# Biosynthesis of Cernuine[1]

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The biosynthesis of cernuine (**9**), an alkaloid of *Lycopodium cernuum*, was studied in intact plants. Specific incorporation of lysine, cadaverine, $\Delta^1$-piperideine, and pelletierine was demonstrated. The alkaloid is not a modified dimer of pelletierine, as might have been anticipated on the basis of structural relations, since only one pelletierine unit is incorporated.

La biosynthèse de la cernuine (**9**), un alcaloide issu du *Lycopodium cernuum*, a été étudiée sur des plantes intactes. L'incorporation spécifique de la lysine, de la cadavérine, de la $\Delta^1$-pipérideine, et de la pelletierine, a été démontrée. L'alcaloide n'est pas un dimère modifié de la pellétierine, comme on pourrait le supposer d'après les relations structurales puisque seule une unité de pellétierine est incorporée.

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## Introduction

In our previous work on the biosynthesis of the Lycopodium alkaloids (2–5) attention was focussed on the precursors of lycopodine (**6**), the major alkaloid of the group. We now report on the origin of cernuine (**9**), a Lycopodium alkaloid whose skeletal type is exceptional in that it does not bear a simple structural relationship to lycopodine.

Lysine (**1**) (3, 4), cadaverine (**2**) (4), $\Delta^1$-piperideine (**3**) (5), and acetate (4) were found to serve as specific precursors of lycopodine. These results lent credence to the hypothesis (Scheme 2) which prompted the work, that lycopodine and its structural relatives were modified dimers of a $C_5N—C_3$ unit, such as pelletierine (**4**), which in turn arises from lysine (Scheme 1). A dinitrogeneous species (*e.g.* **5**) is assumed to be an intermediate in the pathway to lycopodine (**6**). Other alkaloids, such as lycodine (**7**) and α-obscurine (**8**), could arise from the same intermediate. It is now known that pelletierine (**4**) cannot be the monomer unit, $C_5N—C_3$, demanded by the hypothesis, since only one intact pelletierine moiety was incorporated into lycopodine when multiply labelled pelletierine was administered to *Lycopodium tristachyum* (3, 5). It nevertheless appears likely on the basis of the mode of incorporation of the other precursors that a $C_5N—C_3$ unit related to pelletierine is implicated in the biosynthesis of lycopodine, and that a dinitrogeneous species with a lycodine–obscurine type skeleton serves as an intermediate.

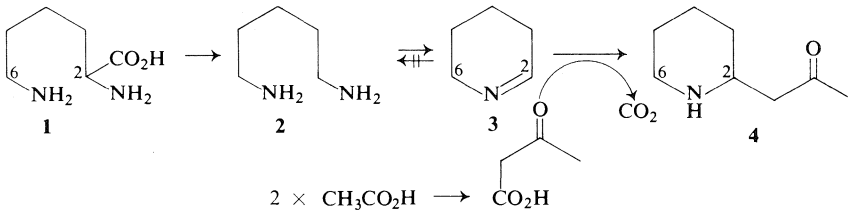
Cernuine (**9**), like lycodine, can be formally dissected into, and could conceivably arise from, two $C_5N—C_3$ units (Scheme 3), but the manner in which the two units are joined is entirely different in the two systems. It is noteworthy that *L. cernuum*, the species which yields cernuine, does not elaborate alkaloids of the lycodine or the lycopodine type. It was of interest, therefore, to determine whether the substrates known to be involved in the biosynthesis of lycopodine are also implicated in cernuine biosynthesis, and, in particular, whether pelletierine (**4**) is incorporated and if so, whether it serves as a precursor of one or of both halves of the cernuine system. The work here reported leads to the conclusion that there is complete analogy in the biosynthesis of cernuine and of lycopodine.

## Methods and Results

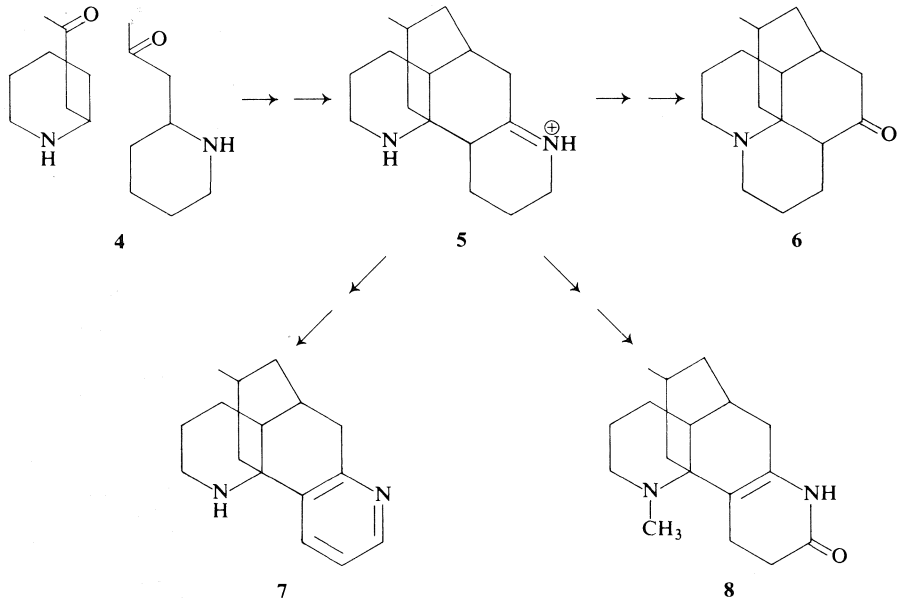
The plant used in this investigation was *Lycopodium cernuum* L., a subtropical Lycopodium species. A voucher specimen, authenticated by Dr. C. D. Adams, Department of Botany, University of the West Indies, is deposited in the University herbarium, Mona, Jamaica. Feeding experiments were carried out at the field station of the Institute of Jamaica, at Mason River.

Specifically labelled samples of lysine, cadaverine, $\Delta^1$-piperideine, and pelletierine were administered to intact plants and also to cuttings. A summary of these experiments is presented in

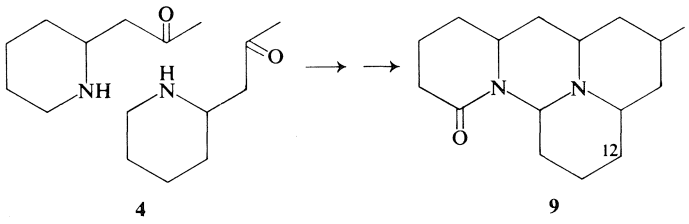
[1]A preliminary account of part of this work has been published (1).



SCHEME. 1. Biosynthesis of pelletierine.



SCHEME. 2. The pelletierine hypothesis of the origin of the Lycopodium alkaloids related to lycopodine.



SCHEME 3. The pelletierine hypothesis of the origin of cernuine.

Table 1. In each case cernuine (**9**) isolated from the plants was radioactive, whereas lycocernuine (12-hydroxycernuine), the major alkaloidal component, contained negligible activity.

The $^{3}H$:$^{14}C$ ratio of the samples of cernuine from experiments 2 and 6 was determined by liquid scintillation counting. Whereas the $^{3}H$:$^{14}C$ ratio of the precursor was maintained in the cernuine derived from the multiply labelled pelletierine (experiment 6), approximately one-third of the tritium, relative to $^{14}C$, was lost in the course of incorporation of the multiply

TABLE 1. Incorporation of precursors into cernuine

| Experiment no. | Compound administered | Nominal total activity (mCi) | Nominal specific activity (mCi/mmol) | Weight of dry plant (g) | Cernuine: Yield* (mg) | Cernuine: Specific activity (counts $min^{-1}$ $mmol^{-1}$) $\times 10^{-4}$ |
|---|---|---|---|---|---|---|
| 1 | 2-$^{14}$C-DL-Lysine† | 0.1 | 3.3 | 77 | 12.5 | 32.1±1.2 |
| 2 | 4,5-$^{3}H_2$,2-$^{14}$C-DL-Lysine | | | 77 | 23 | 16.5±0.8($^{14}$C) |
| | from 4,5-$^{3}H_2$-DL-Lysine† | 1.0 | $5.4 \times 10^3$ | | | |
| | from 2-$^{14}$C-DL-Lysine† | 0.1 | 3.3 | | | |
| 3 | 6-$^{14}$C-DL-Lysine‡ | 0.1 | 48 | 63 | 20 | 33.2±1.3 |
| 4 | 1,5-$^{14}$C-Cadaverine† | 0.1 | 3.2 | 92 | 30 | 27.8±1.2 |
| 5 | 6-$^{14}$C-$\Delta^1$-Piperideine§ | 0.1 | 0.8 | 92 | 31 | 15.8±0.6 |
| | from 6-$^{14}$C-DL-lysine‡ | 0.1 | 48 | | | |
| 6 | 4-$^{3}$H,6,2′-$^{14}C_2$-Pelletierine§ | | | 164 | 45 | 17.8±0.6($^{14}$C) |
| | from 4-$^{3}$H-Pelletierine§ | 0.6 | 10 | | | |
| | from 6-$^{14}$C-Pelletierine§ | 0.03 | 1 | | | |
| | from 2′-$^{14}$C-Pelletierine§ | 0.02 | 0.25 | | | |

*Total yield, obtained by pooling the samples isolated from wick-fed intact plants and from beaker-fed cuttings.
†New England Nuclear.
‡Commissariat à l'Energie Atomique, France.
§See Experimental.

TABLE 2. Incorporation of lysine, cadaverine, and $\Delta^1$-piperideine into cernuine

| Precursor: | 2-$^{14}$C-Lysine | | 4,5-$^{3}H_2$,2-$^{14}$C-Lysine | | 6-$^{14}$C-Lysine | | 1,5-$^{14}$C-Cadaverine | | 6-$^{14}$C-$\Delta^1$-Piperideine | |
|---|---|---|---|---|---|---|---|---|---|---|
| Experiment no.: | 1 | | 2 | | 3 | | 4 | | 5 | |
| Product | SA* | RSA† | SA | RSA | SA | RSA | SA | RSA | SA | RSA |
| Cernuine (**9**) | 2.02±0.06‡ | 100±3 | 3.18±0.04‡,§ | 100±1 | 3.04±0.03‡ | 100±1 | 3.61±0.04‡ | 100±1 | 4.02±0.08‡ | 100±2 |
| 2-*n*-Butyl-4-methyl-6-*n*-pentylpyridine (**10**) | — | | 2.22±0.02§ | 70±1 | 2.22±0.02 | 73±1 | 2.66±0.03 | 74±1 | 2.15±0.02 | 54±1 |
| β-Alanine‖(**11**) | 0.48±0.01 | 24±1 | | | | | | | | |
| γ-Aminobutyric acid‖(**12**) | 0.51±0.03 | 25±2 | | | | | | | | |

*Specific activity (counts $min^{-1}$ $mmol^{-1}$) $\times 10^{-4}$
†Relative specific activity (%) (cernuine = 100).
‡Obtained from the original sample of cernuine, whose specific activity is given in Table 1, by dilution with inactive cernuine.
§Specific activity with respect to $^{14}$C.
‖Isolated as the *N*-2,4-dinitrophenyl derivative.

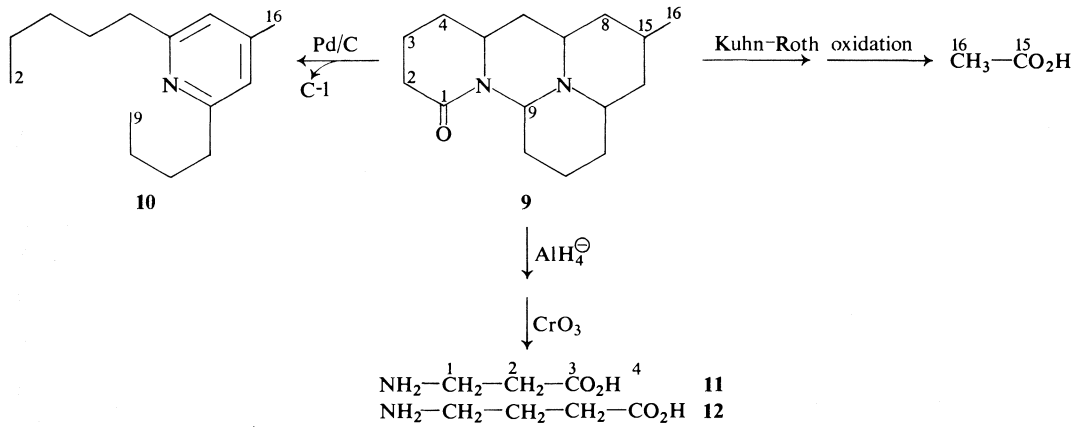
TABLE 3. Incorporation of $^{3}H$,$^{14}C$-labelled precursors

| Experiment no. | Precursor | $^{3}H$:$^{14}C$ Ratio | |
|---|---|---|---|
| | | In precursor | In cernuine |
| 2 | 4,5-$^{3}H_2$,2-$^{14}C$-DL-Lysine | 10.6±0.4 | 6.5±0.04 |
| 6 | 4-$^{3}H$,6,2′-$^{14}C_2$-Pelletierine | 12.4±0.1 | 12.2±0.1 |

TABLE 4. Incorporation of pelletierine into cernuine

| 4-$^{3}H$,6,2′-$^{14}C_2$-Pelletierine | | Cernuine | |
|---|---|---|---|
| Total activity administered ($^{14}C$) (d.p.m.) × $10^{-7}$ in | | Specific activity ($^{14}C$) (counts $min^{-1}$ $mmol^{-1}$) × $10^{-4}$ | |
| 6-$^{14}C$-Pelletierine | 6.77±0.01 | | |
| 2′-$^{14}C$-Pelletierine | 4.64±0.01 | Kuhn–Roth acetate* | 0.81±0.06 |
| Total | 11.41±0.01 | Intact cernuine† | 1.99±0.03 |
| $\frac{2'\text{-}^{14}C}{\text{Total}}$ | (41±1)% | $\frac{\text{Kuhn–Roth acetate}}{\text{Intact cernuine}}$ | (41±1)% |

*Isolated as acetyl-α-naphthylamide.
†Obtained from the cernuine, specific activity (17.8 ± 0.6) × $10^4$ counts $min^{-1}$ $mmol^{-1}$ (experiment 6) by dilution with inactive carrier.

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SCHEME 4. Degradation of cernuine (numbers refer to the carbon atoms of cernuine).

labelled lysine into cernuine (experiment 2) (Table 3). To determine the distribution of $^{14}C$, the labelled samples of cernuine were partially degraded, by reactions to be discussed presently, into the degradation products shown in Scheme 4. The specific activities of the degradation products are listed in Tables 2 and 4. Indicated limits are standard deviation of the mean.

The samples of 2-*n*-butyl-4-methyl-6-*n*-pentylpyridine (**10**) obtained from cernuine derived from 2-$^{14}C$-lysine (experiment 2), 6-$^{14}C$-lysine (experiment 3), and 1,5-$^{14}C$-cadaverine (experiment 4) retained approximately three-quarters of the activity of the intact alkaloid. The pyridine derivative (**10**) from the cernuine derived from 6-$^{14}C$-$\Delta^1$-piperideine (experiment 5), contained approximately one-half of the original activity. β-Alanine (**11**) and γ-aminobutyric acid (**12**), recovered from 2-$^{14}C$-lysine-derived cernuine (experiment 1), contained one-quarter of the original activity. Finally, the fraction of activity recovered in the acetate obtained by Kuhn–Roth

oxidation of pelletierine-derived cernuine was identical with the fraction of activity at the carbonyl carbon atom of the precursor (experiment 6) (Table 4).

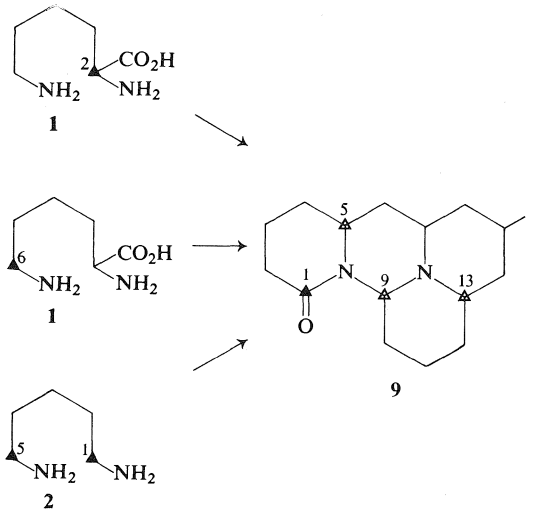
## Discussion

Even though degradation of cernuine was only partial and the activity of the intact molecule was not fully accounted for in terms of the activities of degradation products representing individual labelled sites, the results nevertheless support the view that the radioactive precursors were incorporated into cernuine in a manner which is completely analogous to their mode of incorporation into lycopodine.

Lysine enters each of the two $C_5$ units of lycopodine, C-1 to -5 and C-9 to -13, in a symmetrical manner, and with equal efficiency (2, 4). Cadaverine, a symmetrical molecule and a likely stage of the pathway, is incorporated similarly (4). Analogous entry of lysine and cadaverine into cernuine would be predicted to place activity derived from 2-$^{14}$C-lysine, from 6-$^{14}$C-lysine, and from 1,5-$^{14}$C-cadaverine equally into four sites, C-1, -5, -9, and -13. One quarter of the activity of cernuine, derived from 2-$^{14}$C-lysine (experiment 2), 6-$^{14}$C-lysine (experiment 3), and 1,5-$^{14}$C-cadaverine (experiment 4) was indeed found at C-1 (cernuine minus pyridine derivative (**10**)) (Table 2) (Scheme 5).

In experiment 1 access to C-1 was gained in another manner. Reduction of cernuine with lithium aluminum hydride followed by oxidation of the reduction product with chromic acid gave β-alanine and γ-aminobutyric acid derived, respectively, from C-1 to -3 and C-1 to -4 of cernuine. Both acids, obtained by degradation of cernuine derived from 2-$^{14}$C-lysine, contained one-quarter of the activity of the intact alkaloid. This result confirms those of the previous experiments in which the activity at C-1 was obtained by difference. The finding of one-quarter of the activity of cernuine at C-1 from cernuine derived from 2-$^{14}$C-lysine, 6-$^{14}$C-lysine, and 1,5-$^{14}$C-cadaverine is in complete analogy with the results found for lycopodine.

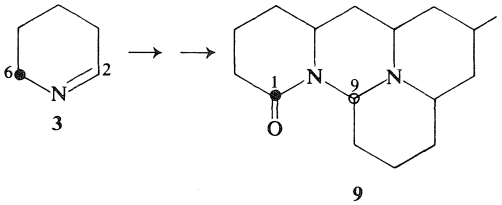
$\Delta^1$-Piperideine is another substrate whose specific incorporation into lycopodine has been demonstrated (5). The precursor enters both "halves" of the alkaloid with equal efficiency. Its mode of incorporation differs from that of



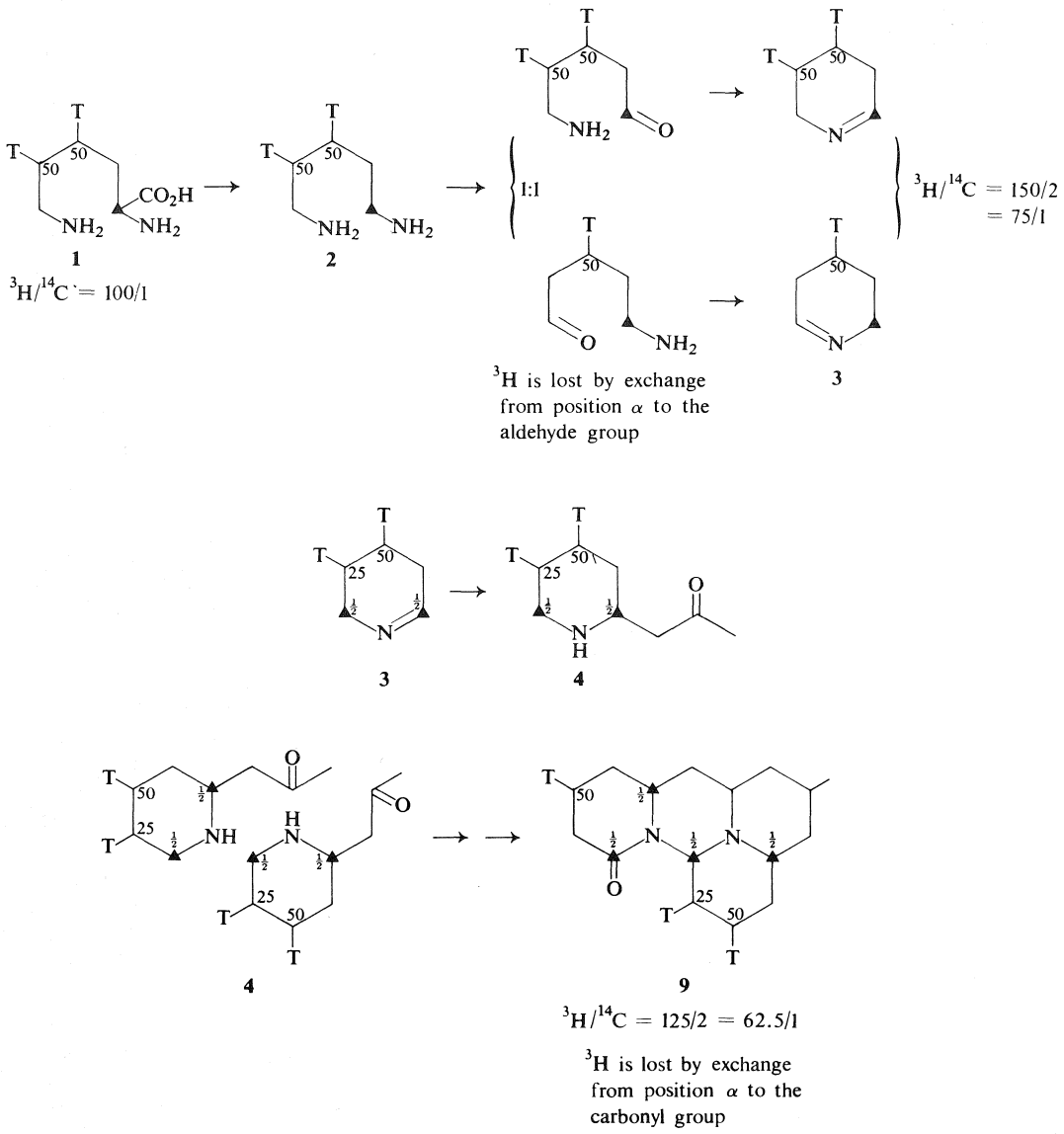

SCHEME 5. Incorporation of lysine (**1**) and cadaverine (**2**) into cernuine. Sites of activity derived from 2-$^{14}$C- and 6-$^{14}$C-lysine and from 1,5-$^{14}$C-cadaverine, established by degradation (▲) (~25% of total activity from each of the three substrates) or inferred (Δ).

lysine in that the individuality of C-2 and -6 of $\Delta^1$-piperideine is preserved in the course of its route into lycopodine. Analogous entry of $\Delta^1$-piperideine into cernuine would be predicted to place label derived from 6-$^{14}$C-$\Delta^1$-piperideine equally into the two sites, C-1 and -9. In accord with this prediction C-1 of cernuine derived from 6-$^{14}$C-$\Delta^1$-piperideine was found to account for approximately half the activity of the intact alkaloid (cernuine minus pyridine derivative (**10**)) (experiment 5) (Table 2) (Scheme 6).

The evidence is clearly consistent with the view that cernuine, like lycopodine, is a modified dimer of an intermediate, $C_5N$—$C_3$, whose piperidine nucleus is, in turn, derived from lysine (**1**) via




SCHEME 6. Incorporation of $\Delta^1$-piperideine into cernuine. Sites of activity from 2-$^{14}$C-$\Delta^1$-piperideine, established by degradation (●) (~50%) or inferred (○).

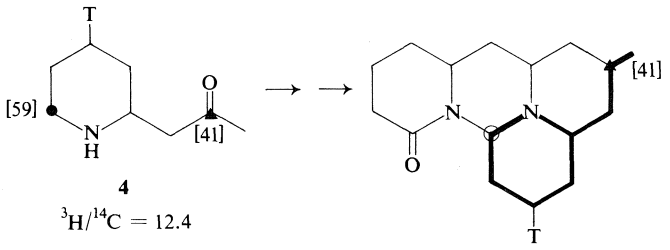



SCHEME 7. Predicted mode of incorporation of 4,5-$^3H_2$,2-$^{14}$C-lysine into cernuine. (Both of the two $C_5N$—$C_3$ units, postulated as cernuine precursors, are here arbitrarily represented as pelletierine).

cadaverine (**2**) and $\Delta^1$-piperideine (**3**) (*cf.* Scheme 1).

Further evidence supporting this sequence to cernuine is provided by the result of the feeding experiment with multiply labelled 4,5-$^3H_2$, 2-$^{14}$C-lysine (experiment 2). As shown in Scheme 7, a loss of 37.5% tritium, relative to $^{14}$C, is predicted by the hypothetical sequence to occur in the course of the incorporation of this lysine into cernuine. The observed loss of 39% (experiment 2) (Table 3) is in remarkable agreement with this prediction.

To throw light on the nature of the postulated intermediate, $C_5N$—$C_3$, a multiply labelled sample of pelletierine (**4**) of known isotope distribution was administered to *L. cernuum* (experi-




SCHEME 8. Incorporation of pelletierine into cernuine. Sites of activity established by degradation (▲) (~41% of total activity) or inferred (○, T).

ment 6). Consistent with the hypothesis (Scheme 3), that pelletierine was the intermediate whose dimerization leads to cernuine, the $^3H$:$^{14}C$ ratio of the precursor was maintained in the product (Table 3). Degradation of the cernuine showed, however, that only one intact pelletierine unit had been incorporated. The acetic acid, obtained from the $C_2$ unit, C-15,16, of the cernuine by Kuhn–Roth oxidation, contained 41% of the activity of the intact alkaloid, a distribution corresponding to the fraction of $^{14}C$-activity within the sidechain of the precursor (Table 4). Entry of two intact pelletierine units would have led to a Kuhn–Roth acetate containing 20% of the activity of the original cernuine. An intact pelletierine moiety thus serves as the precursor of the $C_8$ unit, C-9 to -16, but not of the $C_8$ unit, C-1 to -8, of cernuine (Scheme 8). This biosynthetic relationship is entirely analogous to the mode of entry of pelletierine into lycopodine (3, 5). The problems of interpretation, raised by these experimental results, have been discussed (5).

*The Chemistry of the Degradation Reactions*

The methods used to degrade cernuine are outlined in Scheme 4. Kuhn–Roth oxidation gave C-15 and -16 as acetic acid which was converted to its α-naphthylamide derivative for purification and radioactive assay. Dehydrogenation of cernuine over palladium–charcoal by the method of Ayer *et al.* (6, 7) gave compound **10** which was purified and assayed as its picrolonate. Compound **10** contains all the carbons of cernuine except C-1 and thus the activity at this center is obtained by difference. The third degradation of Scheme 4 in which C-1 is isolated as β-alanine or γ-aminobutyric acid deserves further comment.

The treatment of cernuine with lithium aluminum hydride has been reported to give a single compound, dihydrodeoxycernuine (7). We obtained, however, a small amount (<7%) of an isomer, dihydrodeoxyepiallocernuine (8), in addition to the expected product. Dihydrodeoxyepiallocernuine differs in configuration at C-9 and -13 from dihydrodeoxycernuine but the mechanism by which the isomerization occurs is not known. Fission of the N—$C_9$ bond does not accompany the reduction of the amide as we suggested earlier (1). Since both isomers are expected to yield the same oxidation products the mixture was oxidized without further purification. β-Alanine and γ-aminobutyric acid were isolated, identified, and assayed for radioactivity as their 2,4-dinitrophenyl derivatives. As shown in Scheme 4 the carbon atoms of the amino acids are expected to be derived from C-1 to -3 and C-1 to -4 of cernuine.

To verify that the amino acids come solely from C-1 to -3 and C-1 to -4 of cernuine we prepared a mixture of dihydrodeoxycernuine-1,1-$d_2$ and its isomer, subjected the mixture to oxidation, and examined the amino acids for their deuterium content. Deuterium was incorporated into the dihydrodeoxy compounds by carrying out the reaction of cernuine with lithium aluminum deuteride. Two products, both of mass 250, were obtained from this treatment and the ratio of the two, determined by g.l.c., was the same as that obtained in the hydride reduction. Oxidation of the mixture gave β-alanine-$d_2$ and γ-aminobutyric acid-$d_2$ isolated as their 2,4-dinitrophenyl derivatives. The mass spectra of the 2,4-dinitro-

phenyl derivatives showed no evidence of the presence of undeuterated acids. It can be concluded that the degradation proceeds specifically as shown in Scheme 4.

## Experimental

*Administration of Labelled Compounds to Lycopodium cernuum*

Two methods were used for the administration of tracers to *Lycopodium cernuum* L. Firstly, a solution containing the labelled compound was infused through cotton wicks into the stems of intact plants growing in their natural environment. Cotton thread was inserted into the stems and the end of each thread was placed into a small glass beaker. The tracer solution (10 ml) was divided among the beakers and was absorbed by the plants through the wicks. After the original tracer solution had been absorbed, the beakers were repeatedly refilled with glass-distilled water. The plants were kept in contact with the tracer for 48–72 h and were then harvested.

Secondly, fresh cuttings (12–18 in. high) were packed with cut stems downward into a 100 ml beaker. The labelled compound, dissolved in glass-distilled water (10 ml), was added to the beaker. Refilling and harvesting was carried out in the same manner as above.

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

6-$^{14}$C-$\Delta^1$-Piperideine (experiment 5) was prepared (5) from 6-$^{14}$C-DL-lysine (Commissariat à l'Energie Atomique, France). 6-$^{14}$C-Pelletierine and 4-$^{3}$H-pelletierine were synthesized by condensation of labelled $\Delta^1$-piperideine prepared from 6-$^{14}$C-DL-lysine (Commissariat à l'Energie Atomique, France), and from 4-$^{3}$H-DL-lysine (Commissariat à l'Energie Atomique, France), respectively, with inactive acetoacetic acid (9). 2′-$^{14}$C-Pelletierine was obtained by condensation of 3-$^{14}$C-acetoacetic acid (prepared by hydrolysis of ethyl 3-$^{14}$C-acetoacetate (New England Nuclear)) with $\Delta^1$-piperideine obtained from inactive lysine. An intermolecularly triply labelled sample, 4-$^{3}$H,6,2′-$^{14}C_2$-pelletierine (experiment 6), was obtained by mixing these samples.

*Isolation of Cernuine and Lycocernuine (cf. ref. 7)*

Aerial parts of *L. cernuum* were dried and ground to a fine powder in a blendor (Osterizer Galaxie 500). The powder (500 gm) was moistened with ammonia (0.6 *M*, 1 ml/gm) and was continuously extracted with chloroform for 48 h. The chloroform extract was concentrated to one-tenth of its volume and ether was added. The solution was then extracted with hydrochloric acid (0.6 *M*, 5 × 30 ml). The aqueous extract was washed twice with ether, basified with ammonia (1.2 *M*), and was then extracted with chloroform (5 × 25 ml). The chloroform extract was dried (anhydrous sodium sulfate) and concentrated almost to dryness and a small amount of acetone was added. Lycocernuine (110 mg), which precipitated, was recrystallized from acetone and melted at 228–229° (lit. 230–231°)(7).

The mother liquors from the crystallization were dissolved in benzene and applied to a column of neutral alumina, activity I (40 g). Elution with benzene and benzene–ether (1:1) gave a mixture of minor bases. Anhydrous ether then gave cernuine, and elution with chloroform gave lycocernuine. Finally, methanol gave a mixture of minor bases.

The cernuine was sublimed at 110° and 3 × $10^{-3}$ mm, and was recrystallized from hexane, m.p. 101–102° (lit. 103–104°)(7). In several instances, particularly in the case of radioactive samples, the cernuine was dissolved in ether and the solution was neutralized with an ethereal solution of perchloric acid. The solvent was evaporated and the cernuine perchlorate so obtained was recrystallized from water or from ethanol–ether, and melted at 110° (lit. 110°) (10).

Several variations of the isolation procedure were employed in early experiments. When ether instead of chloroform was used as extracting solvent, the extraction of alkaloids was incomplete. The alkaloid extract, on the basis of g.l.c. analysis, contained twice as much cernuine as lycocernuine whereas the reverse was true when a better solvent such as chloroform (see above) or methanol (see below) was used. Thus when ether was used, only part of the lycocernuine was extracted from the plant material.

Methanol was employed as solvent in large scale batch extraction (7). Finely ground *L. cernuum* was extracted at room temperature with methanol for 36 h and the methanolic extract was concentrated at reduced pressure. Extraction was repeated twice with fresh solvent and the combined extracts were warmed with hydrochloric acid (0.6 *M*). Acid-insoluble material was removed by filtration and was then re-extracted with hydrochloric acid. The filtrates were combined, non-basic material was extracted with ether, and the aqueous layer was made strongly basic with ammonia. The alkaloidal fraction was then extracted into chloroform and worked-up as already described.

*Degradation of Cernuine*

*Reduction of Cernuine (7)*

Cernuine (250 mg) was added to a slurry of lithium aluminum hydride (0.50 g) in anhydrous ether (20 ml, dried over sodium hydride), and the resulting mixture was refluxed for 20 h, and was then cooled in ice. Excess hydride was decomposed by dropwise addition of water (0.5 ml) followed by sodium hydroxide (4 *M*, 0.5 ml). More water (1.5 ml) was then added and the mixture was stirred for 20 min, and filtered with suction. The granular precipitate was washed thoroughly with ether and the ethereal filtrate was dried over sodium sulfate and concentrated at reduced pressure. The crystalline residue was sublimed at 100° and 4 × $10^{-3}$ mm. The mass spectrum of the sublimed product showed a signal, *m/e* 248, which was regarded as due to the molecular ion of the reduction product. However, two components, retention times 2.0 min and 3.2 min, were detected by g.l.c. (1% Carbowax 20 M, coated with 5% KOH on Chromosorb W, 4 ft × 0.125 in., 130–210° at 10°/min and a helium flow rate of 40 ml/min). The retention time of the minor component (< 7%) was 2 min, identical with that of an authentic sample of dihydrodeoxyepiallocernuine.[2] When a sample of the reduction product, mixed with authentic

dihydrodeoxyepiallocernuine,[2] was injected, only two components with retention times 2.0 and 3.2 min, respectively, were observed. The intensity of the peak of the minor component increased with the amount of the authentic dihydrodeoxyepiallocernuine added. The major component was regarded as dihydrodeoxycernuine. These two components were not further separated and were used for the oxidation described below. The same procedure was used to prepare the dideuterated compounds, except that lithium aluminum deuteride was substituted for the hydride.

*β-Alanine and γ-Aminobutyric Acid from Cernuine*

The above reduction product (192 mg) of cernuine was dissolved in hot aqueous sulfuric acid (6 *M*, 4 ml) and chromium trioxide (500 mg) in water (2 ml) was added in several portions. The mixture was heated under reflux with stirring for 48 h. The cooled mixture was extracted several times with ether. The ether layer was rejected. The aqueous layer was diluted with water (10 ml) and warmed, and hot saturated aqueous barium hydroxide solution was added until the solution was neutral. The precipitate was filtered off and washed repeatedly with hot water. The filtrate was evaporated to dryness. The residue was dissolved in water (1 ml) and aqueous sodium bicarbonate (1.2 *M*) was added until the p*H* of the solution was 9. A solution of 1-fluoro-2,4-dinitrobenzene in methanol (10%, *v/v*, 1.5 ml) was then added. The mixture was allowed to stand with occasional shaking for 1 h, the p*H* being maintained at 8–9 with additional bicarbonate solution. After 1 h, water (4 ml) and bicarbonate solution (0.5 ml) were added and excess reagent was removed by extraction with ether (2 × 6 ml). The solution was then acidified with hydrochloric acid (6 *M*) and extracted with ether (3 × 6 ml). The dried (sodium sulfate) ether extract was concentrated and the yellow dinitrophenyl (DNP) amino acids were separated by preparative t.l.c. (silica gel, developed with benzene – pyridine – acetic acid (80:20:2)).

Three yellow bands were observed. The major band showed an $R_f$ value ($R_f$ 0.69) identical with that of an authentic sample of *N*-2,4-dinitrophenyl-β-alanine. A second band ($R_f$ 0.84) corresponded to *N*-2,4-dinitrophenyl-γ-aminobutyric acid. The minor band ($R_f$ 0.28) was not further examined, but may have been *N*-2,4-dinitrophenylglycine. The amino acid derivatives were eluted separately from adsorbent with methanol. The methanolic extract of the β-alanine band ($R_f$ 0.69) which was the most abundant was evaporated to dryness. The residue was dissolved in water (1 ml), hydrochloric acid (6 *M*, 1 ml), was added, and the mixture was extracted with ether (3 × 5 ml). The ether layer was washed with water (2 ml), dried (sodium sulfate), and evaporated to dryness. The residue was sublimed at 160–170° and $1 \times 10^{-3}$ mm when the DNP derivative of β-alanine was obtained as a yellow crystalline solid; yield, 8 mg. The band of the DNP derivative of γ-aminobutyric acid ($R_f$ 0.84) was worked-up similarly; yield, 5 mg.

The same procedure was applied to the product obtained by reduction of cernuine with lithium aluminum deuteride. The DNP derivatives of β-alanine and γ-aminobutyric acid were analyzed mass spectrometrically showing molecular ions at *m/e* 257 and 271, respectively. There were no peaks at 255 and 269 corresponding to undeuterated acid.

*Kuhn–Roth Oxidation of Cernuine*

A solution of chromic acid (2 g) in water (3 ml) was added to a solution of cernuine (100 mg) in sulfuric acid (2 *M*, 5 ml). The mixture was heated under reflux for 2 h. Steam was then passed through the mixture until 200 ml of distillate had been collected. The distillate was neutralized with sodium hydroxide (0.1 *M*) and evaporated to dryness. The sodium acetate so obtained was converted into acetyl-α-naphthylamide (11) which was purified by sublimation at 135° and $1 \times 10^{-3}$ mm. The white sublimate was crystallized from benzene and the product resublimed. Acetyl-α-naphthylamide, m.p. 158–159°, was obtained as colorless needles.

*Dehydrogenation of Cernuine (7)*

Cernuine (100 mg) was dehydrogenated with 5% palladium-on-charcoal, as described by Ayer *et al.* (7), and the basic products separated by preparative t.l.c. on silica gel (0.5 mm thickness, developed with ether/ammonia (75 ml/2 drops)). The most polar component ($R_f$ 0.65), which was the most abundant one (20 mg), was eluted from the adsorbent with chloroform and subjected to high vacuum distillation at 50° and $4 \times 10^{-3}$ mm to yield 2-*n*-butyl-4-methyl-6-*n*-pentylpyridine (14 mg), identical with an authentic sample.[2] The pyridine derivative was converted into the picrolonate, which after recrystallization from ethanol–ether melted at 137–139°.

Anal. Calcd. for $C_{25}H_{33}N_5O_5$: C, 62.09; H, 6.88; N, 14.48. Found: C, 62.10; H, 6.91; N, 14.33.

It was also converted into the chloroplatinate, which was recrystallized from ethanol containing a drop of hydrochloric acid. The orange crystals so obtained melted at 186–188° (lit. 189–190°)(7). The minor components of the dehydrogenation were not examined.

*Radioactivity Measurements*

Radioactivity was assayed on samples of finite thickness on aluminum planchettes with a low background gas flow system (Nuclear Chicago Corporation, Model 4342). Samples were plated with the aid of a 1% solution of collodion in dimethylformamide. The usual corrections for background and self-absorption were applied. The $^3H$:$^{14}C$ ratios were measured by liquid scintillation counting (Mark I, Liquid Scintillation Computer, Model 6860, Nuclear Chicago Corporation). Radioactivity due to $^3H$ and $^{14}C$ was determined simultaneously by external standardization counting with $^{133}Ba$. Samples were dissolved in benzene or methanol and the solution was dispersed in a solution of Liquifluor (Nuclear Chicago Corporation) diluted 25 times with toluene. Duplicate samples of each compound were counted under comparable conditions of quenching. The confidence limits shown in the results (Tables 1–4) are standard deviation of the mean.

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[2]We are greatly indebted to Dr. W. A. Ayer for supplying this sample.

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