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Biosynthetic studies on ambelline and tazettine

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BIOSYNTHETIC STUDIES ON AMBELLINE AND TAZETTINE

by

John Arthur Miller

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Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Head of Major Department

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Dean of Graduate College

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Ames, Iowa

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INTRODUCTION

Extensive studies on the biosynthesis of Amaryllidaceae alkaloids have been carried out during the past five years. It is apparent that the alkaloids are formed from the amino acids, phenylalanine and tyrosine, the former providing the aromatic C_6-C_1 portion of the alkaloid and the latter the C_6-C_2 unit. The role of phenyl-phenyl oxidative coupling and specifically the precursor norbelladine in the biosynthetic scheme is well understood. Because of the availability of the alkaloids and suitable degradative pathways, lycorine, galanthamine, belladine, haemanthamine and tazettine have been studied most completely. All of these alkaloids have two oxygenated functional groups on the aromatic ring. Since many Amaryllidaceae alkaloids have an aromatic ring substituted by three oxygenated groups, it was desirable to determine whether the same biosynthetic pathways held for these alkaloids as well.

It is well known that the alkaloidal one-carbon units such as methoxyl, N-methyl, and methylenedioxy groups are derived from formate or methionine. Anomalous results were found in the incorporation of formate into belladine and ambelline in Nerine bowdenii, where more than 40% of the radioactivity resided in the benzylic methylene group. To examine this result in more detail, radioactive formate and serine incorporation into the alkaloids of Sprekelia formosissima were studied.

HISTORICAL

Ring Systems

The Amaryllidaceae alkaloids (well over one hundred are known)[1] may be divided into eight ring systems (Figure 1). Although a chemical

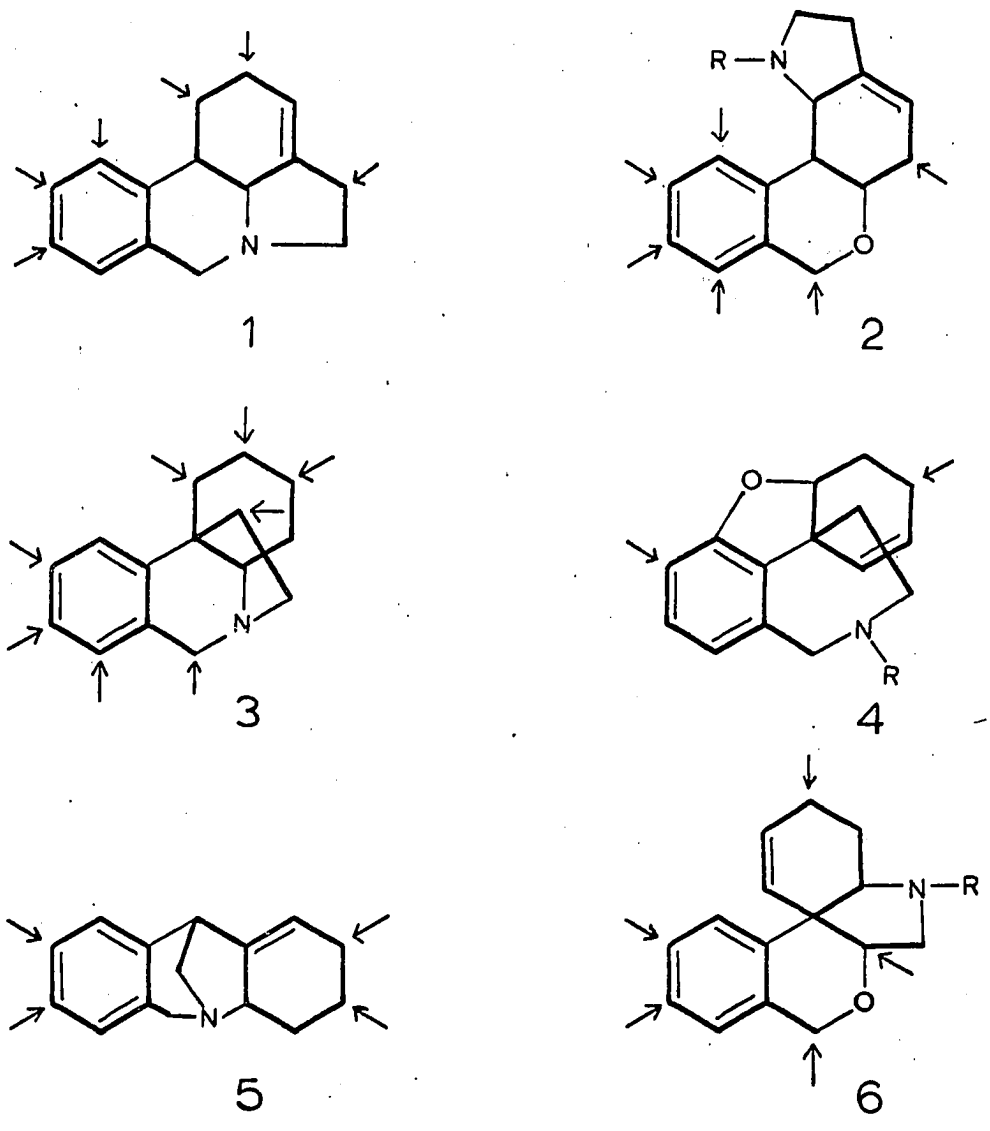
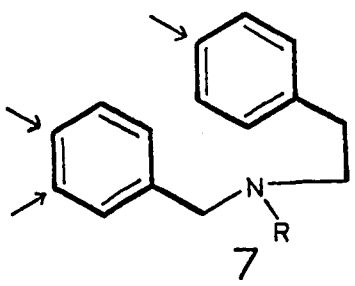


Figure 1. Amaryllidaceae ring systems



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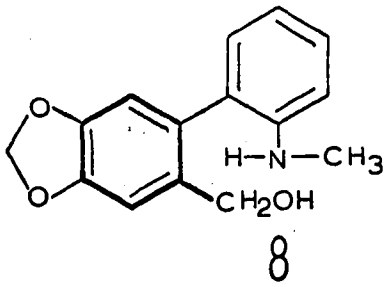

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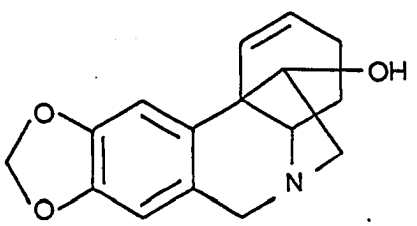
Figure 1. (Continued)

name for each is known, for brevity each ring system is given the name of its most common alkaloid. The eight ring systems are lycorine (1), lycorenine (2), crinine (3) galanthamine (4), montanine (5), tazettine (6), belladine (7) and ismine (8)[2]. Each ring system, except those of belladine and ismine, may be divided into an aromatic C_6-C_1 and a hydroaromatic C_6-C_2 unit represented by the heavy lines. Belladine differs in that it has an aromatic rather than a hydroaromatic C_6-C_2 unit. Ismine, having a biphenyl nucleus, is the only known Amaryllidaceae alkaloid which contains no C_6-C_2 unit.

The large number of alkaloids found in the family can be attributed to the variations in oxygen substitution within these ring systems. The positions at which oxygen substituents may be attached in each nucleus are shown by arrows. The aromatic ring may be di- or tri-oxygenated, and the hydroaromatic ring may be mono- or di-oxygenated. The most common labelling pattern is a di-oxygenated aromatic ring and a mono-oxygenated hydroaromatic ring. The belladine type has only one oxygen

substituent on the aromatic ring of the C_6-C_2 unit. Haemultine (9 or 10), isolated from Haemanthus multiflorus by Boit[3,4] contains no oxygen substituent on the hydroaromatic ring. However, there is some doubt that such an alkaloid exists[5]. Ismine has two oxygen substituents on the aromatic ring of the C_6-C_1 unit, as shown (Figure 1).



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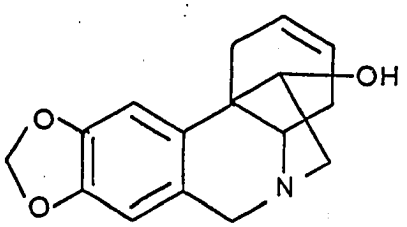

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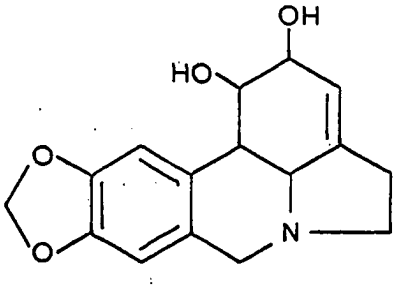
All of the alkaloids contain a tertiary nitrogen except for ismine and some members of the galanthamine and belladine types, which may have either a secondary or tertiary nitrogen atom.

Biogenesis of Amaryllidaceae Alkaloids

Robinson[6] proposed that lycorine (11) and tazettine (12) were derived from a methylenedioxybenzyl amine (13) or from amino acid precursors. He assumed that the first stages of the biosynthesis of lycorine from amino acid precursors was oxidative coupling of one molecule of 3,4-dihydroxyphenylalanine, dopa (14), with a molecule of dihydroxydihydroindone (15a) or the carboxylic acid (15b) derived from dopa. Robinson did not describe the exact manner by which 15a couples



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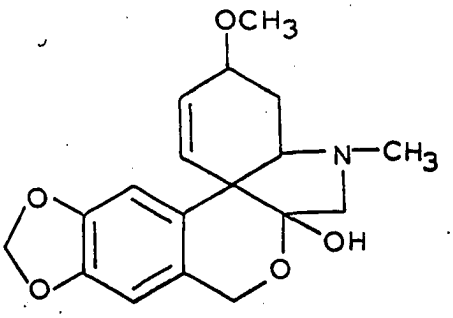

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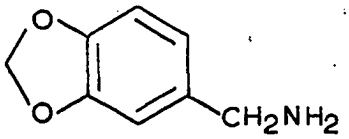

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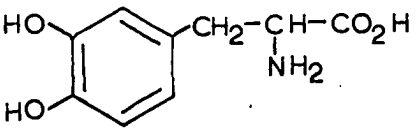

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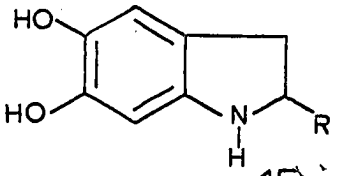

15 a) R = H
b) R = CO_2H

with 3,4-dihydroxyphenylalanine to form the Amaryllidaceae alkaloids or the type of intermediate formed.

In 1957 Steglich[7] proposed a biogenetic scheme based on the condensation of a 3,4-dioxyphenylacetaldehyde (16) and β-keto-glutardialdehyde (17)

with glycine to form 18 and 19, which serve as precursors of the C_6-C_1 and C_6-C_2 units (Figure 2). Caranine (20), lycorine (11), lycorenine (21) and homolycorine (22) may be formed (Figure 3) from the condensation of 3,4-dioxyphenylacetaldehyde and 19, while tazettine (12), buphanamine (23), crinamidine (24), crinine (25) (Figure 4) and galanthamine (26) may be formed from β-ketoglutardialdehyde and 18 (Figure 5).

In 1953 Wenkert[8] proposed a biogenetic scheme (Figure 6) for Amaryllidaceae alkaloids based on the condensation of dioxyphenylethyl amine (27) with dioxyphenylacetaldehyde (16) followed by phenyl-phenyl oxidative coupling. The final step is reduction of one of the aromatic rings to a hydroaromatic ring. Robinson[9] critized this theory on the grounds that there is no reason for one aromatic ring to be reduced in preference to the other following the phenyl-phenyl oxidative coupling.

In 1959 Wenkert replaced his original hypothesis by proposing a shikimic-prephenic biogenetic scheme for the Amaryllidaceae alkaloids[10]. He suggested that shikimic acid (28) serves as the precursor of the C_6-C_1 unit and 29 serves as the precursor of the C_6-C_2 unit in the lycorine (1) and lycorenine (2) systems. The double bond isomer (30) serves as the precursor of the C_6-C_2 unit in crinine (3), galanthamine (4) and tazettine (6) ring systems.

The ketamines (29 and 30) are derived from shikimic acid (28) by reaction with pyruvic acid (31) followed by amination and condensation (Figure 7).

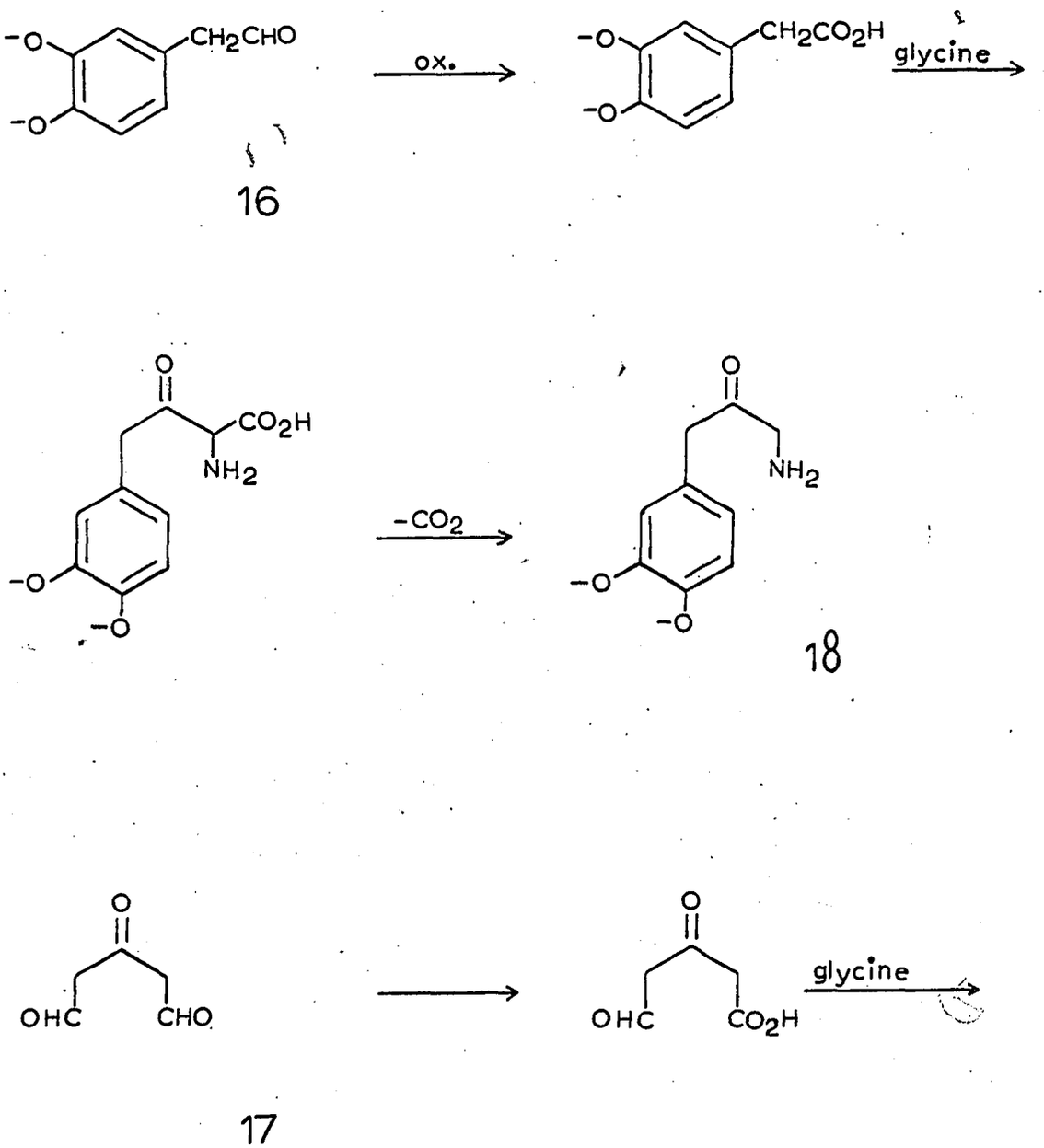

Figure 2. Biogenesis of Amaryllidaceae alkaloid intermediates (Steglich)

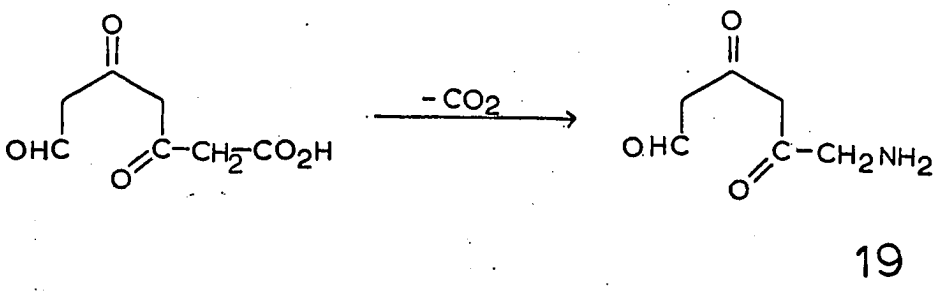

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Figure 2. (Continued)

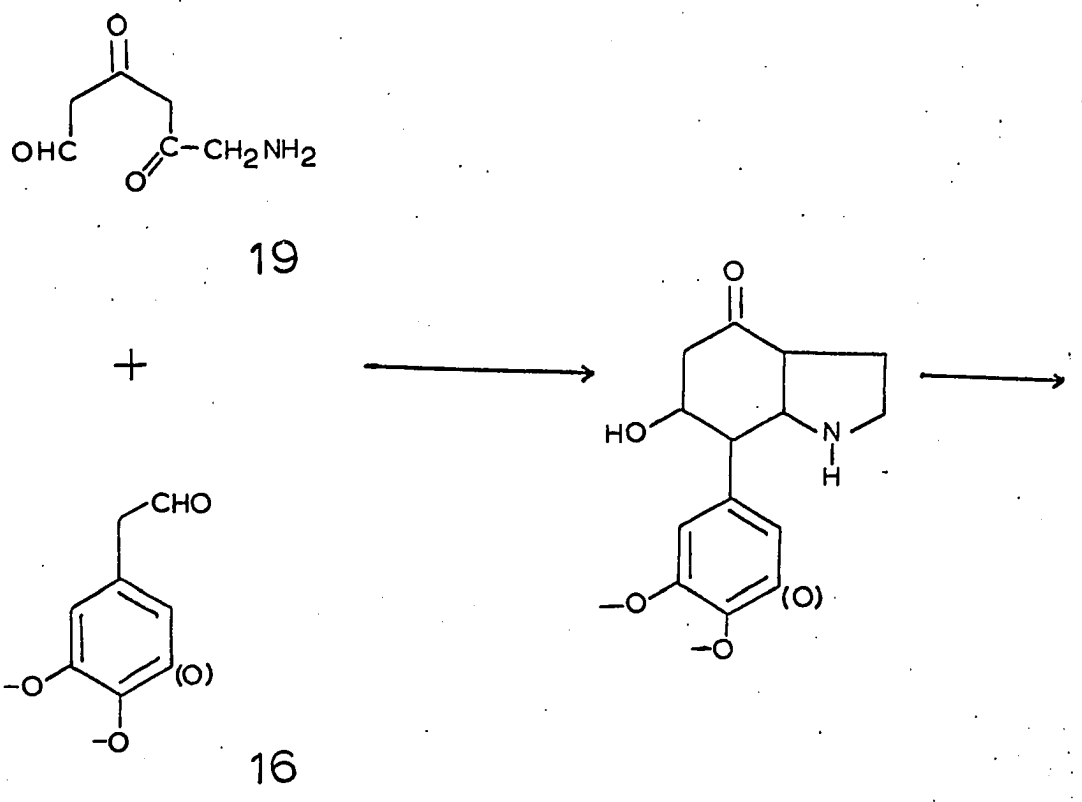


Figure 3. Biogenesis of the lycorine-lycorenine group of Amaryllidaceae alkaloids (Steglich)

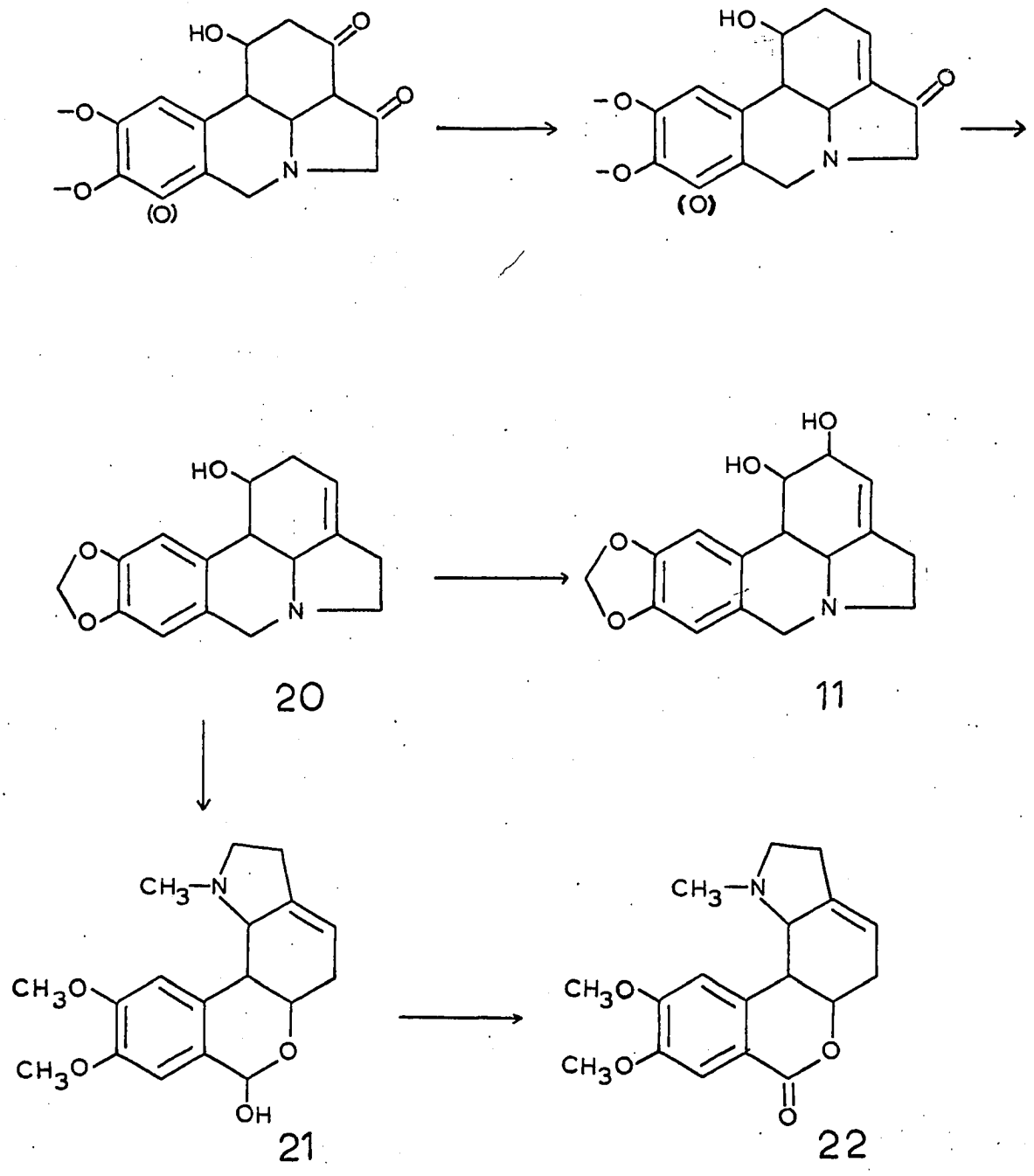


Figure 3. (Continued)

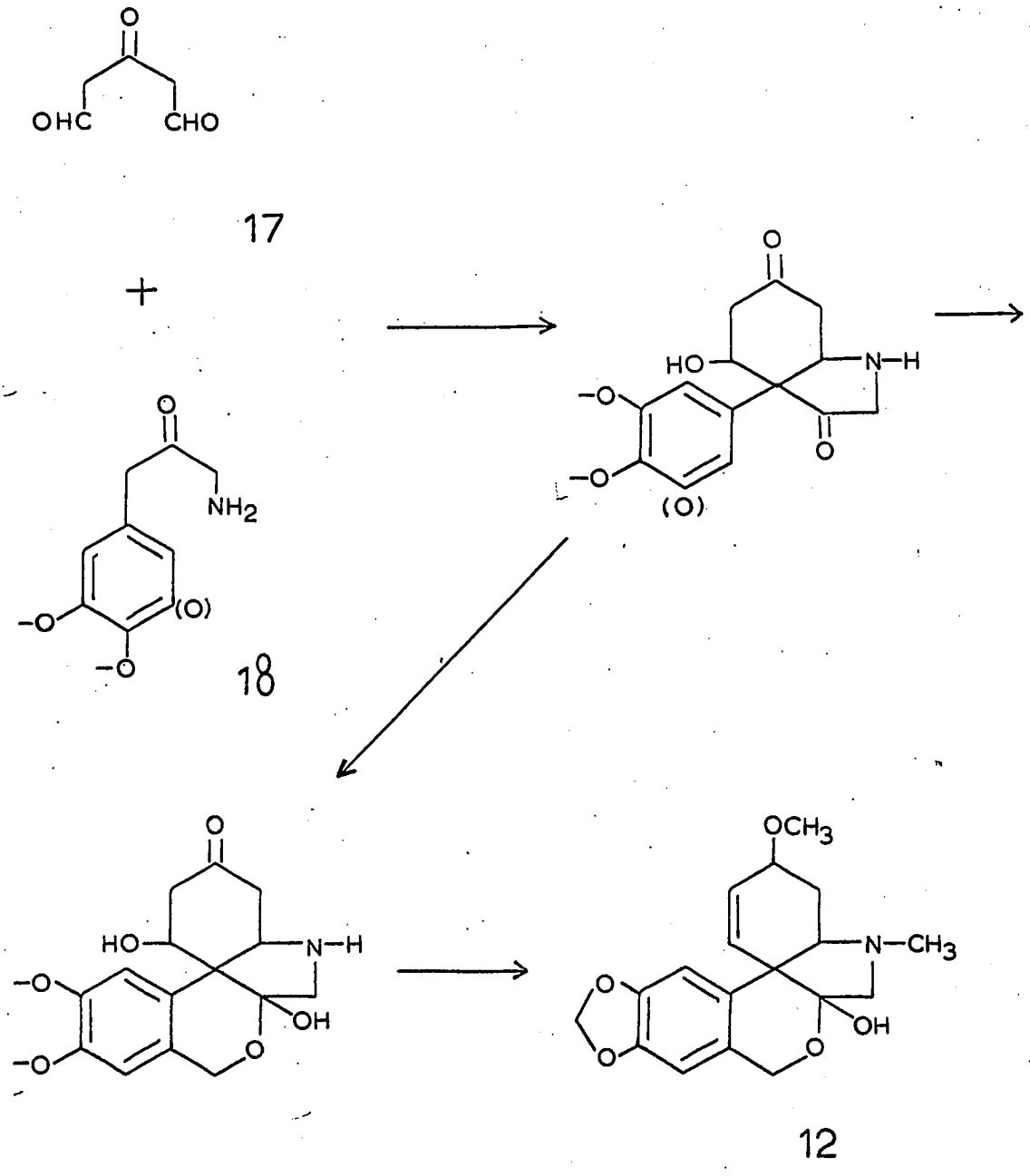


Figure 4. Biogenesis of the tazettine-crinine group of Amaryllidaceae alkaloids (Steglich)

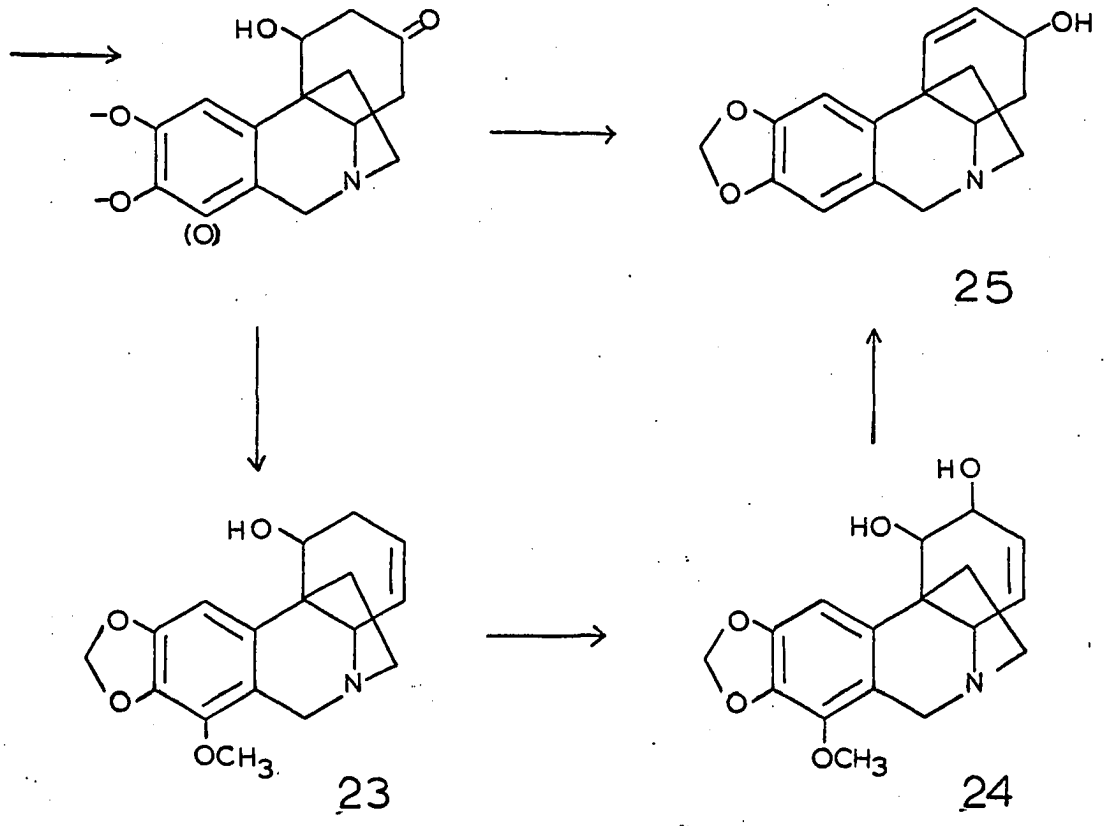


Figure 4. (Continued)

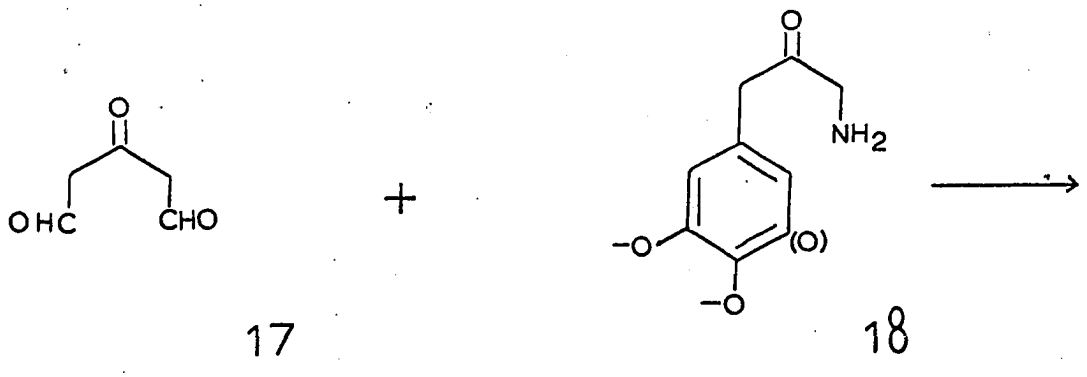


Figure 5. Biogenesis of the galanthamine group of Amaryllidaceae alkaloids (Steglich)

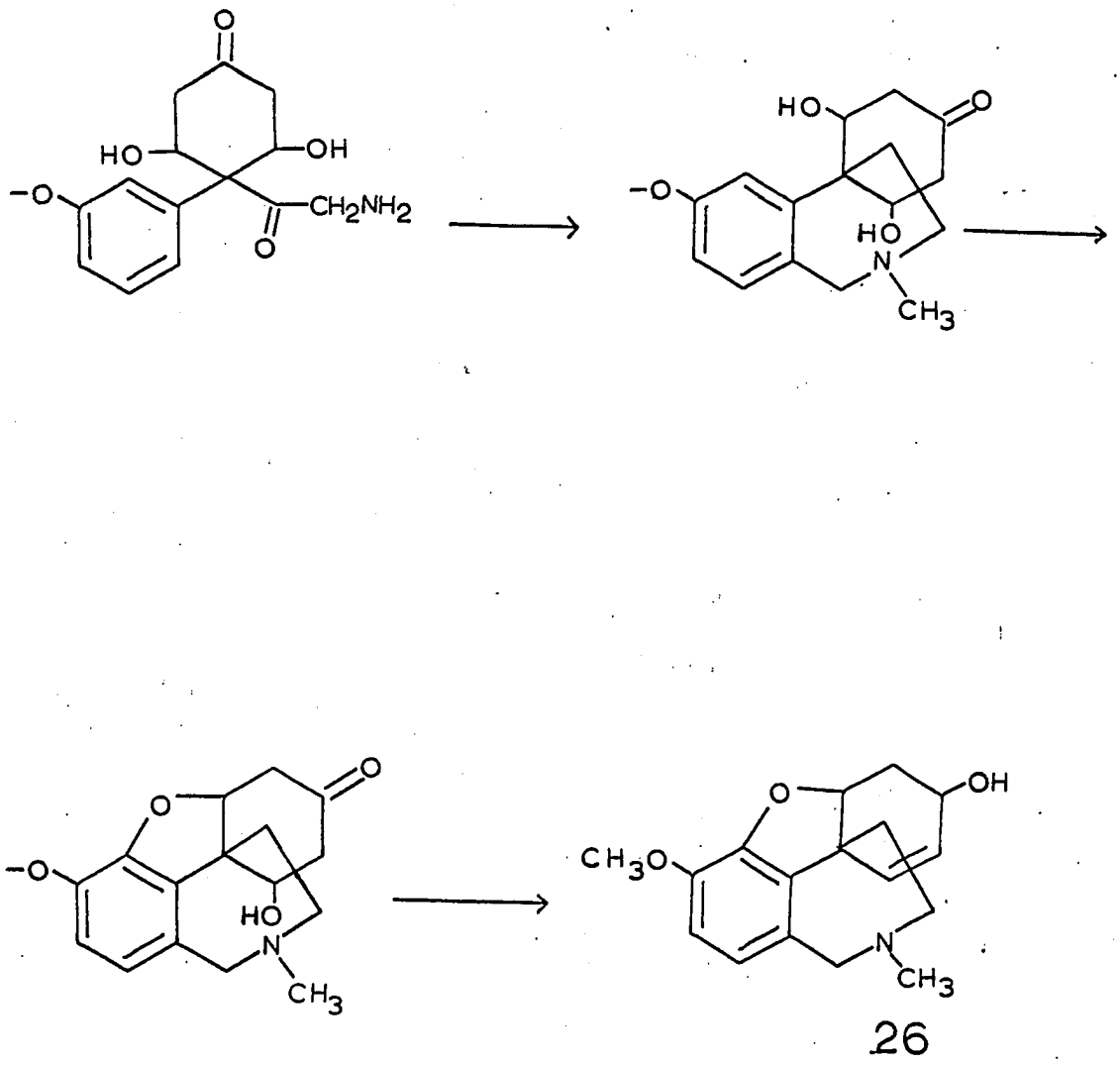


Figure 5. (Continued)

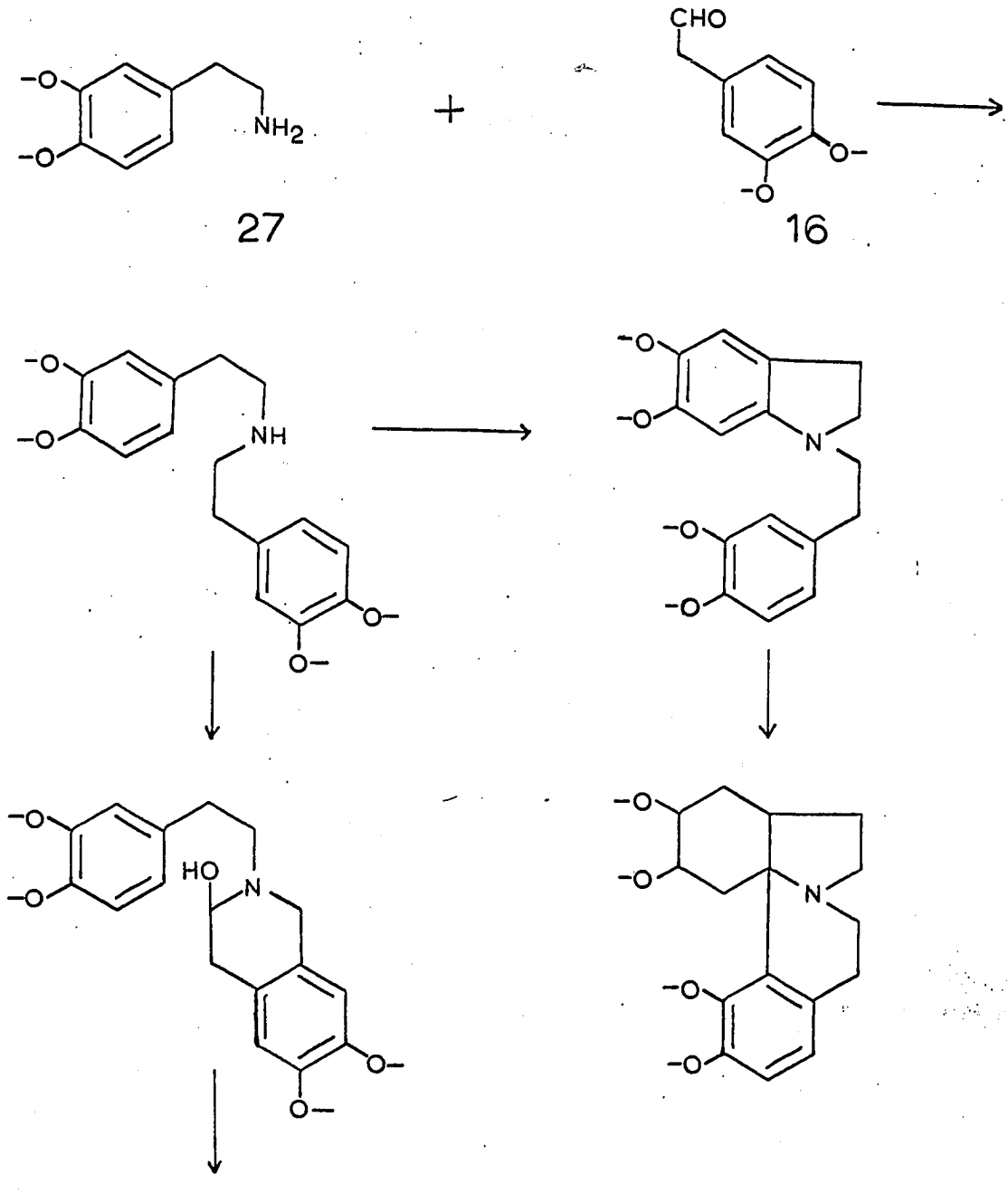


Figure 6. Biogenesis of Amaryllidaceae alkaloids (Wenkert, 1953)

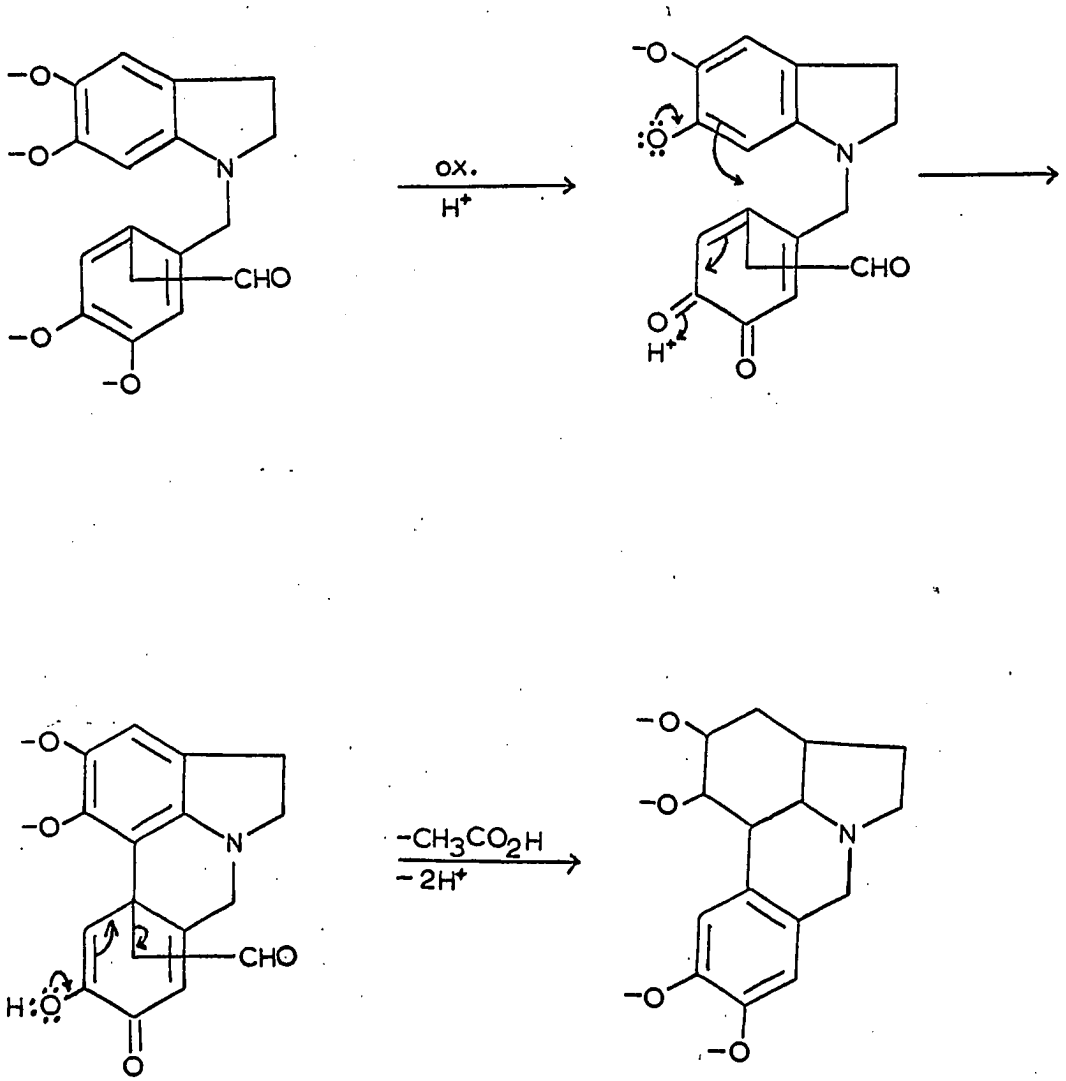


Figure 6. (Continued)

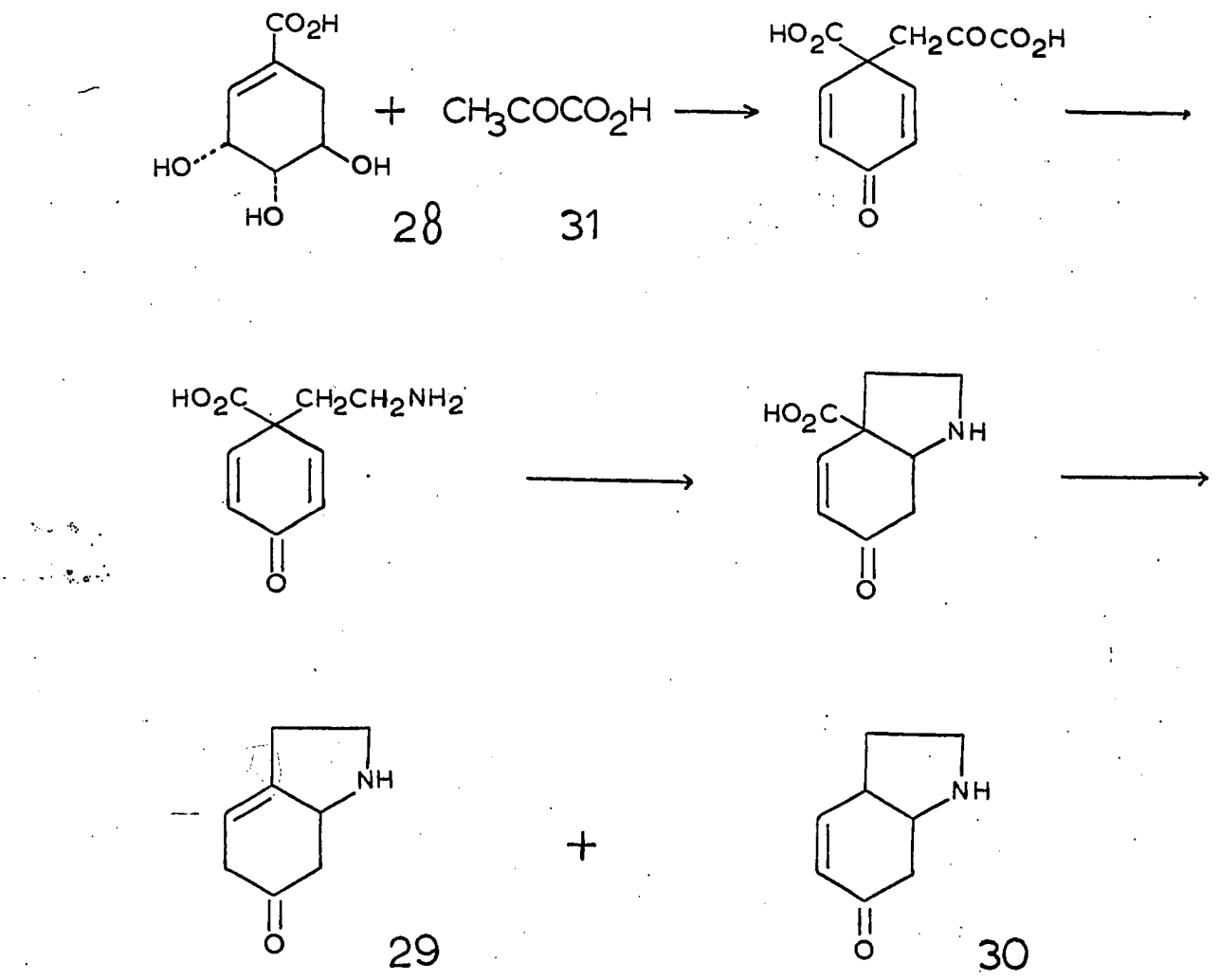


Figure 7. Biogenesis of the prephenates

The derivative (32) formed from shikimic acid and 29 undergoes an internal Michael condensation followed by hydration-dehydration and oxidation-reduction reactions (Figure 8) leading to lycorine (11) and lycorenine (21). The shikimyl derivative (33) of the ketamine (30) is a precursor (Figure 9) to crinine (25), narwedine (34) and tazettine (12).

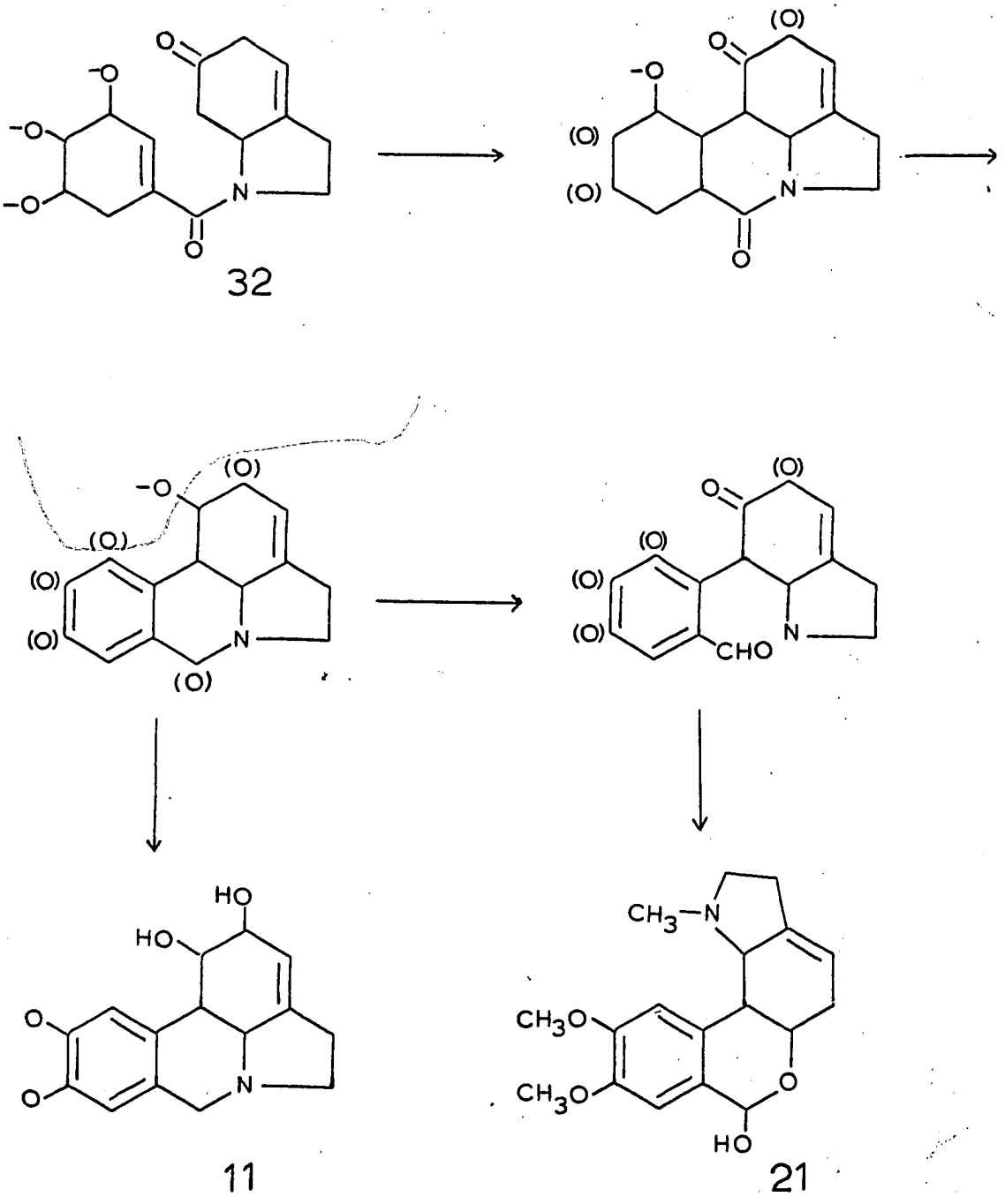


Figure 8. Biogenesis of lycorine and lycorenine (Wenkert, 1959)

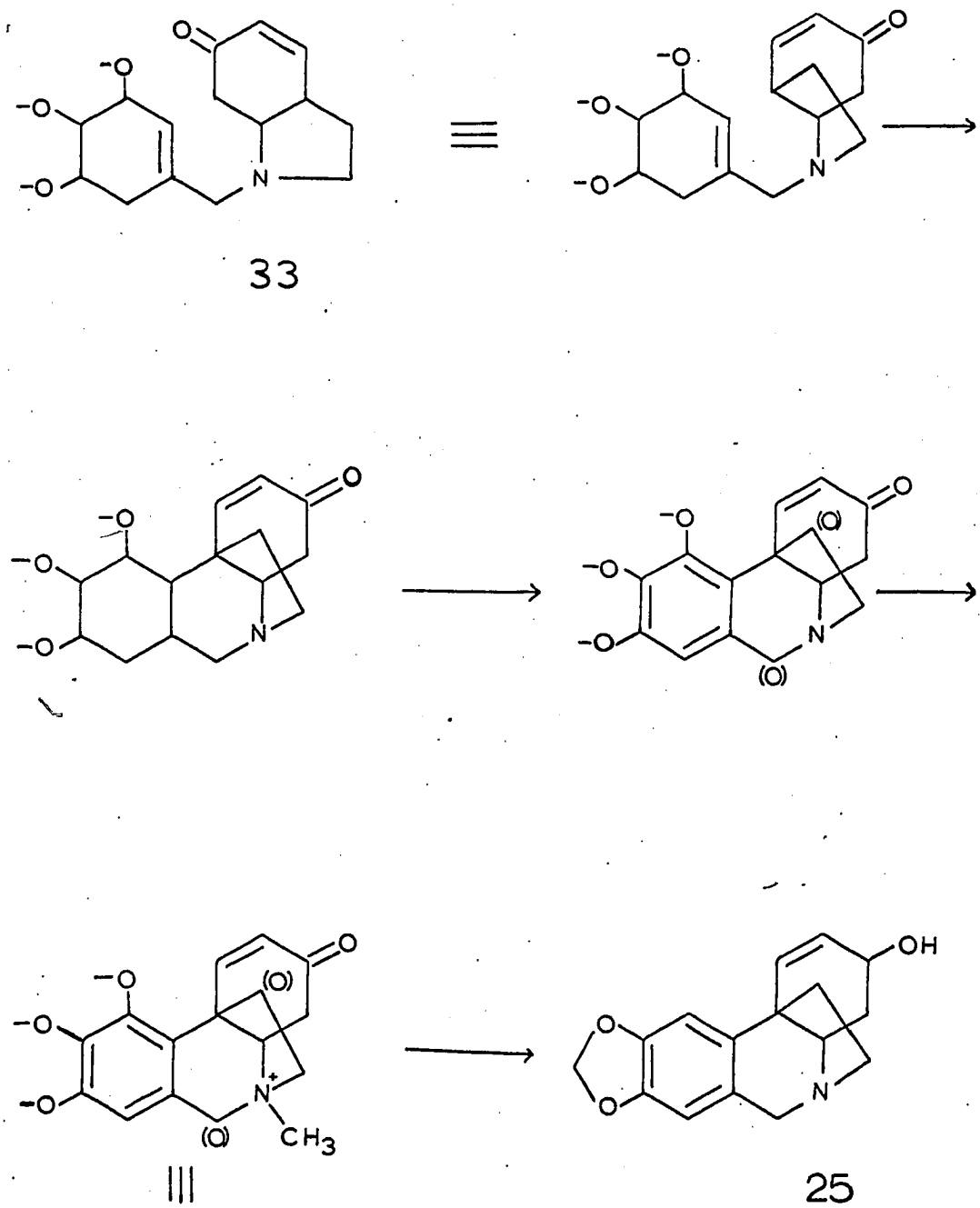


Figure 9. Biogenesis of crinine, narwedine and tazettine (Wenkert, 1959)

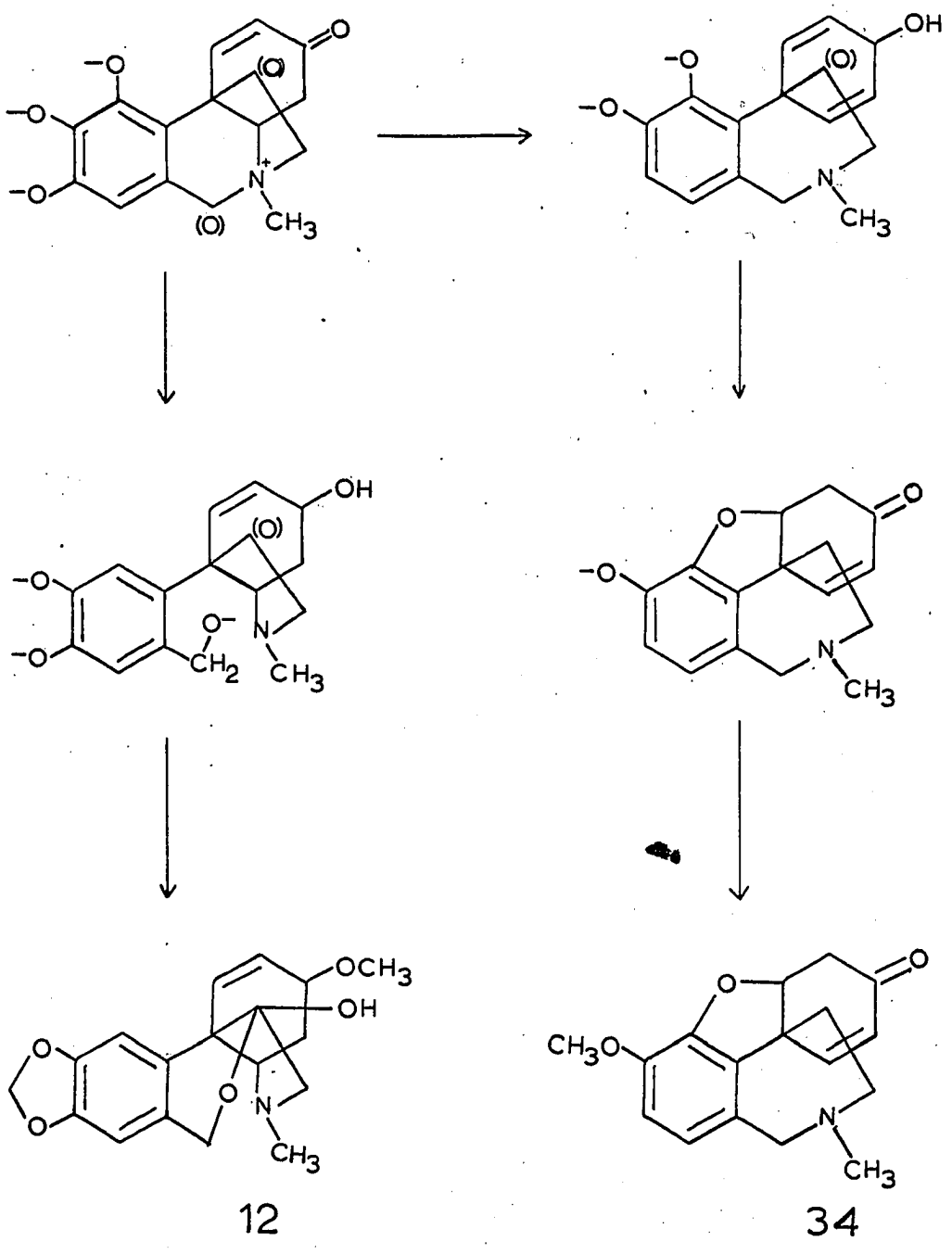


Figure 9. (Continued)

Barton and Cohen[11] have proposed that many natural products may originate from coupling of phenol radicals. The carbon-carbon coupling of these radicals may be ortho-ortho, ortho-para or para-para. Examples of the first type (Figure 10) are shown by formation of dehydrodivanillin (35) from vanillin (36), dehydrodieugenol (37), dehydrodi-o-cresol (38), dehydrodi-p-cresol (39), dehydrodi-2,4-dimethyl phenol (40) and dehydrodi-β-naphthol (41). The best example of the relatively unknown ortho-para coupling (Figure 11) is the formation of Pummerer's ketone (42) from para-cresol (43). Examples of the more common para-para coupling (Figure 12) are shown by the formation of 4,4'-dihydrodiphenyl (44) from lead tetraacetate oxidation of phenol (45), compounds (46 and 47) from 2,6-dimethylphenol (48), the ketone (49) from anthranol (50) and the dihydrophenanthrine (51) from oxidation of 52 via a diketone (53).

Extending the concept of phenol oxidative coupling to the Amaryllidaceae alkaloids, Barton and Cohen[11] postulated that these alkaloids had a common phenolic precursor, norbelladine (54a). At the time that they postulated this phenolic precursor, the alkaloid belladine (54b) was unknown. The subsequent isolation of belladine[12] from Nerine bowdenii and Amaryllis belladonna lent support to their hypothesis.

Barton and Cohen proposed a biogenetic scheme for lycorine (11) and caranine (55) based on their hypothesis (Figure 13). Applying their biogenetic hypothesis to the structure of galanthamine (Figure 14) they predicted the location of the hydroxyl group and double bond in the alkaloid to be as shown in structure 26. Chemical verification for this proposed structure of galanthamine was obtained by other methods[13].

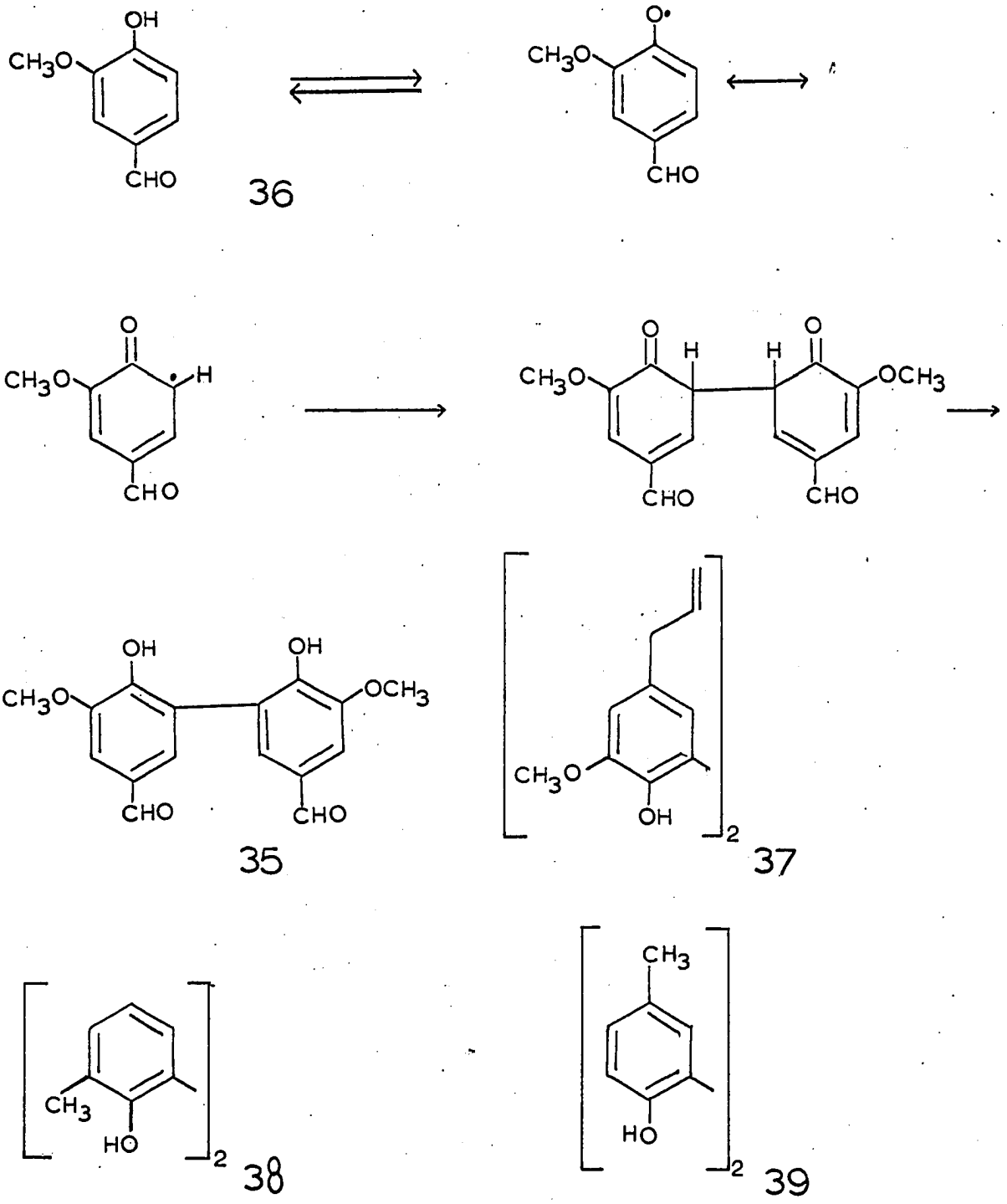
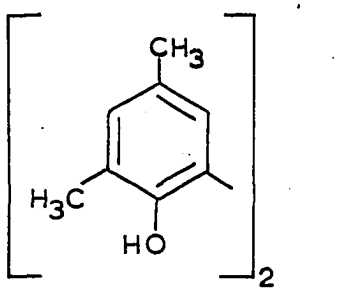


Figure 10. Examples of ortho-ortho phenol-oxidative coupling



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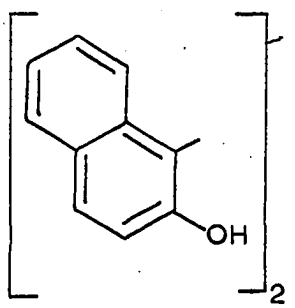

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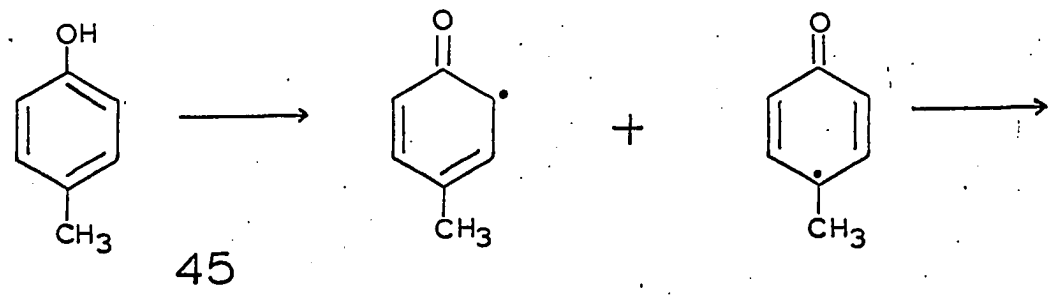
Figure 10. (Continued)



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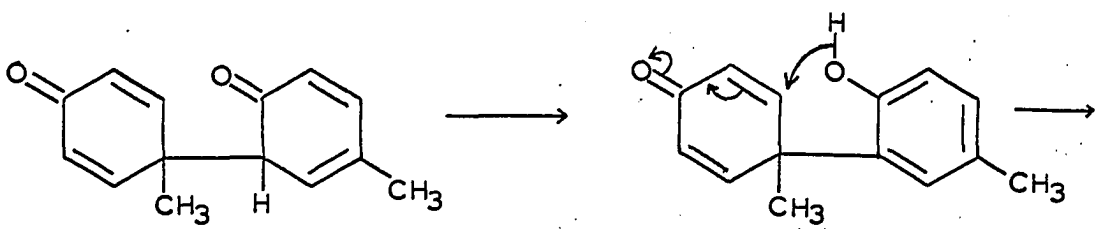

Figure 11. An example of ortho-para phenol-oxidative coupling

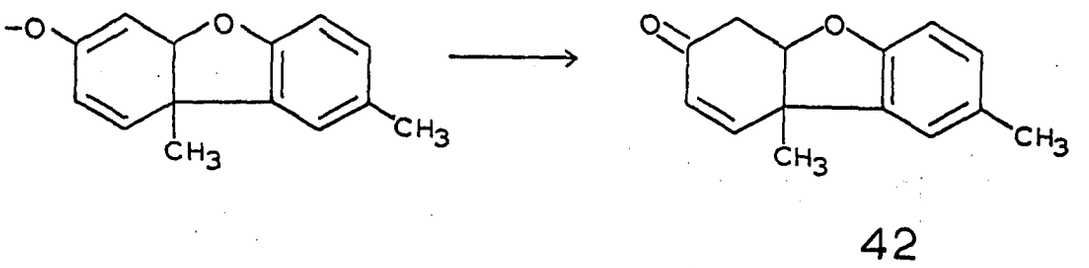

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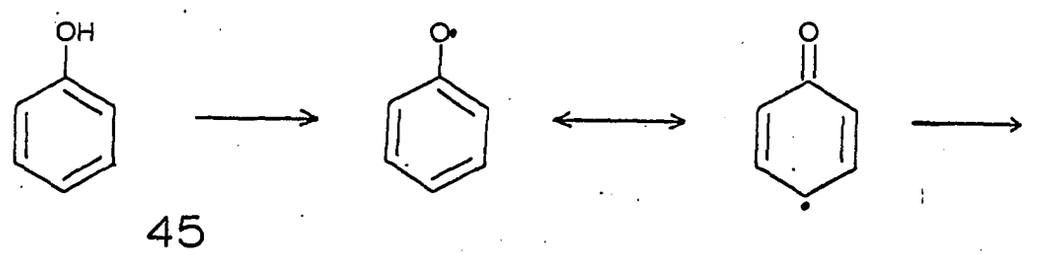
Figure 11. (Continued)



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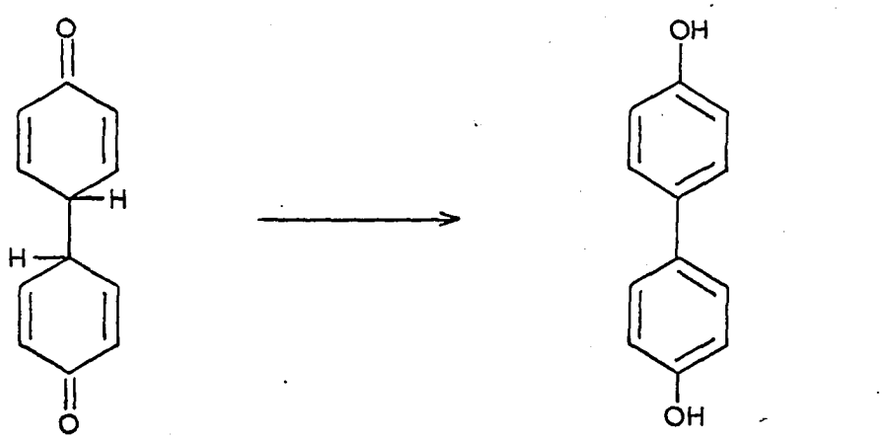

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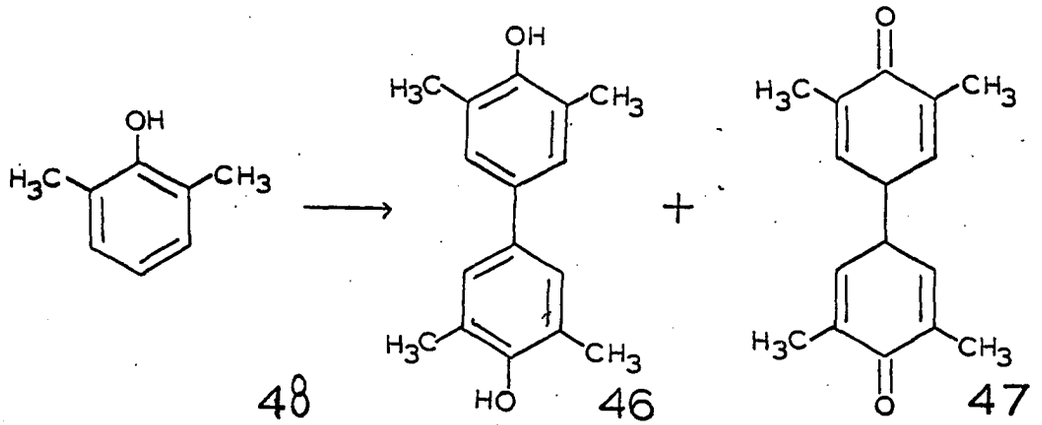
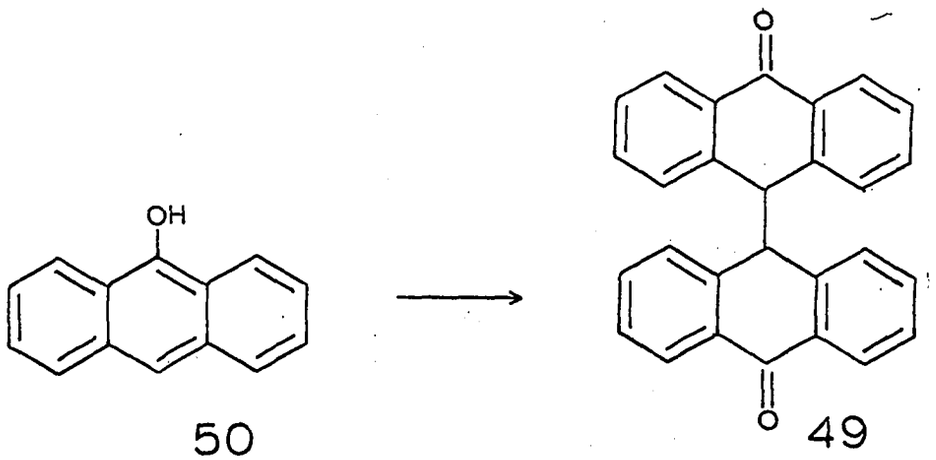
Figure 12. Examples of <u>para</u>-<u>para</u> oxidative coupling of phenol radicals





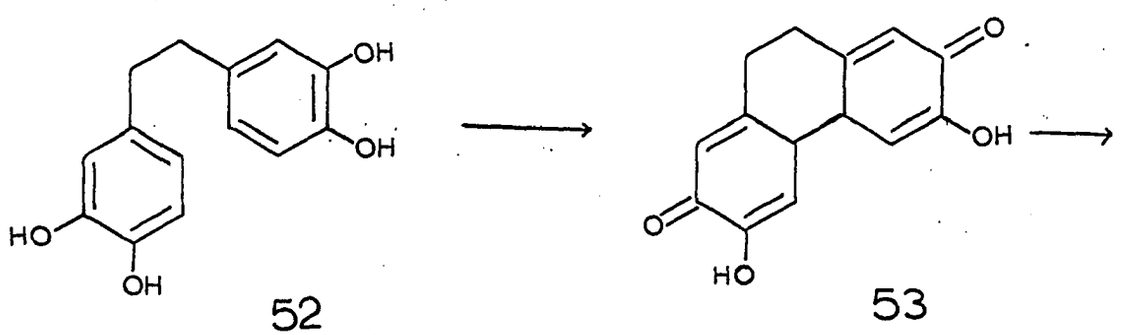


Figure 12. (Continued)

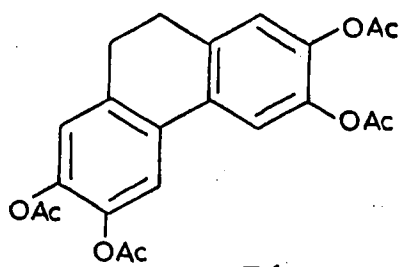


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Figure 12. (Continued)

A biosynthetic type of synthesis of galanthamine has been accomplished[14]. The galanthamine was formed in low yield from the norbelladine derivative (56) by manganese dioxide oxidation and lithium aluminum hydride reduction.

The biosynthesis of lycorenine (21) and tazettine (12) by this theory presented some difficulties. If the nucleus of an alkaloid of this type were to be formed by a simple phenyl-phenyl oxidative coupling reaction, two separate fragments would be required to assume a given orientation prior to being coupled to form the alkaloid nucleus. However, recent biosynthetic studies[15] have shown (Figure 15) that tazettine is formed from haemanthidine (57), which in turn is derived from haemanthamine (58).

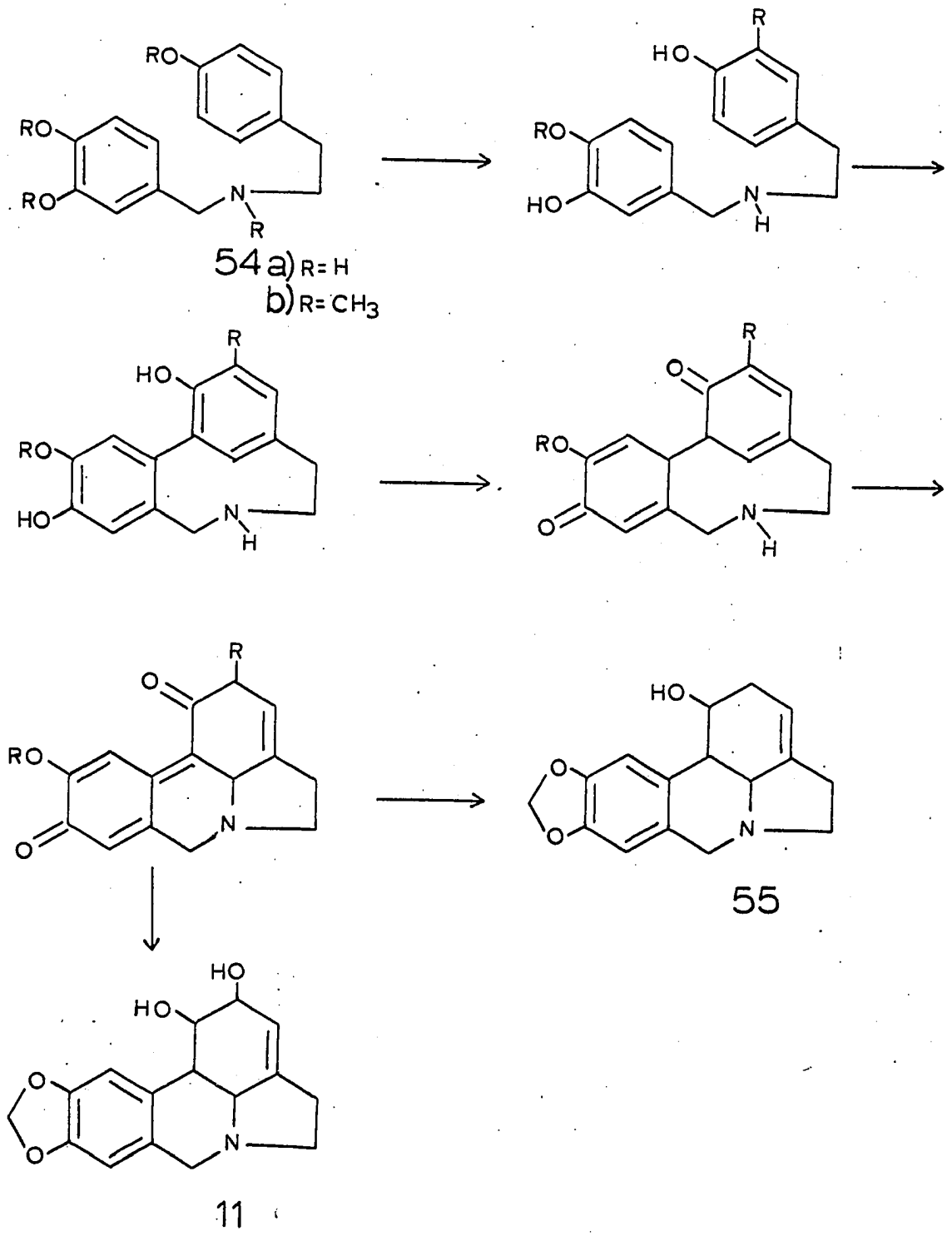

Figure 13. Biogenesis of lycorine and caranine (Barton and Cohen)

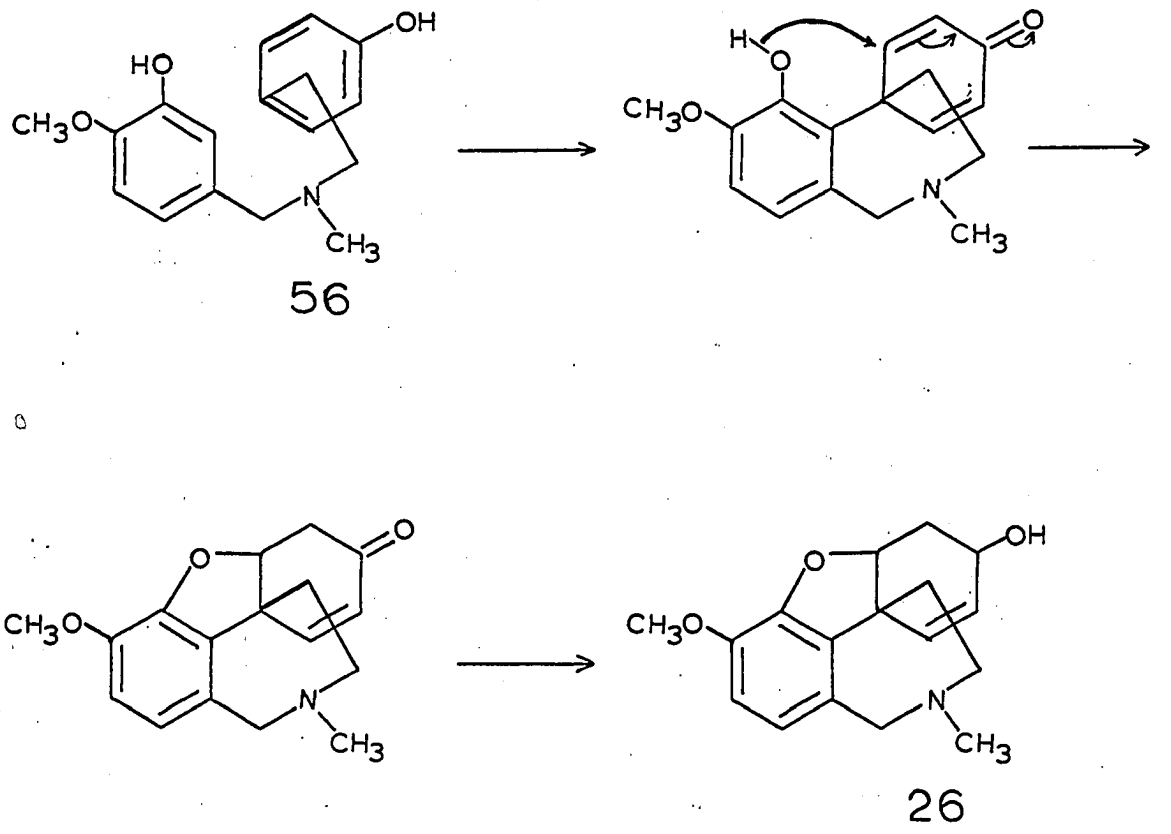

Figure 14. Biogenesis of galanthamine (Barton and Cohen)

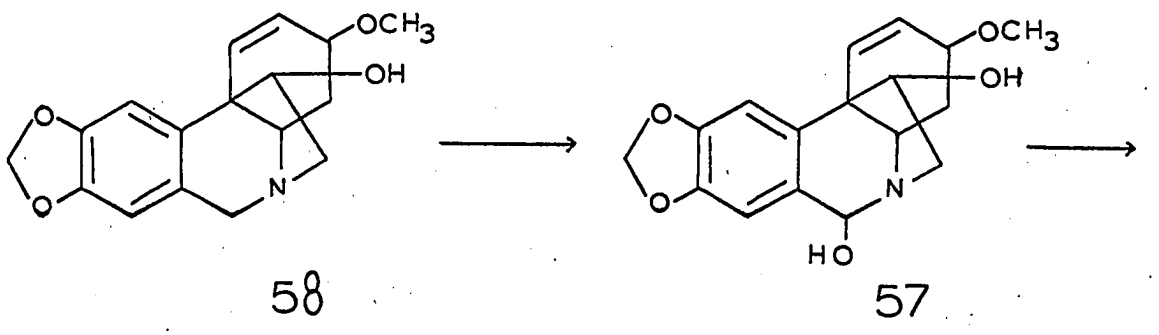

Figure 15. Conversion of haemanthamine to tazettine <u>in vivo</u>

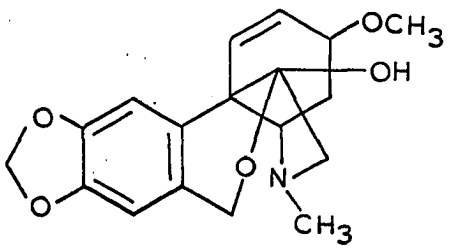

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Figure 15. (Continued)

Biosynthesis of the Amaryllidaceae Alkaloids

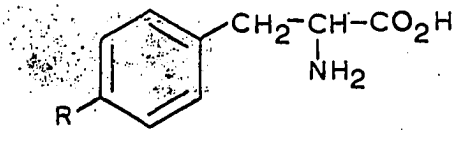
The biosynthesis of various Amaryllidaceae alkaloids was investigated intensively during the period 1960 to 1965. The lycorine (1), crinine (3), galanthamine (4) and tazettine (6) group of alkaloids and the alkaloid belladine (54b) were the primary objects of study. The lycorenine (2) and montanine (5) groups of alkaloids have not been investigated due to their scarcity and the uncertainty of a suitable degradation scheme for the radioactive montanine.

The investigations, summarized in Table 1, have shown that tyrosine (59a) is a precursor of the hydroaromatic C_6-C_2 unit but is never incorporated into the aromatic C_6-C_1 unit of the Amaryllidaceae alkaloids.

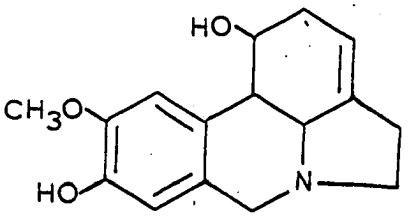
Table 1. Incorporation of amino acids into Amaryllidaceae alkaloids

Precursor	Plant	Alkaloid	% inc.	Ref.
2-C^{14}-Tyrosine	Narcissus "Twink"	Haemanthamine (58)	0.078	16,17
		Norpluvine (60)	0.15	17,18
		Caranine (55)	0.079	17
		Lycorine (11)	0.23	17,18
	Narcissus "King Alfred"	Galanthamine (26)	----	14
		Lycorine (11)	----	14
	Narcissus "Texas"	Galanthamine (26)	----	14
	Narcissus "Deanna Durbin"	Lycorine (11)	----	19
3-C^{14}-Tyrosine	Sprekelia formossisima	Haemanthamine (58)	----	20
		Tazettine (12)	0.16	20
		Haemanthidine (57)	0.20	20
	Haemanthus natalensis	Haemanthamine (58)	0.97	21
		Haemanthidine (57)	0.19	21
		6-Hydroxycrinamine (61)	1.18	21
	Nerine bowdenii	Lycorine (11)	0.11	22
		Belladine (54b)	0.82	22
2-C^{14}-Phenylalanine	Narcissus incomparibilis	Lycorine (11)	0.00	23
3-C^{14}-Phenylalanine	Narcissus incomparibilis	Lycorine (11)	0.18	23
	Nerine bowdenii	Lycorine (11)	0.095	22
		Belladine (54b)	0.42	22
	Narcissus "Deanna Durbin"	Lycorine (11)	----	19
		Pluvine (62)	----	19
		Galanthine (63)	----	19
		Haemanthamine (58)	----	19
		Narcissidine (64)	----	19

Likewise, phenylalanine (59b) is shown to be incorporated only into the aromatic C_6-C_1 unit and is not incorporated into the hydroaromatic C_6-C_2 unit.



59 a) R = OH
b) R = H



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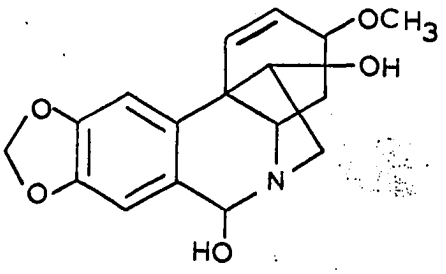

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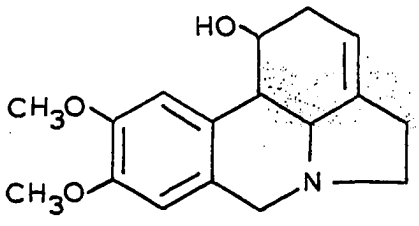

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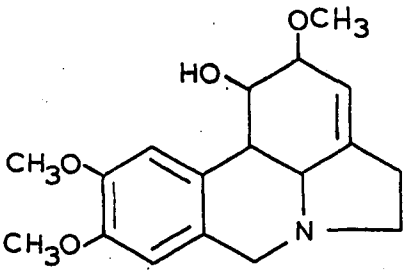

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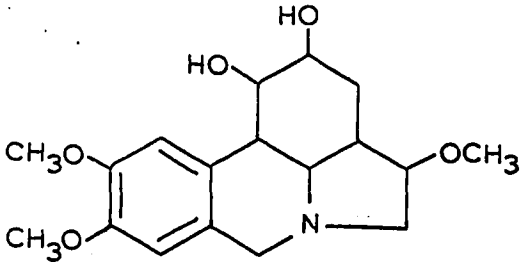

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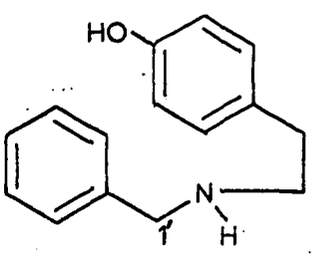
In the biogenetic scheme proposed by Steglich[7], the Amaryllidaceae alkaloids would contain radioactive labels in the hydroaromatic ring of the C_6-C_2 unit, if the 3,4-dioxyphenylacetaldehyde (16) or intermediate (18) was derived from 3-C^{14}-phenylalanine (59b) or 3-C^{14}-tyrosine (59a). Since no biosynthetic experiments using 3-C^{14}-phenylalanine or 3-C^{14}-tyrosine have shown incorporation of carbon-14 into the hydroaromatic ring of Amaryllidaceae alkaloids, Steglich's hypothesis[7] must be discarded.

The hydroaromatic ring of the ketamines (29 and 30) which serve as the precursors of the C_6-C_2 unit in Amaryllidaceae alkaloids (Figure 8 and 9) is derived from shikimic acid (28), and the two-carbon unit of 29 and 30 is derived from pyruvic acid (Figure 7) by the biogenetic scheme proposed by Wenkert[10]. The shikimic acid also serves as a precursor of the C_6-C_1 unit. At present this scheme must be considered as an alternate biosynthetic pathway of the Amaryllidaceae alkaloids.

There have been no investigations using labelled shikimic acid, pyruvic acid or the ketamines (29 and 30) to determine the validity of the Wenkert hypothesis[10]. Evidence proving or disproving this biosynthetic pathway will be difficult to obtain, for shikimic acid and pyruvate are known precursors of phenylalanine (59b) and tyrosine (59a) in microorganisms[24-29].

A series of investigations, summarized in Table 2, have shown norbelladine (54a) to be incorporated intact into the Amaryllidaceae alkaloids. Further studies by Wildman and Battersby[33] have shown negligible incorporation of 1'-C^{14}-bisdeoxynorbelladine (65) and of 1'-C^{14}-hydroxynorbelladine (66) into haemanthamine (58), or any alkaloid of the Amaryllidaceae family (Table 3). These data suggest that, prior to phenyl-phenyl oxidative coupling, the aromatic ring in the C_6-C_1 unit must be di-oxygenated and the aromatic ring in the C_6-C_2 unit of the phenolic precursor must be mono-oxygenated. The data also suggest that further oxygenation of the hydroaromatic ring of the C_6-C_2 unit in the Amaryllidaceae alkaloids



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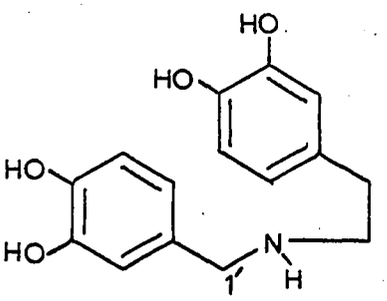

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Table 2. Incorporation of norbelladine into Amaryllidaceae alkaloids

Precursor	Plant	Alkaloid	% inc.	Ref.
1-C^{14}-Norbelladine	Narcissus "Twink"	Lycorine (11)	0.24	30
		Norplurine (60)	0.74	30
		Haemanthamine (58)	0.15	16
	Galanthus elwese	Galanthamine (26)	0.053	31
	Narcissus "King Alfred"	Galanthamine (26)	----	31
		Galanthine (63)	----	31
		Haemanthamine (58)	----	31
1,1'-C^{14}-Norbelladine	Nerine bowdenii	Lycorine (11)	0.07	32
		Crinamine (67)	0.0009	32
		Belladine (54b)	2.64	32

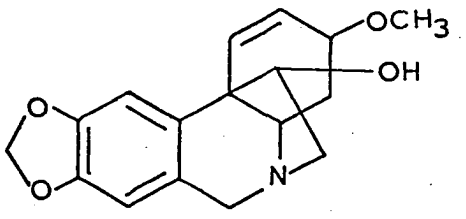

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Table 3. Incorporation of norbelladine derivatives into Amaryllidaceae alkaloids

Precursor	Plant	Alkaloid	% inc.	Ref.
1'-C^{14}-Bisdeoxynorbelladine	Narcissus "Twink"	Tazettine	0.00	33
		Haemanthamine	0.00	33
1'-C^{14}-Hydroxynorbelladine	Narcissus "Twink"	Tazettine	----	33
		Haemanthamine	----	33
		Lycorine	0.00068	33
		Norpluvine	0.0017	33
N-C^{14}-Methyl norbelladine	Narcissus "King Alfred"	Galanthamine	0.18	31,34
		Galanthine	0.00	31,34
		Haemanthamine	0.00	31,34
O-Methyl-N-C^{14}-Methyl norbelladine	Narcissus "King Alfred"	Galanthamine	0.14	31,34
		Galanthine	0.00	31,34
		Haemanthamine	0.00	31,34
O-C^{14}-Methyl-N-C^{14}-Methyl norbelladine	Narcissus "King Alfred"	Galanthamine	0.14	34,35
		Galanthine	0.00	34,35
		Haemanthamine	0.00	34,35
O-C^{14}-Methyl-N-C^{14}-Methyl 1-C^{14}-norbelladine	Narcissus "King Alfred"	Galanthamine	0.018	35
		Galanthine	0.00	35
		Haemanthamine	0.00	35
O-C^{14}-Methylnorbelladine	Narcissus "King Alfred"	Galanthamine	0.00	34,36
		Galanthine	----	34,36
		Haemanthamine	0.036	34,36
O-C^{14}-Methyl-1-C^{14}-norbelladine	Narcissus "King Alfred"	Galanthamine	0.00	34,36
		Galanthine	----	34,36
		Haemanthamine	1.00	34,36

must occur after oxidative coupling. The feeding of tritiated norpluvine (60) to "Deanna Durbin" and isolation of lycorine (11) containing 10.5% of the activity[17] indicates that the oxygenation of the C_6-C_2 unit may occur after oxidative coupling of the phenolic precursor. A probable biosynthetic sequence (Figure 16) was considered to be: norpluvine (60) ⟶ caranine (55) ⟶ lycorine (11).

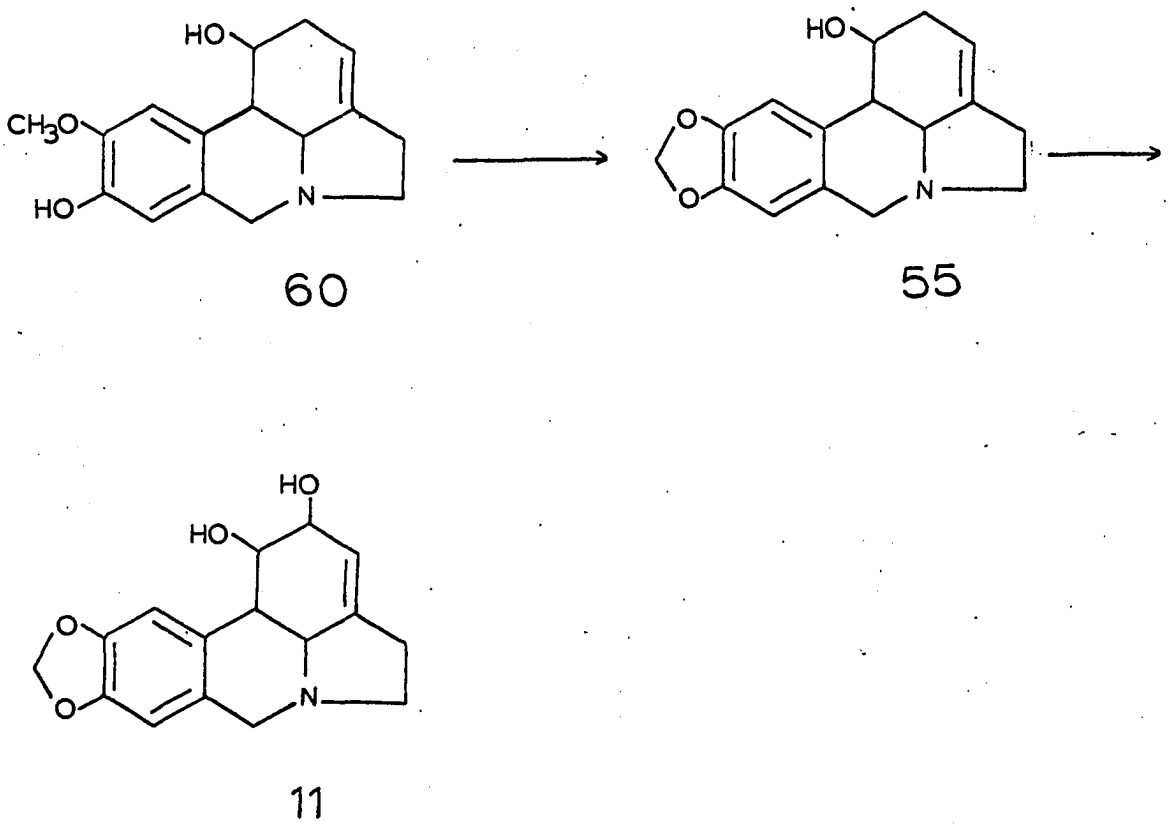


Figure 16. Biosynthetic sequence: norpluvine to lycorine

A series of feeding experiments using labelled methyl derivatives of norbelladine (54a) has been accomplished (Table 3). N-Methyl norbelladine (68) and O,N-dimethyl norbelladine (56) have been shown to be incorporated into galanthamine (26), but they were not incorporated into galanthine (63) and haemanthamine (58). However, O-methyl norbelladine (69) has been shown to be incorporated into haemanthamine but not into galanthamine. The data suggest a definite order of methylation for galanthamine (Figure 17): norbelladine (54a) ⟶ N-methyl norbelladine (68) ⟶ O,N-dimethyl norbelladine (56) ⟶ galanthamine (26). These

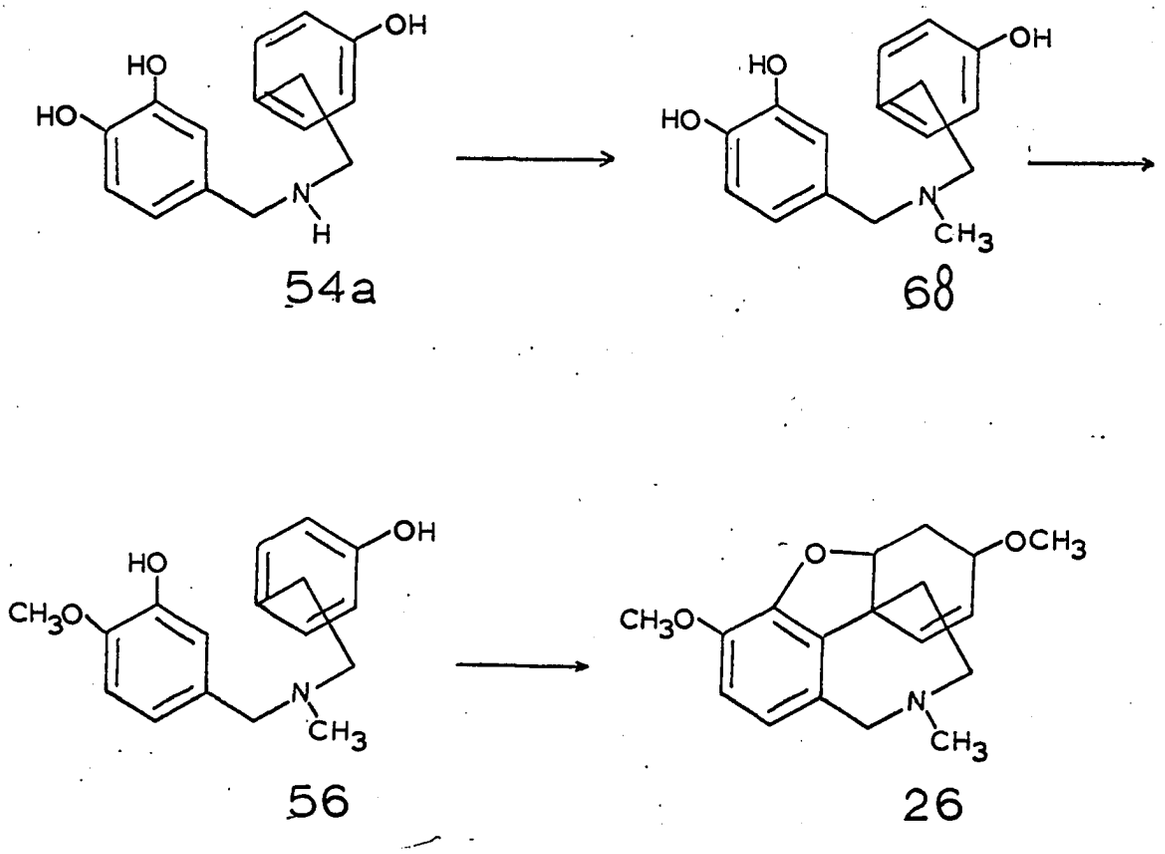
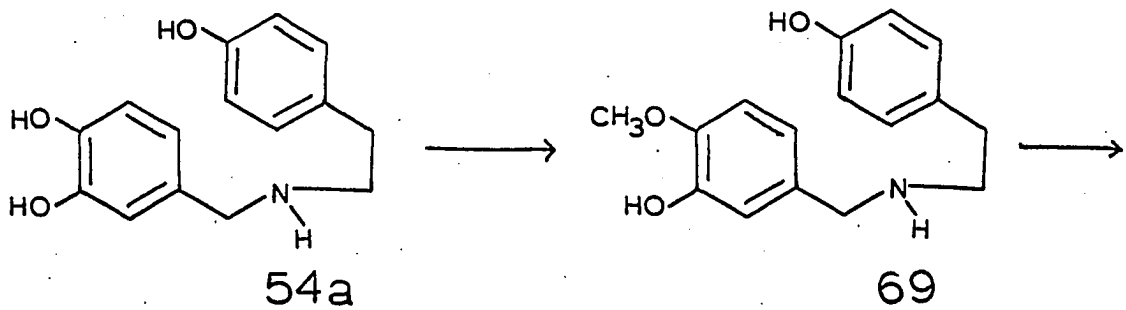


Figure 17. Order of methylation for galanthamine

data also suggest a definite order of methylation for haemanthamine (Figure 18): norbelladine (54a) ⟶ O-methyl norbelladine (69) ⟶ haemanthamine (58).



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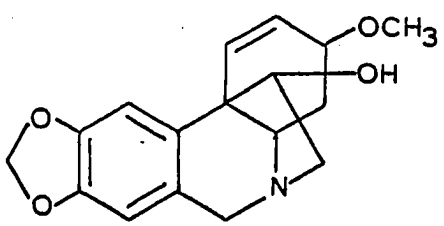

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Figure 18. Order of methylation for haemanthamine

There have been two pathways[37] proposed (Figures 19 and 20) for the incorporation of phenylalanine (59b) into the C_6-C_1 unit of the Amaryllidaceae alkaloids: (1) phenylalanine (59b) ⟶ phenylserine (70) ⟶ benzaldehyde (71) ⟶ p-hydroxybenzaldehyde (72) ⟶

3,4-di-hydroxybenzaldehyde (73) ⟶ norbelladine (54a) and (2) phenylalanine (59b) ⟶ trans-cinnamic acid (74) ⟶ p-hydroxycinnamic acid (75) ⟶ 3,4-dihydroxycinnamic acid (76) or p-hydroxybenzaldehyde (72) ⟶ 3,4-dihydroxybenzaldehyde (73) ⟶ norbelladine (54a).

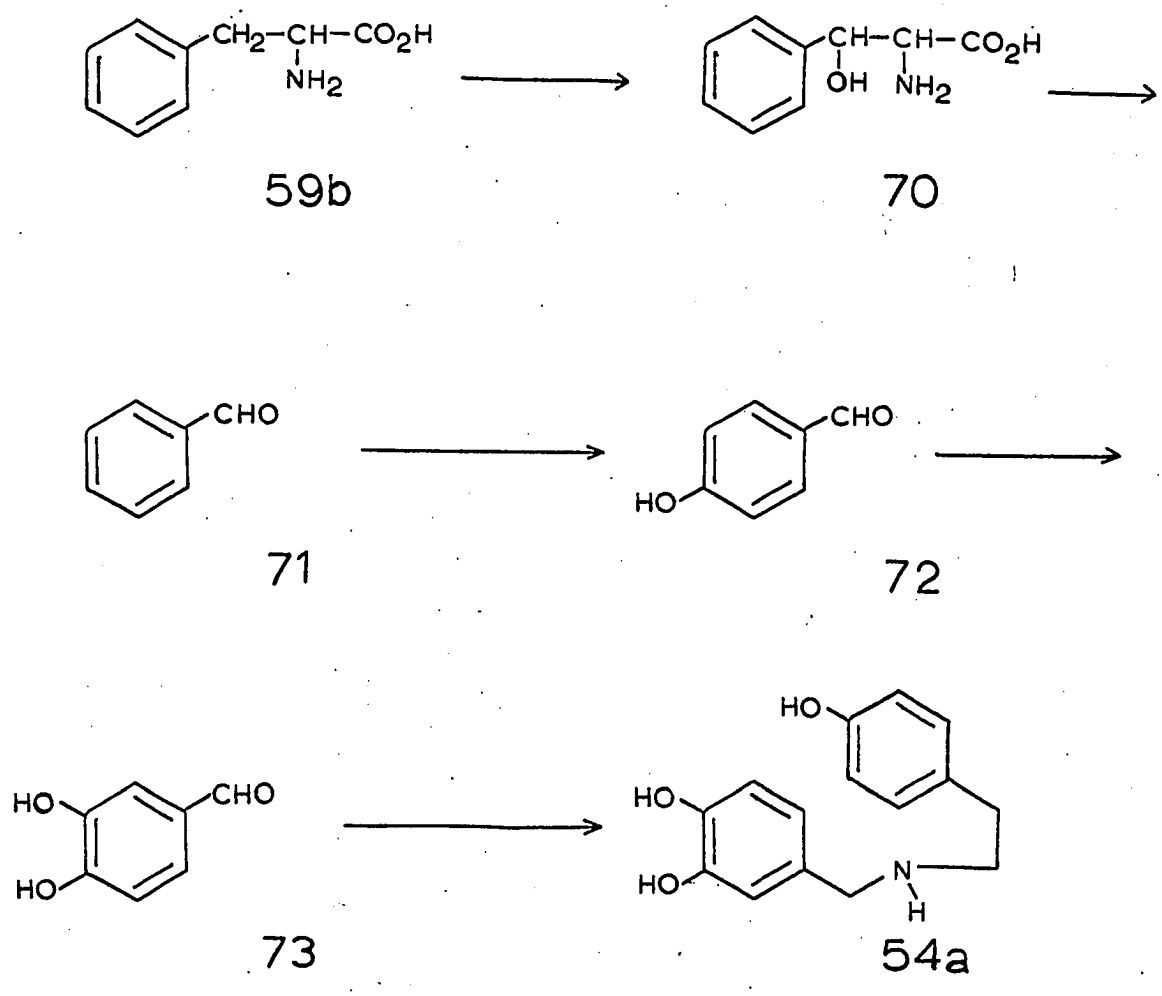


Figure 19. Incorporation of phenylalanine into C_6-C_1 unit of the Amaryllidaceae alkaloids via phenylserine

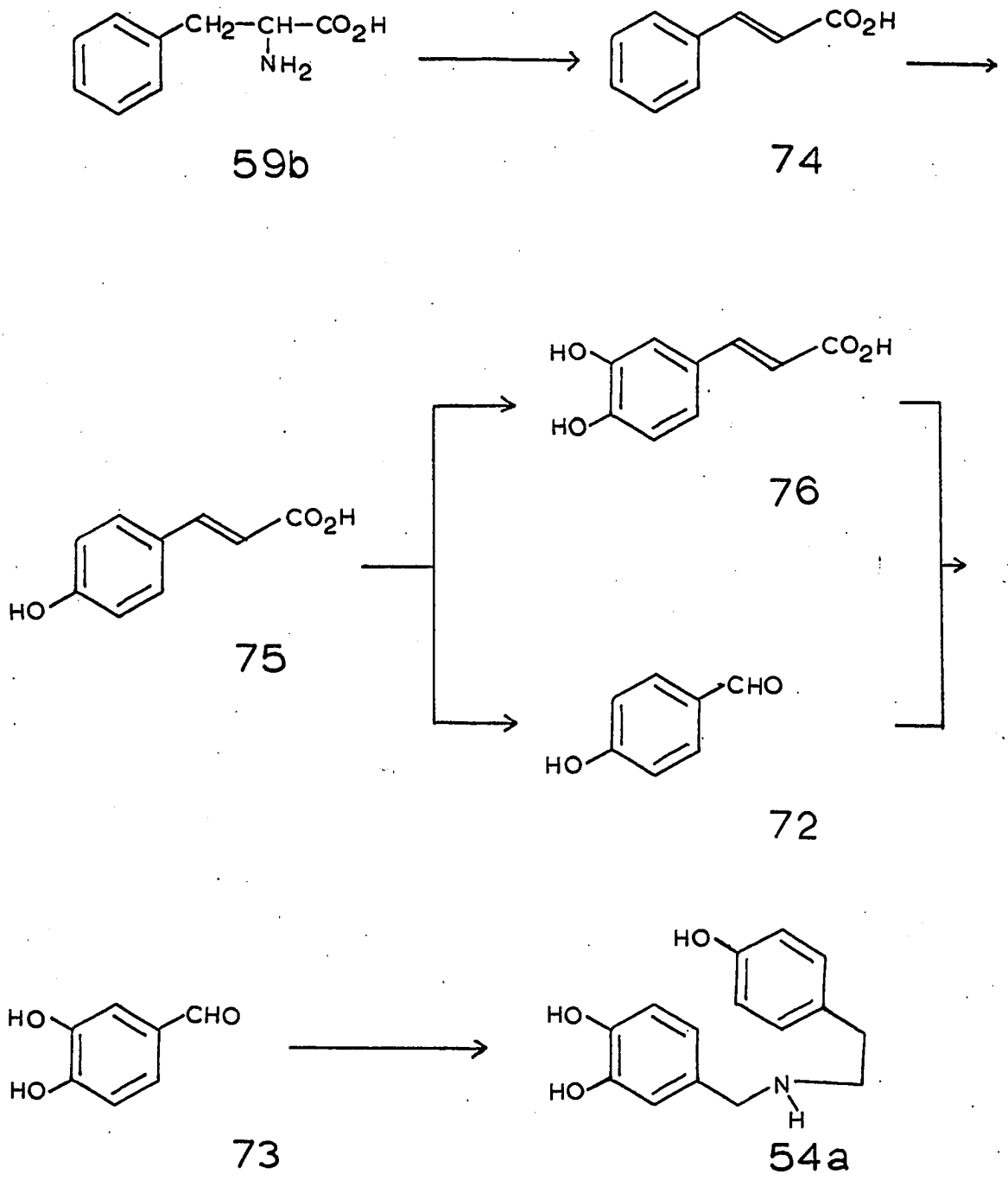


Figure 20. Incorporation of phenylalanine into C_6-C_1 unit of the Amaryllidaceae alkaloids via trans-cinnamic acid

Table 4. Incorporation of other possible C_6-C_1 unit precursors into Amaryllidaceae alkaloids

Precursor	Plant	Alkaloid	% inc.	Ref.
3-C^{14}-Phenylserine	Narcissus pseudonarcissus	Haemanthamine	----	37,38
	Narcissus incomparabilis	Lycorine	0.00	38
7-C^{14}-Benzaldehyde	Narcissus pseudonarcissus	Haemanthamine	0.00	37
	Spekelia formossisima	Tazettine	0.005	33
p-Hydroxy-7-C^{14}-benzaldehyde	Narcissus pseudonarcissus	Haemanthamine	0.00	37,38
		Lycorine	----	37,38
trans-3-C^{14}-cinnamic	Narcissus pseudonarcissus	Haemanthamine	0.35	37-39
		Lycorine	----	37,39
	Nerine bowdenii	Lycorine	0.02	33
		Belladine	----	33
p-Hydroxy-3-C^{14}-cinnamic acid	Narcissus pseudonarcissus	Haemanthamine	0.16	37-39
		Lycorine	----	37-39
3,4-Dihydroxy-3-C^{14}-cinnamic acid	Narcissus pseudonarcissus	Haemanthamine	----	23,38,40
		Lycorine	----	23,38,40
	Narcissus incomparabilis	Lycorine	0.006	38
3,4-Dihydroxy-7-C^{14}-benzaldehyde	Narcissus pseudonarcissus	Haemanthamine	----	40,41
		Lycorine	----	40,41

Negligible incorporation of phenylserine, benzaldehyde, and p-hydroxybenzaldehyde into haemanthamine (58) and tazettine (12) have been reported (Table 4). However, trans-cinnamic acid, p-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, and 3,4-dihydroxybenzaldehyde have been shown to be incorporated in good yield into various Amaryllidaceae alkaloids (Table 4). These data suggest that phenylalanine is incorporated into the C_6-C_1 unit of the Amaryllidaceae alkaloids by the phenylalanine-trans-cinnamic acid pathway (Figure 20) and not by the phenylalanine-phenylserine pathway (Figure 19).

Studies of the incorporation of tyrosine (Table 1) and tyramine (77)[42] into the Amaryllidaceae alkaloids (Table 1) suggest that tyrosine is incorporated into the alkaloids (Figure 21) by the following biosynthetic pathway: tyrosine (63a) ⟶ tyramine (77) ⟶ norbelladine (54a).

The combined biosynthetic pathway for incorporation of phenylalanine (59b) and tyrosine (59a) into norbelladine (54a) is given in Figure 22.

Other investigations have proved that the methylenedioxy group of haemanthamine (58) originates from the O-methyl group of O-methyl norbelladine (69)[34,36].

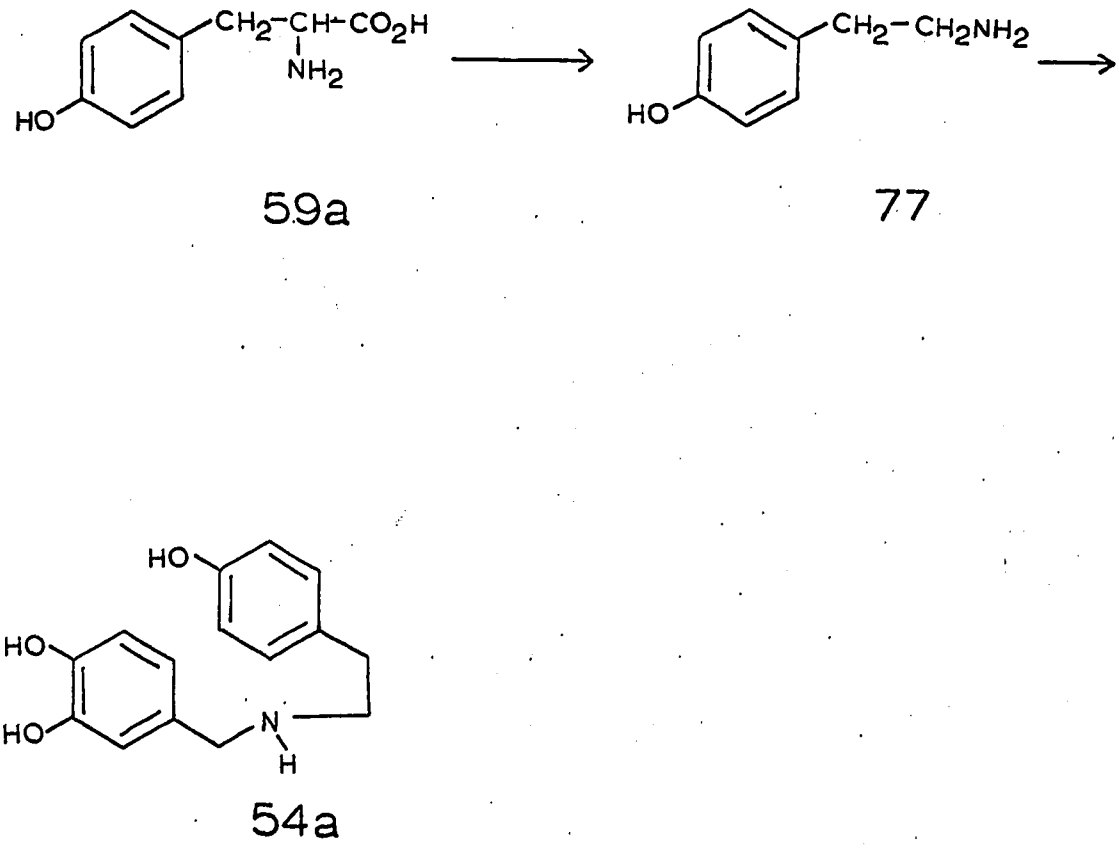


Figure 21. Incorporation of tyrosine into the C_6-C_2 unit of the Amaryllidaceae alkaloids

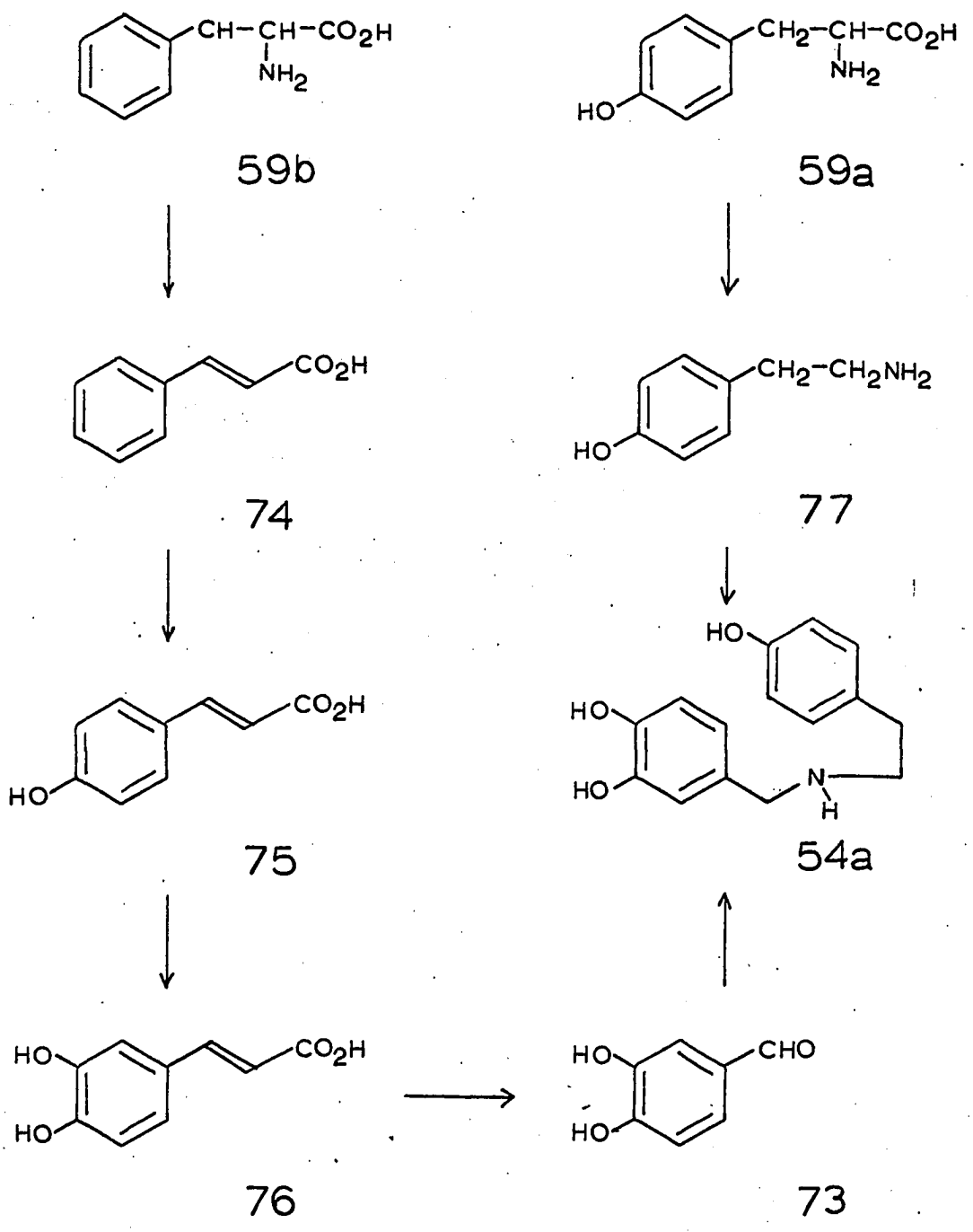
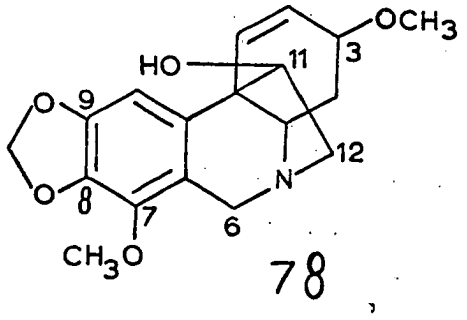


Figure 22. Incorporation of phenylalanine and tyrosine into norbelladine

RESULTS AND DISCUSSION

Ambelline

The structure of ambelline (78), a major alkaloid of Amaryllis belladonna[43] and Nerine bowdenii[44-47], was determined by Wildman in 1963 by degradative and spectroscopic methods[48]. A similar degradation was used in the present studies with radioactive material.



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All feeding experiments (Table 5) used Nerine bowdenii as the plant host and the ambelline was isolated by standard procedures[40].

Table 5. Feeding experiments with Nerine bowdenii as plant host

Feeding experiment	Total act. used	Wgt. amb. isol.	Amb. act. (dpm/mM)	% inc.
3-C^{14}-Phenylalanine	0.3 mc.	70 mg.	4.40×10^{3}	2.33×10^{-3}
3-C^{14}-Tyrosine	0.1 mc.	89 mg.	1.124×10^{4}	1.50×10^{-2}
2-C^{14}-Tyrosine		77.5 mg.	4.465×10^{4}	
1,1'-C^{14}-Norbelladine	0.355 mc.	118 mg.	7.73×10^{3}	3.87×10^{-3}
Sodium C^{14}-formate	0.316 mc.	34 mg.	1.605×10^{4}	2.64×10^{-3}

Degradation of Ambelline

The radioactive precursors fed to Nerine bowdenii cited in Table 5 could yield ambelline (78) labelled in the methoxyl and/or the methylenedioxy groups, as well as at the C_6, C_{11} and C_{12} positions. To isolate and identify each of these carbon atoms in ambelline as obtained from the various feeding experiments, degradation procedures were developed which permitted the determination of the amount of radioactivity at each position.

The total activity present in the two methoxyl groups of ambelline was determined by the Zeisel method (Figure 23). The radioactivity of the methylenedioxy group was determined by acid hydrolysis to formaldehyde[49], (Figure 24), which was isolated as the dimedone adduct (79).

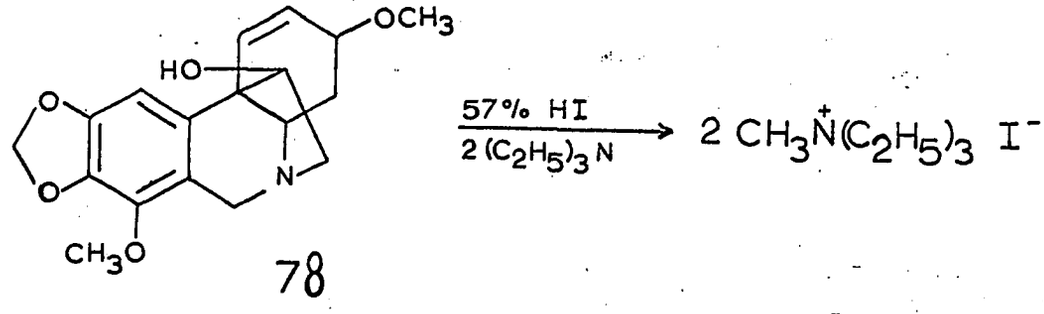

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Figure 23. Methoxyl determination

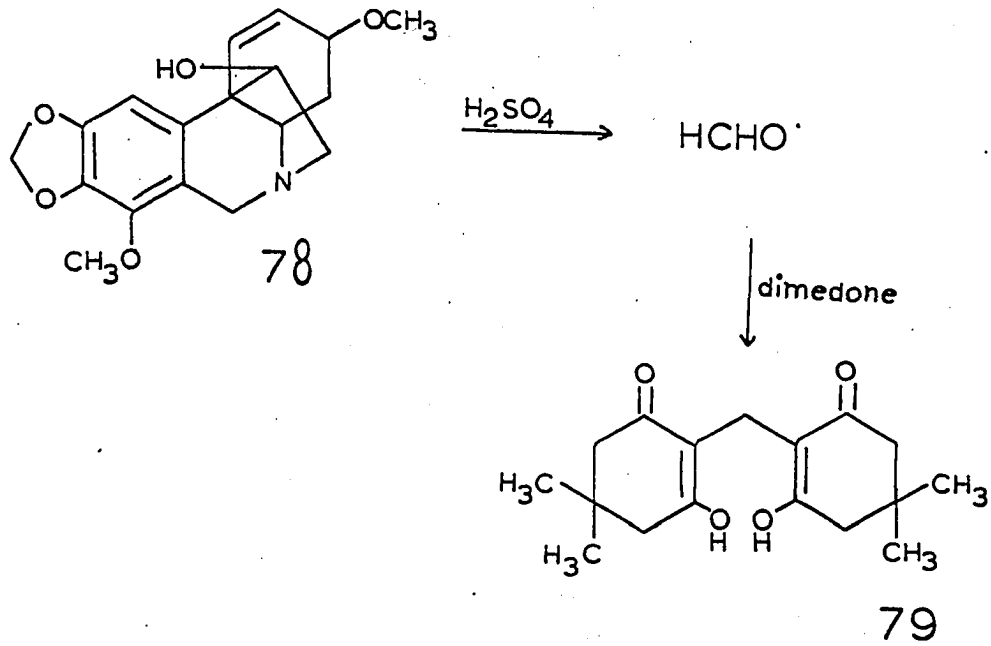

Figure 24. Methylenedioxy determination

Feedings of radioactive phenylalanine and tyrosine labelled in the aliphatic portions of these amino acids should, by biosynthetic prediction, lead to radioactive ambelline, which might be labelled at the C_6, C_{11} and/or C_{12} positions. It was imperative that a degradation scheme be devised which could isolate each of these carbon atoms specifically and in high yield. The carbon atoms C_6, C_{11} and C_{12} in ambelline are designated by a dagger, dot and asterisk in formula 78.

To determine the amount of radioactivity at the C_6, C_{11} and C_{12} positions, ambelline was converted (Figure 25) to N-(2-methoxy-6-

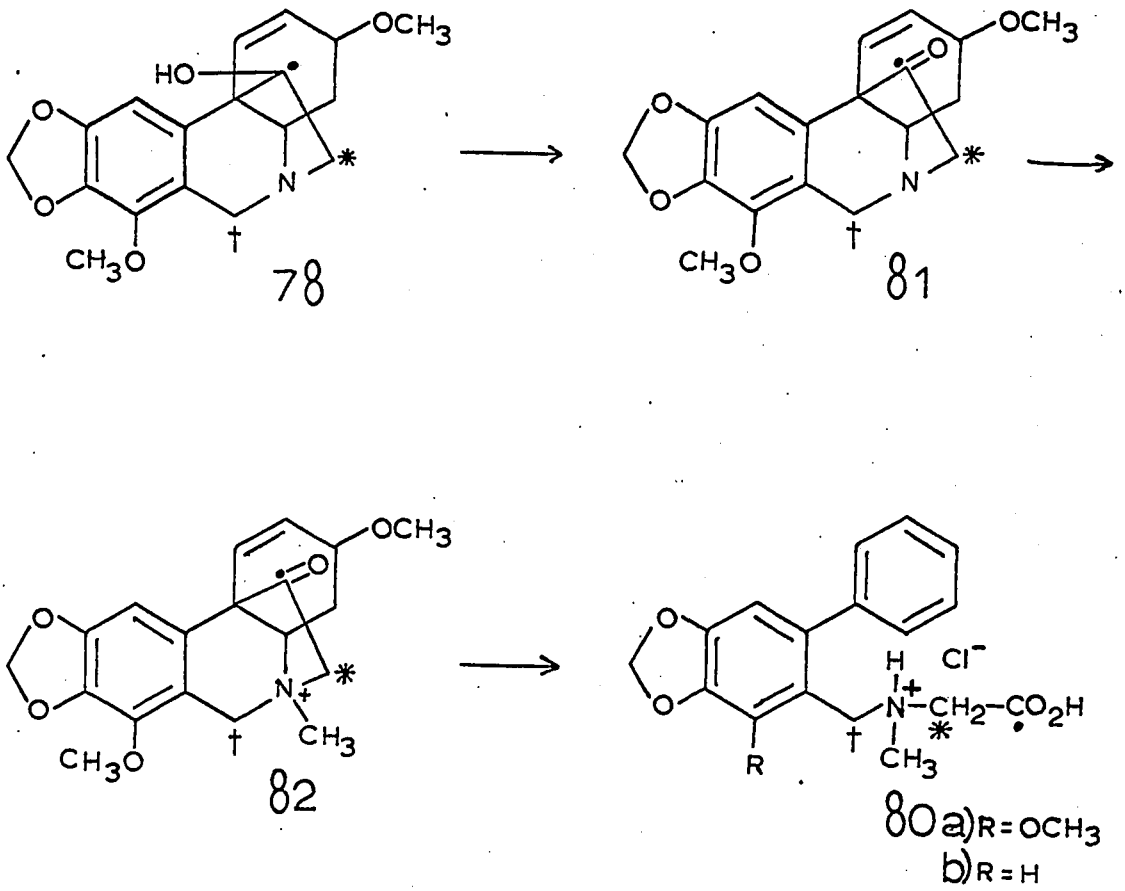

Figure 25. Degradation of ambelline

phenylpiperonyl) sarcosine hydrochloride (80a). This transformation involves the loss of the methoxyl group at the C_3 position of ambelline. There should be no loss of activity in 80a relative to ambelline which was obtained from a 3-C^{14}-phenylalanine, 2-C^{14}- or 3-C^{14}-tyrosine feeding experiment, due to the removal of the C_3 methoxyl group. Oxidation of

ambelline with chromium trioxide in pyridine gave oxoambelline (81), which was quaternized with methyl iodide to give oxoambelline methiodide (82). Treatment of 84a with sodium hydroxide gave oxoambelline methine which was isolated as the hydrochloride (80a). From the hypothetical labelling pattern assigned to 78, corresponding activity is shown in 80a, as would be expected.

No single degradative procedure allowed the simultaneous determination of the radioactivity in the two carbon atoms of the acetic acid group of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride. Although the Kolbe electrolysis was successful for the determination of radioactivity in the methylene and carboxyl groups of 80b[16], the electrolysis proved to be unsatisfactory for the determination for the non-benzlic methylene carbon of 80a. If radioactivity was expected in the carboxylic acid group of 80a, it was most expedient to degrade by procedure A (Figure 26). This procedure could also be used to determine the activity present at the benzylic position of 80a. However, if the activity was present in the methylene group indicated by the asterisk, satisfactory results could be obtained only by use of a second procedure (B), (Figure 26).

In procedure A, hydrogenolysis of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride (80a) gave 3-methoxy-2-methyl-4,5-methylenedioxybiphenyl (83) and sarcosine (84), which was isolated as N-tosylsarcosine (85) by reaction with p-toluene sulfonyl chloride. Kolbe electrolytic oxidation of 85 gave N-methyl-p-toluenesulfonamide (86), carbon dioxide and formaldehyde, the latter being isolated as the

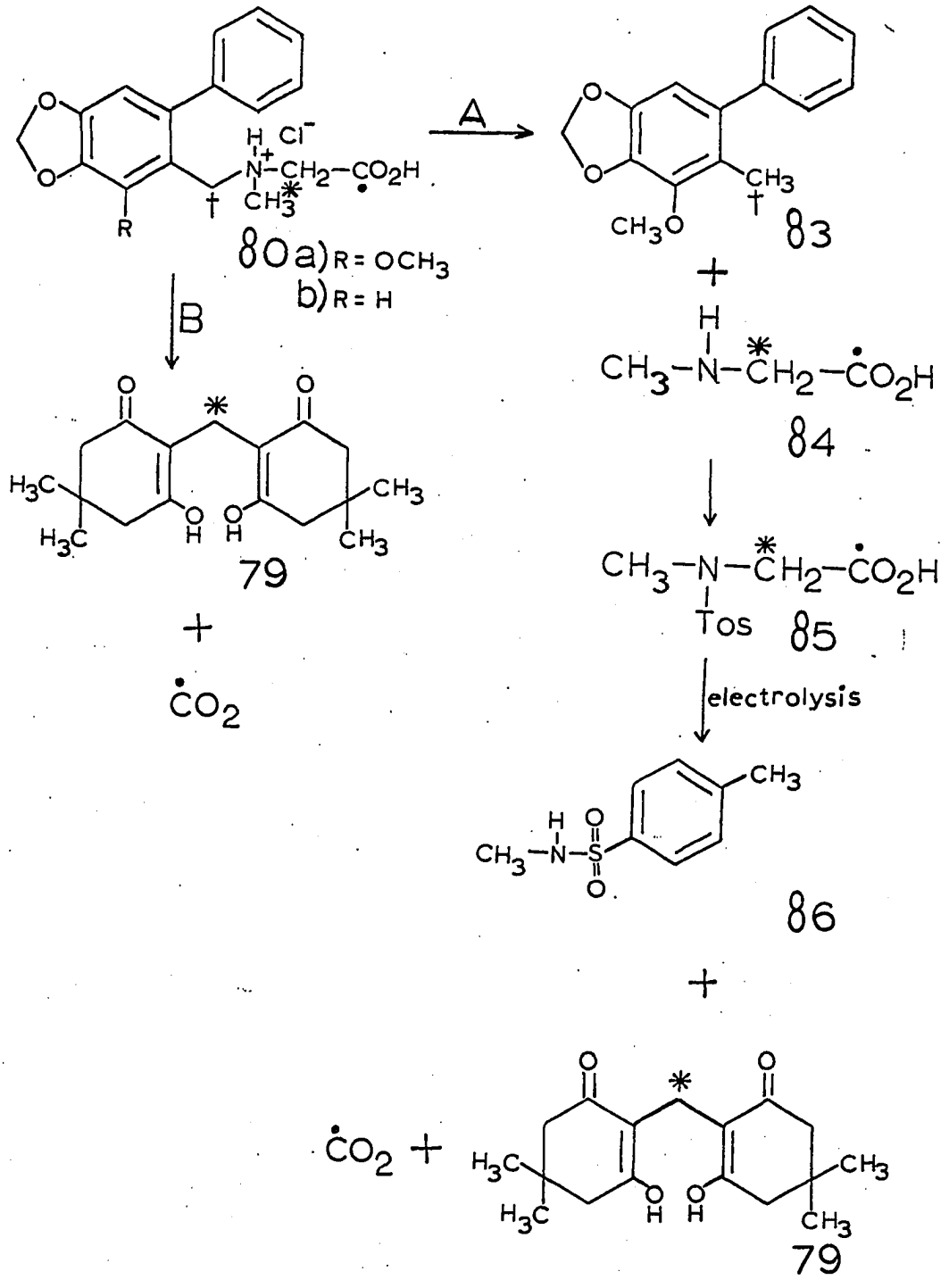

Figure 26. Degradation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride by procedures A and B

dimedone adduct (79). The quantities of radioactive ambelline degraded did not permit the isolation of 86. If the N-tosylsarcosine was labelled in the carboxylate carbon, the carbon dioxide liberated should contain all of the radioactivity. This result was supported by the model degradation of N-tosyl-1-C^{14}-sarcosine (Table 6) synthesized from 1-C^{14}-glycine (87). If 85 was labelled in the methylene group, the carbon

Table 6. Relative activities* of N-tosylsarcosine and degradation products

Compound	Oxidation of N-tosyl-1-C^{14}-sarcosine in ethanol	Oxidation of N-tosyl-2-C^{14}-sarcosine in ethanol	Oxidation of N-tosyl-2-C^{14}-sarcosine in water
N-Tosylsarcosine	1.00	1.00	1.00
Carbon dioxide	0.88	0.00	0.00
Formaldehyde dimedone adduct	0.00	0.34	0.60

*Relative activity is the specific radioactivity in dpm/mM of a compound compared to the specific radioactivity of the parent compound, which is given the value of 1.00 for convenience.

$H_2N-CH_2-CO_2H$

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dioxide contained no radioactivity and the formaldehyde isolated as the dimedone adduct (79) would be labelled. However, in actual experiments the formaldehyde dimedone adduct had less than 70% of the expected radioactivity based on the level of the N-tosylsarcosine. This low relative activity for the formaldehyde dimedone adduct has been attributed to the formation of side products in the electrolytic oxidation of the N-tosylsarcosine[16]. This assumption was supported by electrolytic degration of N-tosyl2-C^{14}-sarcosine (Table 6) prepared from 2-C^{14}-glycine, where model experiments showed that only 60% of the radioactivity of the methylene carbon was recovered as the dimedone adduct. These results necessitated the development of a second procedure (B) which permitted the determination of the activity at the C_{12} position of ambelline. The lead tetraacetate oxidation of amino acids appeared to be a promising alternative.

Wieland and Bergel[50] first reported the catalytic oxidative decarboxylation of amino acids with oxygen to give carbon dioxide, an aldehyde and ammonia. Spenser[51] has shown that oxidation of α-β-H_2^3-valine (88) and α-β-H_2^3-phenylalanine (59b) with sodium hypochlorite, hydrogen peroxide, chloramine-T, ninhydrin and alloxan takes place without loss of tritium to give 1,2-H_2^3-isobutraldehyde (89) and 1,2-H_2^3-phenylacetaldehyde (90), respectively. Spenser proposed that the reaction (Figure 27) involved a carbinolamine intermediate (91) which rapidly decomposes to an aldehyde and ammonia. Support for a carbinolamine intermediate was found in the oxidation of α-N-diphenylglycine (92) with potassium

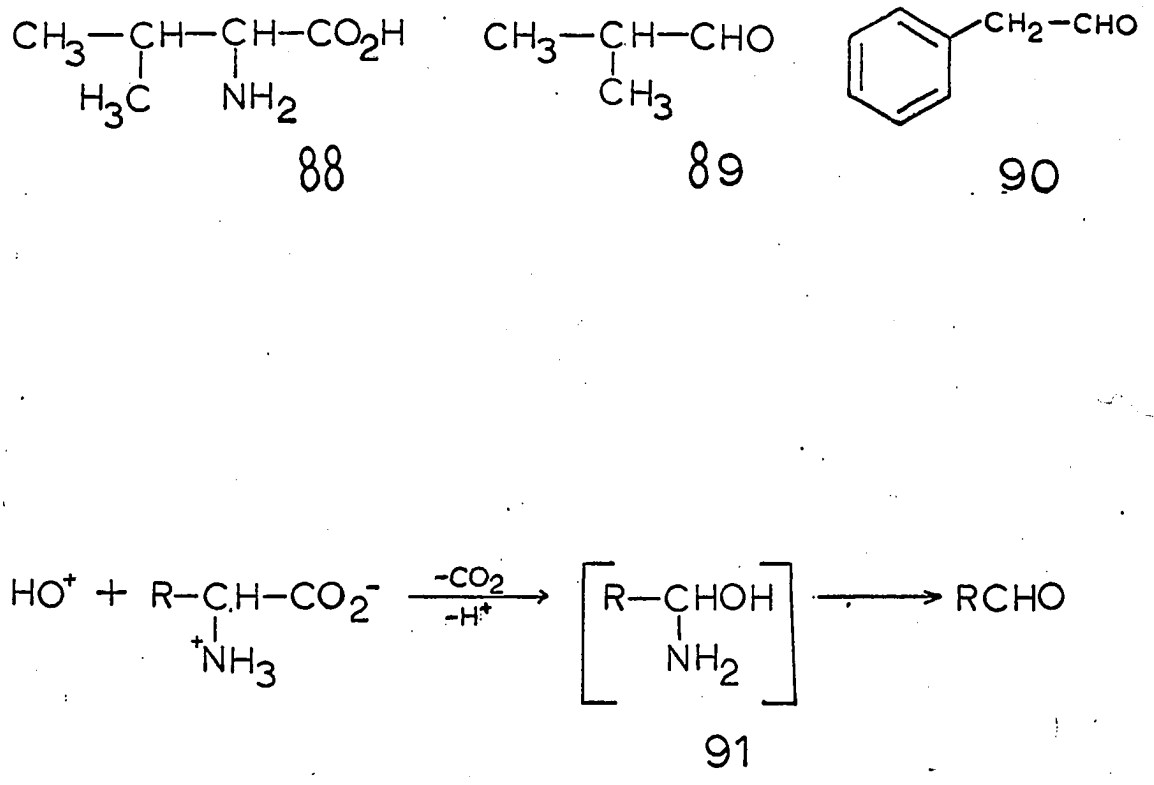

Figure 27. Mechanism of oxidative decarboxylation of amino acids

ferricyanide (Figure 28) to give the isolable Schiff base, benzalaniline (93). Presumably the carbinolamine (94) was formed and underwent rapid elimination.

In procedure B, lead tetraacetate oxidation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride (80a), labelled at the position indicated by the asterisk gave inactive carbon dioxide. The radioactivity of the formaldehyde which was isolated as the dimedone adduct (79) was less than the predicted value. This result suggested that part of the formaldehyde collected as the dimedone adduct was a decomposition

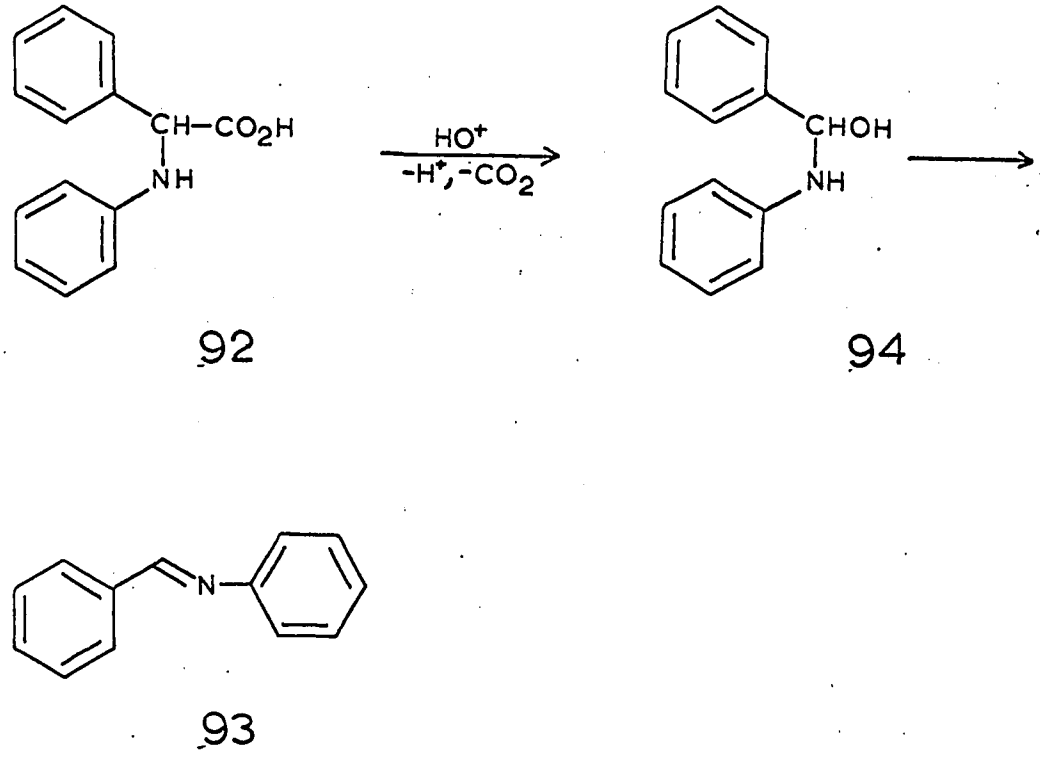

Figure 28. Mechanism of Schiff base formation in oxidative decarboxylation of amino acids

product of the lead tetraacetate-glacial acetic acid solution. This interpretation was found to be correct. Heating the lead tetraacetate-glacial acetic acid solution in the absence of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride afforded formaldehyde which was isolated as the dimedone adduct. In a second experiment lead tetraacetate solution was heated in the absence of 80a and the evolved gases were collected in water. The water solution was shown to contain formaldehyde by the method of Bricker and Vail[52].

A thorough investigation of the amount of formaldehyde produced by 10.0 ml. of 0.67% lead tetraacetate-glacial acetic acid solution at

various reaction times was undertaken (Table 7). The amount of formaldehyde obtained, within experimental error, was directly proportional to the length of time of heating and the age of the solution.

Table 7. Formaldehyde produced by heating 0.67% lead tetraacetate-glacial acetic acid solution

Trial no.	Age of solution	Length of time solution heated	Wgt. of dimedone adduct
1	16 hrs.	2 hrs. 30 min.	---
	16 hrs.	3 hrs. 00 min.	0.26 mg.
	16 hrs.	3 hrs. 45 min.	0.16 mg.
2	2 days 14 hrs. 10 min.	2 hrs. 30 min.	0.20 mg.
	2 days 14 hrs. 10 min.	3 hrs. 15 min.	0.16 mg.
	2 days 14 hrs. 10 min.	4 hrs. 05 min.	0.29 mg.
3	6 days 15 hrs. 40 min.	2 hrs. 30 min.	0.27 mg.
	6 days 15 hrs. 40 min.	3 hrs. 20 min.	0.30 mg.
	6 days 15 hrs. 40 min.	3 hrs. 50 min.	0.33 mg.
4	10 days 16 hrs. 30 min.	2 hrs. 40 min.	0.31 mg.
	10 days 16 hrs. 30 min.	3 hrs. 00 min.	0.53 mg.
	10 days 16 hrs. 30 min.	4 hrs. 05 min.	0.59 mg.
5	24 days 4 hrs. 20 min.	2 hrs. 35 min.	0.75 mg.
	24 days 4 hrs. 20 min.	3 hrs. 15 min.	1.18 mg.
	24 days 4 hrs. 20 min.	3 hrs. 50 min.	1.28 mg.
6	30 days 19 hrs. 30 min.	2 hrs. 30 min.	2.60 mg.
	30 days 19 hrs. 30 min.	3 hrs. 10 min.	5.55 mg.
	30 days 19 hrs. 30 min.	3 hrs. 55 min.	4.99 mg.

A corrected specific activity for the radioactive formaldehyde dimedone adduct (A_c) could be calculated by the following equation:

$$\frac{A_i}{\frac{W_1 - W_2}{W_1}} = A_c$$

where A_i and W_1 are the specific radioactivity and weight, respectively, of the diluted dimedone adduct, and W_2 is the amount of formaldehyde dimedone adduct found to be produced in a blank experiment using 10.0 ml. of 0.67% lead tetraacetate-glacial acetic acid solution.

Ambelline Isolated From a 3-C^{14}-Phenylalanine Feeding Experiment

Previous biosynthetic studies have shown phenylalanine (59b) to be a precursor of the C_6-C_1 unit of the Amaryllidaceae alkaloids (Table 1) having two oxygen substituents on the aromatic ring. If 3-C^{14}-phenylalanine is a precursor of the C_6-C_1 unit of ambelline (78) which is trioxygenated in the aromatic ring, the radioactive label would be expected to occur at the benzylic (C_6) position. Ambelline isolated from the 3-C^{14}-phenylalanine feeding experiment (Table 5) was degraded by procedure A (Figure 26) and shown to contain all its radioactivity at the C_6 position within experimental error (Table 8).

The low specific activity (4,400 dpm/mM) of the ambelline required complete radiochemical purity. The ambelline purity was checked by conversion to the hydrochloride which had the same specific activity as the original ambelline.

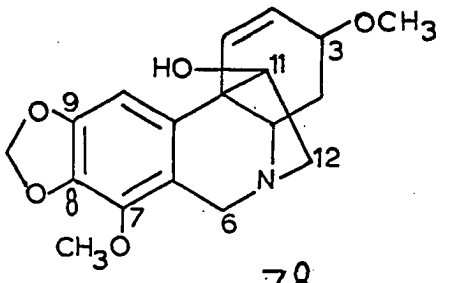

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Table 8. Degradation of ambelline isolated from the 3-C^{14}-phenylalanine feeding experiment

Compound	Yield	Amount used next rctn.	Rel. act.
Ambelline		650 mg.	1.00
Ambelline hydrochloride	---	---	1.04
Oxoambelline	229 mg.	229 mg.	----
Oxoambelline methiodide	220 mg.	200 mg.	1.05
N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride	164 mg.	164 mg.	----
N-Tosylsarcosine	38 mg.		0.04
3-Methoxy-2-methyl-4,5-methylenedioxybiphenyl	61 mg.		0.90

No further degradation was attempted to isolate the label in the methyl group of 3-methoxy-2-methyl-4,5-methylenedioxybiphenyl (83) because any migration of the C_3 atom in phenylalanine into the aromatic ring would be highly improbable.

These data prove that phenylalanine serves as a precursor of the tri-oxygenated aromatic C_6-C_1 unit of ambelline.

Ambelline Isolated From the 3-C^{14}-Tyrosine Feeding Experiment

Previous biosynthetic studies have shown tyrosine (59a) to be a precursor of the hydroaromatic C_6-C_2 unit of the Amaryllidaceae alkaloids (Table 1). If 3-C^{14}-tyrosine is a precursor of the C_6-C_2 unit of ambelline (78) the radioactive label would be expected to occur at the C_{11} position. The ambelline isolated from the 3-C^{14}-tyrosine feeding experiment (Table 5) was degraded by procedure A (Figure 26) and shown to contain all of its radioactivity at position C_{11} (Table 9).

Table 9. Degradation of ambelline isolated from the 3-C^{14}-tyrosine feeding experiment, procedure A

Compound	Yield	Amount used next rctn.	Rel. act.
Ambelline		650 mg.	1.00
Oxoambelline	450 mg.	450 mg.	----
Oxoambelline methiodide	350 mg.	350 mg.	0.99
N-(2-Methoxy-6-phenylpiperonyl) sarcosine hydrochloride	231 mg.	200 mg.	1.00
3-Methoxy-2-methyl-4,5-methylenedioxybiphenyl	82 mg.		0.00
N-Tosylsarcosine	99 mg.	19.75 mg.	1.02
Carbon dioxide	----		0.95
Formaldehyde dimedone adduct	5 mg.		0.00

Ambelline Isolated From a 2-C^{14}-Tyrosine Feeding Experiment

Since feeding 3-C^{14}-tyrosine to Nerine bowdenii was shown to give radioactive ambelline labelled at C_{11}, it would be expected that 2-C^{14}-tyrosine would give rise to ambelline labelled at C_{12}. This was found to be the case.

The ambelline obtained from the 2-C^{14}-tyrosine feeding experiment was degraded twice by procedure A (Table 10). In each degradation the N-tosylsarcosine gave non-radioactive carbon dioxide. The formaldehyde dimedone adduct had a relative specific activity considerably less than acceptable (Table 10, trial 1). Two degradations of ambelline by procedure B (Table 11) gave formaldehyde dimedone adduct with relative specific activities of 0.62 and 0.72 without corrections for impurities. A third degradation, corrected for the extraneous formaldehyde produced according to the method prescribed in procedure B, provided the relative activity (0.97) for the formaldehyde dimedone adduct (Table 12).

Ambelline Isolated From the 1,1'-C^{14}-Norbelladine Feeding Experiment

Previous biosynthetic studies (Table 2) have shown norbelladine (54a) to be incorporated intact into the lycorine, galanthamine, crinine, belladine and tazettine types of Amaryllidaceae alkaloids. The norbelladine used in this feeding experiment had 78.2% of its total radioactivity at the C_1 position and 21.8% of its total radioactivity at the $C_{1'}$ position. If the norbelladine was incorporated intact into

Table 10. Degradation of ambelline isolated from the 2-C^{14}-tyrosine feeding experiment, procedure A

Compound	Trial 1			Trial 2		
	Yield	Amount used next rxtn.	Rel. act.	Yield	Amount used next rxtn.	Rel. act.
Ambelline		824 mg.	1.00		1.3 g.	1.00
Oxoambelline	692 mg.	692 mg.	----	990 mg.	990 mg.	----
Oxoambelline methiodide	350 mg.	350 mg.	1.04	837 mg.	837 mg.	----
N-(2-Methoxy-6-phenylpiperonyl) sarcosine hydrochloride	185 mg.	180 mg.	0.94	435 mg.	200 mg.	0.98
3-Methoxy-2-methyl-4,5-methylenedioxybiphenyl	139 mg.		0.00	32 mg.		0.00
N-Tosylsarcosine	41 mg.	16 mg.	1.08	26 mg.		1.01
Carbon dioxide	----		0.00			
Formaldehyde dimedone adduct	3 mg.		0.46			

Table 11. Degradation of ambelline isolated from the 2-C^{14}-tyrosine feeding experiment, Procedure B

Compound	Trial 1			Trial 2		
	Yield	Amount used next rxtn.	Rel. act.	Yield	Amount used next rxtn.	Rel. act.
Ambelline		750 mg.	1.00		750 mg.	1.00
Oxoambelline	472 mg.	472 mg.	----	472 mg.	472 mg.	----
Oxoambelline methiodide	466 mg.	466 mg.	----	466 mg.	466 mg.	----
N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride	250 mg.	14 mg.	1.06	250 mg.	14 mg.	1.06
Carbon dioxide			0.00	----		0.00
Formaldehyde dimedone adduct	6 mg.		0.72	6 mg.		0.62

Table 12. Corrected values of the relative activities of the formaldehyde dimedone adduct obtained by procedure B

Source of rad. amb.	Amount of HCHO dimedone add.	Amount of HCHO from $Pb(OAc)_4$ ox.	Rel. act.	Cor. rel. act.
2-C^{14}-Tyrosine feeding experiment	5.55 mg.	1.81 mg.	0.66	0.97
1,1'-C^{14}-Norbelladine feeding experiment	4.64 mg.	1.45 mg.	0.53	0.78

ambelline (78) as predicted by the Barton-Cohen hypothesis, the C_{12} position should have 78.2% and position C_6 should have 21.8% of the total radioactivity of the ambelline (Figure 29).

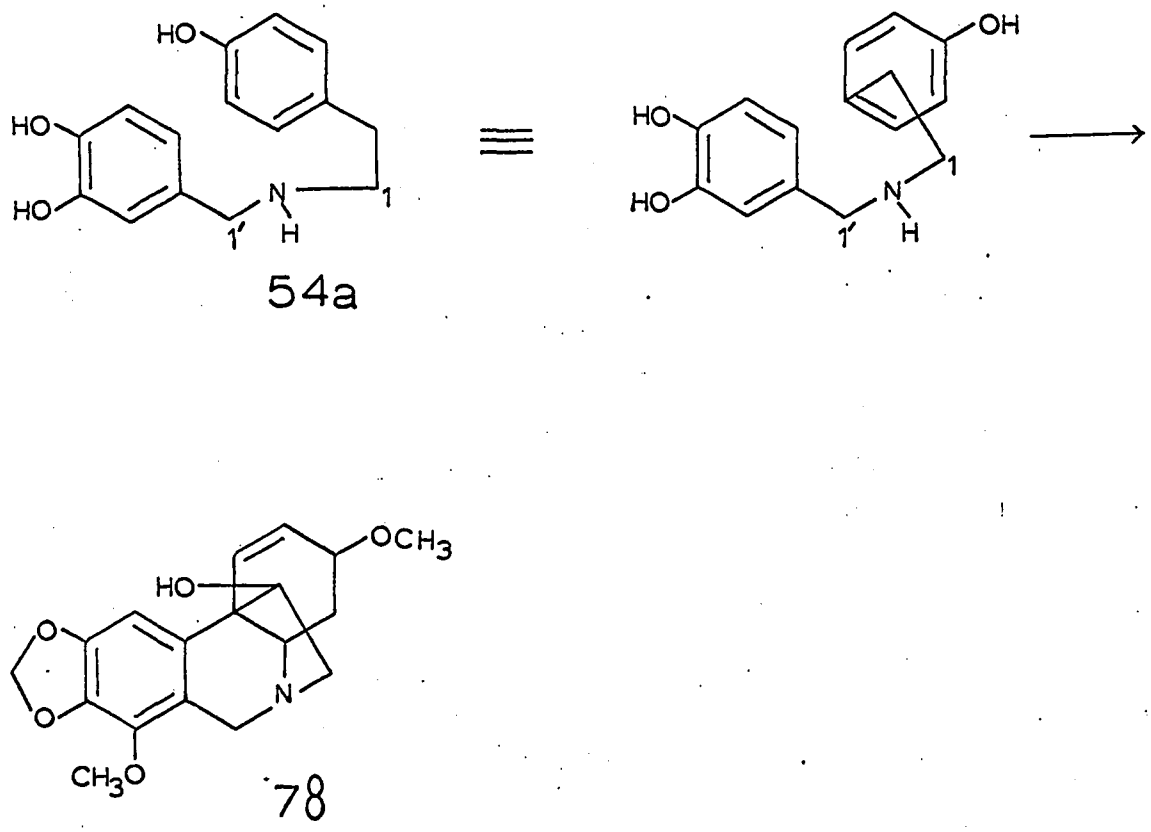

Figure 29. Incorporation of norbelladine into ambelline

The relative activities at the C_{12} and C_6 positions were determined by degradation of ambelline by procedures A (Table 13) and B (Table 14). Degradation of the ambelline by procedure A gave 3-methoxy-2-methyl-4,5-methylenedioxybiphenyl having 20% of the total activity and N-tosyl-2-C^{14}-sarcosine containing 71% of the total activity, which is in good

Table 13. Degradation of ambelline isolated from the 1,1'-C^{14}-norbelladine feeding experiment, procedure A

Compound	Yield	Amount used next rctn.	Rel. act.
Ambelline		836 mg.	1.00
Oxoambelline	573 mg.	573 mg.	----
Oxoambelline methiodide	355 mg.	350 mg.	1.00
N-(2-Methyox-6-phenylpiperonyl) sarcosine hydrochloride	158 mg.	150 mg.	0.93
3-Methoxy-2-methy-4,5-methylenedioxybiphenyl	16 mg.		0.20
N-Tosylsarcosine	26 mg.		0.71

Table 14. Degradation of ambelline isolated from the 1,1'-C^{14}-norbelladine feeding experiment, procedure B

Compound	Yield	Amount used next rctn.	Rel. act.
Ambelline		1.067 g.	1.00
Oxoambelline	660 mg.	660 mg.	----
Oxoambelline methiodide	700 mg.	700 mg.	----
N-(2-Metoxy-6-phenylpiperonyl) sarcosine hydrochloride		14 mg.	1.00
Formaldehyde dimedone adduct	1.36 mg.		0.53
Carbon dioxide	----		

agreement with the theory. The N-tosylsarcosine obtained from degradation of ambelline by procedure A was not subjected to electrolytic oxidation. Lead tetraacetate oxidation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride (Table 14) gave formaldehyde dimedone adduct having a relative specific activity of 0.53, which could be corrected to 0.78 (Table 12). These data prove that norbelladine is incorporated intact into ambelline.

Ambelline Isolated From the Sodium C^{14}-Formate Feeding Experiment to Nerine bowdenii

There is ample evidence that formate serves as a precursor of one-carbon units such as N-methyl, methoxyl and methylenedioxy groups in natural products[53-64]. Thus the ambelline (78) isolated from sodium C^{14}-formate feeding experiments would be expected to be labelled primarily in the methoxyl and methylenedioxy groups.

As a routine experiment, sodium C^{14}-formate was fed to Nerine bowdenii at the same time the feeding experiments with 3-C^{14}-phenylalanine, 3-C^{14}-tyrosine and 1,1'-C^{14}-norbelladine were carried out. The degradation of belladine (54b) isolated from the formate feeding experiment showed extremely peculiar isotopic incorporation. Radioactivity was found to the extent of 27% in the methoxyl and N-methyl groups. However, 73% remained unaccounted for and must be present in the C_6-C_1 and C_6-C_2 units. Degradation of the belladine showed that all remaining radioactivity unaccounted for was at the benzylic position (H. Ishii, Chemistry Department, Iowa State University, Ames, Iowa,

Private Communication, 1965). Although belladine was the major alkaloid isolated from Nerine bowdenii in the sodium C^{14}-formate feeding experiment, it was possible to obtain ambelline at a very low level of activity (1.605×10^4 dpm/mM). Because of the unusual results obtained in the degradation of belladine, it was deemed worthwhile to examine, within the limits of experimental validity, the pattern of incorporation present in ambelline. The pattern of isotopic incorporation in ambelline was in good agreement with that found for belladine.

One-carbon units in ambelline would consist of the methylenedioxy at C_9-C_{10} and methoxyls at the C_3 and C_7 positions. Specific degradations for these groups showed that 57% of the total radioactivity of the ambelline was present in these one-carbon groups (Table 15). The methylenedioxy group contained approximately 3% of the radioactivity, while a Zeisel determination showed that the C_3 and C_7 methoxyl groups contained approximately 54% of the total radioactivity. Since Hofmann degradation of ambelline to 80a occurred with loss of 47% of the total radioactivity, the C_3 methoxyl group contains 47% of the total one-carbon radioactivity and, by difference, C_7 contains about 7%.

Table 15. Per cent of the total activity of the ambelline in the methylenedioxy, the C_3 and the C_7 methoxyl groups

One-carbon units	% act. of amb.
Methylenedioxy group	3.
C_3 Methoxyl group	47.
C_7 Methoxyl group	7.
Total of three groups	57.

Since degradation of the ambelline by procedure B (Table 16) showed no radioactivity at either the C_{11} or C_{12} positions, the remaining radioactivity (43%) in the ambelline must be in the C_6-C_1 unit or in the hydroaromatic ring of the C_6-C_2 unit. Because of the results obtained from belladine, it would be expected that a one-carbon unit would not be incorporated into the hydroaromatic ring of the C_6-C_2 unit or the aromatic portion of the C_6-C_1 unit of ambelline, and it is presumed that the remaining activity is at the benzylic carbon. Unfortunately, insufficient material was available to carry the degradation further.

Table 16. Degradation of ambelline isolated from Nerine bowdenii in the sodium C^{14}-formate feeding experiment, procedure B

Compound	Yield	Amount used next rctn.	Rel. act.
Ambelline		160 mg.	1.00
Oxoambelline	85 mg.	57 mg.	----
Oxoambelline methiodide	27 mg.	25 mg.	1.01
N-(2-Methoxy-6-phenylpiperonyl) sarcosine hydrochloride	--	8 mg.	0.53
Formaldehyde dimedone adduct	1.62 mg.		0.00
Carbon dioxide	---		0.00

Radioactive formate could be incorporated into the C_6 position of ambelline (78) and the $C_{1'}$ position of belladine (54b) by the following biosynthetic pathway (Figure 30).

The formation of serine in Zea mays, wheat, barley, and tobacco from sodium formate and glycine (87) is well established[65-77].

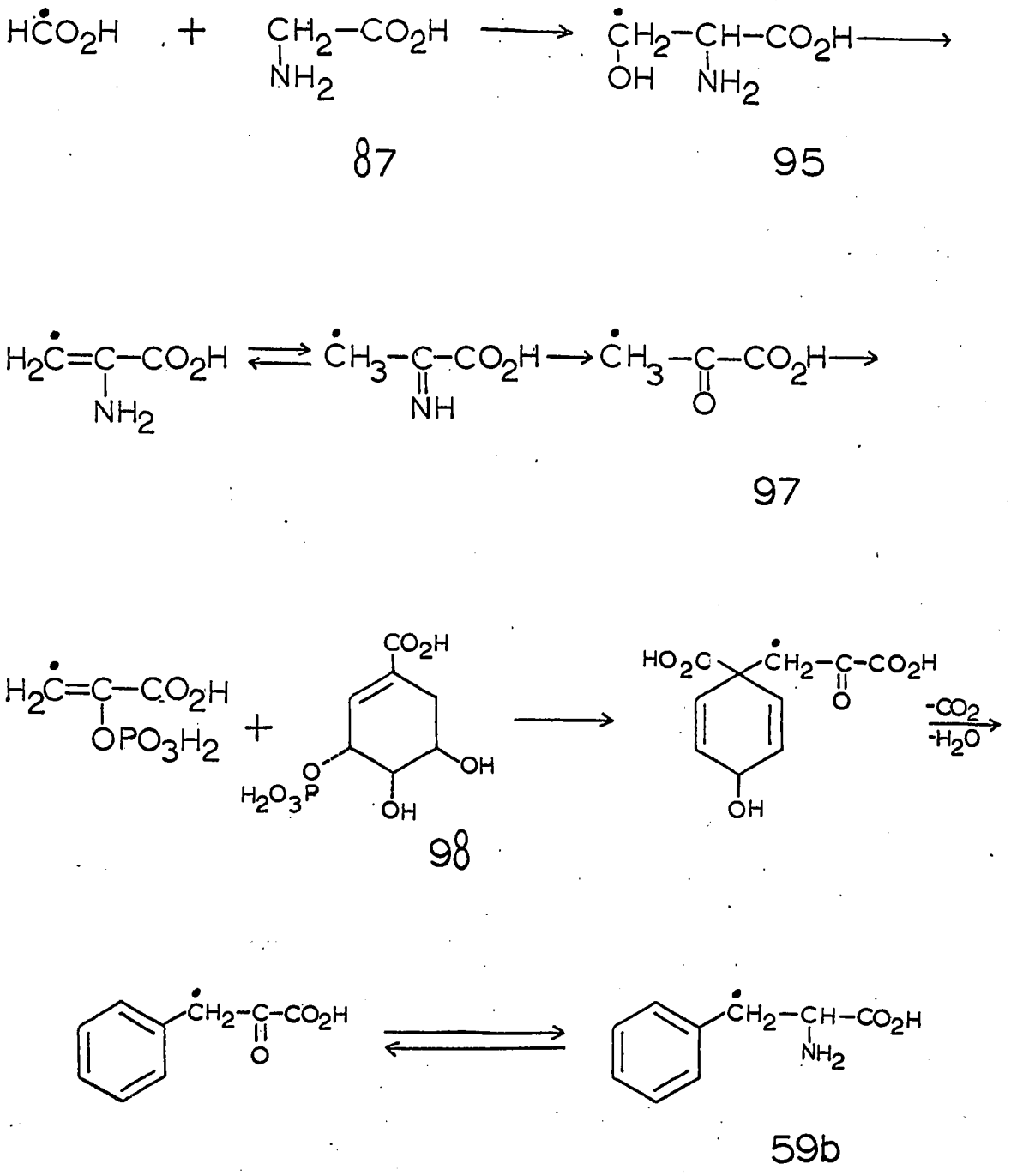

Figure 30. Proposed biosynthesis of phenylalanine

Zelinková and Sorm[73] proposed that the α-carbon of glycine serves as a possible precursor of the C_3 position of serine (95). Sinhad and Cossins[74] have shown that the C_3 position of serine does have varying amounts of radioactivity from 2-C^{14}-glycine feeding experiments. They proposed that serine could be formed by oxidation of the glycine to glyoxalate (96), which reacted with formate to give serine (Figure 31).

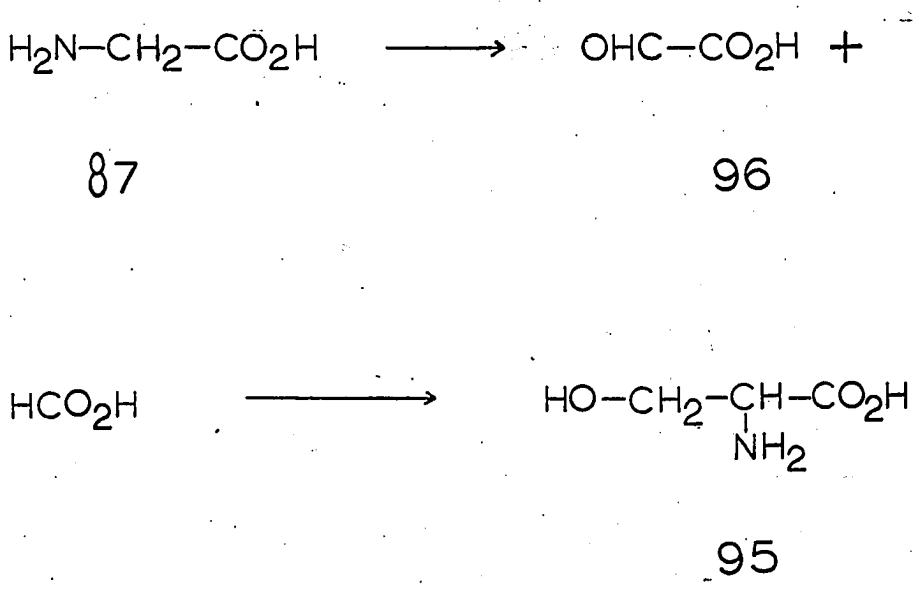

Figure 31. Formation of serine from glycine and formate

The deamination of serine to pyruvic acid (97) has never been demonstrated in botanical specimens, but has been shown to occur in the microorganisms Escherichia coli, Pseudomonos aeruginosa, Proteus ox_{19}, Clostridium welchii and Neurosopora spp[78-82]. The formation of phenylalanine (59b) from 5-phosphoshikimic acid (98) and pyruvic acid has been suggested by studies of the metabolic pathways of the microorganisms Neurospora crassa, Escherichia coli and Aerobacter aerogenes[24-29].

Further Studies on the Incorporation of One-Carbon Unit Precursors into the Amaryllidaceae Alkaloids

The unusual results obtained from the sodium C^{14}-formate feeding experiment with Nerine bowdenii as plant host were completely unexpected. Contrary to the previous biosynthetic experiments in which only members of the phenylalanine-cinnamic acid biosynthetic pathway (Figure 30) have been found to be incorporated into the C_6-C_1 unit of the Amaryllidaceae alkaloids, it now appeared that formate was an excellent precursor of the benzyl position of the C_6-C_1 unit of these alkaloids. To re-examine the pattern of incorporation of sodium C^{14}-formate in Amaryllidaceae alkaloids, further feeding experiments were conducted (Table 17). Sprekelia formosissima was chosen as plant host on the basis of its availability and alkaloid content[83]. Sprekelia formosissima contains

Table 17. Feeding experiments with Sprekelia formosissima as plant host

Feeding experiment	Wgt. taz. iso.	Total act. used	Taz. act. (dpm/mM)	% inc.
Sodium C^{14}-formate	263 mg.	1.0 mc.	6.62×10^{4}	1.15×10^{-2}
3-C^{14}-Serine	220 mg.	0.5 mc.	2.33×10^{4}	8.16×10^{-3}
3-C^{14}-Phenylalanine*	150 mg.	0.5 mc.	2.66×10^{5}	1.00×10^{-2}

* The tazettine obtained from the 3-C^{14}-phenylalanine was not diluted with non-radioactive material prior to determining its activity.

minor amounts of haemanthamine and haemanthidine as well as the major alkaloid, tazettine. Each of these Amaryllidaceae alkaloids has a benzylic carbon in the C_6-C_1 unit. The alkaloids were isolated from Sprekelia formosissima by standard procedures[84]. Tazettine was obtained in sufficient yield for degradative purposes, but only trace amounts of haemanthamine and haemanthidine were obtained. A trace amount of lycoramine also was obtained.

Since 3-C^{14}-serine is a one-carbon unit precursor[85-91] and may be a possible biosynthetic intermediate in the formation of Amaryllidaceae alkaloids, it was also fed to selected bulbs of Sprekelia formosissima at the same time as the sodium C^{14}-formate. Because of previous biosynthetic experiments with 3-C^{14}-phenylalanine, this substance was used as a reference; its incorporation into the C_6-C_1 unit of tazettine is well established (Table 1). In Sprekelia formosissima the levels of incorporation of the radioactive tracers, sodium C^{14}-formate, 3-C^{14}-serine and 3-C^{14}-phenylalanine, were comparable.

Degradation of Tazettine

On the basis of the early experiments with the incorporation of formate into belladine and ambelline, it would be expected that tazettine (12) isolated from the sodium C^{14}-formate and 3-C^{14}-serine feeding experiments, would be labelled at the C_8 position as well as in the C_1 fragments.

The radioactivity present in the C_3 methoxyl group of tazettine was determined by the Zeisel method. The radioactivity of the

methylenedioxy group was determined by acid hydrolysis (Figure 32) to formaldehyde, which was isolated as the dimedone adduct (70).

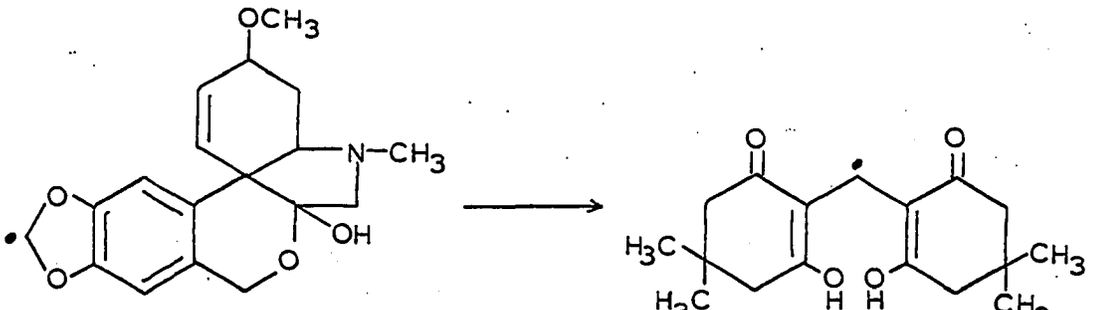

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Figure 32. Methylenedioxy determination

In previous radioactive feeding experiments (Table 1) tazettine was degraded by the procedure[92] given in Figure 33. If the tazettine was labelled at the C_8 position, the radioactivity would appear in the tazettine methine (99) at the position indicated by the dagger. Basic hydrolysis of 99 gave 6-phenylpiperonyl alcohol (100) and dimethylglycine, which was isolated as the hydrochloride (101).

Activity at the position indicated by the dagger in tazettine methine would appear in the benzylic carbon atom of 100. To determine the amount of radioactivity at the benzylic position of 6-phenylpiperonyl alcohol, it may be oxidized to 6-phenylpiperonylic acid (102) with potassium permanganate (Figure 34) and then decarboxylated with copper chromite to give carbon dioxide and 3,4-methylenedioxybiphenyl (103). The carbon

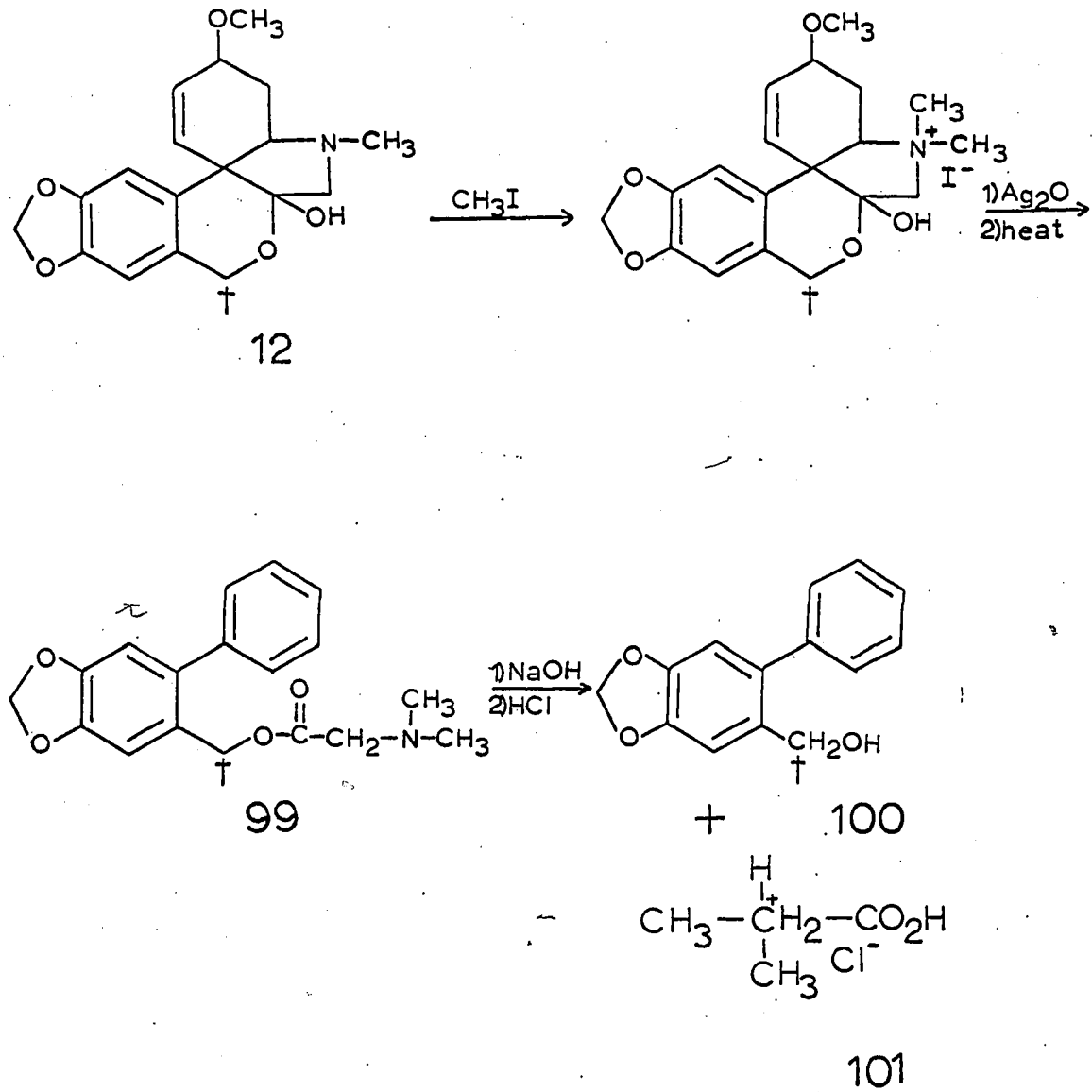

Figure 33. Degradation of tazettine

dioxide should contain all the activity at the C_8 position, and 103 would contain the radioactivity of the methylenedioxy group of tazettine.

One of the N-methyl groups of the dimethylglycine hydrochloride is derived from the N-methyl group of tazettine, and the other arises from

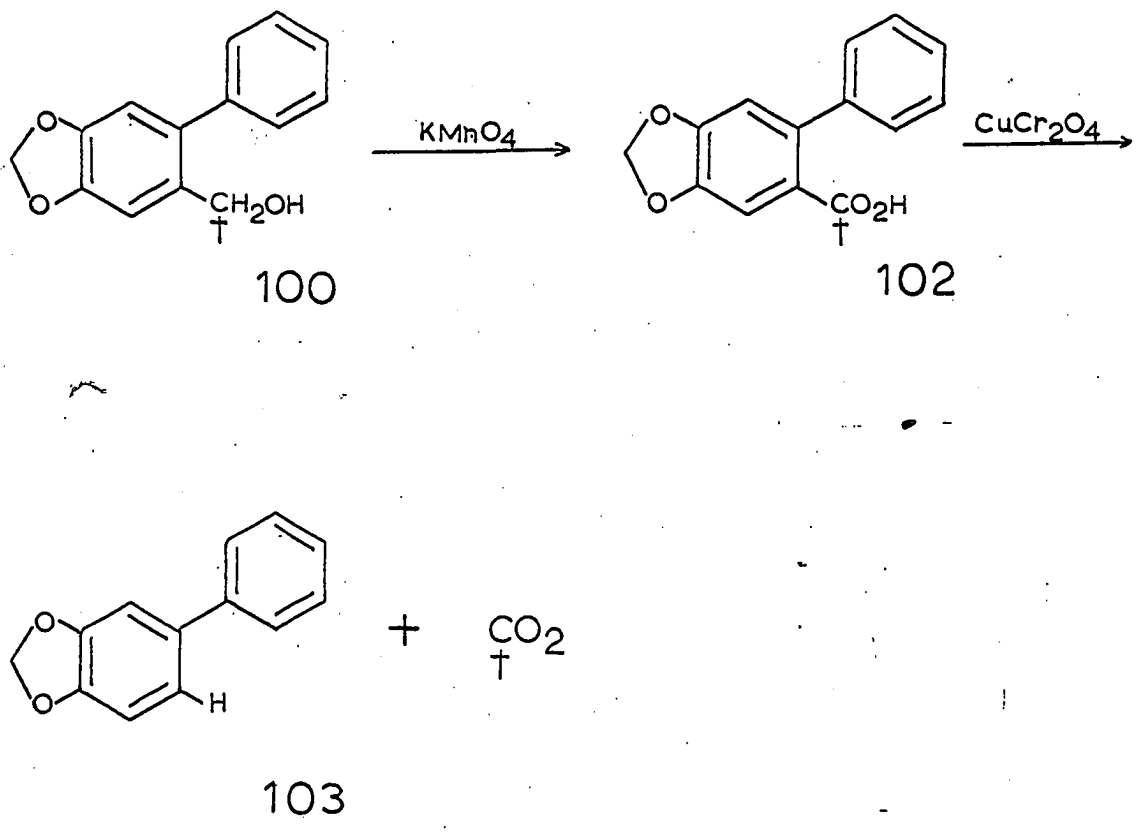

Figure 34. Oxidation-decarboxylation of 6-phenylpiperonyl alcohol

non-radioactive methyl iodide. All radioactivity not in the carboxyl or methylene group of 101 must be in the N-methyl groups. To determine the activity of the N-methyl groups, the dimethylglycine hydrochloride was oxidized with lead tetraacetate to obtain the carboxyl group as carbon dioxide and the methylene group as formaldehyde dimedone adduct (Figure 35). The carbon dioxide and the formaldehyde dimedone adduct were found to be non-radioactive in both the sodium C^{14}-formate and the 3-C^{14}-serine

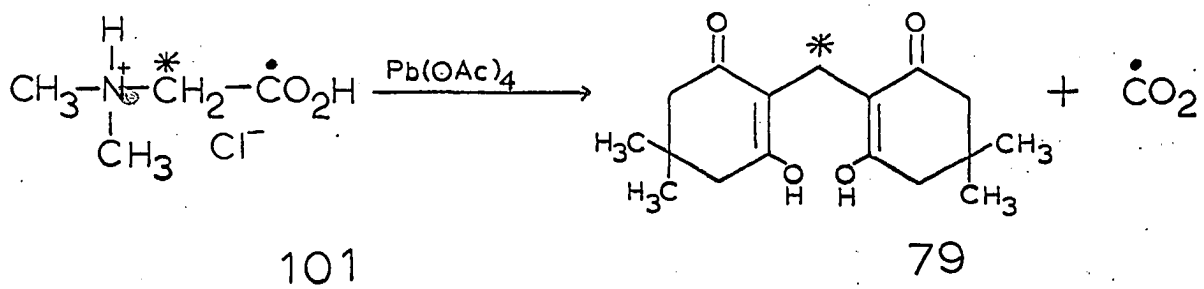

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Figure 35. Lead tetraacetate oxidation of dimethylglycine hydrochloride

feeding experiments. Therefore the activity of the dimethylglycine hydrochloride obtained on degradation of the tazettine isolated from the sodium C^{14}-formate or the 3-C^{14}-serine feeding experiment could only be attributed to the N-methyl group derived from tazettine.

Tazettine Isolated From the Sodium C^{14}-Formate Feeding Experiment

The C_3 methoxyl group was shown to have 8% and the methylenedioxy group was shown to have 3% of the total radioactivity (Table 18) of the tazettine (12). The 6-phenylpiperonyl alcohol (101) obtained on degradation was shown to have only 4% of the total radioactivity of the tazettine. Since this amount of radioactivity, within experimental error, had already been shown to be in the methylenedioxy group, no incorporation of sodium-C^{14}-formate occurred at the C_8 position or in the aromatic or hydroaromatic rings of tazettine. The dimethylglycine hydrochloride obtained in the degradation was found to have 96% of the total

Table 18. Degradation of tazettine from Spekelia formosissima in the sodium C^{14}-formate feeding experiment

Compound	Yield	Amount used next rctn.	Rel. act.
Tazettine		1.25 g.	1.00
Tazettine methiodide	1.58 g.	1.58 g.	0.93
6-Phenylpiperonyl alcohol	350 mg.		0.04
Dimethylglycine hydrochloride	150 mg.	12.49 mg.	0.96
Formaldehyde dimedone adduct from dimethylglycine hydrochloride	4.69 mg.		0.00
Carbon dioxide from dimethylglycine hydrochloride	----		0.00
Methyltriethyl ammonium iodide	6.15 mg.		0.08
Formaldehyde dimedone adduct from methylenedioxy determination	19 mg.		0.03

activity present in the tazettine. Since the C_1 and C_2 positions contained no radioactivity, all of the activity must be in the N-methyl group of the dimethylglycine hydrochloride. These data (Table 19) prove that the major incorporation of sodium C^{14}-formate into tazettine occurred in the N-methyl group.

Table 19. Per cent of total activity of tazettine isolated from the sodium C^{14}-formate feeding experiment in the methoxyl, methylenedioxy and N-methyl groups and at position C_8

Pos. of carbon in comp.	% of total act.
C_3 Methoxyl group	8.
Methylenedioxy group	3.
N-Methyl group	96.
C_8 Position	0.

The different levels of incorporation of formate (Table 19) into the methoxyl, methylenedioxy and N-methyl groups may be explained by considering the metabolic pathway in Figure 36, which is based on previous biosynthetic studies.

A high level of incorporation would be expected in the N-methyl group of the tazettine for incorporation at this position occurs in the last step of the metabolic pathway. A smaller amount of incorporation would be expected from the oxidative coupling of O-methyl norbelladine (69) to form haemanthamine (58), which in turn undergoes oxidation and rearrangement to give tazettine. However, these data do not explain why ambelline (78) and belladine (54b) apparently incorporate a large amount of sodium C^{14}-formate at the benzylic position of the C_6-C_1 unit while tazettine does not. If sodium C^{14}-formate were incorporated into tazettine at positions other than the one-carbon units, the process would presumably occur prior to formation of the norbelladine, since norbelladine is known to be incorporated intact into Amaryllidaceae alkaloids (Table 2). These data indicate such a metabolic sequence (Figure 30) either does not exist or is occurring at such a slow rate in Sprekelia formosissima during the period (7/8/1965 - 8/25/1965) of the sodium C^{14}-formate feeding experiment that detection of the process was unsuccessful.

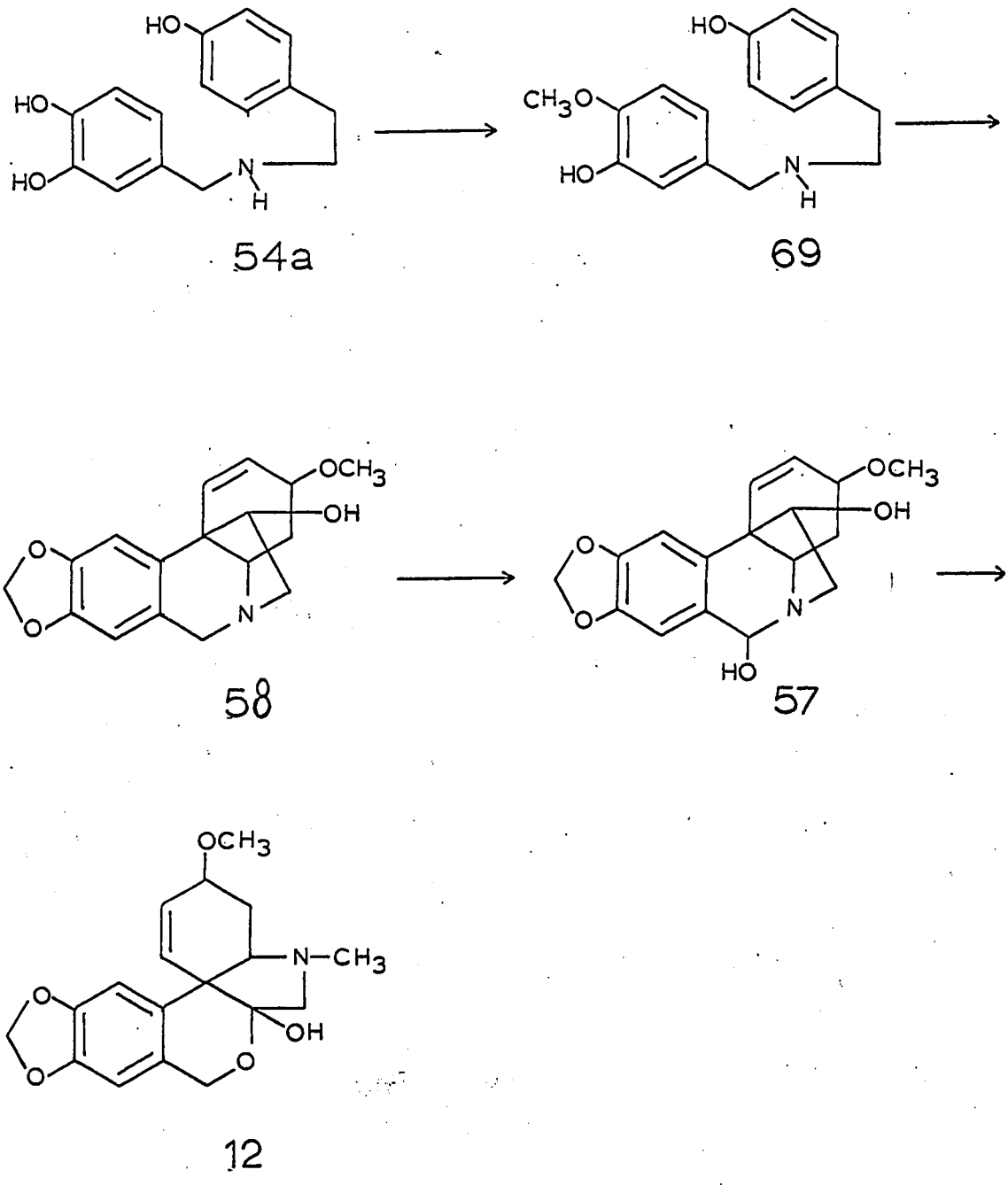
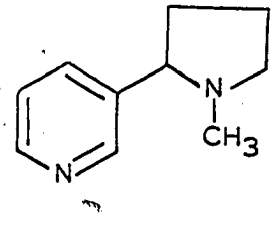


Figure 36. Biosynthesis of tazettine

Tazettine Obtained From the 3-C^{14}-Serine Feeding Experiment

As previously stated, the formation of serine (95) from sodium formate and glycine (87) in plant tissues is well established[67-77], but the initial studies of Tolbert and Cohan[75], designed to show the conversion of serine to glycine and formate, were unsuccessful. However, Wilkinson and Davies[70,77] have been able to show that serine can be cleaved to give formate and glycine in plant tissues. These studies are in agreement with experiments showing 3-C^{14}-serine is an effective precursor of the one-carbon units of nicotine (104)[85-91] and gramine (105)[90,91].



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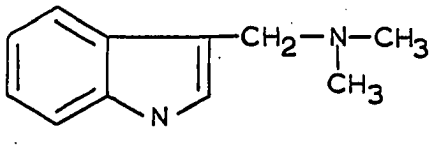

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On the basis of the previous studies with nicotine and gramine, the expected location of the radioactive labels in tazettine (12) obtained from the 3-C^{14}-serine feeding experiment would be the methoxyl, methylenedioxy and N-methyl groups. Furthermore, if the biosynthetic pathway (Figure 30) observed for the incorporation of sodium-C^{14}-formate into ambelline (78) and belladine (54b) is valid, the tazettine would also

be expected to be labelled in the benzylic (C_8) position of the C_6-C_1 unit.

The radioactivities in the methoxyl, methylenedioxy and N-methyl groups and in position C_8 of tazettine were determined in a manner identical to that used in the sodium C^{14}-formate feeding experiment (Table 20). Except for a slight increase in the per cent of radioactivity present in the methylendioxy group and a slight decrease in the activity present in the N-methyl group (Table 21), the results were identical to those obtained for tazettine isolated from the sodium C^{14}-formate feeding experiment (Table 19). These data prove that the 3-C^{14}-serine served as a one-carbon unit precursor almost as effectively as formate. Since sodium C^{14}-formate was not incorporated into the tazettine at the C_8 position, 3-C^{14}-serine would not be expected to be incorporated into this position.

Table 20. Degradation of tazettine obtained from the 3-C^{14}-serine feeding experiment

Compound	Yield	Amount used next rctn.	Rel. act.
Tazettine		901 mg.	1.00
Tazettine methiodide	906 mg.	906 mg.	1.03
6-Phenylpiperonyl alcohol	225 mg.		0.07
Dimethylglycine hydrochloride	55 mg.	12 mg.	0.89
Formaldehyde dimedone adduct from dimethylglycine hydrochloride	3 mg.		0.00
Carbon dioxide	----		0.00
Methyltriethyl ammonium iodide	5.47 mg.		0.08
Formaldehyde dimedone adduct from methylenedioxy determination	6.82 mg.		0.07

Table 21. Per cent of the total activity of the tazettine isolated from the 3-C^{14}-serine feeding experiment in the methoxyl, methylenedioxy and N-methyl groups and the C_8 positions

Pos. of carbon in comp.	% of total act.
Methoxy group	8.
Methylenedioxy group	7.
N-Methyl group	89.
C_8-position	0.

It is clear from the experiments performed with Sprekelia formosissima that both formate and serine serve solely as precursors of C_1 units such as methoxyl, methylenedioxy and N-methyl groups. This finding is in direct contrast to the sodium C^{14}-formate fed Nerine bowdenii, where a significant amount of the radioactivity appeared in the benzylic position on the C_6-C_1 unit. It would seem desirable to repeat the experiments with Nerine bowdenii to confirm the original observations of H. Ishii.

SUMMARY

It has been shown that the Amaryllidaceae alkaloid ambelline, which has three oxygen substituents on the aromatic ring, is synthesized in the plant from the same precursors as those found for alkaloids containing only two aromatic oxygen atoms. Tyrosine was shown to be the precursor of the hydroaromatic C_6-C_2 unit of ambelline, and phenylalanine serves as the aromatic C_6-C_1 precursor. Evidence is presented that norbelladine is incorporated intact into ambelline and does not undergo cleavage to smaller fragments prior to incorporation.

Sodium formate and serine serve as precursors of the methoxyl, N-methyl and methylenedioxy groups of tazettine. These precursors are shown not to be incorporated into either the C_6-C_1 or C_6-C_2 units of tazettine. This result is in contrast with that found previously in the incorporation of sodium formate into ambelline.

EXPERIMENTAL

Source of Plant Materials and Radioactive Precursors

The Nerine bowdenii bulbs were secured from the Walter Marx Gardens, Boring, Oregon. The Sprekelia formosissima bulbs were obtained from Robert D. Goedert, Jacksonville, Florida.

The 1,1'-C^{14}-norbelladine used in the Nerine bowdenii feeding experiment was synthesized by R. J. Highet (National Heart Institute, Bethesda, Maryland)[32]. All other radioactive precursors (Tables 5 and 17) were obtained from New England Nuclear Corp., Boston, Massachusetts. The radioactive precursors were assumed to have the purity established by New England Nuclear Corp.

The author received the radioactive ambelline after isolation and separation of the alkaloids from Nerine bowdenii by Dr. William C. Wildman, National Heart Institute, Bethesda, Maryland.

Measurement of the Radioactivities of the Compounds

To determine the specific radioactivity (dpm/mM) of a compound, other than carbon dioxide, the material (1-6 mg.) was dissolved in 1.0 ml. of methanol and 10.0 ml. of Bray's scintillation solution [60 g. of napthalene, 4 g. of 1,4-bis-2-(5-phenyloxazolyl)-benzene

(Packard) and 100 mg. of 2,5-diphenyloxazole (Packard) in 20 ml. of 1,2-ethanediol, 100 ml. of methanol and sufficient dry, peroxide-free dioxane to make 1.0 l. of solution]. The carbon dioxide was trapped in a solution of 2.0 ml. of hydroxide of Hyamine 10-X (Packard) and 10.0 ml. of toluene POPOP scintillation solution [4 g. of 1,4-bis-2-(5-henyloxazoly)-benzene (Packard) and 100 mg. of 2,5-diphenyloxazole (Packard) in 1.0 l. of toluene]. To determine the relative specific activity of the carbon dioxide, the total disintegrations per minute for the trapping solution were compared with the theoretical disintegrations per minute attainable from a sample of the material being degraded. All measurements of the radioactivities of the compounds were obtained with a Packard Tri-carb Liquid Scintillation Spectrometer System (Model 314 X).

Nerine bowdenii Feeding Experiments

Purification of radioactive ambelline

The radioactive ambelline (Table 5), isolated from a feeding experiment, was diluted with non-radioactive material and crystallized from absolute ethanol to give ambelline having a constant activity in the range 4,400-45,000 dpm/mM (Table 5).

Degradation of ambelline

Ambelline was degraded to N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride by a general procedure in which the ratio of reagents to reactants was held constant in each experiment. All weights quoted were those obtained in the degradation of the ambelline from the 3-C^{14}-tyrosine feeding experiment.

Oxoambelline A solution of 650 mg. of ambelline in 26 ml. of dry pyridine was added to a slurry of 1.3 g. of dry chromium trioxide in 13 ml. of dry pyridine. The reaction mixture was stirred for 20 hours at room temperature and poured over ice. The excess chromium trioxide was reduced with sodium sulfite. The reaction mixture was made basic (pH=9) with sodium carbonate and extracted with chloroform until a negative silicotungstic acid test was obtained. The chloroform extract was washed with water, dried with anhydrous magnesium sulfate and evaporated to give 985 mg. of a brown oil. The oil was chromatographed on 29.4 g. of Florisil packed in 25% ethyl acetate - 75% benzene. Elution with 25% ethyl acetate - 75% benzene gave 540 mg. of oxoambelline, which was not crystalline. The material was identified by its infrared spectrum which was identical with authentic material of oxoambelline.

Oxoambelline methiodide To a solution of 450 mg. of oxoambelline in 5 ml. of acetone was added a large excess of methyl iodide. The

solution was allowed to stand for 12 hours and the precipitated oxoambelline was removed by filtration. The oxoambelline methiodide was crystallized from 95% ethanol to give white crystals (350 mg.), m.p. 246-250°C. (dec.); reported, m.p. 250-254°C. (dec.).

N-(2-Methoxy-6-phenylpiperonyl) sarcosine hydrochloride A solution of 350 mg. of oxoambelline in 5 ml. of hot water was treated with 1.5 ml. of 50% sodium hydroxide. The solution was heated on the steam bath for 1 hour and allowed to cool. The excess alkali was decanted from the brown gum that had formed and the brown gum was dissolved in 3 ml. of 6N hydrochloric acid. The resulting solution was saturated with sodium chloride and extracted five times with chloroform. Evaporation of the chloroform gave 231 mg. of a product which was crystallized from methanol-acetone, m.p. 185-187°C, mixed m.p. 185-187°C. with an authentic sample of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride.

Degradation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride by procedure A

The ratio of reagents to reactants was held constant for each degradation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride by procedure A. All weights quoted were those obtained in the degradation of the ambelline from the 3-C^{14}-tyrosine feeding experiment.

3-Methoxy-2-methyl-4,5-methylenedioxybiphenyl and N-tosyl-sarcosine A solution of 200 mg. of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride in 30 ml. of absolute ethanol and 0.5 ml. of glacial acetic acid was hydrogenated with 833 mg. of pre-equilibrated palladium-

on-charcoal (10%). The reduction was stopped after a period of 48 hours at room temperature. The uptake of hydrogen was 1.5 equivalents. The reaction mixture was filtered. The filtrate was diluted with 20 ml. of water and extracted five times with ether. The ether extract was washed three times with water and dried with anhydrous magnesium sulfate. Evaporation of the ether gave 82 mg. of a brown oil, which was distilled at 110°C. (0.05 mm.). The oil had an infrared spectrum identical with an authentic sample of 3-methoxy-2-methyl-4,5-methylenedioxybiphenyl.

The aqueous raffinate was condensed to one-third of its volume, and the solution was made basic (pH=11) with 2.5N sodium hydroxide. A solution of 1.838 g. of p-toluene sulfonyl chloride in 54 ml. of benzene was added and the emulsion was stirred for 24 hours at room temperature. The alkaline water layer was separated from the benzene layer. The aqueous solution was extracted five times with ether, made acidic (pH=1) with 1.5N hydrochloric acid and extracted five times with chloroform. Evaporation of the chloroform gave 99 mg. of a solid which was crystallized from acetone-cyclohexane, m.p. 144-146°C., mixed m.p. 144-145°C. with an authentic sample of N-tosyl sarcosine.

Electrolytic degradation of N-tosylsarcosine To 8 ml. of commercial ethanol (95%) in a three necked, round-bottomed flask (Figure 37) was added 19.75 mg. of N-tosylsarcosine and 3 mg. of sodium hydride. The 250 ml., three-necked, round-bottomed flask (Figure 37) contained two platinum electrodes (0.5 cm. x 1 cm.), 1 cm. apart and mounted 0.5 cm. from the bottom of the flask. The flask (Figure 37) was fitted with a nitrogen inlet and an outlet attached to a trap containing 2 ml. of

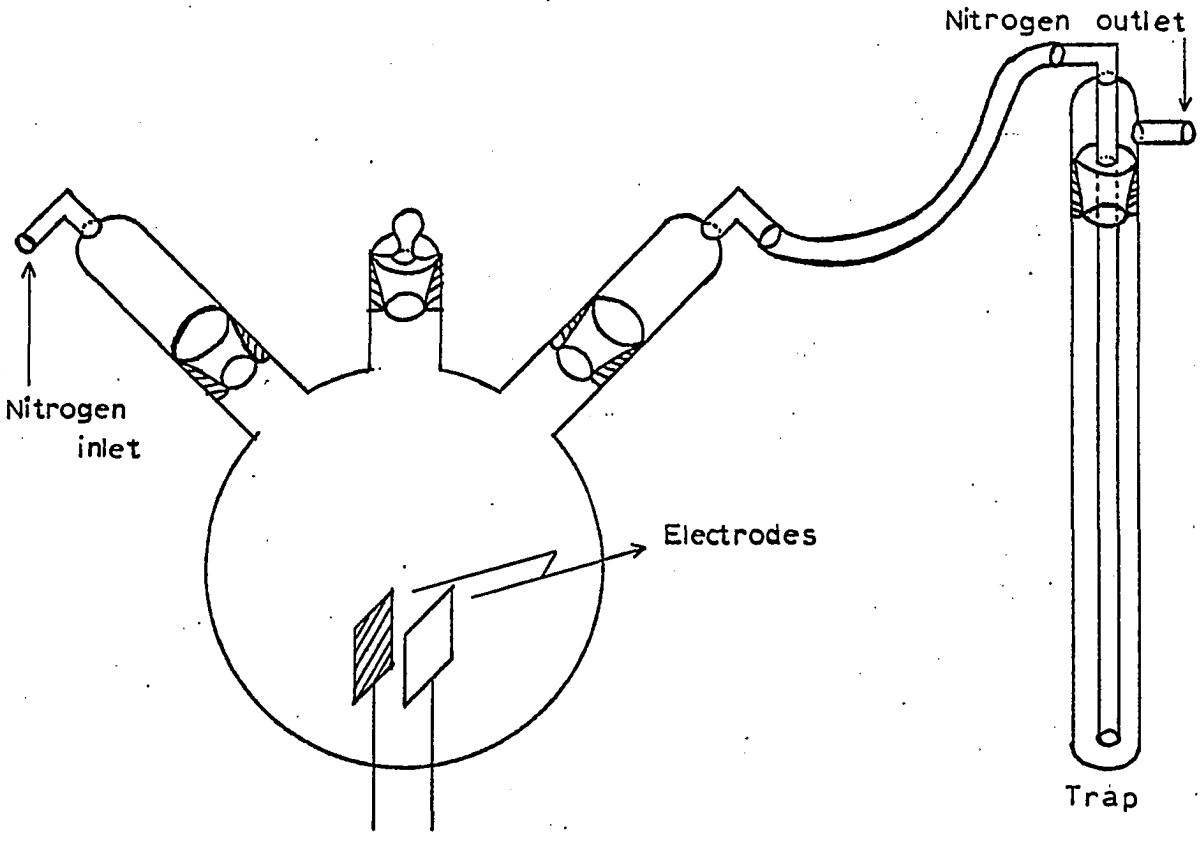

Figure 37. Apparatus for electrolytic degradation of N-tosylsarcosine

Hyamine hydrodide 10-X (Packard) and 10 ml. of standard toluene POPOP scintillation solution. The reaction mixture was electrolyzed for 60 hours at 90 m. amps. The carbon dioxide generated was trapped in the scintillation solution and its total radioactivity (765 dpm) determined.

In a second electrolytic degradation a solution of 50 mg. of N-tosylsarcosine and 6 mg. of sodium hydride in 20 ml. of water was electrolyzed for 16.5 hours at 20 m. amps. To the solution was added 5 ml. of concentrated hydrochloric acid and 100 mg. of dimedone, and the solution was refluxed for 3.5 hours. The solution was allowed to cool and

the cold solution was extracted with chloroform. Evaporation of the chloroform gave a residue which was heated in water to remove excess dimedone. The brown gum remaining after filtration was sublimed to give a solid. The sublimate was heated in water, filtered and resublimed to give white crystals (5 mg.), m.p. 189-191°C., mixed m.p. 187-188°C with an authentic sample of formaldehyde dimedone adduct.

Degradation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride by procedure B

The ratio of reagents to reactants was held constant for each degradation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride by procedure B. All weights quoted were those obtained in the degradation of the ambelline from the 2-C^{14}-tyrosine feeding experiment.

Lead tetraacetate oxidation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride To 15 mg. of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride in a three-necked, round-bottomed flask (Figure 38) was added 10.0 ml. of freshly prepared 0.67% lead tetraacetate-glacial acetic acid solution. The three-necked flask (Figure 38) was fitted with a nitrogen inlet and a condenser. The condenser was fitted with a nitrogen outlet leading to a trap containing a 90% saturated dimedone solution and a second trap containing 2 ml. of Hyamine hydroxide 10-X (Packard) and 10 ml. of standard toluene POPOP scintillation solution. The reaction mixture was heated on a steam bath for 2 hours 38 minutes. The formaldehyde dimedone adduct was separated from the dimedone solution by filtration, heated with water and sublimed at 140°C. (0.01 mm.), to give 5.55 mg. of radioactive formaldehyde dimedone

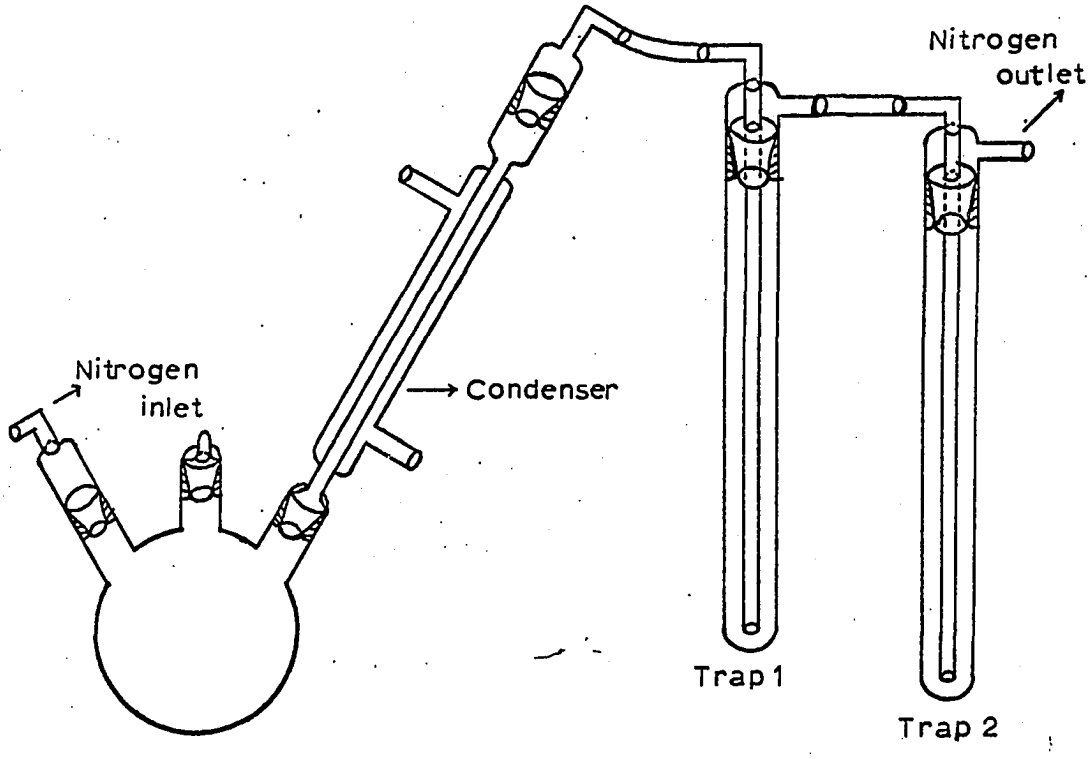

Figure 38. Apparatus for lead tetraacetate oxidation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride

adduct, m.p. 190-191°C., mixed m.p. 190-191°C. with authentic material.

The carbon dioxide produced in the reaction was trapped in toluene POPOP scintillation solution.

Determination of per cent impurities in radioactive formaldehyde dimedone adduct obtained from the lead tetraacetate oxidation of N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride To a three-necked round-bottomed flask connected to apparatus previously described (Figure 38) was added 10.0 ml. of the freshly prepared 0.67% lead

tetraacetate-glacial acetic acid solution used in the oxidation of the N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride. The solution was heated on the steam bath for 2 hours 38 minutes. The non-radioactive formaldehyde generated was trapped in 90% saturated dimedone solution. The non-radioactive formaldehyde dimedone adduct was separated from the dimedone solution by filtration, heated with water and sublimed at 140°C. (0.01 mm.) to give 1.81 mg. of material, m.p. 191-192°C. The non-radioactive formaldehyde dimedone adduct (1.81 mg.) was equivalent to an impurity of 32.6% in the radioactive formaldehyde dimedone adduct obtained in the lead tetraacetate oxidation of the N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride.

In the 1,1'-C^{14}-norbelladine feeding experiment, 4.64 mg. of radioactive formaldehyde dimedone adduct was obtained from the lead tetraacetate oxidation of the N-(2-methoxy-6-phenylpiperonyl) sarcosine hydrochloride and was shown to contain 1.45 mg. of non-radioactive formaldehyde dimedone adduct, equivalent to an impurity of 31.3% in the radioactive formaldehyde dimedone adduct.

Ambelline hydrochloride

Hydrogen chloride was bubbled into 0.75 ml. of an ethanolic solution of ambelline (60 mg.) obtained from the 3-C^{14}-phenylalanine feeding experiment. On evaporation of the ethanol with a stream of air gave crystals of ambelline hydrochloride (66 mg.) which were crystallized from acetone to constant activity (4,580 dpm/mM), m.p. 227-231°C. (dec.); reported, m.p. 227-230°C. (dec.).

A solution of the radioactive ambelline hydrochloride in water was made basic (pH=11) with 10% sodium hydroxide and extracted with chloroform. Evaporation of the chloroform gave 56 mg. of ambelline, (m.p. 259-261°C.); reported, m.p. 260-261°C. (dec.).

Methylenedioxy determination

To 110 mg. of ambelline and 336 mg. of dimedone in a centrifuge tube was added 5.5 ml. of sulfuric acid (60 ml. of concentrated sulfuric acid per 100 ml. of water). The reaction mixture was heated on a steam bath for 15 hours, diluted with 20 ml. of water and extracted five times with ether. The ether extract was dried with calcium chloride and evaporated to give a brown residue. The residue was heated with 10 ml. of water to remove water-soluble material and filtered. The brown residue was sublimed to give 34 mg. of a solid, m.p. 189-191°C. The sublimate was heated with 5 ml. of water for 15 minutes, filtered and resublimed to give white crystals, m.p. 190-191°C., identical with an authentic sample of formaldehyde dimedone adduct.

Zeisel determination

A solution of 0.5 ml. of 5% cadmium sulfate and 0.5 ml. of 5% sodium thiosulfate was placed in the iodine trap B (Figure 3). A solution of 5 ml. of 5% triethyl amine in absolute ethanol was placed in trap C (Figure 39) to trap the methyl iodide generated in the reaction as methyltriethyl ammonium iodide. Trap C (Figure 39) was emersed in a dry ice-chloroform bath at -30°C.

To 18 mg. of ambelline and 500 mg. of phenol in flask A (Figure 39)

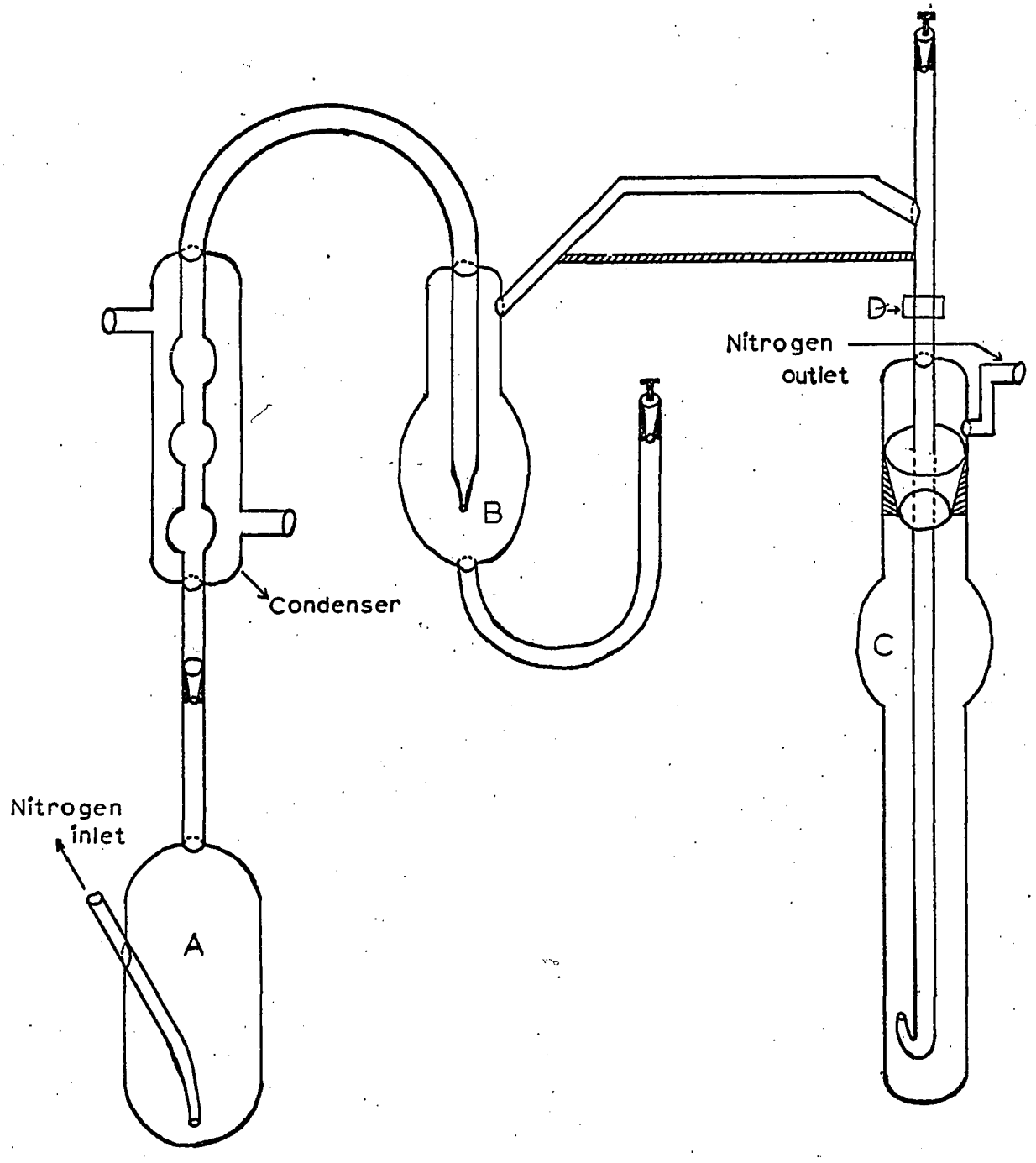

Figure 39. Apparatus for Zeisel determination

was added 2.0 ml. of 57% hydroiodic acid. Flask A was then connected to the nitrogen source and heated 45 minutes at 125-132°C. in a Wood's metal bath. After the reaction was completed, trap C (Figure 39) was detached from the apparatus at point D (Figure 39) -- a rubber joint -- and stored under nitrogen for 12 hours. The ethanol-triethyl amine solution was evaporated by vacuum (aspirator) distillation to give a solid. The material was crystallized from methanol-ether to give 12.5 mg. of methyltriethyl ammonium iodide, m.p. 277-280°C. (dec.), identical with an authentic sample of the material.

Synthesis of 2-C^{14}-N-tosylsarcosine

To a solution of 1 g. of non-radioactive glycine and 0.8 mg. of 2-C^{14}-glycine in 300 ml. of water, adjusted to pH=11 with 10% sodium hydroxide, was added a solution of 20 g. of p-toluene sulfonyl chloride in 600 ml. of benzene. The emulsion was stirred for 20 hours at room temperature. The water layer was separated from the benzene layer. The aqueous solution was made basic (pH=11) with 10% sodium hydroxide and extracted with ether. The water layer was made acidic (pH=1) with 6N hydrochloric acid and extracted with chloroform. Evaporation of the chloroform gave a solid, which was dissolved in 95% ethanol. To the ethanol solution was added 1 ml. of 50% potassium hydroxide and 2 ml. of methyl iodide. White crystals precipitated and were removed by filtration. A solution of the precipitate in water was acidified (pH=1) with 6N hydrochloric acid and the solution was extracted with chloroform. Evaporation of the chloroform gave 7 mg. of material which was sublimed to give white crystals, m.p. 145-148°C., mixed m.p. with N-tosylglycine,

115-140°C., mixed m.p. with N-tosysarcosine 145-148°C. The crystals were diluted with 300 mg. of non-radioactive N-tosylsarcosine and crystallized to constant activity (209,500 dpm/mM) from acetone-cyclohexane.

Determination of formaldehyde with chromotropic acid

To a three-necked, round-bottomed flask connected to the apparatus previously described (Figure 38) was added 10.0 ml. of 0.67% freshly prepared lead tetraacetate-glacial acetic acid solution. The solution was heated on the steam bath for 3.00 hours. The formaldehyde generated was trapped in 10.0 ml. of water.

To 104 mg. of chromotropic acid in a 30 ml. beaker was added 1.00 ml. of the formaldehyde-water solution of unknown concentration. The solution was heated on a hot plate at 175°C. until all of the moisture had disappeared from the beaker. The material in the beaker was heated for an additional 5 minutes at 175° C. The beaker was allowed to cool and 5.0 ml. of concentrated sulfuric acid were added. The material was heated for 30 minutes in boiling water to give a purple solution. The purple solution was allowed to cool and was diluted to 50 ml. in a volumetric flask. The absorption of the purple solution at 570 mμ measured.

The above procedure was repeated with three formaldehyde-water solutions having concentrations of 78 μg./ml., 39 μg./ml. and 19.5 μg./ml. The three purple solutions obtained showed a linear relationship between absorption at 570 mμ and concentration of the formaldehyde-water solution from which they were made. The formaldehyde-water solution of unknown concentration was shown to contain 63.1 μg. of formaldehyde per milliliter.

Sprekelia formosissima Feeding Experiments

The same experimental procedures were used in isolating and degrading tazettine from the sodium C^{14}-formate and the 3-C^{14}-serine feeding experiments. All weights quoted were those obtained in the isolation and degradation of tazettine from the sodium-C^{14}-formate feeding experiment.

Feeding experiments

The sodium C^{14}-formate (1.0 mc., 17.0 mg.) was dissolved in 1.0 ml. of water. Into each of ten Sprekelia formosissima bulbs was injected, with a microsyringe, 0.1 ml. of the sodium C^{14}-formate solution. The radioactive vial was washed with 0.2 ml. of water and the water injected into an eleventh bulb. After allowing the plants to grow for a period of 48 days (7/8/1965 - 8/25/1965) the bulbs (667 g.) were harvested and the alkaloids isolated in the usual manner.

The 3-C^{14}-serine (0.5 mc. 13.7 mg.) was dissolved in 1.0 ml. of water. The 3-C^{14}-serine feeding experiment was identical to the sodium C^{14}-formate feeding experiment in all other respects. The bulbs (643 g.) were harvested and the alkaloids isolated in the usual manner.

General procedure for isolation of the crude alkaloids from Sprekelia formosissima bulbs

The eleven bulbs (667 g.) were homogenized with 2 l. of 95% ethanol in a Waring Blendor. The mixture was allowed to settle for 30 minutes and the solid material removed by filtration. The filter cake was stirred in 2 l. of 95% ethanol and allowed to stand overnight. The solid

material was removed by filtration. The process was repeated a third time. The three filtrates were combined and concentrated to 1.0 l. by aspirator vacuum distillation under a nitrogen atmosphere. The remaining ethanol was removed on a rotatory evaporator to yield 21 g. of residue from the sodium C^{14}-formate feeding experiment and 11.7 g. of residue from the 3-C^{14}-serine feeding experiment.

The crude residue (21 g.) was stirred at 50° C. with 250 ml. of 2N hydrochloric acid and filtered to remove the acid-insoluble material. The insoluble material was heated with 100 ml. of 2N hydrochloric acid and refiltered. The filtrates were combined and extracted 5 times with chloroform. The chloroform extract gave a negative alkaloid test with silicotungstic acid and was discarded.

The 2N hydrochloric acid solution was made basic (pH=12) with 50% sodium hydroxide and extracted with chloroform until the aqueous solution gave a negative alkaloid test. The chloroform extract of the basic solution was evaporated on a rotovaporator to give 646 mg. (0.097% of the total weight of the bulbs) of a crude alkaloid mixture from the sodium C^{14}-formate feeding experiment. The 3-C^{14}-serine feeding experiment afforded 440 mg. (0.069% of the total weight of the bulbs) of a crude alkaloid mixture.

Thin-layer and gas-phase analysis proved the crude alkaloid mixtures to contain primarily tazettine and traces of haemanthamine, haemanthidine and lycoramine.

Separation of tazettine from the crude alkaloid mixture

The crude alkaloid mixture (646 mg.) was dissolved in acetone and allowed to stand for a period of 3 days. The tazettine that had precipitated was removed by filtration. The filtrate was evaporated to give a residue, which was dissolved in 25% benzene-75% chloroform and chromatographed on an alumina (Merck-neutral) column (17.5 cm x 1.5 cm) packed in 25% benzene-75% chloroform. Elution with 25% benzene-75% chloroform gave lycoramine first, haemanthamine second and then tazettine. Elution with chloroform and 1%, 2%, 5% and 10% methanol-chloroform solutions gave no characterizable products. Elution with 20% methanol-80% chloroform gave a mixture containing haemanthidine.

A total yield of 263 mg. of tazettine, m.p. 207-209°C., (317,000 dpm/mM) obtained from the sodium C^{14}-formate feeding experiment was diluted with 1.017 g. of non-radioactive tazettine and crystallized to constant activity, 66,200 dpm/mM (1.25g.), m.p. 209-210°C.; reported m.p. 208-210°C.

A total yield of 220 mg. of impure tazettine (m.p. 204-209°C., 224,100 dpm/mM) was isolated from the crude alkaloid mixture (440 mg.) from the 3-C^{14}-serine feeding experiment. This material was recrystallized from acetone. To the filtrate was added 100 mg. of non-radioactive tazettine, and the material was recrystallized to give a second crop of radioactive tazettine. The process was repeated with the second filtrate to give a third crop of radioactive tazettine. The three crops of radioactive tazettine were combined, diluted with 880 mg. of non-radioactive tazettine and crystallized to constant activity, 23,300

dpm/mM (1.223 g.), m.p. 209-210°C.; reported, m.p. 208-210°C.

Zeisel determination

The Zeisel determination was conducted in the apparatus shown in Figure 39 with the traps B and C containing the prescribed reagents. To 22 mg. of tazettine and 500 mg. of phenol in flask A (Figure 39) was added 2.0 ml. of 57% hydroiodic acid. Flask A (Figure 39) was then connected to the nitrogen source and heated 45 minutes at 125-132°C. in a Wood's metal bath. After the reaction was completed, trap C (Figure 39) was detached from the apparatus at point D (Figure 39) — a rubber joint — and stored under nitrogen for 12 hours.

The ethanol-triethyl amine solution was evaporated under reduced pressure. The methyltriethyl ammonium iodide (6.15 mg.) was crystallized from methanol-ether and dried under vacuum at room temperature.

Methylenedioxy determination

To 103 mg. of tazettine and 308 mg. of dimedone in a centrifuge tube was added 5 ml. of sulfuric acid (60 ml. of sulfuric acid in 100 ml. of water). The reaction mixture was heated on a steam bath for 16 hours, added to 25 ml. of water and heated to dissolve excess dimedone. The hot solution was filtered and the brown residue reheated in 5 ml. of water to remove further dimedone. The hot solution was filtered and the brown residue sublimed at 140°C. (0.01 mm.). The sublimate was heated in water, filtered and resublimed to give white crystals (19 mg.), m.p. 191-192°C., identical with an authentic sample of formaldehyde dimedone adduct.

Degradation of tazettine

Tazettine (850 mg., 66,200 dpm/mM) from the sodium C^{14}-formate feeding experiment was diluted with non-radioactive tazettine (702 mg.) and crystallized from acetone to give tazettine having an activity of 36,100 dpm/mM. The tazettine (23,300 dpm/mM) obtained from the 3-C^{14}-serine feeding experiment was not diluted further prior to degradation.

Tazettine methiodide To a solution of 1.25 g. of tazettine in 50 ml. of acetone was added a large excess of methyliodide, and the solution was allowed to reflux for 2 hours. The acetone solution was concentrated until tazettine methiodide precipitated. The solution was cooled and filtered. The tazettine methiodide (1.58 g.) was recrystallized from methanol-acetone-ether to give white crystals, m.p. 238-239°C. (dec.); reported, m.p. 238°C. (dec.).

Dimethylglycine hydrochloride and 6-phenylpiperonyl alcohol To a solution of 1.58 g. of tazettine methiodide in water was added a large excess of freshly prepared neutral silver hydroxide and the mixture stirred with a magnetic stirrer for 15 minutes. The reaction mixture was filtered. The filtrate gave a negative iodide ion test with acidic silver nitrate. The filtrate was evaporated on a rotovaporator and pyrolyzed 1 hour on the steam bath under aspirator vacuum to give 1.1 g. of tazettine methine, which was not crystalline. The material was identified by its infrared spectrum which was identical with an authentic sample of tazettine methine.

To a methanolic solution (15 ml.) of the methine was added 3 ml. of

2.5N sodium hydroxide. The reaction mixture was heated for 1 hour on the steam bath and added to 20 ml. of cold water. The water emulsion was extracted six times with ether. Evaporation of the ether gave a solid which was crystallized from ether to give 350 mg. of 6-phenylpiperonyl alcohol, m.p. 98-99°C., identical with an authentic sample.

The water raffinate, remaining after the ether extraction, was acidified with 6N hydrochloric acid and evaporated on a rotovaporator to give a mixture of sodium chloride and dimethylglycine hydrochloride. The dimethylglycine hydrochloride was removed from the sodium chloride by sublimation at 145-150°C. (0.01 mm.). Resublimation of the dimethylglycine hydrochloride yielded white crystals (150 mg.), m.p. 184-185°C., identical with an authentic sample of dimethylglycine hydrochloride.

Lead tetraacetate oxidation of dimethylglycine hydrochloride To 12.49 mg. of dimethylglycine hydrochloride in a three-necked, round-bottomed flask connected to the apparatus previously described (Figure 38) was added 10.0 ml. of 0.67% lead tetraacetate-glacial acetic acid solution. The first trap contained a 90% saturated dimedone solution, and the second trap contained a 90% barium hydroxide solution. The flask was heated on the steam bath for 30 minutes.

The formaldehyde dimedone adduct was separated from the dimedone solution by filtration, heated with water and sublimed at 140°C. (0.01 mm.). The sublimed material was heated in water, filtered and resublimed to give 4.69 mg. of formaldehyde dimedone adduct, m.p. 191-192°C., identical with an authentic sample of the material.

The carbon dioxide trapped as barium carbonate was separated from the barium hydroxide solution by filtration, washed with distilled water and then washed with methanol. The barium carbonate was dried under vacuum. To 12.31 mg. of the barium carbonate in a three necked, round-bottomed flask (Figure 38) was added a large excess of 6N hydrochloric acid. The carbon dioxide, generated in an apparatus identical to that used in the lead tetraacetate oxidation reaction (Figure 38), was trapped in 1.0 ml. of Hyamine hydroxide 10-X (Packard) and 10 ml. of toluene POPOP scintillation solution. The nitrogen stream was allowed to bubble into the scintillation solution for a period of 30 minutes following disappearance of the barium carbonate in the reaction flask (Figure 38).

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