BIOSYNTHESIS OF BENZYLISOQUINOLINE ALKALOIDS

by

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ABSTRACT

This research was carried out to obtain a convenient route for the synthesis of [7-^{14}C]-p-hydroxy benzaldehyde.

Section 1 of the thesis includes a route involving intermediates with protecting groups like benzyl and methyl ethers of the phenols. The benzyl ethers afforded the product in relatively better yield. The overall synthesis involves four steps.

Section 2 describes the reactions carried out directly on phenols, and a three step pathway is obtained for the synthesis of p-hydroxy benzaldehyde, which was repeated on labelled compounds to obtain [7-^{14}C]-p-hydroxy benzaldehyde. The synthesis involves the reaction of p-bromo-phenol with Cu^{14}CN to yield [7-^{14}C]-p-cyano phenol, which is then reduced to the aldehyde by means of a simple and clean photolysis method.

The same route was tried out to get 3,4-dihydroxybenzaldehyde and was found to work equally well for the synthesis of this compound.

Section 3 deals with the isolation of labelled alkaloids, corydaline, protopine and reticuline from [2-^{3}H,1-^{14}C]-dopamine (^{3}H/^{14}C ratio = 4) fed Corydalis solida. ^{3}H/^{14}C ratios in the labelled alkaloids were determined. The uncorrected values showed almost 50% loss of ^{3}H relative to ^{14}C in reticuline, and roughly 75% loss of the ^{3}H relative to ^{14}C in corydaline and protopine.

ACKNOWLEDGEMENTS

I am most sincerely grateful to Dr. H. L. Holland for his kind guidance and encouragement, and for the many things that I have learned during the course of this work.

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BIOSYNTHESIS

The term biosynthesis could be applied to the formation of any substance in a living organism, but it is used generally in the more restricted anabolic sense. While "biosynthesis" refers to the actual route of formation of natural products and implies an accurate experimentally confirmed knowledge of the synthesis, the term "biogenesis" generally used by the chemists, refers to an overall synthetic route to a given compound, which has been developed theoretically, but for which there is little or no experimental evidence.

Preliminary clues to the nature of biosynthetic sequences can sometimes be gained by chemical analysis. The pattern of structurally related compounds existing in a species, either at one stage of development or over a period of time can serve as a basis for hypotheses about biosynthetic pathways. But the successive steps in the sequence often proceed so rapidly that it is not possible all the times to distinguish the order of synthesis of individual substances.

The above approach has been most widely developed by Robinson.[1]

In modern practice the fate of possible precursors is followed by means of isotopic labelling. Most labelled atoms are radioactive, with carbon-14 and tritium dominating the field. The proposed labelled precursor is administered to the intact organism. The desired product is then isolated after a certain time period, and is further studied through degradation.

The degradation procedures are certainly very time-consuming and sometimes are not unambiguous either, but generally do provide useful information. Double and even triple labelling can also be applied in order to establish a proposed pathway almost beyond doubt.

The use of some magnetically active nuclei, carbon-13 in particular, has been very helpful in cases where degradation studies could not provide a satisfactory piece of information, biosynthesis of vitamin B_{12} for instance;[2] but in higher organisms, the use of carbon-14 still seems assured mainly due to the low incorporation of labels from precursors into the organism. The incorporation can be as high as 50% in micro-organisms, but in higher organisms it rarely exceeds 0.1%. That much incorporation is not sufficient for NMR studies, because the carbon-13 nucleus has a relatively low sensitivity for NMR signals. Carbon-14, being radioactive, can be detected by several methods with high sensitivity permitting the observation of small numbers of atoms.

ALKALOIDS: OCCURRENCE, FUNCTION IN PLANTS, AND PHARMACOLOGY

The term "alkaloids" applies to a large group of compounds, which occur almost exclusively in plants. Alkaloids are nitrogen-containing organic bases (usually heterocyclic) possessing biological activity. Quinine, for example, which is one of the active principles of cinchona bark extract, has been found to be an effective anti-malarial medicine. Morphine and codeine (poppy alkaloids) are very powerful analgesics, although they prove to be very toxic and addictive if not used in controlled amounts. Reserpine (rauwolfia alkaloid) due to its hypotensive action is exclusively used for the treatment of nervous and mental disorders.[3] Alkaloids like strychnine are very poisonous and are used as pesticides.

As far as the question about the function of alkaloids in plants is concerned, there has been no clear answer to it. They probably act as growth regulators, or being basic, they might participate in the maintenance of the ionic balance in the plants. They are also thought to constitute the part of plants' defence against animals because of their bitter taste.

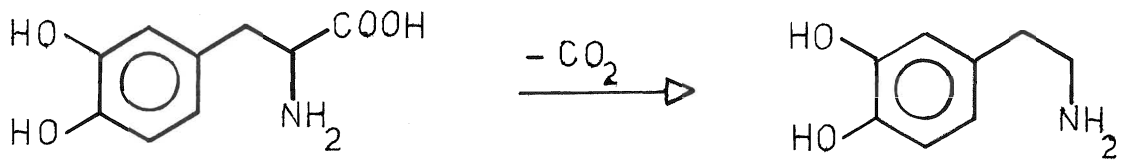
None of the above explanations, however, has been proved to be certain.

THE REACTIONS INVOLVED IN BIOSYNTHESIS OF ALKALOIDS

The chemical reactions going on in biological systems are analogous to the ones carried out in the laboratory. Therefore, in biosynthetic schemes, they can be represented by the normal notations used in organic chemistry. The only difference is that these reactions take place under physiological conditions, and are catalyzed by biological catalysts, "enzymes". It is very unlikely that alkaloids are the products of non-enzymatic reactions, because the majority of them are optically active. It may be that some steps in the formation of an alkaloid occur without enzymes, but key steps are almost certainly enzyme controlled.

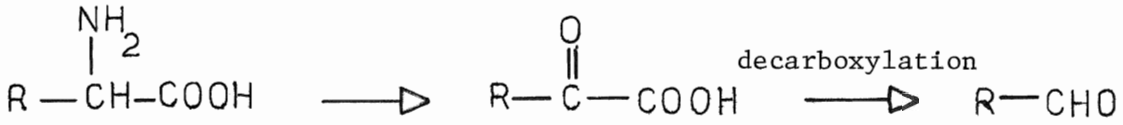
The most important reactions involved in the biosynthesis of alkaloids will be discussed below.

(a) Decarboxylation of α-amino acids



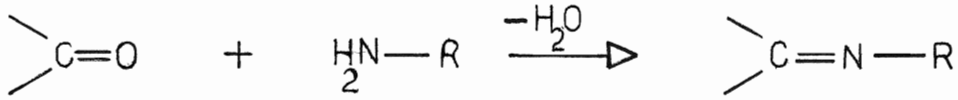
Dopa (1) Dopamine (2)

(b) Deamination of α-amino acids



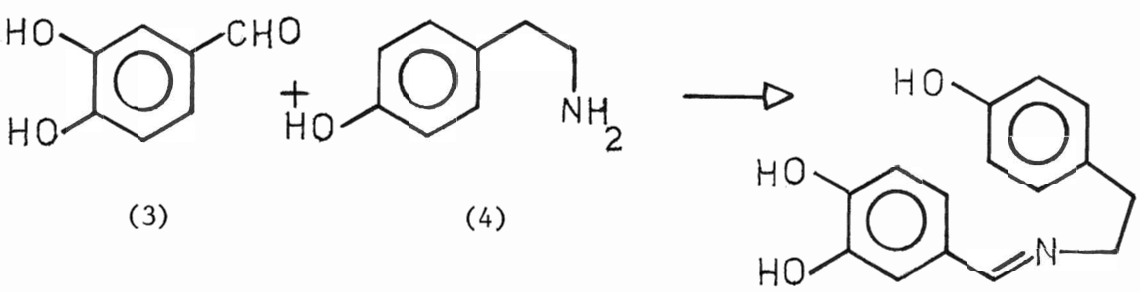
(c) Schiff base formation

Condensation of a primary amine with an aldehyde or ketone gives a Schiff base.

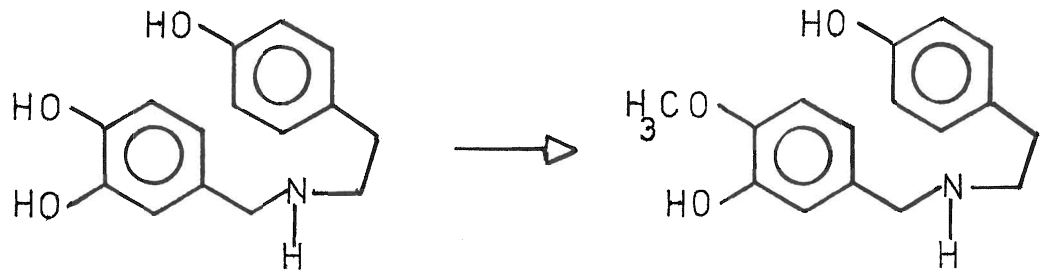


Schiff base

Protocatechuic aldehyde (3) can be derived[4] from phenyl alanine which in turn condenses with tyramine (4) to yield the Schiff base (5). Norbelladine (6) is formed on reduction, which is methylated to O-methyl-norbelladine (7). 7 is the precursor of a number of alkaloids of Amaryllidaceae family.[5]



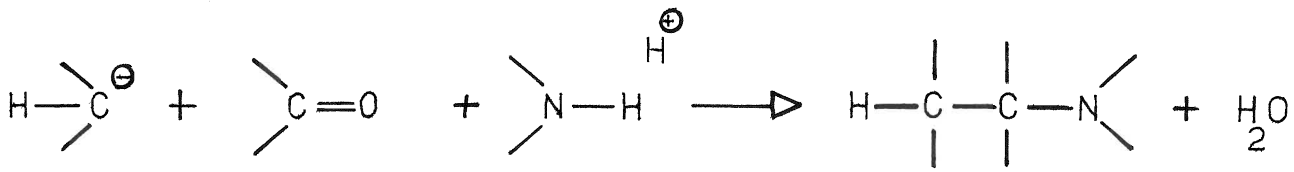
(3) (4) (5)



(6) (7)

(d) Mannich reaction

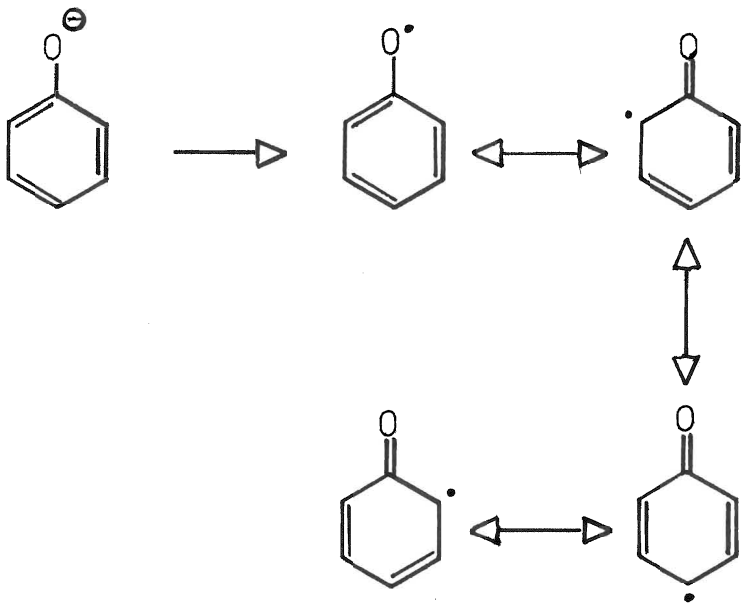
In general terms this reaction involves the formation of two covalent bonds by the condensation between an amine, a carbonyl compound and an electronegative carbon atom.



A large group of alkaloids which contain the 1,2,3,4-tetrahydro isoquinoline ring system are apparently formed by a Mannich reaction involving dopamine (2), and a variety of carbonyl compounds.[6]

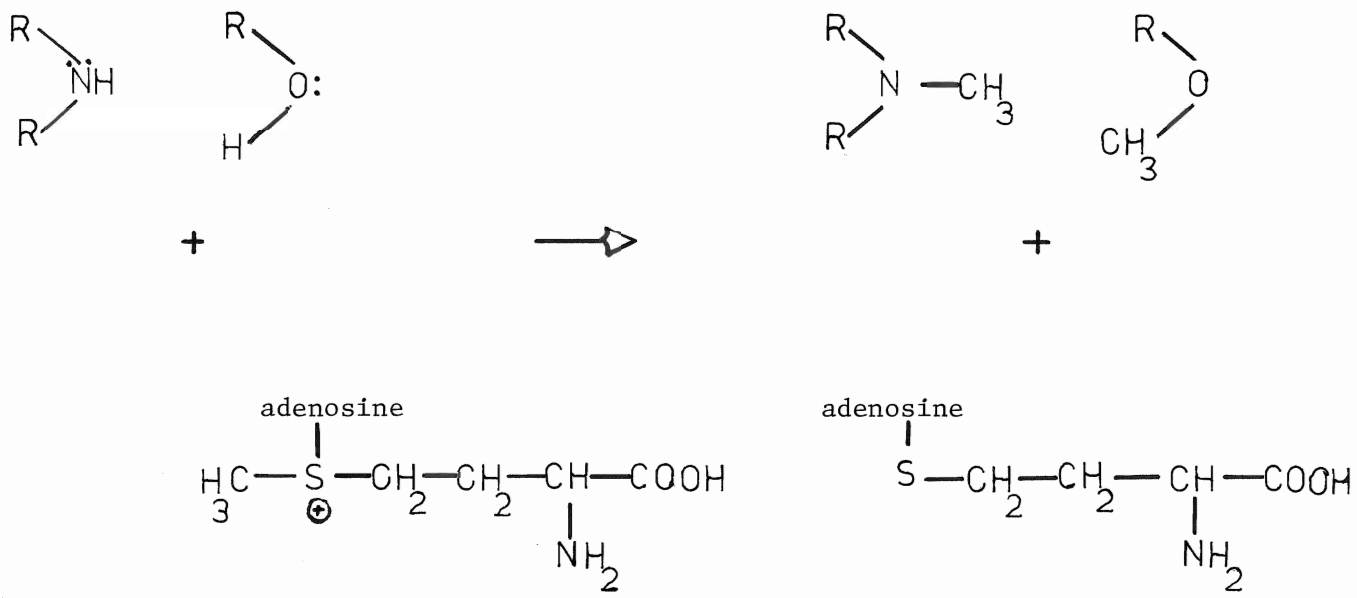
(e) Phenol oxidative coupling

This is one electron oxidation of phenols by radical mechanism[7] leading to a resonance stabilized radical.



There could be three possible modes of coupling, O-O, C-C, or C-O. Of these possibilities, by far the commonest and the most important is C-C coupling, resulting in formation of a carbon-carbon bond. In such a case then there are three possible positional modes for coupling, ortho-ortho, ortho-para, or para-para, and all of these have been observed in alkaloid biosynthesis. Indeed much of the present detailed information on the biosynthesis and biogenesis of isoquinoline alkaloids is a direct consequence of the application of these simple principles.

Besides all above, there could be reactions of minor importance such as the oxidation of an aldehyde to a carboxylic acid, epoxidation of a double bond, dehydration, and the formation of an N- or O-acetyl derivative. The most important of these is the methylation. The biological reagent for this conversion is S-adenosyl L-methionine (8), and the mechanism of this reaction is thought to be as follows.



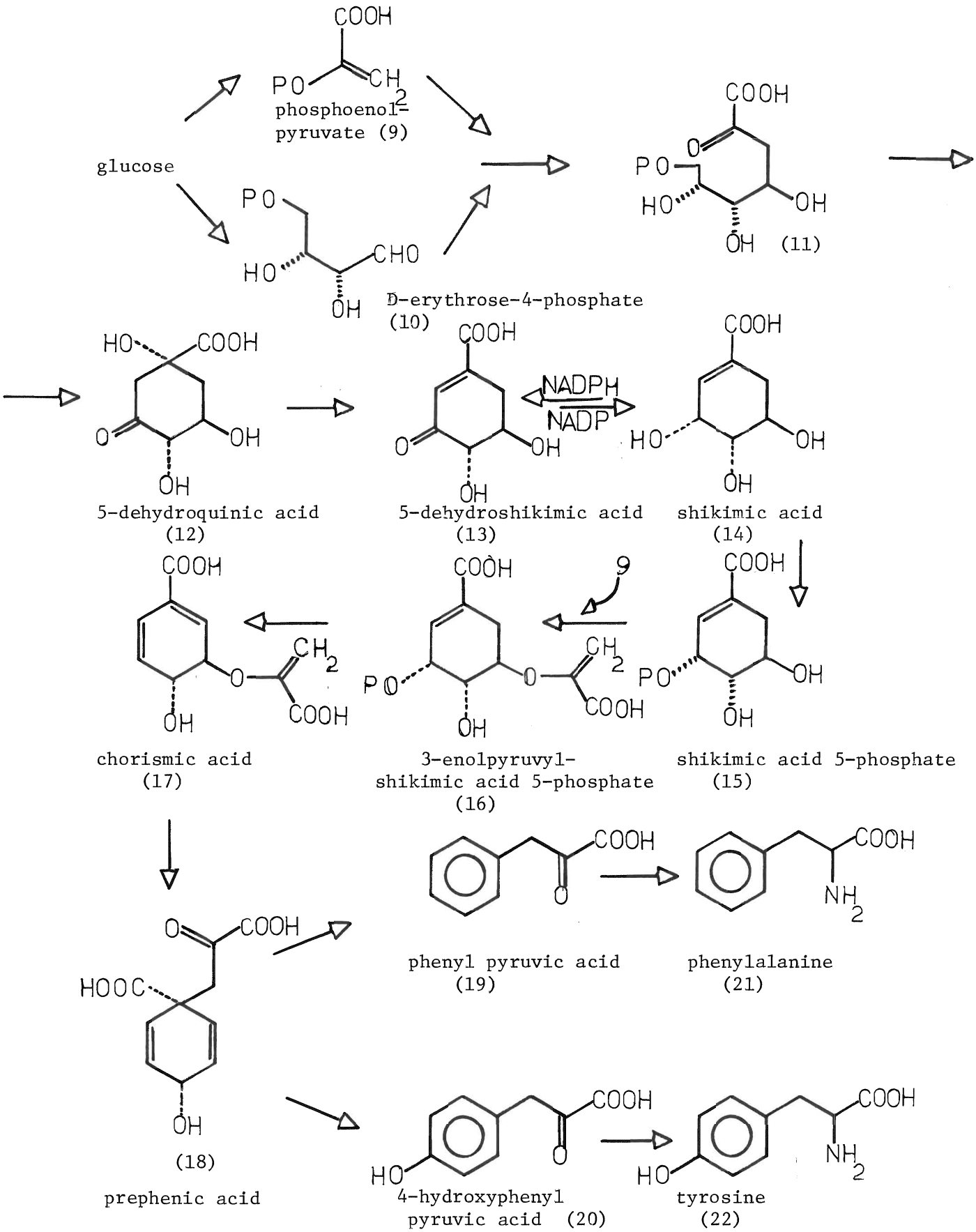
(8)

Most of the reactions discussed above are just hypothetical and their validity can be established only if the biosynthetic experiments are performed at enzymatic level.

AROMATIC AMINO ACIDS AS PRECURSORS AND THEIR BIOSYNTHESIS.

The majority of the alkaloids are derived from a relatively small number of amino acids. These amino acids are not of course, incorporated as such into alkaloids; biological modification of the amino acids leads to reactive intermediates which function as the actual compounds from which the alkaloids are constructed.

Amino acids are biosynthesized from sugars arising independently from the shikimic acid pathway.[8] The major stages of the biosynthetic route in micro organisms are summarized in Scheme II.


glucose
phosphoenol-pyruvate (9)
D-erythrose-4-phosphate (10)
(11)
5-dehydroquinic acid (12)
5-dehydroshikimic acid (13)
NADPH
NADP
shikimic acid (14)
chorismic acid (17)
3-enolpyruvyl-shikimic acid 5-phosphate (16)
shikimic acid 5-phosphate (15)
(18)
prephenic acid
phenyl pyruvic acid (19)
phenylalanine (21)
4-hydroxyphenyl pyruvic acid (20)
tyrosine (22)

Scheme II

BIOSYNTHESIS OF ISOQUINOLINE ALKALOIDS

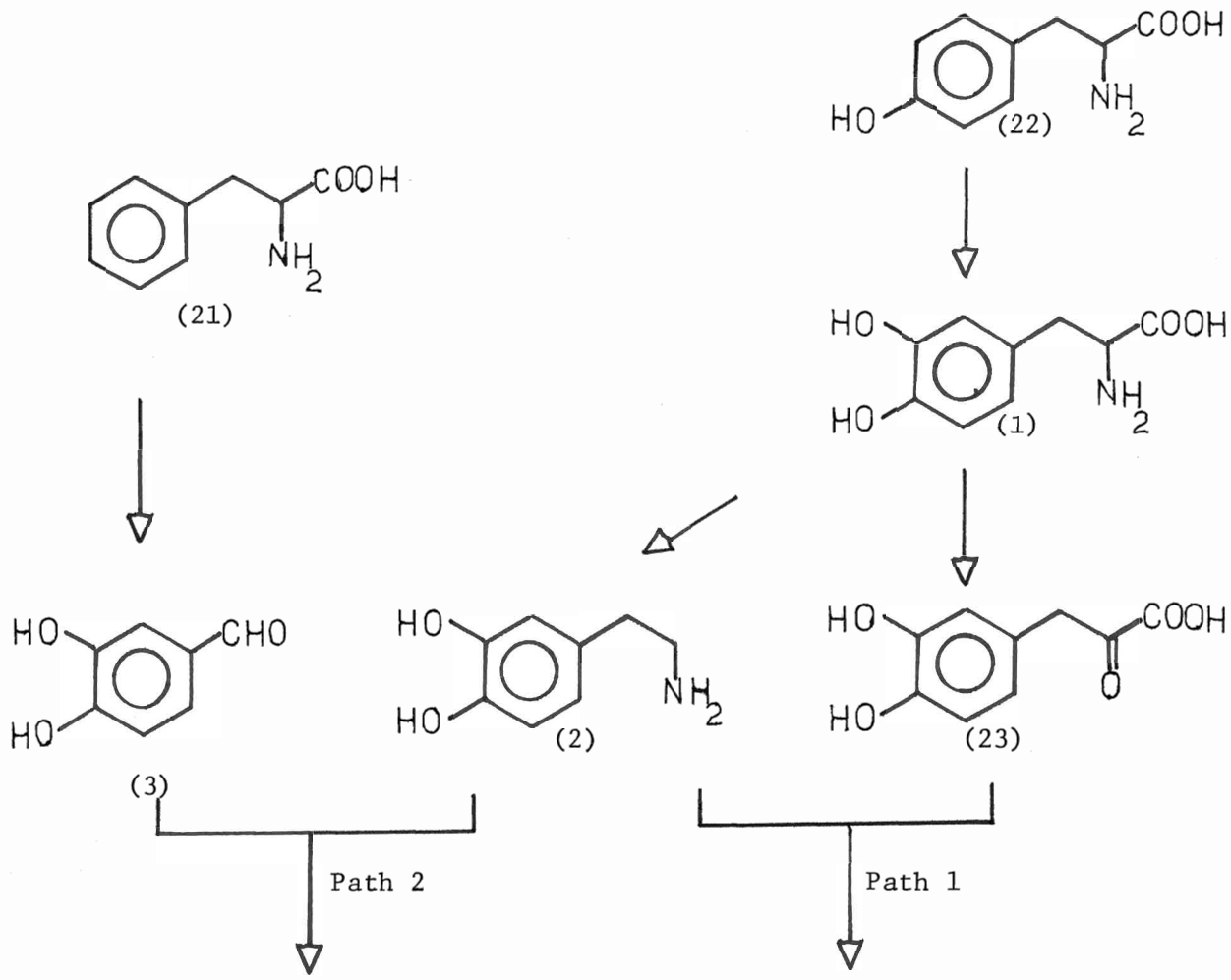
Isoquinoline alkaloids are derived from the aromatic amino acids, phenylalanine (21) and tyrosine (22). Decarboxylation of these acids leads to the corresponding β-aryl-ethyl amines (2), which in turn are considered to incorporate into a large variety of alkaloids by combining either with an aldehyde or the corresponding pyruvic acid. Although the normal pattern is thought to involve a pyruvic acid, the incorporation of aldehyde has also been referenced.[9,10]

Those isoquinolines in which there is no substituent at position 1 are theoretically derivable from formaldehyde as the second component. It is very doubtful whether formaldehyde ever occurs in plants[11] even in very low concentrations. A large number of compounds which may be regarded as formaldehyde equivalent, e.g., glyoxylic acid and formic acid do occur in plants.

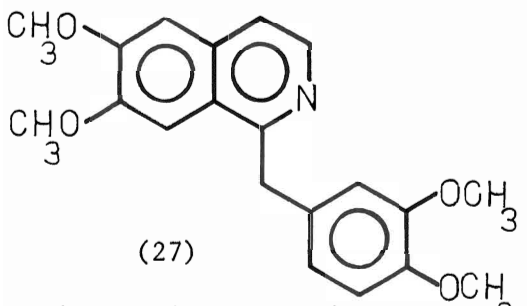
Scheme III is an outline of two of the most important routes by which β-phenyl ethyl amines are further metabolized.

Norlaudanosoline (24) is the parent base among morphine alkaloids. Specific methylation of 24 leads to the key intermediate reticuline (32) (Scheme IV). The incorporation of an active C_1 fragment in plants is a well known synthetic step and is promoted by enzymes involving folic acid as cofactor.

Phenol oxidative coupline of (-)reticuline (32a) gives the alkaloid salutaridine (33), from which thebaine (34), codeine (35) and morphine (26) are successively metabolized (Scheme IV). These transformations have all been substantiated by means of tracer experiments.[12]

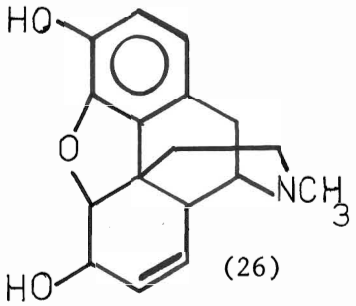

COOH
NH2
(21)
HO
(22)
(1)
CHO
(3)
(2)
(23)
O
Path 2
Path 1
N-benzyl-β-phenylethyl
amines (6)
NH
OH
(24)
1-benzyl-tetrahydroisoquinoline
alkaloids (norlaudanosoline)
Amaryllidaceae alkaloids
Belladine (26)

Scheme IIIa



(27)

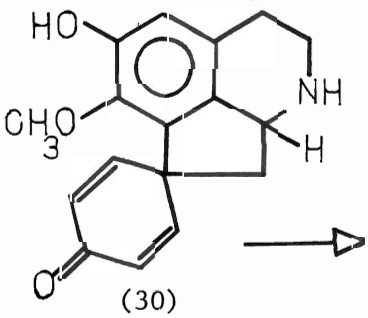
1-benzyl isoquinoline alkaloids (papaverine)



(26)

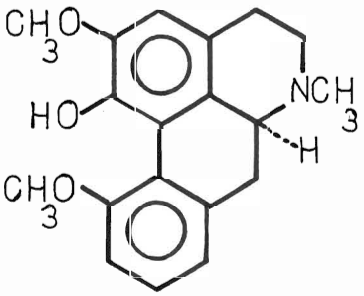
morphine alkaloids (morphine)

norlaudanosoline (24)



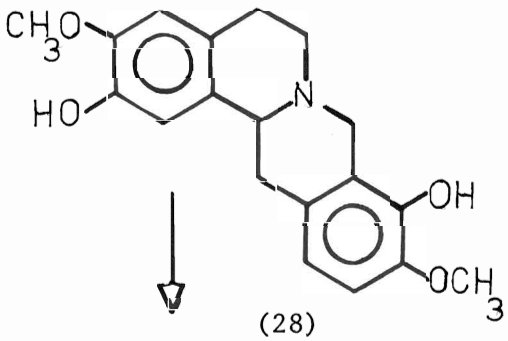
(30)

Proaporphine alkaloids (crotonosine)



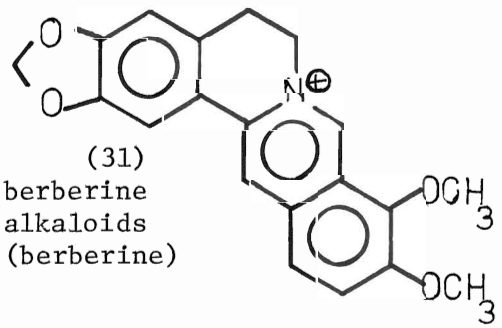
(29)

aporphine alkaloids (isothebaine)



(28)

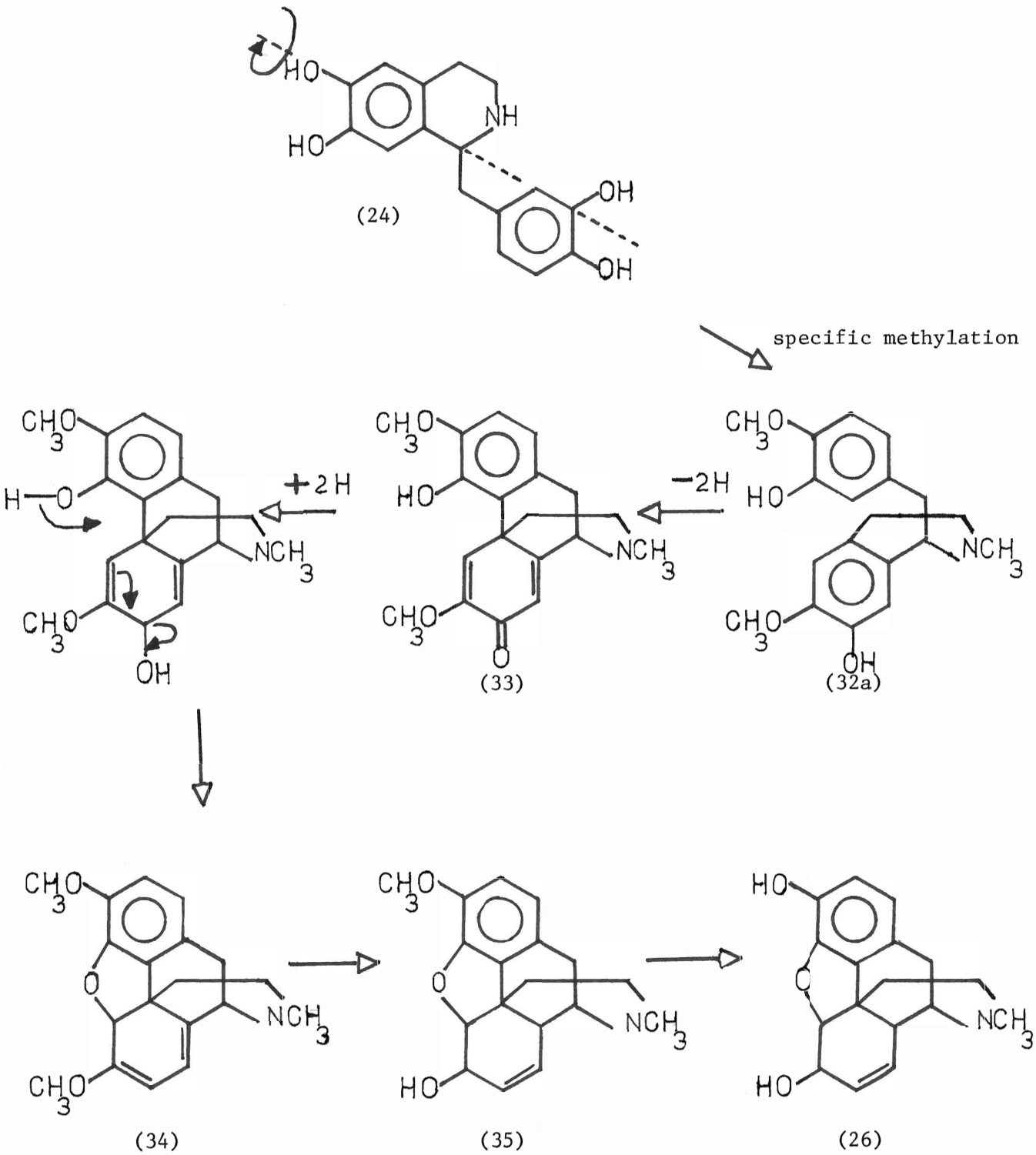
protoberberine alkaloids (scoulerine)



(31)

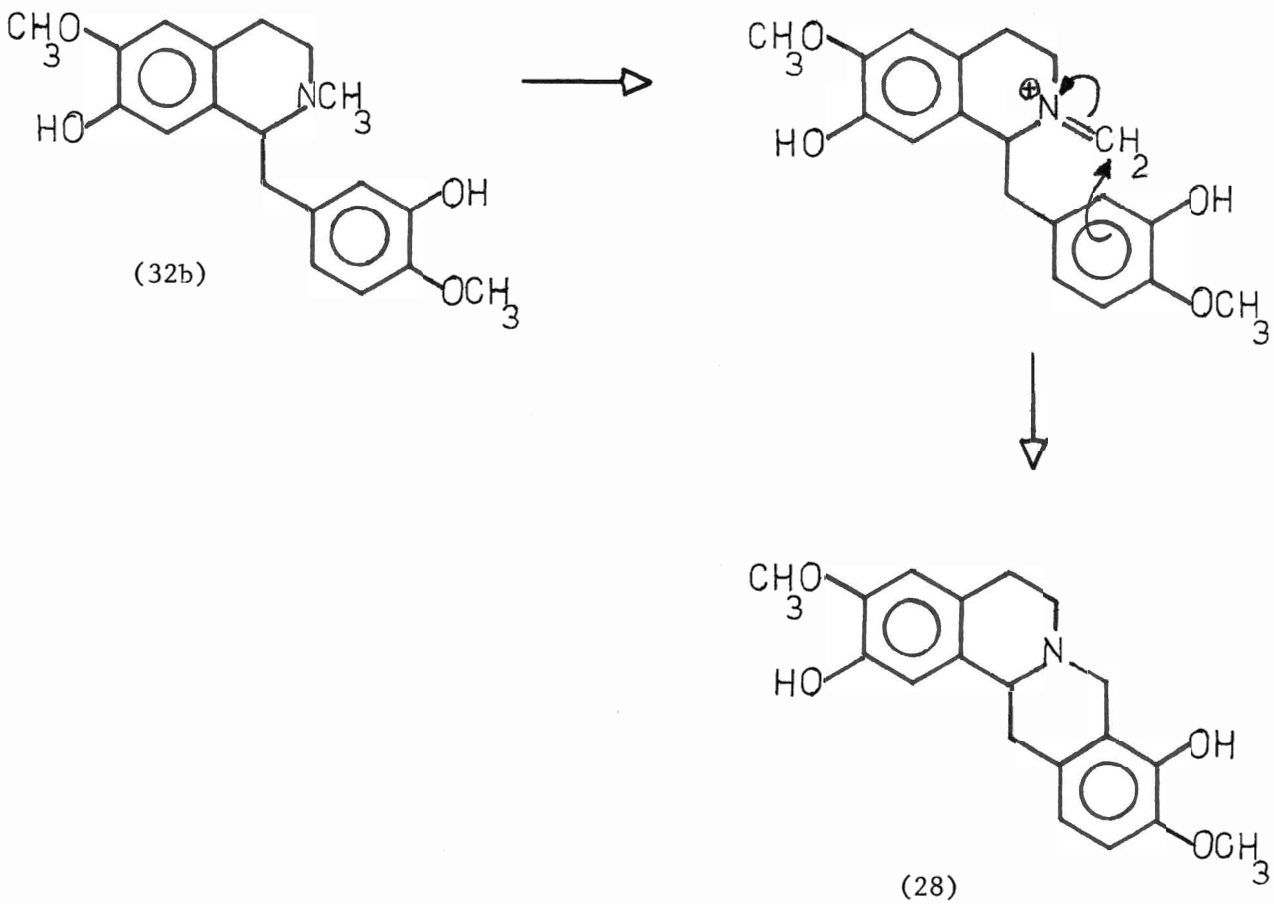
berberine alkaloids (berberine)

Scheme IIIb


HO
HO
NH
OH
OH
(24)
specific methylation
CH3O
H—O
+2H
NCH3
CH3O
OH
CH3O
HO
−2H
NCH3
CH3O
O
(33)
CH3O
HO
NCH3
CH3O
OH
(32a)
CH3O
O
NCH3
CH3O
(34)
CH3O
O
NCH3
HO
(35)
HO
O
NCH3
HO
(26)

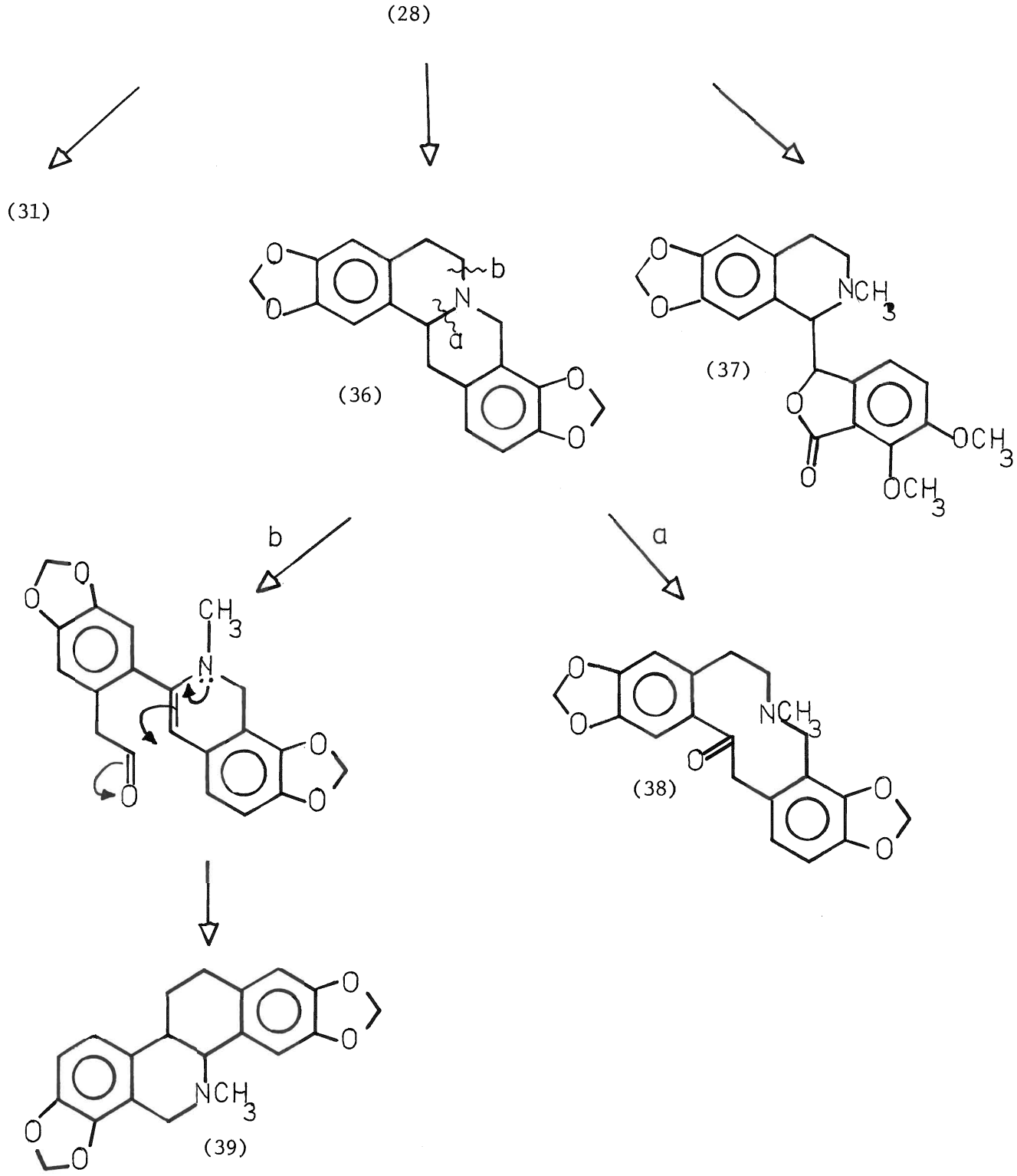
Scheme IV

(+)Reticuline (32b) has been shown (Scheme V) to be an intermediate in the biosynthesis of berberine (31),[13] protopine (38),[13] phthalide-isoquinoline (56),[14] and benzophenanthridine (39)[15] alkaloids. Scoulerine (28) here is the key intermediate that can be transformed into berberine (31), stylopine (36), narcotine (37) and chelidonine (39). Alkaloids of the protopine (38) series are derived from berberine (31) type alkaloids by hydrolysis of the bond between the nitrogen atom and the tertiary carbon atom.



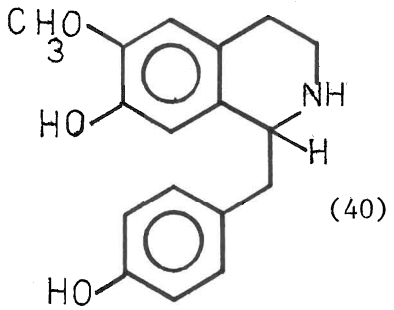
(continued)

Scheme V



Scheme V

There are a number of isoquinoline alkaloids which contain a dienone system, e.g., salutaridine (33) and crotonosine (30). Crotonosine has been shown[16] to be biosynthesized from the 1-benzyltetrahydro isoquinoline alkaloid, (+) coclaurine (40).

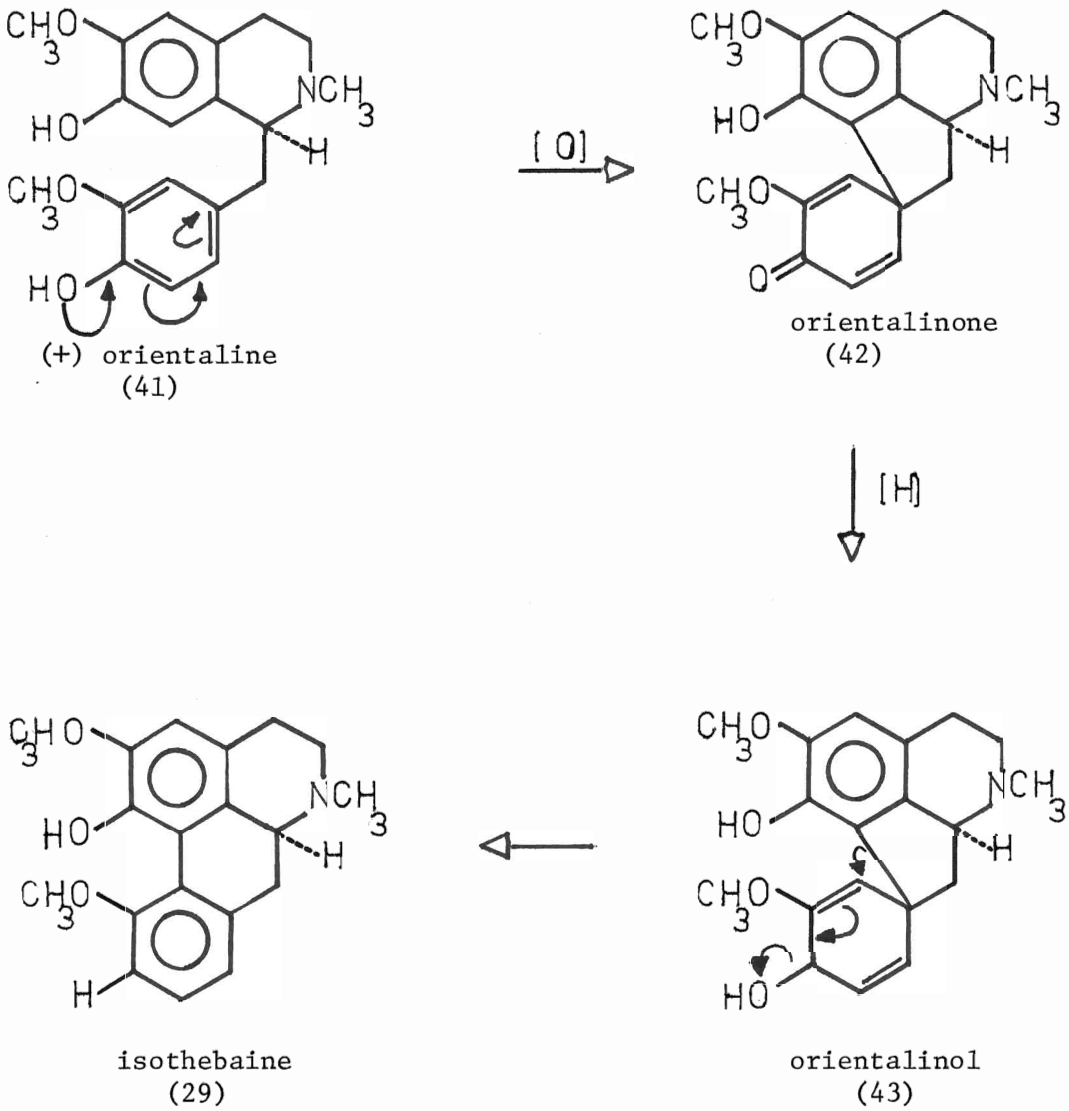


(40)

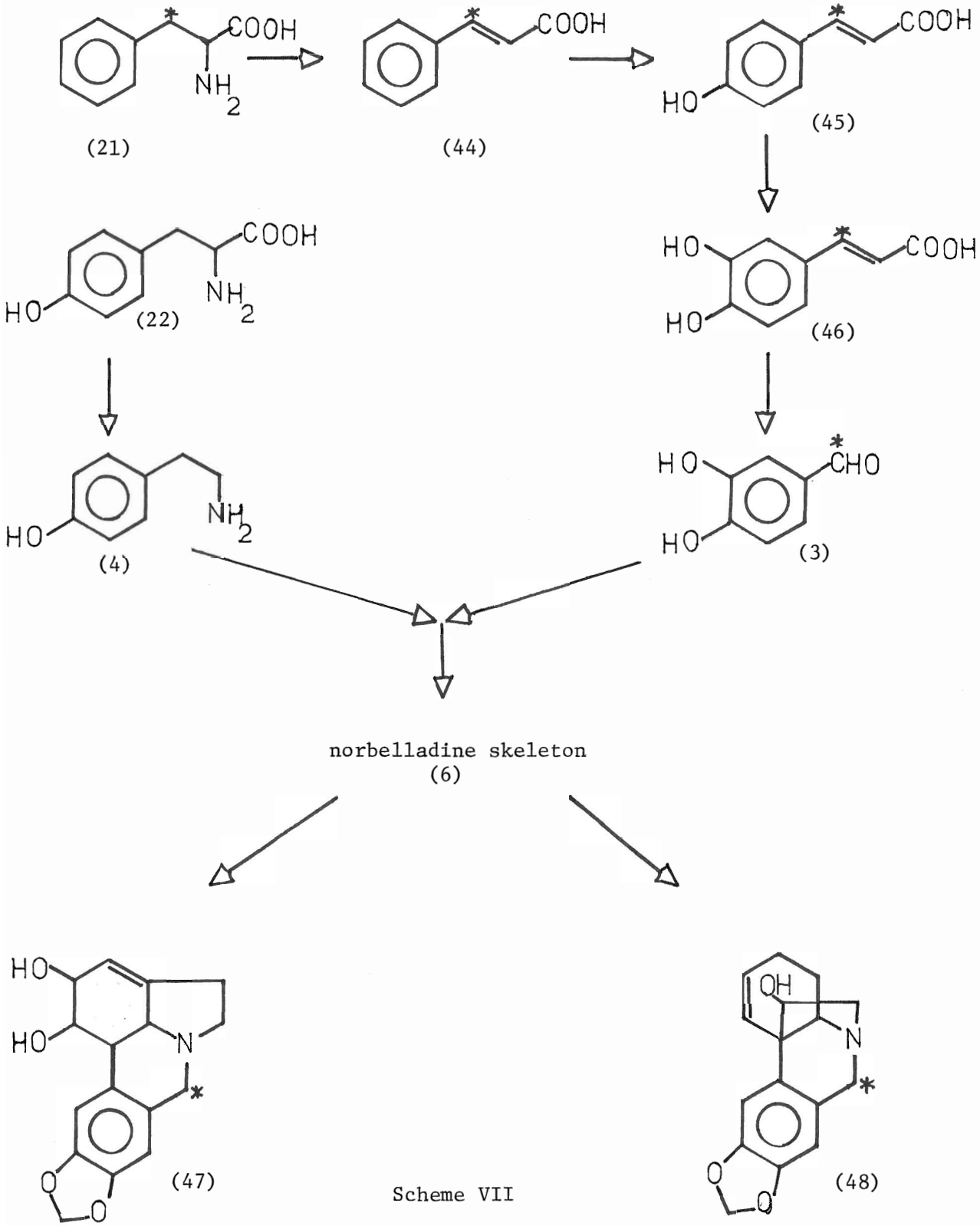
Crotonosine alkaloids are called pro-aporphine alkaloids, as they may undergo dienol benzene rearrangement leading to the aporphine alkaloids. Isothebaine (29) has been shown[17] to be derived from orientaline (41) through the pathway shown in Scheme VI.

The second pathway of β-phenyl ethyl amines involves Norbelladine (6) as key intermediate, which derives from tyrosine (22) and phenylalanine (21). C_6-C_2 unit is tyramine (4), and is derived from tyrosine,[18,19] whereas phenylalanine forms the C_6-C_1 unit through the following pathway (Scheme VII).

The sequence shown in Scheme VII has been established through labelling studies. The activity from [β-^{14}C]phenylalanine, [3-^{14}C]cinnamic acid (44),[20,21] [3-^{14}C] p-coumaric acid (45),[22] [3-^{14}C] caffeic acid (46),[23] and [7-^{14}C] protocatechuic aldehyde (3)[23] has been shown to be specifically incorporated into lycorine (47), and haemanthamine (48) at marked positions.


CH3O
HO
NCH3
H
CH3O
HO
(+) orientaline
(41)
[O]
CH3O
HO
NCH3
H
CH3O
O
orientalinone
(42)
[H]
CH3O
HO
NCH3
H
CH3O
HO
orientalinol
(43)
CH3O
HO
NCH3
H
CH3O
H
isothebaine
(29)

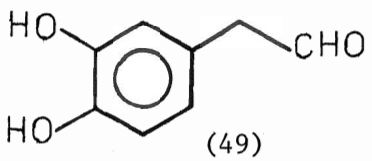
Scheme VI


COOH
NH2
(21)
COOH
(44)
COOH
HO
(45)
COOH
NH2
HO
(22)
HO
HO
COOH
(46)
NH2
HO
(4)
HO
HO
CHO
(3)
norbelladine skeleton
(6)
HO
HO
N
O
O
(47)
OH
N
O
O
(48)

Scheme VII

BIOSYNTHESIS OF BENZYLISOQUINOLINE ALKALOIDS

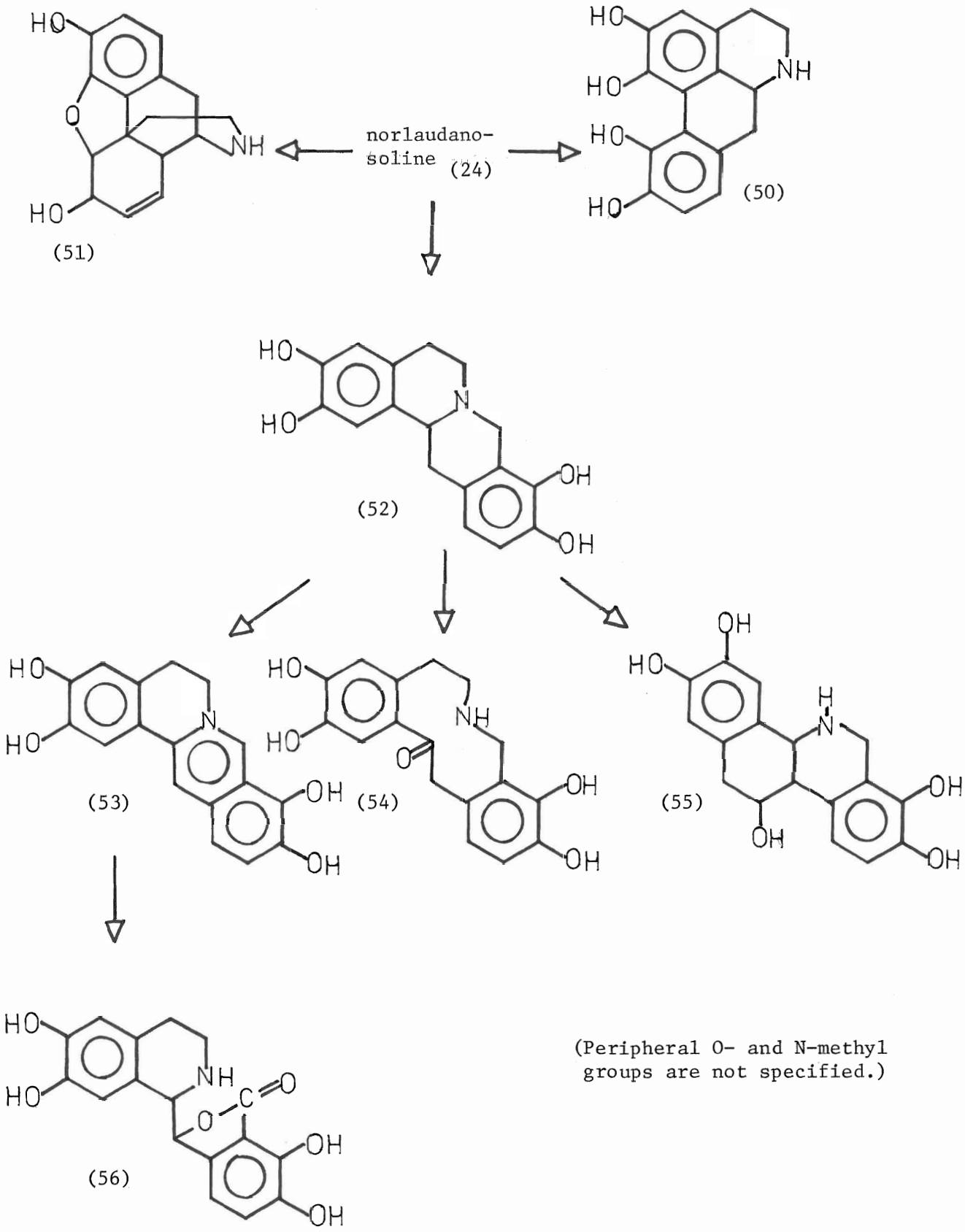
Winterstein and Trier[24] were the first to propose that these alkaloids could be formed in nature from norlaudanosoline (24), which is considered to be derived from dopamine (2), and 3,4-dihydroxyphenylpyruvic acid (23) or 3,4-dihydroxyphenylacetaldehyde (49), each derivable from tyrosine or phenylalanine.



(49)

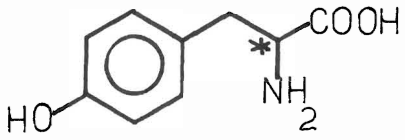
This view was further developed by Robinson[25] who pointed out the relationship between the skeletons of benzylisoquinoline (24), hydrophenanthrene (51), and the aporphine (50) alkaloids (Scheme VIII). He also pointed out that the tetrahydro protoberberine nucleus (52) was related to the skeletons of protopine (54), phthalideisoquinoline (56), and benzophenanthridine (55) alkaloids.

The validity of the Winterstein-Robinson hypothesis have been proved through several tracer experiments.[26] Since two C_6-C_2 units are proposed to be derived from tyrosine, the radioactivity from singularly labelled tyrosine should thus enter two sites in the skeleton of these alkaloids. The radioactive bases isolated from [α-^{14}C] DL tyrosine fed plants did show the predicted labelling pattern. Active samples of papaverine (27),[27] morphine (26),[28] and narcotine[29] isolated from Papaver somniferum; thebaine (34) from Papaver bracteatum[30] hydrastine (57)[31] and berberine (31)[32] from Hydrastis canadensis; and chelidonine (39)[33] from Chelidonium majus were degraded and in each case radioactivity was found to be confined to marked positions (Scheme IX).



(Peripheral O- and N-methyl groups are not specified.)

Scheme VIII



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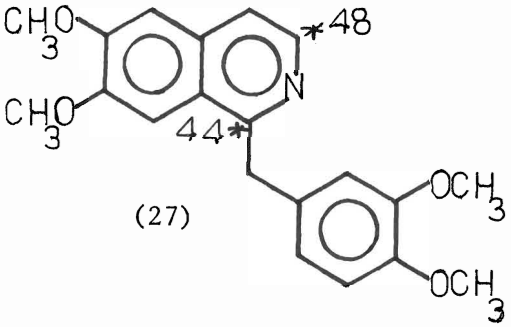

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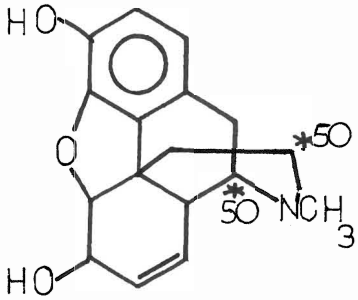

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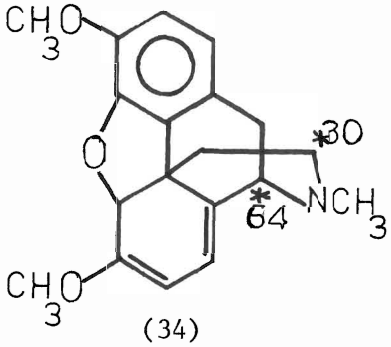
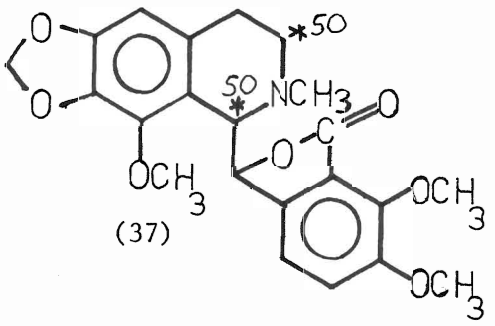
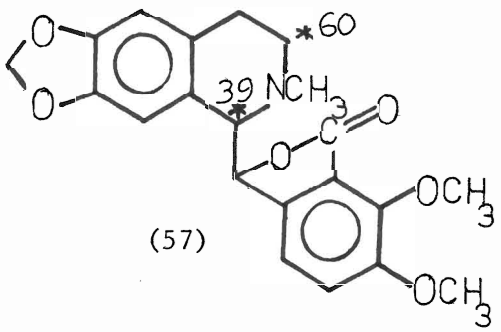


(34)



(37)



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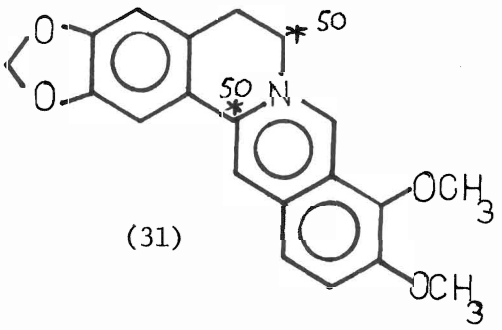
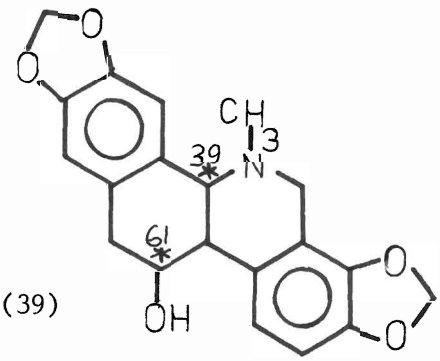


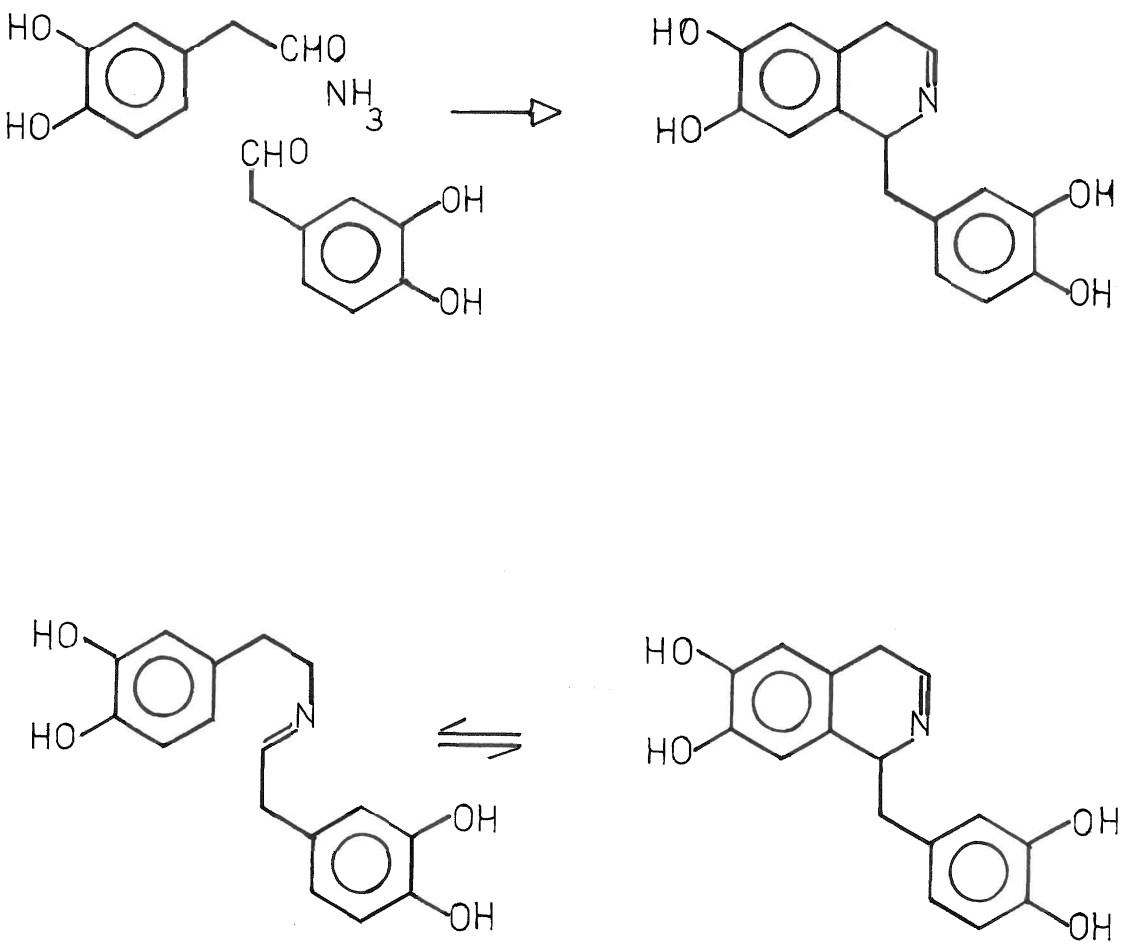
(31)



(39)

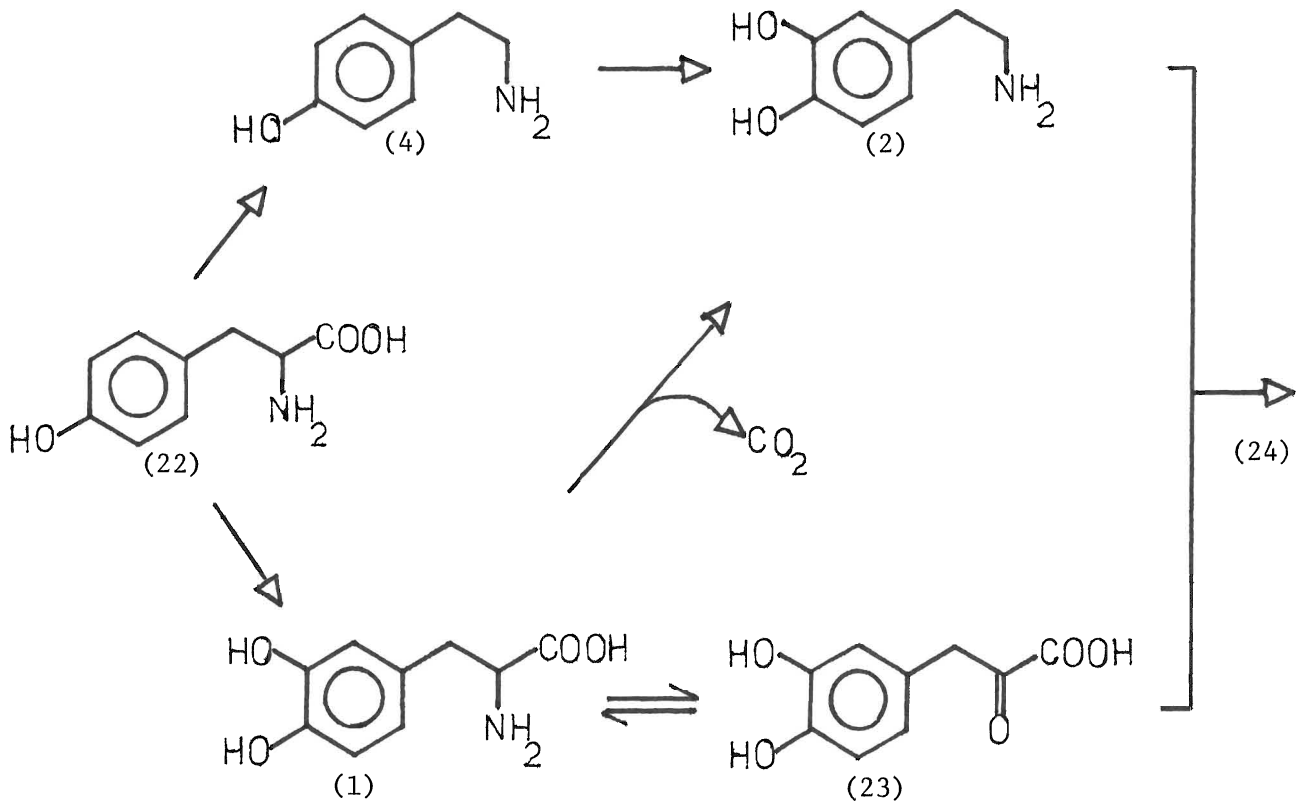
Scheme IX

In several cases, the radioactivity was equally distributed, within the experimental error, between the two labelled centres. In case of equal labelling of two halves, there must be the involvement of two identical intermediates in the "doubling step" or there must be a symmetrical intermediate involved in the pathway (Scheme X). The unequal distribution between the two labelled centres in tyrosine derived hydrastine, chelidonine and thebaine indicates that neither of these alternatives is valid in the present case, and that two C_6-C_2 units differ from each other at a later stage in the pathway, although they both are derived from tyrosine.



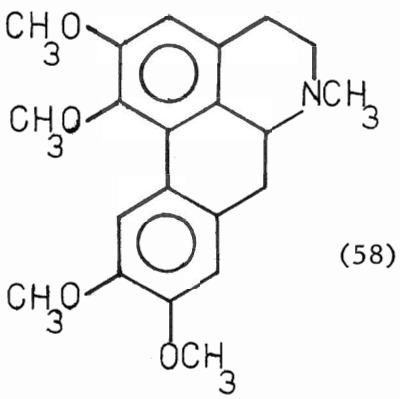
Scheme X

The fate of tyrosine during the biosynthesis of benzylisoquinoline and related alkaloids is not fully established yet, although it goes to both halves of the basic skeleton with equal facility.[34] Dopamine (2) contributes only to the upper portion,[35] and tyramine (4) also is a precursor to this part only.[36] The amino acid dopa (1) has been proposed[37] as the intermediate between tyrosine and 3,4-dihydroxyphenylpyruvic acid (23), the putative precursor[38] of the lower portion of the molecule. The decarboxylation of dopa can lead to dopamine (2). The route shown in Scheme XI can, therefore be proposed for the conversion of two molecules of tyrosine (22) into benzylisoquinoline skeleton (24).



Scheme XI

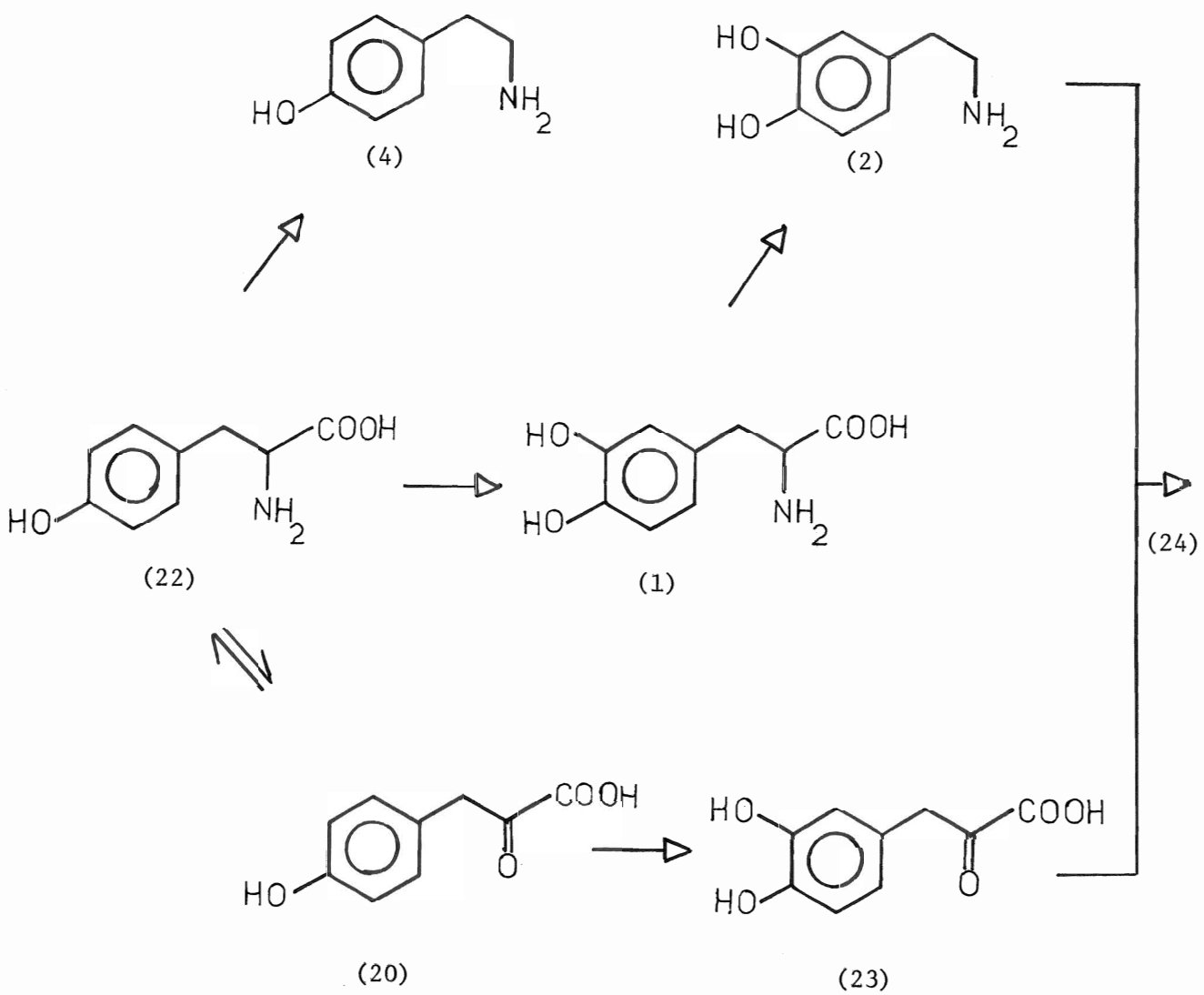
Tracer experiments, however, do not agree with a pathway like that shown in Scheme XI. It has been reported by Battersby et al. that 97 and 96% of the radio label from [α-^{14}C] dopa was located in the upper portions of the morphine (26)[39] and aporphine alkaloid glaucine (58)[40] respectively.



(58)

Tewari et al.[41] observed the 98% incorporation of [α-^{14}C] dopa into the upper portion of reticuline (32) in Litsea glutinosa.

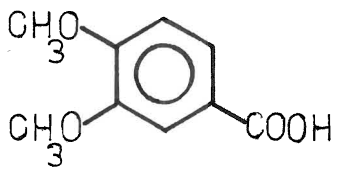
In a separate set of experiments carried out by Holland and MacLean,[42] [3-^{14}C] dopa was fed to the plants which contain protoberberine derived bases as major alkaloid components. In all cases examined the incorporation of dopa was predominantly into the upper portion of the molecule. Since tyrosine incorporates with almost equal ease into both portions of the molecule,[43] it follows that tyrosine cannot be metabolized solely via dopa in its incorporation into both halves of the molecule. There must be an alternative pathway of tyrosine incorporation not involving dopa, and leading to a molecule such as 4-hydroxyphenylpyruvic acid (20) or 3,4-dihydroxyphenylpyruvic acid (23), which can be condensed with dopa (1) or dopamine (2) to form the benzylisoquinoline skeleton (24). This pathway could be like the one shown in Scheme XII.


HO
NH2
(4)
HO
HO
NH2
(2)
COOH
HO
NH2
(22)
HO
HO
COOH
NH2
(1)
(24)
COOH
O
HO
(20)
HO
HO
COOH
O
(23)

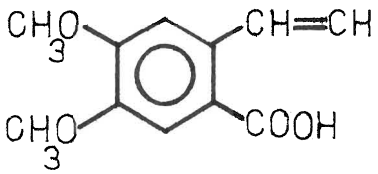
Scheme XII

The observation of Wilson and Coscia,[44] that dopa does indeed contribute to the lower portion of norlaudanosoline (24) in Papaver orientale, means that oxidation of dopa to 3,4-dihydroxyphenylpyruvic acid (23) must occur to an appreciable extent in that plant, whereas in most of other plants studied, this pathway of dopa metabolism is of minor importance.

The incorporation of (20) and (23) into reticuline (32), in fact has been observed in Litsea glutinosa by Bhakuni et al.[45] Feeding of 4-hydroxy-[3,5-$^3H_2$]-phenylpyruvic acid (20) showed that it is efficiently utilized by the plants to form reticuline (32). This biosynthetic reticuline on degradation yielded [5-^{3}H]-veratric acid (59) (42% of the activity of the original base); and vinyl [2-^{3}H]-veratric acid (60) (48.6% of the activity of the original base) indicating that (20) gives rise to both the constituent units of reticuline.



(59)

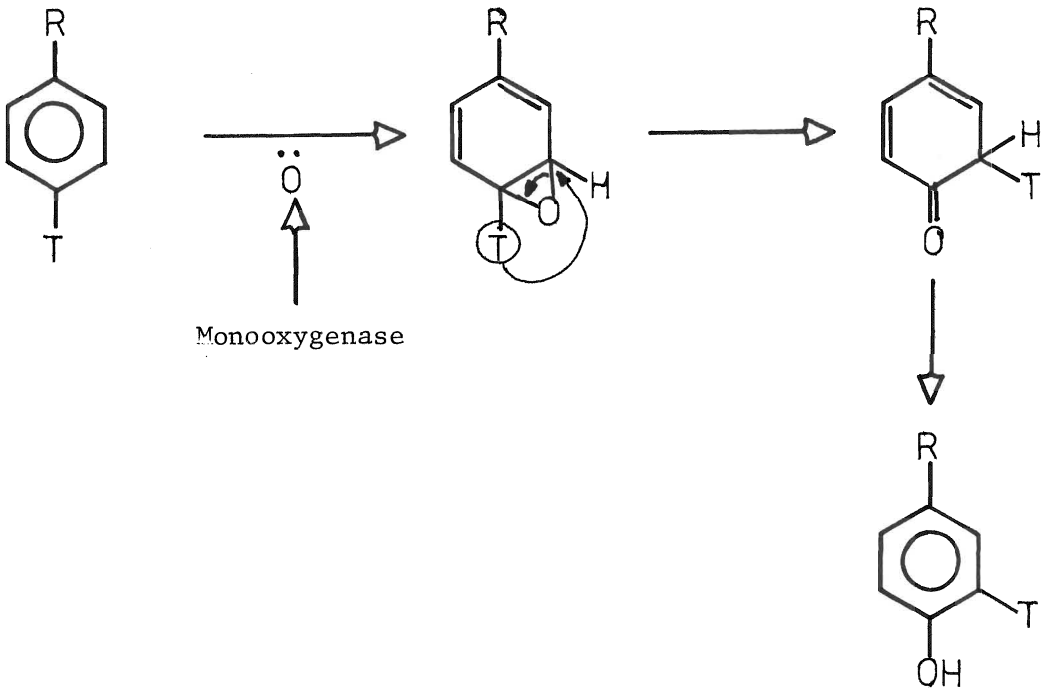


(60)

3,4-Dihydroxy-[2,4,6-$^3H_3$]-phenylpyruvic acid (23) was also shown to be contributed to both halves of the reticuline. Its incorporation into phenyl ethyl portion suggests that it is aminated in the plants to give dopa (1).

The findings of Bhakuni et al.[45] however, cannot confirm the metabolic pathway of tyrosine in plants which contain berberine and related alkaloids as major bases, because tritium could be lost in "berberine bridge" formation,

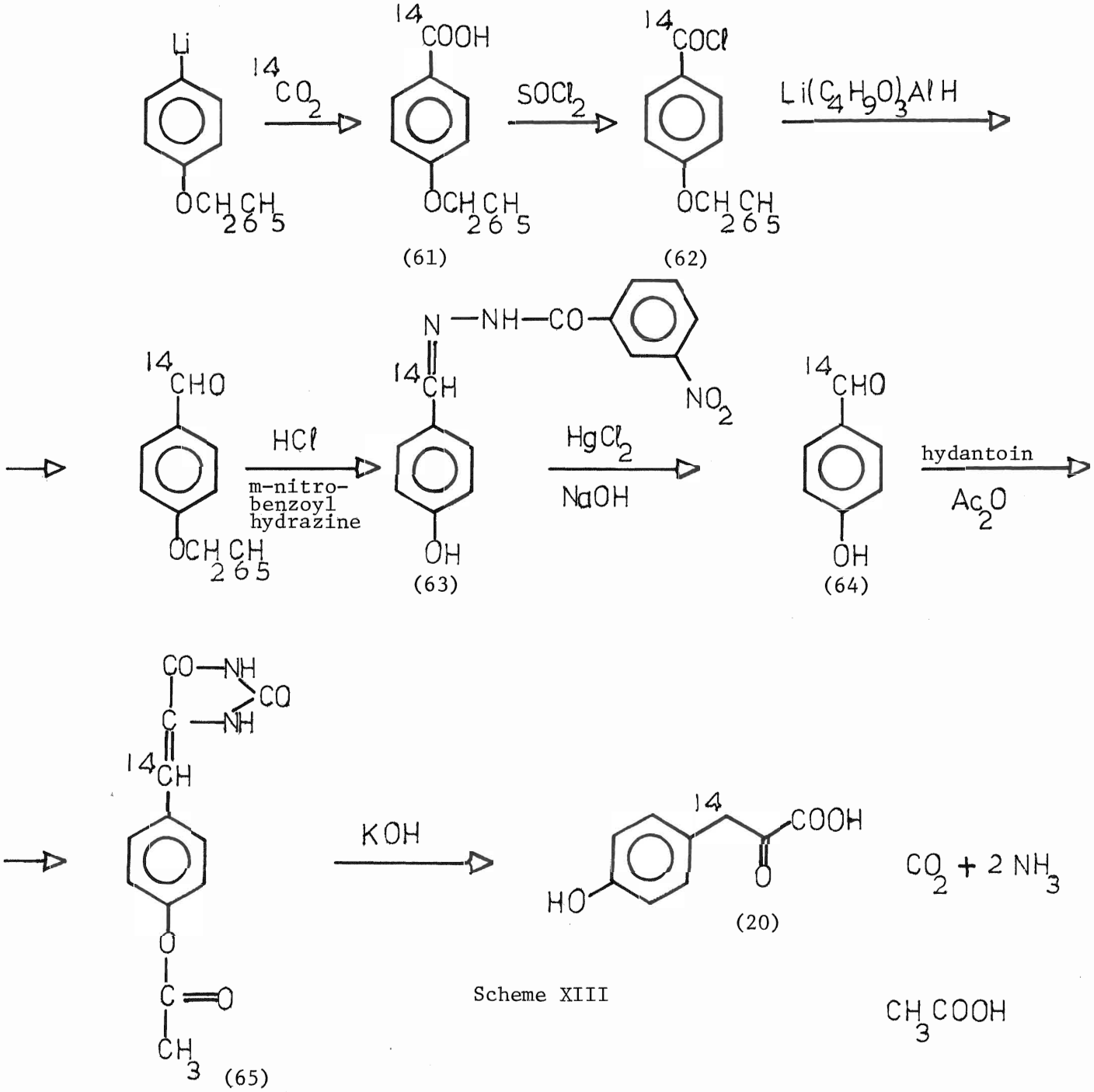
or due to the involvement of possible NIH shifts during aromatic hydroxylation. The mechanism of NIH shift is illustrated in the following figure.



Carbon-14, however cannot be lost during such processes, and the compounds (20) and (23) labelled with carbon-14 can provide more quantitative results. The main purpose of this research was to develop a convenient route for the synthesis of 4-hydroxy-[7-^{14}C]-benzaldehyde and 3,4-dihydroxy-[7-^{14}C]-benzaldehyde which then could be converted into 4-hydroxyphenyl-[3-^{14}C]-pyruvic acid (20), and 3,4-dihydroxyphenyl-[3-^{14}C]-pyruvic acid (23) respectively.

The synthesis of [3-^{14}C] (20) has been reported by Billek and Hermann.[46] The method which involves $^{14}CO_2$ as one of the reagents, is summarized in Scheme XIII.

Haavaldsen and Norseth[47] have described a method for the enzymic preparation of [3-^{14}C] (20). L-[3-^{14}C]tyrosine (22) on subjection to enzyme L-amino oxidase from snake venom yielded [3-^{14}C] (20). The authors have not reported the isolation of product and the yield is calculated on the basis of radioactivity recovered in the solution.



Scheme XIII

DISCUSSION

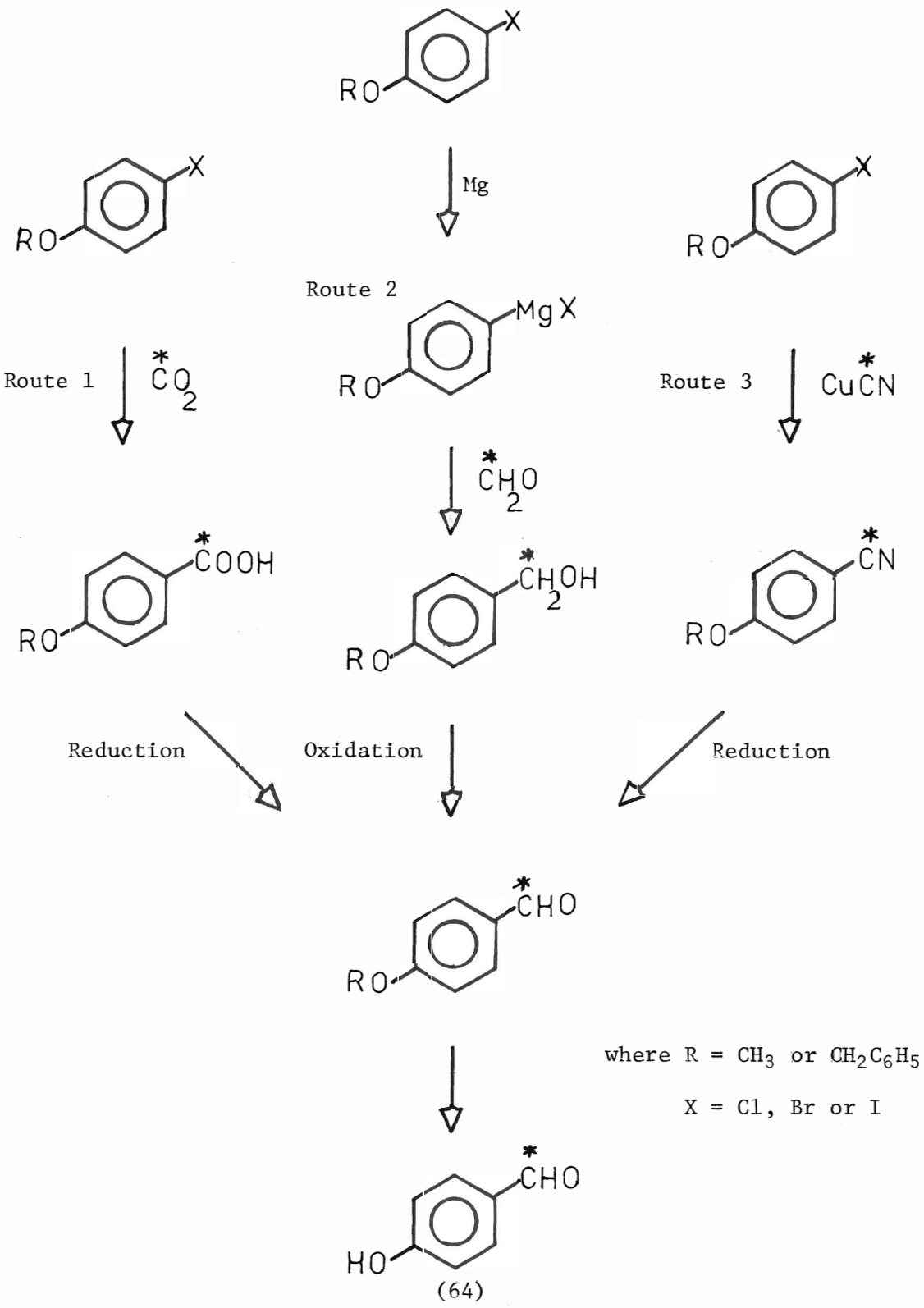
SECTION I

There could be several possible routes to make [7-^{14}C]-p-hydroxy benzaldehyde (64); some of them are shown in Scheme XIV.

Since routes 1 and 2 involve radioactive gases, which cannot be used in an ordinary low level radiation lab, the route 3 was the only alternative for the present work.

Phenols are fairly sensitive compounds and heating them in air even under mild conditions leads to their oxidation, and reactions at phenolic oxygen, which might result in undesired products. Protecting groups are normally used to overcome this problem, but in order to get a desired product in a reasonable yield, the protecting group used should be easily removable. As far as the synthesis of [7-^{14}C]-p-hydroxy benzaldehyde (64) is concerned, most of the references in literature regarding it involve protecting groups.

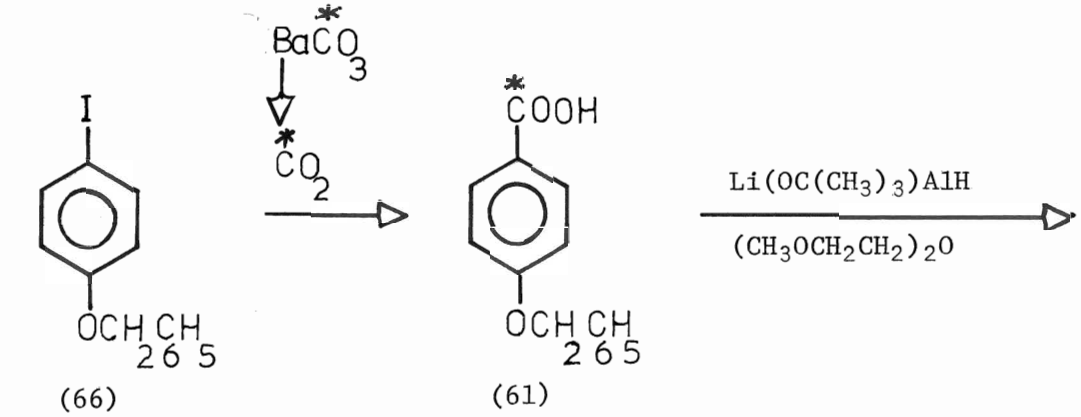
In 1960, Grisebach and Patschke[48] reported the synthesis of [7-^{14}C]-(64). $Ba^{14}CO_3$ in concentrated sulphuric acid produced $^{14}CO_2$, which on reaction with p-iodophenyl benzyl ether (66) yielded [^{14}COOH] p-benzyloxy benzoic acid (61). The acid (61) was then reduced by lithium tri-t-butoxy aluminum hydride ($LiAl(OCMe_3)_3H$) in presence of m-nitrobenzoyl hydrazine to give the hydrazone (63), which on hydrolysis yielded the aldehyde (64). (Scheme XV)



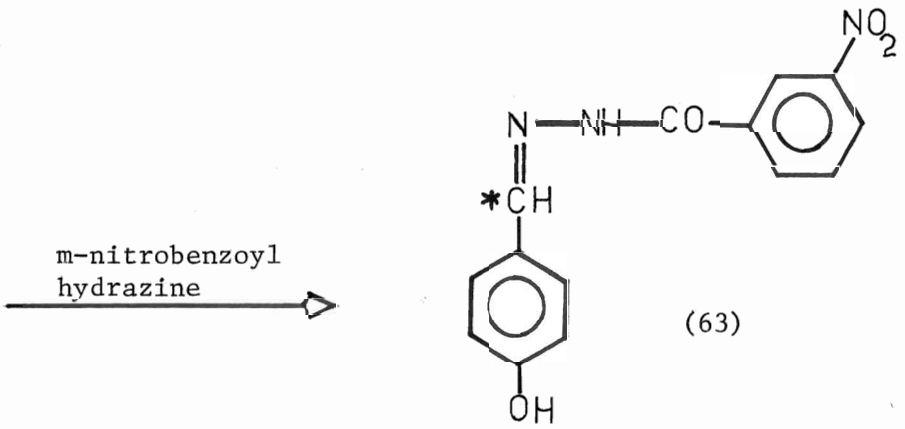
where R = CH_3 or $CH_2C_6H_5$

X = Cl, Br or I

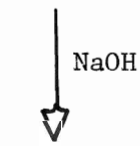
Scheme XIV

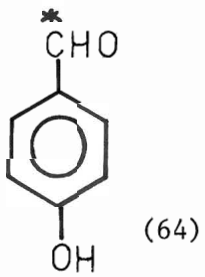


(66) (61)



(63)





(64)

Scheme XV

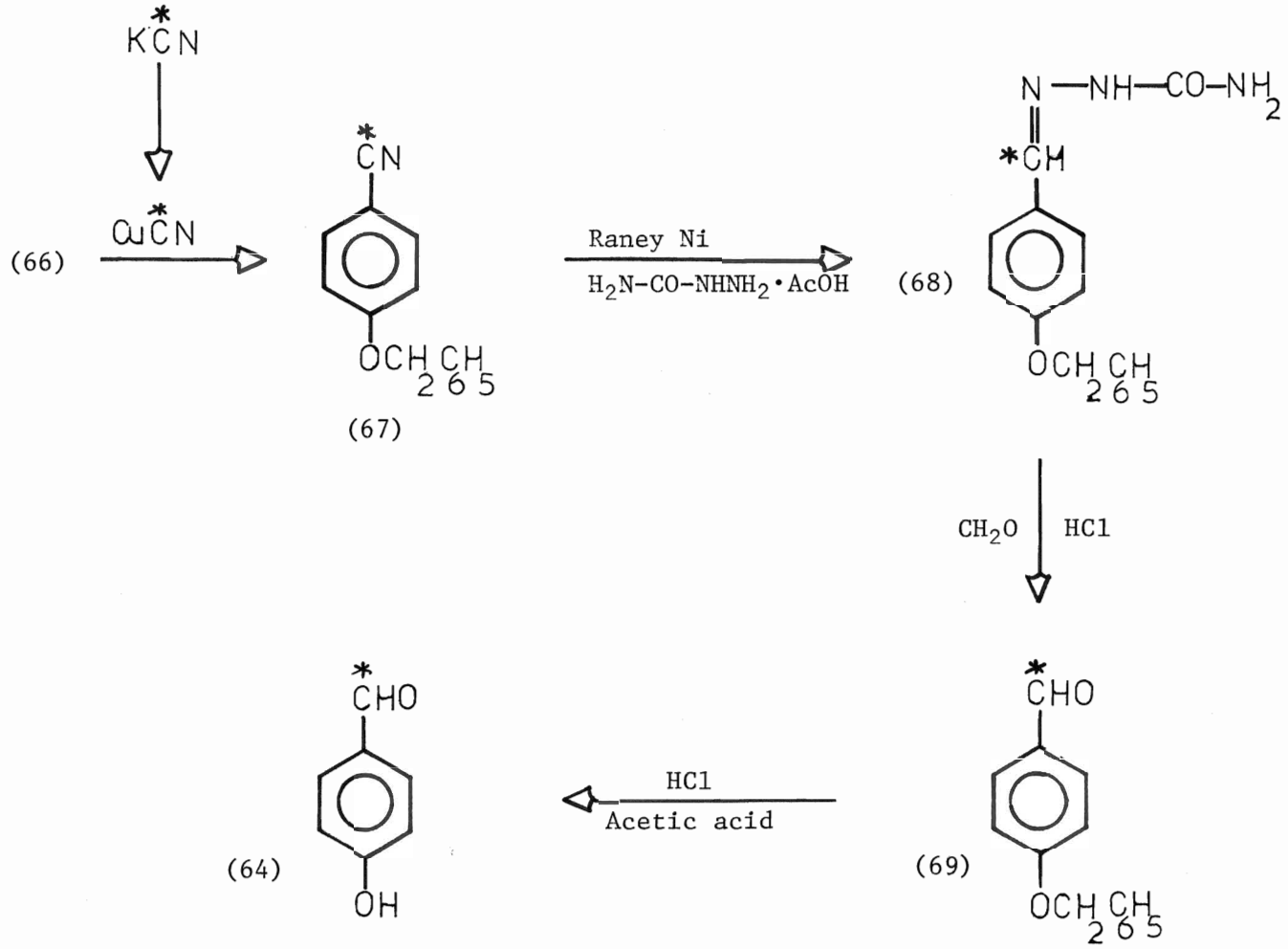
Kratz and Billek[49] previously had made [7-^{14}C]-(64) using the same method, except that (61) was converted to the corresponding acid chloride (62), which on reduction with H_2-Pd in xylene yielded the aldehyde (64). The product was purified by converting it to m-nitrobenzoyl hydrazone (63).

Later on a different route to the synthesis of [7-^{14}C]-(64), not involving any radioactive gases, was forwarded by Griseback and Patschke.[50] Cu^{14}CN was made from K^{14}CN, which on reaction with p-iodophenyl benzyl ether (66) afforded the corresponding nitrile (67). The nitrile (67) was then hydrogenated over Raney Nickel in presence of 35 equivalents of HN_2-CO-NH-NH_2·AcOH to give the semicarbazone (68), which was warmed with aqueous formaldehyde and acetic acid, treated with 2N hydrochloric acid, cooled, diluted with water and filtered to yield [^{14}CHO]-p-benzyloxy-benzaldehyde (69). [7-^{14}C]-p-hydroxy benzaldehyde(69) was obtained by heating (69) in acetic acid and concentrated hydrochloric acid for an hour (Scheme XVI). The overall yield from K^{14}CN to (64) was 26%.

During present work both benzyl and methyl ethers of p-bromophenol were used as starting material. Cuprous cyanide was used throughout to replace the bromo group by a cyano group. Cuprous cyanide itself was prepared according to Barber's method[51] using sodium cyanide, cupric sulphate, and sodium bisulphite. The reaction is illustrated in the following equation:

$$2NaCN + 2CuSO_4 + NaHSO_3 + H_2O \longrightarrow 2CuCN + 3NaHSO_4$$

In this method, cupric sulphate is reduced at the expense of bisulphite instead of cyanide, one half of which otherwise[52] would be lost as cyanogen as shown below.


K*CN
Cu*CN
(66)
*CN
OCH CH
2 6 5
(67)
Raney Ni
H2N-CO-NHNH2 • AcOH
(68)
N —NH—CO—NH2
*CH
OCH CH
2 6 5
CH2O
HCl
*CHO
OCH CH
2 6 5
(69)
HCl
Acetic acid
*CHO
OH
(64)

Scheme XVI

$$2CuSO_4 + 4NaCN \longrightarrow 2CuCN + (CN)_2 + 2Na_2SO_4$$

The yield of cuprous cyanide was found to be highly pH dependent. At lower hydrogen ion concentrations, the product was greenish yellow, which could not be purified by means of repeated washings with boiling water; and excess of acid resulted in lowering of the yield. Under controlled pH conditions, finally, it was possible to obtain the product in almost quantitative yield.

In the first set of experiments, p-bromophenyl benzyl ether (66) was converted into p-cyanophenyl benzyl ether (67), using cuprous cyanide. Cuprous cyanide reacts with aryl halides to yield the corresponding nitriles.[53] The reaction is thought to proceed through an intermediate complex as shown below:

$$2Ar\text{-}X + 2CuCN \longrightarrow [Ar\text{-}CN]_2CuX + CuX$$

$$[Ar\text{-}CN]_2\text{-}CuX \longrightarrow 2Ar\text{-}CN + CuX$$

As the reaction proceeds the mixture becomes dark brown; it is then poured onto ferric chloride hydrate and warmed before isolating the product. The Cu(I) complex is broken by $FeCl_3$, which oxidizes Cu(I) to Cu(II). Cupric ions form no complex with a nitrile.[53] It was observed during present work that by the use of $FeCl_3$, the yield of the product was better than that from the reaction worked up without $FeCl_3$. Cu(I) probably would complex with phenolic oxygen as well.

The nitrile (67), on reduction with diisobutyl aluminum hydride afforded p-benzyloxy benzaldehyde (69), which could not be converted into p-hydroxy benzaldehyde (64) in a yield better than 60%. The overall yield from sodium cyanide to (64) was 22.5%.

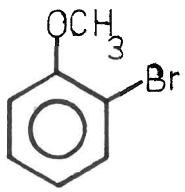
At this point, in hope of getting better overall yield, the methyl ether of p-bromophenol was tried. Bromoanisole (70) on reaction with cuprous cyanide gave p-anisonitrile (71) in 56% yield. p-Anisonitrile could be reduced to corresponding aldehyde (72) by means of diisobutyl aluminum hydride or lithium triethoxy aluminum hydride, but several attempts to convert (72) into p-hydroxy benzaldehyde (64) did not prove to be reasonably satisfactory.

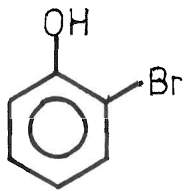
(70)

(71)

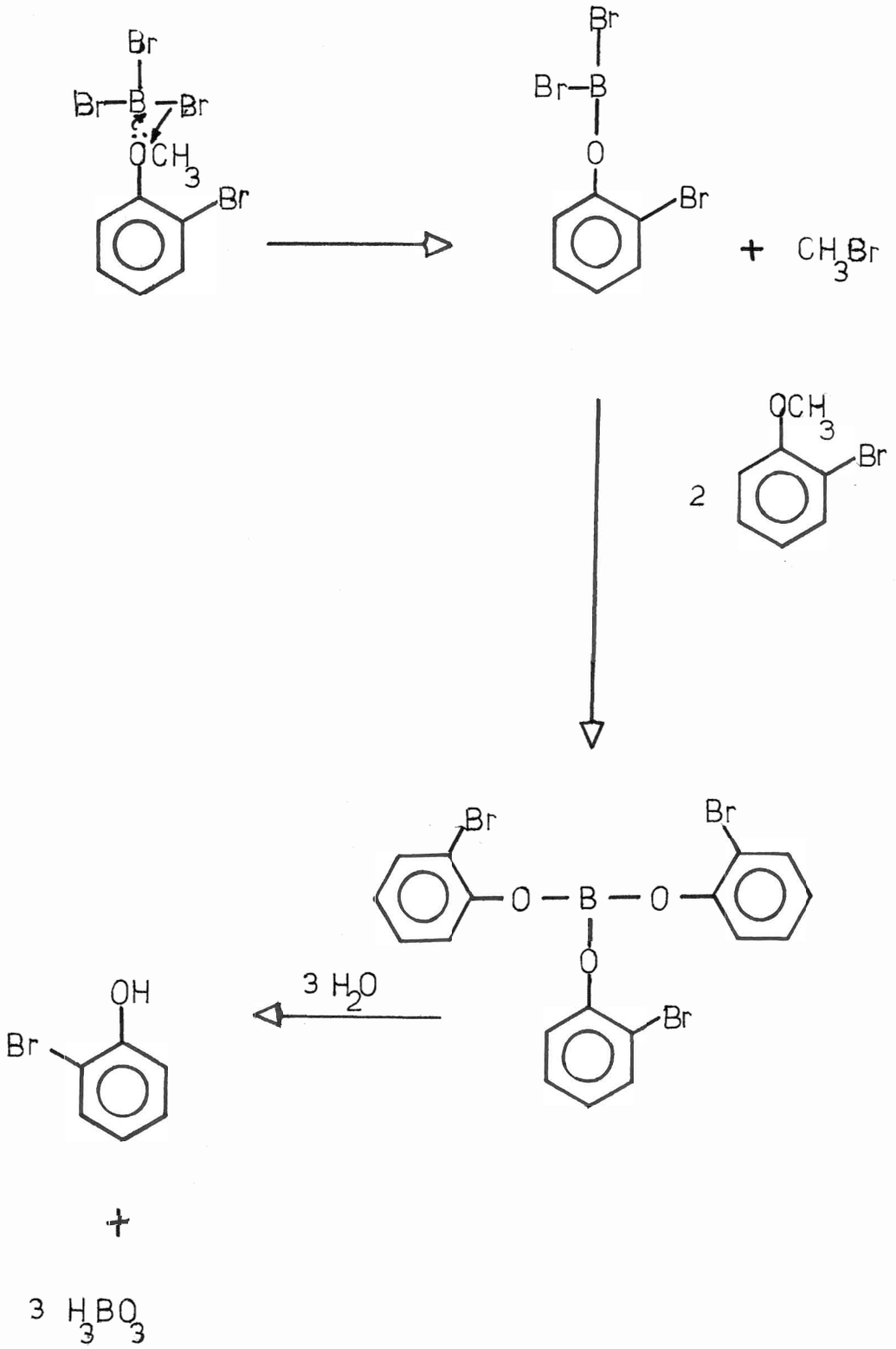
(72)

The reaction of (72) with boron tribromide afforded a dark purple solid which was not characterized further. Boron tribromide has been shown to be a clean and useful reagent for cleaving both aliphatic and phenolic ethers.[54] 2-Bromo anisole has been demethylated in 75% yield.

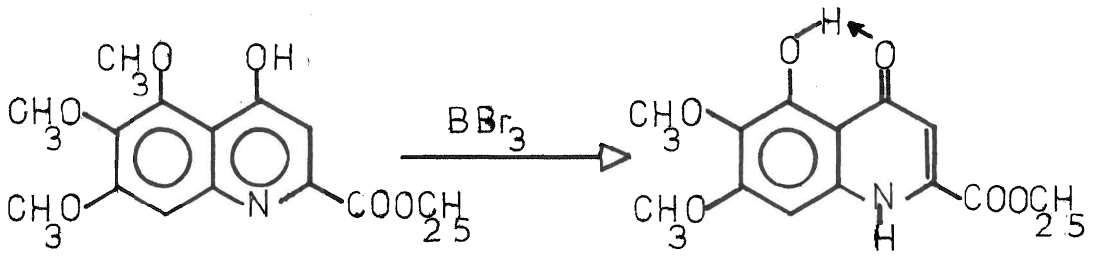




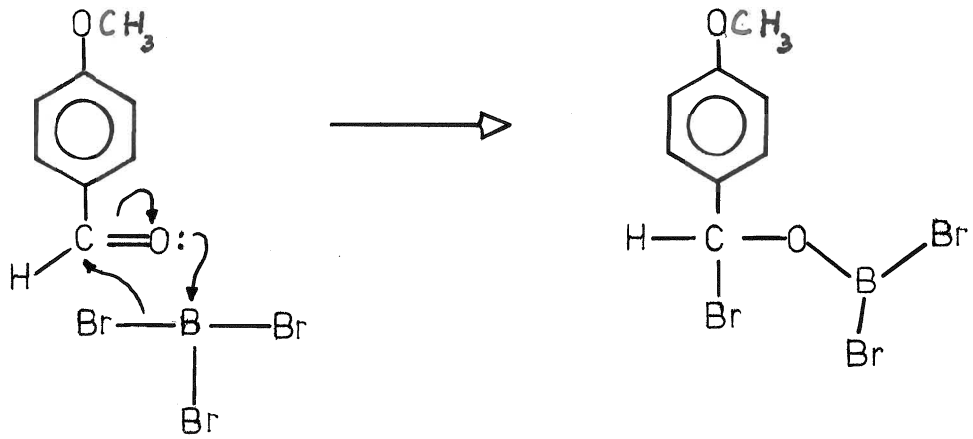
The reaction involves the formation of boron triphenoxide, which in turn is hydrolyzed to 2-bromophenol and boric acid.



Boron tribromide has also been reported[55] to selectively cleave the 5-methoxy group of 4-hydroxy-5,6,7-trimethoxy-2-carbo-ethoxy quinoline. The selectivity however, is attributed to the chelation in the product.



The failure of boron tribromide to demethylate (72) might be due to the possible polymerization of the aldehyde, which could take place in several ways, or due to the complex formation between aldehyde and boron tribromide.



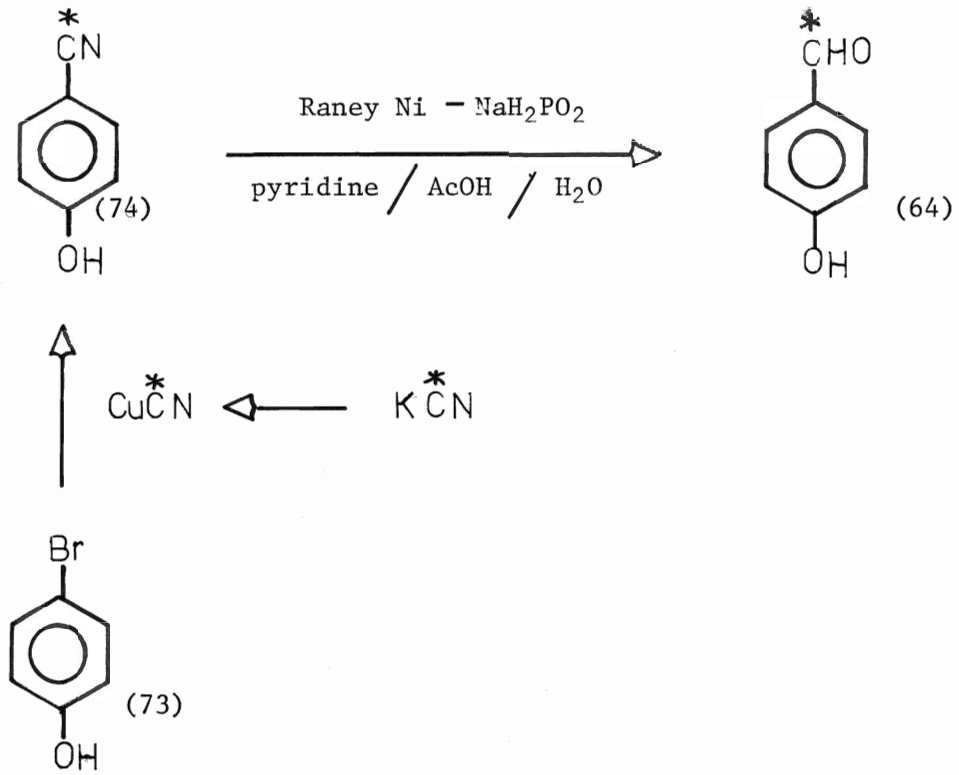
However, pyridine hydrochloride did work, but the product could not be isolated in more than 50% yield, while working on small quantities.

(72) (64)

SECTION 2

SYNTHESIS OF p-HYDROXY BENZALDEHYDE WITHOUT USE OF ANY PROTECTING GROUP

Billek and Kindl[56] have reported a simplified pathway for the synthesis of (64), which is summarized in Scheme XVII.



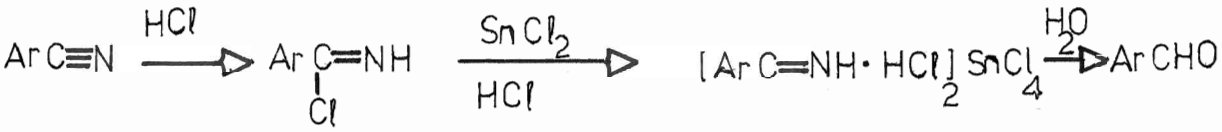
Scheme XVII

From the details given in this publication, it is not clear whether or not the product was actually isolated in the given yield, which is reported as 45%. Specific activities of the individual intermediates are not given either. The total yield is probably based on the recovered activity, which is not good enough to be used for biosynthetic purposes. In order to have a compound

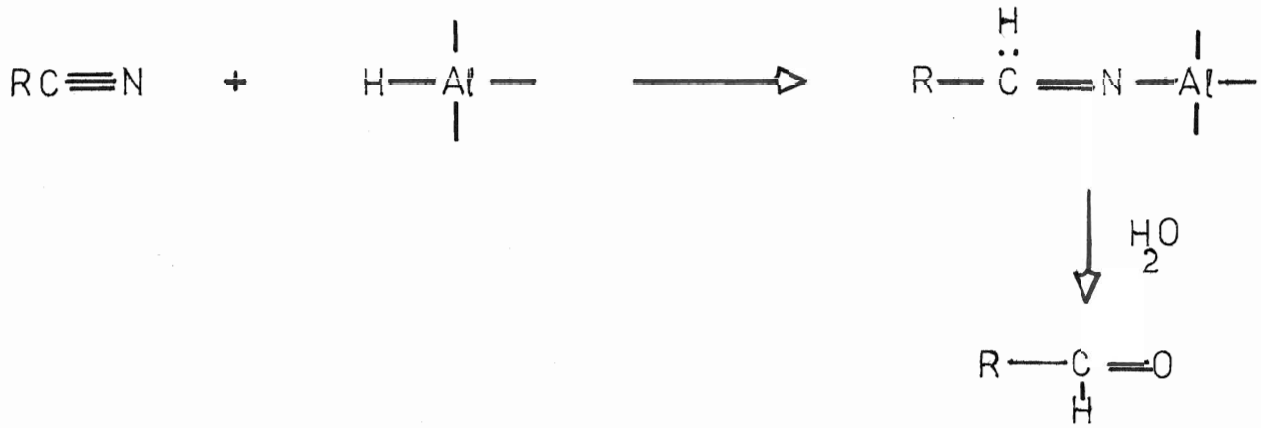
being suitable to be administered into a plant, it must be well characterized and pure, both radiochemically and chemically. Radiochemical purity means that the radioactivity is totally confined to the characterized compound, and not to some other impurity in it. The compound should be in chemically pure form, so that exact amounts can be administered. Another important aspect is that the required radioactivity should be present in minimum amount of the precursor to be fed to the organism. A precursor fed in large quantities might incorporate into a product, but then the studied metabolic pathway cannot be assured to be the naturally occurring one. It might have occurred as a result of a disturbed enzymatic system.

Taking all the above points into consideration, the next step in the present work was to start directly from 4-bromophenol (73). The optimum conditions for the conversion of p-bromophenol to p-cyanophenol (74) were obtained. In order to get the maximum incorporation of the label from $Cu^{14}CN$ into p-cyanophenol, a slight excess of p-bromophenol was used. Prolonged reaction time did not improve the yield, and some unreacted p-bromophenol always accompanied the product, even when the equimolar amounts of $Cu^{14}CN$ and p-bromophenol were used. The product could be crystallized out from benzene-ether (1:1) but the traces of p-bromophenol were still present in the crystallized product. The purification of the p-cyanophenol was therefore effected by means of chromatographic methods.

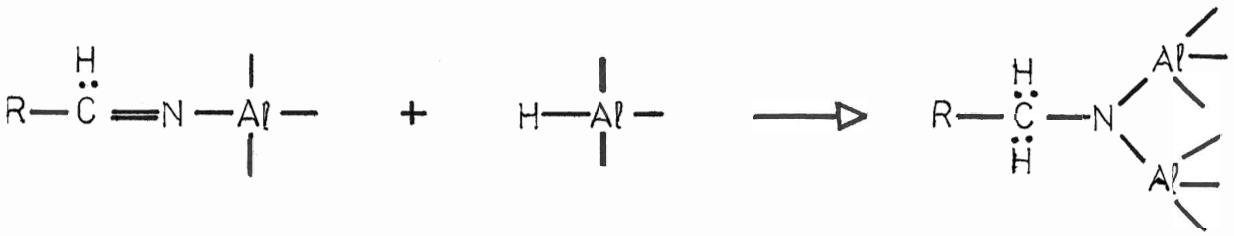
The purified p-cyanophenol was then subjected to reduction by several methods. The Stephens reaction[57] has been a recommended reaction for the reduction of an aromatic nitrile to the corresponding aldehyde. The reaction is conducted by passing hydrogen chloride through a solution of nitrile in ether containing anhydrous stannous chloride.



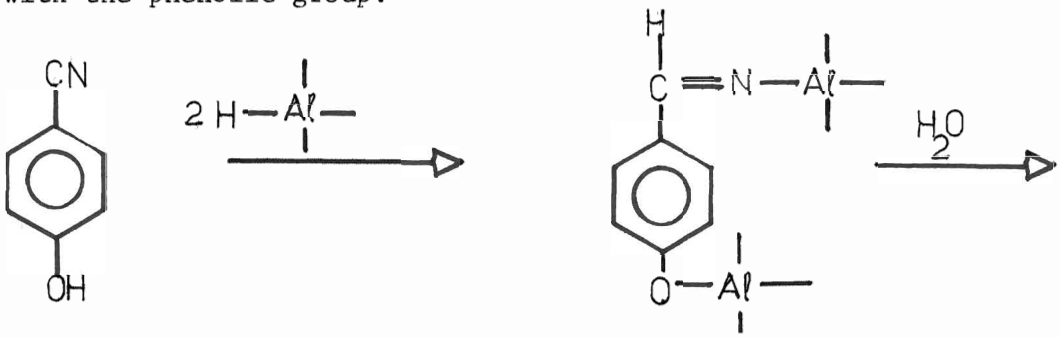
The Stephens reaction gives highly variable yields for different nitriles, whereas lithium triethoxy aluminum hydride[58] and sodium triethoxy aluminum hydride[59] have been reported to reduce nitriles giving better yields of aldehydes. The reaction involves an intermediate imine derivative, which on hydrolysis yields the corresponding aldehyde.

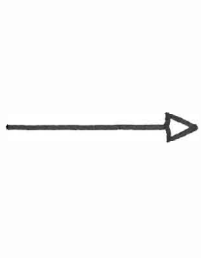


It is evident that excess hydride would lead to further reduction of imine derivative, and hence giving rise to the corresponding amine.



The equimolar amounts of nitrile and hydride in fact have been recommended but in case of p-cyanophenol, one equivalent is required to react with the phenolic group.





(64)

Therefore at least two equivalents of hydride are required to start with. Using two equivalents of lithium triethoxy aluminum hydride, the aldehyde (64) was obtained in 45% yield.

The same procedure applied for sodium triethoxy aluminum hydride failed to afford any (64).

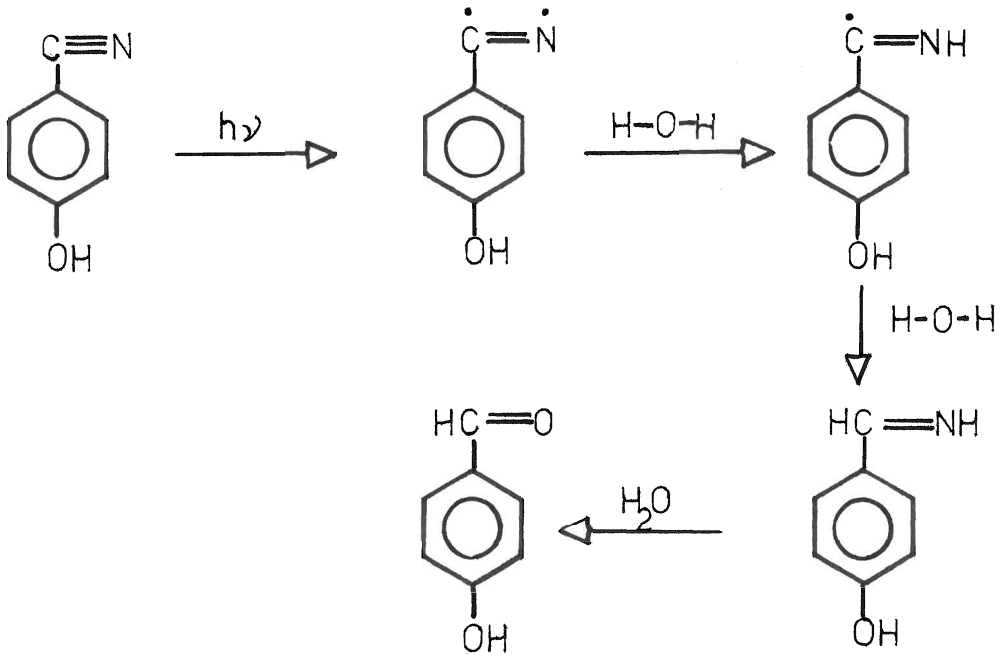
Reduction of p-cyanophenol with diisobutyl aluminum hydride resulted in the corresponding aldehyde in 37% yield. Dialkyl aluminum hydrides are similar to lithium aluminum hydride in their activities, but are easier to handle.

The reduction of p-cyanophenol carried out by means of photolysis[60] proved to be the best one, especially for smaller quantities, as all metal hydrides are extremely hygroscopic, and are quite hard to be handled in milligram quantities. A little exposure of such hydrides to moisture results in lowering of the yield, and if they are used in excess, other side products are obtained, amines for instance in the case of nitrile reduction. Photolysis reaction on the other hand, is effected in presence of relatively much larger volumes of solvent (approximately 1 ml/mg of starting material), and is much easier to be worked up.

During all the photolysis experiments, the only side product formed was an unknown polar compound, which on thin layer chromatography plate corresponded to the impurity present in commercial p-hydroxy benzaldehyde and presumably is the p-hydroxy benzoic acid. Increased irradiation time increased the amount of side product formed. The reaction mixture turned

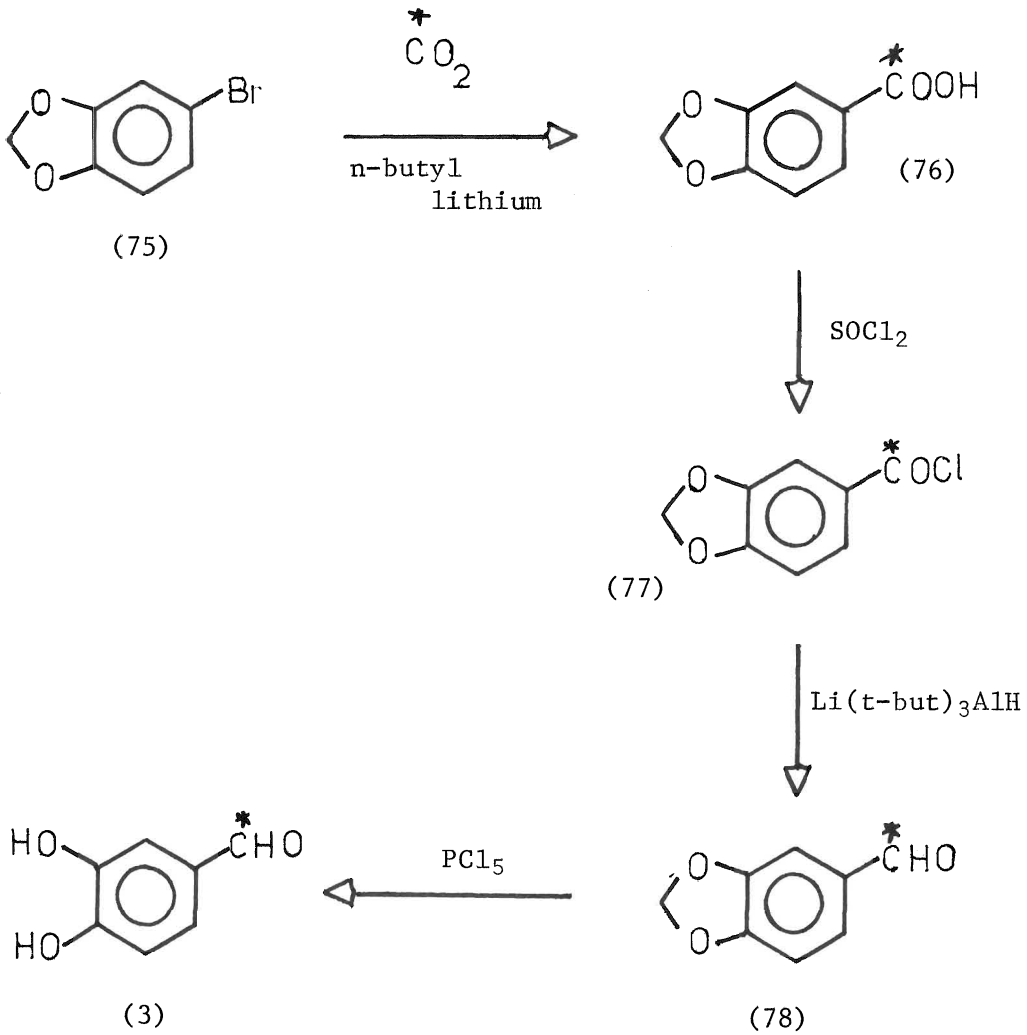
brown on photolysis probably due to free iodine, but most of the colour could be discharged by washing extract with sodium thiosulphate solution. Just before washing the extract with sodium thiosulphate, its pH was brought to 5 by means of ammonium hydroxide. At pH much lower than 5, sodium thiosulphate produces free sulphur, which is quite difficult to remove from organic compounds, as it is fairly soluble in many organic solvents.

The photolysis reaction, probably goes through the mechanism as shown below. Potassium iodide, a rarely used photochemical reducing agent has been shown to improve both the rate of formation and yield of the product.[60] Potassium chloride works as well, but it is not as effective as potassium iodide is.

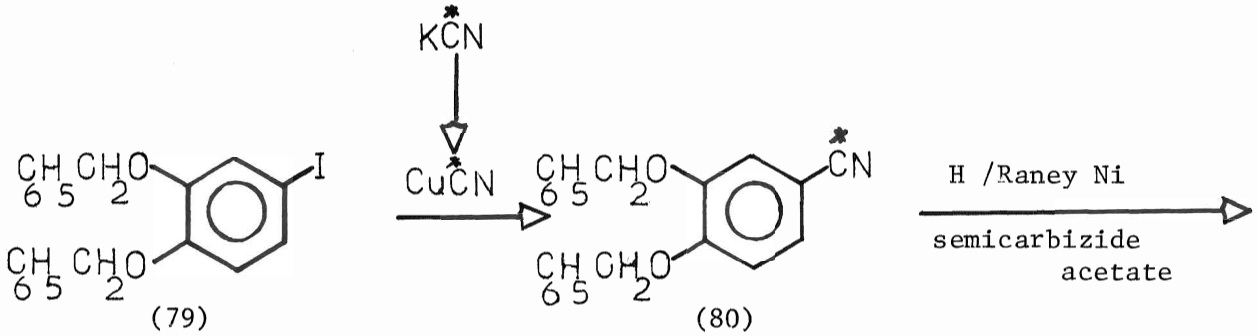


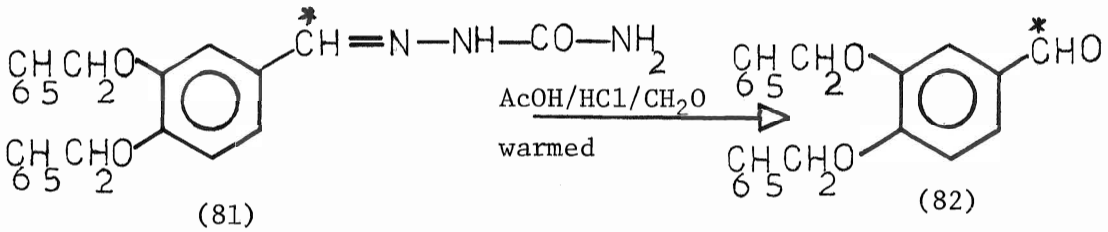
SYNTHESIS OF 3,4-DIHYDROXY BENZALDEHYDE

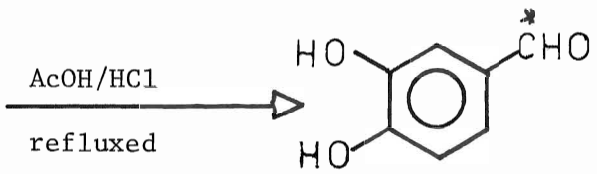
3,4-Dihydroxy benzaldehyde (3) is an extremely sensitive compound, and with [^{14}CHO] has never been made without the use of protecting groups. Zulalian and Suhadolink[61] have synthesized the carbonyl labelled (3) by the route shown in Scheme given below.



This method again involves $^{14}CO_2$, which cannot be used in a low level radiation lab. Patschke and Grisebach[62] have employed the following alternate method, which still is a five step synthesis.

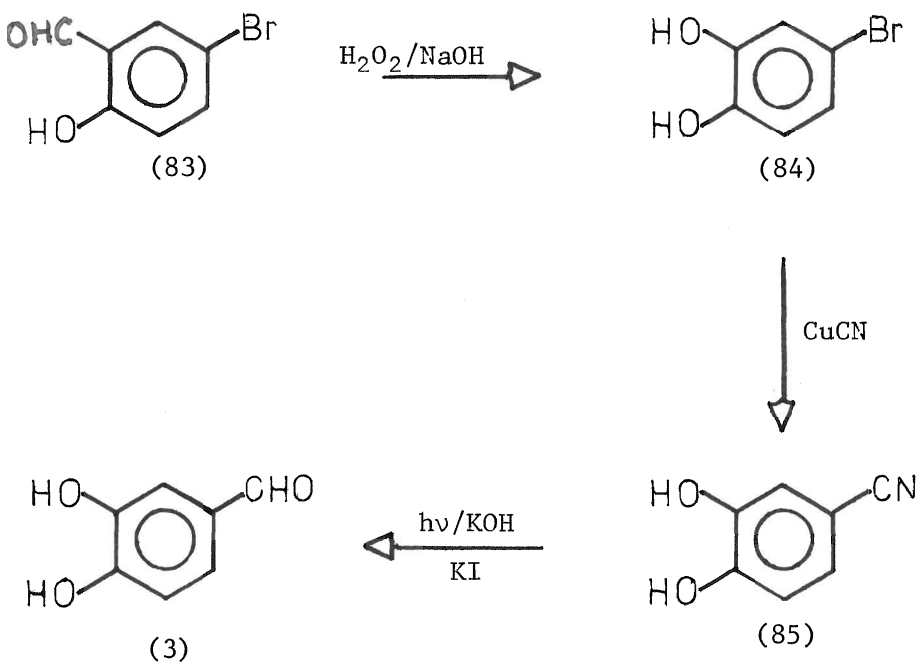






(3)

During the present work, 4-bromo-catechol (84) was found to react with cuprous cyanide, giving 4-cyano-catechol (85), which on photolysis yielded 3,4-dihydroxy benzaldehyde (3).



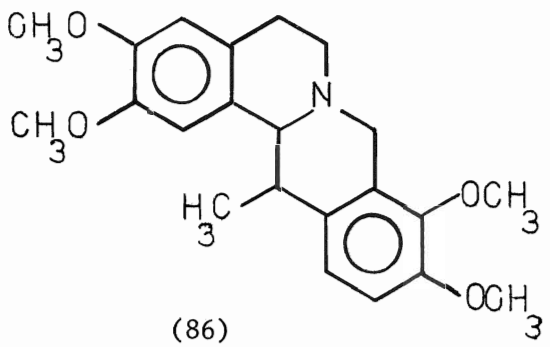
Product from the reaction of 4-bromocatechol and cuprous cyanide was subjected to the next step without purification, although it still contained some unreacted 4-bromo catechol. The purification of 4-cyano catechol could not be effected easily as catechols get oxidized on silica gel. In order to estimate the yield of the product, the crude 4-cyano catechol was subjected to thin layer chromatography several times, and the intensities of both spots (4-bromo catechol and 4-cyano catechol) were approximately equal. Therefore, it was assumed that crude 4-cyano catechol contained

almost 50% unreacted 4-bromo catechol. The reduction of 4-cyano catechol yielded the product 3,4-dihydroxy benzaldehyde (3) in a reasonable yield. It follows from the above results that this simple and clean method for the preparation of (3) can be applied to the synthesis of [7-^{14}C]-3,4-dihydroxy benzaldehyde.

SECTION 3

BIOSYNTHESIS OF CORYDALINE

Corydaline (86) $C_{22}H_{27}O_4N$, is a protoberberine alkaloid which occurs in the plant Corydalis solida, and several other species of genus Corydalis.[63] Corydaline contains the same nuclear structure as berberine (31). It contains four methoxyl groups along with an extra nuclear C-methyl group. The position of C-methyl group in corydaline was established to be C-13, through several reactions by many groups of workers. The structure of corydaline, therefore, is



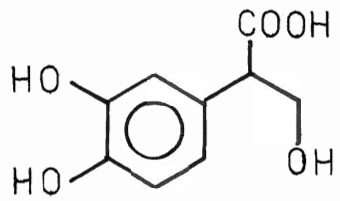
(86)

This structure for corydaline has been established through its synthesis.[64]

During the present work, the alkaloids corydaline (86), protopine (38) and reticuline (32) were isolated from [2-^{3}H,1-^{14}C]-dopamine(T/^{14}C = 4) fed Corydalis solida, to investigate the latter stages of the biosynthesis of corydaline. According to what is known of the biosynthesis of corydaline, one carbon unit is introduced into C-13 of a

protoberberine skeleton,[65] which in turn is derived from two molecules of tyrosine, and one molecule of methionine, although the exact metabolic fate of tyrosine, like in other protoberberine alkaloids, is not known yet.

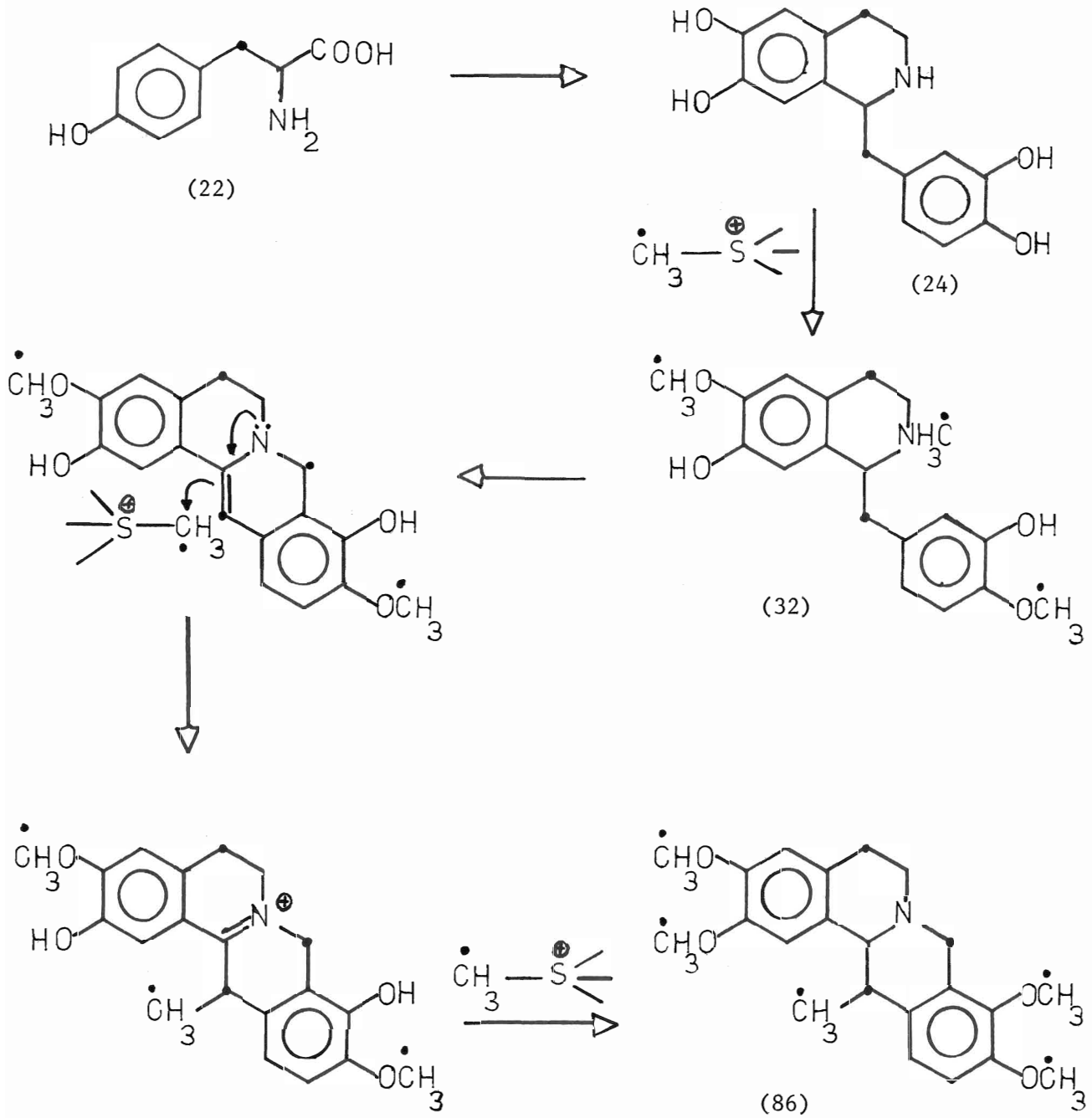
It had also been suggested,[66] that biogenesis of corydaline does not involve a protoberberine system, but is generated by direct combination of amino acid fragments. The segment containing C-methyl group was thought to be derived from a branched chain C_6-C_3 unit like (87).



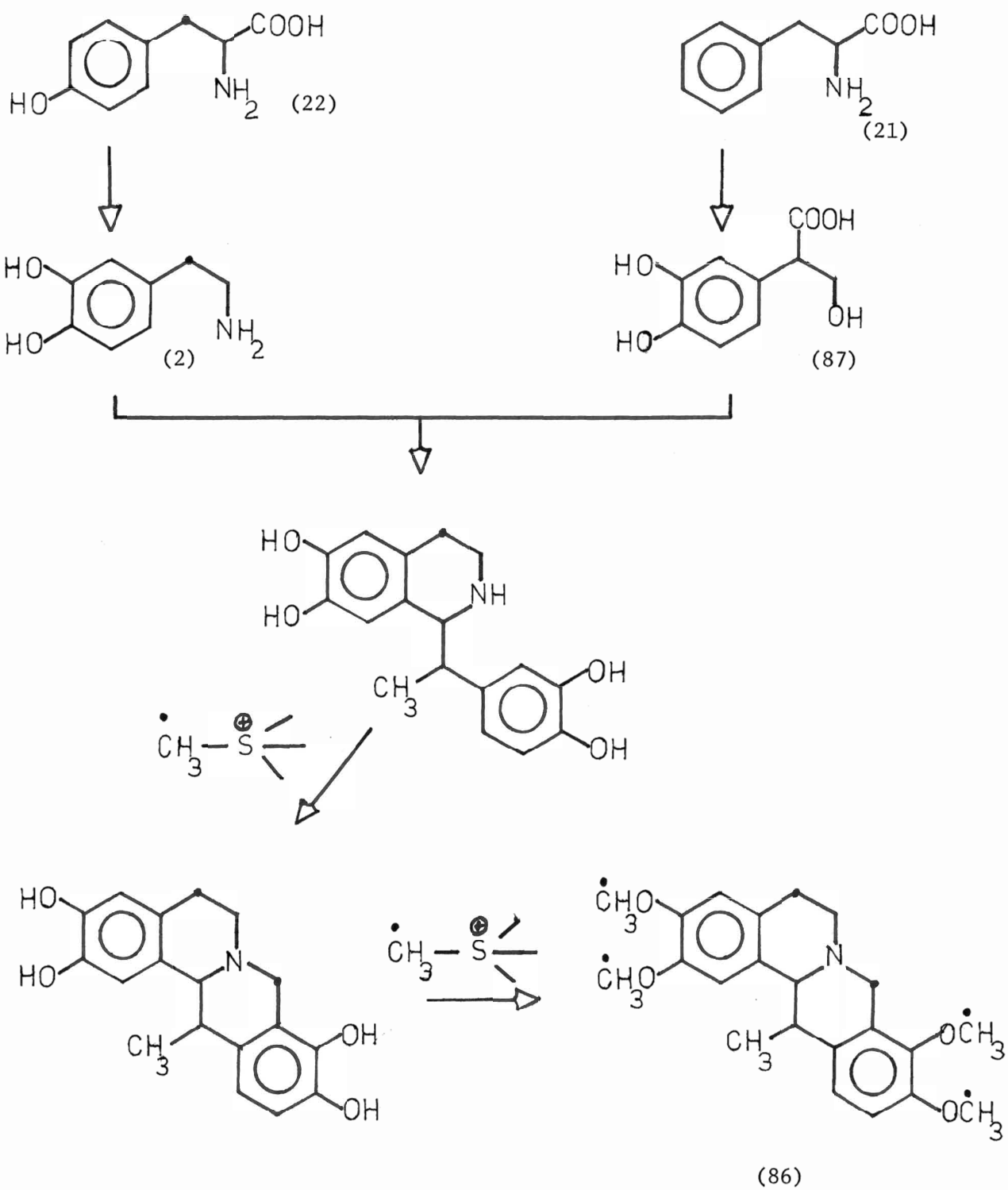
(87)

Later on experiments carried out by Holland et al.[67] tested the validity of these two hypotheses. Samples of L-(methyl-^{14}C) methionine and DL-[3-^{14}C] tyrosine were administered to Corydalis solida, and biosynthetically derived corydaline was degraded. According to the protoberberine route hypothesis, the distribution of radioactivity would take place as given in Scheme XVIII, whereas according to the other hypothesis, the labels would be incorporated as in Scheme XIX.

The results, however, did not agree with the latter one, as the acetate obtained from Kuhn-Roth oxidation of [3-^{14}C]-tyrosine derived corydaline, contained half of the activity of the intact alkaloid. And this was the carboxyl group of the acetate, derived from ring carbon of corydaline,

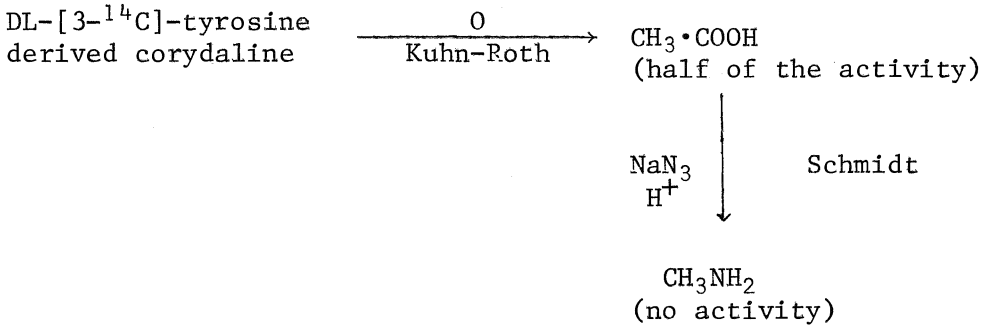


Scheme XVIII


COOH
NH2
HO
(22)
COOH
NH2
(21)
HO
HO
NH2
(2)
COOH
HO
HO
OH
(87)
HO
HO
NH
CH3
OH
OH
CH3 — S
HO
HO
N
CH3
OH
OH
CH3 — S
CH3O
CH3O
N
CH3
OCH3
OCH3
(86)

Scheme XIX

that contained the activity, because further degradation of acetate resulted in methylamine with no activity (Scheme XX).

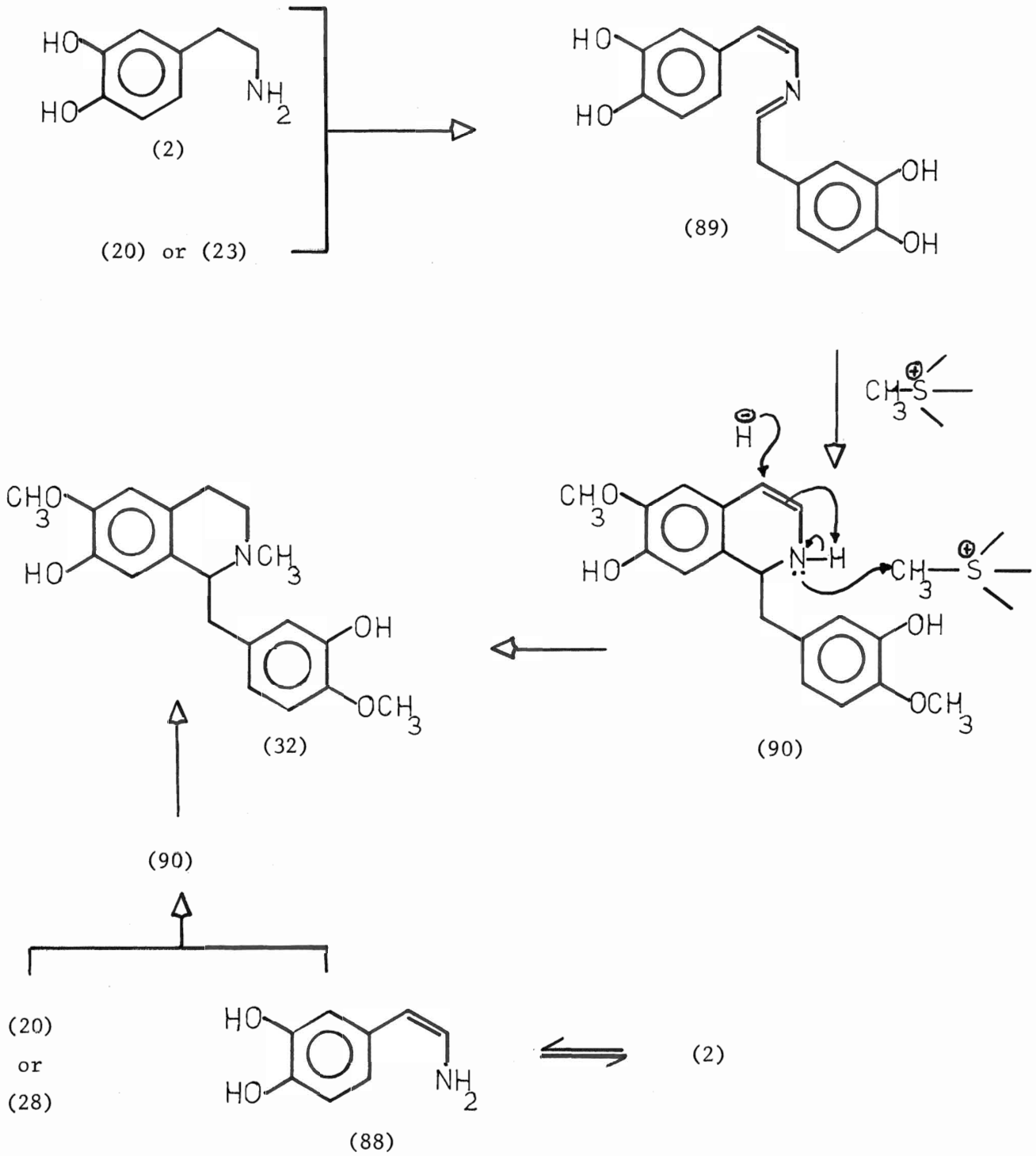


Scheme XX

The above findings support the view that corydaline is derived from nor-laudanosoline (24). Reticuline (32), which can be a possible intermediary in the pathway, in fact, has been shown[68] to incorporate into corydaline, and the occurrence of reticuline with corydaline in the same plant also strengthens this idea.

The $^{3}H/^{14}C$ ratio in the reticuline isolated from [2-^{3}H,1-^{14}C] dopamine, fed plants indicates approximately 50% retention of the tritium, relative to carbon-14. On this basis, it might be assumed that intermediates like (89) or (90) are formed during the incorporation of dopamine into reticuline. The possible route to the formation of reticuline (32) could be like the one shown in Scheme XXI.

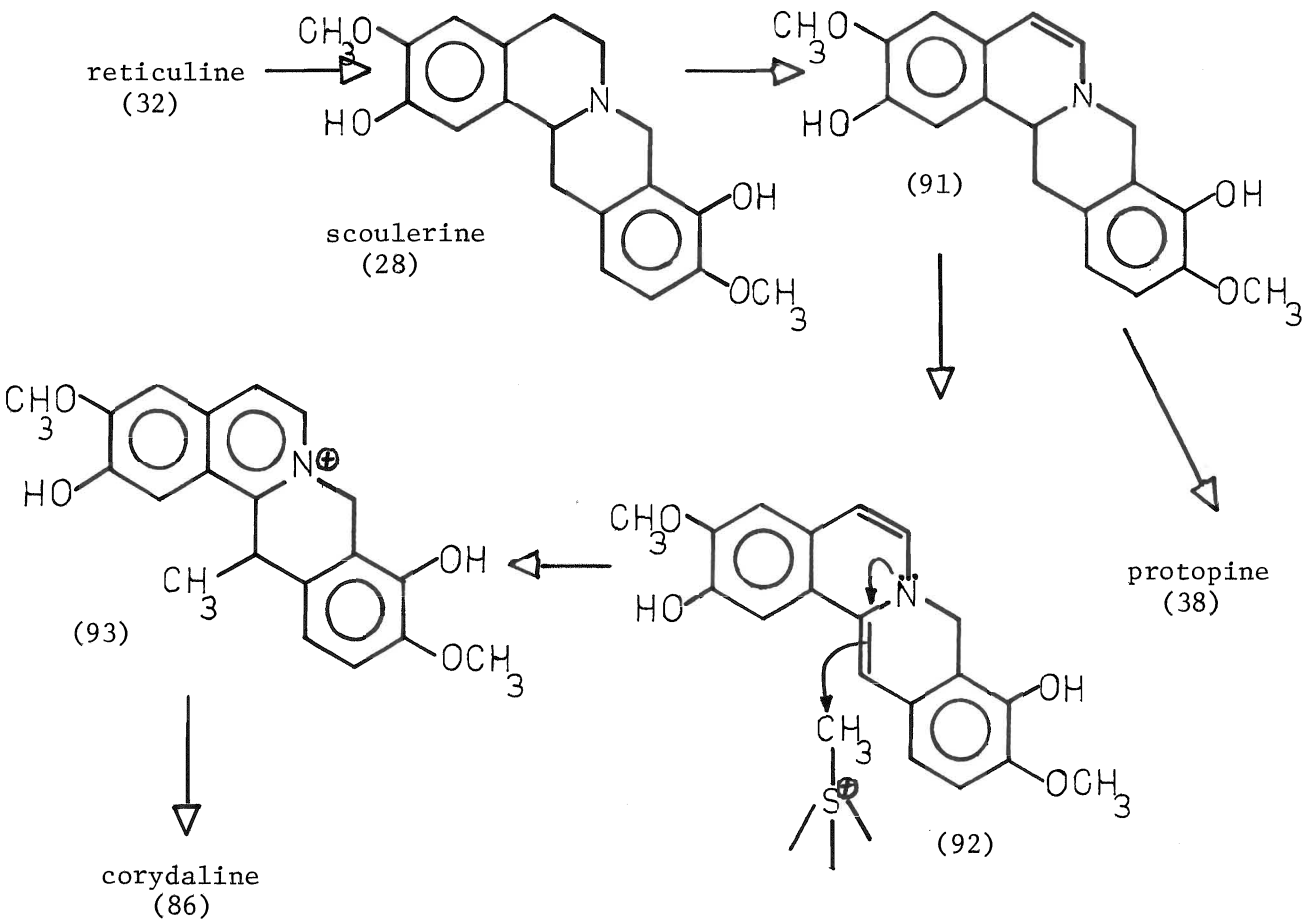
This evidence, however, is certainly not good enough to lead to any specific conclusion, because 50% loss of tritium can be explained in several other ways as well, for instance if dopamine gets oxidized into a molecule like (88), it would result in 50% loss of tritium from dopamine, and there


HO
HO
NH2
(2)
(20) or (23)
(89)
OH
OH
N
CH3
S
H
CH3O
HO
NCH3
OCH3
(32)
(90)
(20)
or
(28)
(88)
(2)

Scheme XXI

is an equal possibility that such an oxidation might occur at nor-reticuline stage.

A similar explanation can be given for another approximate 50% loss of tritium relative to carbon in corydaline and protopine. The reticuline with one half of the original $^{3}H/^{14}C$ ratio, might be converted to the intermediates like (91), (92) and (93) (Scheme XXII) which in turn incorporate into corydaline and protopine (38). If there is no intermediate like (91) or (92), then the oxidation must occur at some other stage, that might be in the alkaloids themselves. However the above results do not provide enough information to say something with certainty about the pathway shown in Scheme XXII. Administration of a compound like (93) to the plant may provide more helpful results.



Scheme XXII

EXPERIMENTAL

Spectroscopic Data

Mass spectra were obtained on an AEI-MS 30 double focusing mass spectrometer. The results are quoted as m/e value for the lower isotopic species unless the isotopic ratios helped to indicate the identity of the fragment.

A Varian A60 spectrometer operating at 60 MHz was used to obtain the ^{1}H n.m.r. spectra, and ^{13}C n.m.r. spectra were obtained on a Bruker WP 60 spectrometer, tetramethyl silane being used as an internal standard.

Infrared spectra were recorded on a Perkin Elmer Model 237B Grating Infrared spectrophotometer. The spectra of all the compounds were recorded as thin films between KBr plates. The spectra were calibrated by means of a polystyrene film, and only the characteristic absorptions are given.

Thin Layer Chromatography

This was carried out on pre-coated silica gel GF-254 slides, which were visualized by exposure to ultra violet light.

Other Physical Data

Melting points were determined on a Gellenkamp melting point apparatus, and the values quoted are uncorrected.

A Searle-Delta 300 liquid scintillation counter was used to measure the ^{3}H and ^{14}C activities, and the fluid used was Aquasol-2 Universal L.S.C. Cocktail.

Photolysis experiments were carried out by means of a Hanovia 140 watt (115 volt) ultraviolet lamp.

SECTION 1

CUPROUS CYANIDE

This was prepared by the procedure used by Reid and Weaver.[69] Cupric sulphate pentahydrate (6 g, 24 millimole) and sodium bisulphite heptahydrate (4.1 g, 16 millimole) were dissolved in water (20 ml). The resulting solution was acidified with 12 N sulphuric acid (3 ml), and to this was added a solution of sodium cyanide (760 mg, 15.6 millimole) in 0.5 N sodium hydroxide (14 ml) dropwise. After 15 minutes, the off-white product was filtered off on a Buchner funnel, washed with boiling water, methanol and ether respectively. The I.R. spectrum of the dried product as nujol mull was idnetical to that of authentic cuprous cyanide; ν_{max} 2170 cm^{-1}. The cuprous cyanide prepared this way was used to convert p-bromophenol to p-cyanophenol; it afforded the same product as authentic cuprous cyanide did.

p-BROMOPHENYL-BENZYL ETHER

In a 200 ml round bottom flask equipped with a reflux condenser, potassium hydroxide (2.8 g, 0.05 mole) and p-hydroxybromobenzene (8.6 g, 0.05 mole) were dissolved in ethanol (30 ml) along with water (2 ml), and benzyl chloride (6.3 g, 0.05 mole) was added to it carefully. The reaction mixture after being refluxed for three hours was brought to room temperature and poured onto ice cold water (40 ml). The crude product was filtered off and crystallized from ethanol (8.38 g, 64%). An analytical sample recrystallized from ethanol as pale yellow crystals, m.p. 60°, (lit.[73] 59.5°) mass spectrum m/e 262 (M^+, 25%), 264 (M + 2, 25%), 171 (M-CH_2-Ph, 5%), 155 (M-OCH_2Ph, 5%), 143 ($C_5H_4Br^+$, 10%), 117 ($C_3H_2Br^+$, 5%), 91 (tropyllium ion, 100%), 77 ($C_6H_5^+$, 5%), and 76 ($C_6H_4^+$, 7%); ^{1}H n.m.r. ($CDCl_3$) 5δ singlet

(2 protons), and 6.7-7.55δ AB pattern overlapped by a broad singlet (9 protons).

p-CYANOPHENYL-BENZYL ETHER

p-Bromophenyl-benzyl ether (2.62 g, 0.01 mole) and cuprous cyanide (1.0 g, 0.011 mole) were heated in refluxing N,N-dimethyl formamide under nitrogen for four hours. The cooled reaction mixture was poured into a solution of ferric chloride hydrate (5 g) in concentrated hydrochloric acid (1.5 ml) and water (8 ml), heated for about 20 minutes at 50-60°, brought to room temperature and extracted with ether (4 x 50 ml). The ether extracts were combined, washed with 5% sodium hydroxide (20 ml), water (40 ml), and dried over anhydrous sodium sulphate. The solvent was removed on a rotary evaporator and the resulting solid on crystallization from ethanol afforded light pink crystals, m.p. 85 (lit.[50] 93°), mass spectrum m/e 290 (M^+, 7%), 181(M-CN, 1%), 180(M-HCN, 2%), 119 (M-CH_2-Ph + H, 3%), 102 (M-OCH_2Ph, 1%), 91 (tropyllium ion, 100%) and 77 ($C_6H_5^+$, 2%); ^{1}H n.m.r. ($CDCl_3$) 5.14δ singlet (2 protons), and 6.8-7.65δ, an AB pattern overlapped by a broad singlet (9 protons); I.R. ν_{max} 2225 (C≡N) cm^{-1}. (Yield 1.15 g, 55%)

p-BENZYLOXY-BENZALDEHYDE

This was prepared by the method of Miller et al.[70] p-Cyanophenyl-benzyl ether (1 g, 0.0048 mole) was dissolved in dry benzene (10 ml) in a three neck round bottom blask equipped with a reflux condenser, a rubber septum and a glass inlet tube for nitrogen stream. Diisobutyl aluminum hydride (4.8 ml of a solution containing 100 g of DIBAL in 700 ml hexane; 0.68 g, 0.0048 mole) was added dropwise through the rubber septum by means of a syringe over a period of an hour. The reaction mixture after being stirred at 45-50° for six hours was cooled down to room temperature and decomposed by the addition of methanol (2.53 ml, 2 g, 0.0625 mole) in

benzene (5 ml), followed by water (1.12 ml, 0.0625 mole). The solid aluminum salts were filtered, and the filtrate was concentrated *in vacuo*. The concentrate was dissolved in ether (100 ml), and benzene (50 ml) and washed with 5% hydrochloric acid, and dried over anhydrous sodium sulphate. This solution on removal of the solvent *in vacuo* afforded p-benzyloxy benzaldehyde, which was crystallized from ethanol, m.p. 70°(lit.[74] 72°) mass spec. m/e 212 (M^+, 5%), 122 ($M-CH_2-Ph + H$, 3%), 121 ($M-OCH_2-Ph$, 3%), 105 ($M-O-CH_2-Ph$, 3%), 104 ($M-HO-CH_2-Ph$, 4%), 91 (tropyllium ion, 100%), 77 ($C_6H_5^+$, 3%) and 76 ($C_6H_4^+$, 3%); ^1H n.m.r. ($CDCl_3$) 5.2δ singlet (2 protons), 7-8δ an AB pattern overlapped by a singlet (9 protons) and 9.94δ singlet (1 proton). (Yield: 0.44 g 43%).

CONVERSION OF p-BENZYLOXY-BENZALDEHYDE TO p-HYDROXY BENZALDEHYDE

Method 1

A solution of p-benzyloxy-benzaldehyde (0.5 g, 0.00235 mole) in acetic acid (8 ml) was added dropwise to refluxing hydrochloric acid (27 ml conc. hydrochloric acid and 12 ml water) by means of a dropping funnel over a period of 45 minutes. The resulting mixture after being cooled to room temperature was taken into water (10 ml) and extracted with ethyl acetate (3 x 50 ml). The ethyl acetate extract was dried over anhydrous sodium sulphate and the solvent was removed *in vacuo* and the solid material thus obtained was crystallized from benzene (0.16 g, 60%). A sample recrystallized from benzene as pink granular crystals corresponded to authentic p-hydroxy benzaldehyde on t.l.c. plate (20 % methanol in benzene, R_f 0.36, standard R_f, 0.36); mass spec, m/e 122 (M^+, 90%), 121 (M-1, 100%), and 93 (M-HCO, 30%); ^1H n.m.r. ($(CD_3)_2CO$) 7.14-7.9 an AB quartet, J = 9 Hz (4 protons) and 9.96 δ singlet (1 proton).

Method 2

p-Benzyloxy benzaldehyde (0.5 g, 0.00235 mole) in ethanol (10 ml) hydrogenated over 10% Pd/charcoal (0.5 g) for two hours at room temperature. The reaction mixture was filtered and the filtrate on removal of the solvent yielded a resinous material. Thin layer chromatography of this resinous material (20% methanol in benzene) showed two spots corresponding to p-hydroxy benzaldehyde (R_f, 0.36; standard R_f, 0.36), and an unknown compound (R_f, 0.08). The ^1H n.m.r. did indicate aldehydic proton as a singlet (at 9.9δ) but the product could not be isolated by means of crystallization.

p-ANISONITRILE

p-Bromoanisole (1.6 g, 0.01 mole) and cuprous cyanide (1.0 g, 0.0115 mole) were heated in refluxing N,N-dimethyl formamide (3 ml) under nitrogen for five hours. The reaction mixture on similar work as given for the preparation of p-cyanophenyl-benzyl ether (p. 61) afforded p-anisonitrile along with traces of p-bromoanisole. The product was crystallized from benzene-ether (1:1). Recrystallization from the same solvents yielded pure p-anisonitrile (0.75 g, 56%), m.p. 55-56° (lit.[75] 59°), not depressed by mixing with an authentic sample; mass spec m/e 133 (M^+, 100%), 118 ($M-CH_3$, 20%, 103 ($M-OCH_3$, 50%), 90 (M-CN and CH_3, 50%), and 76 (C_6H_5, 10%); ^1H n.m.r. ($CDCl_3$) 6.98-7.6δ an AB quartet, J = 8 Hz; I.R. ν_{max} 2210 (C N) cm^{-1}.

p-ANISALDEHYDE

Method 1

This reaction was carried out in a similar fashion as the one described on page 61 for the synthesis of p-benzyloxy-benzaldehyde. p-Anisonitrile (0.66 g, 0.005 mole) was dissolved in benzene (10 ml). Diisobutyl aluminum hydride (6 ml, 0.06 mole) was added dropwise and the reaction mixture was stirred at 45-50° for six hours, when it was decomposed by methanol (2 g,

0.0625 mole) in benzene (5 ml) followed by water (1.12 ml, 0.0625 mole) in methanol 6 ml), and filtered. The filtrate was taken into 5% hydrochloric acid (10 ml) and extracted with ether (4 x 50 ml). The ether extract on drying and removal of the solvent in vacuo yielded a pale yellow liquid (0.36 g, 53%), which on t.l.c. plate (20% methanol in benzene) corresponded to p-anisaldehyde (R_f, 0.5; standard R_f, 0.5); ^1H n.m.r. ($CDCl_3$), 3.8 δ singlet (3 protons), 6.9, 7.8δ an AB quartet, J = 7.5 Hz (4 protons) and 9.9δ singlet (1 proton).

Method 2

In a two neck round bottom flask equipped with a stirrer, a condenser with drying tube on it, and a rubber septum, flushed with nitrogen, was placed lithium aluminum hydride (0.31 g, 0.0083 mole) in ether (8 ml). Into this stirred solution, absolute ethanol (1.43 ml, 1.14 g, 0.0249 mole) was added over a period of 10 minutes at ice bath temperature. After stirring for another 10 minutes, p-anisonitrile (1.1 g, 0.0083 mole) in ether (5 ml) was added to this reagent. There was a rapid exothermic reaction and the soltuion became viscous. The reaction mixture was stirred for an additional hour and then was hydrolyzed with 5N sulphuric acid (5 ml). The organic solvents were removed in vacuo and the resulting residue was extracted with ethyl acetate (3 x 100 ml). The ethyl acetate extract was dried over anhydrous sodium sulphate and the solvent was removed in vacuo, when a pale yellow liquid was obtained (0.54 g, 48%); ^1H n.m.r. ($CDCl_3$) 3.8δ singlet (3 protons) 6.9, 7.8δ an AB quartet, J = 7.5 Hz (4 protons) and 9.9δ singlet (1 proton). The product corresponded to p-anialdehyde on t.l.c. plate (20% methanol in benzene, R_f 0.5; standard R_f 0.5).

CONVERSION OF p-ANISALDEHYDE TO p-HYDROXY BENZALDEHYDE

Attempted Method 1

To a solution of p-anisaldehyde (7.21 g, 0.053 mole) in methylene chloride (50 ml) at dry ice-acetone temperature was added a solution of boron tribromide (5 ml, 0.053 mole) in methylene dichloride (40 ml) over a period of twenty minutes. After stirring at room temperature for twelve hours, the reaction mixture was cooled to 4°, methanol (50 ml) was added slowly and the resulting mixture was evaporated. The residue left behind was taken into water (100 ml), acidified to pH 5 with hydrochloric acid extracted with ethyl acetate (4 x 100 ml), and dried over anhydrous sodium sulphate. The ethyl acetate extract on removal of the solvent gave a dark purple crystalline solid, which decomposed on exposure to atmospheric moisture and was not characterized further.

Method 2

This conversion was achieved by means of pyridine hydrochloride, which was prepared by passing hydrogen chloride through pyridine, and crystallizing the resulting solid from chloroform-ethyl acetate.

p-Anisaldehyde (0.6 g, 0.0044 mole) was refluxed with pyridine hydrochloride (2 g) for 45 minutes, under nitrogen. The hot solution was poured onto water (10 ml), and extracted with ethyl acetate (4 x 50 ml). The ethyl acetate extract on drying over anhydrous sodium sulphate, and removal of the solvent <u>in vacuo</u> afforded p-hydroxy benzaldehyde (0.32 g, 60%); mass m/e 136 (starting material, 10%), 122 (M^+, 90%), 121 (M - 1, 100%), and 93 M-HCO, 90%).

SECTION 2

p-CYANOPHENOL

This was made by the same procedure as described on page 61 for the synthesis of p-cyanophenyl-benzyl ether, except that the reactants were heated for about three and a half hours, and the ether extract, instead of being washed with sodium hydroxide, was washed twice with water. The product was purified by means of column chromatography (details are given on page 69). The purified product on subjection to thin layer chromatography (5% methanol in benzene), showed single spot corresponding to p-cyanophenol (R_f, 0.21; standard R_f, 0.21); mass spectrum m/e 119 (M^+, 100%), 118 (M-1, 5%), 102 (M-OH, 2%), 92 (M-HCN 3%), 90 (5%) and 76 ($C_6H_4^+$, 5%); ^{13}C n.m.r. ($(CD_3)_2CO$) indicated five types of carbons at 161.9δ (-C-OH), 117.9δ (ortho to OH), 134.6δ (meta to OH), 103.4δ(para to OH), and 119.5 (C≡N); I.R. ν_{max} 3250 broad (OH), and 2230 (C≡N)

REDUCTION OF p-CYANOPHENOL

Method 1

p-Cyanophenol (0.57 g, 0.0048 mole) on reaction with two equivalents of diisobutyl aluminum hydride (9.6 ml, 1.36 g, 0.0096 mole) afforded p-hydroxy benzaldehyde (0.22 g, 37%). The product which showed single spot on thin layer chromatography plate (20% methanol in benzene; R_f, 0.36), was characterized by means of ^1H n.m.r. ($(CD_3)_2CO$) 7.14, 7.9δ an AB quartet, J = 9 Hz (4 protons), and 9.96δ singlet (1 proton).

Method 2

This reduction was carried out by means of lithium triethoxy aluminum hydride, which was prepared in a similar fashion to the one described on page 64. Two equivalents of the reagent (0.0166 mole) were allowed to

react with p-cyanophenol (1 g, 0.0083 mole) in ether (10 ml) at room temperature, for two hours, when the reaction mixture was hydrolyzed with 6N sulphuric acid (10 ml) and extracted with ethyl acetate (4 x 50 ml). The ethyl acetate was removed *in vacuo* to leave a solid material, which on crystallization from benzene afforded p-hydroxy benzaldehyde (0.48 g, 45%); ^{1}H n.m.r. ($(CD_3)_2CO$) 7.14, 7.9δ an AB quartet, J = 9 Hz (4 protons), and 9.96δ singlet (1 proton)

In a subsequent series of experiments, sodium triethoxy aluminum hydride was used instead of lithium triethoxy aluminum hydride using a similar procedure, but it failed to reduce the nitrile. The change in solvent from ether to tetrahydrofuran, and prolonged reaction time did not afford any desired product either. ^{1}H n.m.r. did not indicate any aldehydic proton, and starting material was recovered every time which was identified by means of thin layer chromatography and infrared spectra.

4-BROMOCATECHOL

This was prepared by the method of Dakin.[71] 5-Bromo-2-hydroxy benzaldehyde (10 g, 0.05 mole) was dissolved in N sodium hydroxide (90 ml), and 3% hydrogen peroxide (75 ml) was added to this solution at once. The solution became hot and was allowed to stand at room temperature until cool, and acidified with sulphuric acid. A slight excess of sodium bicarbonate was added and the solution extracted with ether. Subsequent drying of the ether extract with anhydrous sodium sulphate and removal of the solvent *in vacuo* yielded a light brown residue, which was triturated with hot petroleum ether. Petroleum ether fraction was concentrated to 50-60 ml *in vacuo*, and kept in fridge overnight, when 4-bromo catechol crystallized out (1 g, 0.0053 mole), which showed single spot of thin layer chromatography plate

(20% methanol in benzene; R_f, 0.43); mass spectrum m/e 188 (M^+), 190 (M + 2), 171 (M-OH), 170 M-H_2O), and 109 (M-Br). The I.R. and ^1H n.m.r. spectra did not show any aldehyde absorption, corresponding to the starting material.

4-CYANOCATECHOL

4-Bromocatechol (188 mg, 1 mmole) and cuprous cyanide (89 mg, 1 mmole) were heated in refluxing N,N-dimethyl formamide (2 ml) under nitrogen for five hours. The reaction mixture was worked up in a similar manner as given on page 69 for the synthesis of p-cyanophenol. Thin layer chromatography (20% methanol in benzene) showed three spots corresponding to starting material 4-bromocatechol (R_f, 0.43; standard by R_f, 0.43); 4-cyanocatechol (R_f, 0.34) and an unknown polar impurity (R_f, 0.05). I.R. indicated the cyano group ν_{max} 2225 cm^{-1}; mass spectrum m/e 188 (starting material), 135 (M^+, 4-cyanocatechol), 117 (M-H_2O), and 109 (M-CN). (Yield 56 mg, 41%)

3,4-DIHYDROXYBENZALDEHYDE

Crude 4-cyanocatechol (25 mg, 0.185 mmole) from the previous step was dissolved in 0.09 N potassium hydroxide. Potassium iodide (1.2 g) was added and the solution was irradiated under nitrogen for twelve hours. A similar workup as given on page 71 for the synthesis of p-hydroxybenzaldehyde yielded a solid (8 mg). Thin layer chromatography (20% methanol in benzene) of this solid showed a single spot corresponding to 3,4-dihydroxybenzaldehyde (R_f, 0.32; standard R_f, 0.32); mass spectrum m/e 138 (M^+, 100%), 137 (M - 1, 90%), 109 (M-CHO) and clusters of peaks due to elemental sulphur; ^1H n.m.r. ($(CD_3)_2CO$) 6.8-7.8 multiplet (3 protons), and 9.95δ singlet (1 proton); I.R. spectrum was identical to that of authentic sample; ν_{max} 3300 (OH), and 1650 (-C(=O)-H) cm^{-1}.

SYNTHESIS OF [7-^{14}C] p-HYDROXYBENZALDEHYDE

$Cu^{14}CN$

In a preweighed 15 ml centrifuge tube, cupric sulphate pentahydrate (375 mg, 1.56 mmole) was dissolved in water (2.5 ml). This solution was acidified with 12 N sulphuric acid (200 µl). [^{14}C]-Sodium cyanide from three vials (containing 1 mg of sodium cyanide each; specific activity 49 mCi/mmole) was transferred carefully to a 25 ml beaker containing cold sodium cyanide (46 mg), and sodium bisulphite heptahydrate (250 mg, 1 mmole) 0.5 N Sodium hydroxide (3.5 ml) was used to wash the vials, and the washings were transferred to the beaker. This solution was then added dropwise to the contents of centrifuge tube by means of a syringe so that the resulting solution was being stirred by means of syringe needle. The product precipitated rapidly. After about 30 minutes, the product was centrifuged, washed with boiling water, methanol, and finally with ether through centrifugation. The [^{14}C] cuprous cyanide was obtained in 100% yield.

[7-^{14}C] p-CYANOPHENOL

In a 10 ml flask [^{14}C] cuprous cyanide (89 mg, 1 mmole), p-bromophenol (180 mg, 1.1 mmole) and dry DMF (2 ml, dried by distillation over phosphorus pentoxide were heated under nitrogen for three and a half hours. The reaction mixture was brought to room temperature and poured into a solution of ferric chloride hydrate (1.5 g) in concentrated hydrochloric acid (1 ml) and water (3 ml). The resulting mixture was warmed at 50-60°C until no oily layer was visible, and while warm was extracted with ether. The ether extract was washed with water (3 x 20 ml), dried over anhydrous sodium sulphate and solvent was removed *in vacuo*. The dark brown residue was left on vacuum line overnight in order to remove water, and subjected to column chromatography. A small burette was used for this purpose. The supporting

medium was silica gel (10 g) slurry in benzene. The brown residue was dissolved in ether (1.5 ml), adsorbed over silica gel (1 g) and transferred to the top of the column. The eluting solvents were benzene, benzene-ether (1:1) and ether respectively. Most of the unreacted p-bromophenol was eluted in the first 100 ml benzene (fractions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) along with an unknown coloured component. The next 50 ml (fractions 11, 12, 13, 14, 15) contained p-cyanophenol with traces of p-bromophenol and unknown compound. The subsequent 50 ml (fractions 16, 17, 18, 19, 20) contained almost pure p-cyanophenol. The column was then eluted with benzene-ether (1:1), and ether respectively until all p-cyanophenol had been eluted (checked on thin layer chromatography; 5% methanol in benzene; R_f, 0.21).

FURTHER PURIFICATION OF p-CYANOPHENOL

Thin layer chromatography (5% methanol in benzene) of fractions 10, 11, 12, 13, 14 and 15 from previous step showed three spots corresponding to p-cyanophenol (R_f 0.21; standard R_f 0.21), starting material p-bromophenol (R_f, 0.41; standard R_f 0.41), and an unknwon impurity (R_f, 0.85). These fractions were combined, evaporated. The resulting residue was dissolved in ether and loaded on a pre-made silica gel thick layer plate. The elution was carried out by 5% methanol in benzene. The fraction corresponding to p-cyanophneol (R_f, 0.21) was scraped off the plate and extracted with ether. The ether extract was combined with fractions 16, 17, 18, 19 and 20 from the previous step, and the solvent was removed in vacuo, when pure p-cyanophenol was obtained (39.5 mg, 33.2%). A little of p-cyanophenol was weighed accurately (0.7 mg) dissolved in 0.5 ml of ether, and 9.5 ml of scintillation fluid was added to it. From this solution 1 ml was taken into a scintillation vial containing 9 ml of scintillation fluid, and the activity in the contents of the vial was

measured in a liquid scintillation counter. Specific activity 2.93 mCi/mmole cold p-cyanophenol (11 mg) was added to [7-^{14}C]-p-cyanophenol (resulting specific activity, 2.33 mCi).

PHOTOLYSIS OF p-CYANOPHENOL [^{14}CN]

The photolysis was effected by the method of Omura and Matsuura.[72] The reaction was done in two batches. A solution of [^{14}CN]-p-cyanophenol (specific activity 2.33 mCi/mmole) and potassium iodide (1.4 g) in 0.09 N aqueous potassium hydroxide (25 ml) was irradiated with an ultraviolet lamp for eight hours under nitrogen. The brown reaction mixture was then acidified with 10% hydrochloric acid to pH 4.5, and extracted with ethyl acetate (4 x 50 ml). The ethyl acetate extract after washing with 1 M sodium thiosulphate solution (40 ml), and water (20 ml), was dried over anhydrous sodium sulphate and freed of solvent in vacuo. The resulting residue was left on vacuum line overnight to sublime the residual sulphur. [^{14}CHO]-p-Hydroxybenzaldehyde (40 mg) was obtained (specific activity 2.321 mCi/mmole). The product was identified by means of thin layer chromatography (20% methanol in benzene) (R_f 0.36, standard R_f 0.36), and was crystalline.

SECTION 3

ISOLATION OF [1-^{14}C,2-^{3}H] DOPAMINE DERIVED CORYDALINE, RETICULINE, AND PROTOPINE FROM *Corydalis solida*

The isolation procedure was adapted from that of Manske.[73] (Table I) The dried whole plant material. *Corydalis solida*, fed with (1-^{14}C, 2-^{3}H]-dopamine; (T/^{14}C = 4) was thoroughly extracted with methanol (250 ml), in a Soxhlet apparatus, for three days when the solvent was removed *in vacuo*. The resulting residue was taken into 5% hydrochloric acid (200 ml), and left overnight. The aqueous solution was then filtered to remove non basic plant materials and the filtrate extracted with chloroform (3 x 100 ml). The chloroform extract (fraction 1), and aqueous fraction (fraction 2) were examined separately.

FRACTION 1 (chloroform soluble hydrochlorides)

The combined chloroform extracts were concentrated to a small volume *in vacuo*, and the concentrate was basified with 10% potassium hydroxide, when it was extracted with ether (4 x 50 ml), to give ether fraction (1a) and aqueous fraction (1b).

The ether fraction (1a) was dried over anhydrous sodium sulphate and the solvent removed *in vacuo*. This fraction contained non-phenolic alkaloids. Thin layer chromatography (chloroform) of 1a showed two major components corresponding to corydaline (R_f, 0.14; standard R_f, 0.14), and tetrahydropalmatine (R_f, 0.04; standard R_f, 0.04). The crude alkaloidal mixture along with cold corydaline (60 mg) was dissolved in minimum amount of methanol and left in fridge for several days, when the corydaline crystallized out, which was filtered off and recrystallized from methanol,

m.p. 134-35°. A sample (1.5 mg) from recrystallized corydaline was dissolved in methanol (1 ml), mixed with scintillation fluid (9 ml) in a vial and the activity due to ^{14}C was measured, 686 d.p.m., or specific activity (d.p.m./mmole), 16.87 x 10^4. $T/^{14}C$ ratio was obtained by the help of efficiency calibration curves (p. 79).

The aqueous fraction (1b), containing phenolic bases was neutralized with solid carbon dioxide, and extracted with ether (4 x 50 ml). This fraction on drying with anhydrous sodium sulphate and removal of the solvent in vacuo gave a resinous solid material (5-6 mg). Thin layer chromatography (chloroform) of 1b showed a spot corresponding to aurotensine (R_f, 0.05), but the alkaloid could not be crystallized out.

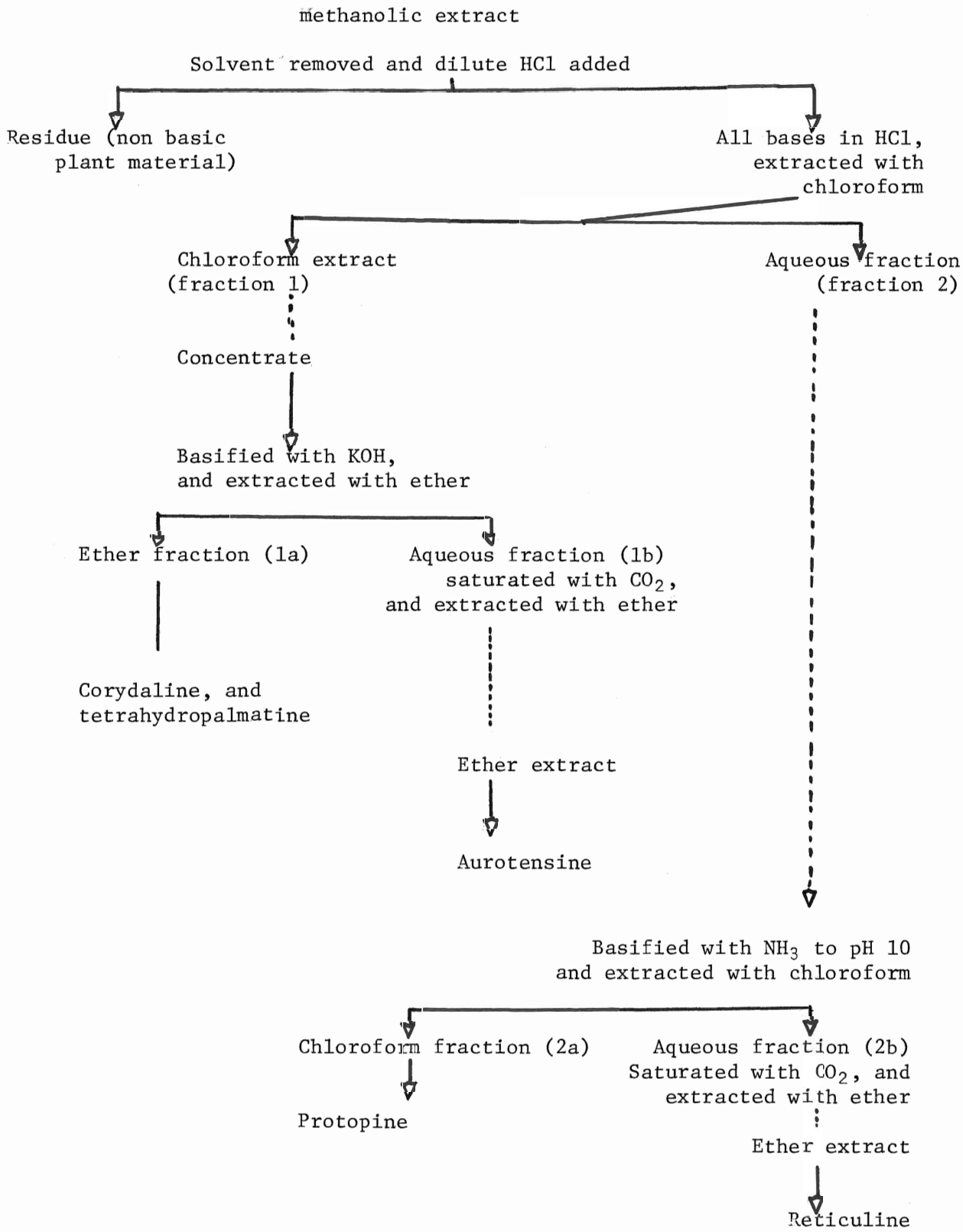
FRACTION 2 (chloroform insoluble hydrochlorides)

The aqueous fraction (fraction 2) was basified with ammonium hydroxide to pH 10 and extracted with chloroform (3 x 100 ml). This gave chloroform fraction (2a) and aqueous fraction (2b). Fraction 2a on drying with anhydrous sodium sulphate, and removal of the solvent in vacuo yielded crude alkaloidal mixture containing protopine as major component. Cold protopine (43 mg) was added to this mixture, and dissolved in methanol. The protopine crystallized out from this solution on slow evaporation, m.p. 205°; specific activity 12.99 x 10^6 dpm/mmole (^{14}C).

Fraction 2b was similarly neutralized by solid carbon dioxide, and extracted with ether. The ether extract on drying over anhydrous sodium sulphate, and removal of the solvent yielded a resinous material, which along with cold reticuline hydrochloride (100 mg) was dissolved in methanol and acidified with hydrochloric acid. This fraction on basification with ammonium hydroxide, and extraction with ether afforded reticuline (R_f, 0.08, chloroform), which did not crystallize out, and, therefore, had to be

subjected to column chromatography. Crude reticuline in chloroform was passed through a column of silica gel. The column was first eluted with chloroform, and then with 1% methanol in chloroform, 2% methanol in chloroform, and 5% methanol in chloroform, respectively. The reticuline came out in the last fraction with 5% methanol in chloroform, specific activity 9.9×10^4 dpm/mmole (^{14}C).

Table 1


methanolic extract
Solvent removed and dilute HCl added
Residue (non basic plant material)
All bases in HCl, extracted with chloroform
Chloroform extract (fraction 1)
Aqueous fraction (fraction 2)
Concentrate
Basified with KOH, and extracted with ether
Ether fraction (1a)
Aqueous fraction (1b) saturated with CO_2, and extracted with ether
Corydaline, and tetrahydropalmatine
Ether extract
Aurotensine
Basified with NH_3 to pH 10 and extracted with chloroform
Chloroform fraction (2a)
Aqueous fraction (2b) Saturated with CO_2, and extracted with ether
Protopine
Ether extract
Reticuline

MEASUREMENT OF RADIOACTIVITY

Liquid scintillation counting was used to measure ^{3}H and ^{14}C activities. The relative efficiencies were obtained by counting the ^{3}H and ^{14}C standards with varying quench. The efficiency curve for ^{14}C only was obtained by plotting the efficiencies versus E.S.R. (Graph 1). To find out $^{3}H/^{14}C$ ratios in doubly labelled compounds, both ^{3}H and ^{14}C standards were counted and the following three efficiency calibration curves were obtained (Graph 2).

(a) The percent efficiency versus E.S.R. curve for ^{3}H as counted in sample analysis channel A.

(b) The percent efficiency versus E.S.R. curve for ^{14}C as counted in sample analysis channel A.

(c) The percent efficiency versus E.S.R. curve for ^{14}C as counted in sample analysis channel B.

The $^{3}H/^{14}C$ ratios for reticuline, corydaline and protopine (Table 2) were obtained by the use of the following formula:

$$^{3}H/^{14}C = \left| \frac{(A \times B_2/B) - B_1}{h} \right|$$

where A = counts per minute in channel A

B = counts per minute in channel B

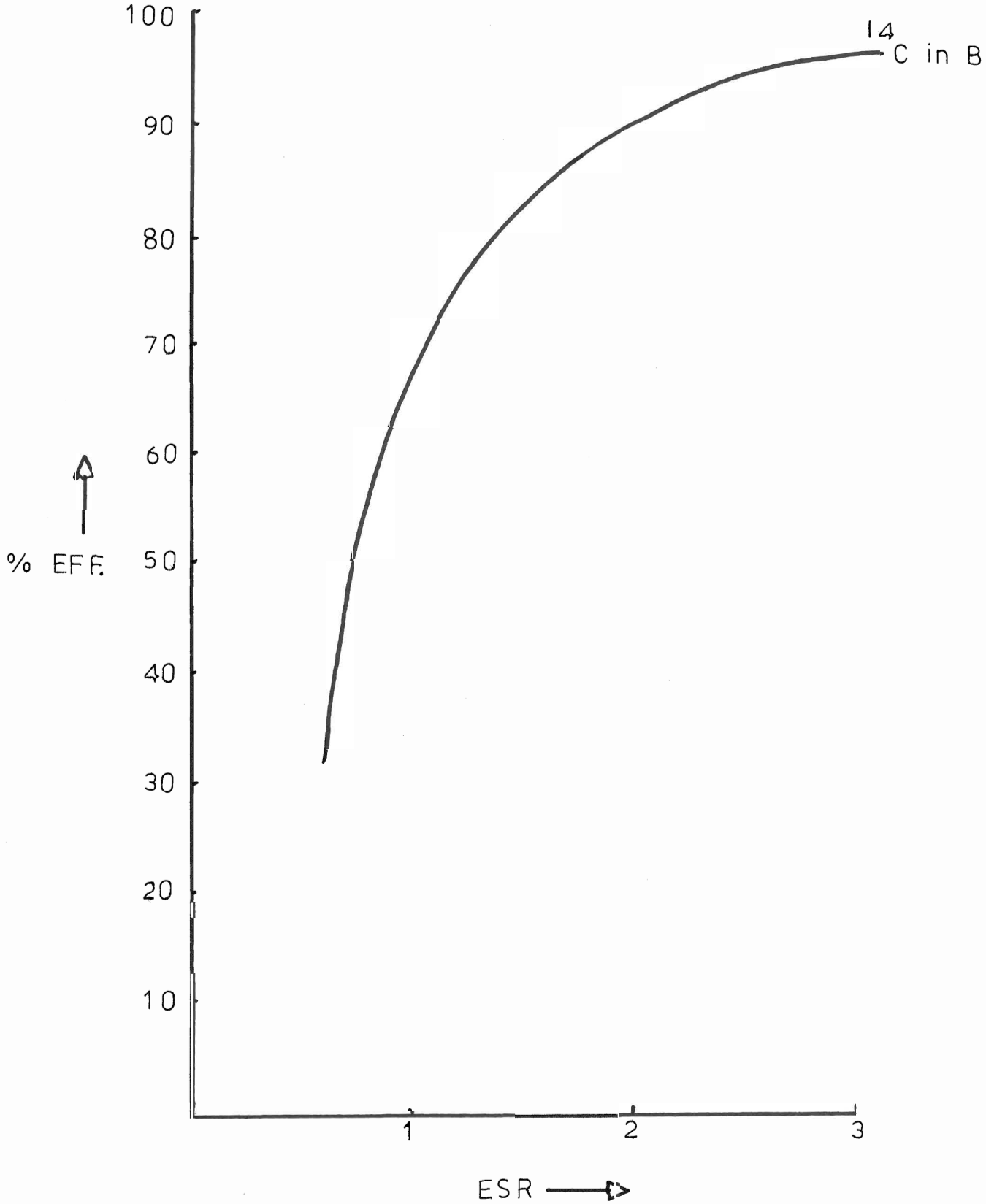
B_1 = ^{14}C efficiency in channel A

B_2 = ^{14}C efficiency in channel B

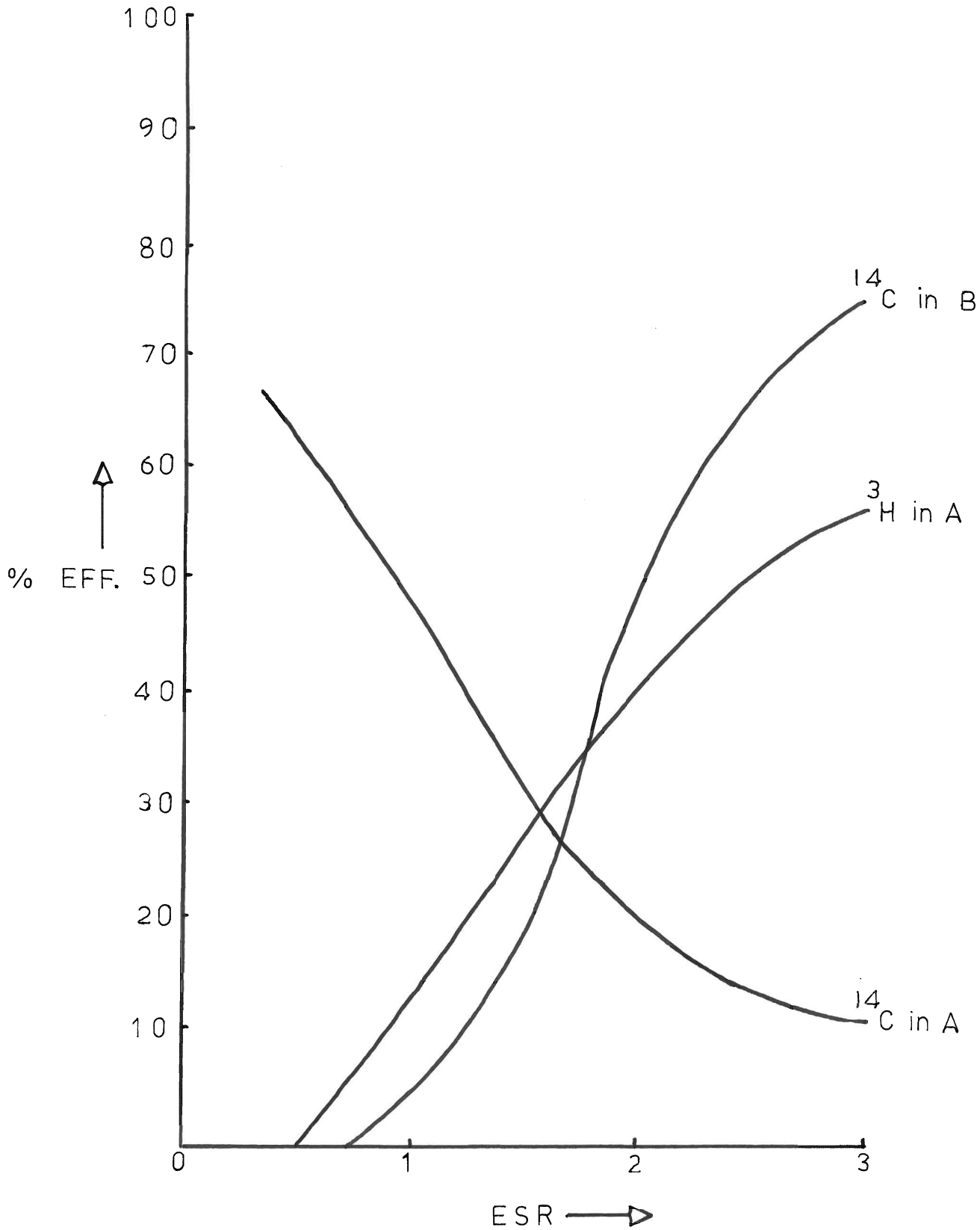
h = ^{3}H efficiency in channel A

Table II

	reticuline	corydaline	protopine
E.S.R.	2.43	1.95	2.41
A	201	452	61227
B	95	304	46431
B_1 (%)	14	20.5	14
B_2 (%)	61.5	45	61.5
h (%)	49	40	49
$^3H/^{14}C$	2.36	1.16	1.37



Graph 1



Graph 2

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