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The biosynthesis of lycorenine, narciclasine, and dimethoxy analogues of lycorine

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THE BIOSYNTHESIS OF LYCORENINE, NARCICLASINE, AND DIMETHOXY ANALOGUES OF LYCORINE

by

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INTRODUCTION

Many experiments have been performed in recent years to determine the biosynthetic routes to the various Amaryllidaceae alkaloids. Only several studies have been concerned with *in vivo* rearrangements and alkaloid interconversions. This study was undertaken to examine heretofore unexamined alkaloid rearrangements and interconversions.

The lycorenine-type alkaloids have been shown to undergo *in vitro* rearrangements to lycorine-type alkaloids--also it is known that lycorine-type alkaloids can be transformed to the lycorenine-type systems. It has been shown that caranine is not an efficient precursor to hippeastrine. Part of this study is concerned with the nature of the lycorine ring system that is capable of undergoing an *in vivo* rearrangement to a lycorenine ring system.

Previous studies have proven that hydroxylation of C-2 of lycorine occurs late in the biosynthetic sequence. The lycorine nucleus is formed and the C-2 alcohol is introduced via an inversion mechanism. Some evidence has been reported to indicate that the same late stage oxidation is occurring in the dimethoxy analogues of lycorine. Part of this study is concerned with further evidence regarding the C-2 oxidation of the dimethoxy analogues of lycorine.

The structure of narcissidine has been recently resolved. A portion of this study is concerned with the late stage *in*

vivo modification of the lycorine-type nucleus to give narcissidine.

It has been hypothesized that narciclasine, due to its structural similarity to the Amaryllidaceae alkaloids, is associated with the biosynthesis of the Amaryllidaceae alkaloids. A part of this study concerns the alkaloid origin of narciclasine.

In vivo oxygen demethylation reactions have not been previously observed in the Amaryllidaceae, although such conversions have been proven to occur in another family of plants. A part of this study involves a possible oxygen demethylation reaction of an Amaryllidaceae alkaloid.

HISTORICAL

The first report of an alkaloid isolation from the Amaryllidaceae appeared in 1877 by Gerrard[1]. This alkaloid was termed "narcissa" and was isolated from Narcissus pseudo-narcissus L. but was not characterized in any way.

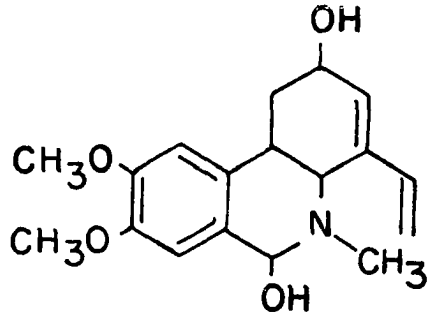
From that time to 1952 only fifteen Amaryllidaceae alkaloids had been isolated and characterized[2]. The following eight years witnessed a substantial increase in interest in the Amaryllidaceae. This was evidenced by the number of isolated and characterized alkaloids reported during that time[3]. By 1960 more than seventy alkaloids had been isolated and approximately one-half of these had structural assignments based on chemical degradations and spectral data.

After 1960, the isolation and characterization of new alkaloids was not of primary interest. The past nine years has been the era of biosynthetic studies in the Amaryllidaceae. Radioactive tracer studies have elucidated the biosynthetic pathways to several of the alkaloids.

Historical discussion will center on the chemistry of the alkaloids of immediate interest to this work. The biosynthesis of the Amaryllidaceae alkaloids will also be discussed.

Lycorenine

The first representative reported of the [2]benzopyrano [3,4g]indole group of alkaloids was lycorenine. It was in 1932 when Kondo and coworkers isolated lycorenine from Lycoris radiata Herb.[4] In a later report these workers proposed structure 1 for lycorenine[5]. It was found that upon hydrogenation, lycorenine formed an O-heterocyclic derivative. It was also found that lycorenine formed an oxime derivative. The hydrogenation product was difficult to justify on the basis of structure 1, since the 3,4 double bond must be reduced in preference to the vinyl side chain, which must be hydrated, and a rearrangement must occur to cause ether formation with the C-2 alcohol. Wenkert and Hansen later reexamined the data of Kondo and Ikeda and proposed structure 2 for lycorenine[6]. Hydrogenolysis of the benzylic hemiacetal function easily rationalized the formation of the O-heterocycle. The hemiacetal functionality was readily accepted



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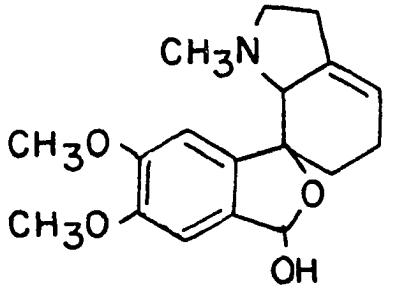
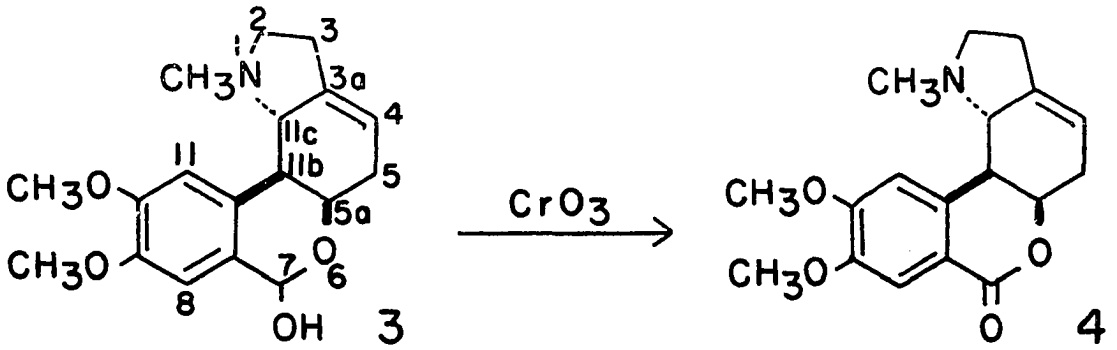


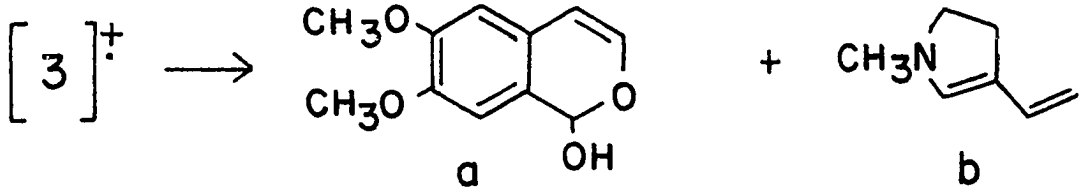
2

because several groups found that oxidation of lycorenine with chromic acid afforded homolycorine, an alkaloid known to be a lactone[7,8]. Homolycorine exhibited a carbonyl absorption at 5.84μ. Since methoxyphthalides absorb near 5.68μ, the structures of lycorenine and homolycorine were revised to 3 and 4, respectively[7,9]. Other chemical degradations supported these assignments for the gross structures of the two alkaloids.



Spectroscopic data also agrees with structure 3 for lycorenine. The 60 MHz. NMR spectra in $CDCl_3$ shows resonances (in ppm downfield from TMS) for the N-Me at 2.85δ(3H, singlet), two methoxyls at 3.90δ(6H, singlet), a vinyl proton at 5.49δ (1H, multiplet), a benzylic proton at 5.88δ(1H, broadened singlet), and two aromatic protons at 6.90δ(1H, singlet) and 6.96δ(1H, singlet). Two broad unresolved multiplets of nine protons appear at 2.34 to 2.75δ and 3.00 to 3.33δ. A broadened doublet centered at 4.37 is exchangeable with D_2O and is attributable to the alcohol proton[10]. Mass spectrometry studies provided additional evidence for the [2] benzopyrano[3,4g]indole ring system for lycorenine.

Burlingame and coworkers reported a mass spectrometric study of several of the lycorenine-type alkaloids[11]. It was found that the dominant mode of fragmentation was cleavage of ring C in a retro-Diels-Alder fashion as shown in 3 to give fragments a and b (m/e=208 and m/e=109, respectively). Fragment b is the base peak of the spectrum with a composition of $C_7H_{11}N$ as determined by high resolution techniques. The remainder of the spectrum consisted mainly of the fragmentation of a and b. These include the loss of 15 mass units from b, the cleavage of the N-Me group; loss of 18 mass units from a, the loss of water from the hemiacetal function (a loss also observed from the molecular ion); and the loss of 30 mass units from a, interpreted as the loss of H_2CO. Thus, chemical



degradations and supporting spectral data established lycorenine as structure 3, without stereochemical implications. The asymmetric centers at C-5a, C-11b, and C-11c remained to be assigned.

The stereochemistry was solved by relating lycorenine to the lycorine-type alkaloids of known configurations. The relation was established by the conversion of lycorenine to a pluviine derivative and dihydrolycorine to hippeastrine.

These transformations allowed the assignment of configurations to carbons 5a, 11b, and 11c of the lycorenine-type alkaloids and are described below.

Reduction of homolycorine with lithium aluminum hydride affords a diol. The diol, with successive treatment with p-toluenesulfonyl chloride and iodide ion gives pluviine β-methiodide(5)[12]. Comparable reactions with α- and β-dihydrohomolycorine (6 and 7, respectively) converted these compounds to the metho salts of α- and β-dihydropluviine (8 and 9, respectively). In both ring systems the α-series has a C:D cis ring fusion. The β-dihydro series is trans at this junction. Figure 1 illustrates these transformations.

It has also been shown that dihydrolycorine(10) can be converted to tetrahydrohippeastrine(13) utilizing the von Braun reaction as the key transformation[13]. The first product isolated is N-cyano-tetrahydrohippeastrine(11), which upon treatment with lithium aluminum hydride affords N-demethyl-tetrahydrohippeastrine(12). N-methylation using formaldehyde in formic acid yields 13. Tetrahydrohippeastrine is obtained from hippeastrine(14) via hydrogenation, reduction of the lactone function with lithium aluminum hydride followed by acid treatment for ether formation. Figures 2 and 3 illustrate the transformations.

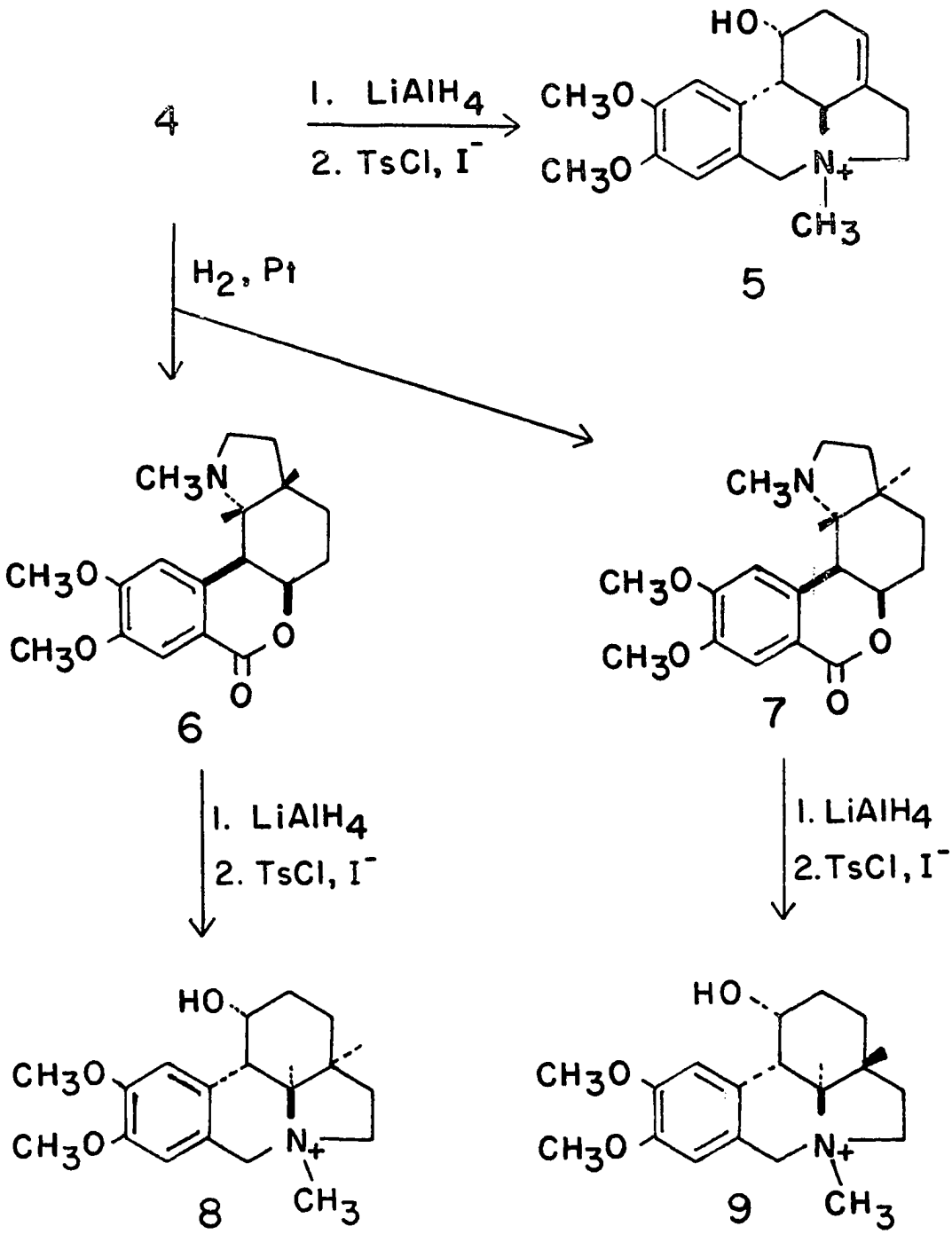


Figure 1. Conversion of homolycorine to pluviine.

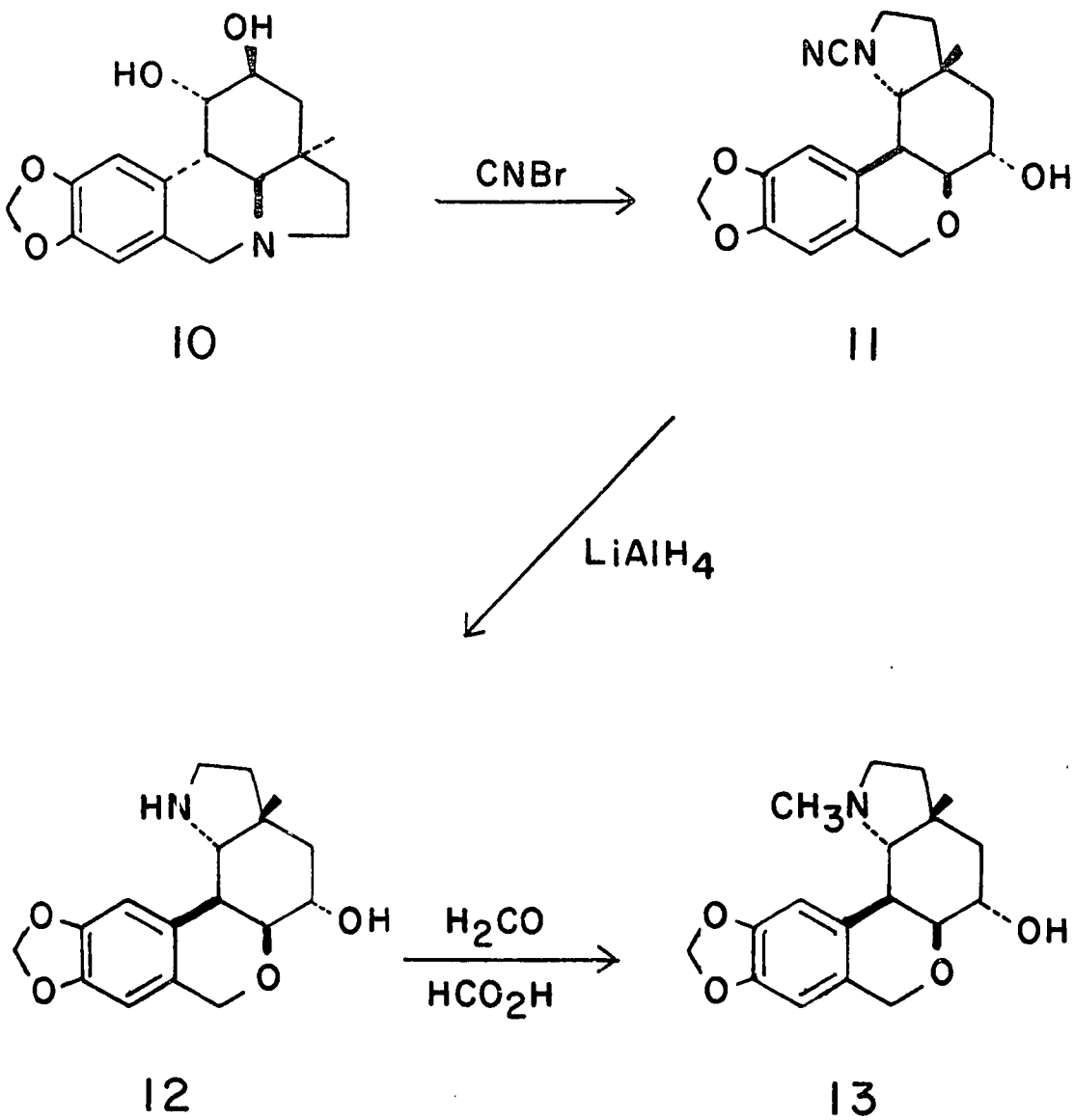


Figure 2. Conversion of dihydrolycorine to tetrahydrohippeastrine

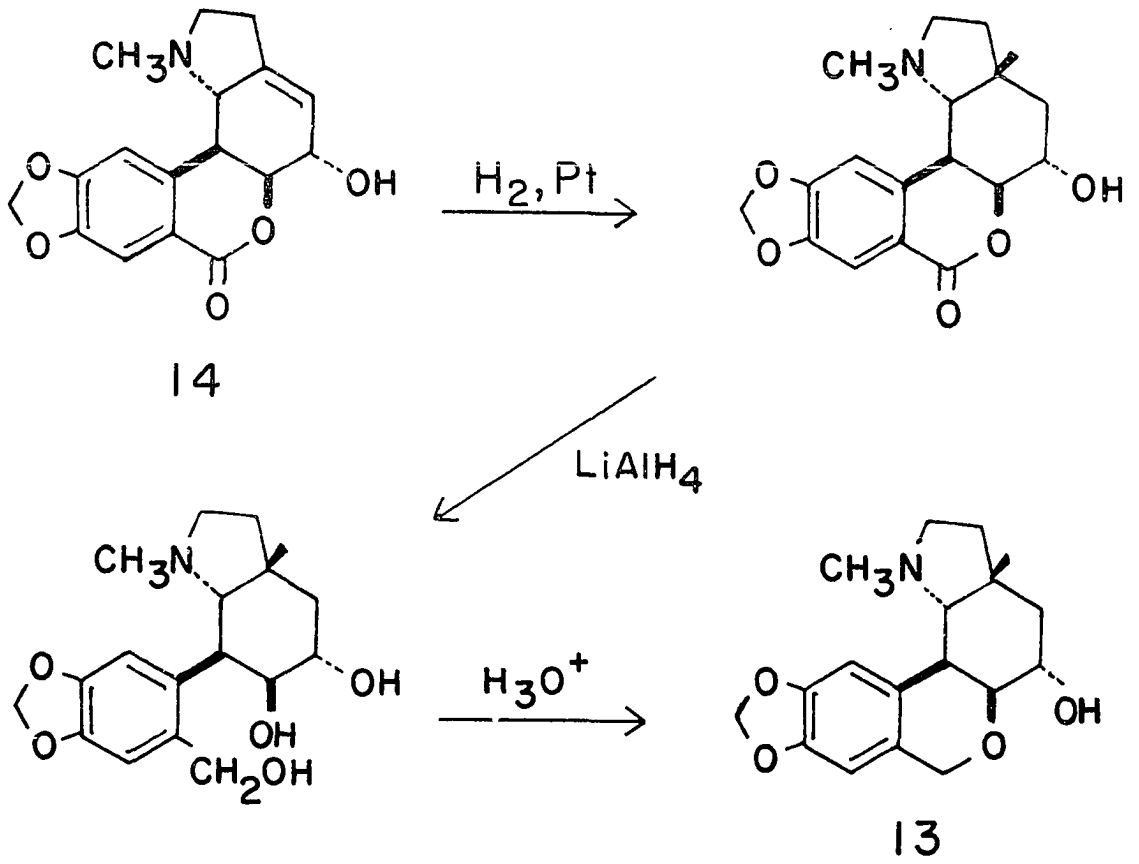


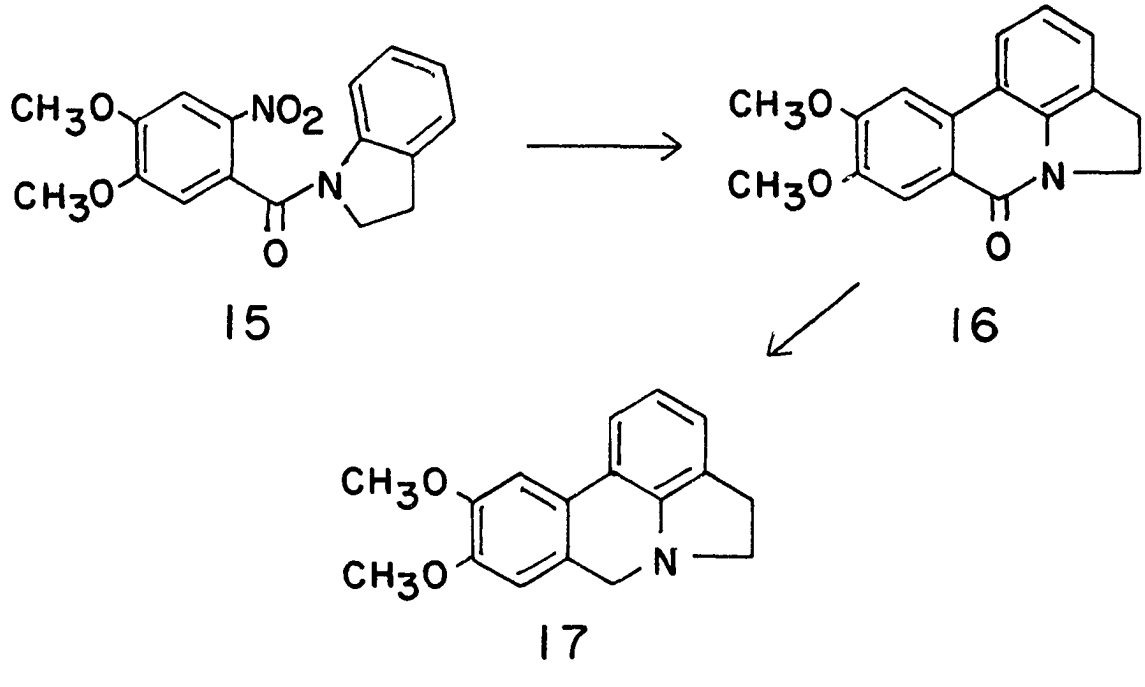
Figure 3. Conversion of hippeastrine to tetrahydrohippeastrine

Methylpseudolycorine, Galanthine, and Pluviine

Since methylpseudolycorine, galanthine and pluviine are closely related in structure and in their chemical transformations, the three alkaloids are discussed as a group rather than individually. Methylpseudolycorine was first isolated by Fales and associates from "King Alfred" daffodils in trace amount[14]. Galanthine was isolated from Galanthus woronowii by Proskurine and Areshkina[15] and

pluviine has been isolated from Lycoris radiata and several varieties of Narcissus pseudonarcissus[16,17].

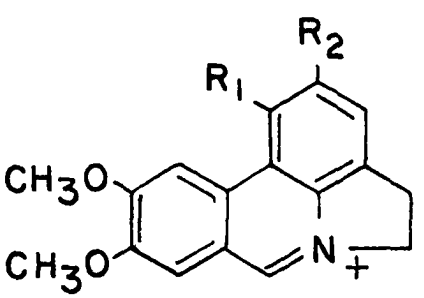
Both methylpseudolycorine and galanthine under pyrolytic conditions yielded the same product, anhydromethylpseudolycorine(17)[14,18]. Pluviine also gave a similar product via acetylation conditions, anhdromethylpseudolycorinium chloride which, when oxidized with potassium ferricyanide yielded the known phenanthridone(16)[19]. The structures of anhydromethylpseudolycroine and the phenanthridone were proven by independent synthesis from 1-(6-nitroveratryl)-indoline(15). This data showed that the three bases were of the pyrrolo[de]phenanthridine type with methoxy groups in the 9 and 10 positions. Other degradative data showed that the alkaloids contained a non-conjugated double-bond

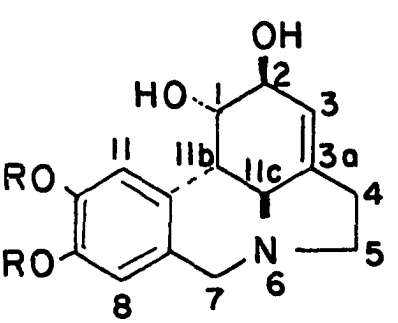


15 16 17

with methylpseudolycorine having two vicinal hydroxyls and galanthine having a hydroxyl and an aliphatic methoxyl as the other functional groups.

Under oxidative conditions (selenium dioxide or Oppenhauer) the three alkaloids yielded different oxyphenanthridinium betaines, 18a(methylpseudolycorine), 18b(galanthine), and 18c(pluviine). The structures of 18a and 18c were assigned by comparison to similar products obtained from lycorine(19b). This established a hydroxyl at C-2 in methylpseudolycorine, a hydroxyl at C-1 for pluviine, and a methoxyl moiety at C-2 in galanthine. Since the hydroxyls of methylpseudolycorine were known to be vicinal and the unsaturation was placed at 3,3a, the remaining alcohol was assigned to C-1. Thus, methylpseudolycorine(19a) is the dimethoxy analogue of lycorine(19b).





18 a) $R_1 = H, R_2 = O^-$

b) $R_1 = H, R_2 = OCH_3$

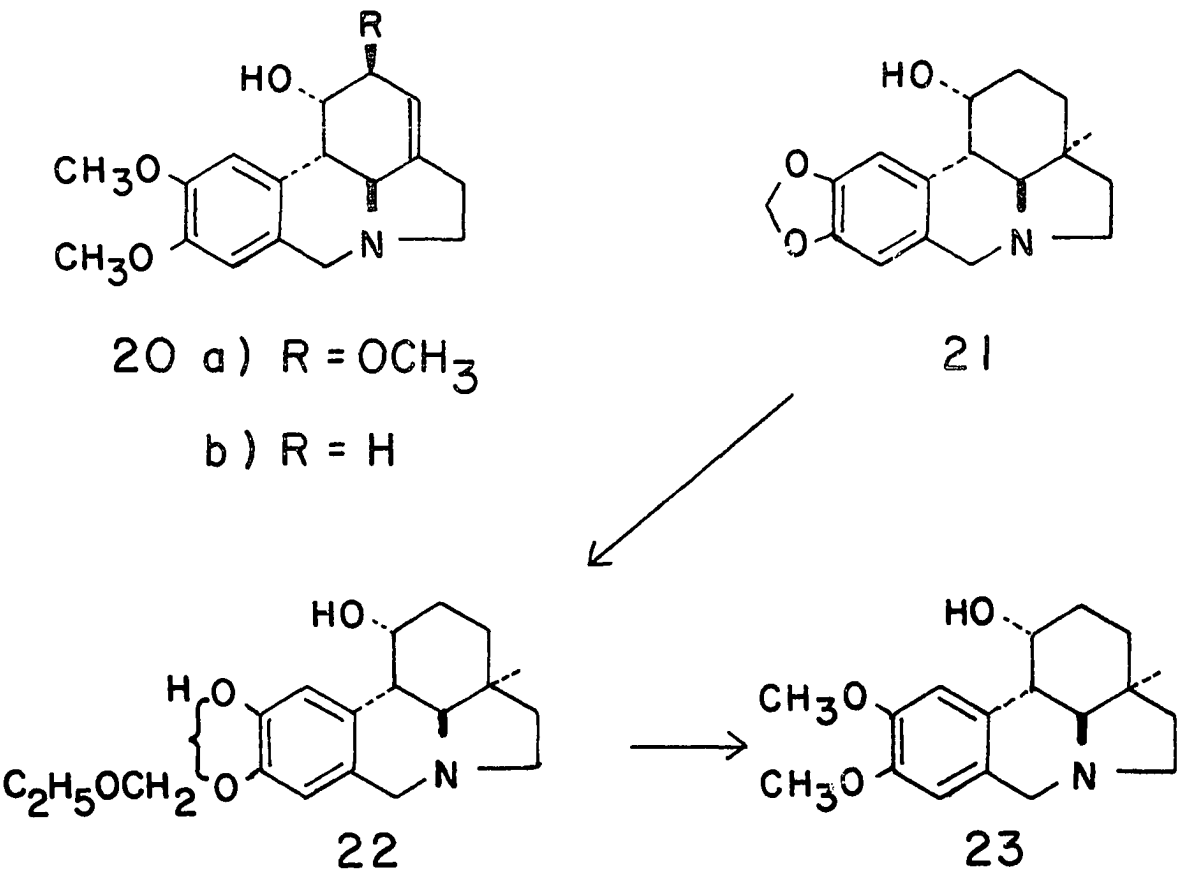
c) $R_1 = O^-, R_2 = H$

19 a) $R = CH_3$

b) $R,R = -CH_2-$

Further interrelationships of these alkaloids was obtained by the reduction of galanthine with sodium and various alcohols[20], and the mild acid hydrolysis of galanthine to methylpseudolycorine[21]. It was found that pluviine was isolated from the sodium/amyl alcohol reduction of galanthine, thereby establishing a C-1 alcohol in galanthine. The C-1 hydroxyl in pluviine was proven in that pluviine is not oxidized with manganese dioxide and the 3,3a unsaturation was assigned on the basis of comparison of other lycorine-type alkaloids[18]. These data then led to the assignment of structures 20a and 20b for galanthine and pluviine, respectively[19]. The remaining problem of stereochemistry was solved easily by converting a methylenedioxy lycorine-type alkaloid to a dimethoxy lycorine-type alkaloid. This was accomplished via ethanolic base cleavage of the methylenedioxy group of α-dihydrocaranine(21, of known configuration) to an ethoxy methyl ether(22) which was converted to α-dihydropluviine(23) via acid hydrolysis followed by treatment with diazomethane[22].

Spectral studies have substantiated the structural assignments. Galanthine and 2-O-methyllycorine show identical hydroxyl stretching frequencies in dilute chloroform solution (3602 and 3624 cm^{-1})[23]. The 3602 cm^{-1} band may be attributed to C-1 hydroxyl bonding to the aromatic π-electron cloud, whereas the latter frequency is typical of an unbonded secondary alcohol. Caranine exhibited comparable values of

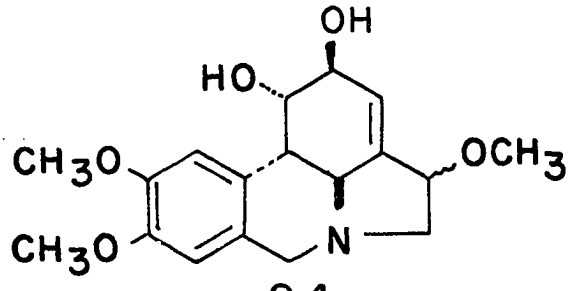


3595 and 3624 cm^{-1}. The mass spectrum of galanthine also supports a <u>trans</u> C-2-oxygenated functionality[24]. The mass spectrum of methylpseudolycorine is very similar to that of lycorine and differs by 16 mass units.

Narcissidine

Tnis base has been isolated from many varieties of <u>Narcissus</u>[16,17,25]. Preliminary characterization indicated the presence of three methoxyls, two hydroxyls, a tertiary base with no N-methyl group. The basic ring system was

determined to be 9,10-dimethoxypyrrolo[de]phenanthridine from the observation that pluviine was isolated from the sodium and amyl alcohol reduction of narcissidine[20]. This also established the presence of a C-1 hydroxyl. Since the hydroxyl groups were determined to be vicinal on the basis of cleavage with periodic acid, structure 24 was assigned to narcissidine by analogy with the structures of lycorine, methylpseudolycorine, and galanthine[3].



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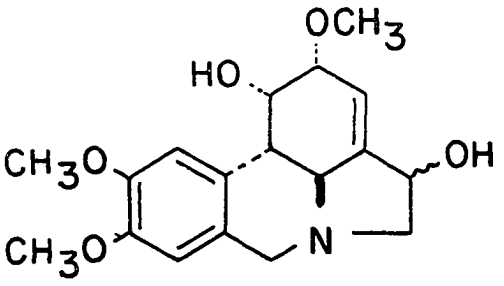
On the basis of mass spectral studies involving pluviine, galanthine and methylpseudolycorine, a revised structure was proposed for narcissidine[24]. In analogy with the three alkaloids above, the mass spectrum of the previously assigned

ıcture of narcissidine(24), there should appear major

ı gments at m/e 273 and 272 due to the loss of C-1, C-2 and the atoms attached. No strong peaks occur at these values. Major fragmentations did occur at m/e 259 and 258 (M-74 and M-75) representing the loss of $C_3H_6O_2$ and $C_3H_7O_2$. A metastable ion at m/e 200.9 ($259^2/333=201.4$) was detected and shows the loss of 74 mass units is derived directly from the parent ion.

Therefore, the aliphatic methoxyl was reassigned to C-2 and the hydroxyl to C-4 as in structure 25. The cis C-1, C-2 arrangement of the groups was indicated by the presence of hydroxyl stretching frequencies in dilute solution of 3544 and 3612 cm^{-1}[23]. The configuration of the C-4 hydroxyl remained undetermined.



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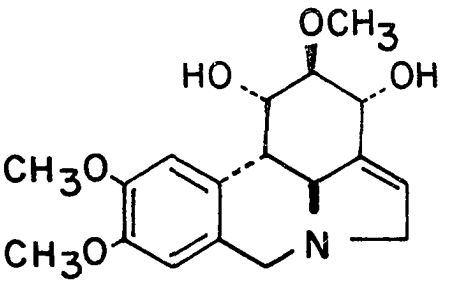


26

The entire structural problem of narcissidine has recently been resolved via the X-ray crystal structure determination of narcissidine hydrobromide. The result of this study shows that narcissidine possesses structure 26[26].

Narciclasine

The neutral substance given the name narciclasine was first reported by Piozzi and colleagues in 1966[27]. Narciclasine was reported to have antimitotic properties and was isolated from several varieties of daffodils. A later report showed that the substance was common to many Narcissus species[28]. Piozzi and coworkers reported chemical

degradations and spectral data in 1968 which led to a proposed structure for narciclasine[29].

Later in 1968 several Japanese workers isolated a substance from Lycoris radiata that possessed physical and chemical properties very similar to narciclasine and it was speculated that the substances were the same[30]. These workers upon examination of their chemical and spectral data proposed a structure different from the structure assigned by Piozzi.

Both groups also isolated compounds similar in structure to narciclasine, but lacking in a phenolic function. The compound isolated by the Italians was called margetine[31]. The comparable compound isolated by the Japanese was given the name lycoricidine and the material comparable to narciclasine was called lycoricidinol. The work of the Italians will be discussed first and the discussion of the Japanese work will follow. It is assumed by this author that narciclasine is identical to lycoricidinol and margetine is identical to lycorididine.

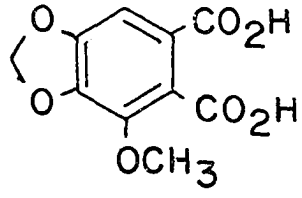
Although combustion analysis was always unsatisfactory, the mass spectrum (molecular ion at m/e 307) and chemical data of narciclasine led to a molecular formula of $C_{14}H_{13}O_7N$. The material possessed no basic properties, but was phenolic in character and a nitrogen in the form of an amide was indicated.

A parent ion of m/e 307 was exhibited in the mass spectrum along with two strong peaks at m/e 289(M-18) and

m/e 271(M-36). The infra-red spectrum indicated the presence of a carbonyl function, hydroxy groups, and a methylenedioxy group. The UV spectrum showed strong absorption maxima, whose wavelengths shifted in basic solution. The 100 MHz. NMR spectrum of narciclasine in DMSO-d_6 showed resonances at 13.23δ(singlet,1H), 4.4-5.5δ(broad,3H), 7.85δ (singlet,1H), 3.7-4.2δ(complex multiplet,4H), 6.78δ (singlet,1H), 6.18δ(broad multiplet,1H) and 6.12δ(singlet,2H). All values are given in ppm downfield from TMS. The singlet at 13.23 was assigned to a phenol proton chelated to the carbonyl. The broad absorption at 4.4-5.5 was assigned to three alcohol protons since the absorption disappeared on addition of D_2O. The singlets at 7.85 and 6.12 were attributed to an aromatic proton and methylenedioxy protons, respectively.

Narciclasine could be converted to a dihydro derivative consuming one mole of hydrogen. The UV spectrum of dihydronarciclasine showed a hypsochromic shift indicating the double bond was conjugated with the aromatic ring. Phenanthridine was the product of zinc dust distillation. An O-methyl derivative was obtained upon treatment with diazomethane. The O-methyl compound exhibited no phenolic properties and oxidation with permanganate yielded cotarnic acid(27). Treatment with pyridine and acetic anhydride afforded an oily O,O,O-triacetyl derivative of O-methylnarciclasine. Mild basic hydrolysis regenerated O-methylnarciclasine.

The spectral data and the chemical transformations suggested the structure 28, with three hydroxyl functions to be positioned to complete the structural determination.



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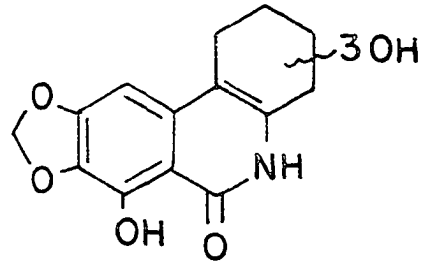
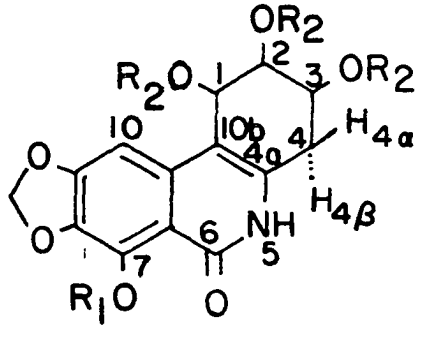


28

The assignment of positions of the hydroxyl groups was based mainly on the analysis of the 100 MHz. NMR data of 0-methyl-0,0,0-triacetylnarciclasine. The assignment of the methylenedioxy, 0-methyl, aromatic, and acetyl protons was straightforward. The remaining five protons showed resonances at 6.27, 5.48, 5.40, 5.18, and 4.58δ, and all were shown to be interacting with each other. The results of the NMR experiments led to a sequence of 1, 2, 3 for the acetoxy groups with C-4 a methylene as shown in structure 29a.



29 a) $R_1 = CH_3$, $R_2 = COCH_3$

b) $R_1 = R_2 = H$

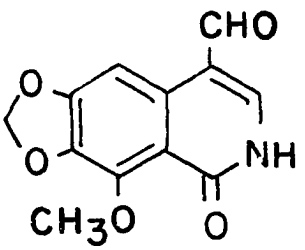
The resonances listed above were assigned to H-1, H-3, H-2, H-4β and H-4α, respectively. Addition of D_2O sharpened the peaks for H-1 and H-4α since exchange of the amide proton removed the coupling between H-1 and H-4α with the N-H. It was assumed from conformational considerations that the H-4β proton was out of the plane of the double bond and consequently the coupling between H-4β and N-H was zero.

A description of the NMR decoupling experiment of O-methyl triacetylnarciclasine is quoted directly from reference 29, p. 1121.

> H-1 couples with H-2($J_{1,2}$=4.4c/s), H-3($J_{1,3}$=1.0c/s), and H-4α($J_{1,4\alpha}$=2.2c/s) giving an eight line pattern. Upon irradiation of H-2($\Delta\nu$=+88c/s) $J_{1,2}$ vanishes and H-1 becomes a quartet with splitting of 1.0 and 2.2 c/s. Irradiation of H-3($\Delta\nu$=+84c/s) also perturbs H-2 according to the small shift difference, while, irradiation of H-1 decouples H-2 with $\Delta\nu$=-86c/s and H-3 with $\Delta\nu$=-82c/s: H-3 changes from a triplet of doublets($J_{2,3}=J_{3,4\alpha}$=2.3-2.4c/s; $J_{1,3}$=1.0c/s) into a triplet; the eight line pattern of H-2($J_{2,3}$=2.4c/s; $J_{1,2}$=4.5c/s; $J_{2,4\alpha}$=1.2c/s) collapses into a slightly broadened doublet, with $J_{2,4\alpha}$ not completely resolved.
>
> The last coupling of H-1($J_{1,4\alpha}$=2.2c/s) is removed by irradiation of H-4α($\Delta\nu$=+170c/s): it leads to a double doublet with splitting of 4.5 and 1.0 c/s. Reversal experiment ($\Delta\nu$=-174c/s) converts the H-4α signal into two doublets of 9.0 and 1.2c/s($J_{4\alpha,4\beta}$ and $J_{2,4\alpha}$, resp.).
>
> The strong coupling on 9.0c/s (gemial interaction of the protons at C-4) vanishes upon irradiation of H-4α($\Delta\nu$=+58c/s): the two doublets of H-4β collapse in one doublet of 2.3c/s ($J_{3,4\beta}$); the reverse experiment ($\Delta\nu$=-58c/s) leads H-4α to a broad signal, the line width of which measures 4.7c/s, being consistent with 1,4α and 2,4α couplings.
>
> The last coupling($J_{2,4\alpha}$=1.2c/s) is proved by irradiation of H-4α(=+87c/s): the H-2 signal goes into a doublet of doublets with splitting of 4.5 and 2.4c/s.

It is argued that the methylene group must be assigned to position 4, since the strong downfield shift of both protons can be explained by the amidic nitrogen, having a strong localized positive charge. Furthermore, the large difference in the chemical shifts of the α and β protons, it is argued, can be caused only by the different orientations to the amidic C=O plane. The final result is structure 29b for narciclasine.

This structural assignment was further supported by the periodic acid oxidation of O-methylnarciclasine. The product of this oxidation was assigned the structure 30 on the basis of its spectral properties and similarity in the UV to the dialdehyde 31 obtained from periodic acid oxidation of dihydrolycorinone.



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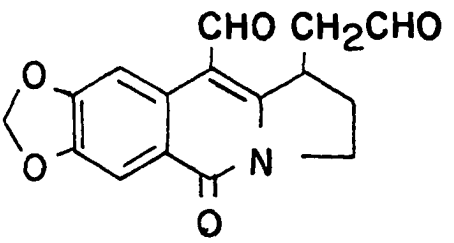
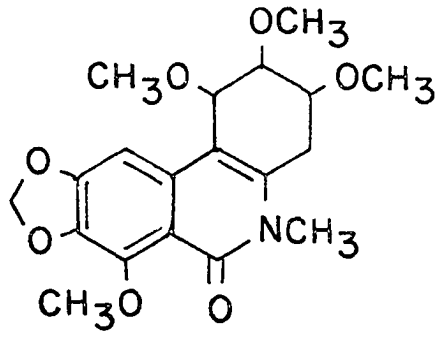


31

It is suggested that the $-CH_2CHO$ group expected at C-3 is removed by further oxidation with excess periodic acid. This is considered reasonable by the authors, because the intermediate with a C-3 $-CH_2CHO$ group is vinylogous with malonic dialdehyde which has been reported to yield three moles of formic acid upon oxidation with periodic acid[29].

Kuhn methylation conditions affords two products, permethyl-narciclasine(32) and permethyl-isonarciprimine(33).



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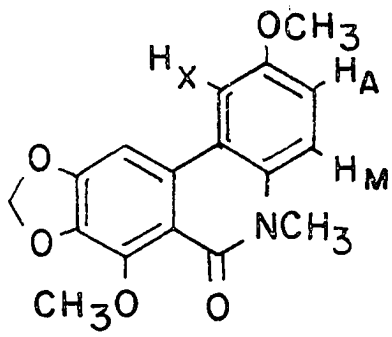
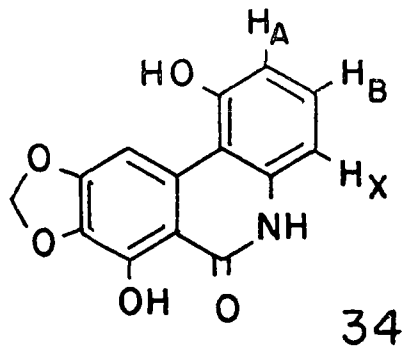


33

The structures of these compounds were based on their NMR spectra. The assignments for structure 33 was that the protons on ring C give an AMX pattern where H_X, a broadened doublet (J_{AX}=2.5c/s, J_{MX}=0.5c/s), H_A a double doublet (J_{AM}=8.5c/s) and H_M a slightly broadened doublet. These are assigned as J_{AM}, an ortho coupling, J_{MX}, a para coupling, and J_{AX}, meta. The NMR spectra of permethylnarciclasine was very similar to the triacetyl O-methyl compound, except that the ring C protons, H-2, H-3, H-4α and H-4β were shifted upfield and H-1 was still remarkably deshielded at 6.13δ.

A substance named narciprimine was believed to be formed from narciclasine during the isolation procedures. It was obtainable from narciclasine by treatment with strong acid. Narciprimine yields a di-O-methyl derivative with diazomethane, a diacetyl derivative with pyridine and acetic

anhydride and cotarnic acid with neutral permanganate. Its structure rests largely on the 100 MHz. NMR which showed a strongly coupled ABX pattern with coupling constants of $J_{BX}=J_{AB}=7.8$c/s (ortho couplings), $J_{AX}=1.5$c/s (meta coupling), where H_A appears as a double doublet, H_B, a triplet and H_X, a double doublet. These data led to structure 34 for narciprimine.



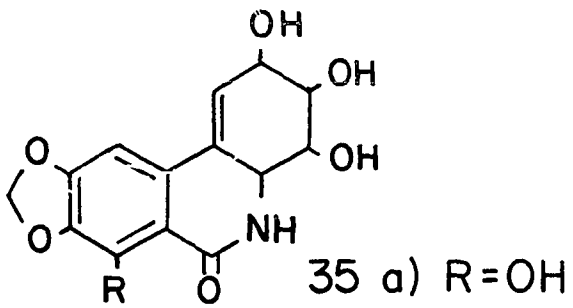
34

The alternative structure with the phenol at C-4 is ruled out since narciclasine produces narciprimine by the loss of two molecules of water. The structure of narciclasine requires a C-1 phenol for narciprimine, rather than a C-4 phenol.

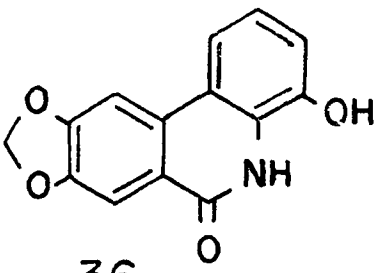
In 1968 Okamoto and coworkers reported the isolation and structure of lycoricidinol and lycoricidine, compounds suspected to be identical with narciclasine and margetine, respectively[30].

The structures assigned to lycoricidinol(35a) and lycoricidine(35b) were based on essentially the same data as that used for narciclasine and margetine. The synthesis of

a degradation product and a different interpretation of the observed NMR spectrum led Okamoto and his colleagues to structures 35a and 35b. The Japanese synthesized a derivative of the double dehydration product from lycoricidine. Lycoricidine, when treated with strong acid, affords a compound assigned structure 36 (comparable to narciprimine but lacking the phenol in ring A). The N-benzyl-O-methyl derivative of 36

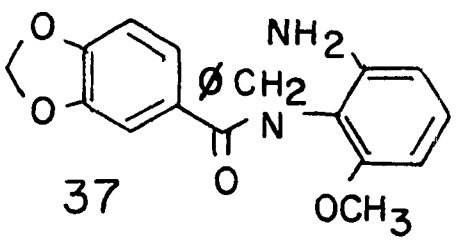


35 a) R=OH
b) R = H



36

was produced by independent synthesis and from 36. Substance 36 upon treatment with diazomethane yields the O-methyl derivative. The N-benzyl-O-methyl derivative was formed by treatment with sodium hydride and benzyl chloride. This same substance was obtained by the Pschorr cyclization of compound 37. It is from this chemical degradation of lycoricidine and synthesis that the sequence of ring C hydroxyls was assigned to C-2, C-3



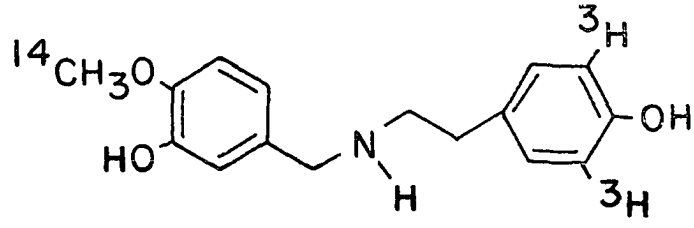
37

and C-4. The NMR spectra were reinterpreted in the following way. The very low field resonance was assigned as a vinyl proton at C-1(6.1δ), Signals at 3.5-4.2δ were assigned as 4 protons at the 2, 3, 4, and 4a positions. These resonance values are for lycoricidinol in DMSO-d_6(60 MHz).

Neither of the proposed structures for narciclasine (lycoricidinol) explain all of the data accumulated. The placement of a hydroxyl at C-4 by the Japanese is required by the synthetic work. But the placement of the olefinic bond at 1,10b does not adequately sustain the NMR decoupling data of the Italian workers. On the other hand, the arguments presented by the Italians for the placement of the hydroxyl at C-1 are at best weak. It is inferred that the structural assignment for narciprimine supports the structure of narciclasine, but the positioning of the phenol of narciprimine was assigned on the basis of the structure of narciclasine. Furthermore, the NMR data does not rigorously require a C-1 hydroxyl.

Both groups report the cleavage of the C-ring with periodic acid. The Italians report as well the product of the oxidation, narciclasic aldehyde(30). The conditions required to give oxidation as reported by Piozzi are much more rigorous than those required for the oxidation of a similar system, shikimic acid. Subjecting narciclasine to those conditions does not bring about cleavage[32].

Experiments have been performed recently to investigate the biosynthesis of narciclasine*. This work was completed before the publication of the Japanese structure for narciclasine(35a) and is therefore based on the Italian structure(29a). The biosynthetic precursor used was the doubly-labeled O-methylnorbelladine(37a).



37a

The O-methylnorbelladine was incorporated intact into narciclasine (0.18%), i.e., no tritium was lost. Degradations proved the ^{14}C label was located in the methylenedioxy group and the tritium resided in ring C. The degradative data is not compatible with the structure assigned to lycoricidinol (35a).

It was proposed that the biosynthetic pathway to narciclasine proceeded through the crinine ring system. It was argued that the degradation data substantiated this hypothesis. Also, norpluviine labeled with tritium (apparently of low activity) was found not to be incorporated.

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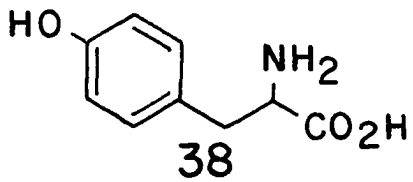
*A. R. Battersby, private communication to Dr. W. C. Wildman, Department of Chemistry, Iowa State University, Ames, Iowa, 1969.

Biosynthesis of the Amaryllidaceae Alkaloids

Biosynthetic investigations of the Amaryllidaceae alkaloids began in 1960 with the use of radioactive tracers. The early work determined the amino acid precursors of the alkaloids. These investigations, summarized in Table 1, showed that tyrosine(38) is a precursor of the hydroaromatic C_6-C_2 unit. It was further shown that phenylalanine(39) is a precursor to the C_6-C_1 fragment of the alkaloids. These incorporations were shown to be highly specific, tyrosine was never incorporated into the C_6-C_1 unit and phenylalanine was never incorporated into the C_6-C_2 unit.



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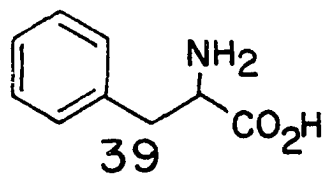


39

Several biogenetic theories have been proposed to account for the biogenesis of the Amaryllidaceae alkaloids [43,44,45,46]. The theory that has received the widest acceptance is that of Barton and Cohen[43] who proposed that many natural products could be rationalized on the basis of ortho-ortho, ortho-para, or para-para oxidative coupling of phenol radicals. They postulated that the Amaryllidaceae alkaloids arise from such oxidative coupling of the precursor norbelladine(40a). The alkaloid belladine(40b) was unknown

Table 1. Incorporation of amino acids into Amaryllidaceae alkaloids

Precursor	Plant	Alkaloid	% Inc.	Ref.
DL-2-^{14}C-Tyrosine	Narcissus "Twink"	Haemanthamine	0.08	33
		Norpluviine	0.15	33
		Caranine	0.08	33
		Lycorine	0.23	33
	Narcissus "King Alfred"	Galanthamine	0.013	34
		Lycorine[a]	----	35
		Haemanthamine	0.13	34
	Narcissus "Texas"	Lycorine	0.44	34
	Narcissus incomparabilis	Lycorine	0.22	36
	Galanthus elvesii	Galanthamine	0.14	34
		Lycorine	0.04	34
DL-3-^{14}C-Tyrosine	Sprekelia formossisima	Haemanthamine[a]	----	37
		Haemanthidine	0.20	37
		Tazettine	0.16	37
	Haemanthus natalensis	Haemanthamine	0.97	38
		Haemanthidine	0.19	38
		6-Hydroxycrinamine	1.18	38
	Narcissus incomparabilis	Lycorine	0.18	36
	Nerine bowdenii	Ambelline	0.015	39
		Belladine	0.82	40
		Lycorine	0.11	40
	Crinum erebescens	Crinamine	0.17	41
		6-Hydroxycrinamine	0.09	41
		Lycorine	0.51	41

[a]Percentage of incorporation was either not reported or quoted in figures that could not be converted to per cent incorporation. In all cases, appreciable incorporations were evident.

Table 1. (Continued)

Precursor	Plant	Alkaloid	% Inc.	Ref.
DL-2-^{14}C-Phenylalanine	Narcissus incomparabilis	Lycorine	0.00	36
DL-3-^{14}C-Phenylalanine	Narcissus incomparabilis	Lycorine	0.18	36
	Nerine bowdenii	Ambelline	0.023	39
		Belladine	0.42	40
		Lycorine	0.095	40
	Sprekelia formosissima	Tazettine	0.01	39
	Narcissus "Deanna Durbin"	Lycorine[a]	----	42
		Pluviine[a]	----	42
		Haemanthamine[a]	----	42
		Galanthine[a]	----	42

at the time that Barton and Cohen proposed their theory. The isolation shortly thereafter of belladine from Nerine bowdenii W. Wats. and Amaryllis belladona L.[47] lent good support to the oxidative coupling theory. A biogenetic scheme for the formation of norpluviine(41), caranine(42), lycorine(19b), and galanthamine(44) is formulated in Figures 4 and 5. In vitro chemical verification for the hypothesis was obtained when manganese dioxide oxidation of O,N-dimethylnorbelladine(45), followed by lithium aluminum hydride reduction yielded galanthamine(44)[48].

Biosynthetic studies have shown that several variously labeled norbelladine derivatives are incorporated intact into several types of alkaloids. These data, summarized in Table 2, lend good support for the phenyl-phenyl oxidative coupling theory. Norbelladine was shown to be incorporated into the lycorine, crinine and galanthamine series. O-methylnorbelladine provides an efficient precursor to the crinine- and lycorine-type alkaloids. If the nitrogen is methylated, the O,N-dimethylnorbelladine(45) appears to incorporate exclusively into the galanthamine series.

Incorporation data has been reported that shows norbelladine is synthesized in vivo via the following pathway: phenylalanine ⟶ trans-cinnamic acid ⟶ caffeic acid ⟶ protocatechuic aldehyde, which couples with tyramine, derived from the decarboxylation of tyrosine[49]. This path-

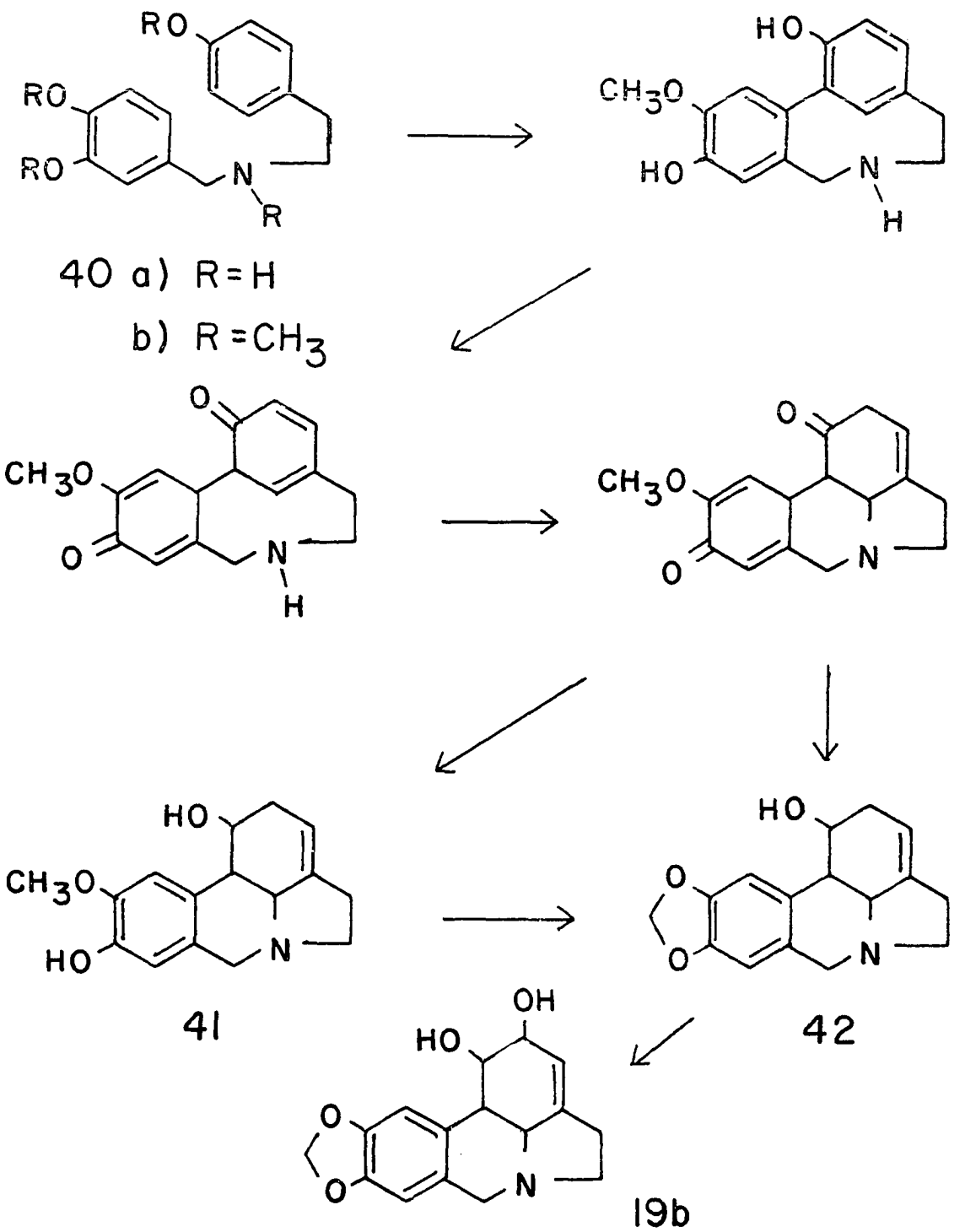


Figure 4. Biogenesis of norpluviine, caranine, and lycorine

Table 2. Incorporation of norbelladine and derivatives into Amaryllidaceae alkaloids

Precursor	Plant	Alkaloid	% Inc.	Ref.
1-^{14}C-Norbelladine	Narcissus "Twink"	Lycorine	0.24	49
		Norpluviine	0.74	49
		Haemanthamine	0.15	50
	Narcissus "King Alfred"	Galanthamine[a]	----	51
		Galanthine[a]	----	51
		Haemanthamine[a]	----	51
1,1'-^{14}C-Norbelladine	Nerine bowdenii	Belladine	2.64	52
		Crinamine	0.0009	52
		Lycorine	0.07	52
1'-^{14}C-Bisdeoxynor-belladine	Narcissus "Twink"	Tazettine	0.00	53
		Haemanthamine	0.00	53
1'-^{14}C-Hydroxynor-belladine	Narcissus "Twink"	Lycorine	0.007	53
		Norpluviine	0.017	53
N-^{14}C-Methylnorbelladine	Narcissus "King Alfred"	Galanthamine	0.18	51
		Galanthine	0.00	51
		Haemanthamine	0.00	51
O-Methyl-N-^{14}C-methyl-norbelladine	Narcissus "King Alfred"	Galanthamine	0.14	51
		Galanthine	0.00	51
		Haemanthamine	0.00	51
O-^{14}C-Methyl-N-^{14}C-methyl-1-^{14}C-norbelladine	Narcissus "King Alfred"	Galanthamine	0.018	54
		Galanthine	0.00	54
		Haemanthamine	0.00	54
O-^{14}C-Methylnor-belladine	Narcissus "King Alfred"	Galanthamine	0.00	55
		Galanthine[a]	----	55
		Haemanthamine	0.036	55

[a]Percentage of incorporation was either not reported or quoted in figures that could not be converted to per cent incorporation. In all cases, appreciable incorporation was evident.

Table 2. (Continued)

Precursor	Plant	Alkaloid	% Inc.	Ref.
O-^{14}C-Methyl-1-^{14}C-norbelladine	Narcissus "King Alfred"	Galanthamine	0.00	55
		Galanthine[a]	----	55
		Haemanthamine	1.00	55
O-^{14}C-Methyl-$^{3}H_2$ norbelladine	Narcissus "Twink"	Lycorine	0.93	56
		Norpluviine	1.50	56
O-Methyl-$^{3}H_4$-norbelladine	Narcissus "Deanna Durbin"	Lycorine	0.46	56
		Norpluviine	0.72	56
	Narcissus "Texas"	Norpluviine	1.40	57
		Narcissidine[b]	----	57
1'-^{14}C-O-Methylmethoxy-norbelladine	Narcissus "King Alfred"	Galanthine	0.00	57

[b]Incorporation not reported.

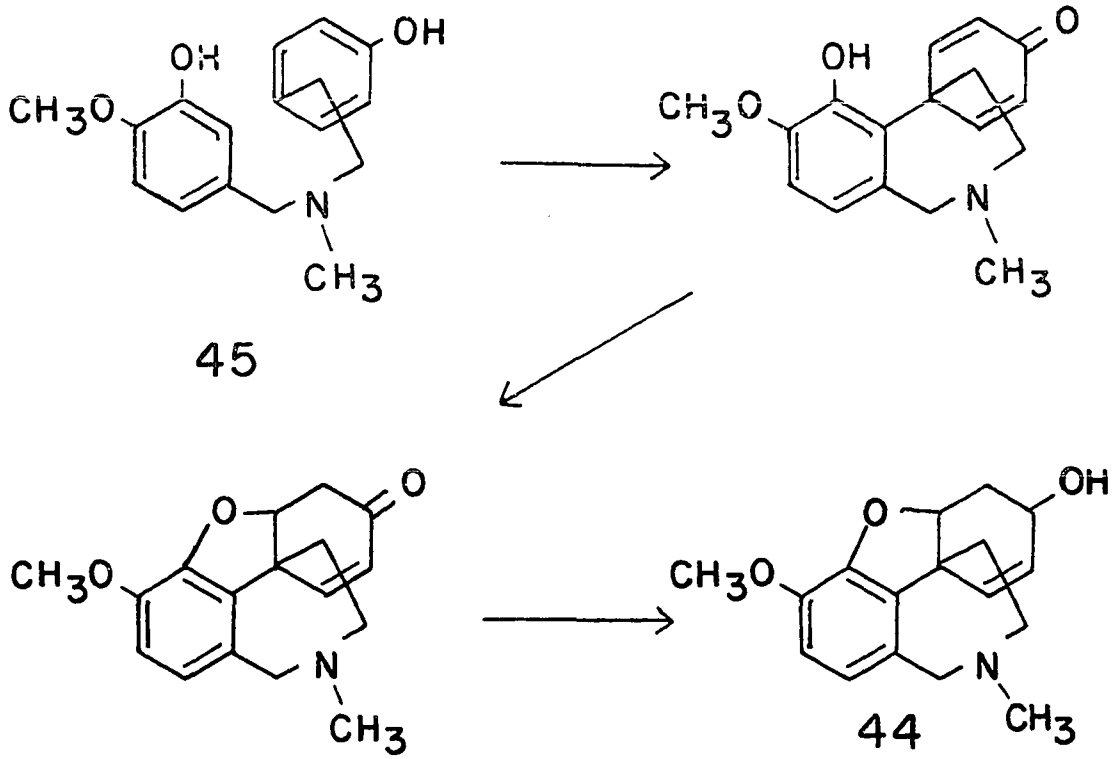


Figure 5. Biogenesis of galanthamine

way is illustrated in Figure 6. An alternate pathway: phenylalanine ⟶ phenylserine ⟶ benzaldehyde ⟶ protocatechuic aldehyde has been ruled out in that phenylserine[58] and benzaldehyde[59] showed negligible incorporation relative to <u>trans</u>-cinammic acid and protocatechuic aldehyde.

Late-Stage Biosynthetic Modifications of the Amaryllidaceae Alkaloids

Although the theory of phenyl-phenyl oxidative coupling is attractive and accounts for the lycorine, galanthamine and crinine ring systems, the lycorenine, montanine and pre-

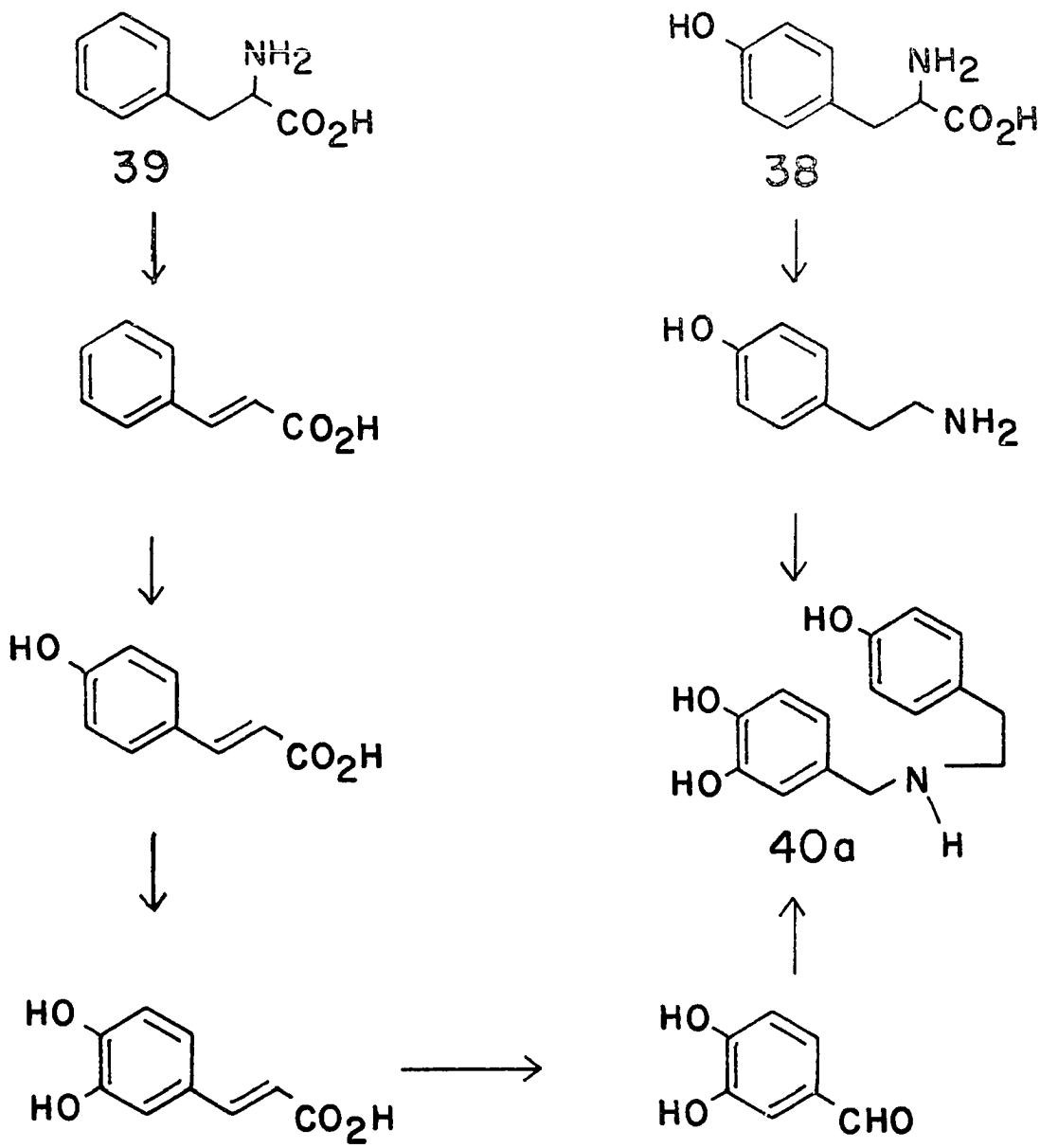
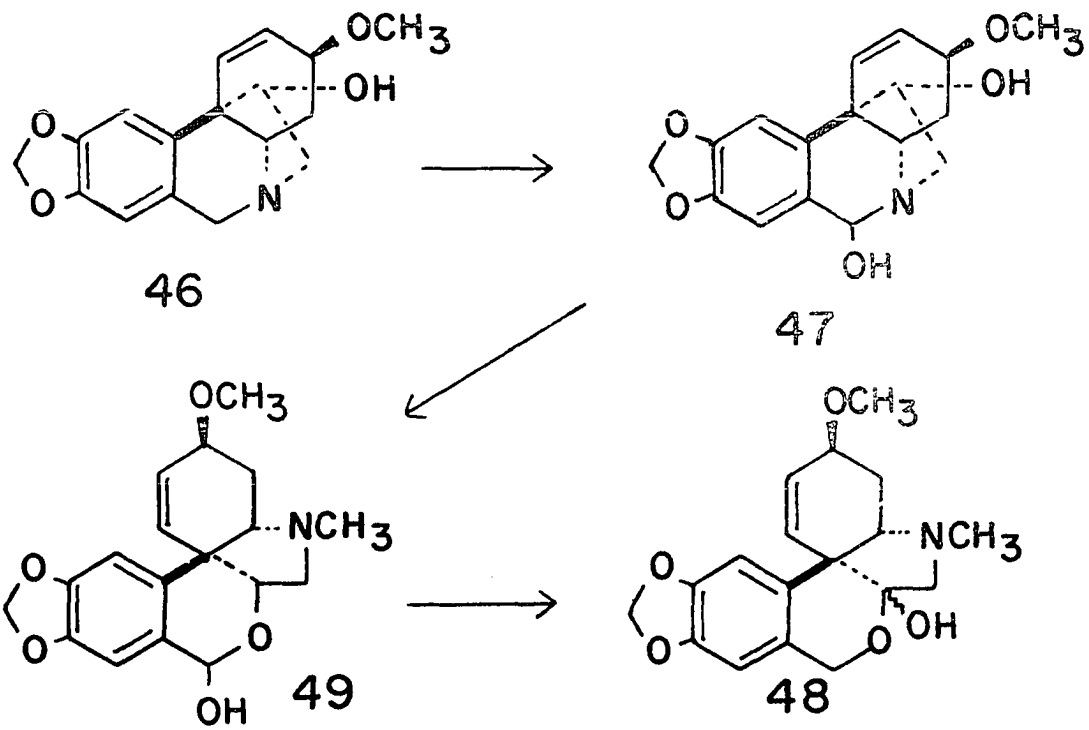


Figure 6. Biosynthesis of norbelladine

tazettine ring systems cannot be accommodated within the framework of the theory. It has been suggested that the most probable biosynthetic route to lycorenine-, montanine-, and pretazettine-type alkaloids involves two essential steps--an oxidation of a preformed alkaloid ring system and a rearrangement involving the oxidized carbon[21]. The alkaloid system required to give the lycorenine-type alkaloids is the lycorine-type nucleus. The montanine and pretazettine ring systems can be rationalized on the basis of oxidation/rearrangement of the crinine nucleus.

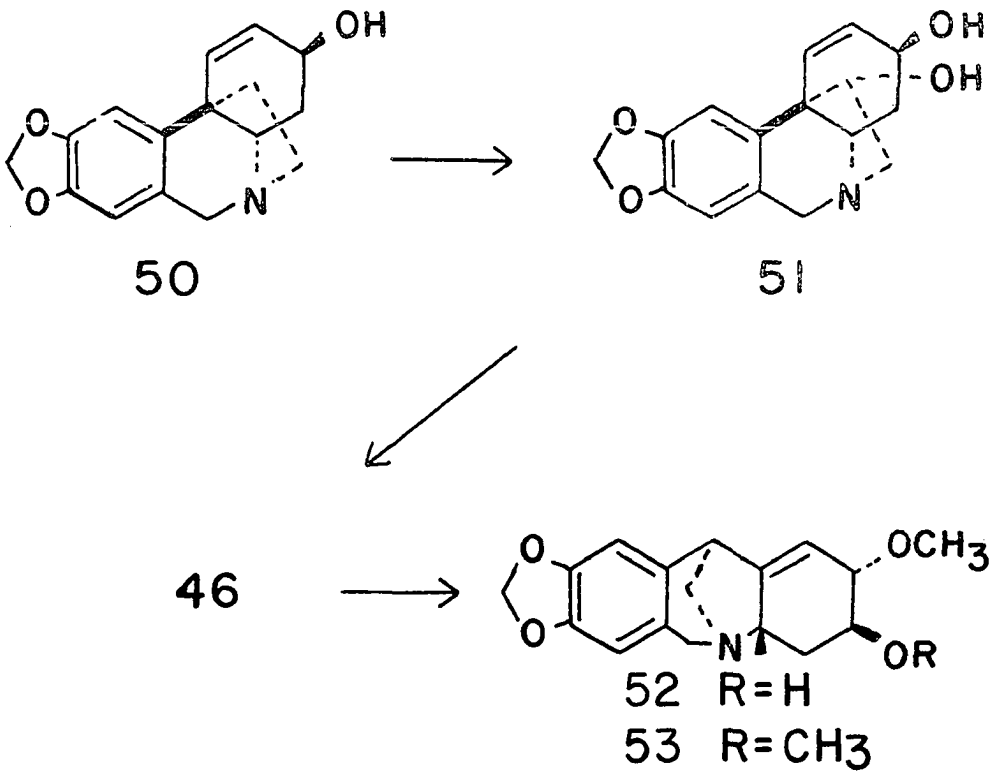
Substantiation of the hypothesized benzylic oxidation and rearrangement process to account for the biosynthesis of tazettine was provided when it was found that ^{3}H-haemanthamine (46) yielded radioactive haemanthidine(47) and tazettine(48) in Sprekelia formosissima[60]. Also, labeled haemanthidine gave radioactive tazettine, but the haemanthamine isolated showed no radioactivity. Since it is now known that tazettine is formed during isolation procedures from pretazettine(49)[61], the biosynthetic pathway from haemanthamine to haemanthidine to pretazettine has become easily rationalized on the basis of the oxidation/rearrangement process.

A different type of rearrangement process involving the crinine nucleus rationalizes the biosynthesis of the montanine alkaloids. In support of this is that aromatic tritiated vittatine(50) was found to be incorporated intact into



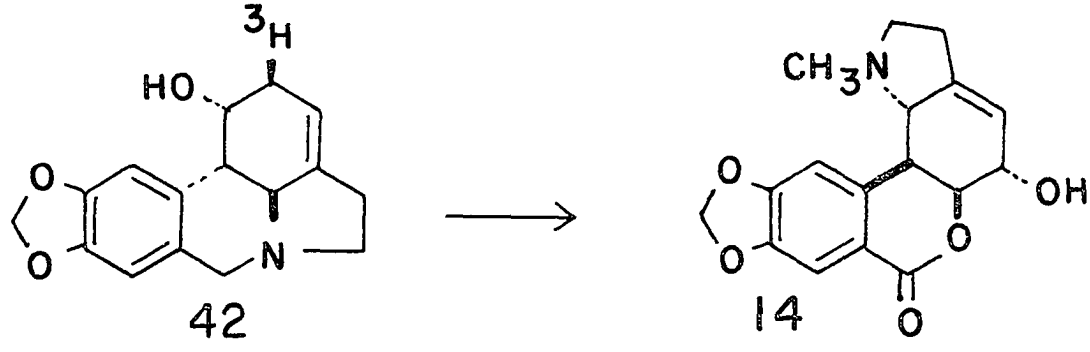
haemanthamine(46) and montanine(52) in Rhodophiala bifida[41]. This biosynthetic conversion is envisaged as a C-11 oxidation of vittatine to yield 11-hydroxyvittatine(51) followed by a phenyl shift displacing the hydroxyl at C-11. Double bond isomerization, capture of the carbonium ion by water and O-methylation completes the sequence from vittatine(50) to montanine(52). Apparently methylation of the C-3 oxygen of 11-hydroxyvittatine to give haemanthamine results in inhibition of the rearrangement process to give montanine alkaloids because ^{3}H-haemanthamine(46) is not incorporated into montanine (52) or manthine(53) in Haemanthus coccineus[62].

A benzylic oxidation and subsequent rearrangement process



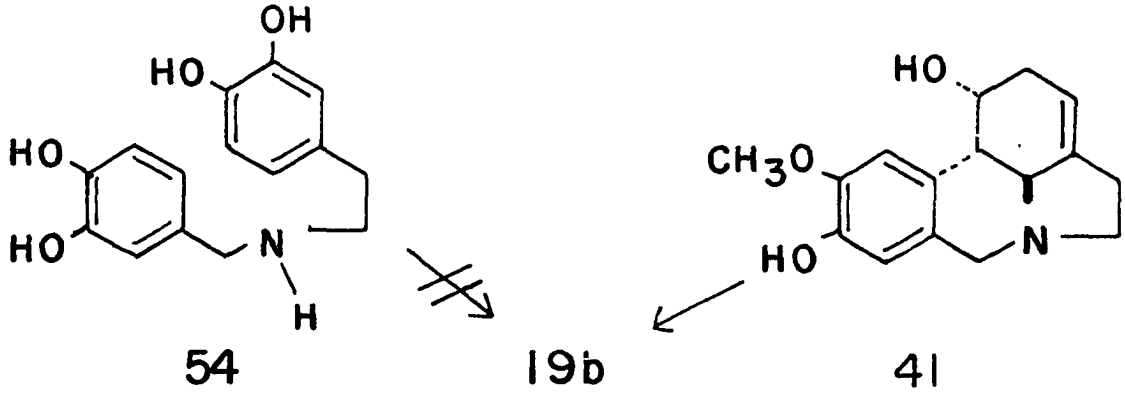
rationalizes the biosynthesis of the lycorenine-type alkaloids. C-7 oxidation of the lycorine nucleus, opening of the amino alcohol to the amino aldehyde, rotation about the 11a-11b bond to bring the alcohol into close proximity of the aldehyde, and closure of the hemiacetal function results in the overall transformation of a lycorine nucleus to a lycorenine nucleus. The first evidence in favor of this type of conversion was the incorporation of norbelladine into homolycorine (per cent incorporation=0.05%) in Narcissus "King Alfred"[63]. It was also found that O,N-dimethylnorbelladine was not incorporated into homolycorine or lycorenine. Further evidence to support the in vivo transformation of a lycorine-type to a lycorenine-

type was the incorporation of 2-^{3}H-caranine(53) into hippeastrine(14) in Hymenocallis americana[64].



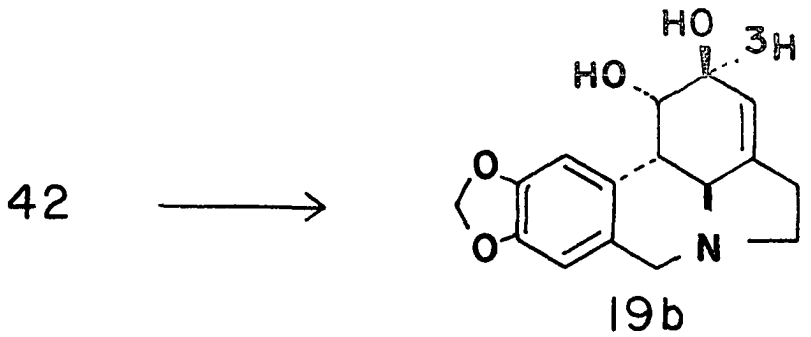
42 14

Another area of late-stage biosynthetic investigations has been the timing sequence of the C-2 hydroxylation in the lycorine alkaloids. The evidence shows that the oxidation reaction occurs late in the biosynthesis of lycorine. It was shown initially that 1'-^{14}C-hydroxynorbelladine(54) was not incorporated into lycorine(19b)[53]. A later investigation using ^{3}H-norpluviine(41) as the precursor showed incorporation into lycorine to the extent of 10.5%[33]. Further study of the



54 19b 41

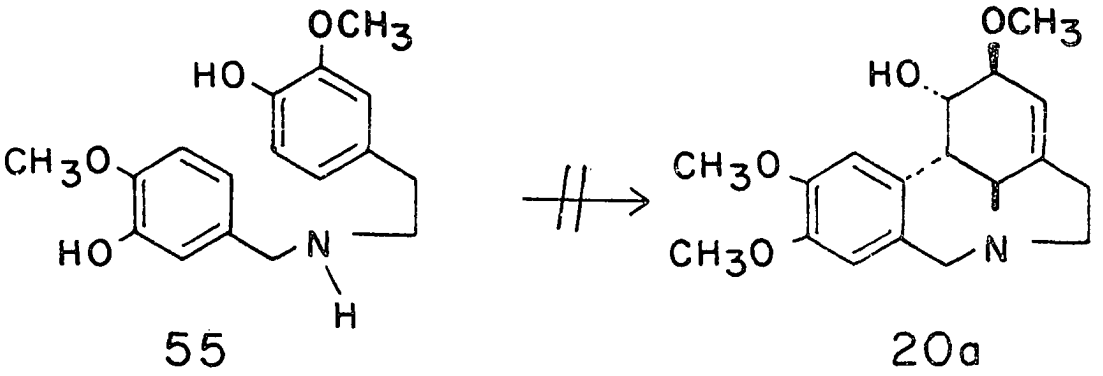
mechanism of the C-2 hydroxylation reaction showed an incorporation of 6.11% of 2β-^{3}H-caranine into lycorine[65]. These incorporations strongly suggest introduction of the hydroxyl function after the formation of the lycorine nucleus. Furthermore, the incorporation of 2β-^{3}H-caranine(42) with retention of the label indicates that the hydroxyl is incorporated via an inversion mechanism.



19b

Biosynthetic studies involving galanthine(20a) a dimethoxy analogue of lycorine, suggest that the C-2 oxygen (in the form of a methoxyl) is introduced late in the biosynthetic pathway of galanthine. It was found that O-methylmethoxynorbelladine (55) was not incorporated into galanthine(20a) in "King Alfred" daffodils[57]. Furthermore 8-^{3}H-norpluviine was incorporated to a small extent (0.006%) into galanthine in the same plant. These results indicate that in the dimethoxy analogues of lycorine, hydroxylation of C-2 appears to occur after the basic ring system is formed.

The origin of the methylenedioxy group of the alkaloids also has been investigated. It has been found that o-

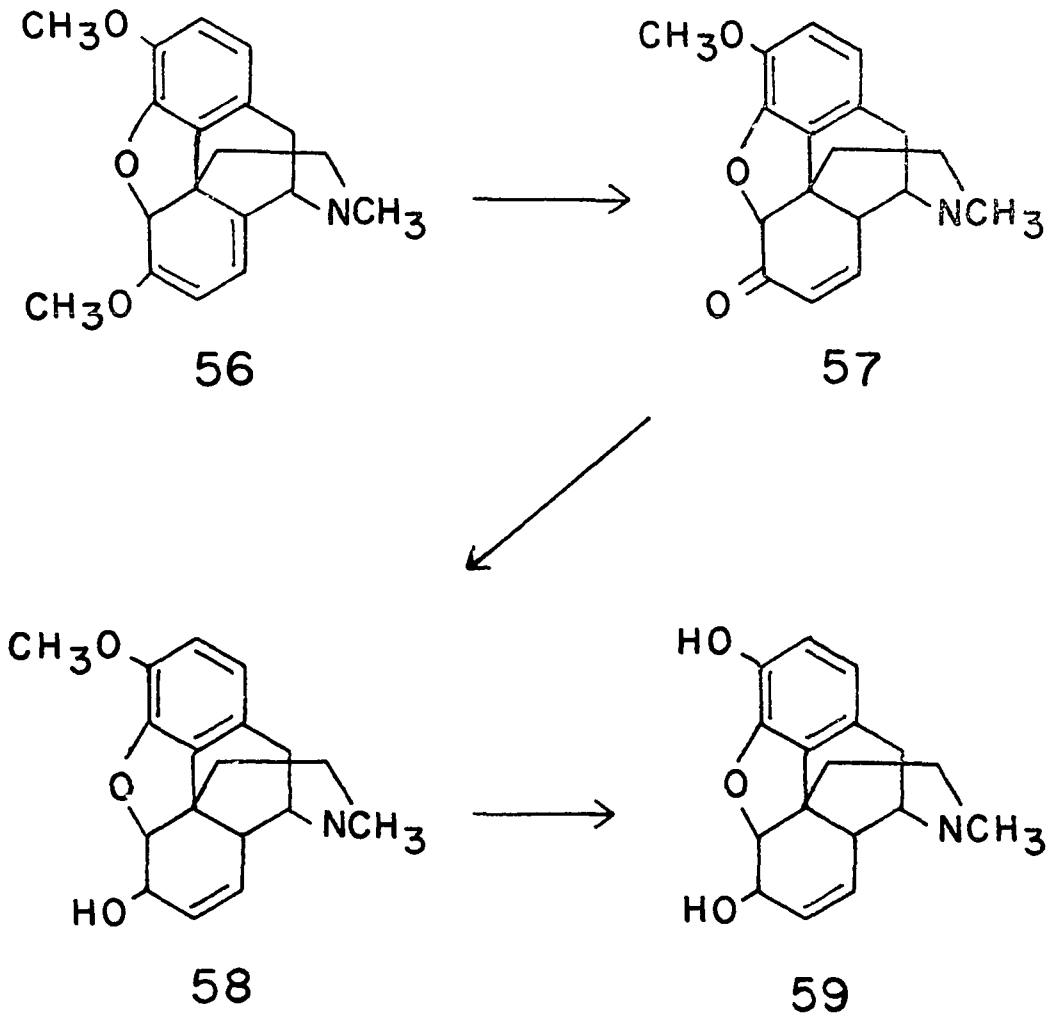


55 20a

methoxy phenols are incorporated intact and thus an O-demethylation process is not operating. The carbon atom of the ortho-methoxy group becomes the carbon of the methylenedioxy group. This has been proven by the intact incorporation of O-^{14}C-methyl-1'-^{14}C-norbelladine into haemanthamine[34], O-^{14}C-methyl-$^{3}H_4$-norbelladine into lycorine[36], and the incorporation of ^{3}H-norpluviine into lycorine[33].

An O-demthylation process in plants containing alkaloids is not unprecedented. The biosynthetic route to morphine in Papaver somniferum has been shown to involve two oxygen demthylations. The biosynthesis of morphine has been proven by several workers to be the following sequence: thebaine(56) $\longrightarrow$ codeinone(57) $\longrightarrow$ codeine(58) $\longrightarrow$ morphine(59)[66,67]. These workers used thebaine, codeinone, and codeine as biosynthetic precursors to morphine. Rapoport and Stermitz also used the rate of incorporation of $^{14}CO_2$ into thebaine, codeine and morphine to colloborate the sequence involving

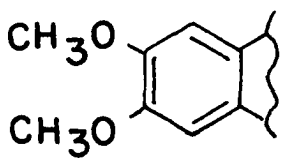
an O-demthylation of thebaine to give codeine and an O-demethylation of codeine to give morphine[68].

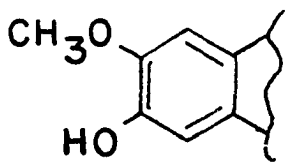


RESULTS AND DISCUSSION

Precursors

One of the objectives of this investigation was to determine the biosynthesis of a [2]benzopyrano[3,4g]indole alkaloid, e.g., lycorenine. Lycorenine was chosen because it is the most abundant of the alkaloids of this type and because it occurs in readily available species of the Amaryllidaceae. Lycorenine possesses a 9,10 dimethoxy substitution pattern on ring A. Therefore, the biosynthetic precursors to be used must have either a 9,10 dimethoxy substitution pattern on the aromatic ring A or functional groups capable of forming a 9,10 dimethoxy aromatic moiety, e.g., 9-hydroxy-10-methoxy.





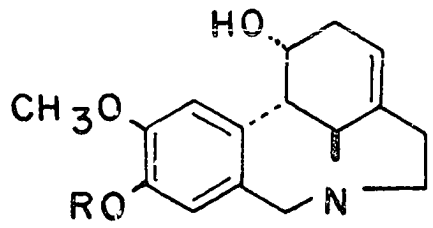
Furthermore, since the alkaloids of the lycorine type had been stereochemically related to alkaloids of the lycorenine type[13], it was thought that a lycorine-type alkaloid would be the logical precursor for one biosynthetic study. It was of interest to determine first, if an alkaloid of the lycorine series might be transformed *in vivo* to lycorenine and second, if the functional groups of the aromatic ring of the lycorine-

type alkaloid played a significant role in the biosynthesis of lycorenine.

The dimethoxy alkaloids related to lycorine have not been thoroughly investigated biosynthetically[57]. Therefore, an alkaloid of the lycorine type possessing a dimethoxy ring A with very little other functionality would be a logical choice as a biosynthetic precursor to more highly functionalized dimethoxy analogues of lycorine. Since a dimethoxy alkaloid of the Amaryllidaceae had not been previously used in a biosynthetic study and since biological oxygen demethylations had been observed in other plants[66,67,68], the dimethoxy alkaloid used would provide another interesting area of investigation.

Alkaloid catabolism is an area of biosynthesis that has received little attention in the Amaryllidaceae. Since plants containing lycorenine also contain a recently discovered compound, narciclasine[29], which appears to be a product of alkaloid degradation, it was of interest to determine whether an alkaloid of the lycorine type might serve as a precursor to this interesting substance.

The factors discussed above led to the choice of norpluviine (60 ≡ 41) and pluviine (61 ≡ 20b) as the precursors for this biosynthetic investigation. These two compounds possess the structural requirements for the biosynthetic study of lycorenine, narciclasine, the dimethoxy analogues of lycorine and the <u>in vivo</u> oxygen demethylation reaction.



60 R = H

61 R = CH_3

Since norpluviine is easily converted to pluviine and since pluviine is not easily converted to norpluviine, it was decided to find a method of preparing a radioactively labeled norpluviine. Two methods are known for the labeling of norpluviine with tritium. Base-catalyzed exchange with tritiated water results in norpluviine with tritium bound to C-8[57]. Norpluviine has also been labeled with tritium by exchange with tritiated acetic acid in the presence of a platinum catalyst[33]. The latter method was used for the preparation of 8-^{3}H-norpluviine. This provided one of the required precursors. Pluviine, the other desired precursor, was obtained from 8-^{3}H-norpluviine by treatment with diazomethane in an ether/methanol solvent. The product, 8-^{3}H-pluviine, provided the necessary complement to achieve the objectives of this investigation.

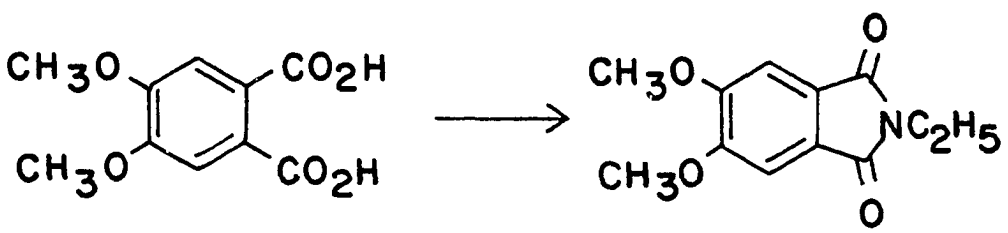
8-^{3}H-Norpluviine was purified by precipitation from dilute aqueous acid solution by neutralization with solid sodium bicarbonate and finally by crystallization from methanol.

This gave 8-^{3}H-norpluviine of constant activity. The purity of 8-^{3}H-norpluviine was established by its constant activity, by thin layer chromatography (TLC) in several solvent systems (one spot with the R_f of pure authentic norpluviine) and vapor phase chromatography. The melting point and mixture melting point determination substantiated the purity of 8-^{3}H-norpluviine. Furthermore, dilution of the labeled norpluviine with inactive norpluviine resulted in no gain or loss of tritium activity (calculated activity of diluted alkaloid = observed activity of diluted alkaloid). When 8-^{3}H-norpluviine was converted to 8-^{3}H-pluviine, the specific molar activities of reactant and product were identical, within the limits of experimental error. The infrared spectrum of 8-^{3}H-norpluviine was identical with the spectrum of authentic norpluviine. These criteria established the purity of the 8-^{3}H-norpluviine used in this study.

8-^{3}H-Pluviine was obtained by treatment of 8-^{3}H-norpluviine with diazomethane and purified by preparative-scale TLC and recrystallization to constant activity. The purity of 8-^{3}H-pluviine was established by TLC in several solvent systems, VPC analysis and the infrared spectrum which was identical with the spectrum of authentic pluviine. The material was also diluted with inactive pluviine with no loss or gain of tritium activity.

The distribution of the tritium label was determined by permanganate oxidation of 8-^{3}H-pluviine. This oxidation

produces m-hemipinic acid(62) which, upon sublimation under reduced pressure, yields the anhydride. Treatment of the anhydride with ethylamine affords N-ethyl-m-hemipinimide(63)[19]. It was found that the specific molar activity of the imide was 0.78 relative to the specific molar activity of 8-^{3}H-pluviine. This result shows that 78% of the tritium was located in the aromatic ring in both norpluviine and pluviine. Since the 8 position is ortho the phenol, it is reasonable that C-8 was the primary site of exchange. Therefore, it is believed that most of the tritium is located at C-8. The remaining 20% of the tritium was not located, but is probably located at the olefinic, allylic and benzylic positions.



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8-^{3}H-Norpluviine Feeding to Narcissus "King Alfred"

The feeding experiment was carried out using Narcissus pseudonarcissus L. var. "King Alfred" as the plant host. This plant was chosen for its ready availability and because it contains enough lycorenine and several dimethoxy lycorine-type alkaloids necessary for the biosynthetic experiment[3].

This feeding experiment had essentially three objectives: 1) to determine whether norpluviine can act as an efficient precursor to lycorenine, 2) to determine the extent that norpluviine is incorporated into pluviine, methylpseudolycorine, and galanthine, and 3) to determine if narciclasine might be derived from the lycorine ring system via removal of the C_2 fragment (C-4 and C-5). The data would provide insights into the biosynthetic pathways of lycorenine, pluviine, methylpseudolycorine, galanthine and narciclasine.

The 8-^{3}H-norpluviine was administered to Narcissus "King Alfred" by injection into the flower stalks as an aqueous acid solution at pH 6. The entire plants were harvested after 18 days of growth. The alkaloid fractions were obtained in the usual manner (see Experimental). An extra separated fraction was obtained from the concentrated acidified ethanolic extract of the plants by extraction with n-butanol. This fraction contained some alkaloids as the tartrate salts and several phenolic compounds including narciclasine. The nonbasic phenols were fractionated as were the alkaloids which were added to the alkaloid fraction obtained at pH 7. All of the alkaloid fractions were combined and subjected to prep-scale TLC silica gel plate and alumina column chromatography for isolation of the alkaloids. Crystallization was used for the final purification procedure. The neutral phenol fraction was chromatographed on a silica gel column to give narciclasine[29].

It was found that 8-^{3}H-norpluviine was efficiently incorporated into lycorenine (0.11%) and to a lesser extent into homolycorine (0.018%). The latter may be an artifact arising from the disproportionation of lycorenine. This result proves that at least one alkaloid of the lycorine series can be converted to lycorenine.

The alkaloids pluviine and galanthine were also found to contain tritium. 8-^{3}H-Norpluviine was incorporated into pluviine to the extent of 0.029% and into galanthine to the extent of 0.017%. The activity of the methylpseudolycorine isolated was found to be low, but still represented a 2.3×10^{-3}% incorporation. This low incorporation may be due to the low concentrations of methylpseudolycorine found in Narcissus "King Alfred".

It was found that narciclasine possessed tritium activity and that the activity represented a 3.4×10^{-3}% incorporation. This incorporation is low, but would appear to be significant because many steps are required for the conversion of norpluviine to narciclasine (discussed below). Furthermore, the incorporation may actually be higher because it is likely some of tritium label was lost during the conversion, since the C-8 of norpluviine becomes the carbon bearing the phenol in narciclasine. Thus, most of the tritium activity would be lost in the conversion of 8-^{3}H-norpluviine to narciclasine. The materials isolated, their activities and per cent incorporations are given in Table 3.

Table 3. 8-^{3}H-Norpluviine[a] feeding to Narcissus "King Alfred"

Alkaloid	Activity (dpm/mM)	Dilution[b]	% Inc.
Lycorenine	34.9×10^{5}	3.50×10^{3}	0.11
Homolycorine	12.3×10^{5}	9.93×10^{3}	0.018
Pluviine	23.4×10^{5}	5.22×10^{3}	0.029
Methylpseudolycorine	2.46×10^{5}	49.6×10^{3}	0.0023
Galanthine	6.79×10^{5}	18.0×10^{3}	0.017
Narciclasine	0.40×10^{5}	145×10^{3}	0.0034

[a]The total activity of 8-^{3}H-norpluviine fed was 0.10 mc.

[b]Dilution = specific activity of precursor ÷ specific activity of alkaloid.

The alkaloids lycorenine and galanthine isolated from this feeding experiment were degraded by oxidation with potassium permanganate. The m-hemipinic acid obtained from each reaction was sublimed to provide the anhydride. The anhydride was then converted to the N-ethyl imide by treatment with ethylamine. The activity of the imide relative to the corresponding alkaloid is given in the last column of Table 4. These results show that the 8-^{3}H-norpluviine was incorporated without scrambling of the tritium label.

Table 4. Degradation of alkaloids isolated from the 8-^{3}H-norpluviine feeding experiment

Alkaloid	Activity (dpm/mM)	N-ethyl-m-hemipinimide activity (dpm/mM)	Imide activity / alkaloid activity
Lycorenine	1.39×10^{5}	1.10×10^{5}	0.79
Galanthine	6.05×10^{4}	4.54×10^{4}	0.75

8-^{3}H-Pluviine Feeding to *Narcissus poeticus*

To complete the study of lycorenine biosynthesis a feeding experiment using a dimethoxy lycorine-type alkaloid was necessary. Since a methylenedioxy lycorine derivative had been fed previously[63] and the *ortho*-methoxyphenol, norpluviine, was fed in the initial part of this study, pluviine, the dimethoxy lycorine-type alkaloid, provides the last dioxygenated aromatic ring type to be fed. It is important to note that norpluviine and pluviine differ only in ring A. Norpluviine is an *ortho*-methoxyphenol and pluviine is the analogous dimethoxy-alkaloid. The remaining functional groups and stereochemistry are identical in the two alkaloids.

There were several objectives for this feeding experiment. One objective was to determine whether pluviine is converted *in vivo* to lycorenine. This data would increase the information concerning the biosynthetic pathway to lycorenine. Another objective was to determine if pluviine was an efficient pre-

cursor to the dimethoxy analogues of lycorine, viz., the alkaloids methylpseudolycorine, galanthine, and narcissidine. Additional knowledge regarding the oxidation of C-2 of galanthine and methylpseudolycorine would be provided. Also, the data would provide insight into the origin of the C-3 alcohol of narcissidine. The last objective of this experiment was to determine whether an O-demethylation reaction may occur. If such a phenomenon were found to occur, it would be the first example of this kind of reaction in the Amaryllidaceae.

The plant host for this experiment was Narcissus poeticus L. It was chosen because of its ready availability and because it contains lycorenine, pluviine, methylpseudolycorine, galanthine and narcissidine in isolable amounts.

The 8-^{3}H-pluviine, in an aqueous hydrochloric acid solution at pH 6, was injected directly into the flower stalks of thirteen Narcissus poeticus plants. After fourteen days of growth, the entire plants were harvested. The isolation was done in the usual manner with the alkaloid fractions obtained by the standard procedure (see Experimental). Isolation of the individual alkaloids of interest was achieved by preparative-scale silica gel plate chromatography. Crystallization afforded the pure alkaloids.

The lycorenine isolated from this feeding experiment did not contain detectable tritium activity. This level of activity (below the limits of detection) requires that the

per cent incorporation of 8-^{3}H-pluviine into lycorenine was 10^{-4} or less. This level of incorporation is considered negligible and it is assumed that the 8-^{3}H-pluviine is not a biosynthetic precursor to lycorenine.

The dimethoxy lycorine-type alkaloids contained appreciable amounts of tritium. Pluviine was converted *in vivo* to methylpseudolycorine to the extent of 0.25%. The incorporation into galanthine was quite efficient, *viz.*, 3.26%. Once again the incorporation into galanthine was appreciably higher than into methylpseudolycorine. This might indicate that methylpseudolycorine is a short-lived intermediate in the biosynthetic pathway. It is interesting to note that the methylenedioxy analogue of methylpseudolycorine, lycorine, is the most abundant alkaloid in the Amaryllidaceae and is certainly not a short-lived intermediate. These data would strongly suggest that the oxygen at C-2 in methylpseudolycorine and galanthine is introduced in the same fashion that the corresponding oxygen introduced into lycorine, i.e., the ring system is formed and then C-2 hydroxylation occurs.

The narcissidine isolated from this feeding experiment of 8-^{3}H-pluviine was found to have a 0.021% incorporation. This indicated that the C-3 oxygen of narcissidine is introduced late in the biosynthetic pathway and is discussed in more detail below.

The lycorine isolated from this feeding experiment contained tritium activity and showed a 0.14% incorporation from

8-^{3}H-pluviine. This is the first observation of a dimethoxy alkaloid being converted to a methylenedioxy alkaloid in the Amaryllidaceae. Further discussion of this phenomenon appears below. The alkaloids isolated, their activities, and the per cent incorporations are summarized in Table 5.

Table 5. 8-^{3}H-Pluviine[a] feeding to Narcissus poeticus

Alkaloid	Activity (dpm/mM)	Dilution	% Inc.
Lycorenine	0	---	0.00
Galanthine	1.09×10^{8}	5.26×10^{1}	3.26
Methylpseudolycorine	1.35×10^{7}	4.25×10^{2}	0.25
Narcissidine	7.50×10^{5}	7.66×10^{3}	0.021
Lycorine	1.91×10^{6}	3.01×10^{3}	0.14

[a]The total activity of 8-^{3}H-pluviine fed was 0.10 mc.

The alkaloids galanthine and narcissidine isolated from this feeding experiment were degraded by oxidation with potassium permanganate, followed by conversion to the anhydride and finally to N-ethyl-m-hemipinimide. Lycorine was also degraded in the same fashion to give N-ethylhydrastimide. The activities of the imides relative to the corresponding alkaloids appear in Table 6.

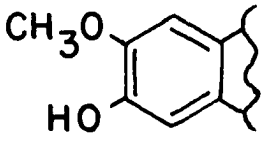
Table 6. Degradation of alkaloids isolated from the 8-^{3}H-pluviine feeding experiment

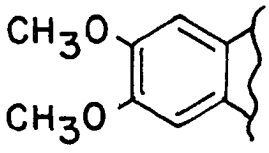
Alkaloid	Activity (dpm/mM)	Imide activity (dpm/mM)	Imide activity / alkaloid activity
Galanthine	2.74×10^{6}	2.08×10^{6}	0.76
Narcissidine	6.73×10^{4}	5.11×10^{4}	0.76
Lycorine	2.87×10^{4}	2.32×10^{4}	0.81

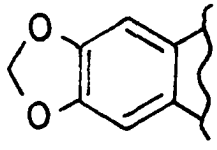
Discussion

Lycorenine

There are three types of functionality at the 9,10 positions in the lycorenine and lycorine ring systems. These are ortho-methoxyphenol, dimethoxy, and methylenedioxy. Lycorenine has the dimethoxy substitution in ring A. There are three







possible biosynthetic alternatives for the biosynthesis of lycorenine. One pathway is that the functional groups on ring A do not affect the biosynthetic pathway of lycorenine as illustrated by path a in Figure 7. This would require the

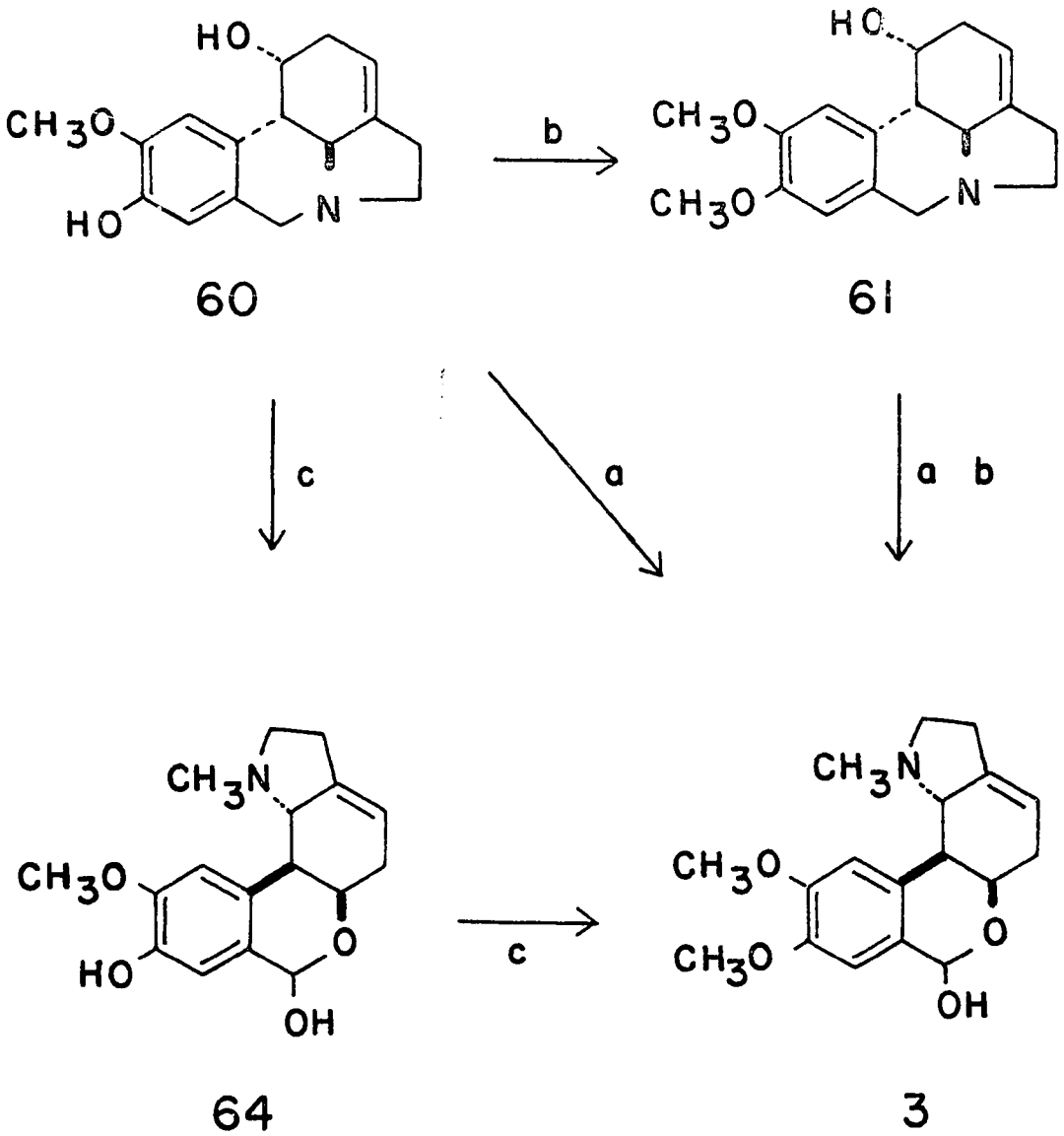


Figure 7. Biosynthetic pathways to lycorenine

incorporation of both 8-^{3}H-norpluviine and 8-^{3}H-pluviine into lycorenine(3). A second possibility (path b) requires the C-9 phenol to be methylated before the oxidation/rearrangement process to give lycorenine(3) can occur. This pathway would require the incorporation of both 8-^{3}H-norpluviine and 8-^{3}H-pluviine with a greater incorporation for 8-^{3}H-pluviine. The third alternative is that the phenolic function is necessary to the oxidation/rearrangement process. This pathway requires the incorporation of 8-^{3}H-norpluviine, but the negative incorporation of 8-^{3}H-pluviine. This route would suggest the existence of an ortho-methoxy phenol alkaloid of structure 64. This biosynthetic pathway is path c in Figure 7.

The data obtained in this investigation--the relatively efficient conversion of norpluviine to lycorenine and the negative incorporation of pluviine into lycorenine--would mitigate against paths a and b. Therefore, it would appear that for oxidation of the lycorine ring system and subsequent rearrangement to the lycorenine ring system to occur, a phenolic function in ring A is required. The phenol could be functioning as a handle for the enzyme (or enzymes) that oxidize the benzylic carbon. The recent isolation of a methoxy hydroxy lycorenine alkaloid[10] (probably of structure 64) offers more credence to path c as the biosynthetic route to lycorenine.

There are several alternatives in the sequence of events during the norpluviine to lycorenine transformation. One such alternative is initial N-methylation to give a quarternary ammonium salt, followed by oxidation of the C-7 benzylic carbon. This intermediate could then open to give an amino aldehyde, capable of 180° rotation about the 11a-11b bond to bring the C-1 alcohol into close proximity to the aldehyde. Closure to the hemiacetal and methylation of the phenolic group completes the transformation. This pathway is not favored since quarternary salts of the Amaryllidaceae alkaloids have not been observed. This scheme is illustrated as path a in Figure 8.

A second possibility (path b, Figure 8) is that oxidation of C-7 occurs first, followed by the quarternization of the nitrogen. The rest of the sequence would be as described above. This would suggest that C-7 hydroxy lycorine-type alkaloids have transient existence, but probably are too unstable for isolation. Lycorine alkaloids of this type have not been observed. α-Hydroxy amines occur in the Amaryllidaceae. Several alkaloids of the crinine-type possess an α-hydroxy amine functionality[21].

Another alternative is to postulate the existence of N-demethyl lycorenine. This would require that the oxidation at C-7 occurs first, followed by the rearrangement and methylation of the phenol. The last step in the process would be the

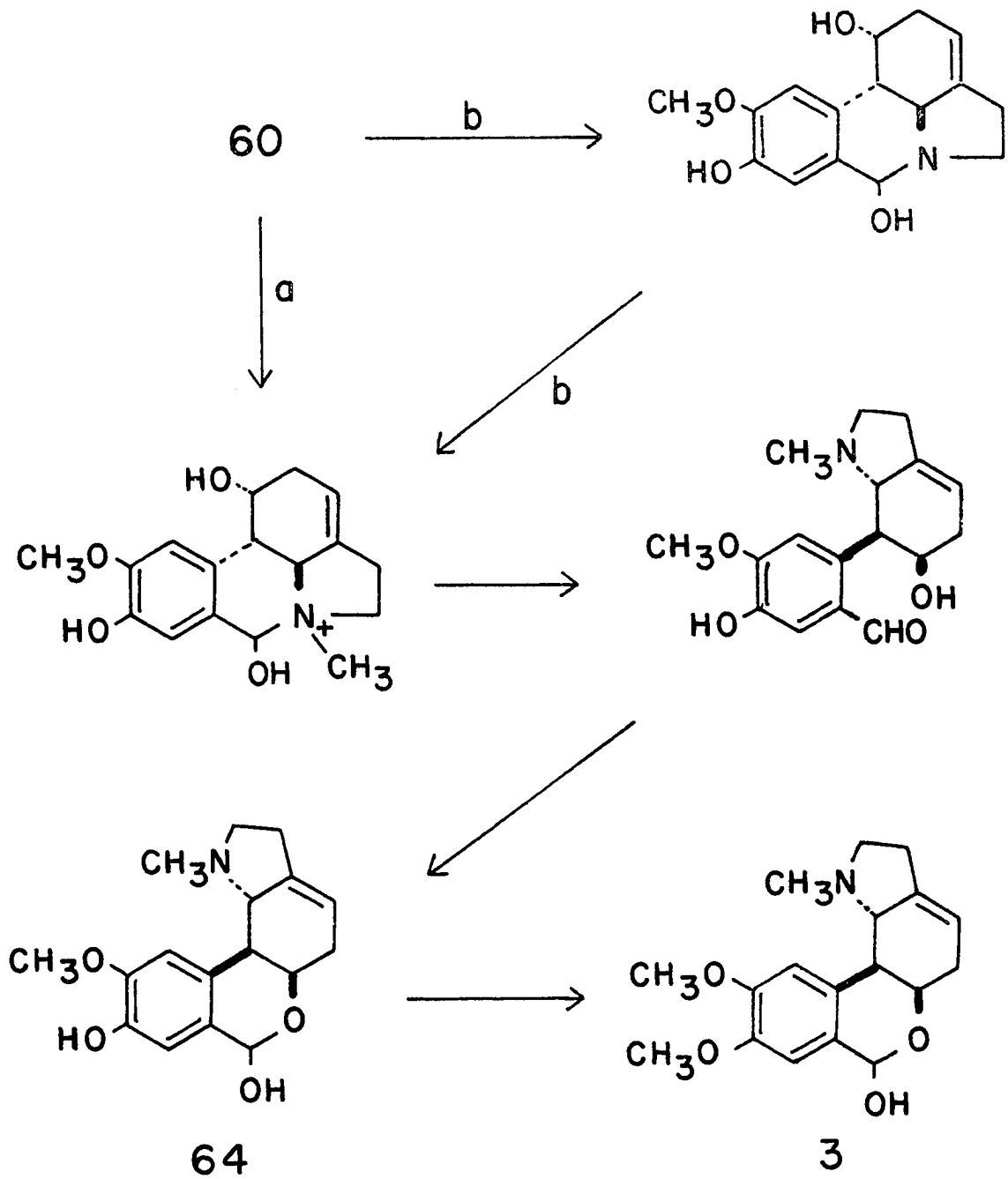


Figure 8. Biosynthetic transformation of norpluviine to lycorenine

methylation of the nitrogen. At this time, no alkaloids of the lycorenine ring system are known to be secondary amines. All of the known alkaloids of this series contain an N-methyl group.

Each of the alternatives is plausible, but each also has shortcomings. Further study into this biosynthetic process would be required to differentiate among the possibilities. A radioactively labeled N-methyl norpluviine would provide an appropriate biosynthetic precursor to lycorenine.

Narciclasine

Our incorporation of 8-^{3}H-norpluviine into narciclasine was low, but would appear to be significant. The conversion of 8-^{3}H-norpluviine to narciclasine would involve a loss of tritium at C-8 of norpluviine, since this carbon becomes a phenol group in narciclasine. Therefore, the incorporation was probably higher than that observed. This result does not agree with the conclusions drawn from the work by Battersby (see Historical).

It does not seem unreasonable that narciclasine might be derived from lycorine (known to be derived from norpluviine[34]) since both substances have a methylenedioxy group, a phenanthridine nucleus, and extensive oxygenation in ring C. Addition of another alcohol to ring C at C-3 would yield an alkaloid comparable in structure to narcissidine. This intermediate

(65 in Figure 9) is not implausible since the methylenedioxy alkaloids of the lycorine-type are all known to possess a C-2 hydroxyl group whereas the dimethoxy lycorine-type alkaloids generally have a C-2 methoxyl. Therefore, the analogy to narcissidine is valid. Oxidation of C-4 to give 66 partially rationalizes the loss of the two carbon fragment by elimination of the keto-amine(66) to give 67. Base attack on the methyl ketone followed by cleavage generates 68. Further oxidation to give the lactam, the phenol, and the double bond completes the transformation to narciclasine(29b). This route rationalizes narciclasine on the basis of the structure assigned by the Italian workers.

The structure of narciclasine, as proposed by the Japanese workers, can be rationalized in a similar manner. Starting from intermediate 68, a dehydration involving the C-1 alcohol and hydroxylation of C-4 gives 69 in Figure 10. Introduction of the phenol and oxidation of the benzylic carbon to give the lactam completes the conversion to the Japanese structure of narciclasine(35a).

The proposed biosynthetic paths to narciclasine through lycorine and the triol 65 (analogous to narcissidine) leads to speculation of the stereochemistry of the alcohol functions in narciclasine. If such a pathway is valid, then the configuration of the C-1 alcohol in narciclasine should be of the α-configuration. The hydroxyl on C-2 should be of the β-config-

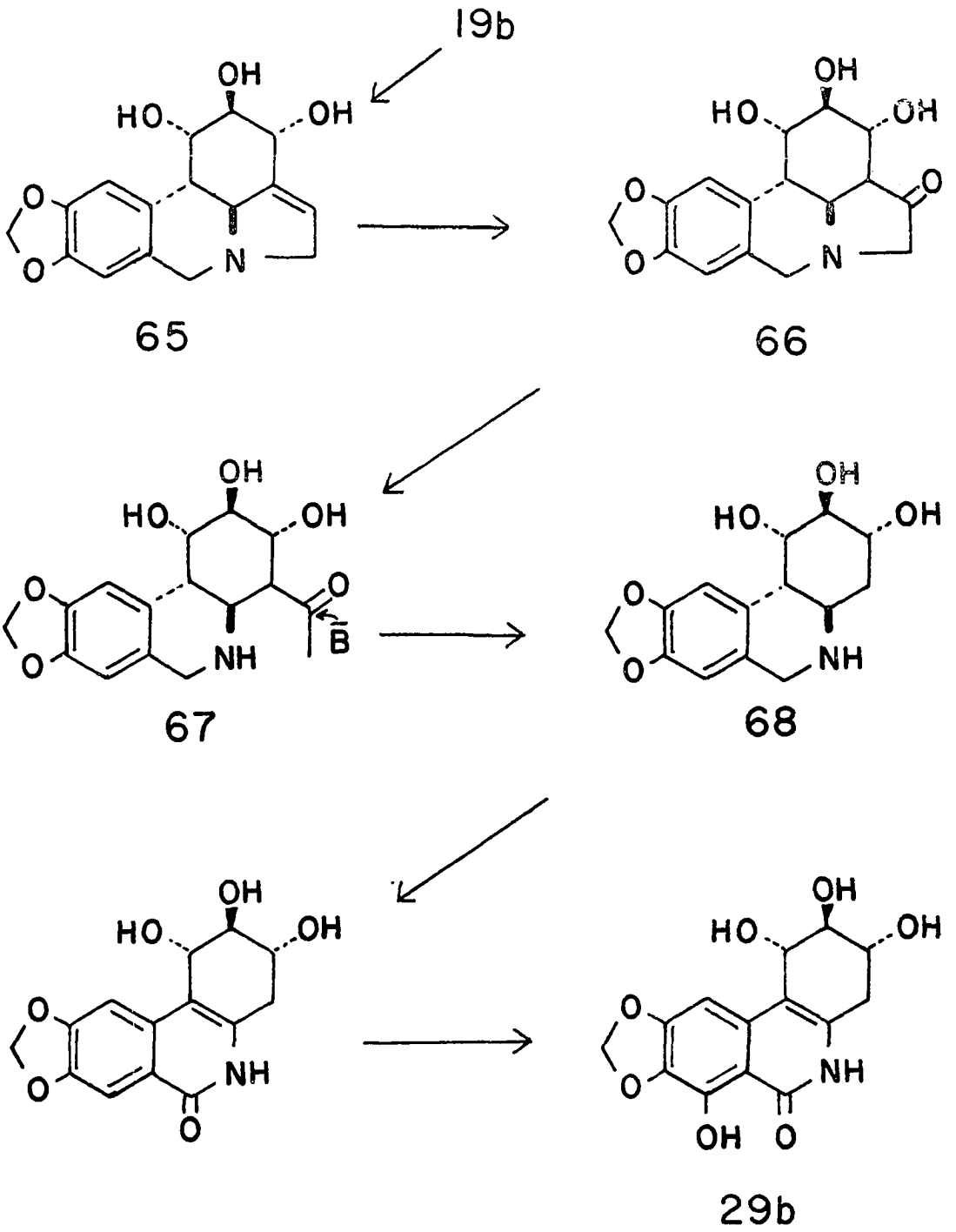


Figure 9. Proposed biosynthetic pathway(I) to narciclasine

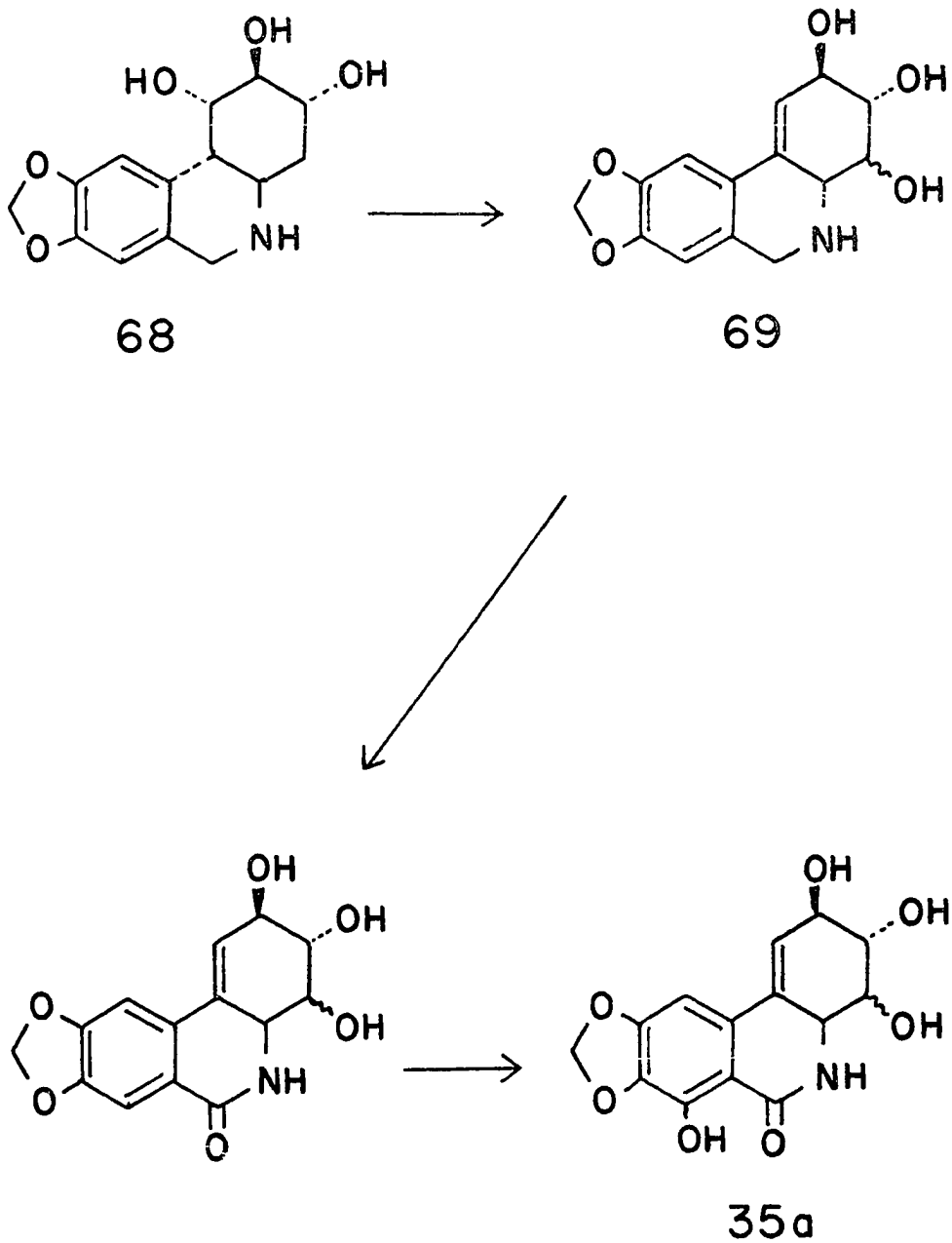


Figure 10. Proposed biosynthetic pathway(II) to narciclasine

uration. If this were correct, it would also explain the nonreactivity of narciclasine to periodic acid under the conditions used for the cleavage of shikimic acid[33]. If the Japanese structure is correct, the configuration of the C-4 alcohol cannot be predicted from biosynthetic considerations, but the C-2 alcohol still should be β and the C-3 alcohol of the α-configuration.

Since the level of incorporation was low, insufficient tritium activity was available for degradation. Therefore, this work does not exclude the possibility that narciclasine may be the product of the catabolism of another alkaloid ring system.

Pluviine, methylpseudolycorine, galanthine and narcissidine

The incorporation of 8-^{3}H-norpluviine into pluviine is not unexpected, since only methylation of the free phenolic group is required. What is somewhat unexpected is the low incorporation. Although the incorporation is in the range of most biosynthetic precursors, such a one-step process would be expected to be considerably more efficient. Extent of incorporation data requires cautious interpretation for it has been found that the per cent incorporation into a given alkaloid will vary widely even for a given plant species using the same precursor[35]. It should also be noted that norpluviine is a branching point for several alkaloids. That is, norpluviine can procede to lycorine, lycorenine and pluviine.

Therefore, at various stages in the plant's development, one particular alkaloid biosynthetic pathway may be favored over the others.

Both norpluviine and pluviine are incorporated into both methylpseudolycorine and galanthine. In the case of galanthine, the incorporation from both precursors was shown to be intact. The efficient conversion of pluviine to galanthine shows that the C-2 oxygen of galanthine is introduced via pluviine. This result is in agreement with the results found for lycorine[65] and previous work with galanthine[58]. Therefore, in the lycorine ring system, regardless of the aromatic substitution type, methylenedioxy or dimethoxy, the hydroxylation of C-2 occurs late in the biosynthetic sequence. The incorporations observed for norpluviine and pluviine into methylpseudolycorine are low relative to galanthine. This might be the result of methylpseudolycorine being a short-lived intermediate between pluviine and galanthine. It is also possible that pluviine is methoxylated at C-2 rather than hydroxylated. This would require a demethylation process to give methylpseudolycorine from galanthine. Such a process might be a minor one and consequently the lower incorporation into methylpseudolycorine. The possible biosynthetic paths to methylpseudolycorine(19a) and galanthine (20a) are summarized in Figure 11. It is interesting to note that lycorine, the methylenedioxy analogue of methylpseudolycorine, is the most abundant Amaryllidaceae alkaloid and does

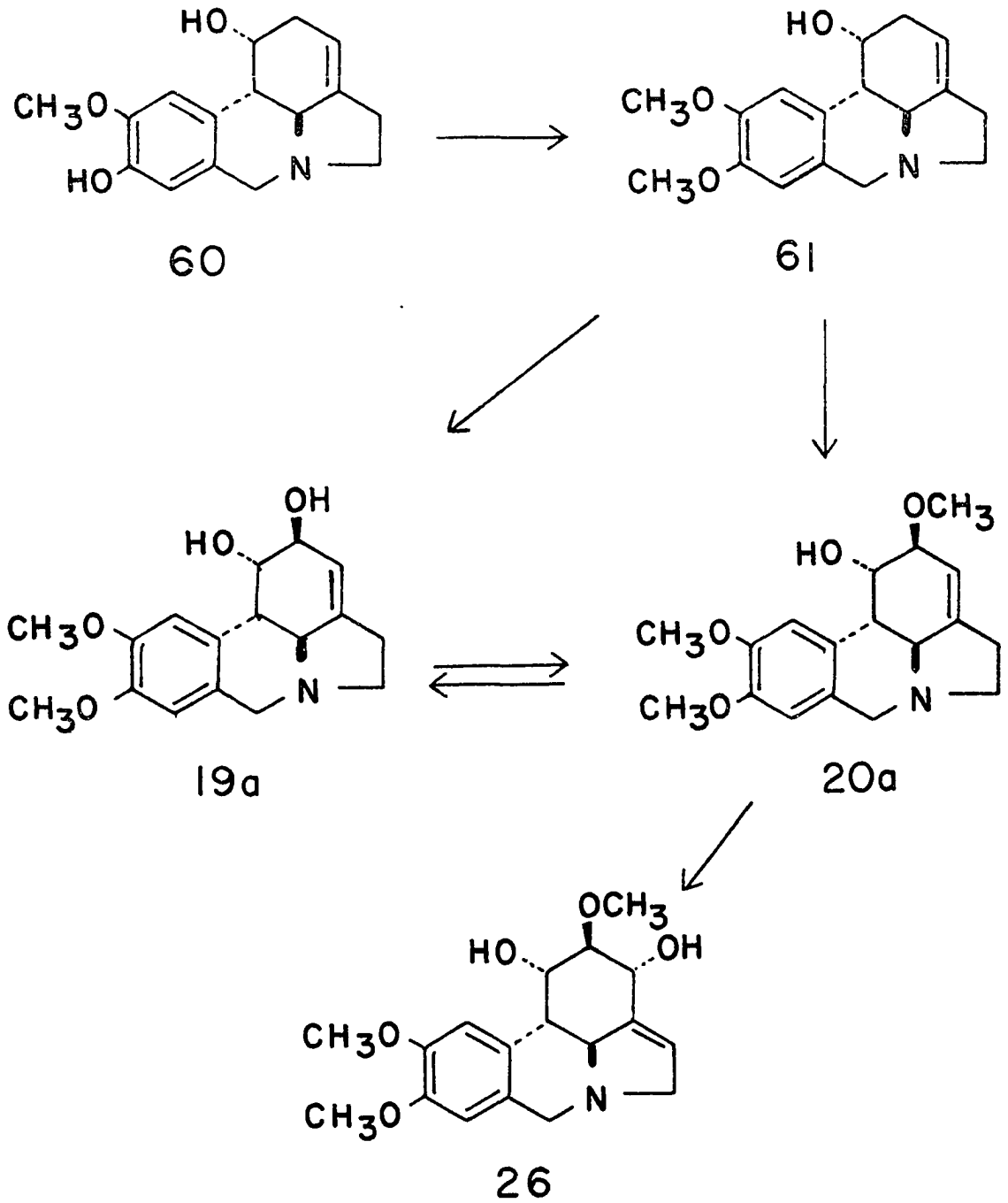


Figure 11. Biosynthetic routes to methylpseudolycorine, galanthine, and narcissidine

not occur in the low concentrations that have been found for methylpseudolycorine[21].

Narcissidine has been a particularly interesting alkaloid because of its unusual structure and the difficulty encountered in its chemical degradation. The intact incorporation of pluviine into narcissidine indicates that C-3 hydroxylation and double bond migration from 3,3a to 3a,4 occurs in a biosynthetic transformation from pluviine. It seems reasonable that this biosynthetic modification at C-3 occurs after the formation of the ring system because it has been established the C-2 oxidation occurs after ring system formation. The late-stage modification of the lycorine-type alkaloids might be envisaged as a sequence beginning with C-2 oxidation followed by double bond migration and C-3 oxidation. To establish with more certainty the biosynthetic pathway to narcissidine, a feeding of labeled galanthine should show an intact, efficient conversion of galanthine to narcissidine. The results of this study suggest that the biosynthetic sequence is pluviine(61) $\longrightarrow$ galanthine(20a) $\longrightarrow$ narcissidine(26), with methylpseudolycorine(19a) occurring in the pathway before galanthine, being formed directly from pluviine, or being biosynthesized by demethylation of galanthine (Figure 11).

There are several possible modes of formation of narcissidine from galanthine. Isomerization of the olefinic linkage from 3,3a to 3a,4 may occur first. Hydroxylation of the

resultant C-3 methylene group to give the α-C-3 alcohol completes the formation of narcissidine. The overall conversion of galanthine to narcissidine may occur in a concerted fashion. Attack of electrophilic oxygen as HO^+ on the double bond concurrent with the loss of a C-4 proton to form the 3a,4 double bond results in the transformation occurring in a one-step concerted manner. Another possibility is a two-step reaction with the removal of hydride from C-4 of galanthine(20a, Figure 12) to form an allylic carbonium ion. Capture of this ion by water by attack at C-3 completes the reaction. This carbonium ion may be stabilized by the C-2 methoxyl by donation of a lone pair of electrons from the oxygen to the C-3 carbon to form an oxonium ion(70). This might also explain the stereochemistry at C-3. Since the C-2 methoxyl is of the β-configuration, the oxonium ion would also have the oxygen on the β-face of the molecule. This configuration then requires the addition of water or hydroxide from the α-side of the molecule and thus the α-configuration of the C-3 hydroxyl group (Figure 12).

Another alternative is that there is initial oxidation at C-4 in galanthine(20a) to give an intermediate(25, Figure 12). This intermediate could then add water to the double bond in an anti-Markovnikov manner to give 1,3,4 triol(71). 3a,4-Dehydration of 71 produces 26, the known structure for narcissidine. This oxidation at C-4 resembles that found in the C-11 hydroxy crinine-type alkaloids since the same carbon is being oxidized.

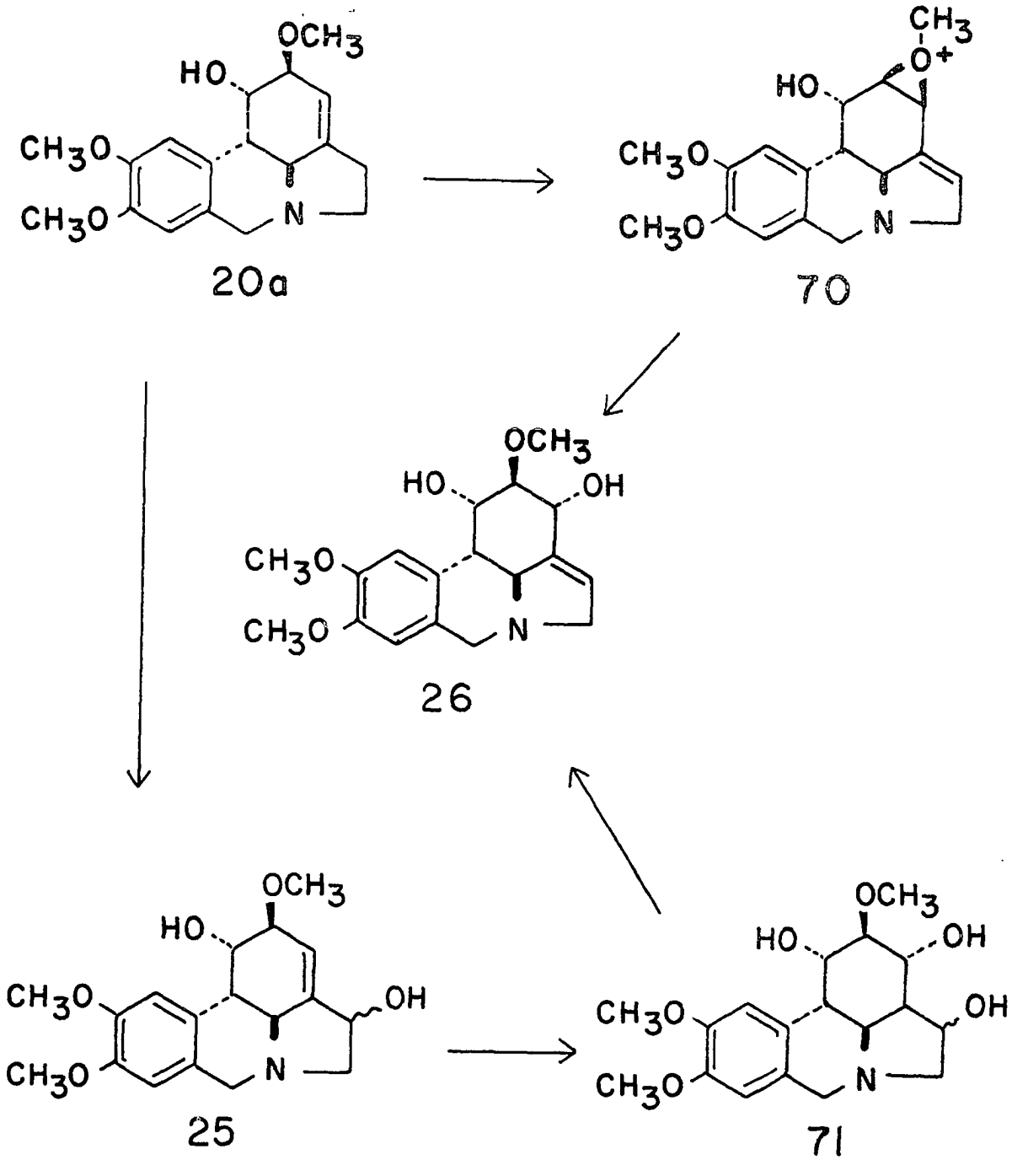


Figure 12. Biosynthetic formation of narcissidine

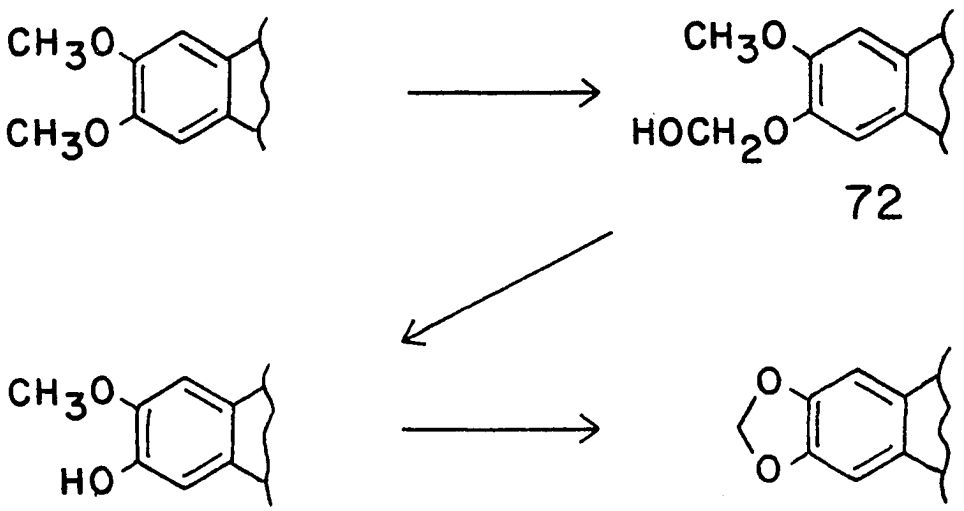
This carbon, C-4 in the lycorine series and C-11 in the crinine series is C-2 in tyrosine, the precursor to the C_6-C_2 unit of both alkaloid systems.

Lycorine

The incorporation of pluviine into lycorine was a highly unexpected result. Previous studies had shown the origin of the 9,10-methylenedioxy group in lycorine to be derived from a 9-hydroxy-10-methoxy moiety[56]. Other biosynthetic studies in the Amaryllidaceae where labeled alkaloids were used as the precursors showed that the biosynthetic pathways studied were one-way routes. For example, the haemanthamine to haemanthidine to tazettine sequence was proven to be a unidirectional biosynthetic pathway[60]. But no previous biosynthetic experiments in the Amaryllidaceae have used an aromatic dimethoxy alkaloid as a precursor. This result indicates there is a process by which the dimethoxy alkaloids are interconvertible biosynthetically with the analogous methylenedioxy alkaloids in the lycorine series. As discussed before, there is precedent for oxygen demethylations in alkaloid systems. Biological O-demethylations produce morphine[67], therefore such a process is not unprecedented in alkaloid biosynthesis. This is the first observation of the phenomenon in the Amaryllidaceae alkaloids.

A logical pathway between pluviine and lycorine would involve norpluviine (or the 9-methoxy-10-hydroxy isomer). This requires an oxygen demethylation of pluviine. If this were to

occur, then the incorporation of pluviine into lycorine is easily explained, since it has been proven that norpluviine is an efficient precursor to lycorine[33]. Since biological hydroxylation of carbon bound methyl groups is known (e.g., terpenes), this may also occur with the oxygen bound methyl groups of pluviine. The hemiformal(72) thus formed by hydroxylation is easily hydrolyzed to formaldehyde and the phenolic alkaloid. Closure of the ortho-methoxyphenol completes the transformation to the methylenedioxy alkaloid.



The mechanism by which the methylenedioxy group is formed might be either radical or cationic. Oxidation may produce a radical as 73 (Figure 13). The isomeric radical 74 is probably somewhat less stable. Either 73 or 74 can lead to 75 which would give rise to the methylenedioxy function. Alternatively,

hydroxylation of the methyl group of the ortho-methoxyphenol followed by the loss of hydroxide ion (probably as water) yields the oxonium ion 76. The alternate cation(77), produced by loss of hydride ion, is somewhat less stable and therefore less favored than the oxonium ion 76. The oxonium ion cyclizes to the methylenedioxy function (Figure 13).

Another possible mechanism from pluviine to lycorine is one in which an ortho-methoxyphenol is not involved. In this mechanism both methyl groups of the two methoxyl groups are hydroxylated. If one of the two hemiformal functions loses hydroxide to give the stabilized oxonium ion(78), the oxygen atom of the other hemiformal that is bound to the aromatic ring is then capable of attack on the carbon atom that will become the methylenedioxy function (Figure 14). This produces 79, a new oxonium intermediate which can collapse to formaldehyde and the methylenedioxy function. The cyclization and loss of formaldehyde may be envisaged as a one-step reaction.

The possibility exists that the conversion of pluviine to lycorine is an artifact. That is, the addition of excess pluviine into the plant during its growing cycle may have induced biological reactions that normally do not occur. Not enough is known regarding this possibility to draw any inferences in this study.

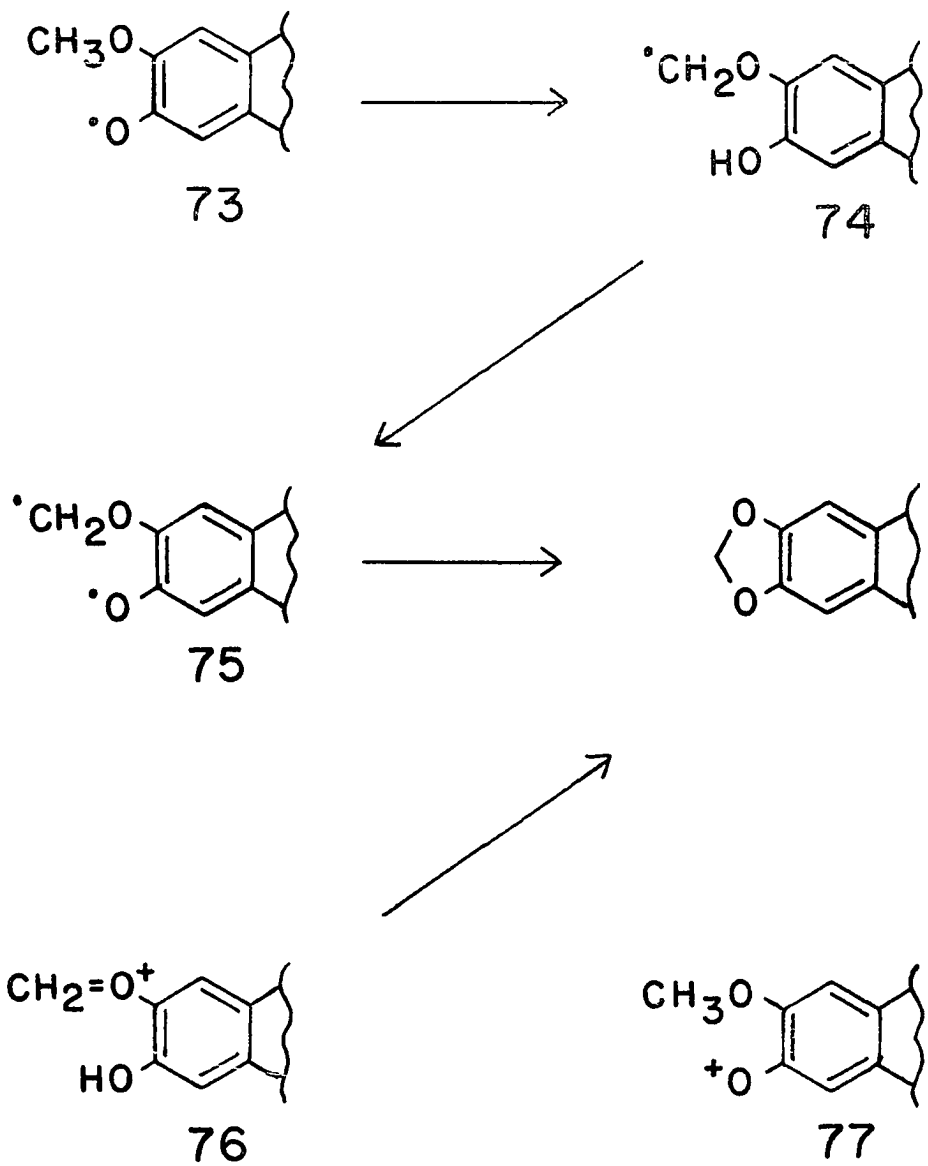


Figure 13. Formation of the methylenedioxy group

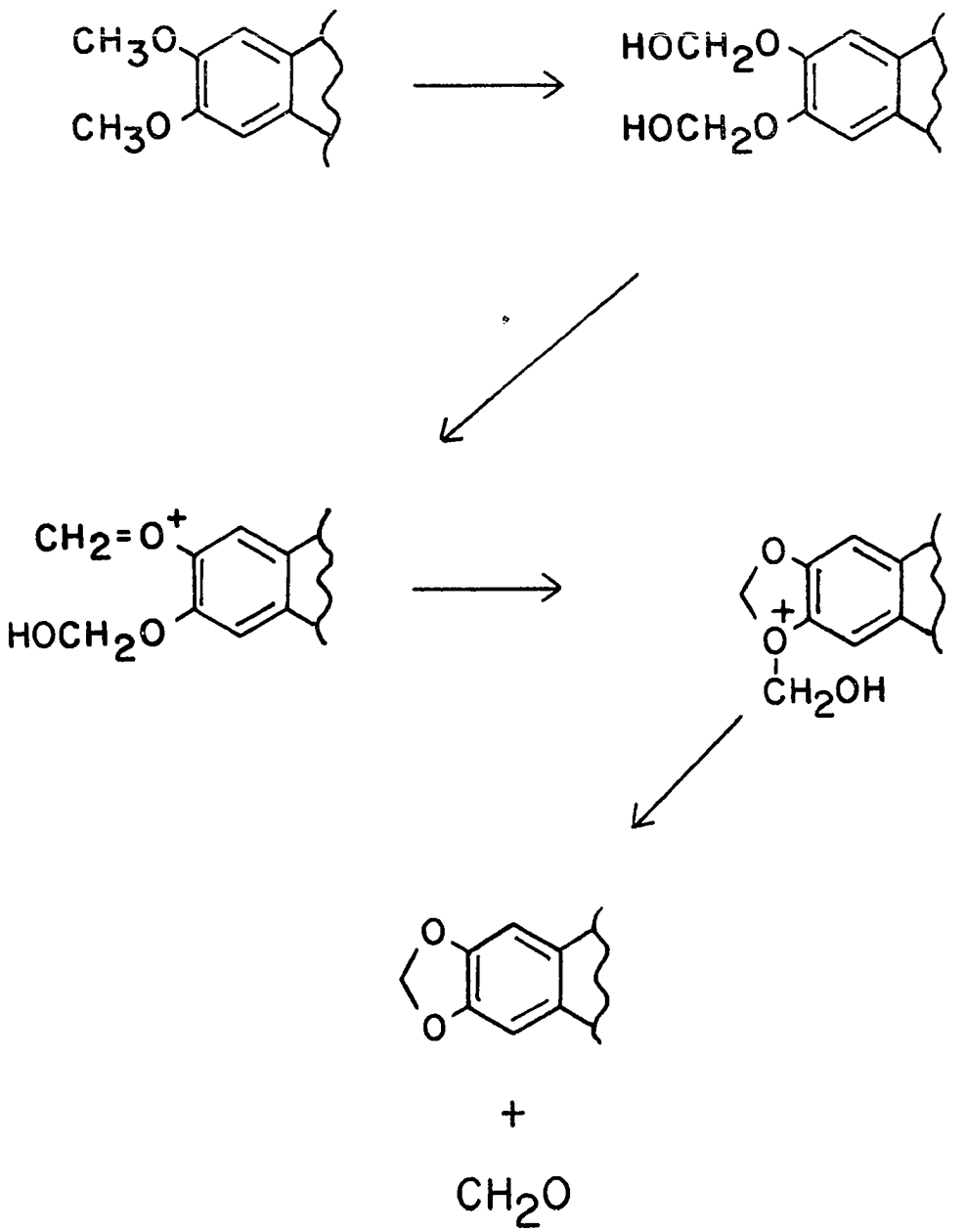


Figure 14. Biosynthetic conversion of dimethoxyphenyl to methylenedioxyphenyl

SUMMARY

The Amaryllidaceae alkaloids norpluviine and pluviine were selected as biosynthetic precursors to study the biosynthesis of lycorenine, narciclasine and the dimethoxy analogues of lycorine. The norpluviine was labeled by an exchange technique using tritiated acetic acid as the source of tritium. Tritiated pluviine was obtained from tritiated norpluviine by treatment with diazomethane.

It has been shown that norpluviine is an efficient precursor to lycorenine and that pluviine is not a biosynthetic precursor to lycorenine in Narcissus species of the Amaryllidaceae. A biosynthetic route from norpluviine to lycorenine involving benzylic oxidation and rearrangement is discussed.

It has been shown that pluviine is derived from norpluviine and that pluviine is an efficient biosynthetic precursor to the alkaloids methylpseudolycorine, galanthine and narcissidine. This work substantiates the hypothesis that C-2 hydroxylation of the alkaloids methylpseudolycorine, galanthine and narcissidine occurs late in the biosynthetic sequence. Furthermore, C-3 hydroxylation and double bond isomerization to give narcissidine is also shown to be a late-stage biosynthetic reaction.

It was found that norpluviine was incorporated into the neutral substance narciclasine. A biological alkaloid degradation pathway is proposed and discussed.

The conversion of pluviine, an aromatic dimethoxy analogue of lycorine, to lycorine, an aromatic methylenedioxy alkaloid, is presented and discussed in terms of a methoxyl hydroxylation pathway.

EXPERIMENTAL

Methods

Melting points were taken on a Köfler microscope hot stage apparatus and are corrected. Infrared spectra were taken on a Beckman Model IR-12 recording spectrophotometer in chloroform solution or as the solid on a KRS-5 internal reflectance crystal using a Wilks Scientific internal reflectance apparatus (Model 9).

The radioactivities of tritiated samples were measured by scintillation counting in solution. A sample (1-6 mg.) was counted in 15 ml. of Bray's scintillation solution [60 g. of naphthalene, 4 g. of 1,4-bis-2-(5-phenyloxazolyl)-benzene (Packard Scintillation Grade) and 0.20 g. of 2,5-diphenyloxazole (Packard Scintillation Grade) in 100 ml. methanol, 20 ml. 1,2-ethanediol, and sufficient dry, peroxide-free p-dioxane to make 1 l. of solution]. Efficiency of counting tritium was 9-14% and was determined for each sample by means of an internal standard of ^{3}H-toluene. Measurements of the radioactivities were obtained with a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3002). Reproducibility of the assays was ±3%.

Preparative-scale layer chromatography used 20 cm. x 20 cm. glass plates coated with silica gel(Merck PF254+366) 0.5 mm. in thickness. The plates were eluted once with the solvent system specified. The alkaloids were detected by ultraviolet light. The bands of silica gel and alkaloid were removed from the plate

and covered with 80-100 ml. of 10% HCl for a minimum of one hour. The acid was neutralized with 10% aqueous ammonia and the aqueous layer including the silica gel was extracted with chloroform until the aqueous layer gave a negative silico-tungstic acid test.

The alkaloids were identified by their TLC behavior, melting point, mixed melting point and comparison of their infrared spectra with known reference spectra. The alkaloids were purified from chromatographic fractions via recrystallization from appropriate solvents to constant activity. The per cent incorporation was calculated as (100 x total activity of isolated alkaloid) divided by (total activity fed). For this purpose, the final constant activity of the alkaloid per mg. was multiplied by the quantity of alkaloid isolated (in mg.) which was of good chemical purity as determined by TLC, melting point, and the infrared spectrum.

Precursors

8-^{3}H-norpluviine

Tritiated norpluviine was obtained from the National Institutes of Health, Bethesda, Maryland. The material was prepared by New England Nuclear Corporation, Boston, Massachusetts, by heating norpluviine in tritiated acetic acid in the presence of a platinum catalyst. A portion of the 8-^{3}H-norpluviine was purified by reprecipitation from aqueous acid

solution using solid sodium bicarbonate, and finally, by crystallization from methanol. This purification procedure gave 15 mg. of 8-^{3}H-norpluviine (mp. 237-239°, lit. mp. 239-241°[57]) with a specific activity of 1.22 x 10^{10} dpm/mM, 5.51 mc./mM. The infrared spectrum of this material was identical with the infrared spectrum of authentic norpluviine. Dilution with pure inactive norpluviine resulted in no gain or loss of total activity.

8-^{3}H-Pluviine

A diluted sample of 8-^{3}H-norpluviine was purified as above to give 29 mg. (5.62 x 10^{9} dpm/mM). This material was suspended in 2 ml. of methanol. This suspension was treated with 10 ml. of ethereal diazomethane (prepared from Aldrich "Diazald") at room temperature for six hours. The solvent was removed and the residue resuspended in 2 ml. methanol and treated again with 10 ml. of ethereal diazomethane for four hours at room temperature. The solution was concentrated and chromatographed on a preparative-scale TLC plate, and eluted with chloroform/methanol/diethylamine, 93/2/5. This afforded 20 mg. (67%) of crude 8-^{3}H-pluviine which was recrystallized from benzene/hexane (mp. 218-219°, lit. mp. 220-221°[57]) to a constant activity of 5.68 x 10^{9} dpm/mM, 2.55 mc./mM. The specific molar activity of 8-^{3}H-norpluviine relative to the specific molar activity of 8-^{3}H-pluviine was 1.01. The infrared spectrum of 8-^{3}H-pluviine was identical with the spectrum of pure authentic pluviine. Dilution

of the sample with inactive pluviine resulted in no gain or loss of total activity.

Degradation of 8-^{3}H-Pluviine

Pluviine (200 mg., 1.33 x 10^{7} dpm/mM) was dissolved in 30 ml. of 10% hydrochloric acid. The solution was basified with solid sodium carbonate to pH 8. With stirring at room temperature, a solution of 1.20 g. of potassium permanganate in 100 ml. of water was added dropwise over a period of 35 minutes. Stirring was continued for an additional four hours. Sulfur dioxide was bubbled through the solution until the precipitate had completely disappeared and the solution was clear. The solution was acidified to pH 2 with 50% H_2SO_4. The solution was concentrated to a volume of approximately 50 ml. under reduced pressure. Ether was used to continuously extract the solution for 36 hours. The ether was removed under reduced pressure to give a wet residue. This material was partitioned between ethyl acetate and 5% sodium bicarbonate solution. The aqueous basic layer was acidified with 50% H_2SO_4 to pH 2 and extracted with eight 30 ml. portions of ethyl acetate. The organic layer was dried over $MgSO_4$ and filtered. The ethyl acetate was removed under reduced pressure and the residue evaporated to dryness with acetic anhydride. The brown gum was transferred to a sublimation tube and sublimed at 120° (0.10 mm.). Recrystallization of the sublimed material from

ethyl alcohol (95%) gave 11 mg. of pure N-ethyl-m-hemipinimide (mp. 230-231°, lit. mp. 229-230°[69]) with a specific molar activity of 1.04 x 10^7 dpm/mM. The specific molar activity of the imide relative to the specific molar activity of 8-^{3}H-pluviine was 0.78.

8-^{3}H-Norpluviine Feeding to Narcissus "King Alfred"

Five mg. of 8-^{3}H-norpluviine (2.22 x 10^8 dpm, 0.10 mc.) in 1.1 ml. of aqueous HCl solution at pH 6 was injected with a fine hypodermic syringe in equal amounts into the flower stalks of thirteen blooming Narcissus pseudonarcissus var. "King Alfred" plants. The plants were allowed to grow for 18 days. The entire group of plants (1.47 kg.) was processed by grinding in a Waring Blendor using 6 l. of 95% ethanol. The solid material was filtered, covered with 3 l. of ethanol and allowed to stand overnight. This treatment was repeated twice with 3 l. of ethanol. The combined ethanolic extracts were concentrated in vacuo to 1.5 l. The aqueous suspension was acidified to pH 4 with 10% tartaric acid. The aqueous solution was washed five times with 200 ml. portions of diethyl ether. This organic fraction contained neutral substances and was not investigated further. Three extractions with 200 ml. portions of n-butanol yielded, after removal of the butanol, 9.5 g. of brown gum. Alkaloid fractions were obtained by extraction with six 200 ml. portions of chloroform at pH 7, 9 and 12. Ammonium hydroxide

was used to basify the solution to pH 7 and 9, while 15% NaOH was used to bring the pH to 12. The combined chloroform extracts yielded 2.6 g. of crude alkaloid mixture. The crude alkaloid mixture (2.6 g.) was chromatographed on preparative-scale TLC plates eluting with 70/30/1 ethyl acetate/methanol/ammonia to give four fractions (1-4). Fraction 2, R_f=0.7, gave 172 mg. of haemanthamine (mp. 198-200°, lit. mp. 200-201°[41]) that was devoid of tritium activity. The mother liquors and fraction 3 were rechromatographed on plates in 90/10/0.5 chloroform/methanol/ammonia. The lycorenine containing fractions (as determined by analytical TLC) were combined and chromatographed on a column of 600 mg. of alumina(Merck). Lycorenine was eluted in ethyl acetate and ethyl acetate/chloroform mixtures. Trituration with acetone yielded 12 mg. of impure lycorenine. The material was diluted with 30 mg. of non-radioactive lycorenine and recrystallized several times from ethyl acetate to yield 23 mg. (mp. 197-199°, lit. mp. 198-200°[42]). Constant activity was attained at 1.11×10^4 dpm/mg. The total activity was 0.11 μc.

Fractions containing homolycorine from the thick layer plate and column chromatography were combined and purified further on preparative-scale TLC plates using chloroform/methanol/acetone 60/20/20 as the solvent system (R_f=0.8). The homolycorine was obtained in trace quantities and was diluted with 40 mg. of non-radioactive homolycorine. Recrystallization from ethanol afforded 10 mg. of homolycorine (mp. 174-176°,

lit. mp. 174-176°[73]) with constant activity of 3.92 x 10^3 dpm/mg. and a total activity of 0.018 μc.

Column and TLC fractions containing pluviine were combined and further purified via preparative-scale TLC eluting with 60/20/20 chloroform/methanol/acetone (R_f=0.7). Recrystallization several times from acetone gave 8 mg. of pluviine (mp. 217-219°). The activity was constant at 8.16 x 10^3 dpm/mg. with a total activity of 0.029 μc.

By analytical TLC, the mother liquor of pluviine crystallization contained methylpseudolycorine. Dilution with 10 mg. of inactive methylpseudolycorine and recrystallization initially from ethanol and then from acetone yielded 7 mg. (mp. 234-237°, lit. mp. 234-242°[14]). Constant activity was attained at 7.11 x 10^2 dpm/mg. with a total activity of 2.3 x 10^{-3} μc.

Fractions rich in galanthine were rechromatographed on preparative-scale TLC plates using 60/20/20 chloroform/acetone/methanol (R_f=0.6). Recrystallization from ethyl acetate/hexane afforded 18 mg. of galanthine (mp. 135-136°, lit. mp. 134-136°[14]). Radioactivity was constant at 2.14 x 10^3 dpm/mg. The total activity was 0.017 μc.

The butanol extract was further fractionated in the following manner. The gum was dissolved in 200 ml. of chloroform and 50 ml. of ethanol. This solution was extracted with three 50 ml. portions of 15% sodium hydroxide. The combined basic layers were washed with three 50 ml. portions of chloroform. These

chloroform extracts were added back to the initial chloroform/ethanol solution. The chloroform/ethanol solution was evaporated to dryness and taken up in 10% HCl (150 ml.). The acidic solution was extracted with ether and basified to pH 7 and extracted with chloroform. The chloroform extracts were added to the alkaloid fractions. The initial aqueous basic layers were acidified with 20% HCl and extracted four times with 100 ml. portions of ethyl acetate to give 0.70 g. of material. This material, in ethyl acetate, was chromatographed on a 20 g. column of silica gel, packed in ethyl acetate. Narciclasine was eluted in ethyl acetate and recrystallized from acetic acid to give 59 mg. of pure narciclasine (mp. dec. above 220°, lit. mp. 232-234°[29]). Its activity was constant at 1.29×10^2 dpm/mg. Its total activity was 3.4×10^{-3} μc.

A summary of this tracer experiment appears in Table 7.

Degradation of Tritiated Lycorenine and Galanthine from the 8-^{3}H-Norpluviine Feeding to Narcissus "King Alfred"

The lycorenine and galanthine isolated from the 8-^{3}H-norpluviine feeding were each diluted with inactive alkaloid to a total weight of 200 mg. The alkaloids were oxidized with potassium permanganate to m-hemipinic acid and converted to the N-ethyl imide derivative by the same procedure described for the oxidation of 8-^{3}H-pluviine. The activities of the imides relative to the corresponding alkaloids appears in the last column of Table 8.

Table 7. Alkaloids isolated from the 8-^{3}H-norpluviine feeding to Narcissus "King Alfred"

Alkaloid	Amt. isolated (mg.)	Activity (dpm/mg.)	Yield, %
Lycorenine[a]	23	1.11×10^{4}	---
Homolycorine[b]	10	3.91×10^{3}	---
Pluviine	8	8.16×10^{3}	0.00054
Methylpseudolycorine[c]	7	7.11×10^{2}	---
Galanthine	18	2.14×10^{3}	0.0012
Narciclasine	59	1.29×10^{2}	0.0040

[a]Isolated by dilution with 30 mg. of inactive alkaloid.

[b]Isolated by dilution with 40 mg. of inactive alkaloid.

[c]Isolated by dilution with 10 mg. of inactive alkaloid.

Table 8. Permanganate oxidation of alkaloids from the 8-^{3}H-norpluviine feeding to Narcissus "King Alfred"

Alkaloid	Activity (dpm/mM)	Yield of N-ethyl-m-hemipinimide (mg.)	Imide activity (dpm/mM)	Imide activity / alkaloid activity
Lycorenine[a]	1.39×10^{5}	5	1.10×10^{5}	0.79
Galanthine[a]	6.05×10^{4}	4	4.54×10^{5}	0.75

[a]The oxidation used 0.200 g. of alkaloid.

8-^{3}H-Pluviine Feeding to Narcissus poeticus L.

The 8-^{3}H-pluviine (2.22×10^{8} dpm, 11 mg.) was dissolved in 1 ml. of aqueous HCl acid at pH 6 and introduced into the flower stalks of thirteen blooming Narcissus poeticus L. plants with a fine hypodermic needle. The plants were harvested after growing fourteen days.

The entire plants (800 g.) were ground in 3 l. of ethanol four times with a Waring Blendor, filtering the solids after each extraction. The ethanol solution was concentrated under reduced pressure to approximately 0.5 l. Acidification with 10% tartaric acid solution and extraction with five 100 ml. portions of ether removed the neutral materials. The combined ether layers were washed with two 100 ml. portions of 10% HCl and the aqueous washes were added to the original aqueous layer. Extractions of the aqueous acid layer with six 100 ml. portions of chloroform gave the chloroform-soluble hydrochloride fraction (0.17 g., free bases 0.07 g.). The pH of the aqueous layer was adjusted to 7 with solid sodium bicarbonate and extracted with nine 100 ml. portions of chloroform to give 0.94 g. of crude alkaloids. At pH 9 and 12, six 100 ml. washes with chloroform yielded 0.13 g. of alkaloids.

The chloroform-soluble hydrochloride fraction was chromatographed as the free bases on preparative-scale TLC plates in 70/30 chloroform/methanol. In this solvent lycorenine has an R_f=0.5. Removal of this area from the plates and trituration

of this fraction with acetone and recrystallization of the resultant crystals from acetone yielded 13 mg. of lycorenine. Analysis of this sample for radioactivity showed that there was no detectable tritium activity.

Removal of the area about R_f=0.6 yielded a fraction containing pluviine. Dilution with 15 mg. of inactive pluviine and recrystallization from acetone gave 11 mg. of pluviine (mp. 218-220°). Constant activity was attained 2.43×10^3 dpm/mg. The total activity was 0.014 μc.

The chloroform extract at pH 7 yielded 0.94 g. of crude alkaloids. Trituration with chloroform afforded crude lycorine. The lycorine was purified via recrystallization of the hydrochloride salt from ethanol. The activity of lycorine hydrochloride (mp. 216-217°, lit. mp. 212-214°[45]) was 7.65×10^3 dpm/mg. The total amount of lycorine hydrochloride isolated was 30 mg. and the total activity was 0.10 μc.

The residue of the pH 7 fraction was chromatographed on preparative-scale TLC plates using the solvent system 60/20/20 chloroform/methanol/acetone. From the fraction of R_f=0.5, 21 mg. of galanthine was obtained by crystallization from ethyl acetate/hexane. Recrystallization from this solvent gave galanthine (mp. 113-135°) of constant activity of 3.45×10^5 dpm/mg. The total activity of galanthine isolated was 3.26 μc. The area of R_f=0.4 afforded a fraction containing methylpseudolycorine. Dilution of this fraction with 15 mg. of inactive methyl-

pseudolycorine and recrystallization from acetone afforded 13 mg. of methylpseudolycorine (mp. 234-238°). The activity was 4.27×10^4 dpm/mg. and the total activity isolated was 0.25 μc. Narcissidine was found to be in the fraction at R_f=0.0 to 0.1, but was contaminated with other material. The alkaloid was purified by dilution with 35 mg. of inactive narcissidine and recrystallization from ethyl acetate. This yielded 21 mg. of narcissidine (mp. 198-200°, lit. mp. 201-203°[20]) with a specific activity of 2.25×10^3 dpm/mg. The total activity was 0.021 μc.

Table 9 summarizes the isolation of alkaloids from this feeding experiment.

Table 9. Alkaloids isolated from the 8-^{3}H-pluviine feeding to Narcissus poeticus

Alkaloid	Amt. isolated (mg.)	Activity (dpm/mg.)	Yield, %
Lycorenine	13	0	0.0016
Pluviine[a]	11	2.43×10^3	---
Lycorine	30[b]	7.65×10^3	---
Galanthine	21	3.45×10^5	0.0025
Methylpseudolycorine[a]	13	4.27×10^4	---
Narcissidine[a]	21	2.25×10^3	---

[a]Isolated by dilution with inactive alkaloids.

[b]Isolated as the hydrochloride salt.

Degradation of Tritiated Galanthine, Narcissidine, and Lycorine from the 8-^{3}H-Pluviine Feeding to *Narcissus poeticus*

The galanthine and narcissidine isolated from the 8-^{3}H-pluviine feeding were each diluted to a total weight of 200 mg. The alkaloids were degraded to N-ethyl-*m*-hemipinimide by the same procedure described for the degradation of 8-^{3}H-pluviine. The activities of the imides relative to the corresponding alkaloids appears in the last column of Table 10.

The tritiated lycorine obtained from the 8-^{3}H-pluviine feeding experiment to *Narcissus poeticus* was diluted to 300 mg. with inactive lycorine. The diluted alkaloid (activity=2.87 x 10^4 dpm/mM) was oxidized with potassium permanganate to hydrastic acid by the same procedure described for the oxidation of 8-^{3}H-pluviine to *m*-hemipinic acid. The hydrastic acid was converted to its anhydride derivative by sublimation at 120° (0.10 mm.). Trituration of the anhydride with 70% ethylamine in water in a sublimation tube and sublimation afforded N-ethyl hydrastimide. Recrystallization from ethanol (95%) yielded the pure imide (mp. 168-170°, lit. mp. 168-169°[70]). The mixed melting point was 168-170°. The activity of N-ethyl hydrastimide was found to be 2.32 x 10^4 dpm/mM. The activity of the imide relative to the activity of the lycorine was 0.81.

Table 10 summarizes the results of the degradations of galanthine, narcissidine, and lycorine.

Table 10. Permanganate oxidation of alkaloids from the 8-^{3}H-pluviine feeding to Narcissus poeticus

Alkaloid	Activity (dpm/mM)	Yield of Imide (mg.)	Imide activity (dpm/mM)	Imide activity / alkaloid activity
Galanthine[a]	2.74×10^{6}	7[b]	2.08×10^{6}	0.76
Narcissidine[a]	6.73×10^{4}	12[b]	5.11×10^{4}	0.76
Lycorine[c]	2.87×10^{4}	4[d]	2.34×10^{4}	0.81

[a]The oxidation used 0.200 g. of alkaloid.

[b]The product is N-ethyl-m-hemipinimide.

[c]The oxidation used 0.300 g. of alkaloid.

[d]The product is N-ethyl hydrastimide.

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