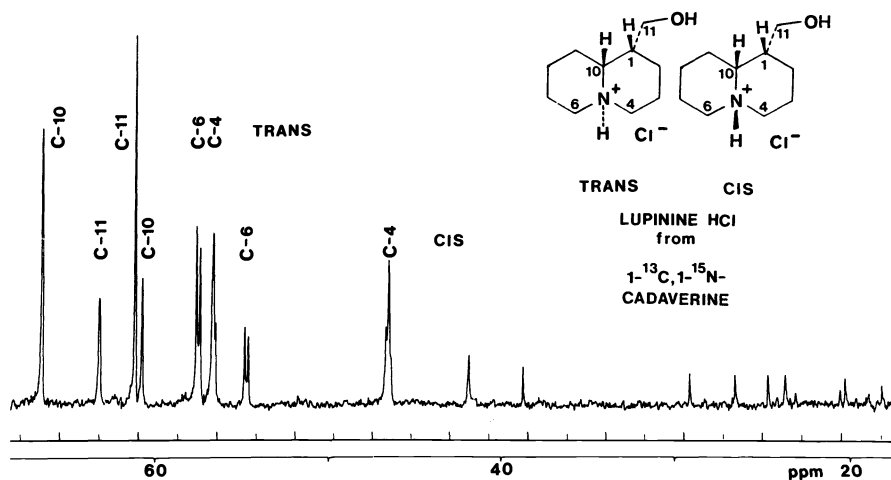


Figure 2: 20.15 MHz  $^{13}\text{C}$  NMR (PND) spectrum of  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched sample of lupinine hydrochloride derived from  $(1-^{13}\text{C}, 1-^{15}\text{N})$ cadaverine (Reference 5).

The situation is somewhat complicated by the fact that new C-N bonds, not present in the administered doubly labelled substrate, are formed in the course of the biosynthetic sequence. It is unavoidable that some of these new bonds will be between  $^{13}\text{C}$ - and  $^{15}\text{N}$ -enriched atoms (rather than  $^{12}\text{C}^{14}\text{N}$ ,  $^{12}\text{C}^{15}\text{N}$  or  $^{13}\text{C}^{14}\text{N}$ ), derived intermolecularly from two enriched monomer units.  $^{13}\text{C}$  atoms, other than those transferred from doubly labelled substrate as intact  $^{13}\text{C}^{15}\text{N}$  units, will thus give rise to  $^{13}\text{C}$  NMR doublets. In general the intensity of such doublets, relative to that of the singlets they straddle, is much lower than that of the signal due to  $^{13}\text{C}$  of the  $^{13}\text{C}^{15}\text{N}$  unit that was transferred intact.

The  $^{13}\text{C}$  NMR spectrum of a sample of retronecine<sup>4</sup> obtained from *Senecio vulgaris* plants to which intramolecularly doubly labelled (1- $^{13}\text{C}$ ,1- $^{15}\text{N}$ )putrescine had been administered is shown in Fig. 1, and that of a sample of lupinine<sup>5</sup> from *Lupinus luteus* fed with intramolecularly doubly labelled (1- $^{13}\text{C}$ ,1- $^{15}\text{N}$ )cadaverine in Fig. 2.

In each case four carbon atoms, three of them those adjacent to nitrogen and the fourth the carbinol carbon of the sidechain, are enriched with  $^{13}\text{C}$ . This enrichment pattern complements and completes the evidence of the distribution of label, obtained from experiments with the corresponding  $^{14}\text{C}$ -labelled substrates, but does not solve the question concerning the structure of the "dimeric" intermediate. This question is answered by the coupling patterns shown in the two spectra.

In the retronecine spectrum (Fig. 1) two of the carbon atoms adjacent to nitrogen, C-3 and C-5, appear as doublets superimposed on singlets. The two sets of signals show identical signal areas and identical ratios of the doublet and singlet areas. It follows that a dimeric intermediate with  $\text{C}_{2v}$  symmetry lies on the pathway from putrescine into cadaverine.<sup>4</sup> This pathway is then represented by Route A, Scheme 3.

In the corresponding spectrum of lupinine (Fig. 2) the ratio of the areas due to doublet and singlet in the signal due to C-6 is much larger than that in the signal due to C-4 (Fig. 3). This finding disproves the intermediacy of a "dimeric" compound with  $\text{C}_{2v}$  symmetry on route from cadaverine into lupinine<sup>5</sup> (Route A, Scheme 4). Route B, Scheme 4<sup>7</sup> on the other hand, is entirely consistent with the experimental observation.

#### trans-LUPININE HYDROCHLORIDE

FROM  $^{15}\text{NH}_2\text{---}^{13}\text{CH}_2\text{---CH}_2\text{---CH}_2\text{---CH}_2\text{---CH}_2\text{---NH}_2$

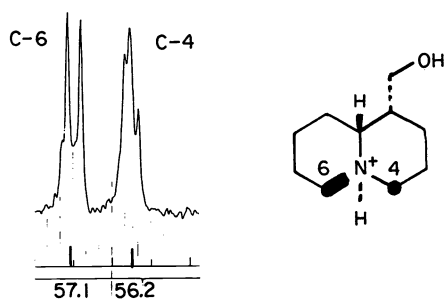
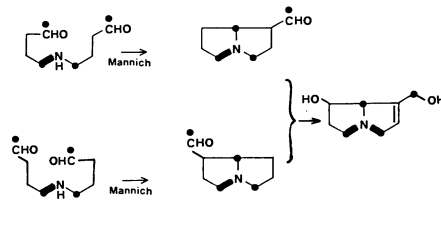


Figure 3: Expanded segment of Figure 2.

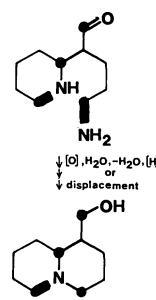
#### RETRONECINE

via  
Route  
A



#### LUPININE

via  
Route  
B



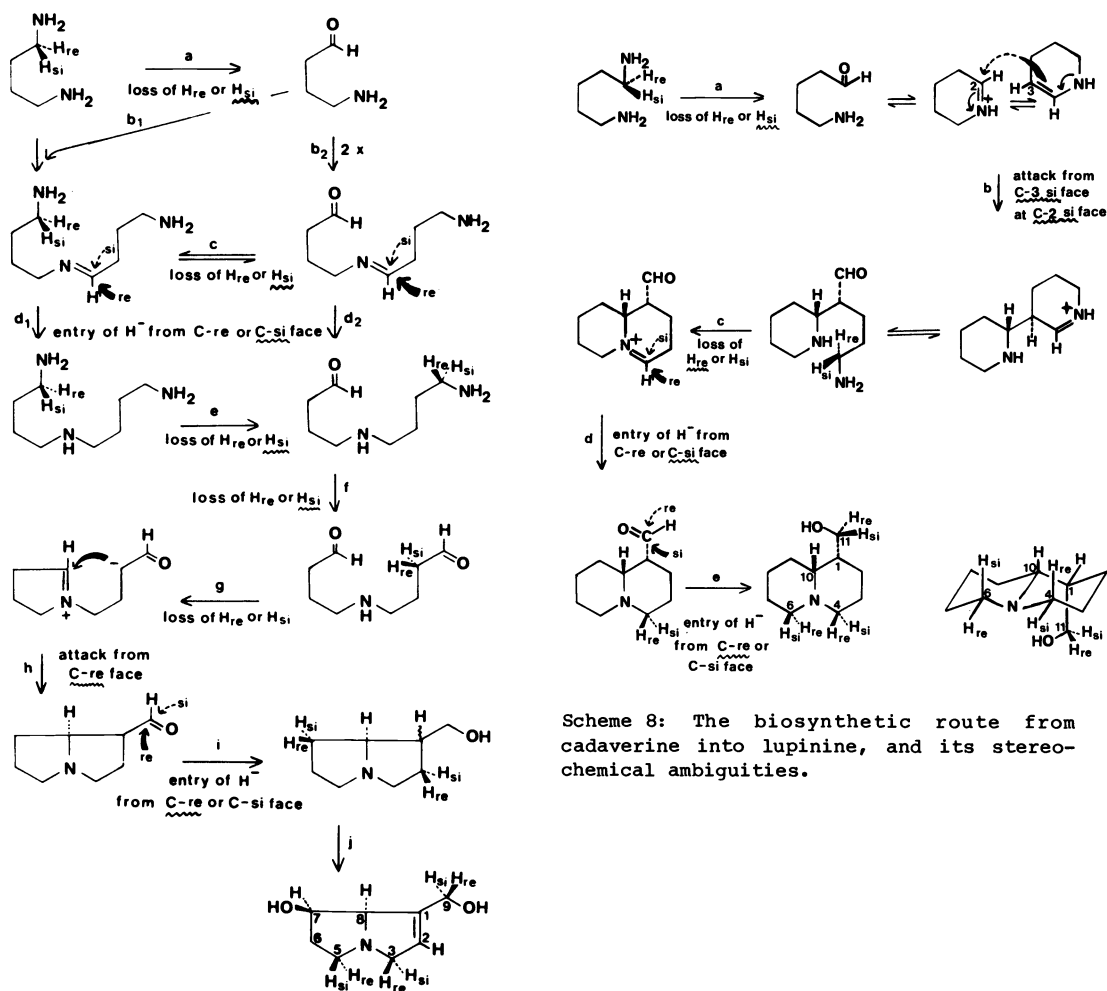
Scheme 6

Retronecine and lupinine are structurally homologous and this homology is reflected in their origins from homologous primary precursors, ornithine and putrescine in the one case and lysine and cadaverine in the other. But that is as far as homology extends. The biosynthetic route leading from putrescine into retronecine is entirely different from that leading from cadaverine into lupinine. This difference is summarized in Scheme 6.

Contrary to conventional wisdom, structural analogy is not a guide to biosynthetic processes.

## (ii) Stereochemistry in the biosynthetic routes to retronecine and to lupinine.

There is more to be learned about the routes from the diamines into the alkaloids. In particular there are, in each case, several stereochemical questions that must be answered. These questions are posed in Schemes 7 and 8, which show the most likely steps from putrescine into retronecine via a "dimeric"  $C_{2v}$  intermediate (Scheme 3, Route A) and from cadaverine into lupinine by a sequence which excludes a dimeric  $C_{2v}$  intermediate (Scheme 4, Route B). Each of the two sequences shown in Schemes 7 and 8 includes a step in which the stereochemistry of the ring junction of the product is determined. The stereochemical course of these steps can be inferred from the known chirality of the products. But, in addition, there are, in each of the two sequences, several steps which involve hidden stereochemistry. In each of these steps a diastereotopic proton is lost from or added to a prochiral centre.



Scheme 8: The biosynthetic route from cadaverine into lupinine, and its stereochemical ambiguities.

Scheme 7: The biosynthetic route from putrescine into retronecine and its stereochemical ambiguities

We have determined the stereochemistry of these steps by means of experiments with chirally deuterated precursors. In separate feeding experiments, samples of  $R$ - and  $S$ -(1- $^2H$ )putrescine<sup>6</sup> were administered to *Senecio vulgaris*<sup>7</sup>, and samples of  $R$ - and  $S$ -(1- $^2H$ )-cadaverine<sup>6</sup> to *Lupinus luteus*.<sup>8</sup> The sites of deuteration within the alkaloids that were isolated from these experiments were determined by  $^2H$  NMR spectroscopy. Assignment of the signals in the  $^2H$  NMR spectra of the *Senecio* alkaloids and of lupinine was facilitated by published data.<sup>7,8</sup>

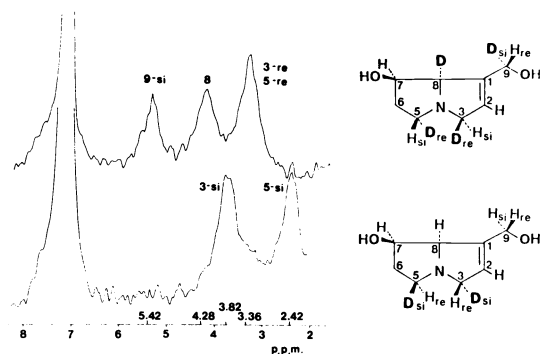


Figure 4: 38.40 MHz  $^2\text{H}$  NMR spectra of (top) a sample of Senecio alkaloids obtained from administration of (R)-(1- $^2\text{H}$ )putrescine dihydrochloride and of (bottom) a sample obtained from administration of (S)-(1- $^2\text{H}$ )putrescine dihydrochloride (Reference 7).

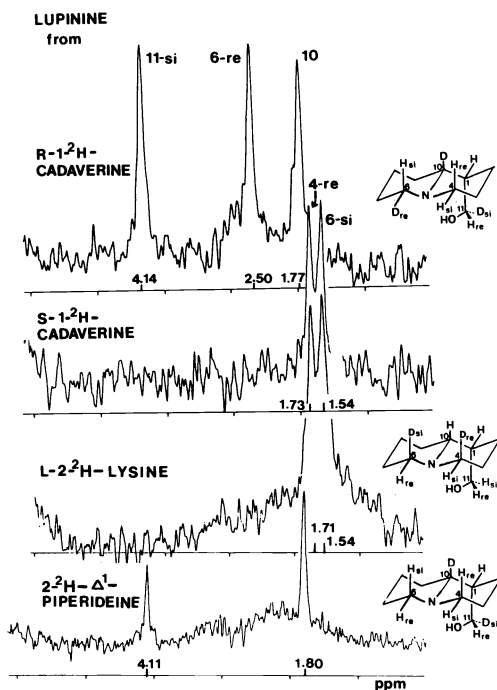


Figure 5:  $^2\text{H}$  NMR spectra (38.40 MHz) of lupinine samples obtained from administration of  $^2\text{H}$  labelled substrates (Reference 8 and unpublished results).

The  $^2\text{H}$  NMR spectra of the retronecine-containing Senecio alkaloids isolated in these experiments are shown in Fig. 4 and the  $^2\text{H}$  NMR spectra of lupinine in Fig. 5. From these spectra the stereochemistry of the biosynthetic steps that implicate the carbon atoms adjacent to nitrogen and the carbon atom of the carbinol sidechain can be deduced. The stereochemical questions answered by these experiments are shown in Scheme 7 and Scheme 8. The answers to the stereochemical questions are shown in bold face (e.g.  $\text{H}_{\text{si}}$ ). In each case loss of the si proton accompanies the conversion of the diamine into the aminoaldehyde (step a). This is the stereochemistry which would be expected if removal of the proton were catalyzed by a plant diamine oxidase<sup>6</sup> or by an L-amino acid transaminase.

In each case entry of hydride in the reduction of a  $\text{C}=\text{N}$  or  $\text{C}=\text{N}^+$  bond (step d) takes place from the C-si face. In each case entry of hydride in the reduction of the sidechain aldehyde group (Scheme 7, step i; Scheme 8, step e) takes place from the C-re face of the carbonyl carbon.

The one significant difference in the deuterium distribution of the alkaloids reflects the difference in the pathways, one via a "dimeric" intermediate with  $\text{C}_{2v}$  symmetry, the other via a dissymmetric dimer. The two alkaloid samples derived from the S-isomer of the chirally deuteriated (1- $^2\text{H}$ )diamine both show a deuterium at each of the two methylene groups adjacent to nitrogen. In the case of the Senecio alkaloids (Fig. 4) both these deuterons are in the si position, one  $\beta$  (at C-5), the other  $\alpha$  (at C-3). This distribution is a necessary consequence of the intermediacy of a "dimer" with  $\text{C}_{2v}$  symmetry. In the case of lupinine (Fig. 5) one of these deuterons is in the si position ( $\beta$ , at C-6) but the other is in the re position ( $\beta$ , at C-4). This distribution provides supporting evidence that a "dimer" with  $\text{C}_{2v}$  symmetry cannot lie on the route to lupinine.

The results of the experiments with  $^2\text{H}$ -labelled and with  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labelled diamines are complementary and in full agreement with one another.

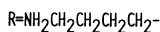
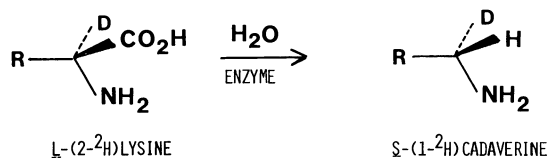


The proposed reaction sequence (Scheme 8) postulates that  $\Delta^1$ -piperidine serves as an intermediate between cadaverine and lupinine. The validity of the proposed sequence, and its stereochemistry, can be further tested by means of a feeding experiment with deuterium labelled  $\Delta^1$ -piperidine. If the conclusions drawn from the experiments with chirally deuterated cadaverine are correct, it can be predicted that deuterium from C-2 of  $\Delta^1$ -piperidine must enter at C-10 and at C-11si of lupinine. The  $^2\text{H}$  NMR spectrum of a sample of lupinine derived from (2- $^2\text{H}$ )- $\Delta^1$ -piperidine is shown in Fig. 5. It is evident that the predictions are fully substantiated. This result provides further evidence in support of the sequence shown in Scheme 8.

Two further stereochemical questions, associated with the biosynthesis of the two alkaloids under study, should be answered. The first, a question of chirality, concerns the stereochemistry, D or L, of the amino acids, ornithine and lysine, respectively, which serve as precursors of the alkaloids. The second question concerns the prochirality of the decarboxylation of the amino acids to yield the diamines, putrescine and cadaverine. Does this decarboxylation take place with net retention or with net inversion of configuration?

We have answered the first question for retronecine as well as for lupinine biosynthesis. By experiments with intermolecularly doubly  $^3\text{H}$ ,  $^{14}\text{C}$ -labelled samples of ornithine and of lysine, respectively, it was shown that in each case it is the L-amino acid which serves as the precursor of the alkaloids. Thus, administration of a sample of L-[4- $^{14}\text{C}$ ]lysine in admixture with DL-[6- $^{14}\text{C}$ ]lysine ( $^3\text{H}/^{14}\text{C}$  ratio  $4.1 \pm 0.1$ ) to *L. luteus* gave a sample of lupinine with a  $^3\text{H}/^{14}\text{C}$  ratio  $8.4 \pm 0.1$  (predicted  $^3\text{H}/^{14}\text{C}$  ratios of lupinine if L-lysine were the precursor: 8.2; for D-lysine: 0). Analogous experiments with doubly  $^3\text{H}$ ,  $^{14}\text{C}$ -labelled samples of ornithine showed that the L-isomer of ornithine serves as the precursor of retronecine.

The question of the prochirality of the decarboxylation of lysine in *Lupinus luteus* was solved by a feeding experiment with DL-(2- $^2\text{H}$ )lysine. The  $^2\text{H}$  NMR spectrum of the lupinine obtained in this experiment (Fig. 5) was identical with that obtained when S-(1- $^2\text{H}$ )-cadaverine served as the substrate. The experiment with doubly  $^3\text{H}$ ,  $^{14}\text{C}$ -labelled lysine established that only L-lysine serves as the precursor of lupinine. The experiment with DL-(2- $^2\text{H}$ )lysine shows that this L-lysine enters lupinine via S-(1- $^2\text{H}$ )cadaverine. It follows that, in *L. luteus*, decarboxylation of L-lysine to yield cadaverine takes place by replacement of the carboxyl group by a proton with net retention of configuration (Scheme 9). This is in accord with the known stereochemistry of other amino acid decarboxylases.<sup>6</sup>



Scheme 9

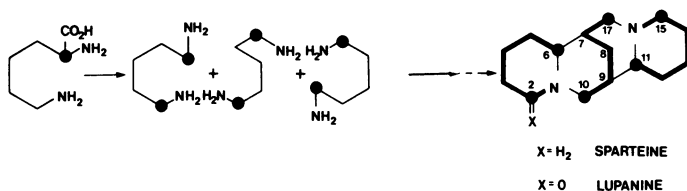
### (iii) Conclusion

The experiments with  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labelled and the  $^2\text{H}$ -labelled substrates yield results which fully substantiate the sequences, outlined in Scheme 3, Route A, for the biosynthesis of retronecine and in Scheme 4, Route B, for the biosynthesis of lupinine. Stable isotopes, in concert with  $^{13}\text{C}$  NMR and  $^2\text{H}$  NMR spectrometry, have yielded insights into the details of the biosynthetic sequences leading to these alkaloids, far beyond what was possible to observe by classical radioactive tracer methods.

## 3. THE BIOSYNTHESIS OF SPARTEINE AND LUPANINE

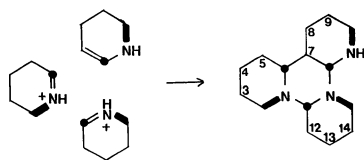
(i) The regiochemistry of biosynthesis

Early tracer experiments carried out by H. R. Schütte and his associates<sup>9</sup> substantiated the view of Sir Robert Robinson<sup>1b</sup> that the bisquinolizidine lupine alkaloids, e.g., sparteine, originated from three C<sub>5</sub> chains derived from lysine or cadaverine, and that the two nitrogen atoms were also supplied by these precursors. These experiments disposed of many of the early biogenetic hypotheses of the origin of these bases, hypotheses which implicated such diverse building blocks as acetonedicarboxylic acid, nicotinic acid or formaldehyde.

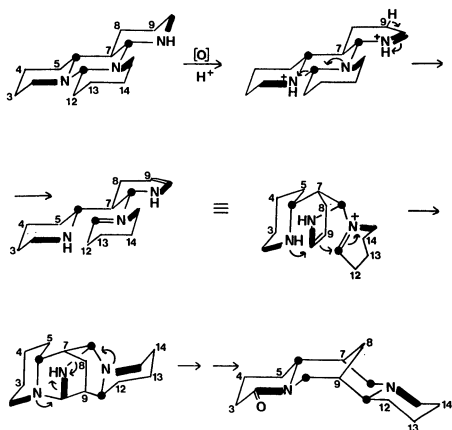


Scheme 10: Incorporation of lysine and cadaverine into sparteine and lupanine.

The model for the biosynthetic anatomy of sparteine and lupanine (Scheme 10) which was put forward on the basis of these tracer results did not address questions of mechanism -- clearly cadaverine itself does not possess the chemical reactivity required to generate the C<sub>15</sub>N<sub>2</sub> skeleton of the alkaloids -- or questions of stereochemistry. Almost ten years ago we advanced a biogenetic hypothesis of the origin of the C<sub>15</sub>N<sub>2</sub> lupine alkaloids<sup>10</sup> which accounted for the steps of their origin from cadaverine, as well as for their stereochemistry. This hypothesis, which is entirely consistent with the tracer evidence on the mode of incorporation of lysine<sup>9</sup> (or, more specifically, L-lysine<sup>11</sup>) and cadaverine<sup>9</sup>, views the C<sub>15</sub>N<sub>2</sub> lupine alkaloids as modified trimers of  $\Delta^1$ -piperideine, a compound which, as was seen earlier in this lecture, is derivable from cadaverine by oxidative deamination or by transamination. The observed stereochemistry of the alkaloids is accounted for by their derivation, in a four step sequence (Scheme 11), from the favoured all-trans stereoisomer of isotripiperideine, one of the trimers of  $\Delta^1$ -piperideine.

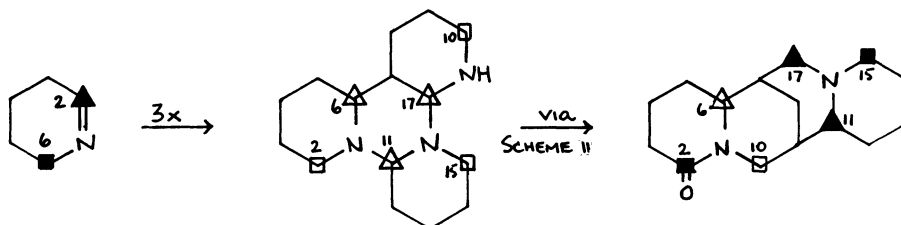
Trimerization of  $\Delta^1$ -piperideine

## Formation of lupanine skeleton



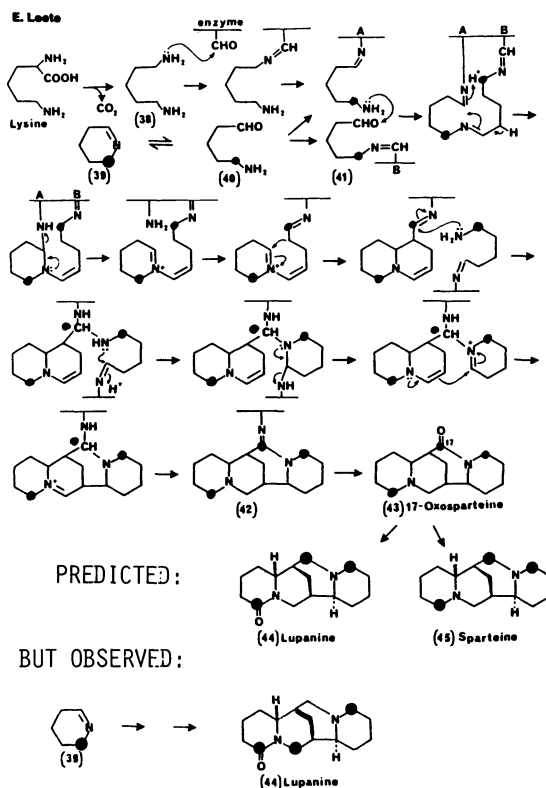
Scheme 11

The observed<sup>10</sup> mode of incorporation, into lupanine, of label from [2-<sup>14</sup>C]- and from [6-<sup>14</sup>C]- $\Delta^1$ -piperidine supported this biogenetic model. As predicted, label from [6-<sup>14</sup>C]- $\Delta^1$ -piperidine enters C-2, C-15 and, presumably, C-10 of lupanine, whereas label from [2-<sup>14</sup>C]- $\Delta^1$ -piperidine enters C-11, C-17 and, presumably, C-6 (Scheme 12). This mode of entry of  $\Delta^1$ -piperidine invalidates a recent biogenetic proposal<sup>12</sup> (Scheme 13), which predicts a different distribution of label from  $\Delta^1$ -piperidine. It also invalidates the early notion<sup>9</sup> that the central C<sub>5</sub>-unit of the alkaloids was derived via glutardialdehyde, a molecule with C<sub>2v</sub> symmetry, since the mode of incorporation of  $\Delta^1$ -piperidine is inconsistent with the intermediacy, beyond cadaverine, of any compound with C<sub>2v</sub> symmetry.



Scheme 12

### Biosynthesis of the Quinolizidine Alkaloids via Enzyme-Bound Intermediates



Scheme 13

The  $\Delta^1$ -piperidine trimer model of the biosynthesis of lupanine and sparteine is entirely consistent with the experimental observations and also accounts<sup>10</sup> for the correct relative stereochemistry at the four chiral centres of sparteine and lupanine.

Experiments with intramolecularly doubly  $^{13}\text{C}$ ,  $^{15}\text{N}$  labelled cadaverine in *Lupinus luteus*, to yield sparteine<sup>5</sup>, and in *L. angustifolius*, to yield lupanine<sup>13</sup>, lead to results which further substantiate the model. In each case six carbon atoms, those adjacent to the two nitrogen atoms, are enriched in  $^{13}\text{C}$ . This confirms, complements and completes the evidence generated with  $^{14}\text{C}$ -labelled precursors. But only two of the six carbon atoms adjacent to nitrogen within lupanine (Fig. 6) and sparteine (Fig. 7), namely C-2 and C-15, yield  $^{13}\text{C}$  NMR signals which show intense doublets due to  $^{13}\text{C}$ - $^{15}\text{N}$  coupling. The piperidine-trimer model is indeed consistent with these observations. The model predicts that the only C-N bonds within the alkaloids which are transferred intact from cadaverine are C-2, N-1 and C-15, N-16.

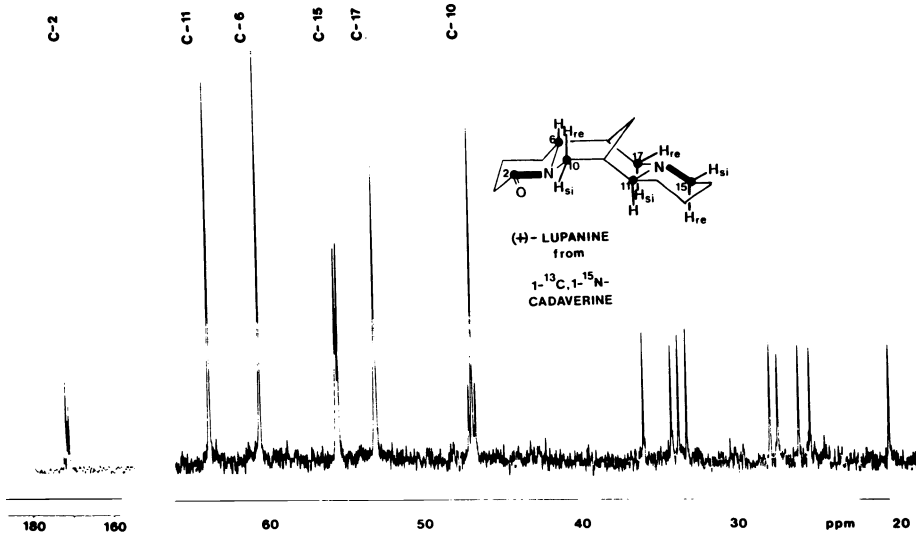


Figure 6: 20.15 MHz  $^{13}\text{C}$  NMR (PND) spectrum of  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched sample of lupanine derived from (1- $^{13}\text{C}$ , 1- $^{15}\text{N}$ )cadaverine (Reference 13).

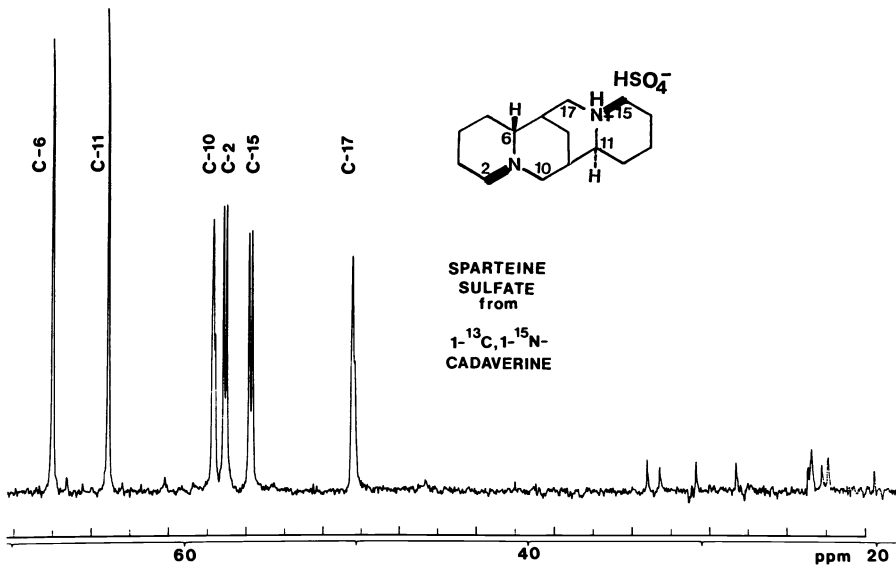
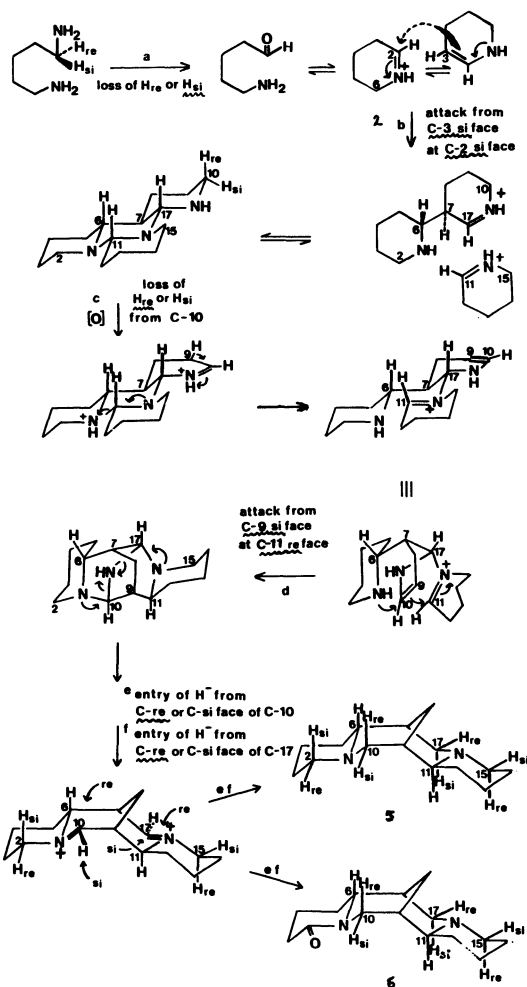


Figure 7: 20.15 MHz  $^{13}\text{C}$  NMR (PND) spectrum of  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched sample of sparteine sulfate derived from (1- $^{13}\text{C}$ , 1- $^{15}\text{N}$ )cadaverine (Reference 5).

(ii) The stereochemistry of biosynthesis

As in the case of retronecine and lupanine, the biosynthetic sequence raises a number of stereochemical questions. These are posed in Scheme 14. Two of the steps (steps b and d) determine the stereochemistry at the four chiral centres of the alkaloids. The steric course of these steps can be predicted from the known chirality at C-6, C-7, C-9 and C-11 of sparteine and lupanine.

But in addition, as in the previous instances, there are several steps involving hidden stereochemistry which can be unmasked by means of experiments with chirally deuteriated substrates.



Scheme 14

The  $^2H$  NMR spectra of the samples of lupanine, from *L. angustifolius*, and of sparteine, from *L. luteus*, obtained in these experiments<sup>13</sup>, are shown in Figs. 8 and 9. Assignment of the signals was again facilitated by published data. Lupanine from  $R$ -(1- $^2H$ )cadaverine maintains  $^2H$  at C-6, C-11, C-17<sub>si</sub> and at C-15<sub>re</sub>. Sparteine, from the same substrate, is labelled at the same sites, as well as at C-2<sub>re</sub>. Lupanine from  $S$ -(1- $^2H$ )cadaverine and from  $L$ -(2- $^2H$ )lysine maintains  $^2H$  at C-10<sub>si</sub> and C-15<sub>si</sub>. Sparteine, from the same substrates, is labelled at the same protons, as well as at C-2<sub>si</sub>. Interpretation of these labelling patterns yields the answers to the stereochemical questions posed. These answers are underlined (e.g.,  $H_{si}$ ) in Scheme 14.

On route to sparteine and to lupanine, cadaverine is converted into the aminoaldehyde with loss of the si-proton from C-1 (step a) as was the case in the biosynthesis of lupanine. As in the previous instances, entry of hydride in the reduction of  $C=N^+$  bonds takes place from the C-re face (steps e and f).

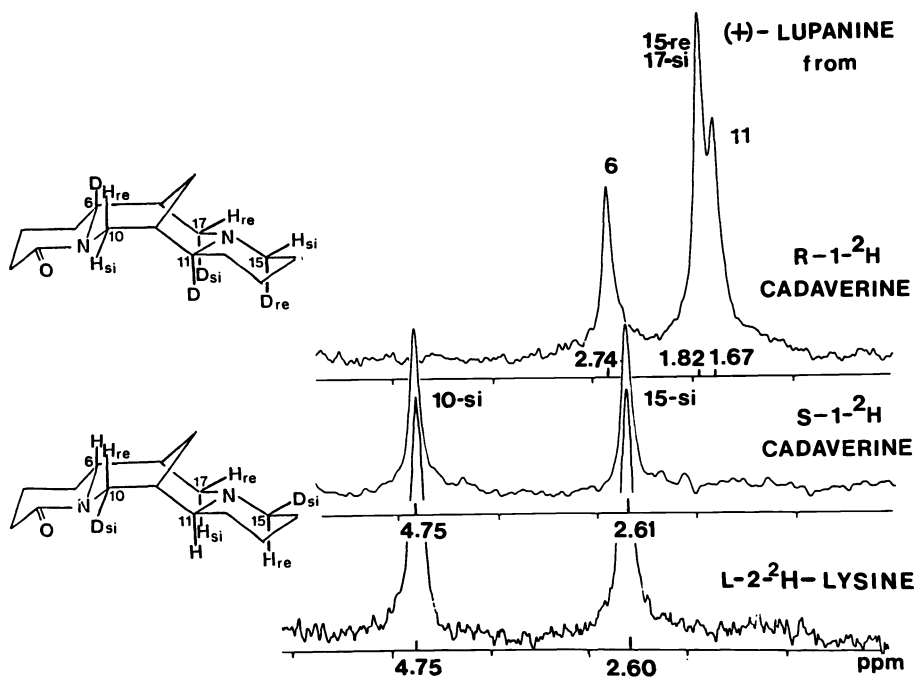


Figure 8:  $^2\text{H}$  NMR spectra (38.40 MHz) of lupanine samples obtained from administration of  $^2\text{H}$  labelled substrates (Reference 13).

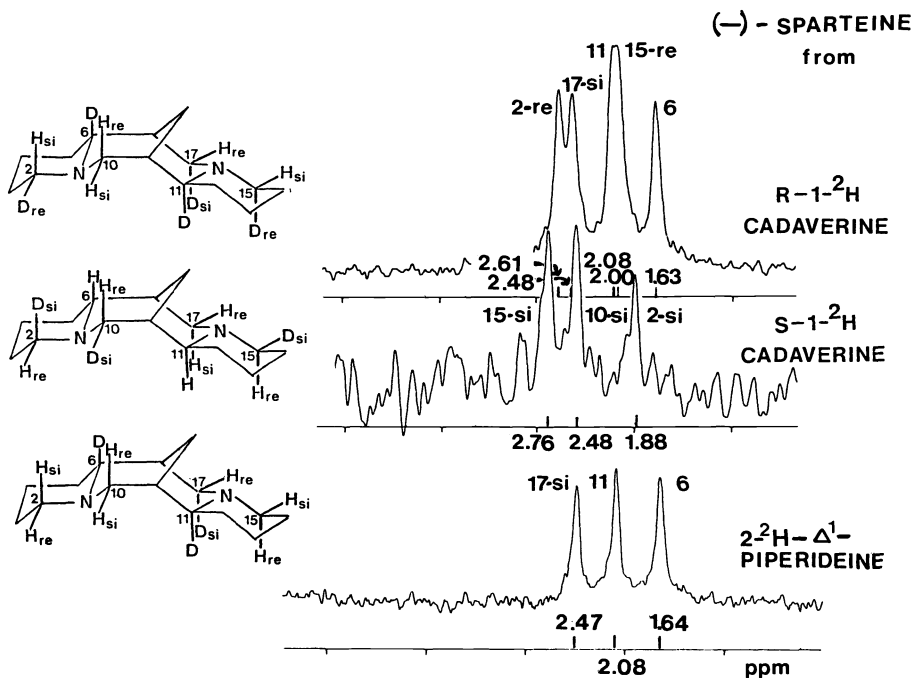


Figure 9:  $^2\text{H}$  NMR spectra (38.40 MHz) of sparteine samples obtained from administration of  $^2\text{H}$ -labelled substrates (Reference 13).

A crucial feature in the biosynthesis of sparteine and lupanine is the removal of the re proton from the carbon atom destined to become C-10 of the alkaloids (step c). This observation will be referred to again in the sequel.

If the sequence shown in Scheme 14 is correct, then (2-<sup>2</sup>H)- $\Delta^1$ -piperideine should deliver deuterium into C-6, C-11 and C-17<sub>si</sub>, but into no other sites of sparteine. Fig. 9 shows that this prediction is indeed confirmed, further validating the proposed biosynthetic sequence.

The fact the L-(2-<sup>2</sup>H)lysine yields samples of sparteine and lupanine with a deuterium distribution identical with that obtained when S-(1-<sup>2</sup>H)cadaverine served as the substrate confirms that decarboxylation of lysine takes place with net retention of configuration in L. luteus, the plant that elaborates lupanine (*vide supra*) and sparteine, and establishes the same course of reaction in L. angustifolius, the plant that generates lupanine.

### (iii) Interrelation among the alkaloids

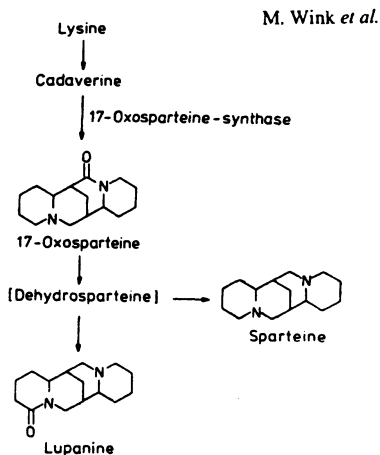
The deuteration patterns within the alkaloids derived from chirally deuteriated cadaverine establish the stereochemistry of the steps of the biosynthetic route. They also provide critical evidence which controverts several suggestions which have been made concerning the biosynthetic interrelationship among various C<sub>15</sub>N<sub>2</sub> lupine alkaloids.

It has been suggested that sparteine originates by reduction of lupanine<sup>14</sup> or by reduction of 1,2-dehydrosparteinium ion<sup>15</sup>. Since deuterium from S-(1-<sup>2</sup>H)- and from R-(1-<sup>2</sup>H)-cadaverine is stereospecifically maintained at C-2 of sparteine, the former at the si-site, the latter at the re-site, these suggestions are invalidated.

It has also been suggested<sup>10</sup> that 10-oxosparteine may be a biosynthetic precursor of sparteine and lupanine. Maintenance of deuterium from S-(1-<sup>2</sup>H)cadaverine and from L-(2-<sup>2</sup>H)lysine at the si-site of C-10, in both alkaloids, controverts this suggestion.

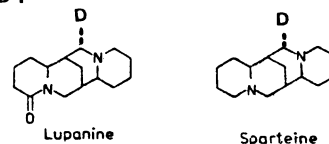
It has been claimed<sup>16</sup> that 17-oxosparteine is the first C<sub>15</sub>N<sub>2</sub> alkaloid to be synthesised by Lupinus polyphyllus, and that in this plant all other C<sub>15</sub>N<sub>2</sub> alkaloids, including sparteine and lupanine, are derived from 17-oxosparteine by further processes (Scheme 15). Stereospecific maintenance of deuterium from R-(1-<sup>2</sup>H)cadaverine at the si-site of C-17 of lupanine in L. angustifolius<sup>13</sup> and from R-(1-<sup>2</sup>H)cadaverine as well as from (2-<sup>2</sup>H)- $\Delta^1$ -piperideine at the si-site of C-17 of sparteine in L. luteus<sup>13</sup> shows that in these two lupine species 17-oxosparteine cannot be an intermediate on route from L-lysine, via cadaverine, 5-aminopentanal and  $\Delta^1$ -piperideine, into sparteine or into lupanine. It is, of course, possible that lupine alkaloid biosynthesis in L. polyphyllus differs from that in the two lupine species we have used in our investigations. It is more likely, however, that the 17-oxosparteine, which in L. polyphyllus is claimed to be the primary product of alkaloid biosynthesis, is in fact an artefact of isolation.

Possible pathway of lupanine biosynthesis.



PREDICTED: NO RETENTION OF <sup>2</sup>H  
AT C-17

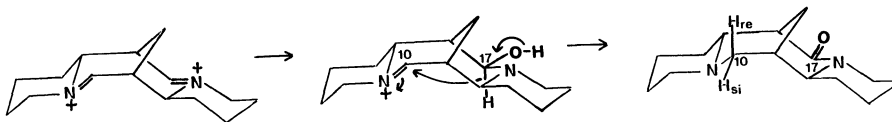
BUT OBSERVED:



RETENTION OF <sup>2</sup>H FROM  
(R)-(1-<sup>2</sup>H)CADAVERINE  
AT C-17

(iv) Conclusion

A rational reaction sequence whereby 17-oxosparteine may be generated as an artefact of isolation from the actual primary  $C_{15}N_2$  compound, 1,10;16,17-didehydrosparteinium ion (see Scheme 14) is shown in Scheme 16. It involves hydration of the C-17, N-16 bond, followed by intramolecular hydride transfer from C-17 to C-10 via a six membered transition state. This transformation is under study in our laboratory.



Scheme 16

Generation of 17-oxosparteine from three molecules of cadaverine is claimed<sup>16</sup> to take place in a single enzymic step which involves a concerted condensation sequence that is accompanied by loss of four of the six amino groups of the three cadaverine units, by reactions akin to transamination (Schemes 13 and 15).

Evidence has been presented in this lecture that, in the generation of sparteine and lupanine, precursor nitrogen remains attached to the carbon atoms destined to become C-2 and C-15, but is lost from the carbon atoms destined to become C-6, C-10, C-11 and C-17.

If nitrogen were to be removed from these four carbon atoms by a single enzyme, it would be expected that the stereochemical course of the reaction would be the same at each of the four sites. Our experiments show that this is not so. Whereas deuterium from *R*-(1-<sup>2</sup>H)-cadaverine is maintained at C-6, C-11 and C-17 of sparteine and lupanine, while deuterium from *S*-(1-<sup>2</sup>H)-cadaverine does not enter these positions, the opposite is the case with C-10. Here, deuterium from *S*-(1-<sup>2</sup>H)-cadaverine is maintained whereas deuterium from *R*-(1-<sup>2</sup>H)-cadaverine does not enter this site (Figs. 8 and 9). It is difficult to accept the notion that one and the same enzyme should exert one stereospecificity in the removal of three of four equivalent amino groups, but the opposite stereospecificity in the case of the fourth. The claim<sup>16</sup> that, in *L. polyphyllus* tissue, 17-oxosparteine is generated, as the first  $C_{15}N_2$  alkaloid, from cadaverine in a concerted reaction sequence catalyzed by a single enzyme clearly needs re-examination.

The  $\Delta^1$ -piperideine-trimer model for the biosynthesis of sparteine and lupanine, on the other hand, is consistent with all the evidence accumulated from radioactive tracer studies with <sup>14</sup>C and <sup>3</sup>H, bond labelling studies employing <sup>13</sup>C and <sup>15</sup>N, and from the stereochemical studies using chirally deuteriated substrates. A key feature of this hypothesis is the intermediacy of 1,10;16,17-didehydrosparteinium ion, from which (+)-lupanine and (-)-sparteine are separately derived.

## 4. CONCLUSION

In this lecture I have attempted to show that studies of natural product biosynthesis are very much alive. The incisiveness of the new methods which employ the stable isotopes of hydrogen and carbon as markers, rather than the radioactive isotopes of these elements, is due entirely to the availability of <sup>2</sup>H and <sup>13</sup>C NMR technology of a power which a few years ago was still undreamed of. Problems of regio- and stereochemistry of biosynthesis can now be tackled which were not accessible to investigation with radioactive labelling, and which, indeed, until a few years ago, were not even recognized as questions to be answered.

For this reason, as well as for some of the other reasons outlined in the introduction to this lecture, it will be profitable to reinvestigate many biosynthetic problems which appear to have been solved, or rather, whose solutions are accepted even though the experimental evidence on which these solutions appear to be based is, in fact, incomplete and often unsubstantiated.

New methods bring new insights but they can also bring new uncertainties. The methods employing <sup>13</sup>C NMR and <sup>2</sup>H NMR spectrometry in biosynthetic studies are no exception. I would like to conclude by illustrating one of the weaknesses of the application of stable isotopes, exemplified by a comparison of the results of two experiments on the incorporation of labelled samples of lysine into lupanine in *L. angustifolius*, one with (6-<sup>13</sup>C)-lysine, the other with [6-<sup>14</sup>C]lysine.



The  $^{13}\text{C}$ -NMR spectrum of the sample of lupanine isolated from the former experiment is shown in Fig. 10. As expected, six carbon atoms, C-2, -6, -10, -17, -11 and -15, are enriched. From the relative intensities of the enriched signals (after correction for the different relaxation time of the C=O signal at C-2) it is evident that, within experimental error, there is equal enrichment at the six centres, a result which is similar to that obtained when labelled samples of cadaverine served as precursors (Fig. 6).

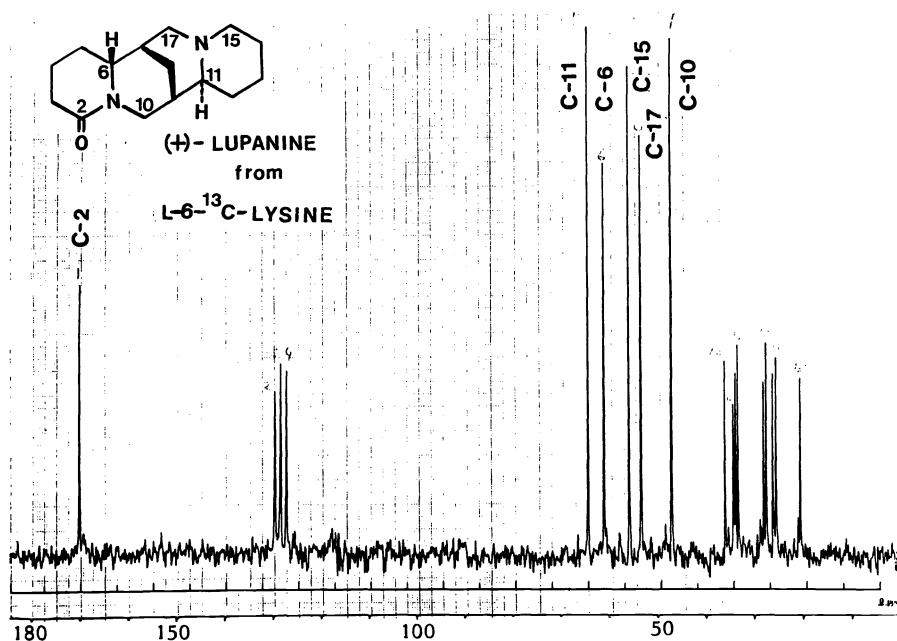
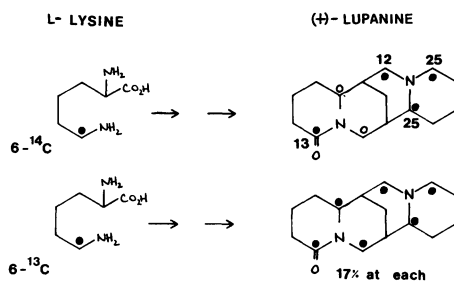


Figure 10: 20.15 MHz  $^{13}\text{C}$  NMR (PND) spectrum of  $^{13}\text{C}$ -enriched sample of lupanine, derived from  $\underline{\text{L}}$ -(6- $^{13}\text{C}$ )lysine (unpublished result).



Scheme 17

The result of the experiment with [6- $^{14}\text{C}$ ]lysine was qualitatively identical, in that the same six carbon atoms contained radioactivity (established by chemical degradation: C-2, C-17, C-11, C-15; inferred, C-6, C-10), but it was quantitatively different: Two of the labelled carbon atoms, C-11 and C-15, were each found to contain one quarter of the molar specific activity (dpm per mmole) of the intact alkaloid, whereas each of the other four (established by chemical degradation, C-2, C-17; inferred, C-6, C-10) contained one eighth of the total activity (Scheme 17).

The latter distribution is readily explicable in terms of the  $\Delta^1$ -piperidine-trimer model (Scheme 14) of the biosynthesis of lupanine: Carbon atoms 2, 6, 10 and 17 of the alkaloid arise from the  $\Delta^1$ -piperidine dimer which is formed in the initial stages of the pathway. The concentration of label at each of the four labelled centres of the dimer, derived from lysine via cadaverine and  $\Delta^1$ -piperidine, must be equal, i.e., the specific activity at these four centres must be the same. Trimer is formed by condensation of dimer with a third unit of  $\Delta^1$ -piperidine. If the steady state concentration of monomer within the plant is smaller than that of dimer, endogenous dilution of dimer with unlabelled material will be higher than that of monomer, so that the specific activity of dimer will be lower than that of monomer. It follows that, when trimer is formed, a monomer unit of higher specific activity (which will eventually generate the segment of lupanine containing C-11 and C-15) condenses with a dimer unit of lower specific activity. The distribution of label within the alkaloid formed from [6- $^{14}\text{C}$ ]lysine is thus accounted for. Differences in

$^{14}\text{C}$  specific activity between closely related substances can be maintained in an intact plant system since  $^{14}\text{C}$ -labelled materials are administered in trace amounts, so small that the endogenous concentration of metabolites is not changed and homeostasis is preserved. It is often overlooked that maintenance of the internal environment is one of the desirable conditions when a normal biosynthetic process is to be studied. It is possible to employ trace amounts of radioactive tracers in a biosynthetic study and thereby to maintain homeostasis, because of the supreme sensitivity of the modern counting equipment used for the quantitative assay of radioactivity. The sensitivity of this equipment is such that even after  $10^8$  fold dilution of the  $^{14}\text{C}$ - or  $^3\text{H}$ -concentration normally present within the labelled precursor, quantitative assay of radioactivity within the product is still reliable.

The sensitivity of assay of  $^2\text{H}$ -enrichment by  $^2\text{H}$ -NMR spectroscopy and of  $^{13}\text{C}$  enrichment by  $^{13}\text{C}$ -NMR spectroscopy is lower by several orders of magnitude. As a consequence, massive doses of  $^2\text{H}$ - or  $^{13}\text{C}$ -labelled substrates must be administered if there is to be any hope of detecting enrichment within individual sites of the biosynthetic product. Such massive doses will swamp the system under study, will disturb homeostasis, and are likely to lead to metabolic recycling of heavy isotopes. Whereas the qualitative distribution of  $^{13}\text{C}$  and of  $^2\text{H}$  in the product will probably be the same as that of  $^{14}\text{C}$  and of  $^3\text{H}$ , respectively, and stable isotopes are thus suitable tracers for establishing sites of labelling, the kinetic course of the biosynthetic process will be obscured in the case of heavy isotopes, due to the swamping of the living system with tracer. Radioactive tracers on the other hand, since they are employed in trace amounts, can be used to study the kinetics of biosynthetic processes.

Notwithstanding their power, stable isotopes and nuclear magnetic resonance spectrometry in biosynthetic studies do not constitute a panacea and certainly do not represent the final word in methodology.

#### ACKNOWLEDGEMENTS

The work reported in this lecture was carried out by two highly capable collaborators: Dr. Gunnar Grue-Sørensen, Technical University of Denmark, Lyngby and now at Leo Pharmaceuticals, Copenhagen, was responsible for the investigations on the biosynthesis of the Senecio alkaloids. Dr. W. Marek Golebiewski, University of Warsaw, performed the studies of the biosynthesis of the Lupine alkaloids. I am grateful to them for their devoted efforts. Thanks are due to Brian G. Sayer and to J. Ian A. Thompson, Department of Chemistry, McMaster University, for recording NMR spectra, and to Thelma Leech, Greenhouse Supervisor, for cultivating plants and providing facilities for our experiments. I am indebted to Professor Dr. M. Wiewiorowski, Polish Academy of Sciences, Poznań, for supplying seeds of Lupinus luteus and L. angustifolius.

The work was supported in its entirety by a grant from the Natural Sciences and Engineering Research Council of Canada.

#### REFERENCES

1. R. Robinson. The structural relations of natural products. Clarendon Press, Oxford. 1955. a. pp. 72-74; b. pp. 74-76.
2. N. J. Leonard and S. W. Blum. J. Am. Chem. Soc. 1960, 82, 503-504.
3. E. E. van Tamelen and R. L. Foltz. J. Am. Chem. Soc. 1960, 82, 502-503; 1969, 91, 7372-7377.
4. G. Grue-Sørensen and I. D. Spenser. Can. J. Chem. 1982, 60, 643-662, and references cited therein.
5. W. M. Golebiewski and I. D. Spenser. J. Chem. Soc., Chem. Commun. 1983, 1509-1511, and references cited therein.
6. J. C. Richards and I. D. Spenser. Tetrahedron 1983, 39, 3549-3568.
7. G. Grue-Sørensen and I. D. Spenser. J. Am. Chem. Soc. 1983, 105, 7401-7404, and references cited therein.
8. W. M. Golebiewski and I. D. Spenser. J. Am. Chem. Soc. 1984, 106, 1441-1442, and references cited therein.
9. H. R. Schütte in "Biosynthesis of Alkaloids"; K. Mothes and H. R. Schütte, Eds.; VEB Deutscher Verlag der Wissenschaften, Berlin. 1969. pp. 324-347.
10. W. M. Golebiewski and I. D. Spenser. J. Am. Chem. Soc. 1976, 98, 6726-6728.
11. E. Leistner and I. D. Spenser. Polish J. Chem. 1979, 53, 49-56.
12. E. Leete in "Biosynthesis, Specialist Periodical Report" 1984, 7, 110-112.
13. W. M. Golebiewski and I. D. Spenser. J. Am. Chem. Soc. 1984, in press.
14. M. Wink, L. Witte and T. Hartmann. Planta Medica 1981, 43, 342-352.
15. Y. D. Cho, R. O. Martin and J. N. Anderson. J. Am. Chem. Soc. 1971, 93, 2087-2089.
16. M. Wink, T. Hartmann and H. W. Schiebel. Z. Naturforsch. 1979, 34c, 704-708, and nine subsequent papers.