THE BIOSYNTHESIS OF TROPANE AND TOBACCO ALKALOIDS IN DATURA AND NICOTIANA TRANSFORMED ROOT CULTURES

Thesis presented in part fulfilment of the requirement for the degree of Doctor of Philosophy.

by

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September 1991

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"Free at last, free at last.

Thank God Almighty,free at last"

Martin Luther King

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors Professor D. J. Robins (Glasgow) and Dr N. J. Walton (Norwich) for their help and support throughout my Ph.D. project. I would like to express my thanks to all the technicians and fellow students who supported my research especially Mr J. Gall, Dr. I. Colghoun (n.m.r.) and Miss I. Freer (root cultures).

My sincere thanks also go to Mrs B. Phillips and Miss J. Brown for their patience in the excellent typing of the thesis.

Finally, I would like to thank my mother and father for their support throughout my acadmic career.

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The biosynthesis of tropane and tobacco alkaloids has been extensively studied since the 1950's. Their importance is due to two main reasons:

(i) use in medicine e.g. scopolamine, and;

(ii) mis-use in society e.g. nicotine and cocaine.

The advent of transformed root cultures (hairy root cultures) has allowed the expansion of these studies.

The introduction of this thesis contains descriptions of tropane alkaloid structure and occurrence, tobacco alkaloid structure and occurrence, pharmacology of tropane alkaloids, pharmacology of tobacco alkaloids and finally, the aims of the project.

A critical review of the biosynthesis of the tropane and tobacco alkaloids is presented in conjunction with a simplified description of the production and manipulation of transformed root cultures.

Putrescine is incorporated into the carbon skeleton of (-)-hyoscyamine and (-)-scopolamine in the Datura plant species and (-)-nicotine in the Nicotiana plant species. It has been used as the starting block to study the biosynthesis of (-)-hyoscyamine and (-)-scopolamine and, in analogue form to study the effect on the biological systems of interest.

Enantiomerically labelled putrescines, (R)-[2-^{2}H]- and (S)-[2-^{2}H]putrescine were synthesisd in order to study epoxide ring formation in the biosynthesis of (-)-scopolamine. The powerful technique of ^{2}H n.m.r. spectroscopy was used to examine this biosynthetic step. These results are presented in Chapter Five.

The enantiomerically labelled putrescines, (R)-[1-^{2}H]- and (S)-[1-^{2}H]putrescine were synthesised in order to study the biosynthesis of the tropane ring. The use of ^{2}H n.m.r. spectroscopy was used to examine this biosynthetic step, firstly, in hygrine and secondly, in (-)-scopolamine. These results are presented in Chapter Six.

The biosynthesis of anabasine in Nicotiana transformed root cultures was examined by the feeding experiments with (R)-[1-^{2}H]- and (S)-[1-^{2}H]cadaverine and analysis by ^{2}H n.m.r. spectroscopy. This experiment has determined the optical purity of anabasine in Nicotiana transformed root cultures. These results are presented in Chapter Seven.

Various analogues of putrescine were synthesised and administered to Datura and Nicotiana transformed root cultures. Their effect on the production of the natural occurring alkaloids was examined. In addition the selectivity of the enzymes involved in tropane and tobacco alkaloid biosynthesis was studied. They were also supplied for enzymic studies on the oxidation of putrescine derivatives by Diamine Oxidase.

INTRODUCTION TO THE TROPANE AND TOBACCO ALKALOIDS

Alkaloids are basic nitrogen-containing compounds of either plant or animal origin. "**True**" alkaloids as they were classed by Winterstein and Trier[1] fulfilled in addition the following criteria:-

i) They contained a nitrogen atom within a heterocyclic system.

ii) They had a complex molecular structure.

iii) They had pharmacological activity.

iv) Occurrence was limited to the plant kingdom.

1.1 Tropane alkaloid structure and occurrence

Tropane alkaloids are a well defined group of structurally related natural products, with pharmacological activity, which contain a common structural element, the azabicyclo[3.2.1]octane system which is hydroxylated in position three at least. The systematic name for the tropane ring system is 8-methyl-8-azabicyclo[3.2.1]-octane (Figure 1), shown with position three hydroxylated.

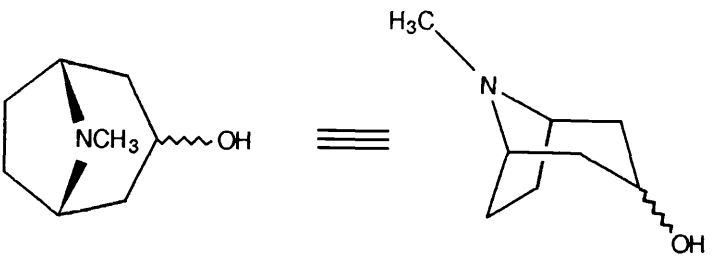


Figure 1 : The tropane ring system

Throughout this thesis the bridge head carbon denoted C-1 has the absolute configuration as above.

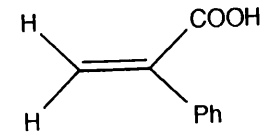
Tropane alkaloids occur mainly in the plant family Solanaceae but are also found in other families i.e. Convolvulaceae, Erythroxylaceae, Proteaceae and Rhizophoraceae. The Solanaceae family of plants contains species from the following genera of plants : **Atropa** (especially **A.belladonna**), **Datura**, **Hyoscyamus**, **Mandragora**, **Duboisia** and **Scopolia** (Table 1).

The tropane alkaloids are all esters of organic acids (e.g. atropic, benzoic, cinnamic, isovaleric, 2-methylbutyric, tiglic, tropic and veratric). These are combined with one of a series of cyclic bases (e.g. ecgonine, nortropine, scopine, pseudotropine and tropine) (Figure 2).

Table 1: Examples of plants producing tropane alkaloids [2].

Family	Sub-family	Genus
Solanaceae	Solanoideae	Atropa
		Datura
		Hyoscyamus
		Mandragora
		Nicandra
		Scopolia
		Withania
	Cestroideae	Duboisia
		Schizanthus
Erythroxylaceae	-	Erythroxylon
Proteaceae	Grevilleoideae	Knightia
Rhizophoraceae	Rhizophoroideae	Bruguiera

Acids



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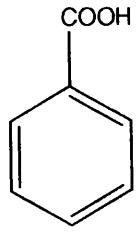

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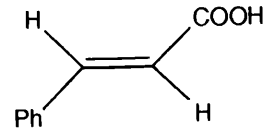

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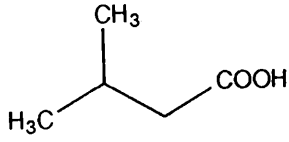

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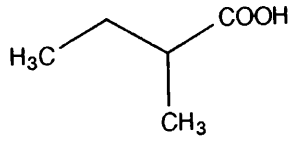

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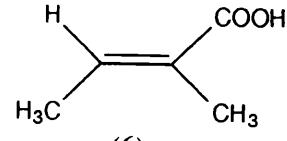

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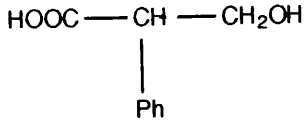

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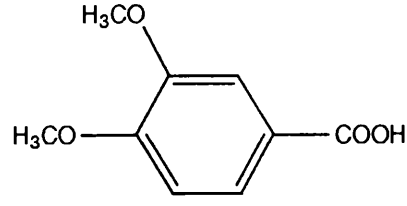

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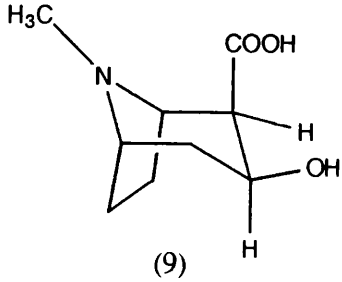
Bases



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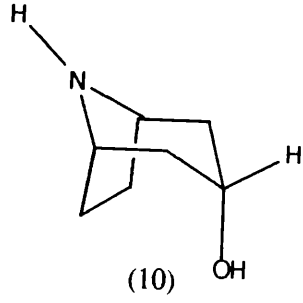

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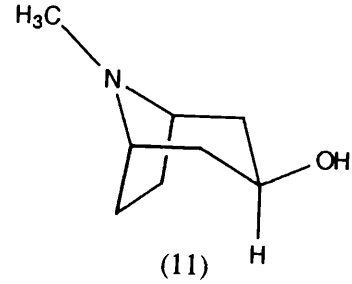

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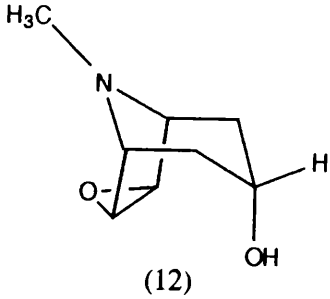

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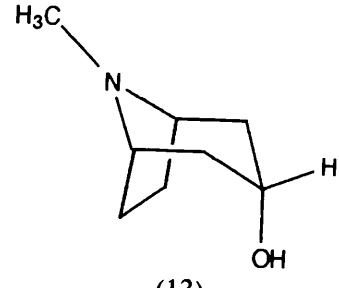

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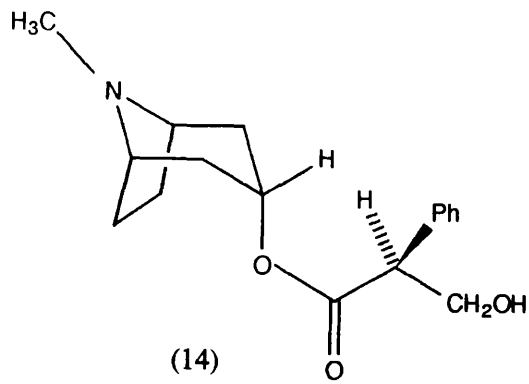
Figure 2 : Common acids and base found in tropane alkaloids

The best known alkaloids of the Solanaceae are (-)-hyoscyamine (14) and (-)-scopolamine (15). The esterification of (S)-tropic acid (7) with the bases tropine (13) and scopine (12) gives (-)-hyoscyamine (14) and (-)-scopolamine (15) respectively. The optical activities of both these alkaloids are due to the presence of the (S)-tropic acid (7) residue as the bases possess a plane of symmetry through positions three and eight.



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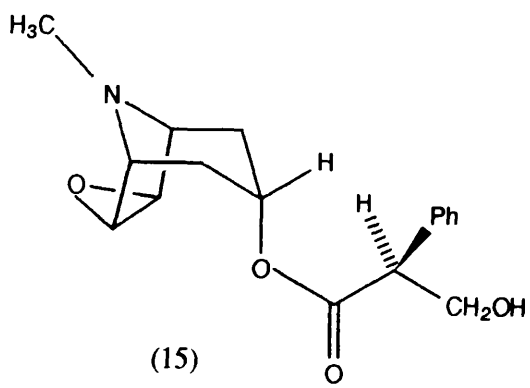

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1.2 Tobacco alkaloid structure and occurrence

The physiological affects of smoking and the economic importance of tobacco cultivation in many countries, especially those in the Third World, has brought about a dramatic expansion in the literature of tobacco constituents. The literature on nicotine and related tobacco substitutes is supplemented by information about their pyrolysis and combustion products. Over the last thirty years an extensive number of reviews has appeared [3-7].

The tobacco alkaloids are the best known of the pyridine alkaloids and have the following structural requirements:-

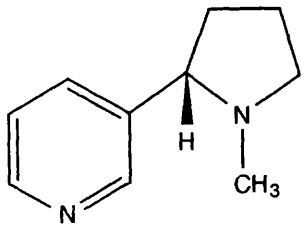
i) They contain a pyridine ring.
ii) The pyridine ring is substituted only at C-3.
iii) They contain a pyrrolidine or piperidine ring substituted only at C-2.
iv) The ring systems are joined by a carbon-carbon bond from C-3 of the pyridine ring to C-2 of the pyrrolidine or piperidine ring.

Tobacco (Nicotiana tabacum) has been extensively studied with (-)-nicotine (16) as its major alkaloid. More than twenty minor alkaloids have been identified in tobacco leaves or air-cured tobacco[8,9]. Many plant families have been found to contain (-)-nicotine (16) as well as all Nicotiana species (Table 2). For the natural product chemist the major tobacco alkaloids of interest (and publications) are nicotine (16), anabasine (17), nornicotine (18) and anatabine (19).

Table 2

Family	Species
Solanaceae	Datura stramonium
	Duboisia myoporoides
	Solanum melongena
Lycopodiaceae	Lycopodium flabelliforme
	Lycopodium sabinaefolium
Crassulaceae	Sedum oppositifolium
Erythroxylaceae	Erythoxylon coca
	Erythroxylon truxillense

The major tobacco alkaloids



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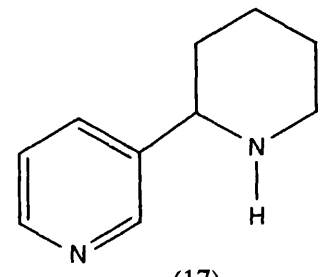

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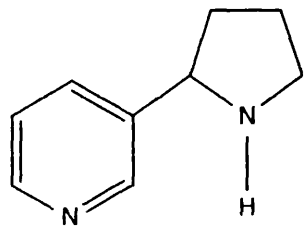

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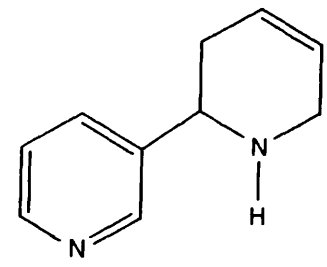

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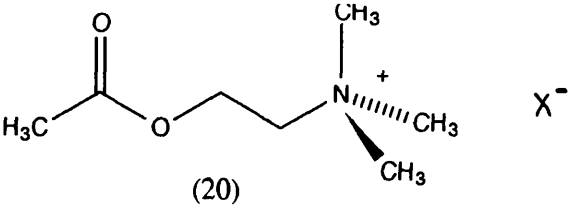
1.3 Pharmacology of Tropane Alkaloids

It is outwith the scope of this thesis to give a detailed description of the pharmacology of tropane alkaloids. The following text gives a general overview of their applications and suggested mode of action.

The pharmacology of tropane alkaloids has been studied for many years and they have been found to exhibit a wide range of properties. They act at the peripheral autonomic nervous sytem as blocking agents for nerve pulse transmission. The natural products (±)-hyoscyamine (21) (atropine) and (-)-scopolamine (15) are the best known of these blocking agents and are believed to interact with the muscarinic recepters [10], which are activated by acetylcholine (20). Acetylcholine (20) is the chemical responsible for nerve pulse transmission across synapses between nerve cells.



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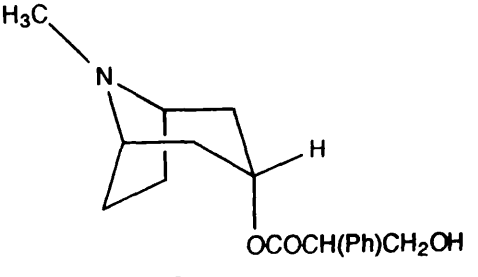


(21)

Other tropane alkaloids act in a similar manner [11] to atropine (21) and (-)-scopolamine (15), except cocaine (33) which acts on

adrenergic receptors. The actions of acetylcholine (20) presumably depend on its interaction with a receptor. It may be inferred that the receptor contains an anionic site which binds to the quaternary nitrogen of acetylcholine (20) and another site which binds to the carbonyl group of the ester. Compounds which mimic acetycholine (20) not all of which contain a quaternary centre, may attach to this site without activation. In tropane alkaloids it is presumably the nitrogen and the ester carbonyl which interact with the receptor.

Tropane alkaloids and synthetic analogues act as mydriatics, anaesthetics, central nervous system stimulants and some synthetic tropanes show curare-like activity [12]. Atropine (21) has been shown to reduce heart rate and blood pressure [13], and relieves a form of angina [14]. It also acts as an anti-viral agent on the multiplication of enveloped viruses [15]. Scopolamine (15), or its quaternary salt, has been shown to provide rapid and substantial relief from motion sickness [16] and it possesses broncospasmolytic activity [17].

The pharmacology of tropane alkalods will be investigated further as more synthetic analogues become available and their mode of action is better understood.

1.4 Pharmacology of tobacco alkaloids

Approximately thirty per cent of the four thousand known products from smoking tobacco contain nitrogen atoms. They have clearly

defined biological activity and have been identified in both leaf and smoke [18]. The best known constituent of tobacco to the consumer and non-consumer is nicotine (16) which has an acute toxicity of approximately 60 mg in adults. Acute poisoning causes convulsions, nausea and vomiting, following by depression and respiratory paralysis of both central and peripheral origin.

Nicotine (16) activates receptors in the aorta causing tachycardia, vasoconstriction and resultant elevation of blood pressure. It also affects receptors in the gastrointestinal tract, mesentery, stomach, tongue and lung. It has also been implicated in the etiology of some cardiovascular diseases and chronic obstructive lung disease, but not in the propagation of certain neoplastic diseases. Nicotine (16) does not produce congenital defects of the skeletal system in farm animals [19]. This observation was also found with anabasine (17)[20].

Nornicotine (18) is three times more toxic than nicotine (16) whereas anabasine (17) has the same toxicity as nicotine (16). These alkaloids have different biological properties to nicotine (16) and cause depression not stimulation of receptors, as is the case with nicotine (16). Research will continue intensively on the pharmacological effects of these compounds and on their modes of action.

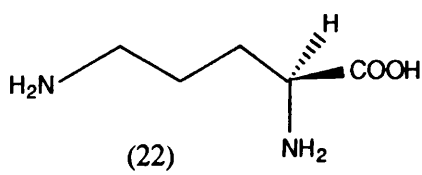
1.5 Aims of project

The biosynthesis of the tropane alkaloids (-)-hyoscyamine (14) and (-)-scopolamine (15) has been extensively studied. Their biological activities mean that the way they are produced in

plants is of fundamental interest. However, some very important steps in the biosynthesis have yet to be elucidated. The stereochemistry of a number of the enzymic processes involved in tropane ring formation remains to be resolved, and the mechanisms of these processes need to be understood. The tropane ring system is formed in plants from L-ornithine (22) via the unsymmetrical diamine N-methylputrescine (23). This area is reviewed in Chapter 2.

The oxidation of N-methylputrescine (23) is a key reaction in the biosynthesis of the tropane ring system. The use of enantiomeric [1-^{2}H]putrescines allowed the stereochemistry of this process to be studied. The results of these investigations are presented in Chapter 6.

The mechanism of epoxide formation in (-)-scopolamine (15) is an area of great interest and the use of enantiomeric [2-^{2}H]-putrescines allowed investigation of the stereochemistry of hydrogen retention and removal on the epoxide carbon atoms. The results are presented in Chapter 5.



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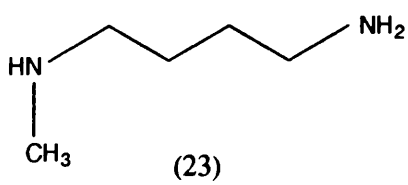

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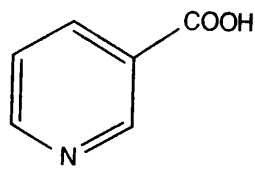
The opportunity arose to study the biosynthesis of anabasine (17). There is debate as to whether the pyridine ring of this alkaloid is derived from nicotinic acid (24) or L-lysine (25). These results are presented in Chapter 7.



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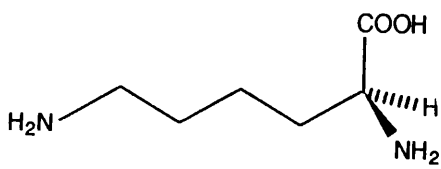

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Tropane and tobacco alkaloids have a wide range of biological activities and analogues of these alkaloids are useful for biological evaluation. Total synthesis of these alkaloid analogues in optically active form can be difficult therefore a different strategy was considered. A range of putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) derivatives were prepared and fed to transformed root cultures in an attempt to produce analogues of their normal metabolites from these structually modified biosynthetic precursors. These precursors can also be used to test the selectivity and specificity of the enzymes found in the biosynthetic pathway.

The precursors may act as enzyme inhibitors or produce analogues with biological activities different from the natural products. In addition, these compounds were supplied and tested as substrates of diamine oxidase (DAO) in a separate project supported by the SERC. These results are discussed in Chapter 8.

CHAPTER TWO

BIOSYNTHESIS OF TROPANE ALKALOIDS

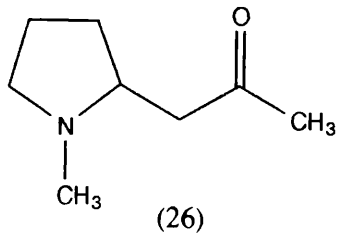
2.1 Introduction

Since the 1950's the use of radioactive isotopes (^{3}H, ^{14}C) and more recently stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O) has become one of the best tools for the natural product chemist. The use of labelled compounds has allowed recognition of precursor-product relationships in many classes of compounds. The use of possible precursors highly enriched with stable isotopes has increased dramatically as more sensitive spectral techniques which do not require degradations of samples have been developed. This has assisted in the identification of intermediates which lie on the route from precursor to product and has allowed an insight into the mechanism and stereochemistry of enzyme catalysed steps on the biosynthetic pathway. Many important advances in the biosynthesis of the tropane alkaloids have occurred and these will be reviewed in this chapter.

2.2 Biosynthesis of the tropane ring

The tropane alkaloids were amongst the first to have their biosynthetic pathways investigated with radioactive percursors. This provided confirmation of many of the hypotheses advances by Sir Robert Robinson more than forty years previously[21]. He suggested that hygrine (26) is formed from ornithine (22), proline (27) or glutamic acid (28) and an acetone equivalent. This acetone equivalent could be acetoacetic acid (29), acetone

dicarboxylic acid (30) or citric acid (31) with an oxidising system.



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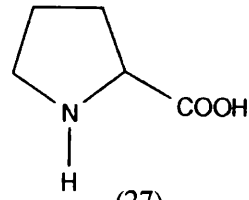

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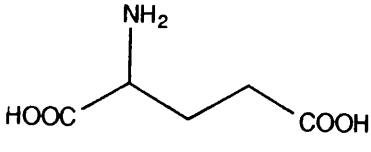

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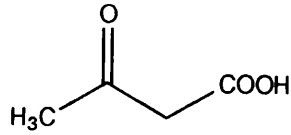
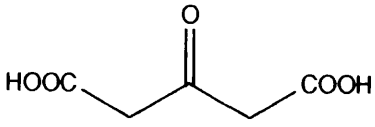


(29)

Tropinone (32) was subsequently suggested to arise from a pyrrolidine ring attached to the acetone equivalent by two links whereas hygrine (26) had one link between the two units. Robinson extrapolated the reasoning to suggest that the amino acid involved was ornithine (22) [or arginine] or glutamic acid (28) and not proline (27). By analogy with his famous synthesis of tropinone (32) in 1917 he dismissed the possibility of an internal oxidation of hygrine (26) and cyclisation to tropinone (32).



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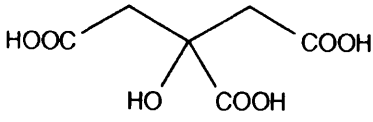
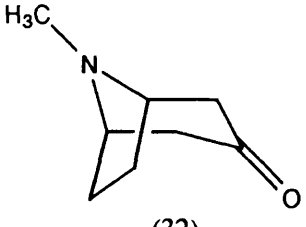


(31)

The biosynthesis of cocaine (33) has became an extensive area of research due to its misuse in society. Cocaine (33) was believed to arise in an analogous manner with the acetone equivalent being carboxylated. Acetoacetic acid (29) was the or Robinson's suggested acetone equivalent.



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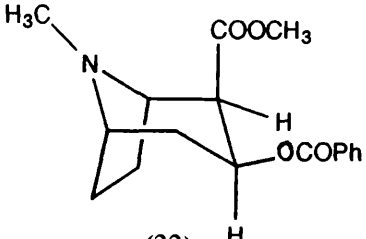
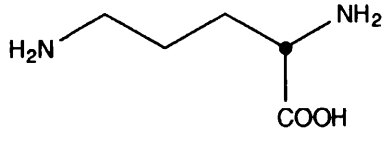
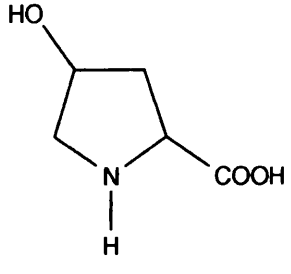


(33)

The first attempt to test Robinson's ideas was by Leete et al[22] in 1954. They synthesised DL-[2-^{14}C]ornithine (34) and fed it to mature Datura stramonium plants by the hydroponics technique. They found by systematic degradation that all the activity in (-)-hyoscyamine (14) was located at positions one or five or both. Their degradation method did not allow the last possibility to be excluded. They found (-)-scopolamine (15) contained no activity and suggested hydroxyproline (35) as its biosynthetic precursor.



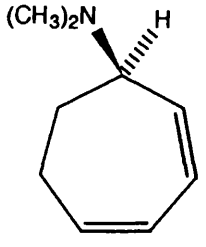
(34) • = ^{14}C .



(35)

In order to determine which carbon of the tropane ring was labelled Leete[23] used a new degradation scheme developed by Bothner-By and co-workers[24]. This allowed differentiation of the two bridgehead carbons of the tropane ring. Leete fed DL-[2-^{14}C]ornithine (34) to D.stramonium plants and showed that only one of the bridgehead carbons was labelled. This carbon contained all the activity of the isolated (-)-hyoscyamine (14) but no distinction could be made as to which bridgehead carbon was labelled.

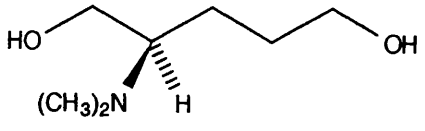
This experiment also gave labelled (-)-scopolamine (15) in contrast to Leete's earlier findings[22]. The unsymmetrical labelling pattern was complimentary to that published[24] by Bothner-By and co-workers who fed sodium [1-^{14}C]acetate to D.stramonium root cultures. Sodium acetate is converted into ornithine (22) via established metabolic pathways of the Krebs cycle and amino acid interconversions. Leete[25] showed that C-1 was the labelled bridgehead carbon by the determination of the absolute configuration of the degradation product (+)-1-dimethylamino-2,4-cycloheptadiene (α-methyltropidine) (36).



(36)

This product was converted into 2-dimethylamino-1,5-pentanediol (37). The rotation of this sample was compared to that of an authentic sample of (+)-(S)-2-dimethylamino-1,5-pentanediol (38). They were found to have equal and opposite rotations, therefore Δ^1-methyltropidine (36) has the (R)-configuration at its asymmetric centre.

In 1969 Gros and Barelle[25] found DL-δ-N-methyl-[^{3}H]ornithine (39) to be an excellent precursor for (-)-hyoscyamine (14) when fed to Atropa belladona plants. This was in accordance with the results of Schütte et al.[25,27] but in conflict with the results of Neumann and Schröter[28] who suggested α-N-methylornithine (40) as the precursor of (-)-hyoscyamine (14).



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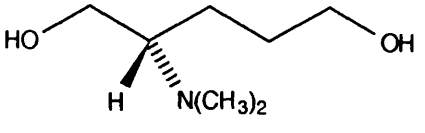

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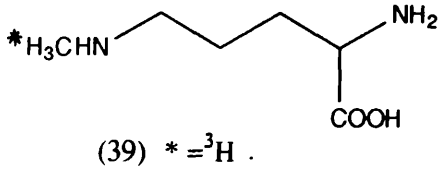
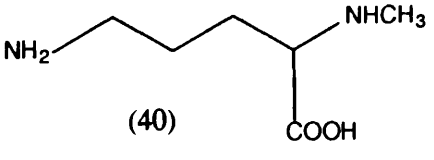
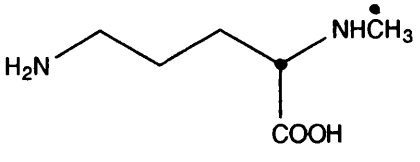


(39) * = ^{3}H .

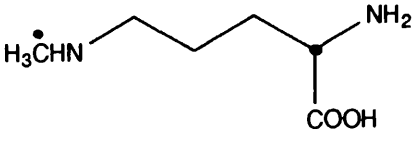


(40)

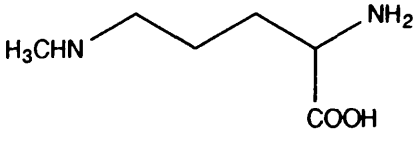
Leete and Ahmad[29] resolved this mystery by feeding α- and δ-N-[$^{14}CH_3$]methyl[2-^{14}C]ornithines (41) and (42) to D.stramonium plants. Isolated (-)-hyoscyamine (14) fed with α-N-[$^{14}CH_3$]methyl[2-^{14}C]ornithine (41) was inactive whereas δ-N-[$^{14}CH_3$]methyl[2-^{14}C]ornithine (42) gave labelled (-)-hyoscyamine (14) and (-)-scopolamine (15). The specific activities obtained from the feeding of δ-N-[$^{14}CH_3$]-methyl[2-^{14}C]ornithine (42) were higher than those obtained from the administration of [2-^{14}C]-ornithine (34) to D.stramonium plants. Therefore, δ-N-methylornithine (43) was proposed to be the natural precursor of (-)-hyoscyamine (14) and(-)-scopolamine (15), δ-N-methylornithine (43) has been



(41)● = ^{14}C .



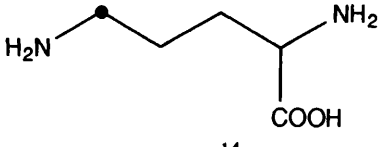
(42) ● = ^{14}C .



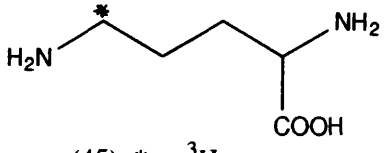
(43)

found to be a constituent of A.belladonna[30]. Labelled δ-N-methylornithine (43) was obtained from the feeding of DL-[5-^{14}C]ornithine (44) and DL-[5-^{3}H]ornithine (45); therefore δ-N-methyl-ornithine (43) fulfills the usual criteria to establish it as an intermediate in the tropane alkaloids pathway.

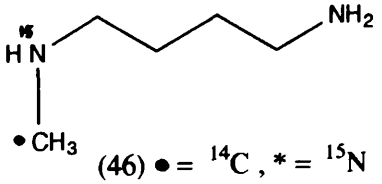
Liebisch and co-workers[31] fed [^{15}N]-N-[$^{14}CH_3$]methylputrescine (46) to root cultures of Datura metel. The ratio of the specific incorporations of ^{15}N to ^{14}C was measured and found to be 0.93 in (-)-hyoscyamine (14) and 0.91 in (-)-scopolamine (15). This indicated incorporation of the intact precursor with no demethylation of the precursor to the symmetrical compound putrescine.



(44)● = ^{14}C .

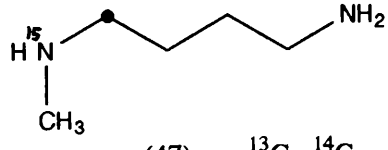


(45) * = ^{3}H .



(46) ● = ^{14}C , * = ^{15}N .

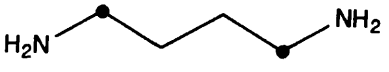
No degradations of the isolated alkaloids were undertaken to show the labelling of the bridgehead carbons, Leete and McDonell[32] answered this question with the synthesis and feeding of [1-^{13}C, ^{14}C methylamino-^{15}N]-N-methylputrescine (47) to Datura innoxia plants. The ^{13}C n.m.r. spectrum showed one bridgehead carbon was labelled. The bridgehead carbons, (C-1 and C-5), have a chemical shift difference of 0.09 ppm and only one of these signals contained satellites due to ^{13}C-^{15}N coupling ($^{1}J_{^{13}C-^{15}N}$=2.9Hz). As expected N-methylputrescine (23) was incorporated into (-)-hyoscyamine (14) and (-)-scopolamine (15) unsymmetrically. No conclusive proof was given as to which of the bridgehead carbons was labelled but the results of earlier work[25,29] strongly suggested C-1.



(47) • = ^{13}C , ^{14}C .

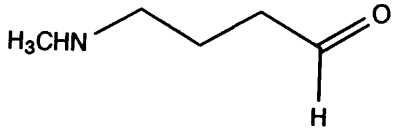
From these results it was proposed that N-methylputrescine (23) is a precursor of the tropane alkaloids. N-methylputrescine (23) is produced by the decarboxylation of the known precursor δ-N-methylornithine (43).

It is worth noting that other compounds have been incorporated into tropane alkaloids although the process is believed to occur by an indirect pathway. When [1,4-^{14}C]putrescine (48) was fed to excised root cultures of D. metel[33,34] labelled (-)-hyoscyamine (14) and (-)-scopolamine (15) were isolated and degradation showed that both bridgehead carbons were labelled. Putrescine is believed to be incorporated via N-methyl-putrescine (23).



(48) • = ^{14}C .

N-methylputrescine (23) is oxidised by a diamine oxidase (DAO) to 4-methylaminobutanal (49) which has been found in D.stramonium. Mizusaki et al.[35] administered [2-^{14}C]ornithine (34) and in this trapping experiment isolated labelled 4-methylaminobutanal (49). An equilibrium exists between 4-methylaminobutanal (49) and its N-methyl-Δ'-pyrrolinium salt (50) which by the completion of a Mannich reaction attaches the remaining three carbons required for the tropane ring structure. Leete studied[36] the possible tautomerism in the N-methyl-Δ'-pyrrolinium salt (50) (figure 3). The reaction of [2-^{14}C]N-methyl- Δ '-pyrrolinium chloride (51) with acetone dicarboxylic acid (30) in vitro gave hygrine (26) labelled only at C-2. The 1,2-double bond in the pyrrolinium salt therefore was not able to tautomerise to the 1,5-position.



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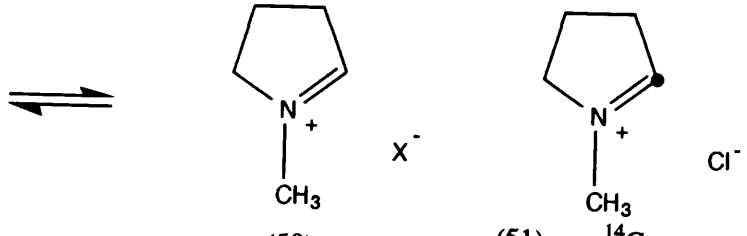
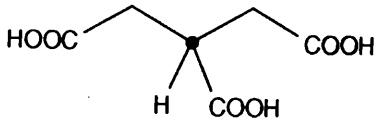


(50) (51) • = ^{14}C .

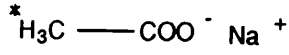
Figure 3

This result substantiates the unsymmetrical labelling pattern observed in earlier work[22,23] from the administration of [2-^{14}C]ornithine (34) to D.stramonium plants.

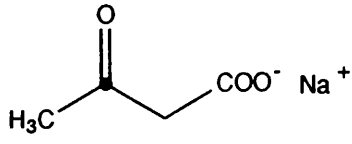
Extensive investigations have been carried out on the origin of the "acetone equivalent" proposed by Robinson[21]. He suggested that C-2, C-3 and C-4 of the tropane ring are derived from citric acid (31) but this has been proved incorrect by Robertson and Marion[37]. They synthesised and fed [3-^{14}C]citric acid (52) to D.stramonium plants and found an insignificant level of activity in the alkaloidal extract. Acetoacetate (53) was suggested as the precursor for the "acetone equivalent" by Liebisch et al.[35] who



(52) • = ^{14}C .



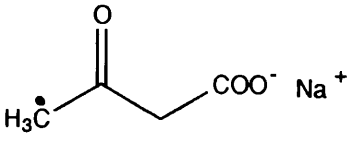
(54) * = ^{3}H .



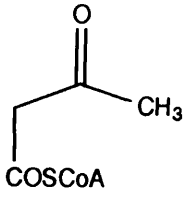
(53) • = ^{12}C .
(55) • = ^{14}C .

fed sodium [2-^{3}H]acetate (54) and sodium [3-^{14}C]acetoacetate (55) to D.metel. The incorporation of the ^{14}C label was five times greater than that of the ^{3}H label in the isolated (-)-hyoscyamine (14), therefore acetoacetate (53) was suggested as the precursor of the "acetone equivalent". O'Donavan and Keogh[39] fed [2-^{14}C]ornithine (34) and sodium[1-^{14}C]acetate to Nicandra physaloides plants in separate experiments. They found [2-^{14}C]ornithine (34) labelled only C-2 and [1-^{14}C]acetate

labelled C-2' of hygrine (26), but the question still arose as to which carbon of acetoacetate (53) became attached to C-2 of the N-methyl-Δ^1-pyrrolinium salt (50). In 1979 McGaw and Woolley[40] synthesised and fed sodium [4-^{14}C]acetoacetate (56) to Nicandra physaloides plants and by degradation found that C-2 of acetoacetate (53) was the site of reaction in the formation of hygrine (26). The unsymmetrical labelling of hygrine (26) by [2-^{14}C]ornithine (34) as found by O'Donavan and Keogh[39] was



(56) • = ^{14}C .

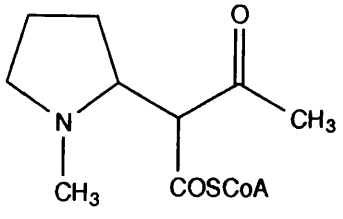


(57)

further examined by Leete[41] who administered DL-[5-^{14}C]ornithine (44) to N.physaloides plants. The specific incorporation of the isolated hygrine (26) was higher than that reported by O'Donovan and Keogh (2.2% compared to 0.1%) and symmetrical labelling of hygrine (26) was observed.

Leete pointed out serious discrepancies and mistakes in the previous paper[39] over degradation products and radiochemical analysis, therefore the symmetrical labelling of hygrine (26) would seem the more probable.

Acetoacetate (53) probably exists as its coenzyme A ester (57) in plants and could combine with the N-methyl-Δ¹'-pyrrolinium salt (50) to give the coenzyme A ester of hygrine-1'-carboxylic acid (58). Hydrolysis and instantaneous decarboxylation of hygrine-1'-carboxylic acid (58) would yield hygrine (26).



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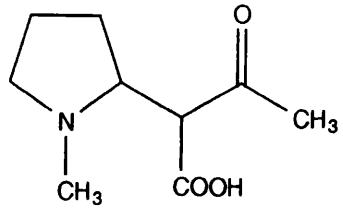

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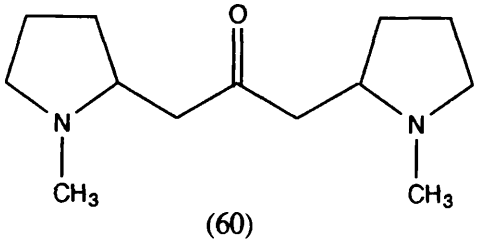

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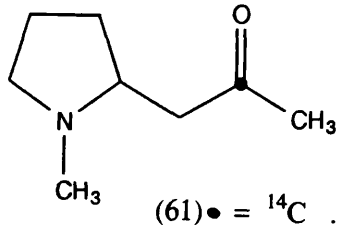


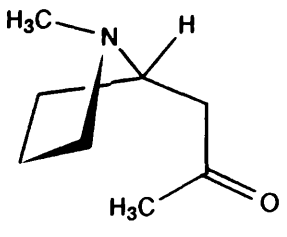
(61)• = ^{14}C .

Hygrine (26) and hygrine-1'-carboxylic acid (59) are important intermediates in the tropane alkaloid biosynthetic pathway. Their involvement in the biosynthesis of cuscohygrine (60) and cocaine (33) has been studied.

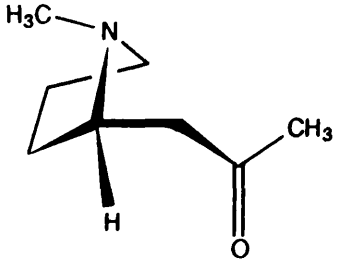
Hygrine (26) has been shown to be a precursor of the tropane alkaloids by the feeding of DL-[2'-^{14}C]hygrine (61) to D.stramonium[39]. This gave (-)-hyoscyamine (14) labelled in the C-3 position.

Hygrine (26) has two enantiomeric forms and the occurrence and utilisation of these enantiomers is dependent on the plant species. McGaw and Woolley synthesised DL-[2'-^{14}C]hygrine (61) and separated each enantiomer as its (+)-tatrate salt. After feeding experiments were carried out their results indicated[42] that Datura plants used (+)-hygrine (62) as the precursor of (-)-hyoscyamine (14) whereas in Hyoscyamus niger and A.belladonna both the (+)- and (-)-enantiomers (62) and (63) were used. In D.innoxia, which also produces (-)-scopolamine (15), it was found that (+)-hygrine (62) was a more efficient precursor of the alkaloid.

McGaw and Woolley[43] continued their studies by investigating whether hygroline (64), a known plant constituent, was an intermediate between hygrine (26) and tropane ring formation. In two separate experiments they fed [1",2'-^{14}C]tigloyl hygroline (65) and [2'-^{14}C]hygrine (61) to Datura meteloides plants and measured the incorporations into the alkaloids. Isolated (-)-hyoscyamine (14) and (-)-scopolamine (15) had higher specific incorporations from the feeding of [1",2'-^{14}C]tigloyl hygroline (65) than the [2'-^{14}C]hygrine (61) control feed. The [1",2'-^{14}C]tigloyl hygroline (65) had undergone extensive hydrolysis to [2'-^{14}C]hygroline (66) as (-)-hyoscyamine (14) and (-)-scopolamine (15) were both labelled. It was proposed that either Datura plants were able to convert hygroline (64) to hygrine (26) or hygroline (64) was the immediate tropane ring precursor.

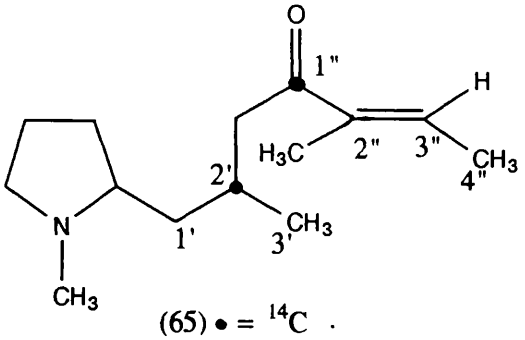


(62)

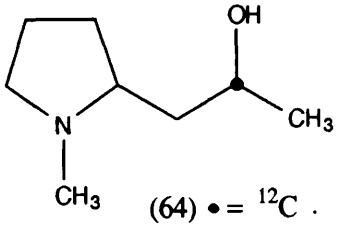


(63)

The synthesis and administration of [2'-^{3}H;2'-^{14}C]hygroline (67) to D.innoxia Mill plants[44] showed hygroline (64) was not a precursor of the tropane ring. The ratio of ^{3}H to ^{14}C showed that 90% of the tritium had been lost from the isolated (-)-hyoscyamine (14) and (-)-scopolamine (15). It was claimed that the remaining 10% of the tritium was retained at other positions in the compounds due to proton exchange occurring during synthesis of the labelled precursor.

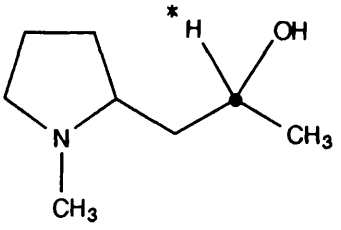


(65) • = ^{14}C .



(64) • = ^{12}C .

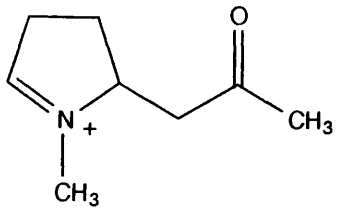
(66) • = ^{14}C .



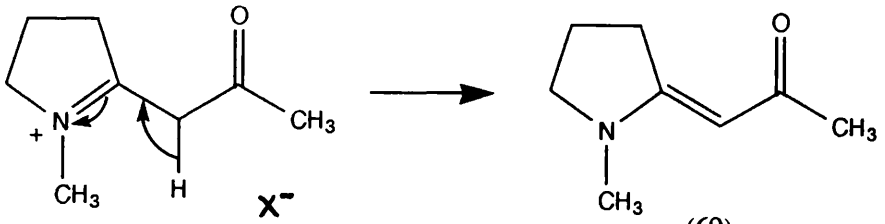
(67) • = ^{14}C , * = ^{3}H .

It has been suggested[45] that the intermediate between hygrine (26) and tropinone (32) is dehydrohygrine (68) which yields tropinone (32) via completion of an intramolecular Mannich type reaction. Leete and Kim[46] examined the oxidation of hygrine (26) with mercury(II) acetate. They synthesised tropinone (32) and 2,1'-dehydrohygrine (69) which is the stable product formed from

the 2-acetonyl-1-methyl-Δ^1-pyrrolinium salt (70). This biomimetic synthesis adds credance to the proposed biosynthesis of tropine (13) from hygrine (26) via tropinone (32). Indeed, tropinone (32) has been isolated from several plant species[47,48].

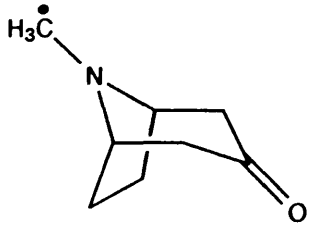


(68)



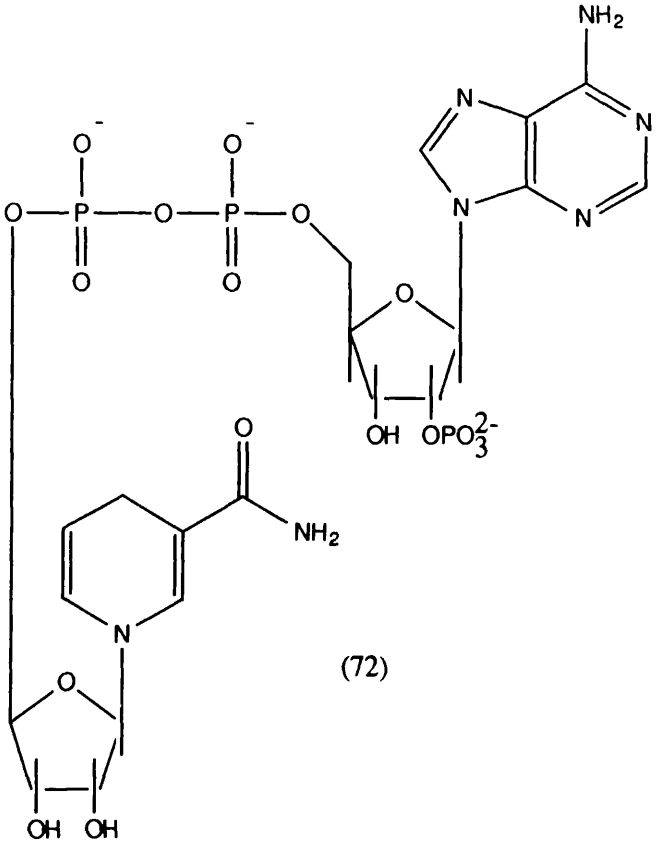
(70) (69)

Leete and Landgrebe[49] synthesised and administered [methyl-^{14}C]-tropinone (71) to D.innoxia plants. The isolated (-)-hyoscyamine (14) and (-)-scopolamine (15) showed high specific incorporations (2.5% and 7.3% respectively). Degradation of each alkaloid indicated that essentially all the activity was present in the N-methyl group. These results are consistent with the proposed hypothesis that tropinone (32) is an intermediate in tropane alkaloid biosynthesis.



(71) • = ^{14}C

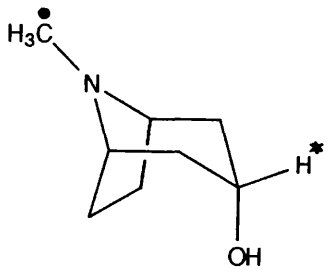
The reduction of tropinone (32) to tropine (13) has been studied in a cell-free system obtained from a root culture of D.stramonium[50]. The crude enzymes, in the presence of the cofactor NADPH (72), catalysed the formation of tropine (13). The so-called tropine dehydrogenase enzyme catalyses the interconversion of tropine (13) and tropinone (32).



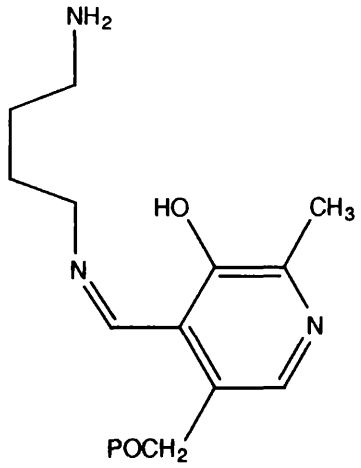
(72)

Leete synthesised and fed [3ß-^{3}H,^{14}C-methylamino]tropine (73) to D. meteloides plants by the wick method.[51] The tropane alkalods were isolated and their ^{3}H:^{14}C ratios were analysed. In all cases no change in this ratio was detected which strongly suggested intact incorporation of the fed precursor. No evidence

was found of a reversible reaction between tropine (13) and tropinone (32) because such a reaction would involve the loss of tritium.



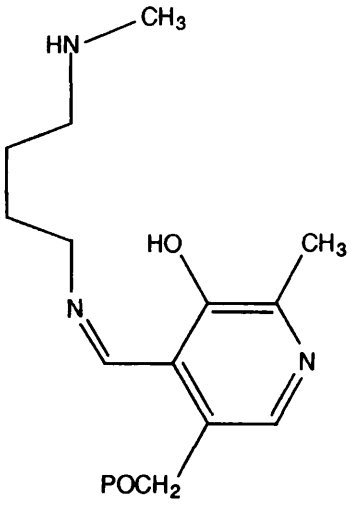
(73) • = ^{14}C , * = ^{3}H .



(74)

Recent publications have cast doubt over earlier observations that ornithine (22) is incorporated unsymmetrically into the pyrrolidine ring of tropane alkaloids. When [5-^{14}C]ornithine (44) was fed[52] to a root culture of Hyoscyamus albus equal labelling of C-1 and C-5 was observed. This observation was seen again with the feeding of DL-[5-^{14}C]ornithine (44) to Dubioisia leichhardtii[53]. These results were rationalised by the proposal that the biosynthetic pathway in Datura species operated via 'bound' putrescine which undergoes methylation to 'bound' N-methylputrescine (23). Possible forms of the 'bound' putrescine

and 'bound' N-methylputrescine are the Schiff bases (74) and (75). In species where symmetrical labelling of the tropane ring occurs the hydrolysis of the Schiff base (74) takes place to yield 'free' putrescine prior to N-methylation.



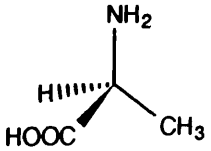
(75)

In conclusion, it has been established that L-ornithine (22) is incorporated unsymmetrically into (-)-hyoscyamine (14) and (-)-scopolamine (15) in Datura plants. Various studies with stable and radioactives ornithines have established their incorporation into the pyrrolidine ring of the tropane ring structure. The remaining three carbons of the tropane ring have also been shown by labelling studies to be derived from acetoacetate (53). The study by McGaw and Woolley[40] where they

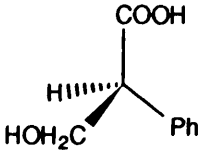
administered sodium [4-^{14}C]acetoacetate (56) to N.physaloides plants showed C-2 to be the site of reaction in the biosynthesis of hygrine (26). Recently, doubt was voiced over the established biosynthetic pathway to the tropane alkaloids due to an experiment carried out by Leete[54]. He fed DL-[5-^{14}C]ornithine (44) by the leaf painting technique to Erythroxylon coca and established by degradation of the isolated cocaine (33) that both C-1 and C-5 of the tropane ring were labelled. This result suggested that cocaine (33) was biosynthesised from ornithine (22) via the symmetrical intermediate putrescine. This observation may be explained by the subsequent hypothesis[53] that 'free' and 'bound' putrescine are responsible for either symmetrical or unsymmetrical incorporation in different plant species.

2.2 The biosynthesis of tropic acid and the esterification of tropine

Combination of the bases tropine (13) and scopine (12) with (S)-tropic acid (77) yields (-)-hyoscyamine (14) and (-)-scopolamine (15). The absolute configuration of the tropic acid moiety was determined by Fodor and Csepreghy[55] who carried out a correlation with (-)-alanine (76). Natural tropic acid was found to possess the (S)-configuration (77).

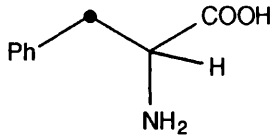


(76)



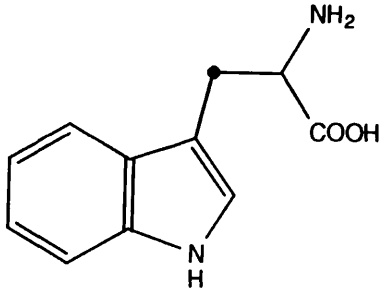
(77)

In 1960 an initial investigation into the biosynthesis of (S)-tropic acid (77) was carried out by Leete[56]. The administration of DL-[3-^{14}C]phenylalanine (78) to D.stramonium plants gave labelled (-)-hyoscyamine (14) and (-)-scopolamine (15). Hydrolysis of each of these alkaloids yielded (S)-tropic acid (77) which by degradation was shown to contain essentially all of the activity at C-2 of (S)-tropic acid (77). The bases tropine(13) and scopine (12) were shown to contain negligible activity. This result strongly suggested phenylalanine (79) as the biosynthetic precursor of (S)-tropic acid (77).



(78) • = ^{14}C .

(79) • = ^{12}C .

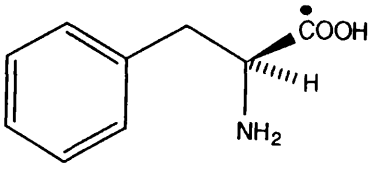


(80) • = ^{14}C .

(81) • = ^{12}C .

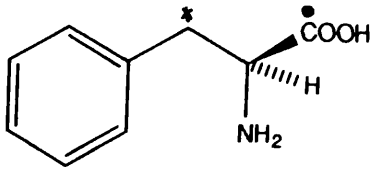
Later workers confirmed this result in D.stramonium (intact plants)[57] and D.metel (sterile root cultures)[58]. Goodeve and Ramstad reported incorporation of DL-[3-^{14}C]tryptophan (80) used for the study did not show that tryptophan (81) was able to furnish the entire carbon skeleton of (S)-tropic acid (77)[59]. Leete and Louden[60] proposed that the DL-[3-^{14}C]tryptophan (80) was broken down to give radioactive carbon dioxide which then

participated in a carboxylation reaction with a metabolite derived from phenylalanine (79).



(82) • = ^{14}C .

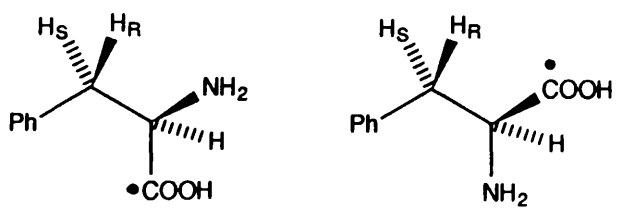
Louden and Leete[60] observed a reasonable specific incorporation (0.23%) in (-)-hyoscyamine (14) from the feeding of L-[1-^{14}C]phenylalanine (82) to D.stramonium plants. Hydrolysis gave (S)-tropic acid (77) which was degraded to show that only the carboxyl group was labelled. An intramolecular rearrangement of the side-chain of phenylalanine (79) was suggested with migration of the carboxyl group.



(83) • = ^{13}C , * = ^{13}C , ^{14}C .

Leete and co-workers provided evidence for this theory[61] by feeding DL-[1-^{14}C;1,3-^{13}C]phenylalanine (83) to D.innoxia plants. Satellites were observed in the ^{13}C n.m.r. spectra of (-)-hyoscyamine (14) and (-)-scopolamine (15) around C-1' and C-2'. The migration of the carboxyl group is intramolecular as endogeneous unlabelled phenylalamine (79) would have diluted the coupling beyond visibility if an intermolecular migration had occurred.

Leete used the synthesis of Battersby and co-workers[62] to prepare the four possible stereoisomers of [1-^{14}C, 3-^{3}H]-phenylalanine (84)-(87). Administration[63] of a mixture of equal amounts of the four possible steroisomers to D.innoxia and D.stramonium plants was carried out. Isolated (S)-tropic acid (77) obtained by hydrolysis of (-)-hyoscyamine (14) and (-)-scopolamine (15) showed that more than 50% of the tritium was retained relative to ^{14}C. Leete made no comment over this observation. Degradation showed that the methylene group of (S)-tropic acid (77) was labelled. An intramolecular shift of hydrogen occurs from C-3 of phenylalanine (79) to the carbon which ultimately becomes the hydroxymethyl group of (S)-tropic acid (77) (figure 4).



(84) $H_R = H$, $H_S = {}^3H$. (86) $H_R = {}^3H$, $H_S = H$.

(85) $H_R = {}^3H$, $H_S = H$. (87) $H_R = H$, $H_S = {}^3H$.

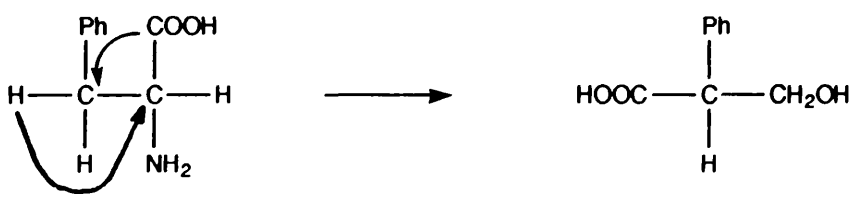
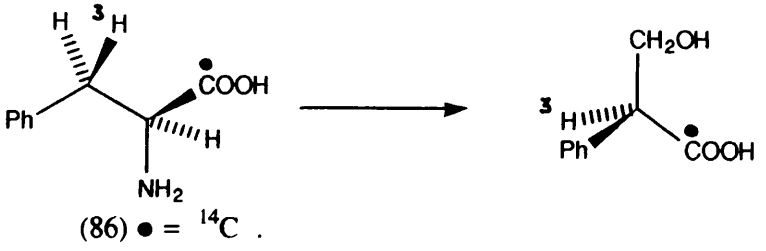


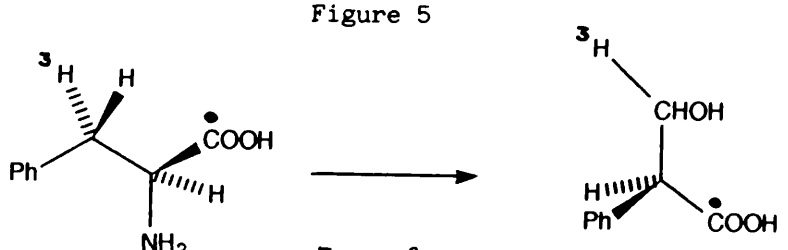
Figure 4

Leete[64] took this work further by making use of a synthesis of Battersby and co-workers[62]. Samples of (2S,3R)- and (2S,3S,)-[1-^{14}C,3-^{3}H]phenylalanine (86) and (87) were prepared to study the migration of the carboxyl group of phenylalanine (79) to the prochiral C-3 position in phenylalanine (79) on the pathway to (S)-tropic acid (77). The doubly labelled phenylalanines, (86) and (87) were fed to D.innoxia plants with isolation of (-)-hyoscyamine (14) and (-)-scopolamine (15). Mild hydrolysis of the alkaloids yielded (S)-tropic acid (77). It was found that migration had occurred with retention of configuration. Tritium was retained from the administration of (2S,3R)-[1-^{14}C,3-^{3}H]phenylalanine (86) and its position was determined by racemisation of (-)-hyoscyamine (14) with aqueous sodium hydroxide. Only 10% of the tritium was retained in the (±)-tropic acid (77) which indicated that most of the tritium was located at C-2 of (S)-tropic acid (77) (figure 5).
Tritium was also retained from the administration of (2S,3S)-[1-^{14}C,3-^{3}H]phenylalanine (87). Racemisation of (-)-hyoscyamine (14) with aqueous sodium hydroxide showed 85% retention of tritium. Degradation of the resultant (±)-tropic-acid (7) showed that the hydroxymethyl group of (S)-tropic acid (77) was labelled (figure 6).



(86) ● = ^{14}C .

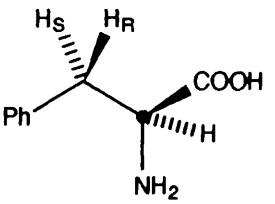
Figure 5



(87) ● = ^{14}C .

Figure 6

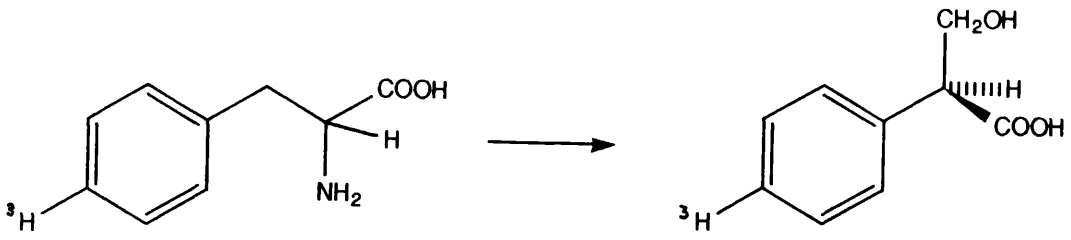
Leete's results[64] were in direct contrast with those of Haslam et al[65] who fed (2S,3S)-[2-^{14}C,3-^{3}H]- and (2S,3R)-[2-^{14}C,3-^{3}H]phenylalanine (88),(89) to D.stramonium plants. The results of Haslam and co-workers[65] were explained by Leete[64] in his subsequent paper as follows. The hydrolysis of the alkaloid fraction with 10% sodium hydroxide solution heated at reflux resulted in racemisation of the (S)-tropic acid (77). No optical rotation measurement of the isolated tropic acid was undertaken by Haslam et al.[65] The high loss of tritium observed after the administration of (2S,3R)-[2-^{14}C,3-^{3}H]-phenylalanine (89) was not due to carboxyl migration with inversion of configuration as claimed by the authors but because of the racemisation of (S)-tropic acid (77). The low loss of tritium observed after the administration of (2S,3S)-[2-^{14}C,3-^{3}H]phenylalanine (88) was explained by the hydride shift as observed by Leete[64]. The intact incorporation



(88) $H_R = {}^{3}H$, $H_S = H$, $\bullet = {}^{14}C$.

(89) $H_S = {}^{3}H$, $H_R = H$, $\bullet = {}^{14}C$.

of the phenyl group of phenylalanine (79) into (S)-tropic acid (79) has been shown by Leete[66] who fed DL-[4-^{3}H]phenylalanine (90) to D.innoxia plants. Degradations on the DL-[4-^{3}H]phenylalanine (90) and the derived (S)-tropic acid showed that tritium was located in both cases at the para-position (figure 7).

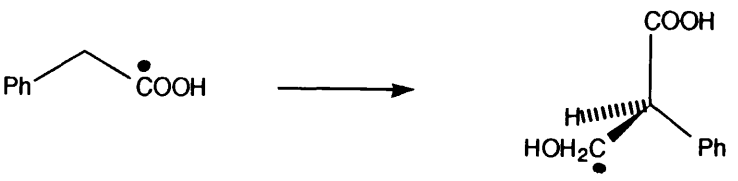


(90)

Figure 7

Intermediates between phenylalanine (79) and (S)-tropic acid (77) have been sought with little success. Many putative precursors have been synthesised and administered to Datura plants either alone or in competitive feeding experiments. The status of these putative precursors will be reviewed as follows.

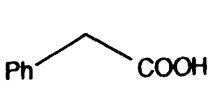
High incorporations of [1-^{14}C]phenylacetic acid (91) into (S)-tropic acid (77) have been reported[67] with degradations showing that essentially all the activity was located at C-3 of (S)-tropic acid (77) (figure 8). However it would seem unlikely



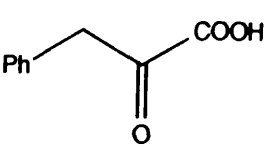
(91) • = ^{14}C .

Figure 8

that phenylacetic acid (92) is an intermediate between phenylalanine (79) and (S)-tropic acid (77) because of the results obtained from experiments using doubly labelled phenylalanines (83), (86) and (87). It was postulated that phenylacetic acid (92) underwent a carboxylation to phenylpyruvic acid (93) and then yielded phenylalanine (79) by transmination. This series of reactions has been observed by Allison and

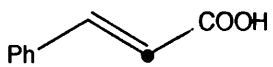


(92)

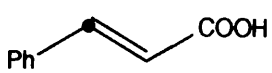


(93)

co-workers in ruminal bacteria[68] and photosynthetic anaerobic bacteria[69]. The administration of [1-^{14}C]phenylacetic acid (91) to both groups of bacteria resulted in phenylalanine (79) labelled solely at C-2.



(94) • = ^{14}C .

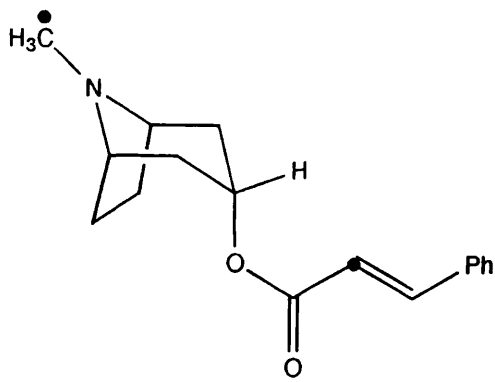


(95) • = ^{14}C .

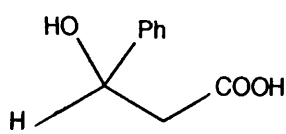
The enzyme L-phenylalanine ammonia-lyase (EC 4.3.1.5) has been found to occur widely in higher plants and in some fungi[70]. It catalyses the elimination of a proton and ammonia from phenylalmine (79) to give cinnamic acid (3).

Cinnamic acid (3) was suggested as an intermediate between phenylalanine (79) and (S)-tropic acid (77). Evans and Woolley[71] fed [2-^{14}C]cinnamic acid (94) to D.innoxia plants and isolated (-)-hyoscyamine (14) and (-)-scopolamine (15). Hydrolysis showed no incorporation into either the base or acid moieties. In the

same year Prabhu et al.[72] claimed that [2-^{14}C]cinnamic acid (94) was incorporated into atropine (21) in D.innoxia plants. Leete[66,73] re-investigated the proposed biosynthesis of (S)-tropic acid (77) from cinnamic acid (3). In two experiments Leete and Kirven[73] fed a mixture of [3-^{14}C]cinnamic acid (95) with [4-^{3}H]phenylalanine (90) as an internal control and secondly [2-^{14}C]cinnamoyl-[N-methyl-^{14}C]tropine (96) to D.innoxia plants.



(96) • = ^{14}C .



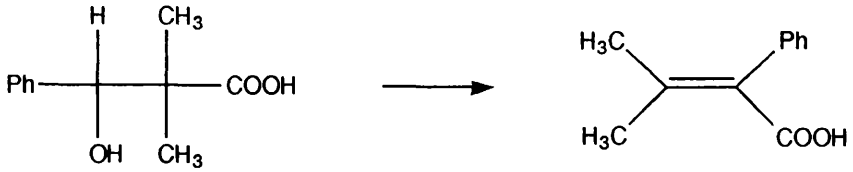
(97)

The first experiment yielded (-)-hyoscyamine (14) and (-)-scopolamine (15) containing only tritium which by hydrolysis

was shown to be confined to the (S)-tropic acid (77) residue. The second experiment yielded labelled (-)-hyoscyamine (14) and (-)-scopolamine (15) with degradations showing that all the activity was confined to the N-methyl groups. These results showed that cinnamic acid (3) was not a precursor of (S)-tropic acid (77). A possible explanation of the reuslts of Prabhu et al[72] advanced by Leete is that their isolated atropine (21) was not radiochemically pure or their degradation scheme did not unequivically determine the position of the ^{14}C label. The majority of evidence strongly suggests that cinnamic acid (3) is not a precursor of (S)-tropic acid (77).

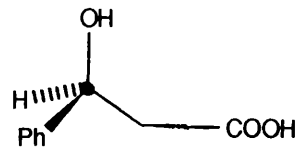
Leete[66] proposed 3-hydroxy-3-phenylpropanoic acid (97) as an intermediate between phenylalanine (79) and (S)-tropic acid (77). It was shown by Berner and co-workers[74] that the *in vitro* rearrangement of 3-hydroxy-2,2-dimethyl-3-phenylpropanoic acid (98) to 3-methyl-2-phenyl-2-butenoic acid (99) involved a 1,2-intramolecular carboxyl group shift (figure 9). Leete[66] synthesised and resolved both enantiomers of [3-^{14}C]-3-hydroxy-3-phenylpropanoic acid (100) and (101) by means of morphine and brucine salts. Administration of each enantiomer to *D.innoxia* plants with [4-^{3}H]phenylalanine (90) as an internal control yielded (-)-hyoscyamine (14) and (-)-scopolamine (15) labelled only with tritium. This showed the failure of both

isomers of 3-hydroxy-3-phenylpropanoic acid (97) to serve as precursors of (S)-tropic acid (77).

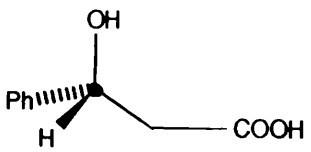


(98) (99)

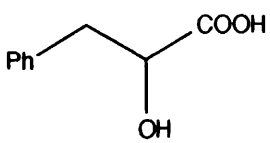
Figure 9



(100) (R) - (+) - isomer.



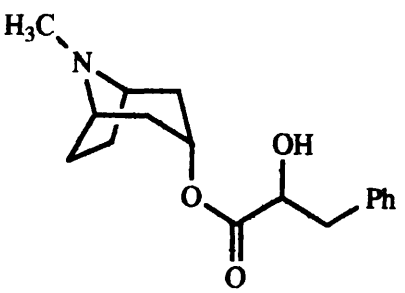
(101) (S) - (-) - isomer.



(102)

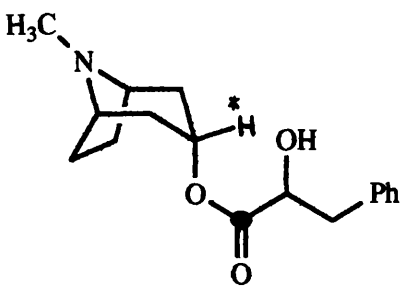
Phenyllactic acid (102) has also been postulated as an intermediate on the pathway to (S)-tropic acid (77). Deamination of phenylalanine (79) yields phenylpyruvic acid (93) which on reduction yields phenyllactic acid (102). The relationship between phenylalanine (79) and phenyllactic acid (102) has been shown by Evans and Woolley[75].

They administered [3-^{14}C]- and [1-^{14}C]phenylalanine (78) and (82) in separate experiments to Datura sanguinea plants and isolated the tropane alkaloid littorine (103). The littorine (103) was hydrolysed and its phenyllactic acid (102) residue was shown to be labelled. In addition they isolated (-)-hyoscyamine (14) and (-)-scopolamine (15) from the same experiment. The (S)-tropic acid (77) residue of these alkaloids was also labelled but with a lower specific incorporation than littorine (103). Evans and Woolley[75] proposed that phenylalanine (79) was a more immediate precursor of phenyllactic acid (102) than of (S)-tropic acid (77).



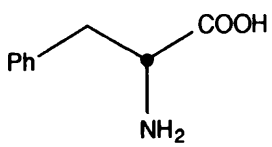
(103)

Leete[73] examined the ability of littorine (103) to serve as a direct precursor of atropine (21). He synthesised DL-[1-^{14}C]-(2-hydroxy-3-phenylpropionyloxy)-[3-^{3}H]tropane (104) and administered it to D.stramonium plants. Atropine (21) was isolated and its ^{3}H to ^{14}C ratio was determined. The large change in the ^{3}H to ^{14}C ratio (from 6.75 in the fed compound to 33.0 in the isolated atropine) indicated that littorine (103) was not directly converted into atropine (21). It was suggested that hydrolysis occurred in vivo to tropine (13) and phenyllactic acid (102), the latter undergoing rearrangement to (S)-tropic acid (77) before re-esterification with tropine (13).

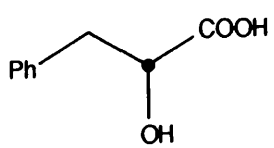


(104) • = ^{14}C , * = ^{3}H .

Evans and Woolley[76] studied the incorporation of phenyllactic acid (102) in D.innoxia plants. They fed [2-^{14}C]phenyllactic acid (106) and isolated samples of (-)-hyoscyamine (14) and (-)-scopolamine (15) after nine days which were both labelled. In a concurrent experiment they fed [2-^{14}C]phenylalanine (105) and isolated (-)-hyoscyamine (14) and (-)-scopolamine (15). They found higher specific incorporations in the alkaloids from the [2-^{14}C]phenyllactic (106) feed than from the [2-^{14}C]phenylalanine (105) feed. However, no suggestion that phenyllactic acid (102) was an intermediate between phenylalanine (79) and (S)-tropic acid (77) was made due to three possible explanations. Phenyllactic acid (102) may either be: (1) the acid which rearranges to (S)-tropic acid (77), is (2) involved in equilibria with phenylalanine (79) and phenyllactic acid (102), or (3) it may induce a greater competition for available phenylalanine (79).

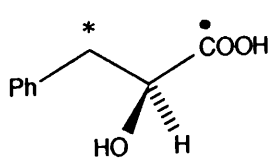


(105) • = ^{14}C .

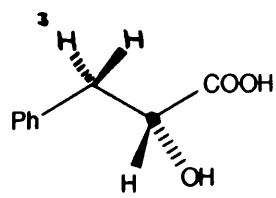


(106) • = ^{14}C .

In conclusion the biosynthesis of (S)-tropic acid (77) has not been fully elucidated. Various feeding experiments with labelled phenylalanines have shown that a 2,3-carboxyl group shift occurs with a hydride shift from C-3 to C-2. The 2,3-carboxyl group shift has been shown to be intramolecular. In D.innoxia the 2,3-carboxyl group shift has been shown to occur with retention of configuration. The pro-R proton at C-3 of phenylalanine (79) migrates to C-2 as feeding experiments with (2S,3R)- and (2S,3S)-[1-^{14}C,3-^{3}H]phenylalanines (86) and (87) have shown. The detailed mechanism for the conversion of phenylalanine (79) into (S)-tropic acid (77) is at present still unknown. Woolley and co-workers[77] have reported at a conference new findings which may help to elucidate the pathway to (S)-tropic acid (77). They synthesised a number of radio-labelled compounds and fed them to various Datura species. After carrying out feeds with (-)-(2S)-[1-^{14}C; 1,3-^{13}C]phenyllactic acid (107) and (2R,3S)-[3-^{3}H,2-^{14}C]phenyllactic acid (108). Woolley and co-workers[77] claimed that the migration of the carboxyl group in phenyllactic acid (102) occurs with inversion of configuration at C-3. Greater experimental detail is required to substantiate these unexpected results. A major problem in the investigation of intermediates on the pathway to (S)-tropic acid (77) is the proposed equilibria between phenylalanine (79), phenylpyruvic

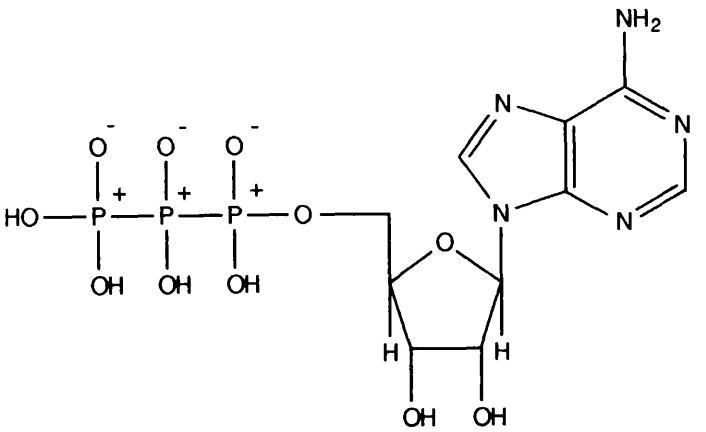


(107) • = ^{13}C , ^{14}C . * = ^{13}C .



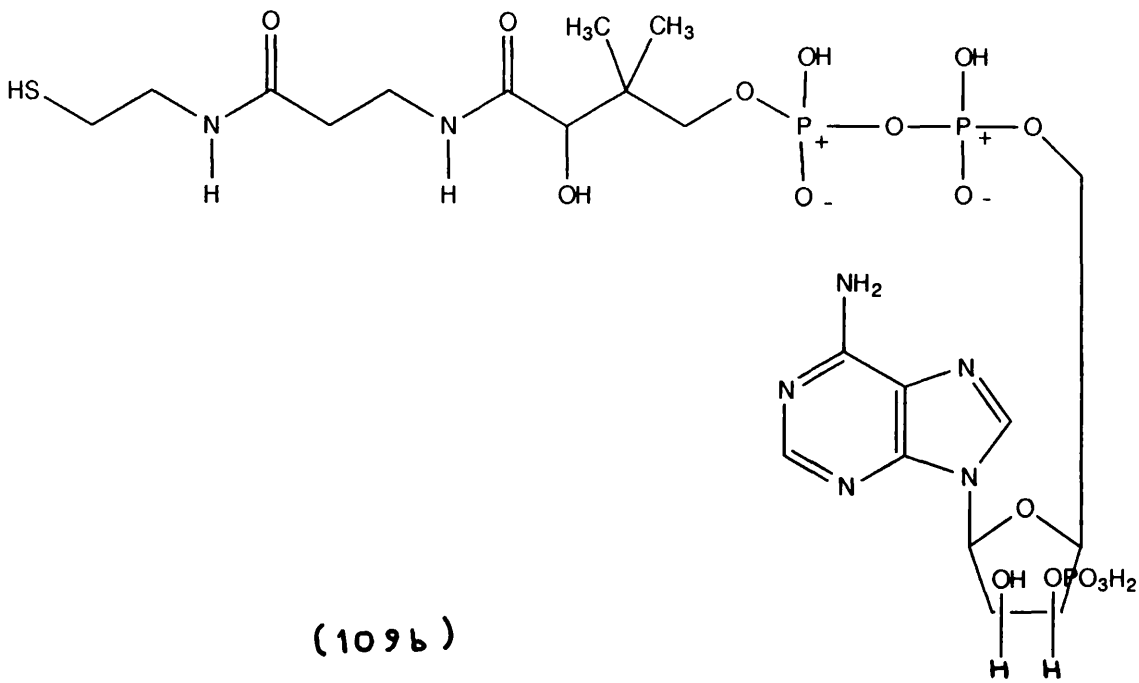
(108) • = ^{14}C .
* = ^{3}H .

acid (93) and phenyllactic acid (102). It is highly probable that the biosynthesis of (S)-tropic acid (77) is completed prior to esterification with tropine (13). because of the Failure of various acids to act as precursors of tropane alkaloids.



(109a)

The *in vitro* synthesis of atropine (21) from tropine (13) and (±)-tropic acid (7) in the presence of ATP (Adenosine triphosphate) (109) and CoA (coenzyme A) (109b) has been reported[78].

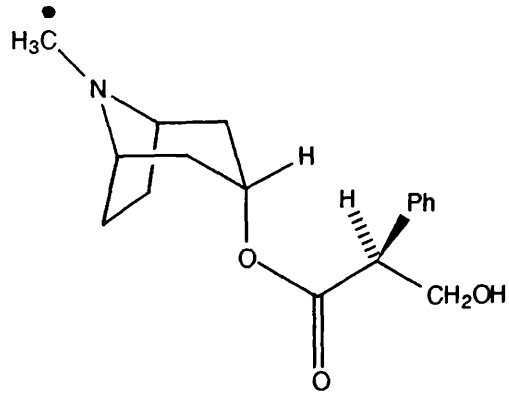


(109b)

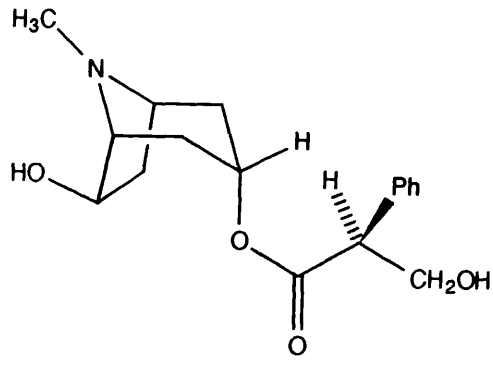
2.3 The biosynthesis of (-)-scopolamine

Literature on the biosynthesis of (-)-scopolamine (15) has increased recently due to the partial purification of enzymes responsible for the conversion of (-)-hyoscyamine (14) into (-)-scopolamine (15) in Datura species. These results have led to a re-evaluation of previous findings and a greater understanding of the mechanism involved.

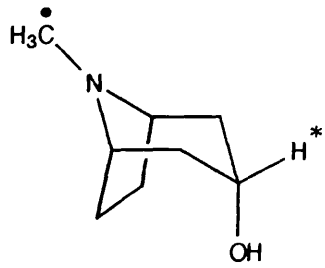
Romeike and co-workers have shown[79] that (-)-scopolamine (15) is derived from (-)-hyoscyamine (14). They synthesised [$^{14}CH_3$]hyoscyamine (110) and administered it to D.stramonium plants. Paper chromatography indicated incorporation into (-)-scopolamine (15) and an unknown alkaloid which was proposed to be 6-hydroxyhyoscyamine (111). Leete synthesised [$^{14}CH_3$,3-^{3}H]tropine (112) and fed it to D.meteloides plants[80]. Incorporation of tropine (13) into (-)-scopolamine (15) was



(110) • = ^{14}C .

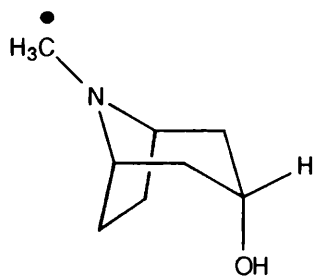


(111)



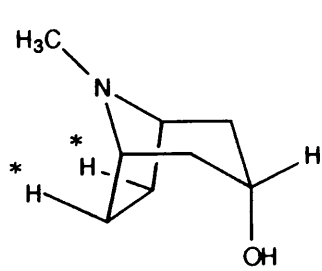
(112) • = ^{14}C , * = ^{3}H .

observed with essentially the same ^{3}H to ^{14}C ratio thus suggesting that tropine (13) is incorporated intact.



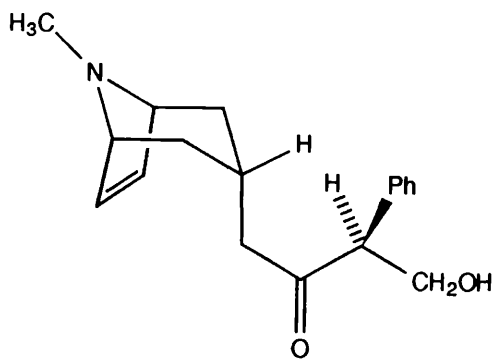
(113) ● = ^{14}C .

Leete and Lucast[81] studied the stereochemical outcome for the epoxide ring formation in (-)-scopolamine (15). They administered a mixture of [$^{14}CH_3$]tropine (113) and [6,7-$^{3}H_2$]tropine (114) to D.innoxia and D.meteloides plants (initial ^{3}H:^{14}C ratio of 7.3). They isolated tropine (13), (-)-hyoscyamine (14) and (-)-scopolamine (15) from both species. The tropine (13) and (-)-hyoscyamine (14) had essentially the same ^{3}H to ^{14}C ratio as administered. However, (-)-scopolamine (15) was found to retain only small amounts of tritium (^{3}H:^{14}C ratio of 0.3). Interpretation of these results was related to the results of Fodor et al.[82] who demonstrated the conversion of



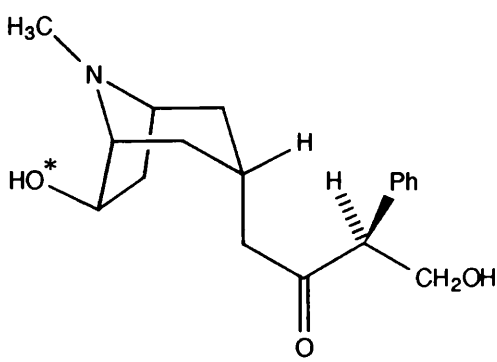
(114) * = ^{3}H .

6,7-dehydrohyoscyamine (115) into (-)-scopolamine (15) in scions of Datura ferox grafted on Cyphomandra betacca. Leete and Lucast[81] proposed that the cis-dehydration of 6-hydroxyhyoscyamine (111) occurred to form 6,7-dehydrohyoscyamine (115) which was then transformed into (-)-scopolamine (15).



(115)

In 1987 Yamada and Hashimoto reported[83] the isolation and partial purification of the enzyme hyoscyamine 6-hydroxylase which was classed as a 2-oxoglutarate-dependent dioxygenase. The hydroxylase was found to require (-)-hyoscyamine (14), 2-oxoglutarate, iron (II), catalase and a reductant for maximum activity. In order to study the proposals, Leete and Lucast[81] synthesised [6-^{18}O]-6-ß—hydroxyhyoscyamine (116) from (-)-hyoscyamine (114) and hyoscyamine 6ß-hydroxylase incubated in an $^{18}O_2$ atmosphere. Administration of [6-^{18}O]-6ß-hydroxyhyoscyamine (116) to shoot cultures of Duboisia myoporoides[84] was carried out and the isolated alkaloids were studied by GC-MS. This showed that 82.5% of the (-)-scopolamine (15) contained one atom of ^{18}O, thus disproving that 6,7-dehydrohyoscyamine (115) is an intermediate between (-)-hyoscyamine (14) and (-)-scopolamine (15) (Figure 10).



(116) * = ^{18}O .

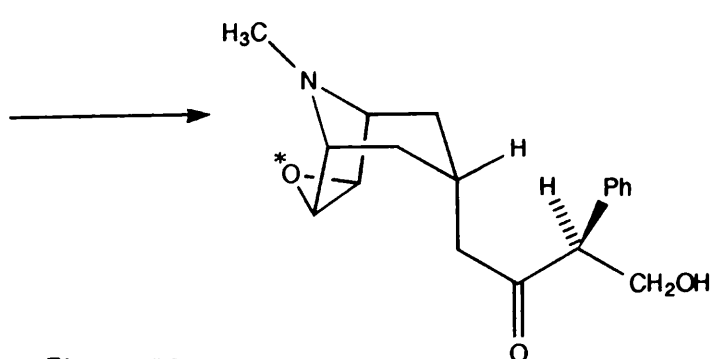
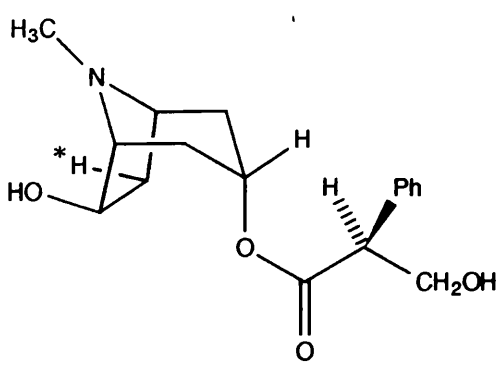
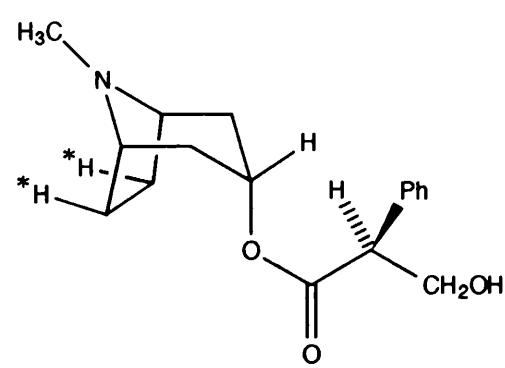


Figure 10 .

Yamada and Hashimoto used their enzyme preparation to study which hydrogen at C-7 of (-)-hyoscyamine (14) is lost in the conversion into (-)-scopolamine (15). They synthesised [7-^{2}H]-6-hydroxyhyoscyamine (117) from [6,7-$^{2}H_{2}$]-hyoscyamine (118), by using hyoscyamine 6ß-hydroxylase, and administered it to shoot cultures of D.myoporoides[85]. The mass spectrum of the isolated (-)-scopolamine (15) was identical to that of unlabelled (-)-scopolamine (15) with no indication of deuterium in the isolated alkaloid. This result strongly suggests the loss of the 7ß-hydrogen in the biosynthesis of (-)-scopolamine (15) from (-)-hyoscyamine (14) and substantiates the findings of Leete and Lucast[81]. Hashimoto and co-workers have recently partially purified 6-hydroxyhyoscyamine expoxidase from cultured roots of Hyoscyamus niger[86]. This has also been classed as a 2-oxog lutarate-dependent dioxygenase. They have proposed a reaction mechanism for the conversion of 6-hydroxyhyoscyamine (111) into (-)-scopolamine (15) which involves the insertion of a highly reactive ferryl enzyme species (119) into the C-7 bond of 6-hydroxyhyoscyamine (111) with retention of configuration. This iron-carbon species (120) could be

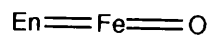


(117) * = ^{2}H .

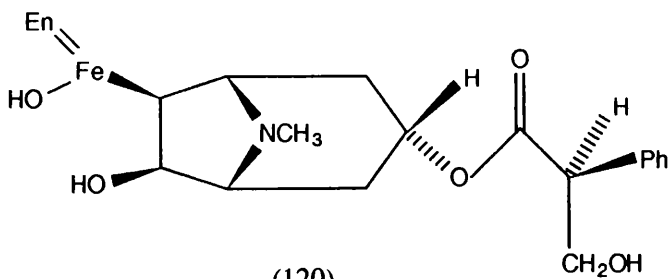


(118) * = ^{2}H .

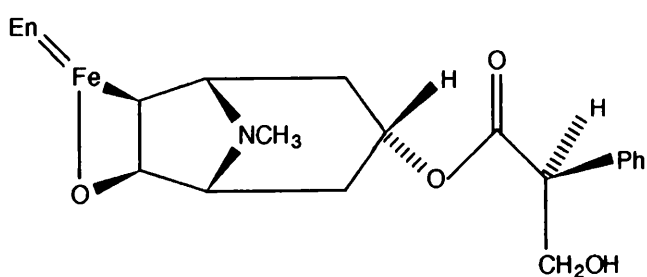
attached intramolecularly by the 6-hydroxy group from the same exo face to produce a metallocycle (121). This could collapse to (-)-scopolamine (15) and the initial ferrous enzyme.



(119)



(120)



(121)

The biosynthesis of (-)-scopolamine (15) has become better understood due to the isolation of enzymes responsible for this transformation. The work of Hashimoto and co-workers[83-86] has allowed earlier findings with whole plants to be invalidated [81].

They have shown that 6,7-dehydrohyoscyamine (115) is not an intermediate in the pathway. The conversion of (-)-hyoscyamine (14) into (-)-scopolamine (15) occurs *via* 6-hydroxyhyoscyamine (111) with loss of the 7ß-hydrogen.

CHAPTER THREE

THE BIOSYNTHESIS OF TOBACCO ALKALOIDS

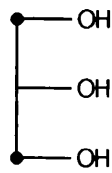
3.1 Introduction to tobacco alkaloids

Extensive studies on the tobacco alkaloids have been carried out over many years. Commercial tobacco is obtained from Nicotiana tabacum, while the vast majority of biosynthetic studies have been carried out on other Nicotiana species eg rustica, glauca and glutinosa. The tobacco alkaloids have a common structural relationship. They all contain a pyridine ring joined directly from C-3 to C-2 of a pyrrolidine or piperidine ring system. The pyridine ring of tobacco alkaloids has been shown to be derived from nicotinic acid (24) by various labelling experiments. The pyrrolidine and piperidine ring systems are derived from L-ornithine (22) or L-lysine (25) respectively, with the exception being anatabine (19) which is derived from two molecules of nicotinic acid (24).

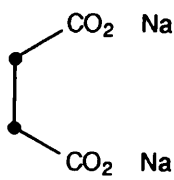
3.2 The biosynthesis of Nicotinic acid and its incorporation into the tobacco alkaloids

Nicotinic acid (24) is derived biogenetically by at least two different pathways. Tryptophan (81) is used by animals, certain bacteria, fungi, and algae to biosynthesise nicotinic acid (24). Some micro-organisms do not utilize tryptophan (81) to

biosynthesise nicotinic acid (24) eg Escherischia coli and Mycobacterium tuberculosis. In higher plants nicotinic acid (24) is not furnished by tryptophan (81) as shown by the non-incorporation of trypotophan (81) into the alkaloids of Nicotiana [87,88].



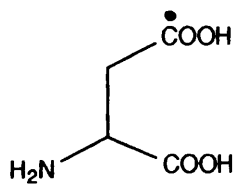
(122) • = ^{14}C .



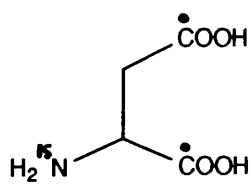
(123) • = ^{14}C .

The non-tryptophan pathway to nicotinic acid (24) has been studied predominantly with different bacteria eg E.coli and M.tuberculosis. Ortega and Brown [89] fed various [^{14}C]-labelled putative precursors to E.coli and showed the incorporation of [1,3-^{14}C]glycerol (122) and [2,3-^{14}C]succinate (123) into nicotinic acid (24). Investigations into the so-called C_3 + C_4

pathway were continued by Mothes and co-workers [90] who administered DL-[4-^{14}C]aspartic acid (124) to Mycobacterium bovis strain BCG. Isolated nicotinic acid (24) was labelled solely at C-7. The origin of the ring nitrogen was shown [91] by the incorporation of DL-[1,4-^{14}C, ^{15}N]aspartic acid (126) into nicotinic acid (24) in M.tuberculosis strain BCG. The ^{14}C:^{15}N ratio, obtained by degradation of the isolated nicotinic acid, was 91% of that expected for incorporation of the administered precursor with loss of C-1. Thus C-2, C-3 and C-4 of aspartic acid (125) are incorporated into positions 2,3 and 7 of nicotinic acid (24).

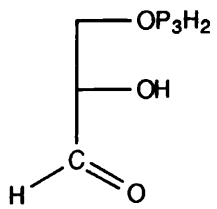


(124) • = ^{14}C .
(125) • = ^{12}C .

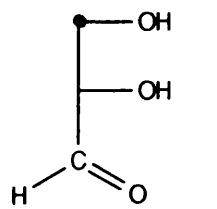


(126) • = ^{14}C .

The remaining carbons of nicotinic acid (24) were proposed to originate from a glycerol derived unit. Mothes and co-workers[91] confirmed earlier findings with the incorporation of [1,3-^{14}C]glycerol (122) into nicotinic acid (24) in M.tuberculosis. Leete[92] proposed that the glycerol equivalent was glyceraldehyde-3-phosphate (127) which condensed with aspartic acid (125) and by a series of transformations produced nicotinic acid (24). Fleeker and Byerrum[93] administered [3-^{14}C]glyceraldehyde (128) to Nicotiana glauca and isolated (-)-nicotine (16) which was shown by degradation to have essentially all its activity located at C-4 of (-)-nicotine (16).



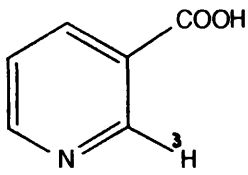
(127)



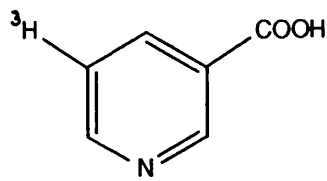
(128) • = ^{14}C .

However, the validity of this proposed route is under debate due to the lack of evidence on the manufacture of nicotinic acid (24) in higher plants.

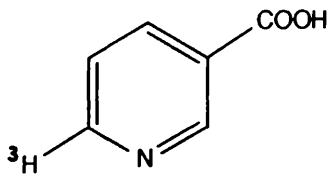
Dawson and co-workers[94] demonstrated the incorporation of nicotinic acid (24) into (-)-nicotine (16) in N.tabacum by the administration of a series of deuterium and tritium labelled nicotinic acids (129)-(131). Incorporations found for [2-^{3}H]- and [5-^{3}H]nicotinic acid, (129) and (130), were ten times higher than that for the incorporation of [6-^{3}H]nicotinic acid (131). These workers proposed that the loss of tritium from [6-^{3}H]nicotinic acid (129) strongly suggested unsymmetrical incorporation of nicotinic acid (24) into (-)-nicotine (16). Scott and Glynn substantiated the above observations with



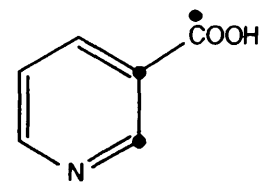
(129)



(130)



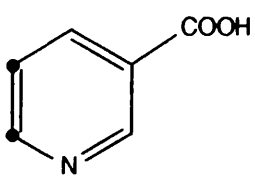
(131)



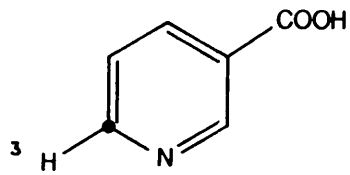
(132) • = ^{14}C .

the administration of [2,3,7-^{14}C]nicotinic acid (132) to N.tabacum plants[95]. Isolated (-)-nicotine (16) was oxidised to

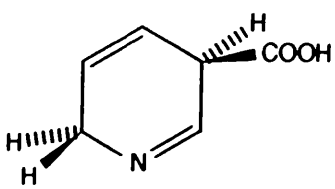
nicotinic acid (24) with potassium permanganate. Stepwise degradation showed that C-2 and C-3 were labelled, with negligible activity detected for C-4, C-5 and C-6. Leete confirmed these findings[96] with the administration of [5,6-^{14}C:5,6-$^{13}C_2$]nicotinic acid (133) to N.tabacum and N.glauca plants. The ^{13}C n.m.r. spectra of isolated (-)-nicotine (16) and nornicotine (18) showed satellites due to ^{13}C-^{13}C spin-spin coupling around the signals for C-5 and C-6 of each of the alkaloids. The finding of Dawson and co-workers[94] concerning the loss of H-6 from nicotinic acid (24) in the biosynthesis of (-)-nicotine (16) was further examined by Leete and co-workers[96]. They administered [6-^{14}C, 6-^{3}H]nicotinic acid (134) to N.tabacum plants and isolated (-)-nicotine (16). Measurement of the ^{14}C to ^{3}H ratio indicated a 98% loss of tritium relative to ^{14}C. However, re-isolated nicotinic acid (24) retained most of the tritium which strongly suggested that the loss of tritium was a consequence of the activation of nicotinic acid (24) prior to condensation to manufacture (-)-nicotine (16). Leete[96] and Dawson[97] have both proposed 3,6-dihydronicotinic acid (135) as



(133) • = ^{13}C , ^{14}C .

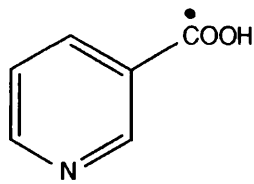


(134) • = ^{14}C .



(135)

the activated form of nicotinic acid (24) with the hydrogen which is added stereospecifically at C-6 of nicotinic acid (24) retained in (-)-nicotine (16). The 3,6-dihydronicotinic acid (135) is believed to be the activated form required for coupling to form (-)-nicotine (16). Chandler and Gholson[98] have partially



(136) • = ^{14}C .

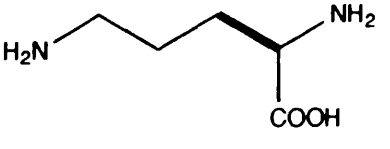
purified nicotinic acid decarboxylase which catalyses the oxygen dependent release of $^{14}CO_2$ from [7-^{14}C]nicotinic acid (136) in N.rustica.

In conclusion, it has been established that nicotinic acid (24) is incorporated into (-)-nicotine (16) in an unsymmetrical manner with the point of attachment of the N-methylpyrrolidine ring

being the carbon which was substituted with a carboxyl group. Various labelling experiments have shown the loss of hydrogen from C-6 and the point of attachment of the pyrrolidine ring occurs with loss of the carboxyl group at C-3 of nicotinic acid (24).

3.3 The biosynthesis of the pyrrolidine and piperidine rings of (-)-nicotine, nornicotine and anabasine.

In 1955 the incorporation of [2-^{14}C]ornithine (34) into (-)-nicotine (16) in N.tabacum and N.rustica plants was observed by the groups of Leete[99] and Byerrum[100]. Leete[101] administered [2-^{14}C]ornithine (34) to N.glutinosa plants and by a series of unambiguous degradations showed equal labelling of C-2' and C-5' of (-)-nicotine (16). In a subsequent paper Leete and Yu[102] synthesised DL-[2,3-$^{13}C_2$]ornithine (137) and fed it to N.glutinosa plants. Satellites due to ^{13}C-^{13}C spin coupling were observed in the ^{13}C n.m.r spectrum of (-)-nicotine (16) around the signals corresponding to C-2', C-3', C-4' and C-5'. This substantiated, by a non-degradative method, incorporation of ornithine (22) via a later symmetrical intermediate into the N-methylpyrrolidine ring of (-)-nicotine (16).



(137) ——— = ^{13}C - ^{13}C .

Mizusaki and co-workers have detected the presence of L-ornithine decarboxylase (ODC)[103], putrescine N-methyl transferase (PMT)[103] and N-methylputrescine oxidase (MPO)[104] in Nicotiana roots. Decarboxylation of L-ornithine (22) produced putrescine, the proposed symmetrical compound responsible for equal labelling of C-2' and C-5' of (-)-nicotine (16). Leete[105] has shown by the administration of [1,4-^{14}C]putrescine (48) to Nicotiana species a relatively high incorporation of putrescine in (-)-nicotine (16). Spenser and Richards[106] have studied the stereochemical course of the decarboxylation of L-ornithine (22) which is catalysed by L-ornithine decarboxylase. They have shown that decarboxylation proceeds with retention of configuration. Putrescine N-methyl transferase (PMT) catalyses the methylation of putrescine to yield N-methylputrescine (23). The source of the methyl group has been shown to be S-adenosyl methionine[107]. Leete and McDonell[32] administered [1-^{13}C,1-^{14}C, methylamino-^{15}N]-N-methylputrescine (47) to N.tabacum plants. Isolation and ^{13}C n.m.r. spectroscopy of (-)-nicotine (16) showed satellites around the signal for C-5' due to ^{13}C-^{15}N spin-spin coupling ($^{1}J_{^{13}C-^{15}N}$=4.2Hz). This suggested the incorporation of intact N-methylputrescine (23) in the biosynthesis of (-)-nicotine (16). N-Methylputrescine oxidase (MPO) catalyses the oxidation of N-methylputrescine (23) to the N-methyl-Δ'-pyrrolinium ion (50). This enzyme has recently been partially purified and it is thought to be a quinoprotein[108]. The N-methyl-Δ'-pyrrolinium ion (50) has been isolated in labelled form after the administration of

[2-^{14}C]ornithine (34) which strongly suggests its natural occurrence in plants. Leete[109] fed [2-^{14}C]-N-methyl-Δ'-pyrrolidinium chloride (51) to N.tabacum plants and isolated labelled (-)-nicotine (16) with all of its activity located at C-2'. Spenser and co-workers[110] used their synthesis[106] of (R)-[1-^{2}H]putrescine (138) to elucidate the stereochemical course of the conversion of N-methylputrescine (23) into N-methyl-4-aminobutanal (49) by feeding it to N.tabacum.

The ^{2}H n.m.r spectrum of the isolated (-)-nicotine (16) showed two singlets in a 1:1 ratio. These signals corresponded to C-2' and C-5' of (-)-nicotine (16) which indicated the stereospecific loss of the pro-S hydrogen in the oxidation of N-methylputrescine (23) to N-methyl-4-aminobutanal (49) (Figure 11). The N-methyl-4-aminobutanal (49) is the proposed active form which combines with 3,6-dehydronicotinic acid (135), the proposed activated form of nicotinic acid (24) to produce (-)-nicotine (16). Leete and Friesen[111] have recently proposed a mechanism where the reduction of nicotinic acid (24) is aided by NADPH which acts as a hydride donor. The resultant 3,6-dihydronicotinic acid (135) readily decarboxylates to give 1,2-dihydropyridine (139) which attacks the N-methyl-Δ' -pyrrolinium salt (50) (Figure 12) Nornicotine (18) has been shown to be the demethylation product[64] of (-)-nicotine (16). Mizusaki and co-workers[112] administered [2-^{14}C]ornithine (34) to N.tabacum plants and isolated nornicotine (18). Degradations showed unequal labelling of C-2' and C-5' (2:1) in nornicotine

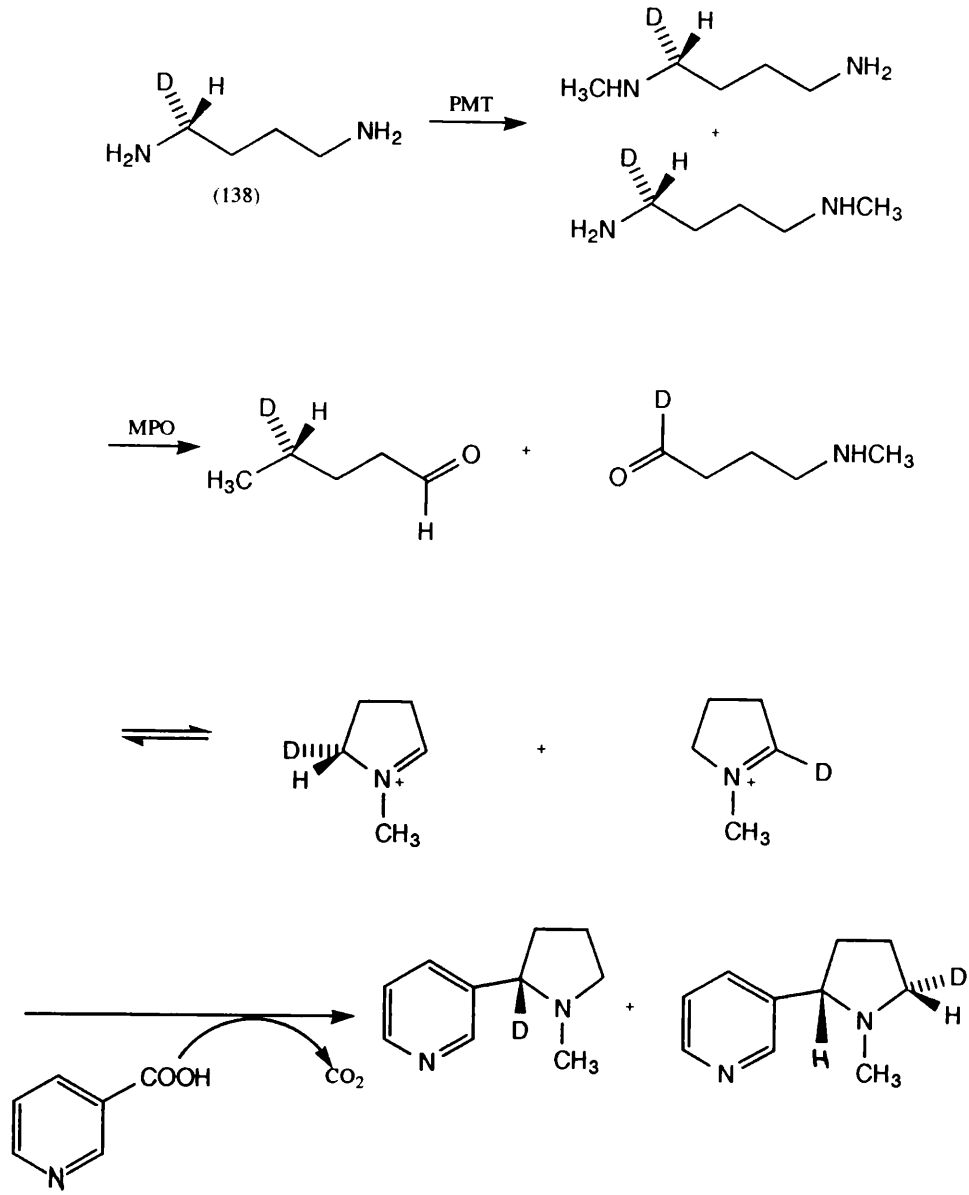
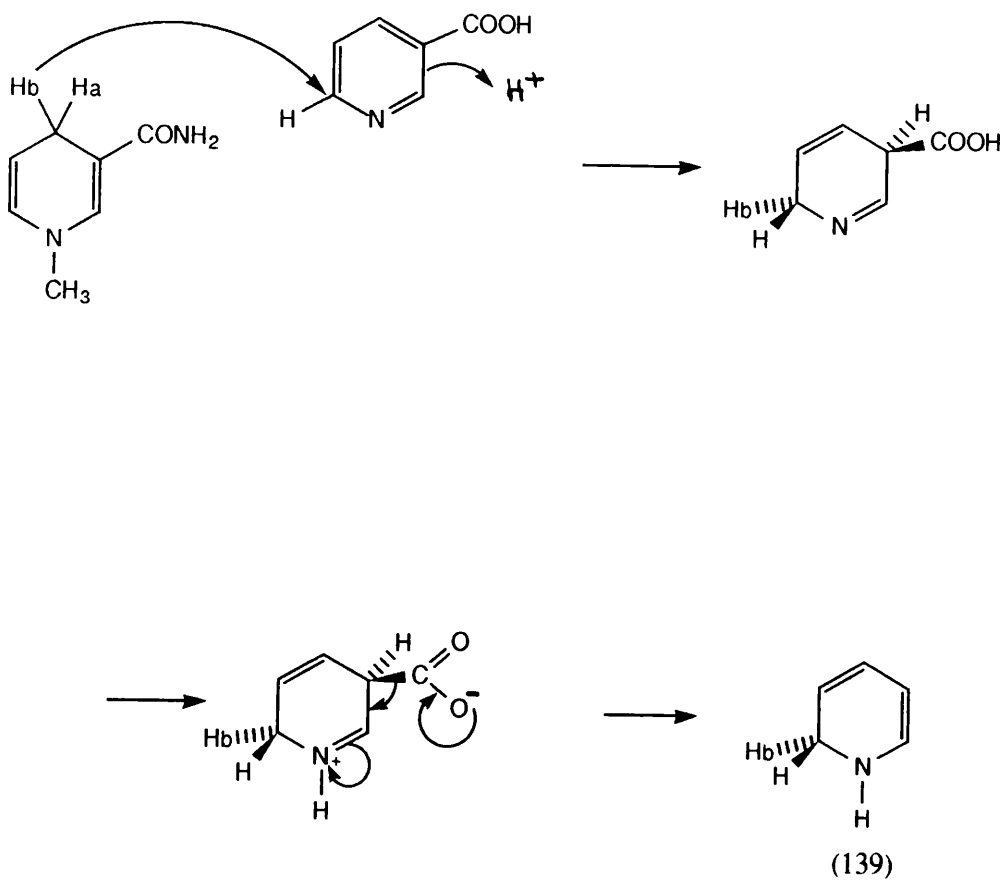

D
H
H2N
NH2
(138)
PMT
D
H
H3CHN
NH2
+
D
H
H2N
NHCH3
MPO
D
H
H3C
O
H
+
D
O
NHCH3
D
H
N+
CH3
+
N+
D
CH3
COOH
CO2
N
N
D
N+
CH3
+
N
D
H
H
N
CH3

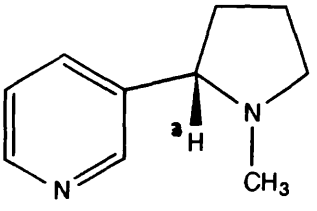
Figure 11



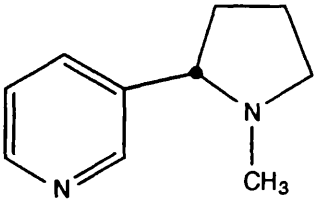
(139)

Figure 12

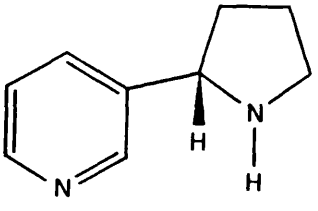
(18). They suggested nornicotine (18) biosynthesis occurred by two distinct routes, the first by demethylation of (-)-nicotine (16) and the second directly from L-ornithine (22). Leete and Chedekel[113] administered a mixture of [2'-^{3}H]-(-)-nicotine (140) and [2'-^{14}C]-(+)-nicotine (141) to N.glauca plants. Isolation and resolution of each enantiomer of nornicotine (142) and (143) showed almost equal quantities of each enantiomer. Degradations of each enantiomer showed retention of tritium in (-)-nornicotine (18a) and loss of tritium in (+)-nornicotine (18b). Thus, if (+)-nornicotine is formed by the demethylation of (-)-nicotine (16) it occurs with the loss of hydrogen from C-2' of (-)-nicotine (16).



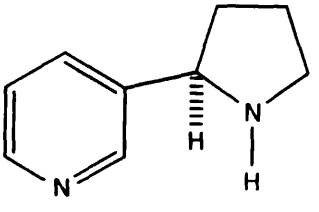
(140)



(141) • = ^{14}C .



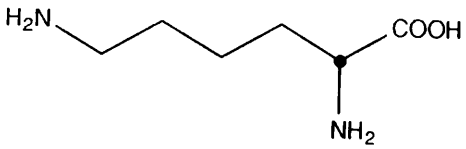
(142)



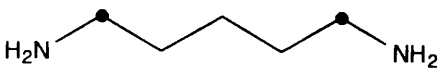
(143)

The interpretation of the above results suggest further experimental evidence is required to elucidate the mechanism for the conversion of (-)-nicotine (116) into nornicotine (18).

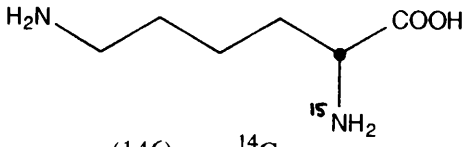
The piperidine ring of anabasine (17) is derived from lysine (25). Leete[114] fed [2-^{14}C]lysine (144) to N.glauca plants and isolated anabasine (17) which was shown to contain all its activity at the C-2' position. Hence free cadaverine is not an intermediate between lysine (25) and anabasine (17), although [1,5-^{14}C]cadaverine (145) was incorporated into the piperidine ring of anabasine (17). Leete and co-workers[115] administered [2-^{14}C,α-^{15}N]- and [2-^{14}C, ε-^{15}N]lysine (146) and (147) to excised roots of N. glauca. The nitrogen of the piperidine ring of anabasine (17) was shown to originate from the ε-amino group of lysine (25). An analogy to the biosynthesis of (-)-hyoscyamine (14) and (-)-scopolamine (15) was used to explain the above observations. Methylation of the ε-amino group of lysine (25) occurred before decarboxylation to N-methylcadaverine (148). This hypothesis was examined by feeding [2-^{14}C]-N-methyl-Δ^{1}-piperideinium chloride (149) to N.tabacum and N.glauca plants. Isolated (-)-[2'-^{14}C]-N-methylanabasine (150) was shown to have essentially the same specific activity as the administered compound whereas isolated anabasine (17) had negligible activity. This experiment showed N-methylanabasine (151) was not an intermediate between lysine (25) and anabasine (17). N-Methylanabasine (151) is probably formed by an aberrant biosynthesis.



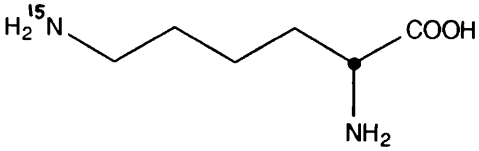
(144) • = ^{14}C .



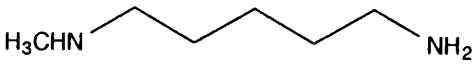
(145) • = ^{14}C .



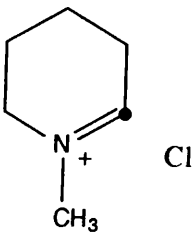
(146) • = ^{14}C .



(147) • = ^{14}C .



(148)



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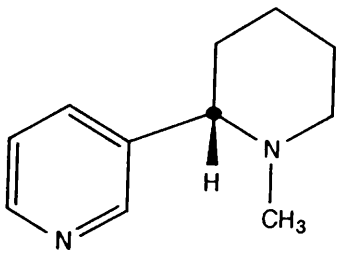
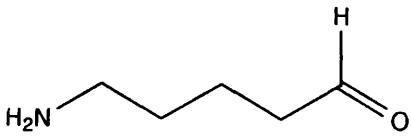


(149) • = ^{14}C .

(150) • = ^{14}C .
(151) • = ^{12}C .

A more recent model[115] proposes a concerted decarboxylation of L-lysine (25) and subsequent oxidation to δ-aminovaleraldehyde (152) in complexation with pyridoxal phosphate. This model proposes that 'bound' cadaverine exists in the biosynthesis of anabasine.



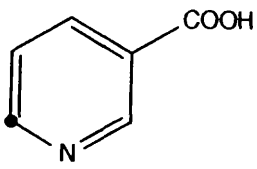
(152)

3.4 The biosynthesis of Anatabine

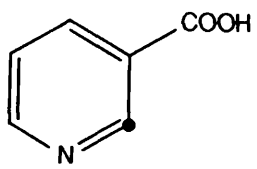
Anatabine (19) is formed by two molecules of nicotinic acid (24). Leete and Slattery[117] administered [5-^{14}C]nicotinic acid (153) to N.glutinosa plants and [2-^{14}C]nicotinic acid (154) to N. tabacum plants. Degradation of anatabine (19) isolated from N.glutinosa indicated equal labelling of C-6 and C-6'.

Degradation of anatabine (19) isolated from N.tabacum showed equal labelling of C-2 and C-2'. Leete[118] has shown by the administration of a mixture of [5,6-^{14}C;5,6-$^{13}C_2$]- (133) and [5-^{3}H]nicotinic acid (131) that both rings were ^{13}C labelled. Degradation of anatabine (19) showed that the pyridine ring was almost devoid of tritium whereas the reduced ring system retained essentially 100% of its tritium. The tritium was shown to be located in the pro-S position at C-6'.

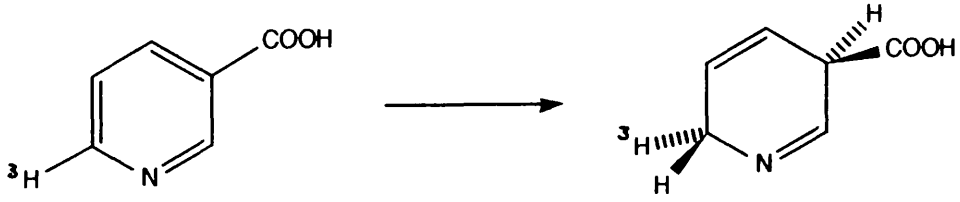
The configuration of this tritium suggested that in the reduction of of nicotinic acid (24), hydrogen was introduced at the pro-R position (Figure 13).



(153)• = ^{14}C .

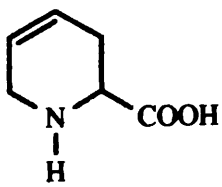


(154)• = ^{14}C .



(131)

Figure 13



(155)

The intermediacy of a dihydropyridine is supported by a biomimetic synthesis[119] of anabasine (17) from baikianin (155). The regiochemistry of the synthesis was confirmed by the use of a mixture of [2-^{13}C]baikianin (156) and [2-^{2}H]baikianin (156) (Figure 14).

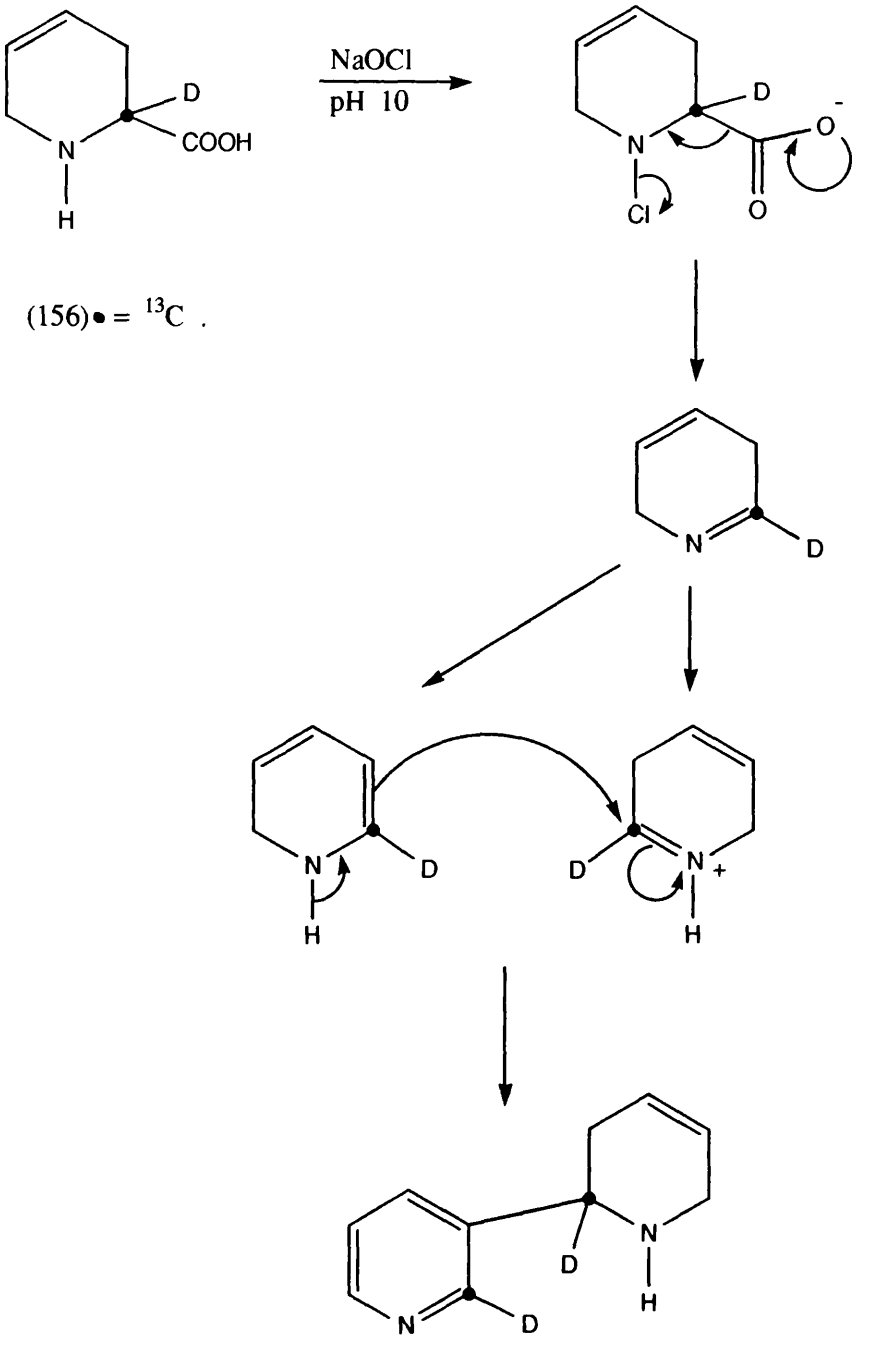


Figure 14

3.5 Conclusions

The common precursor of the tobacco alkaloids has been shown to be nicotinic acid (24) which is incorporated unsymmetrically. The proposed activated form of nicotinic acid (24) is 3,6-dihydro-nicotinic acid (135) which by combination with either an N-methyl-Δ^{1}-pyrrolinium ion or Δ^{1}-piperideinium ion produces either (-)-nicotine (16) or anabasine (17), respectively. The aforementioned ions have their origins in either L-ornithine (22) or L-lysine (25). Anatabine (19) is unique amongst the tobacco alkaloids in that it is produced biosynthetically from two molecules of nicotinic acid (24).

The incorporation of L-ornithine (22) in *Nicotiana* species has been shown to occur unsymmetrically which is in direct contrast with the incorporation of L-ornithine (22) into (-)-hyoscyamine (14) and (-)-scopolamine (15) in *Datura* species. Distinct mechanisms for the incorporation of L-ornithine (22) in *Nicotiana* and *Datura* have been shown to take place.

CHAPTER FOUR

TRANSFORMED ROOT CULTURES IN BIOSYNTHETIC STUDIES

4.1 Introduction

Plants remain a major source of a number of pharmaceuticals and other fine chemicals due to uneconomic synthesis of most structurally complex natural products. The use of plant cells in culture has long been identified as a worthwhile alternative to extraction of plant tissue. Major problems have been the instability and slow rate of production of these cultures. Alkaloid production usually tails off after subculturing cells for a period of time. In the 1980's it was discovered that genetic transformation of plant tissue by the pathogenic soil bacterium Agrobacterium rhizogenes[120] gave rapidly-growing, productive and stable hairy root cultures (Figure 15).

4.2 The genetic effect of transformation

The infection of plants with A.rhizogenes causes one or two pieces of transfer-DNA (T_L and T_R) to be transferred from the bacterial plasmid to the plant genome. Activation of the vir genes on an area of the bacterial plasmid not transferred is required before this transfer of DNA takes place. Integration of the DNA is entirely random with alteration of the auxin

metabolism of the transformed tissue. This causes the hairy root phenotype. The transfer-DNA may include genes which contain information relating to the formation of specific metabolites. The most vigorous hairy root response occurs when both T_L and T_R are transferred to the plant genome. Hairy root cultures are superior to untransformed root cultures and most cell suspension cultures because of their fast growth rates in culture and high rates of production of alkaloids.

Robins et al.[121] have successfully transformed Datura candida X Datura aurea hybrid plants with A. rhizogenes strain LBA 9202. This transformed root culture, which is named line DB5, is highly competent in the biosynthesis of (-)-hyoscyamine (14) and (-)-scopolamine (15). In contrast to a number of other Datura hairy root cultures this hairy root culture accumulates a high level of (-)-scopolamine (15). The maximum rate of biosynthesis of (-)-hyoscyamine (14) and (-)-scopolamine (15) was observed 10 - 14 days after sub-culture. The administration of various established intermediates in the pathway had marked effects on overall (-)-hyoscyamine (14) and (-)-scopolamine (15) production. The administration of tropinone (32) and tropine (13) at various concentrations resulted in increased alkaloid production whereas DL-tropic acid (7) and DL-ß-phenyllactic acid (102) led to a decrease in overall alkaloid production. It was suggested that the ability of the hairy root culture to accumulate (-)-hyoscyamine (14) and (-)-scopolamine (15) was restricted by the esterifying acids not being in activated forms.

Walton and Belshaw[122] have studied the effect of cadaverine on the formation of anabasine (17) from lysine (25) in hairy root cultures of Nicotiana hesperis. The administration of unlabelled cadaverine with L-[U-^{14}C]lysine did not diminish the incorporation of lysine (25) into anabasine (17) despite causing a stimulation of anabasine production. When cadaverine was supplied at 1.0 mM in solution, in conjunction with L-[U-^{14}C]lysine, incorporation was undimished although anabasine (17) production was increased three-fold. The effect of cadaverine (1,5-diaminopentane) on anabasine (17) production in N. rustica hairy root cultures will be reviewed in a later chapter.

4.3 Exploitation of hairy root cultures

Secondary metabolites of commercial importance are potentially available from hairy root cultures. Presently no hairy root culture meets the criteria required for commercial exploitation. The criteria include: (1) high production levels of the desired secondary metabolite; (2) release of secondary metabolite from cells to culture medium in order to use the biomass in subsequent alkaloid accumulations, and (3) formation of simple mixtures of secondary metabolites which are easily separable. Hairy root cultures have been found to reflect the biosynthetic capacity of their parent plants. Highly productive hairy root cultures may arise from plants of high productivity[123].

Genetic manipulation of hairy root cultures is possible during the transformation of plant material with A. rhizogenes. The insertion of foreign genes, in conjunction with genetic material supplied from A. rhizogenes, has been achieved. The insertion of foreign genes may increase the amount of a regulatory enzyme in the biosynthetic pathway and cause increased production of secondary metabolites.

The commercial exploitation of highly productive hairy root cultures will follow two outlets. The first approach is the cultivation of hairy roots in bioreactors. Secondary metabolites of high value which are not released into the medium could be isolated by harvesting the hairy roots and direct extraction. It may only be economically viable to obtain secondary metabolites of lower value by their isolation from the exchange of culture medium continuously. An alternative approach would be plant regeneration. The passage of highly productive hairy roots through media which promote shoot growth and subsequent establishment could allow the cultivation of highly productive plants in the field.

In conclusion, hairy root cultures are in the very early stages of development. Further advancement is dependent on the discovery of highly-productive hairy root cultures and an understanding of the mechanism of secondary metabolite formation at the genetic level. The use of labelled compounds of established precursors has allowed the stereochemistry of a

number of the enzymic processes to be elucidated. Various analogues of established precursors are needed which produce new secondary metabolites in culture. They may also allow the isolation and characterisation of enzymes responsible for the flux through the biosynthetic pathway.

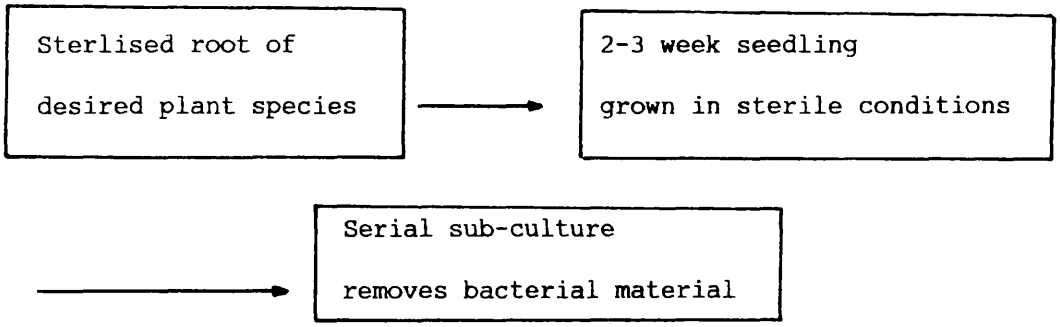


Figure 15: Establishment of hairy root cultures.

CHAPTER 5

THE BIOSYNTHESIS OF (-)-SCOPOLAMINE

5.1 Introduction

Investigations into the biosynthesis of (-)-scopolamine (15) have been concerned with the two main areas: (a) the biosynthesis of the tropane ring structure, and (b) the biosynthesis of the (S)-tropic acid (77) moiety. The vast majority of these studies have been carried out in conjunction with investigations into the biosynthesis of (-)-hyoscyamine (14).

Until recently very few publications had appeared on the biosynthesis of (-)-scopolamine (15) from (-)-hyoscyamine (14) in Datura plants. At the outset of these investigations no publications were available on the stereochemical fate of the hydrogens involved in the epoxide ring formation of (-)-scopolamine (15). The work of Hashimoto and co-workers, as reviewed in Chapter 2, was published after the initiation of these investigations. The work outlined in this Chapter has used the powerful technique of deuterium n.m.r spectroscopy to establish the fate of hydrogen atoms during epoxide formation in the biosynthesis of (-)-scopolamine (15).

5.2 Investigations on the incorporation of putrescine into (-)-scopolamine (15) in the hairy root culture Datura candida x Datura aurea hybrid (DB5)

In order to determine the optimum feeding conditions required for the feeding experiments with various deuterium labelled precursors it was decided to feed different concentrations of unlabelled putrescine to the Datura hairy root culture. To measure the incorporation of the fed precursor we decided to add a tracer, [1,4-^{14}C]putrescine (48), to each solution of unlabelled putrescine. Feeding experiments with radiolabelled precursors usually involve feeding experiments with small amount of material with high specific activity. Feeding experiments with precursors labelled with stable isotopes involve the administration of a critical amount of material.

In order to determine this critical amount we established four separate batches of the hairy root culture. Feeding experiments were carried out by dividing the samples equally amongst the flasks containing the hairy root culture. The final concentrations of putrescine after administration to each batch were 0.1 mM, 0.5 mM, 1.0 mM and 2.0 mM.

After ten days incubation, and fourteen days after sub-culturing, each batch of hairy root culture was removed and the mixture of alkaloids was isolated by an acid-base extraction procedure. For

each batch of hairy root culture its medium was also extracted by an acid-base extraction procedure. The results of this experiment can be seen in Table 3. The medium was found to contain putrescine which had not been taken up by the hairy root culture.

Table 3

Concentration (mM)	Total ^{14}C Incorporation (%)	
	roots	medium
0.1	2.8	1.8
0.5	4.4	2.1
1.0	2.9	2.5
2.0	1.24	4.11

Each batch of extracted medium yielded a low weight of combined alkaloids, as shown by h.p.l.c. analysis. The vast majority of ^{14}C "incorporation" in the medium was associated with putrescine which had not been taken up by the roots. In all our subsequent feeding experiments with deuterium labelled precursors the culture medium was discarded.

The total incorporation of [1,4-^{14}C]putrescine (48) into the alkaloid extract reached a peak at 0.5mM in these experiments and was suggested as the concentration at which the deuterium labelled putrescines should be fed. In order to test this hypothesis each of the extracts obtained from acid-base extractions of the roots were purified by preparative thin-layer chromatography. A reference sample was used to identify the region corresponding to (-)-scopolamine (15). This was removed and submitted for radio-counting in order to estimate the specific incorporation of the fed precursor. The quantity of (-)-scopolamine (15) was determined by h.p.l.c. analysis. These results can be seen in Table 4.

Table 4

Concentration (mM)	Specific incorporation of ^{14}C into (-)-scopolamine(15) isolated from the roots	Weight of (-)-scopolamine (μg)
0.1	0.4	317
0.5	3.3	127
1.0	11.5	20
2.0	13.5	15

The relatively high specific incorporations obtained for the (-)-scopolamine (15) isolated from the hairy root cultures administered with 1.0mM and 2.0mM solutions of putrescine can possibly be explained by the following.

Putrescine is a known inhibitor of ornithine decarboxylase (ODC)[124]. Thus, the flux of the biosynthetic pathway may have been affected by the administration of these high concentrations. This hypothesis is suggested due to the low weight of (-)-scopolamine (15) isolated from both these concentrations. The concentration selected for the feeding of the deuterium labelled precursors was 0.5mM. This allowed reasonable amounts of label present to be incorporated with no overload of the cultures, thus avoiding the wasting of valuable or scarce precursors.

5.3 The rigorous assignment of the ^{1}H n.m.r. spectrum of (-)-scopolamine

To assist the assignment of signals in the ^{2}H n.m.r. spectra, a detailed assignment of the ^{1}H n.m.r. spectrum of (-)-scopolamine (15) was required. Chazin and Colbrook[125] used proton spin-lattice relaxation and nuclear Overhauser effect (NOE) data to determine the chemical shifts of each proton in (-)-scopolamine (15). The critical chemical shifts in (-)scopolamine (15) for the following biosynthetic studies are those of H-6 and H-7. They have shown by observa tion of N.O.E. enhancements at H-6 and H-7 when the equatorial protons of H-2 and H-4 were irradiated that H-6 and H-7 sit in an endo conformation.

Their experiments showed by an unambiguous method the stereochemistry of the epoxide ring located at C-6 and C-7 of (-)-scopolamine (15).

The ^{13}C n.m.r. spectrum of (-)-scopolamine (15) was tentatively assigned by Feeney and coworkers[126] who voiced reservations for the assignments of C-1, C-5, C-6 and C-7.

The powerful technique of heteronuclear ^{1}H-^{13}C chemical shift correlation spectroscopy (2D COSY) was employed in our work to confirm firstly the previously published assignment[125] of the ^{1}H n.m.r. spectrum of (-)-scopolamine (15). Secondly, to remove the ambiguities in the assignment of the ^{13}C n.m.r. spectrum by Feeney and co-workers[126]. The results of the 2D COSY experiments are shown in **Figure 16.** The ^{1}H n.m.r. spectral assignment arising from this experiment was confirmed by Difference Nuclear Overhauser Effect spectroscopy. The ^{1}H n.m.r. spectral assignment is in general agreement with that made by previous workers. The rigorous assignment of the signals for H-6 and H-7 in the ^{1}H n.m.r. spectrum is crucial for the interpretation of the ^{2}H n.m.r. spectra associated with our investigations. The chemical shifts for H-6 and H-7 were found to be 3.31 and 2.66 ppm respectively. The above n.m.r. spectroscopy experiments have shown the assignment made by Feeney and co-workers[126] of the ^{13}C n.m.r. spectrum of (-)-scopolamine (15) to be correct.

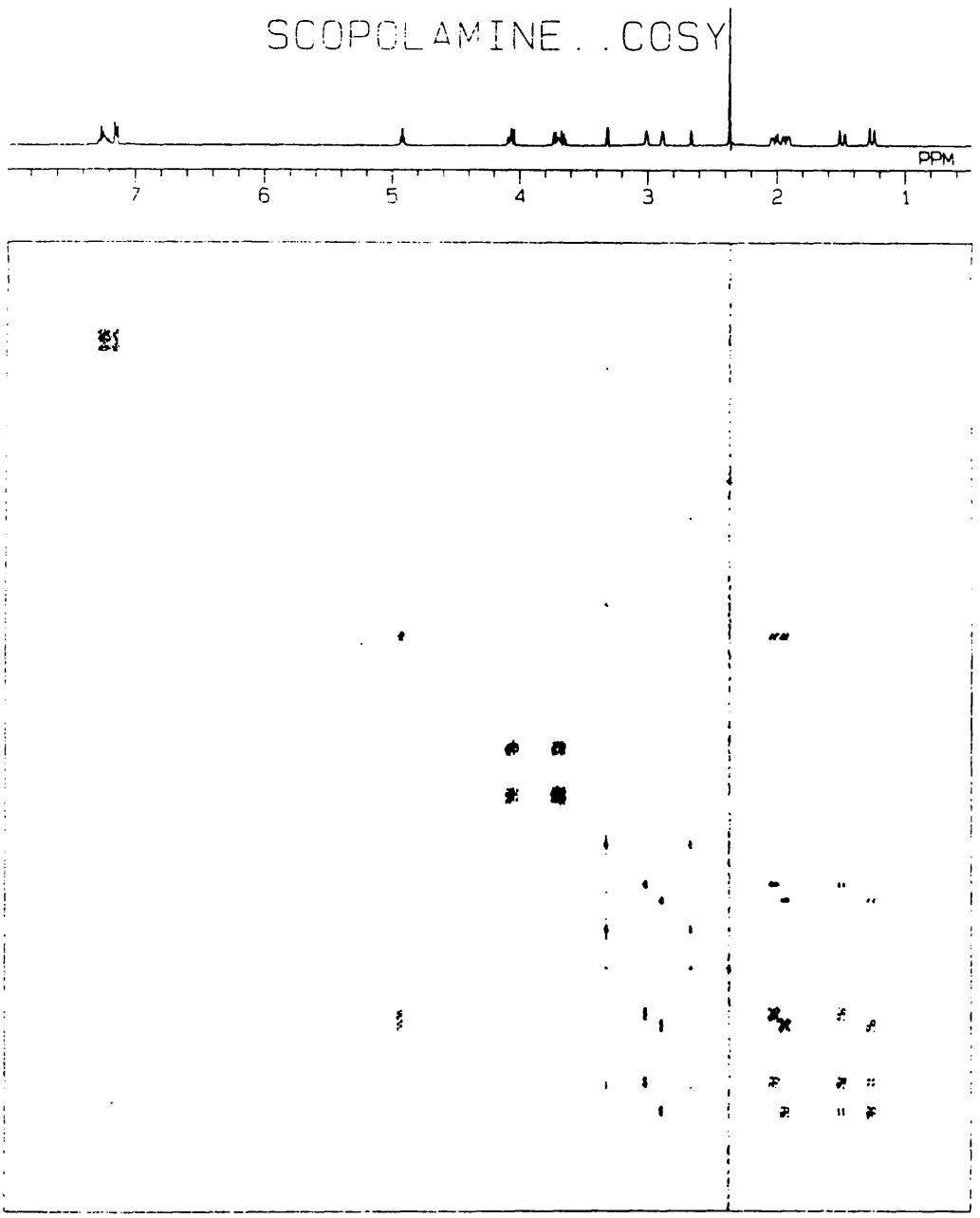


Figure 16

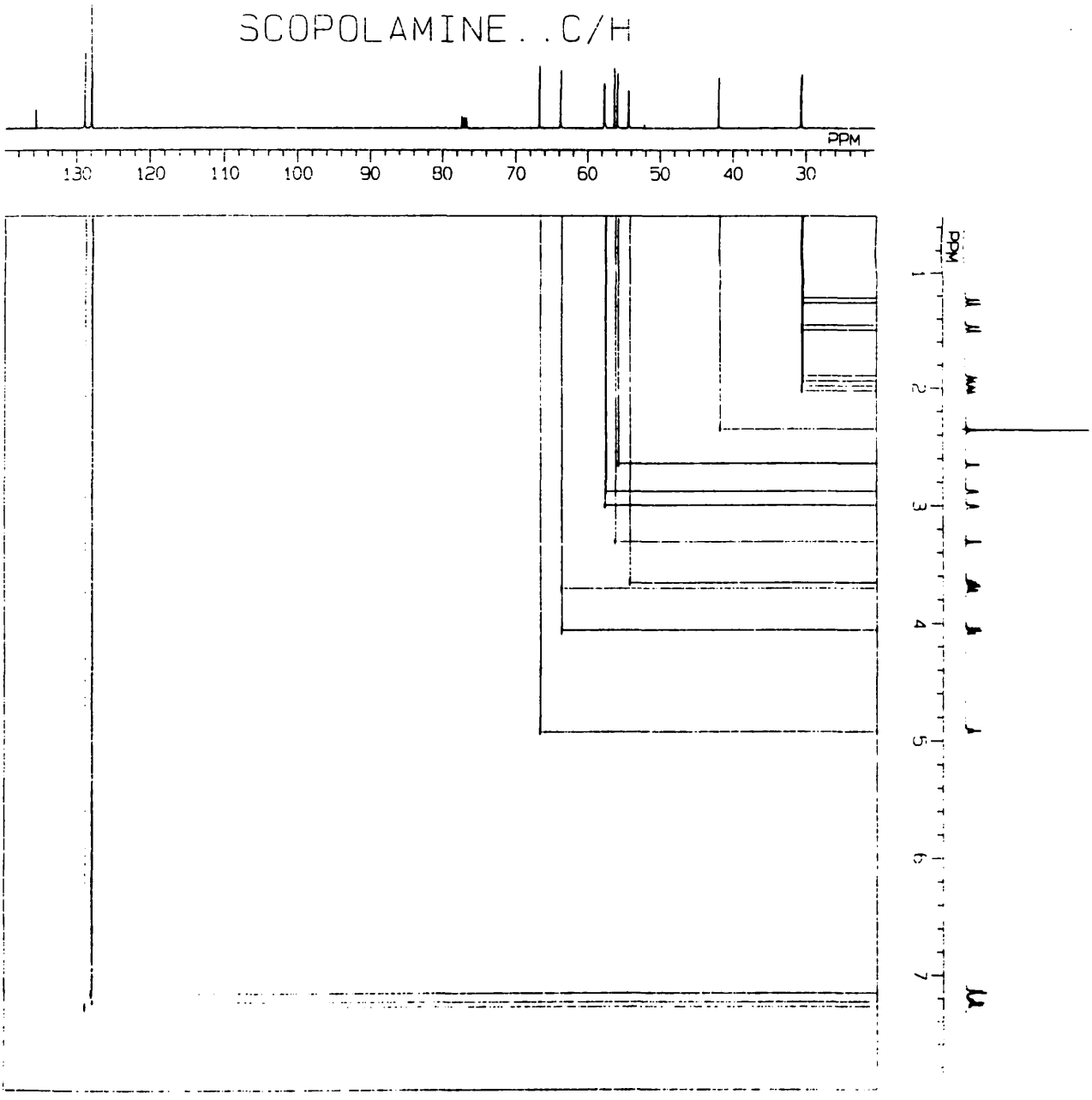

SCOPOLAMINE. .C/H
PPM
130
120
110
100
90
80
70
60
50
40
30
PPM
1
2
3
4
5
6
7

Figure 16

5.4 Incorporation of ^2H-labelled precursors into (-)-scopolamine (15)

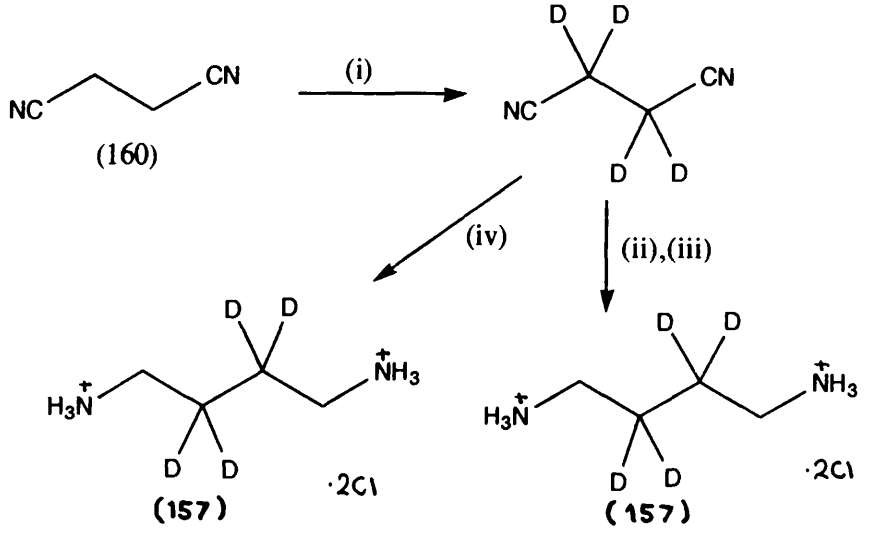
A combination of ^2H-labelled precursors and ^2H n.m.r. spectroscopy has allowed examination of the stereochemistry of the enzyme processes involved in epoxide ring formation in (-)-scopolamine (15) to be undertaken. The ^2H-labelled derivatives of putrescine chosen for this work were [2,2,3,3-$^2H_4$]- (157), (R)-[2-^2H]- (158) and (S)-[2-^2H]putrescine (159).

The [2,2,3,3-$^2H_4$]putrescine (157) was prepared by the exchange of the α-protons of succinonitrile (160) in ^2H_2O. It was found beneficial to heat the above solution at reflux in the presence of 1,4-dioxan and the non-nucleophilic base DBU. Purification and subsequent reduction of the dinitrile was carried out. The reduction step was carried out by two distinct methods. Firstly, the dinitrile was reduced by a solution of borane-dimethyl sulphide in THF. The product was isolated as its dihydrochloride salt by the passage of HCl gas through an ethereal solution a) the free base. Secondly the dinitrile was reduced by hydrogenation at atmospheric pressure over Adams' catalyst (PtO_2). It was isolated as its dihydrochloride salt by dissolving the residue in a 1M HCl solution and removing the solvent *in vacuo* (Figures 17 and 18). The (R)- and (S)-[2-^2H]putrescines, (158) and (159) were prepared as their dihydrochlorides by modification of the route reported by Kunec and Robins[127]. The ^2H content of each compound was found to be *ca.*100% and 98% respectively. For synthesis of (R)-[2-^2H]putrescine (158) dihydrochloride the starting material was (S)-aspartic acid (161). Sequential chlorination, methylation and reduction with DIBAL-H of (S)-aspartic acid (161) gave rise to

(S)-2-chlorobutan-1,4-diol (162). Reaction with $LiAl^2H_4$ gave (R)-[2-^{2}H]butan-1,4-diol (163). The modification of the route involved the conversion of the primary hydroxyl groups of (163) into primary amino groups using a procedure published by Fabiano and Golding[128]. This involved a Mitsunobu-type reaction[129], which gave the diazide (164), and an in situ Staudinger reaction[130] which yielded a phospho-imine species such as (165) (Figure 21).

Aqueous acidic hydrolysis afforded the product, (R)-[2-^{2}H]putrescine (158) which was isolated as its dihydrochloride salt in a reasonable yield from the starting material (Figure 19).

The enantiomeric (S)-[2-^{2}H]putrescine (159) was prepared in a similar manner but employing (R)-aspartic acid (166) as the starting material (Figure 20).



Reagents : (i) D_2O , DBU ; (ii) BMS , THF ; (iii) HCl ; (iv) PtO_2 , H_2 .

Figures 17 and 18

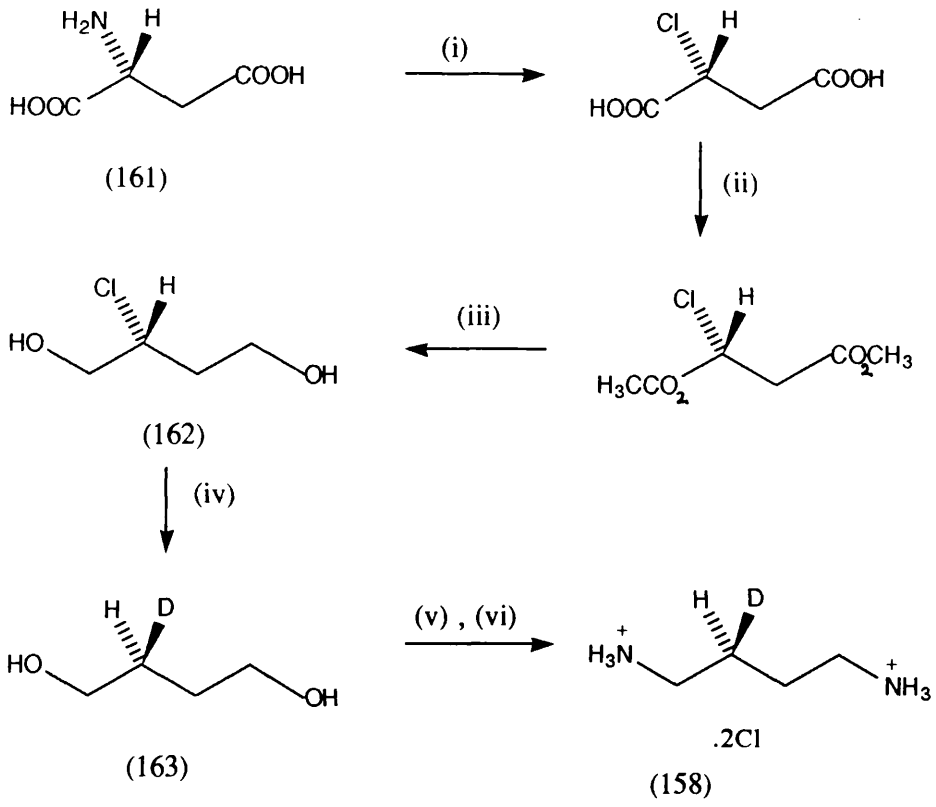
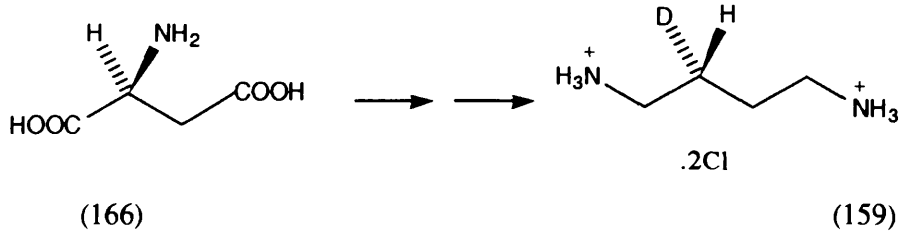


Figure 19

Reagents :

(i) HCl ,HNO_3 ; (ii) MeOH , $SOCl_2$; (iii) DIBAl-H , toluene ;
(iv) $LiAl^2H_4$; (v) HN_3 , $iPrCO_2N{=}NCO_2iPr$, Ph_3P ; (vi) H_2O / HCl .



(166) (159)

Figure 20

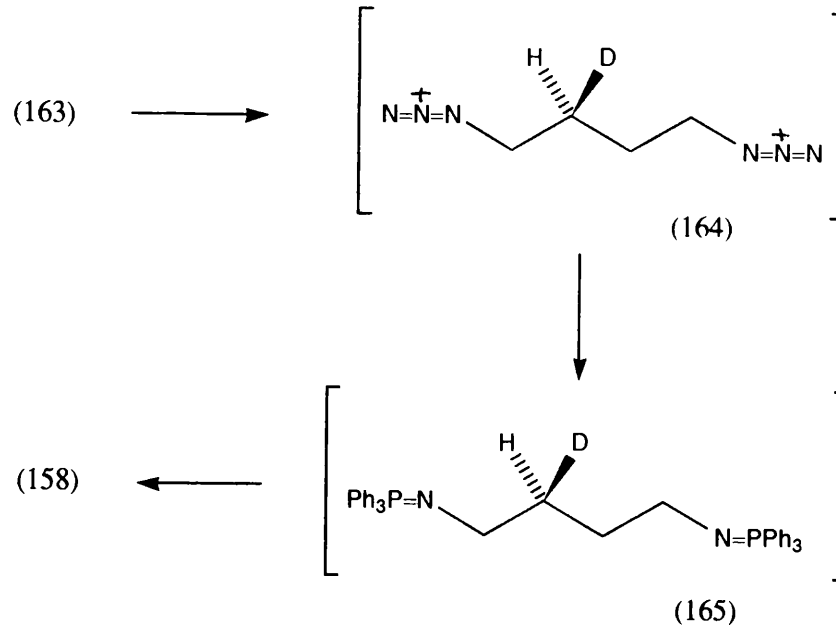
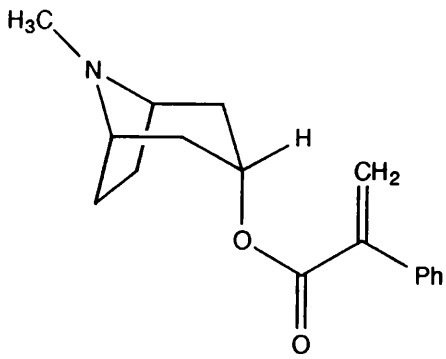
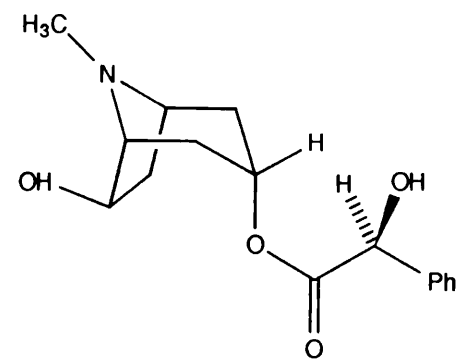


Figure 21



(167)



(168)

The feeding of [2,2,3,3-$^{2}H_{4}$]putrescine (157) to the Datura hairy root culture DB5 was carried out in conjunction with a sample of [1,4-^{14}C]putrescine (48) dihydrochloride as an internal refernce. Feeding was carried out under the conditions laid out previously with the roots being harvested then the alkaloids isolated after an acid-base extraction.

Purification of the crude extract was carried out by preparative thin layer chromatography with the bands for (-)-hyoscyamine (14) and (-)-scopolamine (15) being identified by reference samples. Both bands were removed and the alkaloids were isolated. The ^{2}H n.m.r. spectrum of (-)-hyoscyamine (14) had a broad signal at 1.53 ppm which corresponded to ^{2}H present at H-6 and H-7. A broad singlet is seen due to the closeness of the chemical shifts for the protons at H-6 and H-7 (see Figure 22). Values for the incorporation of ^{2}H into (-)-hyoscyamine (14) were estimated from the ^{2}H n.m.r. spectrum by comparison of the size of peaks due to the administered sample and the signal due to natural abundance for ^{2}H in $CHCl_{3}$. For (-)-hyoscyamine (14) the specific incorporation of ^{2}H was comparable to that of ^{14}C. The ^{14}C specific incorporation of (-)-hyoscyamine (14) was found to be 6.12% and the ^{2}H specific incorporation of (-)-hyoscyamine (14) was 7.84%. The feeding of [2,2,3,3-$^{2}H_{4}$]putrescine (157) yielded labelled (-)-scopolamine (15). This showed two broad singlets in the ^{2}H n.m.r. spectrum at 2.63 ppm and 3.36 ppm in a 1:1 ratio. These values correspond to the chemical shifts of H-6 and H-7 of (-)-scopolamine (15) found in our 2D COSY experiment (Figures 16 and 23). The specific incorporation of ^{14}C was found to be 13.8% in the isolated (-)-scopolamine (15).

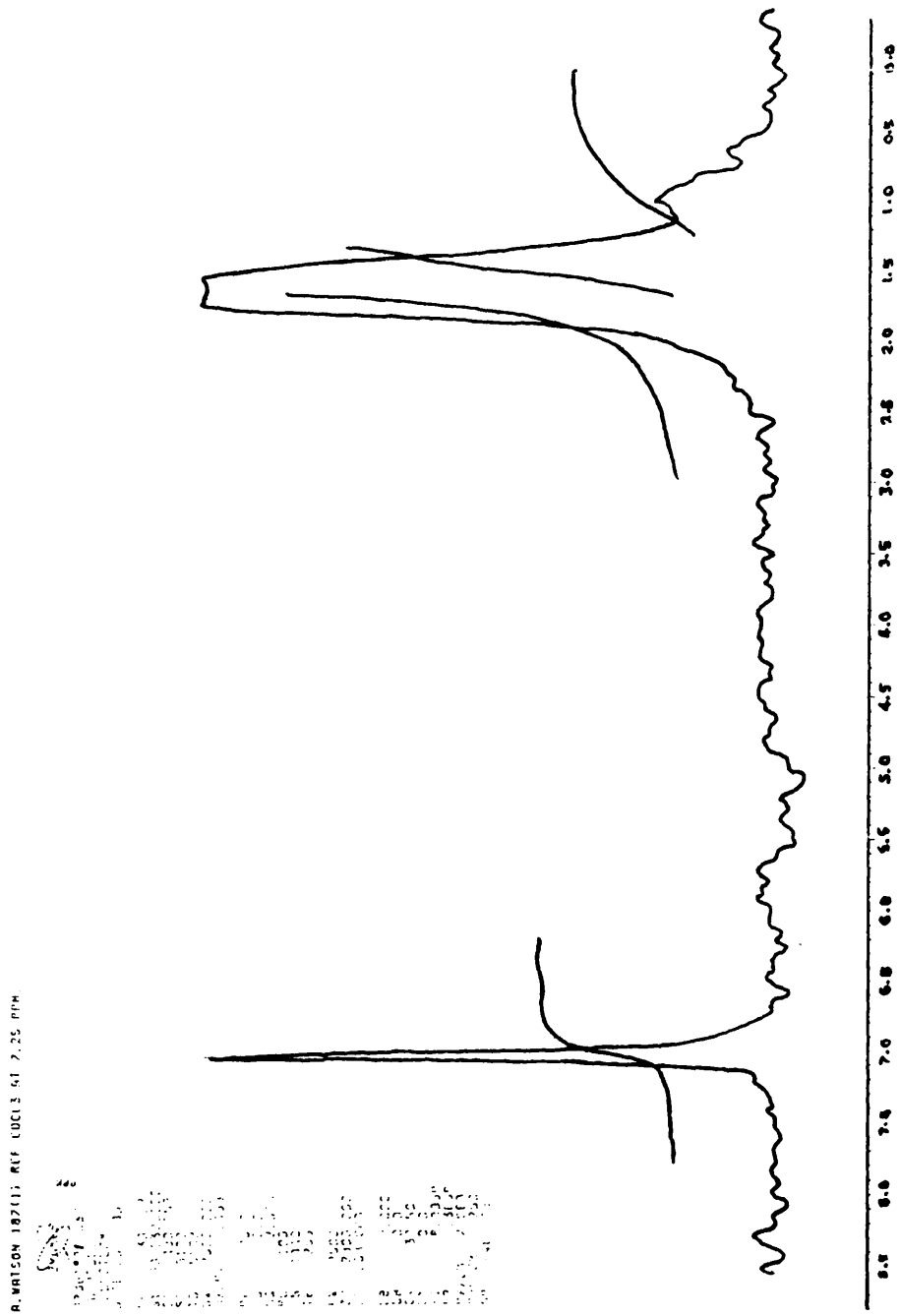

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Figure 22

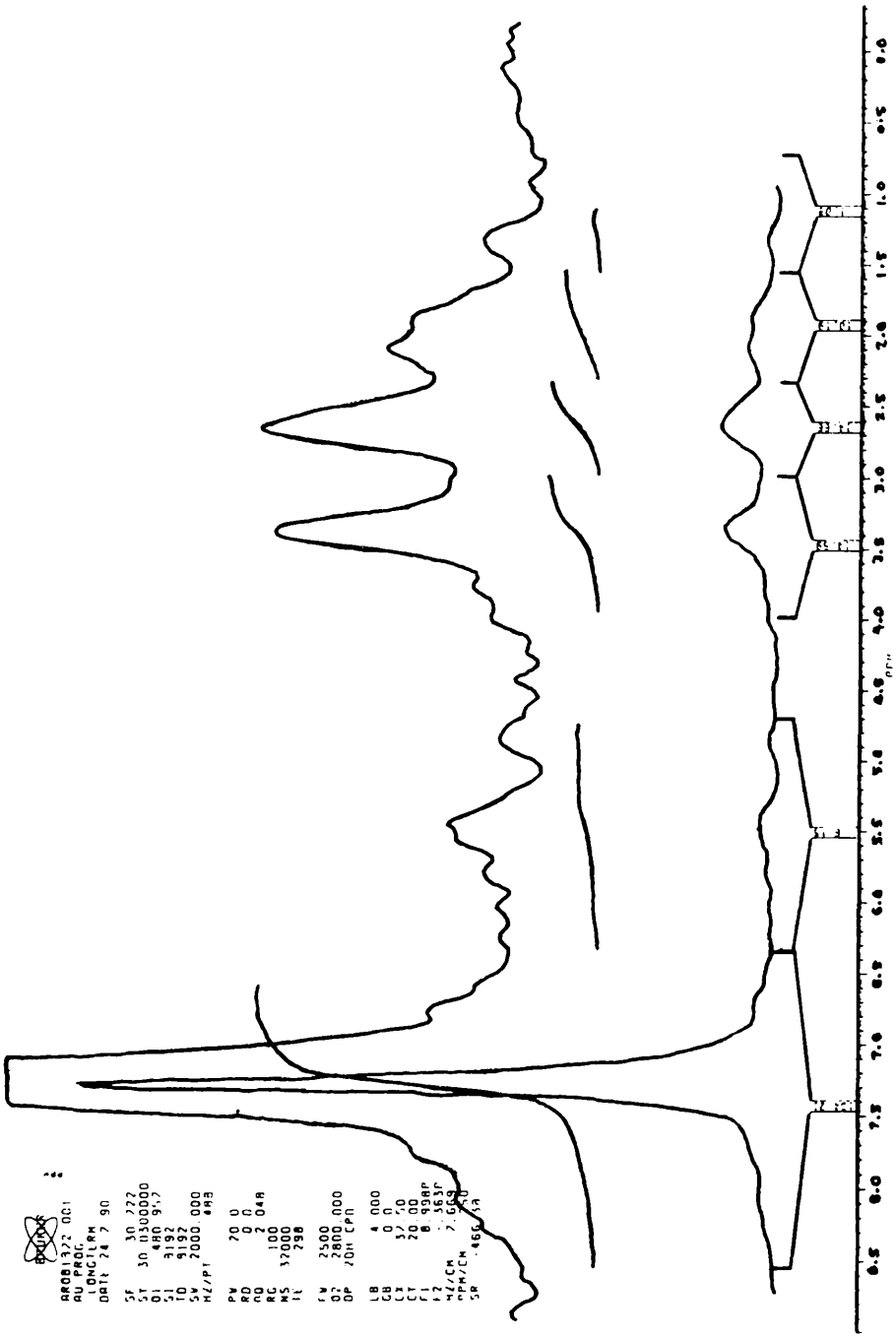


Figure 23

Further information about the purity of (-)-hyoscyamine (14) and (-)-scopolamine (15) was obtained by gas-chromatography (g.c.) and h.p.l.c. analysis. Isolated (-)-hyoscyamine (14) showed two peaks in its gas-chromatography trace in the ratio of approximately 1:2. The two peaks corresponded to apoatropine (167) and (-)-hyoscyamine (14) respectively. For (-)-scopolamine (15) a single peak, which corresponded to a reference sample of (-)-scopolamine (15), was found.

The specific incorporation of ^{2}H from the fed precursor [2,2,3,3-$^{2}H_{4}$]putrescine (157) was also estimated from the ^{2}H n.m.r. spectrum of (-)-scopolamine (15). This was found to be 0.84% which strongly suggested that the isolated (-)-scopolamine (15) was not radiochemically pure.

This preliminary experiment has shown the purification technique employed is not adequate for the isolation of pure (-)-hyoscyamine (14). The isolation of (-)-scopolamine (15) containing ^{2}H has shown that the planned feeding experiments with the (R)- and (S)-[2-^{2}H]putrescines, (158) and (159), are viable.

Feeding experiments with the (R)- and (S)-[2-^{2}H]putrescines, (158) and (159), were carried out under the conditions outlined previously. Purification was again carried out by preparative thin layer chromatography. In both experiments a sample of [1,4-^{14}C]putrescine (48) dihydrochloride was added as an internal marker.

The administration of (R)-[2-^{2}H]putrescine, (158) dihydrochloride gave a sample of (-)-scopolamine (15) which had a singlet at 3.37 ppm in the ^{2}H n.m.r. spectrum. This corresponded to the chemical shift of 3.31 ppm assigned to H-6 of (-)-scopolamine (15) in the 2D COSY experiment. This spectrum is shown in Figure 24A. The specific incorporation of (R)-[2-^{2}H]putrescine (158) was estimated from the incorporation of [1,4-^{14}C]-putrescine (48) to be 3.5%. From the resultant ^{2}H n.m.r. spectrum, the specific incorporation of ^{2}H was estimated to be 0.6%. The administration of (S)-[2-^{2}H]putrescine (159) yielded a sample of (-)-scopolamine (15).

The ^{2}H n.m.r. spectrum (Figure 24B) showed a peak at 2.66 ppm which corresponded to the chemical shift assigned to H-7 in the 2D COSY experiment. The specific incorporation of ^{2}H was estimated be 2.2%. This was in reasonable agreement with the observed specific incorporation of ^{14}C (3.2%). The purity of (-)-scopolamine (15) was confirmed by g.c., h.p.l.c. and mass spectroscopy.

The above observations can be rationalised in the following manner. An intermediate between (-)-hyoscyamine (14) and (-)-scopolamine (15) is 6-hydroxyhyoscyamine(168)[131]. The hydroxylation at C-6 of (-)-hyoscyamine (14) proceeds with the loss of the pro-S hydrogen and retention of configuration. This was shown by the absence of a deuterium signal corresponding to H-6 in the ^{2}H n.m.r. spectrum of (-)-scopolamine (15) obtained after the administration of (S)-[2-^{2}H]putrescine (159).

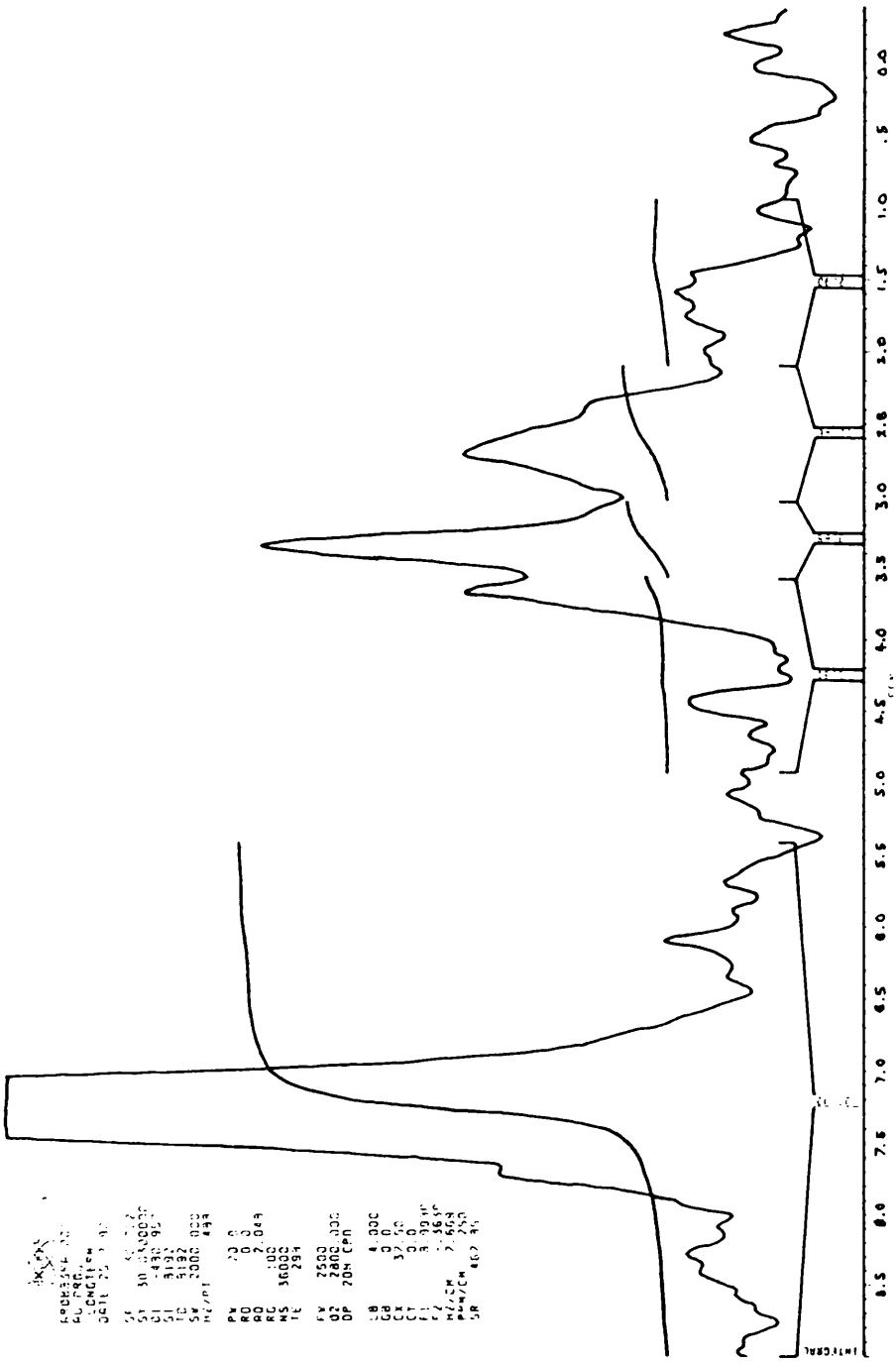
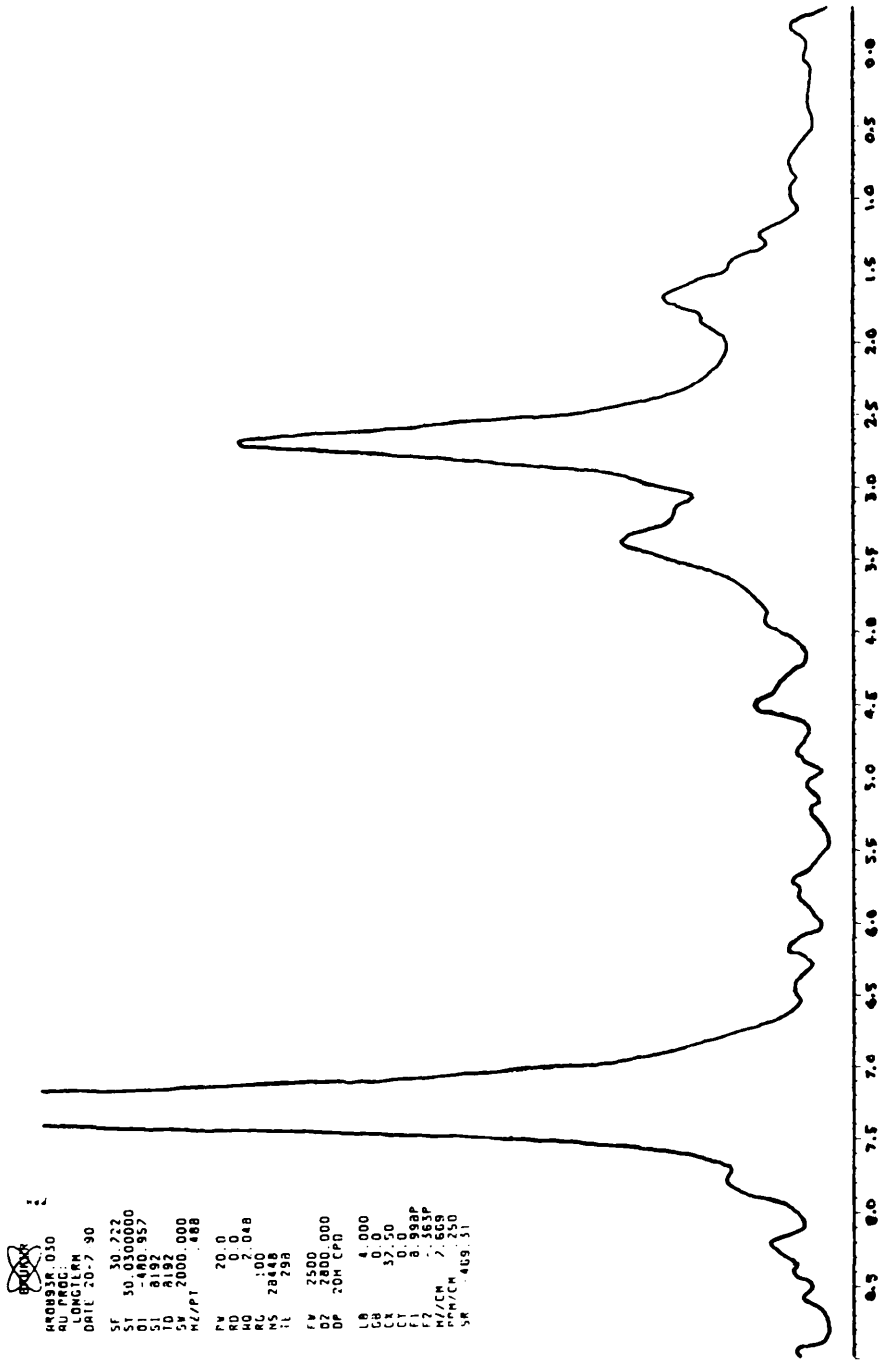


Figure 24A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SI 8192
TD 8192
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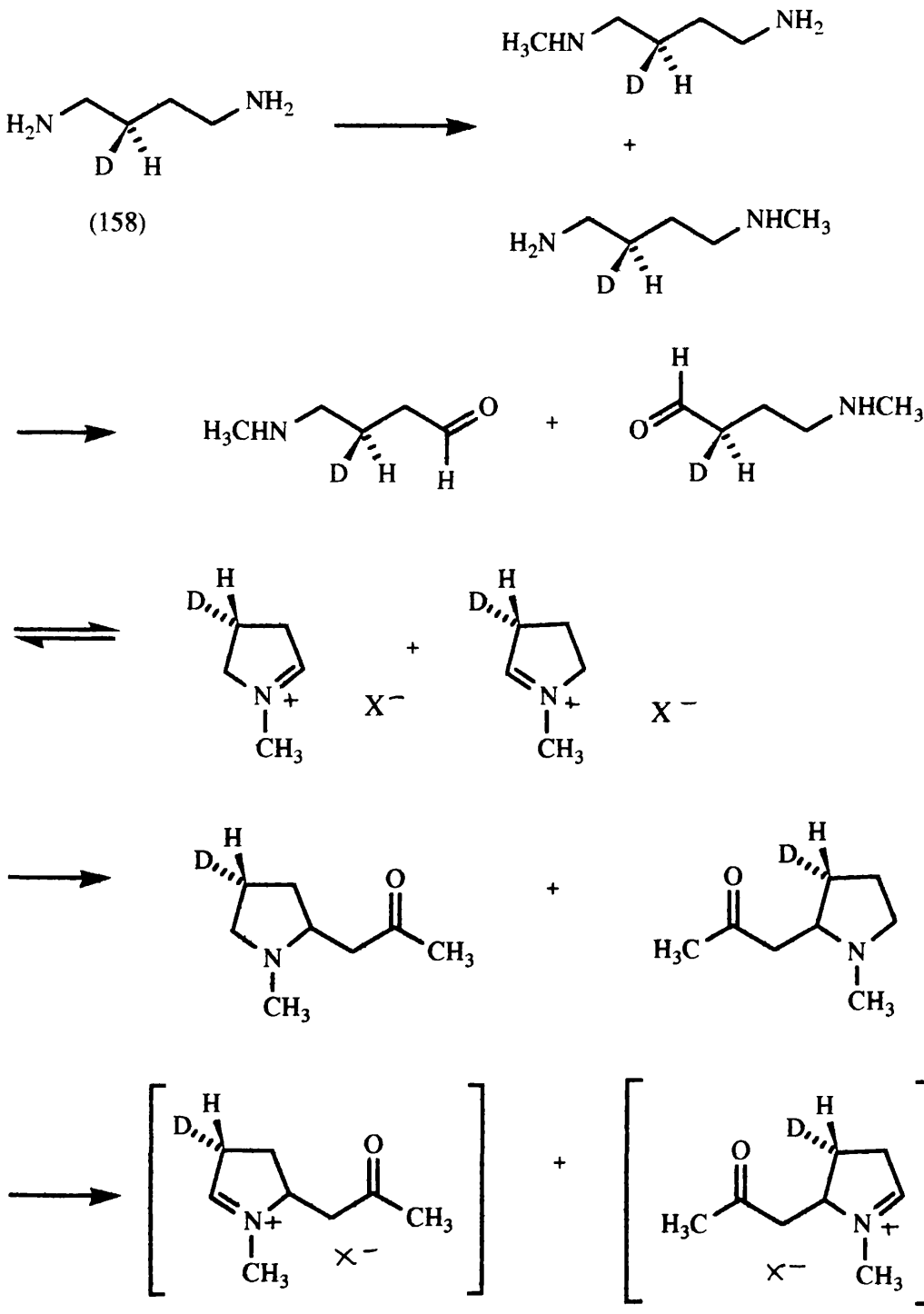
PW 20.0
RD 0.0
NO 2.048
RG 100
NS 28448
TL 298
FW 2500
D7 2800.000
DF 20H CPD
LB 4.000
GB 0.0
CX 32.00
CT 8.993P
F1 -.365P
F2 2.669
PPM/CM .250
SR 469.51
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

Figure 24B

By analogy, the deuterium signal corresponding to the retention of the pro-R hydrogen at C-6 was seen in the ^{2}H n.m.r. spectrum of (-)-scopolamine (15) obtained after the administration of (R)-[2-^{2}H]putrescine (158). The stereochemical course of epoxide ring formation at C-7 of (-)-scopolamine (15) can be explained in an analogous manner. The loss of the pro-R hydrogen at C-7 occurs in the formation of the epoxide ring of (-)-scopolamine (15). These observations can be seen in schematic form (Figures 25 and 26).

The experiments with (R)- and (S)-[2-^{2}H]putrescine (158) and (159) have substantiated the findings of Hashimoto and co-workers[83-86] who used partially purified enzymes in their studies. The formation of the epoxide ring of (-)-scopolamine (15) proceeds in Datura hairy root cultures with the loss of two hydrogens from the same side in the biosynthesis of (-)-scopolamine (15). Both hydrogens are lost from the β face of (-)-hyoscyamine (14). The stereochemical course of this process has been partially elucidated by the administration of enantiomerically deuteriated putrescines and the subsequent analysis of the isolated (-)-scopolamine (15).

The Incorporation of (R)-[2-^{2}H]putrescine (158)



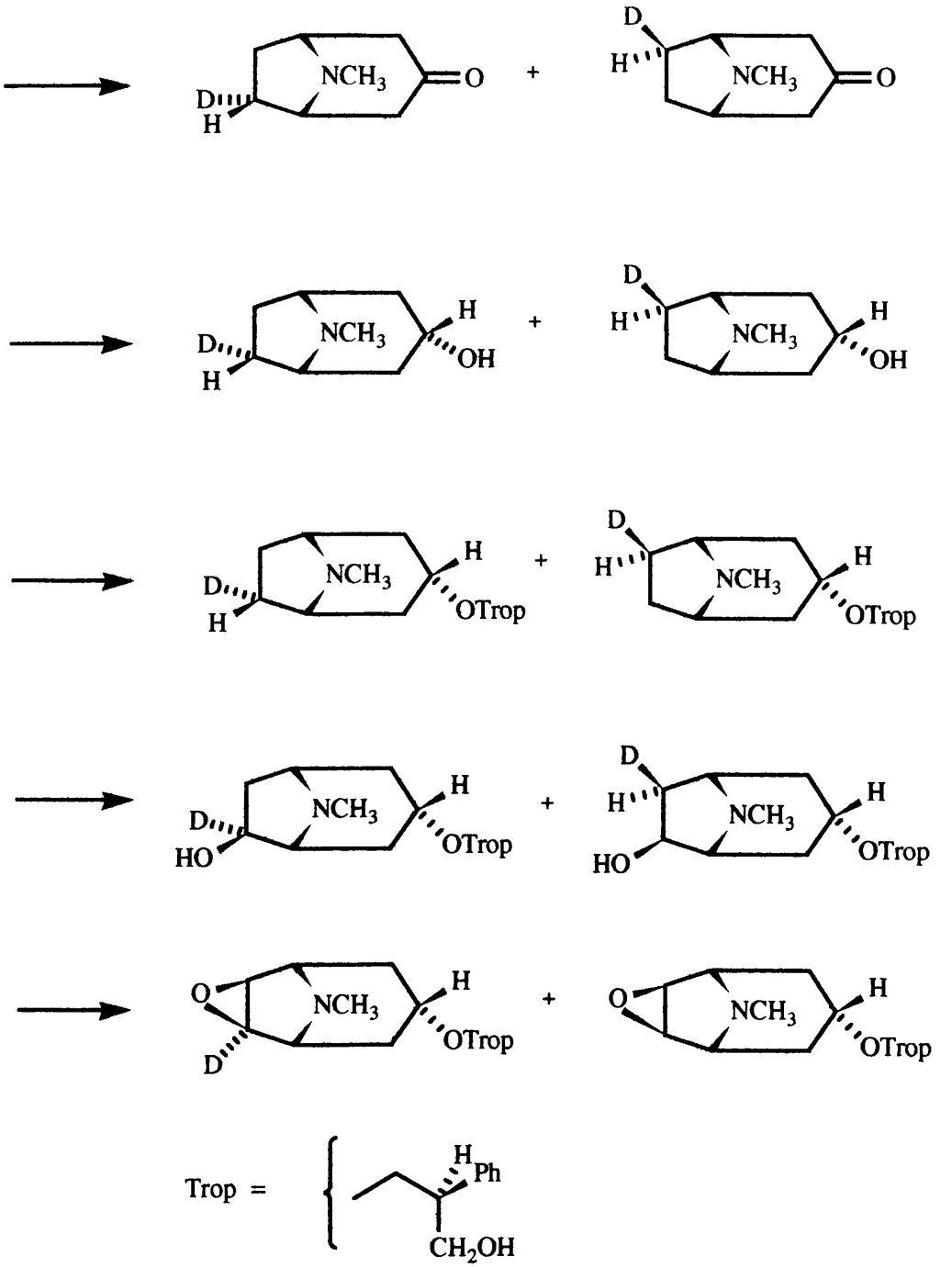
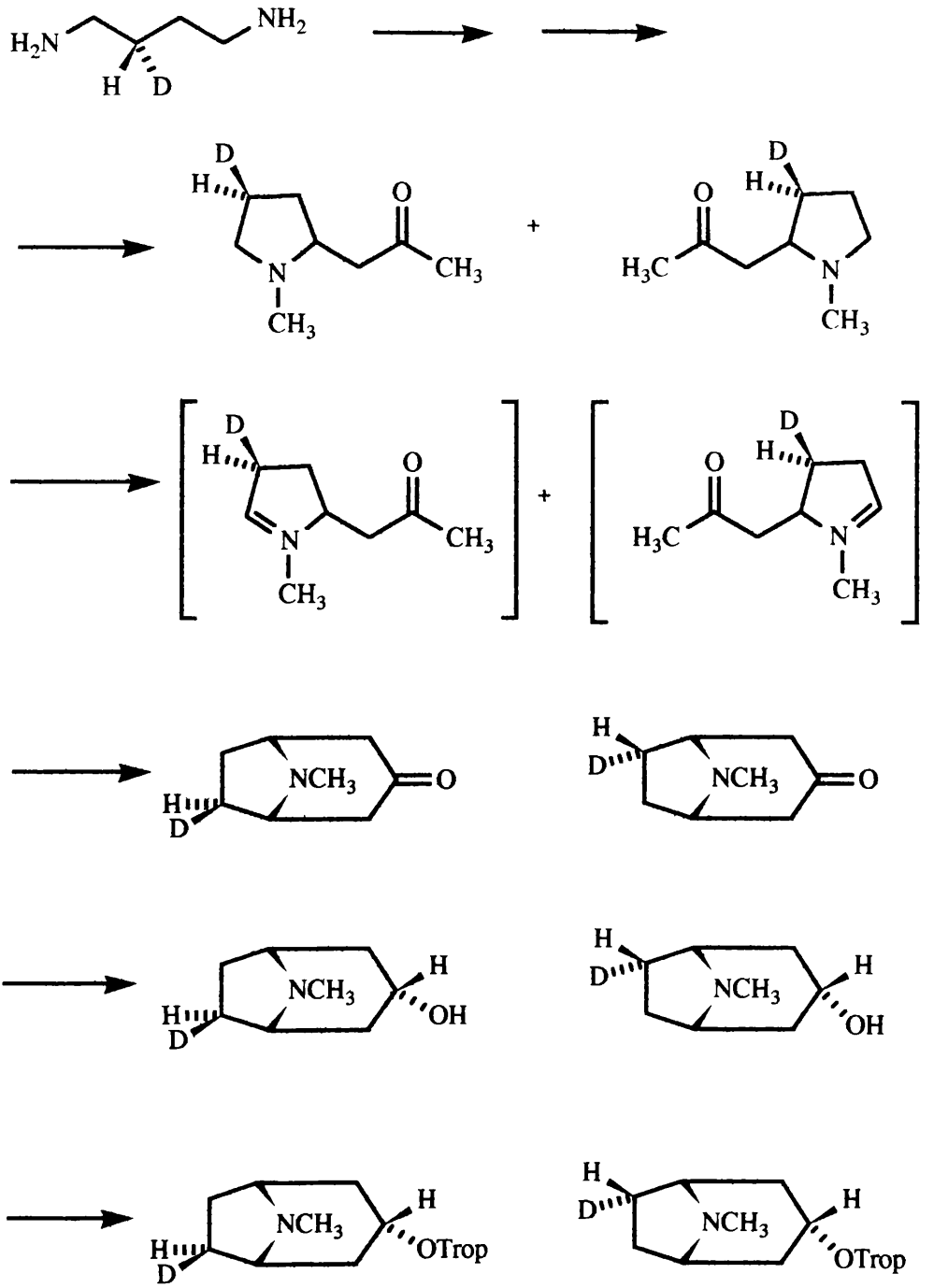


Figure 25

The Incorporation of (S)-[2-^{2}H]putrescine (159)



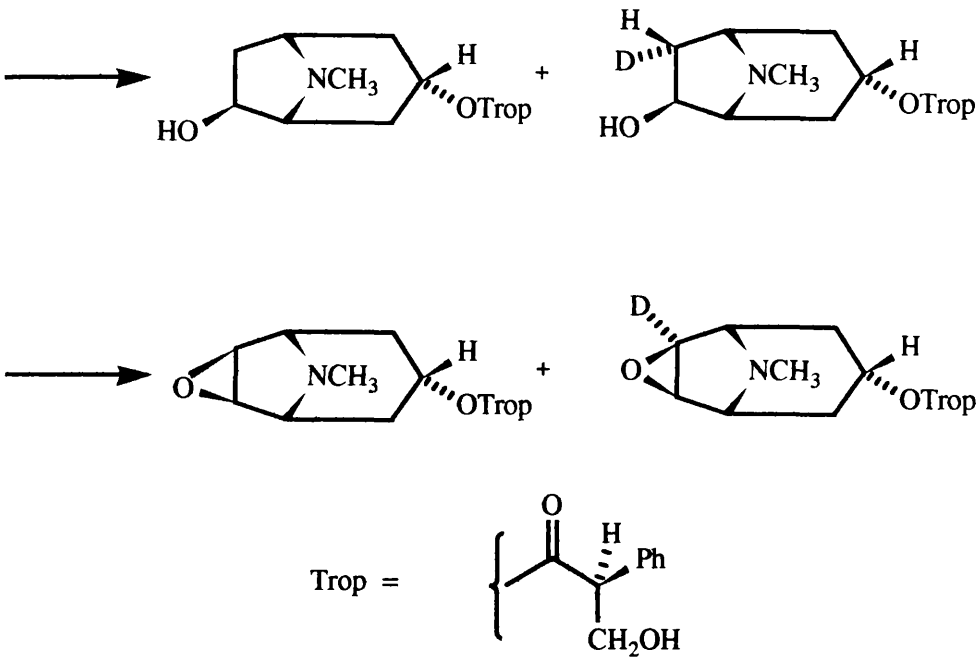

NCH3
H
OTrop
HO
+
H
D
NCH3
H
OTrop
HO
NCH3
H
OTrop
O
+
D
O
NCH3
H
OTrop
Trop =
O
H
Ph
CH2OH

Figure 26

CHAPTER SIX

THE OXIDATION OF PUTRESCINE BY *NICANDRA PHYSALOIDES* AND *DATURA CANDIDA* X *DATURA AUREA* ROOT CULTURES

6.1 Introduction

Oxidation of putrescine by diamine oxidase (EC 1.4.3.6) in various plant species is an area which has gained greater prominence in the last decade. The enzyme is of low substrate specificity and acts upon a wide range of primary amines. It catalyses the oxidative deamination of a primary amino group (**Figure 27**).

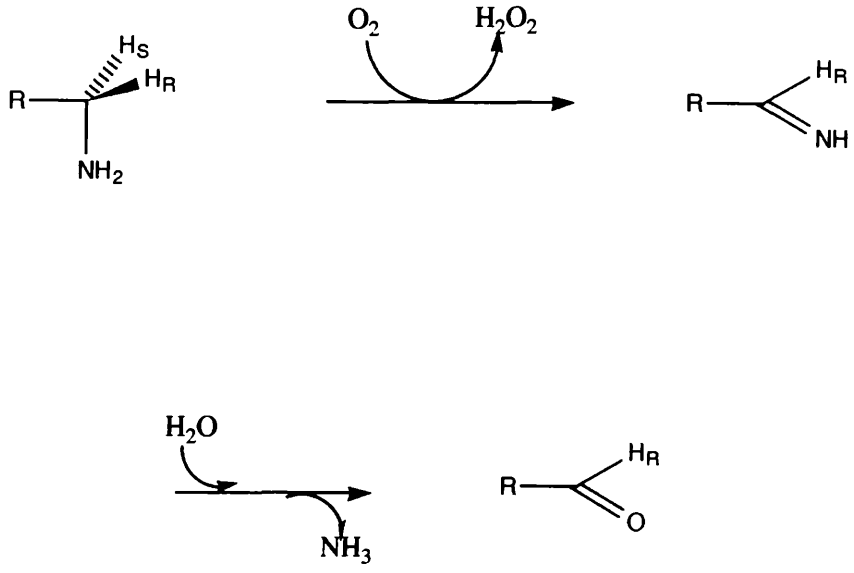
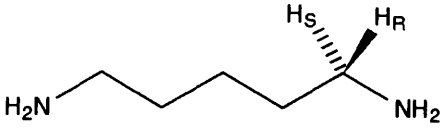
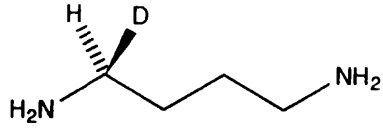


Figure 27

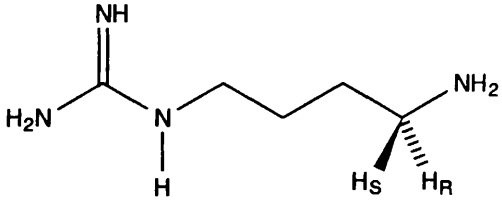
Tabor and Tabor[124] have shown that diamine oxidase is a key enzyme in the metabolism of polyamines. Polyamines are essential for the growth and replication of all living cells. The first publication to deal with the stereospecificity of diamine oxidase was written by Spenser and Richards[132]. They studied the oxidation catalysed by partially purified hog kidney oxidase of a range of substrates. They synthesised (R)-[1-^{2}H]putrescine (171) and (R)-[1-^{2}H]-(138) and (S)-[1-^{2}H]agmatine (173). They showed that the enzymic oxidation of all the above substrates proceeds with the stereospecific removal of the pro-S hydrogen.





(169) $H_R = {}^2H$, $H_S = {}^2H$.
(170) $H_R = H$, $H_S = {}^2H$.
(174) $H_R = H$, $H_S = {}^3H$.

(171)



(172) $H_R = {}^2H$, $H_S = H$.
(173) $H_R = H$, $H_S = {}^2H$.

Diamine oxidase isolated from pea seedlings has also been used to study the stereospecific course of diamine oxidation. Gerdes and Leistner[133] synthesised (S)-[1-^{3}H]-(174) and (R)-[1-^{2}H]cadaverine (169) to study their oxidative deamination. Their results showed the loss of the pro-S hydrogen from each substrate.

The loss of the pro-S hydrogen in the oxidation of polyamines by diamine oxidase has been generally accepted.

6.2 Investigations into the incorporation of putrescine in Nicandra physaloides transformed root cultures

In order to determine optimum feeding conditions for Nicandra physaloides it was necessary to carry out experiments similar to those reported in Chapter Five for Datura candida x Datura aurea transformed root cultures.

Three separate experiments were carried out in order to establish the best conditions for the incorporation of putrescine. These were:

(i) determination of the time lag between sub-culturing of the N.physaloides hairy root cultures and administration of the precursor;

(ii) optimisation of putrescine incorporation; and

(iii) optimisation of hygrine (26) yield.

For the three experiments [1,4-^{14}C]putrescine (48) was employed as an internal marker. Transformed root cultures of N.physaloides were established by using the techniques outlined in Chapter 4.

For the first experiment putrescine dihydrochloride with [1,4-^{14}C]-putrescine (48) dihydrochloride added, was administered to N.physaloides hairy root cultures at a concentration of 0.5 mM. Feeding commenced two, five and seven days after sub-culture. In all cases the roots were harvested fourteen days after sub-culture. Crude alkaloid extracts were obtained by a standard acid-base extraction method and analysed by radio-counting techniques. The total incorporation of ^{14}C showed a peak in the hairy root cultures fed five days after sub-culture. These results can be seen in Table 5.

Table 5

No of days after sub-culture	Total Incorporation (%)	Wt of alkaloid extract (mg)
2	0.35	3.7
5	7.03	8.0
7	3.24	10.8

No attempt was made to determine the specific incorporation of putrescine into the two major alkaloids found in N.physaloides plants, namely hygrine (26) and cuscohygrine (60).

In addition it was also noted that root growth was affected by the length of time between sub-culturing and administration of the precursor. The weight of roots isolated from N.physaloides hairy root cultures fed two days after sub-culture was significantly lower than those isolated five and seven days after sub-culture. The weight of roots isolated from N.physaloides hairy root cultures fed five and seven days after sub-culture was comparable.

The second experiment on the optimisation of putrescine incorporation was carried out using the dihydrochlorides of putrescine and [1,4-^{14}C]putrescine (48), fed at a concentration of 0.5mM five days after sub-culture. Flasks of the transformed root culture were removed after a further seven, ten, fifteen and twenty days respectively. The roots were again subjected to an acid-base extraction procedure and aliquots were removed for radio-counting. These results can be seen in Table 6.

Table 6

No of days after sub-culture	Total Incorporation (%)	Wt of alkaloid extract (mg)
12	3.6	9.2
15	7.4	22.3
20	7.9	13.9
25	5.1	13.6

The medium from each experimental set of flasks was also subjected to an acid-base extraction procedure and aliquots were removed for radio-counting analysis. This was undertaken to determine the uptake of putrescine by the roots. The uptake of putrescine was found to be very high in all cases with less than 1% of the precursor left in the medium. It was decided to discard the medium in subsequent experiments.

Further analysis of each extract from the hairy roots was undertaken using thin layer chromatography and radio-scanning techniques. An authentic sample of hygrine (26) hydrochloride was used as a reference. The solvent system employed was developed by Romeike[134].

The activity of each extract was located on the base line. In order to determine the location of the authentic sample of hygrine (92) each plate was stained by the Dragendorff reagent[135]. No orange bands were found in any extract corresponding to the authentic sample of hygrine (26). In order to discount the possibility of N-oxide formation in the root cultures a zinc/hydrochloric acid reduction procedure was used for each extract. Subsequent radio-scans showed no change in the distribution of activity. The conclusion reached was that no hygrine (26) was produced by the transformed root cultures when fed with putrescine at a concentration of 0.5 mM.

The third, and final experiment was an investigation into the feeding of putrescine, at various concentrations, and the effect on hygrine (26) production.

Samples of putrescine dihydrochloride at concentrations of 0.1 mM, 0.5 mM, 1.0 mM and 2.0 mM were fed to N.physaloides hairy root cultures. In addition [1,4-^{14}C]putrescine (48) dihydrochloride was added as a tracer. In each case the samples of putrescine dihydrochloride were fed five days after sub-culture and the roots were isolated ten days later. The initial results of this experiment showed variance in the weight of alkaloid extract, the weight of roots and total incorporation of the precursor. These findings can be seen in Table 7.

Table 7

Concentration of putrescine fed (mM)	Weight of roots (g)	Weight of extract (mg)	Total Incorporation (%)
0.1	36.1	32	3.9
0.5	41.5	107	3.4
1.0	42.7	128	2.1
2.0	33.6	12	0.7

The initial conclusion reached was that the administration of putrescine dihyrochloride at 0.5 mM was the best concentration for our subsequent feeding experiments. However, radio-scans of each of the extracts showed that all the activity was again located upon the base-line. A reference sample of hygrine (26) hydrochloride was again used. When the plate was stained with the Dragendorff reagent[135] no hygrine (26) was visualised except in the extract obtained after the feeding of putrescine dihydrochloride at 2.0 mM. When the total incorporation figure, the weight of total extract and lack of activity

from the radio-scan except at the base-line were considered it was decided that feeding experiments with ^{2}H-labelled precursors would be unrealistic in N.physaloides hairy root cultures.

A final check on hygrine (26) production within N.physaloides hairy root cultures was undertaken. The transformed root culture was grown under normal conditions, without the administration of any precursor and harvested 30 days after sub-culture. No hygrine (26) was detected.

The conclusion reached from this experiment was that our transformed root cultures of N.physaloides did not produce hygrine (26) or cuscohygrine (60). These cultures had been reported to contain hygrine (26) by workers at Norwich.

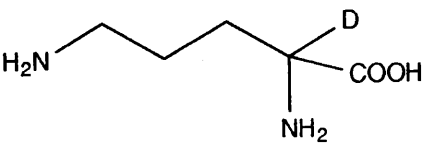
6.3 The incorporation of (R)-[1-^{2}H]-(138) and (S)-[1-^{2}H]putrescine (171) in a D.candida x D.aurea hybrid transformed root culture

Failure to find hygrine (26) or cuscohygrine (60) within transformed root cultures of N.physaloides led to a re-evaluation of this work.

It was decided to use the information supplied by the heteronuclear ^{1}H-^{13}C chemical shift correlation spectroscopy (2D COSY) of (-)-scopolamine (15). This showed that the hydrogen signals of interest (namely H-1 and H-5) were separable. The signals for H-1 and H-5 were δ2.88 and 3.00, respectively.

The work on (R)-[2-^{2}H]-(158) and (S)-[2-^{2}H]putrescine (159) indicated the optimum feeding conditions required for the incorporation of putrescine into (-)-scopolamine (15). (See Chapter 5).

The synthesis of (R)-[1-^{2}H]-(138) and (S)-[1-^{2}H]putrescine (171) dihydrochloride followed the procedure of Richards and Spenser[133]. The (R)-[1-^{2}H]putrescine (138) dihydrochloride was produced by the action of L-ornithine decarboxylase (EC 4.1.1.17) on L-ornithine (22) while incubated in deuterium oxide. This reaction is known to proceed with retention of configuration. The (S)-[1-^{2}H]putrescine (171) dihydrochloride was produced by the action of L-ornithine decarboxylase upon the L-component of DL-[2-^{2}H]ornithine (175) monohydrochloride.



(175)

Each enantiomer of [1-^{2}H]putrescine ((138) and (171)) dihydrochloride showed a deuterium content of greater than 95%, as determined from the ^{1}H n.m.r spectrum of each enantiomer. The ^{2}H n.m.r spectra are shown in Figure 28.

The feeding of (R)-[1-^{2}H]putrescine (138) dihydrochloride to D.candida x D.aurea transformed root cultures was carried out under the conditions used in a work with (R)-[2-^{2}H]-(158) and (S)-[2-^{2}H]putrescine (159) dihydrochloride. Once again the use of [1,4-^{14}C]putrescine (48) dihydrochloride, as an internal marker, was employed.

Isolation of the crude alkaloid extract was carried out by the standard acid-base extraction procedure. The presence of (-)-hyoscyamine (14) and (-)-scopolamine (15) was determined by thin layer chromatography and comparison with reference samples of (-)-hyoscyamine (14) and (-)-scopolamine (15). The alkaloids were visualised with the Dragendorff reagent[135] which showed the extract to contain both (-)-hyoscyamine (14) and (-)-scopolamine (15). Purification of the alkaloid extract was undertaken by preparative thin layer chromatography and (-)-hyoscyamine (14) and (-)-scopolamine (15) were isolated.

The ^{14}C specific incorporation for the (R)-[1-^{2}H]putrescine (158) dihydrochloride feed into (-)-hyoscyamine (14) was found to be 4.2%. For (-)-scopolamine (15) the ^{14}C specific incorporation was 5.6%.

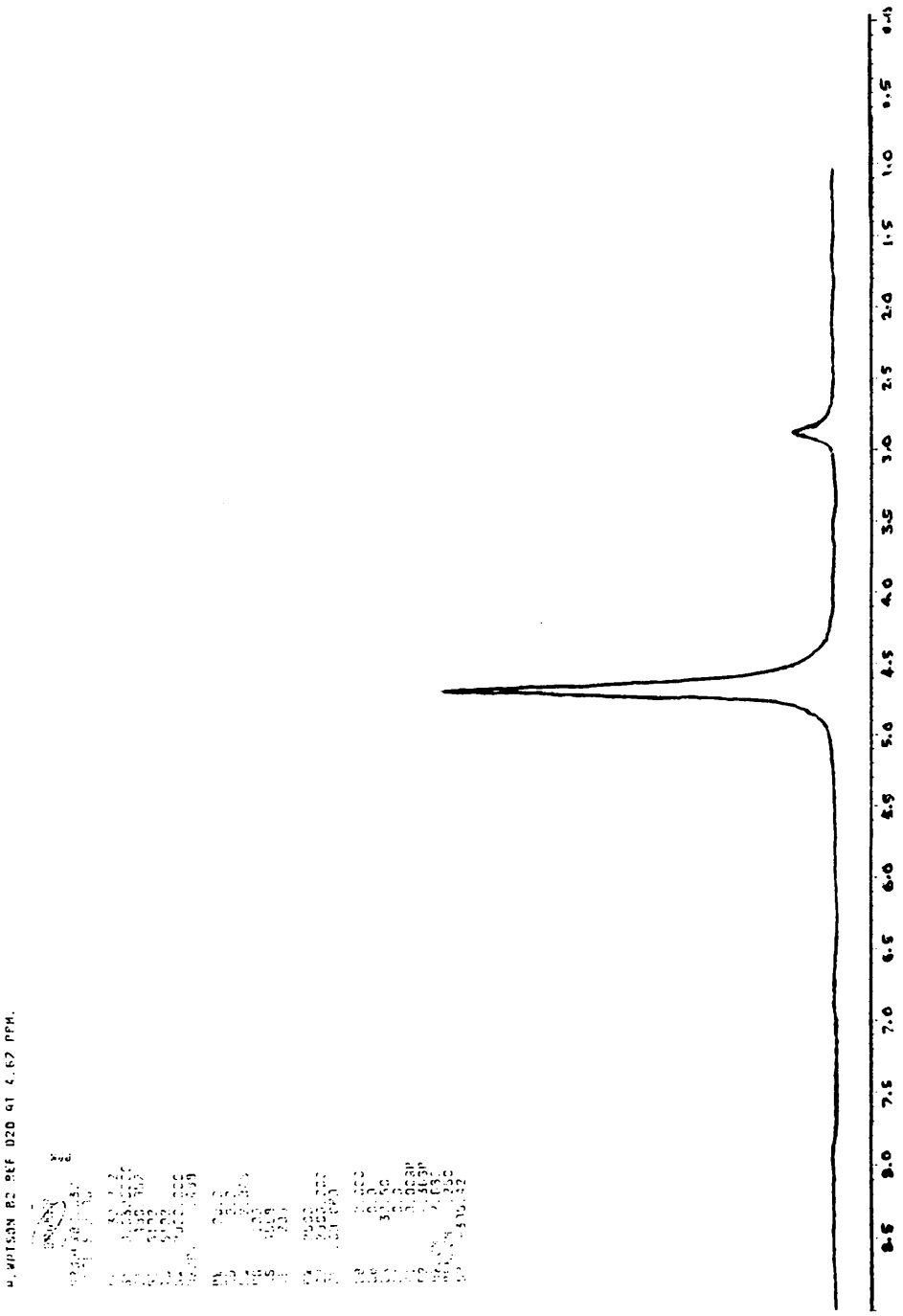


Figure 28

(S)-[1-^{2}H]putrescine

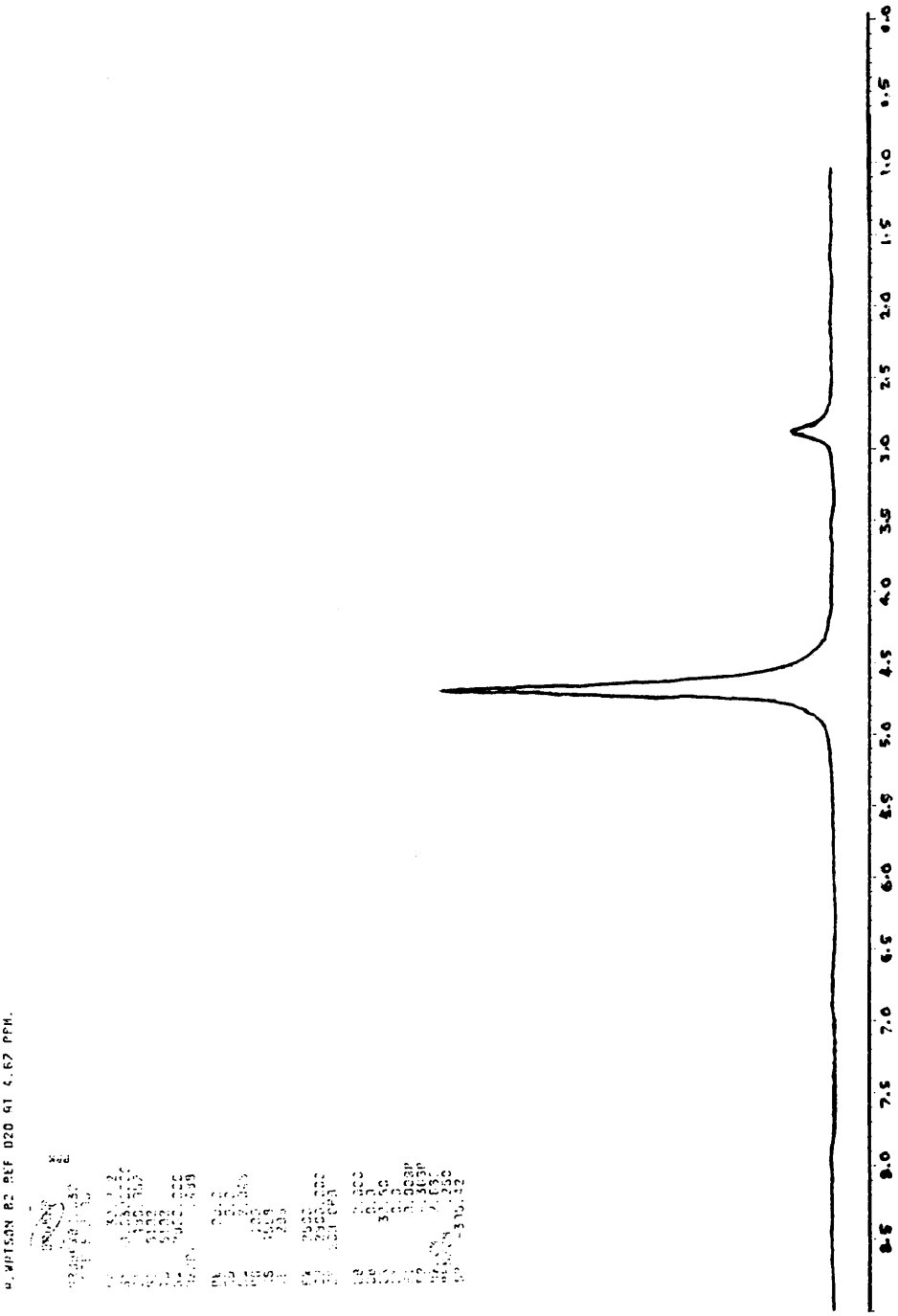


Figure 28

(R)-[1-^{2}H]putrescine

The isolated (-)-scopolamine (15) showed an single broad peak in the ^{2}H n.m.r spectrum at 3.12 ppm. This is in better agreement with the chemical shift of 3.00 ppm assigned to H-5 of (-)-scopolamine (15) in the 2D COSY experiment described in Chapter 5 (**Figure 16**), and suggests that there is no deuterium at H-1 (δ2.88).

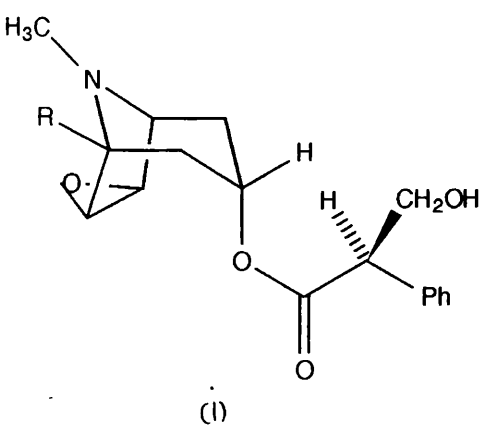
The analogous experiment with (S)-[1-^{2}H]putrescine (171) dihydrochloride was carried out under the same conditions. [1,4-^{14}C]-putrescine (48) dihydrochloride as an internal marker was also employed. The (-)-scopolamine (15) was isolated using the procedure outlined above and radioactivity measurement was carried out. The ^{14}C specific incorporation for the (S)-[1-^{2}H]putrescine (171) dihydrochloride feed into (-)-hyoscyamine (14) was 2.1%. However, the ^{14}C specific incorporation from this (S)-[1-^{2}H]putrescine (171) dihydrochloride feed into (-)-scopolamine (15) was only 0.8%.

The subsequent ^{2}H n.m.r spectrum of the isolated (-)-scopolamine (15) failed to show any distinguishable peaks. Time restrictions did not allow this experiment to be repeated.

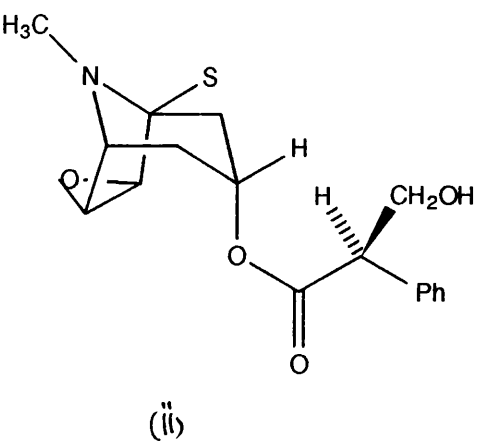
The inconclusive labelling results permit two possible interpretations. Firstly, if ^{2}H is present only at H-5 of (-)-scopolamine (15), it suggests that ^{2}H from the (S)-[1-^{2}H]putrescine feed should have appeared at H-1 of (-)-scopolamine (15) leading to labelling pattern (ii) in **Figure 29 A**. On the other hand this result would indicate that loss of the pro-R

hydrogen at C-1 of putrescine occurs in the formation of (-)scopolamine (15) in Datura hairy root cultures. However, the general concensus is that the pro-S hydrogen at C-1 of putrescine is lost in the oxidation catalysed by diamine oxidase leading to the appearance of ^{2}H from the R-isomer at C-1[25]. If this is true for Datura transformed root cultures it suggests another interpretation of these results is more likely.

The signal observed in the deuterium n.m.r spectrum obtained for (-)-scopolamine (15) after feeding the R-isomer (138) could in fact be due to ^{2}H present at both H-1 and H-5. The broadness of the peak is responsible for the non-resolution of both peaks. This would explain why no signals were observed in the ^{2}H n.m.r. spectrum of (-)-scopolamine (15) obtained after the feeding of (S)-[1-^{2}H]putrescine (171) dihydrochloride to Datura hairy root cultures. This leads to the possible labelling pattern (B) as shown in Figure 29B



(I)



(II)

Figure 29A

In conclusion this work should be continued in the following ways:

(i) transformed root cultures or plants of N.physaloides should be established which produce adequate quantities of hygrine (26);

(ii) samples of (R)-and (S)-[1-^{2}H]putrescines, (138) and (171), should be fed to transformed root cultures or plants of N.physaloides so that the stereochemistry of enzymic processes can be elucidated;

(iii) samples of (R)-and (S)-[1-^{2}H]putrescines, (138) and (171) should be administered to transformed root cultures of Datura species which produce (-)-scopolamine (15). Good deuterium n.m.r. spectra from both feeding experiments are required.

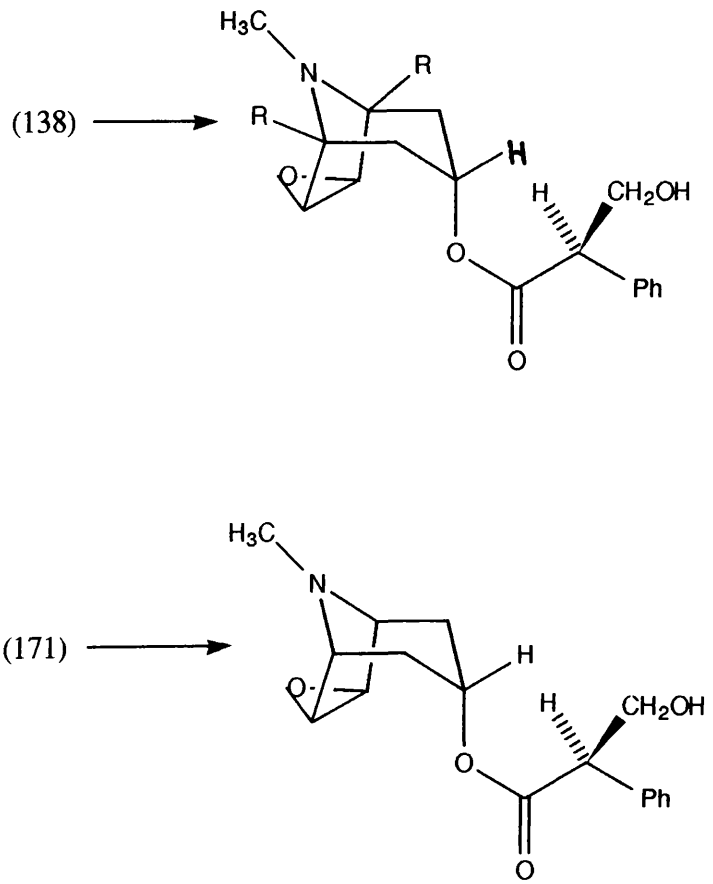


Figure 29B

CHAPTER 7

THE BIOSYNTHESIS OF ANABASINE (17)

7.1 Introduction

Anabasine (17), although known as a tobacco alkaloid, is found in many plant species of different families[137]. It is found in many members of the Nicotiana genus and is the main alkaloid of Nicotiana glauca. Anabasine (17) is so called as it was first isolated from Anabasis aphylla, a member of the Chenopodiaceae plant family.

Anabasine(17) has been isolated from various plant species, and its optical rotation has been measured. Different values have been found depending on the plant species from which anabasine (17) was isolated. In Anabasis aphylla it was found that anabasine (17) had the (S)-configuration[138] with reported optical rotations of -52 degrees to -80 degrees whereas in Nicotiana glauca anabasine (17) is almost racemic.

In the 1950's evidence from pea and lupin extracts[139,140] suggested that cadaverine was the precursor of both the unsaturated and saturated rings of anabasine (17). This evidence has, however, never been obtained in vivo.

Cadaverine has been shown to have an important role in the biosynthesis of anabasine(17). A publication by Walton et al.[141] reported that anabasine (17) production is stimulated in transformed root cultures of Nicotiana rustica when fed with cadaverine.

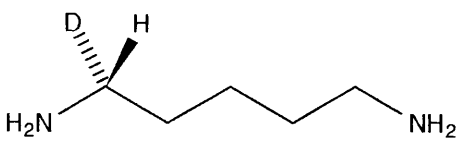
Transformed root cultures of Nicotiana rustica have been shown to produce (-)-nicotine (16) as their major product under normal conditions. The administration of cadaverine results in the production of anabasine (17) at the expense of (-)-nicotine(16).

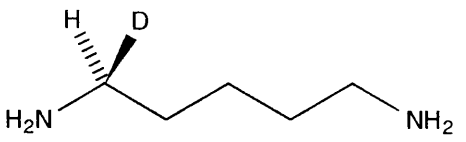
7.2 Investigations into the biosynthesis of Anabasine(17) in Nicotiana hairy root cultures

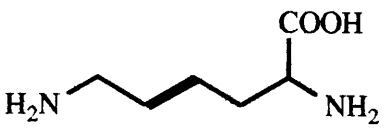
In order to study the biosynthesis of anabasine (17) in Nicotiana hairy root cultures it was decided to use the synthesis of (R)-[1-^{2}H]-(169) and (S)-[1-^{2}H]cadaverine (170) as developed by Richards and Spenser[133]. This has been discussed previously in Chapter Six. The enantiomeric deuterium labelled cadaverines (169) and (170) were synthesised by Dr. A.M. Brown.

The administration of these precursors (169) and (170) was carried out by myself using the facilities of the AFRC institute of Food Resarch in Norwich.

In order to identify sites of deuterium incorporation in anabasine (17) in our biosynthetic studies it was necessary to obtain a complete assignment of the ^{1}H n.m.r. spectrum of anabasine (17). A publication by Leete[142] described the synthesis and administration of DL-[4,5-$^{13}C_2$]lysine (177) to Nicotiana glauca plants. Subsequent isolation of anabasine (17) allowed the complete assignment of the ^{13}C n.m.r. spectrum to be made.

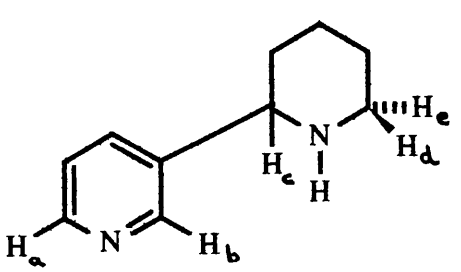






(177) —— = ^{13}C-^{13}C .

It was decided to use Leete's[142] rigorous assignment of the ^{13}C n.m.r. spectrum in our heteronuclear ^{1}H-^{13}C chemical shift correlation spectroscopic study. This allowed a complete assignment of the ^{1}H n.m.r. spectrum of anabasine (17) to be made. This spectroscopic study is summarised in Figure 30. The peaks in the ^{1}H n.m.r. spectrum of anabasine of special interest are those at δ3.62, 2.76, 3.15, 8.43 and 8.55. These signals correspond to H-2' H-6' (axial), H-6' (equatorial), H-6 and H-2 respectively.



H_a = H-6 ; H_b = H-2 ; H_c = H-2' ;
H_d = H-6' (ax.) ; H_e = H-6' (eq.) .

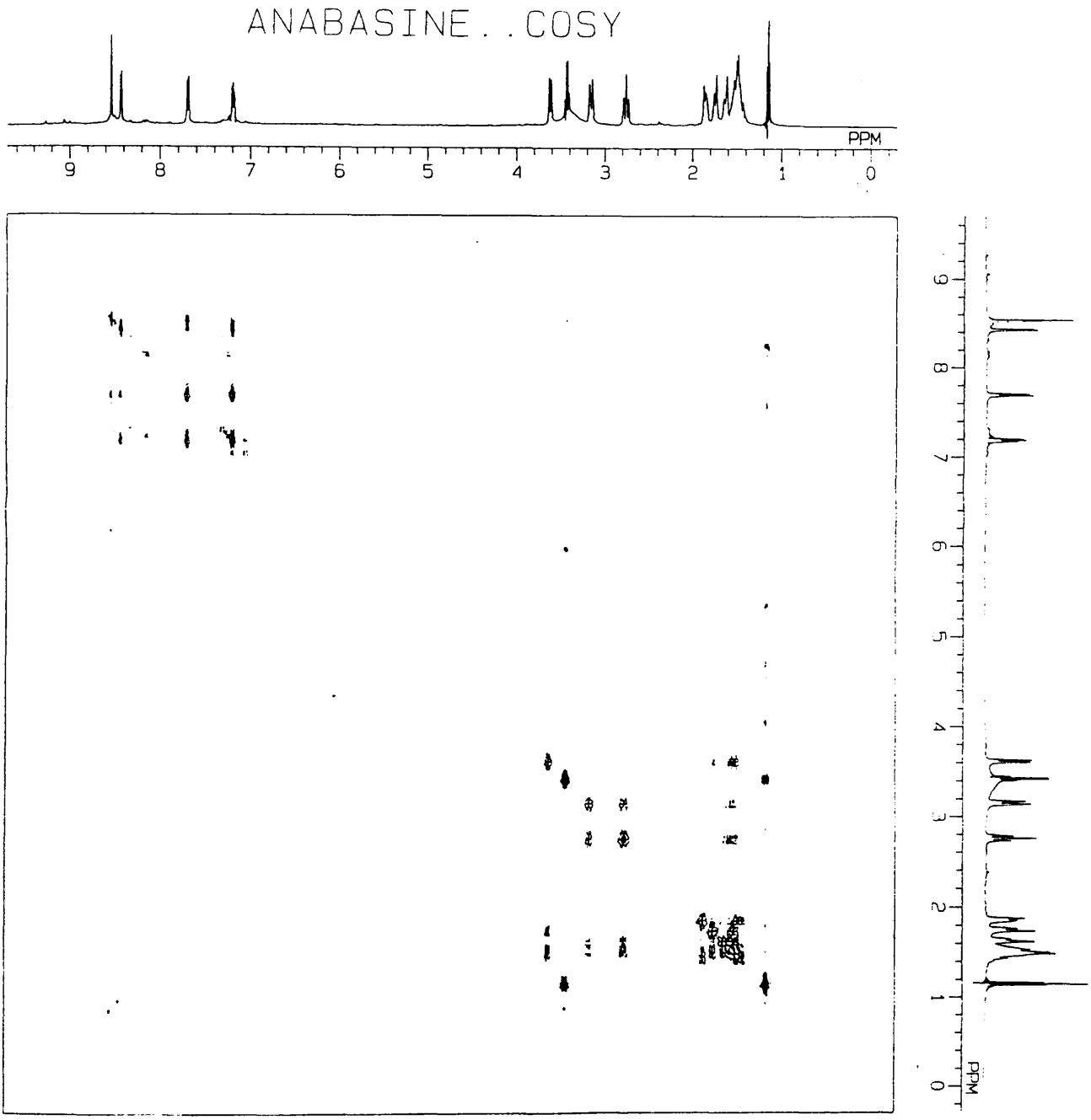


Figure 30

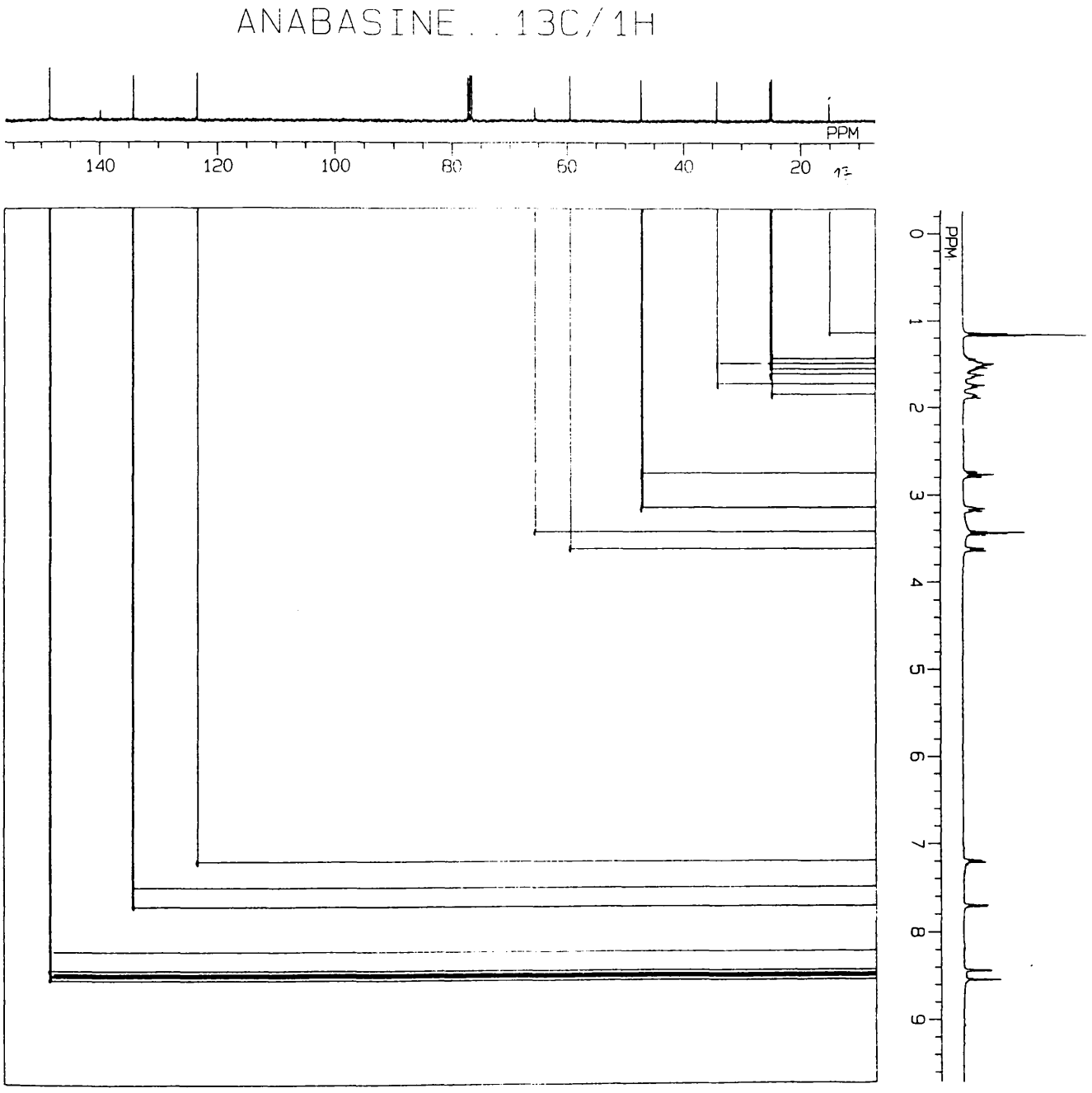


Figure 30

The assignment of these proton signals was confirmed by the use of homonuclear (^{1}H) chemical shift correlation spectroscopy and N.O.E. spectroscopy. The administration of the enantiomerically labelled precursors (169) and (170) was carried out with two strains of hairy root cultures. These precursors were fed at 0.5 mM to Nicotiana tabacum and Nicotiana rustica hairy root cultures six days after sub-culturing. Feeding was continued for a further twelve days before harvesting of the roots.

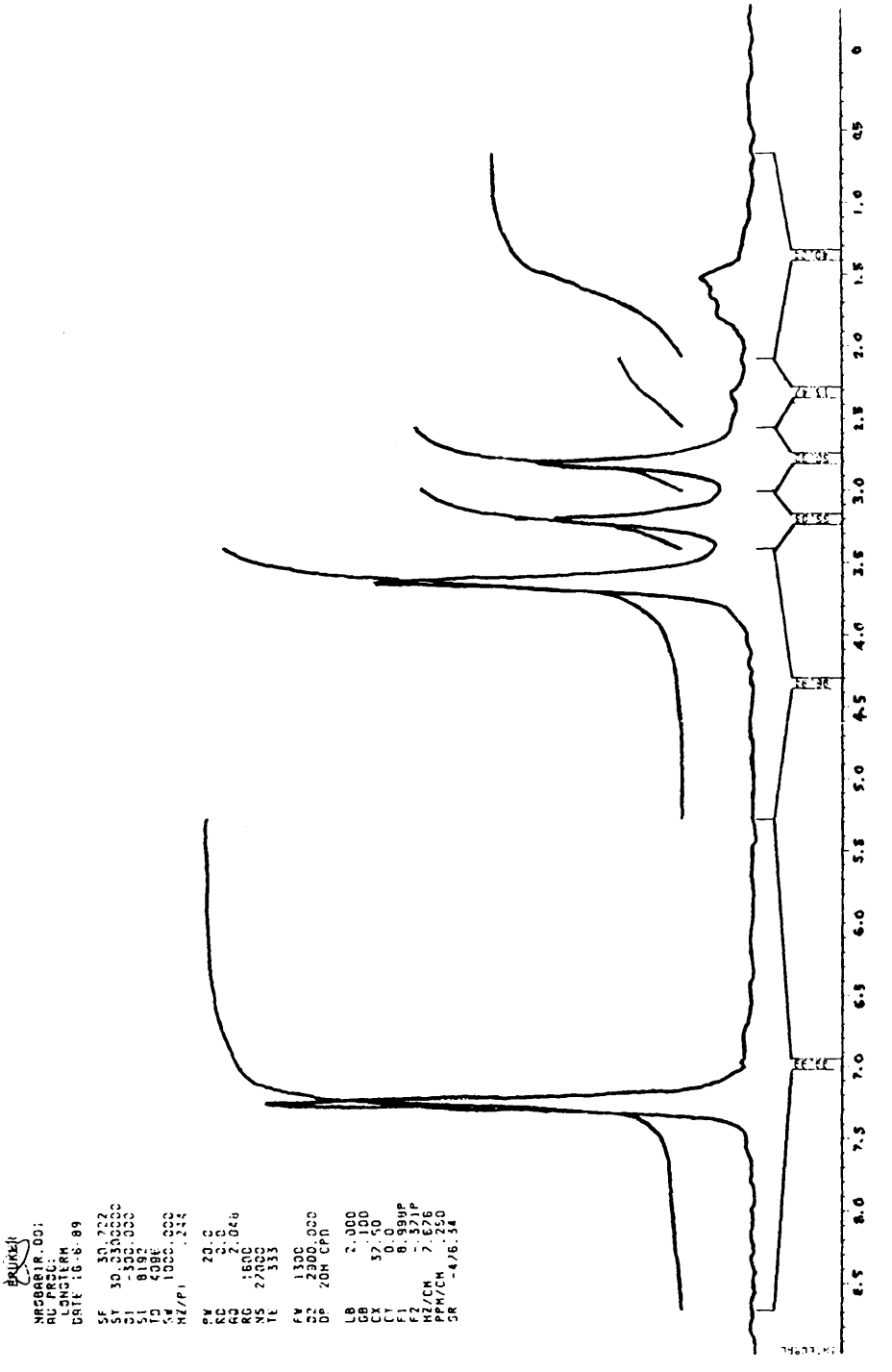
Each strain of hairy root culture yielded a mixture of alkaloids which was subjected to g.c. and h.p.l.c. analysis. The ratio of alkaloids was measured and found to be similar for each experiment. The alkaloids were identified by the comparison of these traces with traces from reference samples of (-)-nicotine (16), anabasine (17), nornicotine (18) and anatabine (19). Typical percentages of (-)-nicotine (16), anabasine (17) and anatabine (19) were 64%, 23% and 6% respectively. The quantity of nornicotine (18) found in each case was not deemed significant. In both experiments no radio-labelled precursor was adminstered as the quantity of anabasine (17) found in intact hairy root cultures is minor. This means that almost 100% of anabasine (17) isolated after a feeding experiment should be labelled.

The crude alkaloid mixtures were submitted for ^{2}H n.m.r. spectroscopy. The spectrum obtained from the administration of (R)-[1-^{2}H]cadaverine (169) to Nicotiana rustica hairy root cultures is shown in Figure 31. Analysis of this spectrum shows three broad singlets at δ3.63, 3.21 and 2.61 in the ratio of 2:1:1 respectively. These peaks correspond to deuterium labelling of C-2' C-6' (equatorial) and C-6' (axial) of anabasine (17), respectively.

In contrast the ^{2}H n.m.r. spectrum obtained from the adminstration of (S)-[1-^{2}H]cadaverine (170) to Nicotiana rustica hairy root cultures is shown in Figure 32. Analysis of this spectrum showed two broad singlets in a 1:1 ratio at δ3.21 and 2.61. These peaks correspond to equal enrichment of the axial and equatorial positions of C-6' of anabasine (17). Both spectra obtained from the feeding of (R)-[1-^{2}H]- (169) and (S)-[1-^{2}H]cadaverine (170) to Nicotiana rustica hairy root cultures gave deuterium n.m.r. signals in good accordance with the proton assignments obtained from the heteronuclear ^{1}H-^{13}C chemical shift correlation spectroscopy experiment. The ^{2}H n.m.r. spectra obtained from the experiment using Nicotiana rustica hairy root cultures shows no peaks in the aromatic region and thus indicates that there is no incorporation of cadaverine into the pyridine ring of anabasine (17). Therefore, cadaverine does not furnish the carbon skeleton of the pyridine ring of anabasine (17) in Nicotiana rustica hairy root cultures. The signals observed in the deuterium n.m.r. spectra appear to be due entirely to anabasine (17), with little or no contribution from the other alkaloids of the crude extract. Thus, cadaverine is not incorporated into any other alkaloid apart from anabasine (17).

The deuterium n.m.r. spectra obtained from the administration of (R)-[1-^{2}H]- (169) and (S)-[1-^{2}H]cadaverine (170) to Nicotiana tabacum hairy root cultures were almost identical to those obtained from the experiment with Nicotiana rustica hairy root cultures.

For both experiments the distribution of ^{2}H signals in the spectrum of anabasine (17) can be explained in the following way.


BRUKER
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SW 1000.000
HZ/PT .244
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RD 0.0
AQ 2.048
RG 1600
NS 22000
TE 333
FW 1300
SD 2900.000
DP 20M CPD
LB 2.000
GB .100
CX 37.50
CY 0.0
F1 8.990P
F2 7.371P
PPM/CM 7.676
HZ/CM 1.250
SR -476.34
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8.0
7.5
7.0
6.5
6.0
5.5
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2.5
2.0
1.5
1.0
0.5
0

Figure 31

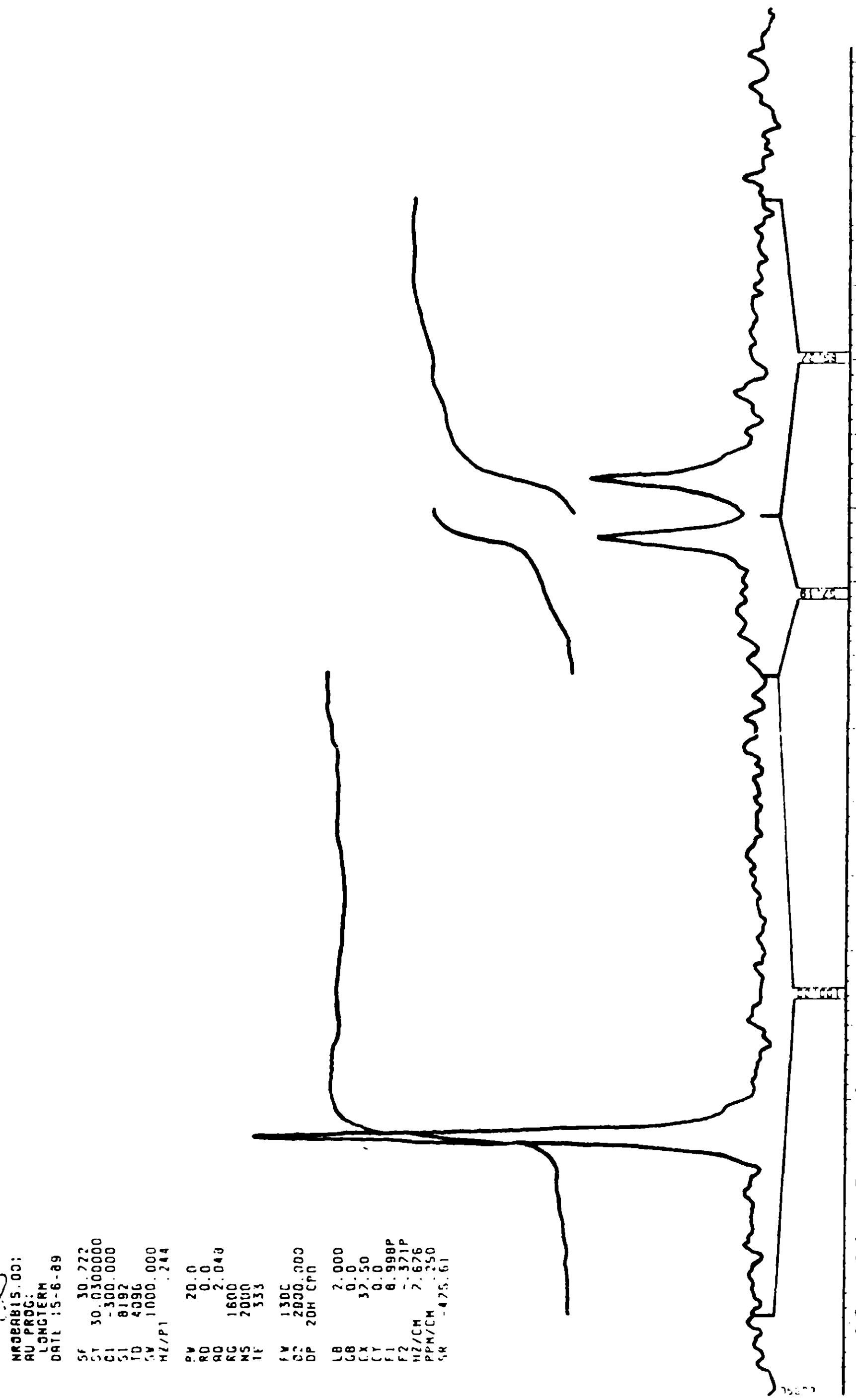

Figure 32

The adminstration of (R)-[1-^{2}H]cadaverine (169) and subsequent analysis of the ^{2}H n.m.r. spectrum shows that equal amounts of the two enantiomers and two diastereomers of labelled anabasine (17) are produced by the hairy root cultures (Figure 33) The observed ^{2}H n.m.r. spectrum is due to a combination of the signals from the four labelled species.

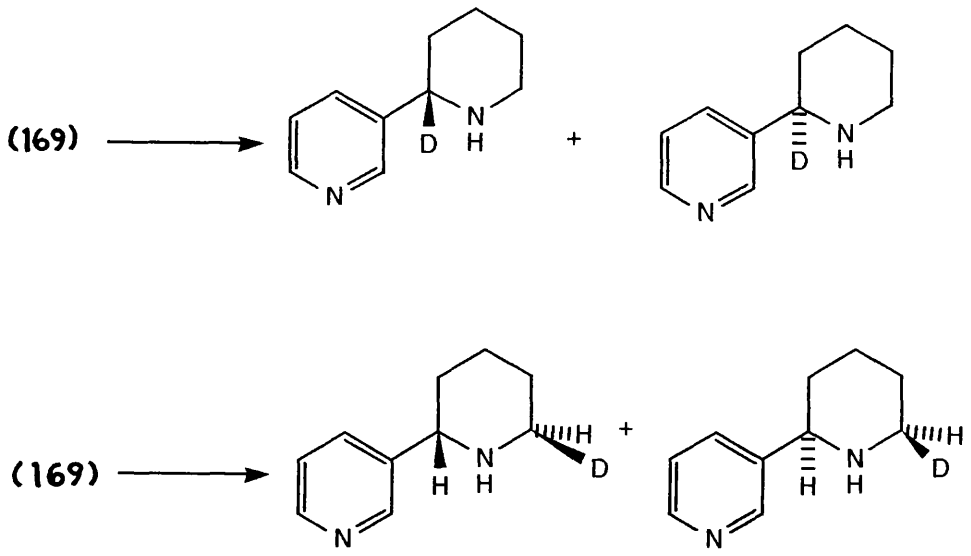

Figure 33 - Anabasine (17) derived from the feeding of (R)-[1-^{2}H]cadaverine.

In the more stable chair conformation, the pyridine ring adopts the equatorial position. Therefore, the administration of (R)-[1-^{2}H]cadaverine (169) results in the deuterium which is incorporated at the 2'-position of anabasine (17) adopting the axial position. The deuterium incorporated at the 6'- position of anabasine (17) is equally distributed at the axial and equatorial positions. This is a consequence of the formation of racemic anabasine (17). The stable chair conformations can be seen in Figure 34.

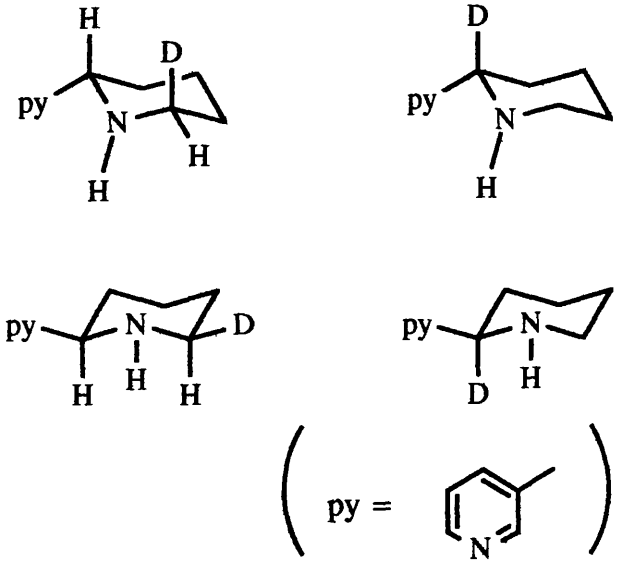

Figure 34

An analogous argument can be used to explain the ^{2}H n.m.r. spectrum obtained after the administration of (S)-[1-^{2}H]cadaverine(170). The equal intensity of ^{2}H signals in the ^{2}H n.m.r. spectrum of anabasine (17) indicates equal labelling of the axial and equatorial sites of C-6'. The stable chair conformations can be seen in Figure 35.

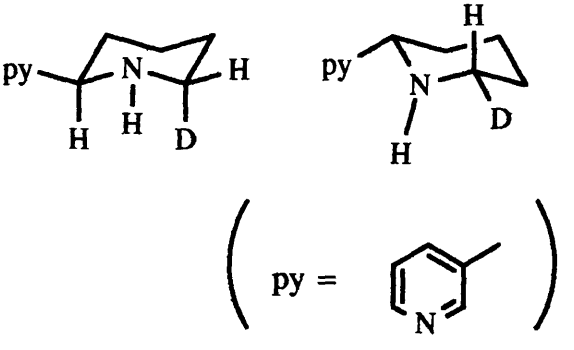

Figure 35

DS90 Chromatogram report Run: ZGT3960001, 4-Oct-89 09:28
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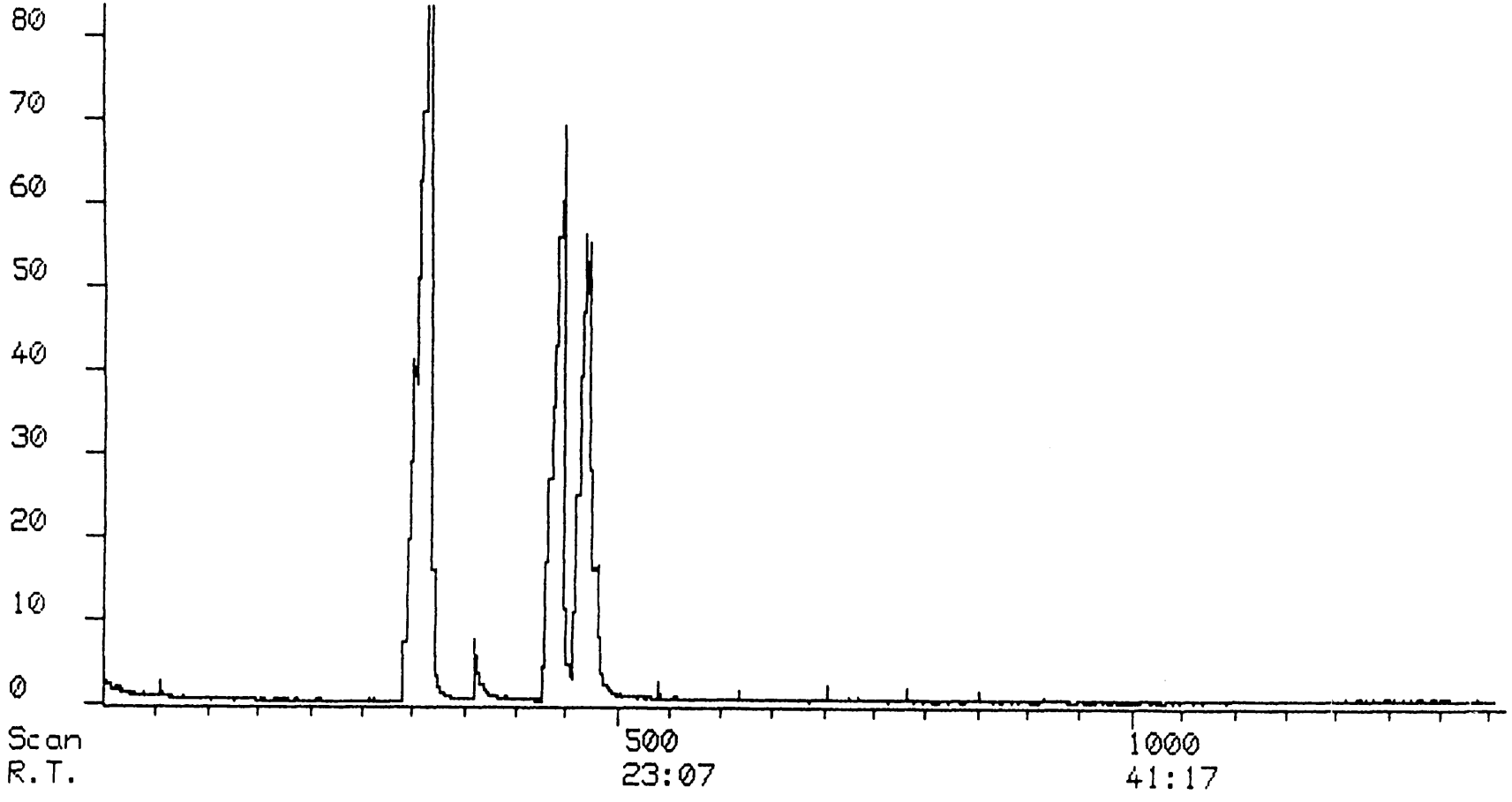

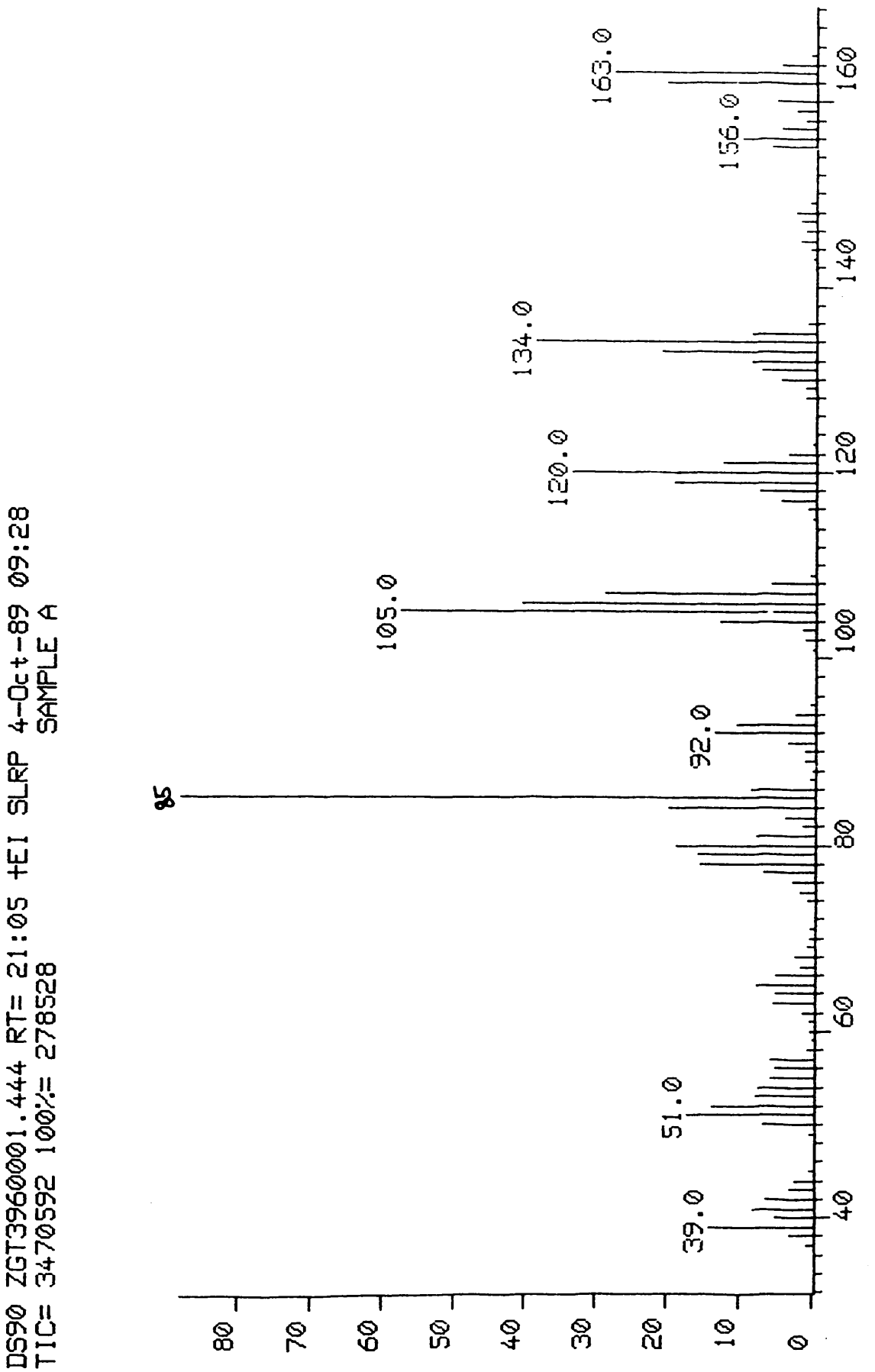
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TIC= 3470592 100%= 278528
SAMPLE A
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92.0
120.0
134.0
163.0
156.0
51.0
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10
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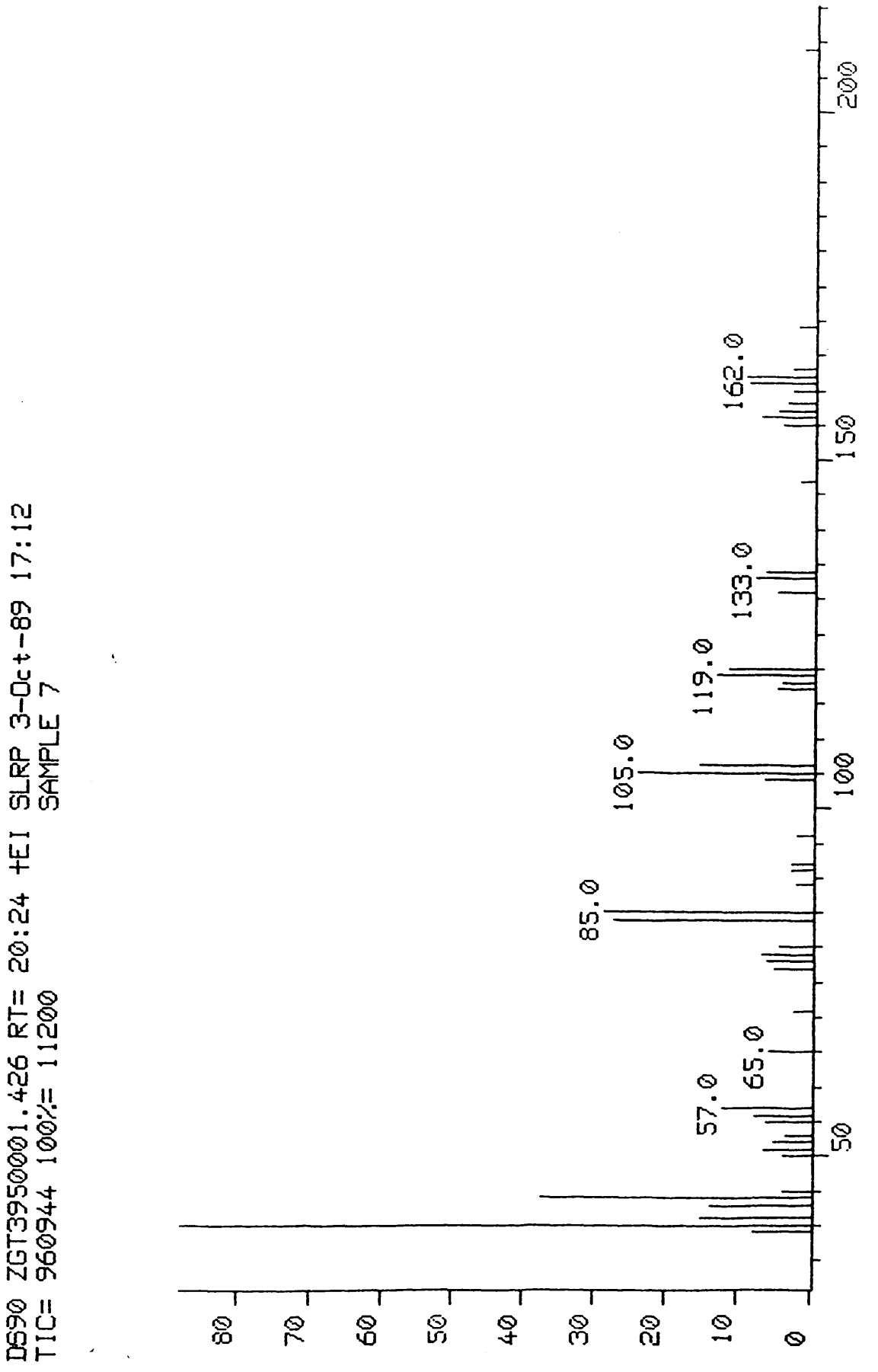
30
40
50
60
70
80
40
60
80
100
120
140
160

Figure 36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105.0
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162.0
57.0
65.0
0
10
20
30
40
50
60
70
80
50
100
150
200

Figure 37

In order to confirm the presence of deuterium in our isolated alkaloid extracts we used the powerful technique of gas chromatography-mass spectroscopy (g.c-m.s). Peaks corresponding to anabasine (17) were identified by the use of a reference g.c-m.s trace of anabasine (17). Anabasine (17) derived from the administration of (S)-[1-^{2}H]cadaverine (170) showed two peaks of almost equal intensity at m/z 163 and 162. The ratio of these peaks is consistent with the presence of $^{2}H_{1}$ species in approximately 50% of anabasine (17) molecules. In contrast, anabasine (17) isolated after the administration of (R)-[1-^{2}H]cadaverine (169) had a major peak at m/z 163 which suggests that a high percentage of anabasine (17) molecules contain one deuterium atom. The mass spectra of these labelled samples can be seen in Figures 36 and 37. Further analysis of these mass spectra shows a major fragment at m/z 85 for anabasine (17) derived from (R)-[1-^{2}H]cadaverine (169). Possible structures for this fragment are drawn in Figure 38.

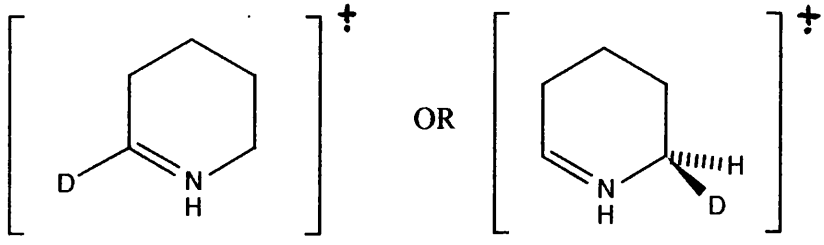


Figure 38

The very high incorporation of the deuterium labelled precursors (169) and (170) allowed a further investigation to be undertaken. An attempted purification of both crude alkaloid extracts was undertaken. Each alkaloid extract isolated from the independent feeding of (R)-[1-^{2}H]- (169) and (S)-[1-^{2}H]cadaverine (170) to Nicotiana rustica hairy root cultures was subjected to preparative t.l.c. Each extract yielded 3-5 mg of "anabasine". The very high incorporation of deuterium labelled precursors should lead to the absence of some ^{1}H signals and collapse of ^{1}H-^{1}H coupling in the ^{1}H n.m.r. spectra of these samples, with respect to unlabelled material. In particular, anabasine (17) isolated after the administration of (R)-[1-^{2}H]cadaverine (169) should show changes in the signals due to H_2-3' and H_2-5'. By contrast anabasine (17) isolated after the administration of (S)-[1-^{2}H]cadaverine (170) should yield changes in the signals corresponding to H-6' and H_2-5'.

The corresponding ^{1}H n.m.r. spectra were obtained but showed significant amounts of impurities which were not removed by subsequent attempts at purification by preparative t.l.c. An explanation of these impurities is that amounts of anatabine (19) and nornicotine (18) were also isolated. This is highly probable as the three bands, as identified by the Dragendorff reagent[135], are very close together on preparative t.l.c. plates. It was therefore impossible to observe changes in the coupling of either H_2-3', H_2-5' or H-6', H_2-5' respectively because of the presence of these impurities.

In contrast the mass spectrum of anabasine (17) derived from (S)-[1-^{2}H]cadaverine (170) shows two peaks of approximately equal intensity at m/z 84 and 85. Possible structures to account for this observation are indicated in Figure 39.

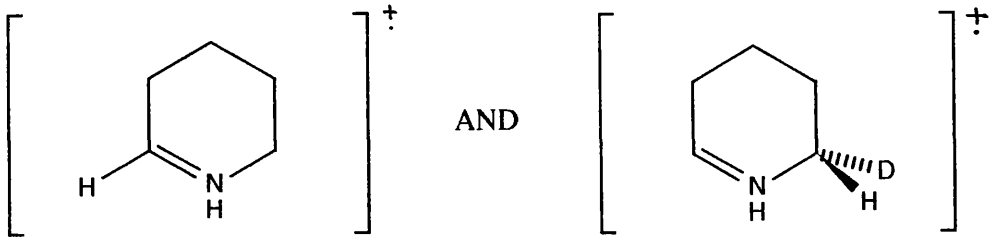


Figure 39

Further analysis of these peaks by accurate mass techniques was not available although our proposed structures were based on information supplied by accurate mass data of unlabelled anabasine (17).

7.3 Conclusions

Several conclusions can be reached from the above results on the biosynthesis of anabasine (17) in Nicotiana species:-

i) Cadaverine is not a precursor of the pyridine ring of anabasine (17). This has been shown by the non-incorporation of (R)-[1-^{2}H]- (169) and (S)-[1-^{2}H]cadaverine (170) into the pyridine ring of anabasine (17). No deuterium signals were observed in the aromatic region of both spectra. This finding is in direct contrast to that reported earlier in this Chapter[139,140].

ii) The conversion of cadaverine into 1-aminopentanal(178) [piperideinium salt] proceeds with the loss of the pro-S proton from C-1 and the retention of the pro-R proton. This substantiates the findings of Spenser and Richards [133] who observed the same stereochemical course of reaction on oxidation of cadaverine with kidney diamine oxidase.

iii) The nicotinic acid (14) attacks the Δ'-piperideinium moiety at C-2 from either the re or si face to produce racemic anabasine (17). If this process was stereospecific only one signal for H-6' would be seen in each ^{2}H n.m.r. spectrum. The stereospecific attack of nicotinic acid (17) would result in deuterium adopting either an axial or an equatorial site.

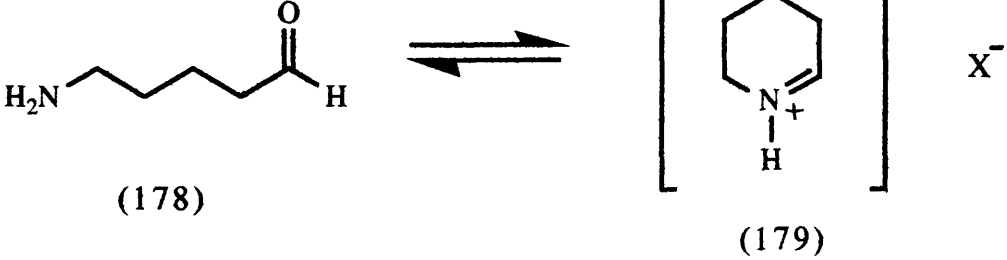
The above observations allow the following proposals to be made on the biosynthesis of anabasine (17) in Nicotiana species. The condensation of nicotinic acid (24) and Δ^1-piperideinium(179) occurs due to:-

i) a non-enzymic process;

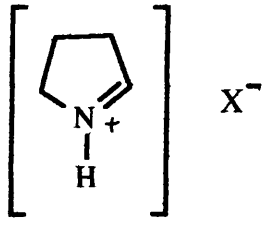
ii) a non-stereospecific enzymic process; or

iii) the process being a mixture of (i) and (ii).

The ^2H n.m.r. spectrum obtained after the administration of (R)-[1-^{2}H]cadaverine (169) indicates that racemisation occurs during the biosynthesis and not during the isolation of anabasine (17). It would seem highly probable that the final stages of anabasine (17) and nicotine(16) biosynthesis differ substantially; Wigle et al.[110] have shown from feeding experiments with (R)-[1-^{2}H]- (138) and (S)-[1-^{2}H] putrescine (171) that only (S)-nicotine is formed in Nicotiana tabacum. It has also been reported by Endo and co-workers[143] that the formation of hygrine (26) from acetoacetic acid (29) and Δ^1-pyrroline (180) can be non-enzymic.



(178) (179)



(180)

These investigations can and should be extended by the purification of labelled anabasine (17) from crude alkaloid extracts and subsequent analysis of the ^{1}H n.m.r. spectra. However, a new purification method, rather than preparative t.l.c., will have to be used. A further improvement would be the production of anabasine (17) on a larger scale after feeding Nicotiana species with (R)-[1-^{2}H]- (169) and (S)-[1-^{2}H]cadaverine (170).

Finally the feeding of the enantiomeric deuterium labelled samples to other species that produce anabasine (17), in tandem with ^{2}H n.m.r. spectroscopy would be revealing in determining the optical purity of the anabasine (17). This would allow proposals on the condensation of nicotinic acid(24) with Δ^{1}-piperideinium (179) to be made.

This work on anabasine (17) biosynthesis has been published:-

A.B.Watson, A.M.Brown, I.J.Colquhoun, N.J.Walton and D.J.Robins, J.Chem. Soc., Perkin Trans.1, 1990, 2607.

CHAPTER EIGHT

INVESTIGATIONS INTO ALKALOID ANALOGUE BIOSYNTHESIS IN *NICOTIANA* AND *DATURA* TRANSFORMED ROOT CULTURES

8.1 Introduction

There are a variety of reasons for making analogues of precursors of natural products. Such reasons include:

(i) the elucidation of enzyme mechanisms;

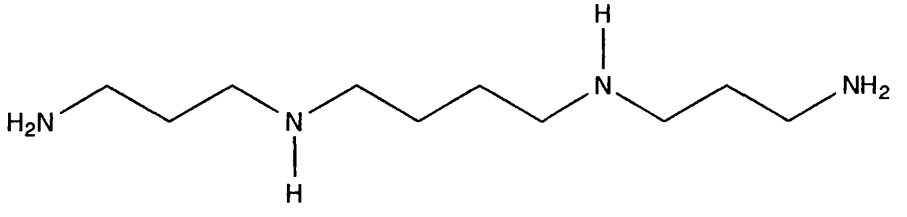
(ii) the design of specific enzyme inhibitors; and

(iii) the biosynthesis of unnatural natural products.

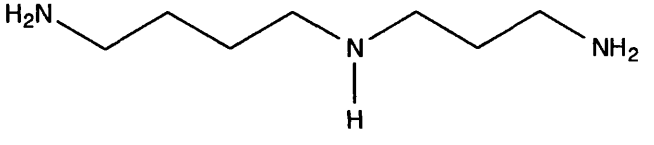
Publications have appeared steadily over the last twenty years on topics within the groups outlined above. There are many possible opportunities within the tropane and tobacco alkaloid biosynthetic pathways to study the effects of precursor analogues. In this work it was decided to study the effects of various putrescine analogues on the production of alkaloids in transformed root cultures.

Putrescine is one of a number of ubiquitous polyamines which are essential for both normal and pathological cell growth[124]. It is also an essential growth factor for many micro-organisms and an intermediate in the biosynthesis of spermine (181) and spermidine (182). Any reduction of putrescine availability will have serious consequences on cell growth and function. The biosynthesis of putrescine in many cells is from either ornithine (22) or agamatine

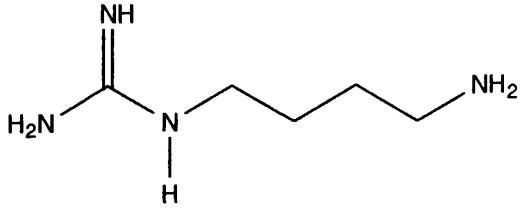
(183), but in certain lower micro-organisms, notably fungi, the source of putrescine is only from ornithine (22). The inhibition of the decarboxylation of ornithine (22) (catalysed by ornithine decarboxylase) would stop cell growth within fungi. There are several compounds which are known to inhibit this enzyme, for example, α-difluoromethylornithine (184), its methyl ester (185), and putrescine itself. Putrescine acts as a regulator of ornithine decarboxylase with a higher concentration of putrescine resulting in a lower enzyme reaction rate.



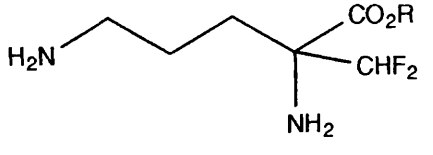
(181)



(182)



(183)



(184) R = H .

(185) R = CH_3 .

No definitive mechanism is known which explains the action of (184) and (185) but their proposed mode of action is the interaction of the substrate with a pyridoxal phosphate cofactor bound to the enzyme. The decarboxylation of these compounds, (184) and (185), may leave highly polarized C-F bounds which interact with a nucleophile at the enzyme active site. Thus, the enzyme could become bound to the substrate irreversibly.

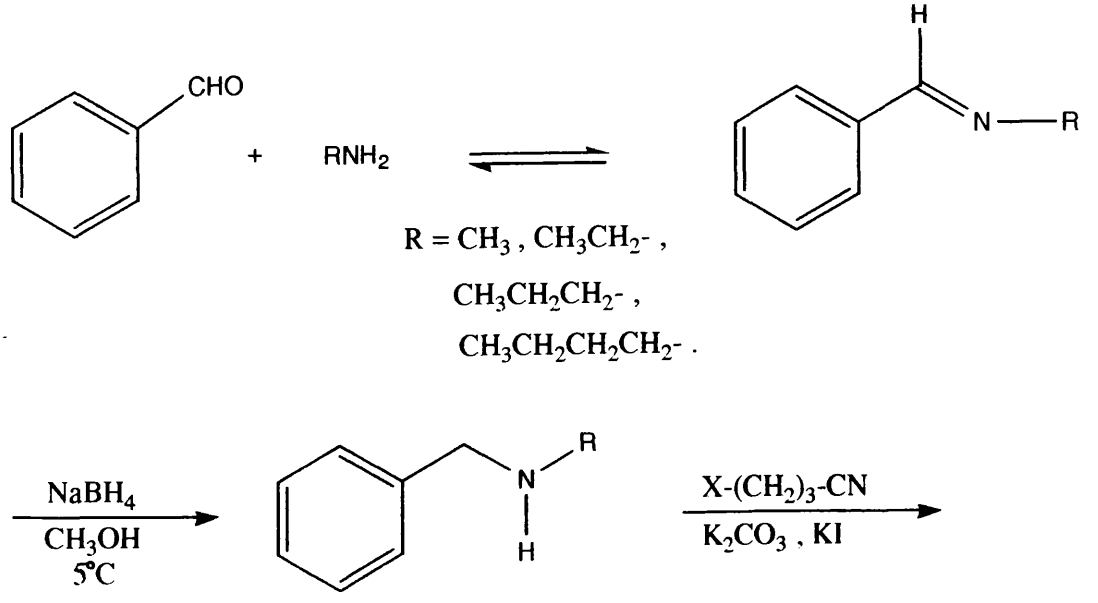
It was our intention to produce analogues of putrescine which might interfere with the action of ornithine decarboxylase and inhibit the decarboxylation of ornithine (22). Alternatively these putrescine analogues might have an effect on another enzyme in the biosynthetic pathway i.e diamine oxidase. The specificity of the enzyme systems involved in tropane and tobacco alkaloids was also to be studied. Analogues of known alkaloids might be produced by plants or root cultures.

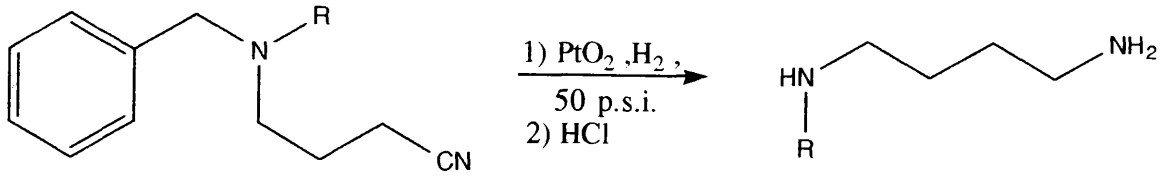
8.2 The synthesis of N-alkylputrescines and subsequent feeding experiments

The proposal that N-methylputrescine (23) is a precursor of (-)-hyoscyamine (14) and (-)-scopolamine (15) was first made by Liebisch and co-workers[31] and later substantiated by Leete and McDonell[32]. It was decided to synthesise analogues of this natural precursor and feed them to Datura transformed root cultures. The publication of Leete and McDonell[32] also showed that N-methylputrescine (23) is a natural

precursor of (-)-nicotine (16). Higher homologues of N-methylputrescine (23) were therefore also fed to Nicotiana transformed root cultures.

Frydman and co-workers[144] published a general synthesis of N-alkylputrescines (alkyl = methyl (23), ethyl (186), propyl (187) and butyl (188)). This general synthesis was used to produce the above mentioned compounds with varying degrees of success. For each compound the intial step was imine formation which involved the condensation of benzaldehyde (189) with the required primary amine. Subsequent reduction of the imine with sodium borohydride afforded the corresponding N-alkylbenzylamines. The alkylation of the N-alkylbenzylamines was carried out with either 4-bromobutyronitrile (190) or 4-chlorobutyronitrile (191) in the presence of potassium carbonate and a catalytic amount of potassium iodide. No difference was noted in purity and yield obtained when either alkylating agent was used. The final step involved a high pressure hydrogenation reaction, in the presence of 10% palladium on charcoal, which should reduce the nitrile group to a primary amino group and remove the benzyl protecting group. The above steps are summarised in Figure 40.





(192) R = CH_3 -
(193) R = CH_3CH_2 -
(194) R = $CH_3CH_2CH_2$ -

(186) R = CH_3CH_2-
(187) R = $CH_3CH_2CH_2$-
(188) R = $CH_3CH_2CH_2CH_2$-

Figure 40

However, great difficulty was found with the final hydrogenation step of Figure 40. Repeated hydrogenations on samples of N-methyl-N-(3-cyanopropyl)benzylamine (192) yielded a white crystalline product which, on re-crystallisation, gave accurate analytical data. However, the yield of this hydrogenation was only 12%.

Changes to the published procedure, for example, the purification of N-methyl-N-(3-cyanopropyl)benzylamine (192) gave no improvement on the yield of N-methylputrescine (23) dihydrochloride isolated.

The subsequent hydrogenations of N-ethyl-N-(3-cyanopropyl)benzylamine (193) and N-propyl-N-(3-cyanopropyl)benzylamine (194) yielded, in both cases, a thick green oil which did not crystallise. No crystalline material was found after prolonged standing. Analysis of each of the thick green oils by ^{1}H n.m.r. spectroscopy did not show either starting material or product to be present.

The above general procedure (Figure 40) was once again followed except that the final hydrogenation step was carried out at atmospheric pressure. The molar ratio of all reactants was retained. On isolation a whitish crystalline material was recovered from both the hydrogenation of N-ethyl-N-(3-cyanopropyl)benzylamine (193) and N-propyl-N-(3-cyanopropyl)benzylamine (194). In both cases the yield was 4-6%. Subsequent recrystallisation gave white crystalline material which exhibited the expected analytical data. However, the yield (4-6%) of each hydrogenation made this synthesis (Figure 40) unrealistic for large scale feeding experiments. The procedure, incorporating atmospheric hydrogenation, was repeated in order to give enough material for limited feeding experiments.

It was decided to feed N-ethylputrescine (186) dihydrochloride first at a concentration of 2 mM to flasks of N.rustica transformed root cultures. Administration of N-ethylputrescine (186) dihydrochloride was commenced fourteen days after sub-culture and the roots were harvested after a further seven days. These conditions were provided by the research team based at the Food Research Institute in Norwich

using their experimental evidence. The isolation of the crude alkaloid extract was obtained by a standard acid-base extraction procedure. In addition a blank control flask was also extracted by an acid-base extraction procedure. T.l.c. was used to analyse the crude alkaloid extracts obtained from the control flask extraction and the crude alkaloid mixture obtained after the adminstration of N-ethylputrescine (186) dihydrochloride. A reference sample of (-)-nicotine (16) was also applied. The alkaloids were visualised by the Dragendorff reagent[135]. The crude alkaloid extract obtained after the feeding of N-ethylputrescine (186) dihydrochloride showed the normal distribution of alkaloids. This was shown by comparison with the alkaloid distribution obtained from the control flask of N.rustica hairy root cultures. However, in addition a new Dragendorff positive product was visualised. This suggested the formation of a new alkaloid analogue.

The analysis of the crude alkaloid extract (obtained after feeding N-ethylputrescine (23) dihydrochloride) was continued by h.p.l.c. and g.c. techniques.

The h.p.l.c. trace obtained showed a new peak that was distinguishable from the normal alkaloids isolated from N.rustica transformed root cultures. Comparison of the traces obtained from the blank and N-ethylputrescine (186) dihydrochloride fed transformed root cultures showed no distinguishable decrease in the amount of (-)-nicotine (16) produced. Assuming that the new alkaloid had the identical response on h.p.l.c. as (-)-nicotine (16) it was estimated that the weight of the new alkaloid analogue was 110 μg (from the administration of 51 mg of N-ethylputrescine (186) dihydrochloride). Therefore, purification of the crude alkaloid extract was not attempted. The

g.c. trace showed a new peak with a retention time of 18.4 minutes (retention time of (-)-nicotine (16) is 15.4 minutes). The new peak was not found in the g.c. trace of the blank alkaloid extract. The h.p.l.c. traces obtained from both the blank alkaloid extract and N-ethylputrescine (186) dihydrochloride feeding experiment can be seen in Figures 41 and 42.

Analysis was continued by employing h.p.l.c.-m.s. and g.c.-m.s. techniques. The h.p.l.c.-m.s. trace obtained from the administration of N-ethylputrecine (186) dihydrochloride showed a peak with a retention time of 27.0 minutes which was not seen in the trace obtained from the blank alkaloid extract. The new peak had a m/z with a value of 177 amu. This is proposed to be the N-ethyl analogue of (-)-nicotine (16) i.e. N-ethylnornicotine (195). The m/z for this alkaloid analogue in the g.c.-m.s. spectra was also found to be 177 amu. The major fragment in the m.s. of (-)-nicotine (16) corresponds to a m/z of 84 amu which was suggested to be the N-methylpyrrolinium ion (196). The major fragment in the m.s. of the alkaloid analogue was found to have a m/z of 96 amu. This was proposed to be the N-ethylpyrrolinium ion (197). The m.s. spectra obtained in both cases are shown in Figures 43 and 44. Further credance was given to these proposals by accurate mass analysis of these peaks. No parent ion was observed for (-)-nicotine (16) but the m/z of 84 amu had an accurate mass of 84.0826 amu. The calculated value for the accurate mass of the N-methylpyrrolinium ion (196) is 84.0813 amu. This strongly suggested that the major fragment was indeed the N-methylpyrrolinium ion (196). The accurate mass of the alkaloid analogue showed a parent ion with a m/z of 176.1310 amu. This corresponds to a calculated value for N-ethylnornicotine (195) of 176.1314 amu and strongly suggests that the alkaloid analogue is N-ethylnornicotine (195). The accurate mass also showed a major

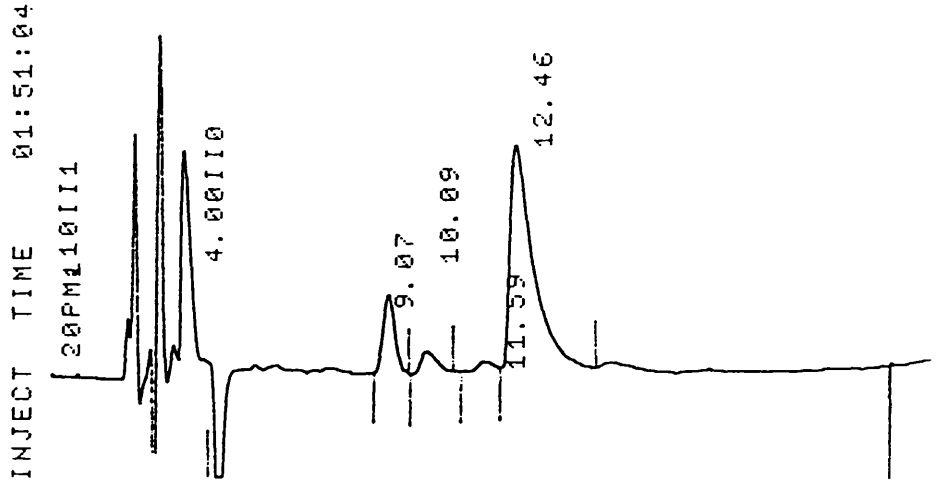


Figure 41

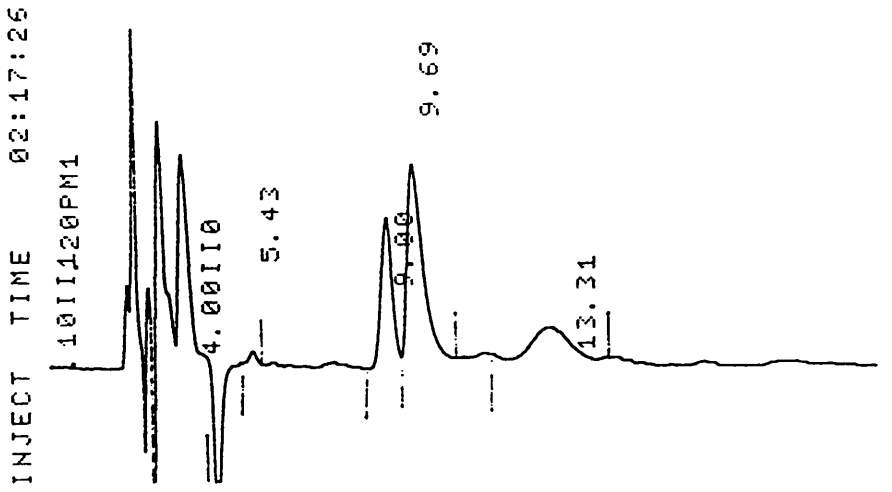
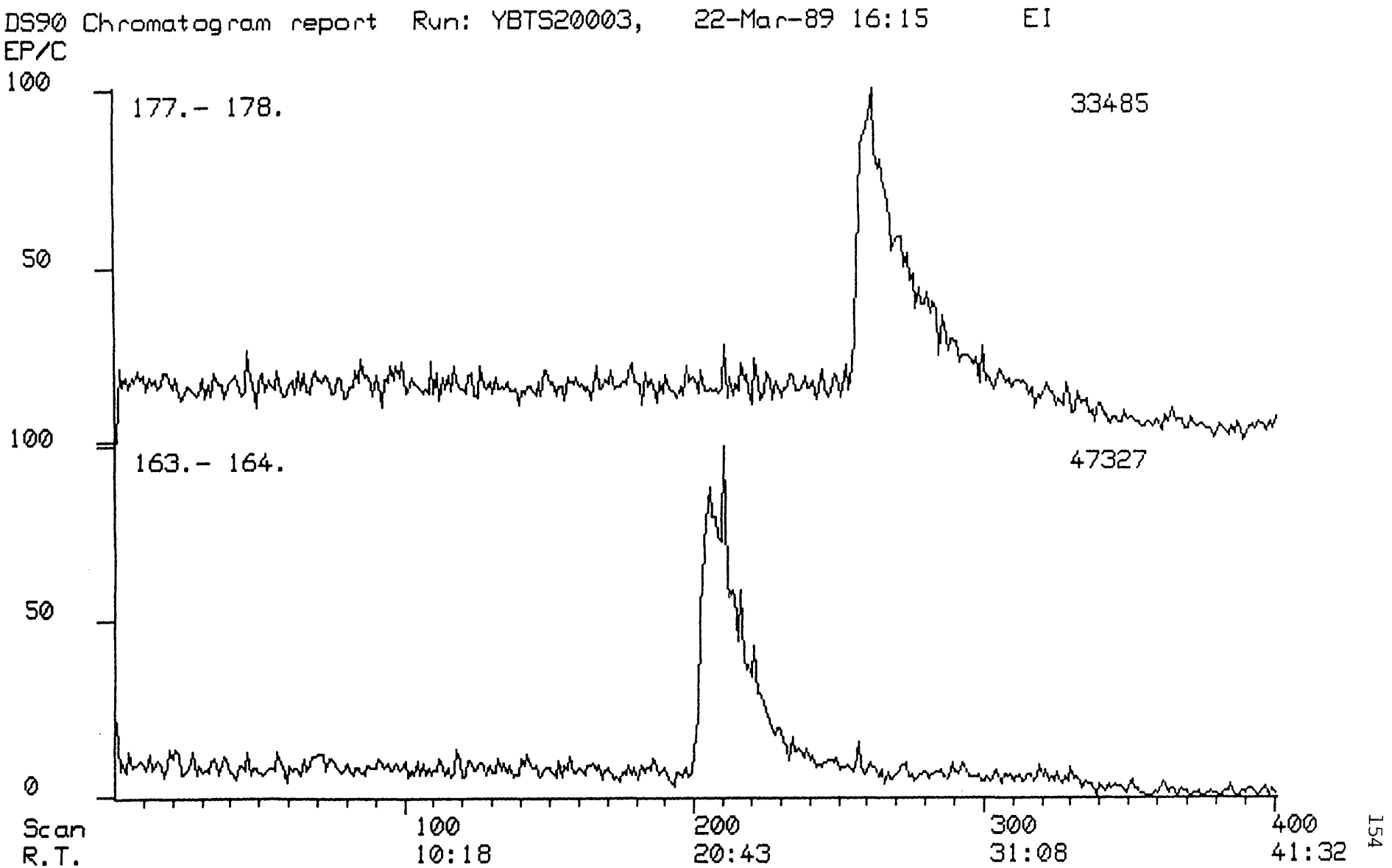


Figure 42


DS90 Chromatogram report Run: YBTS20003, 22-Mar-89 16:15 EI
EP/C
100
177.- 178.
33485
50
100
163.- 164.
47327
50
0
Scan
R.T.
100
10:18
200
20:43
300
31:08
400
41:32

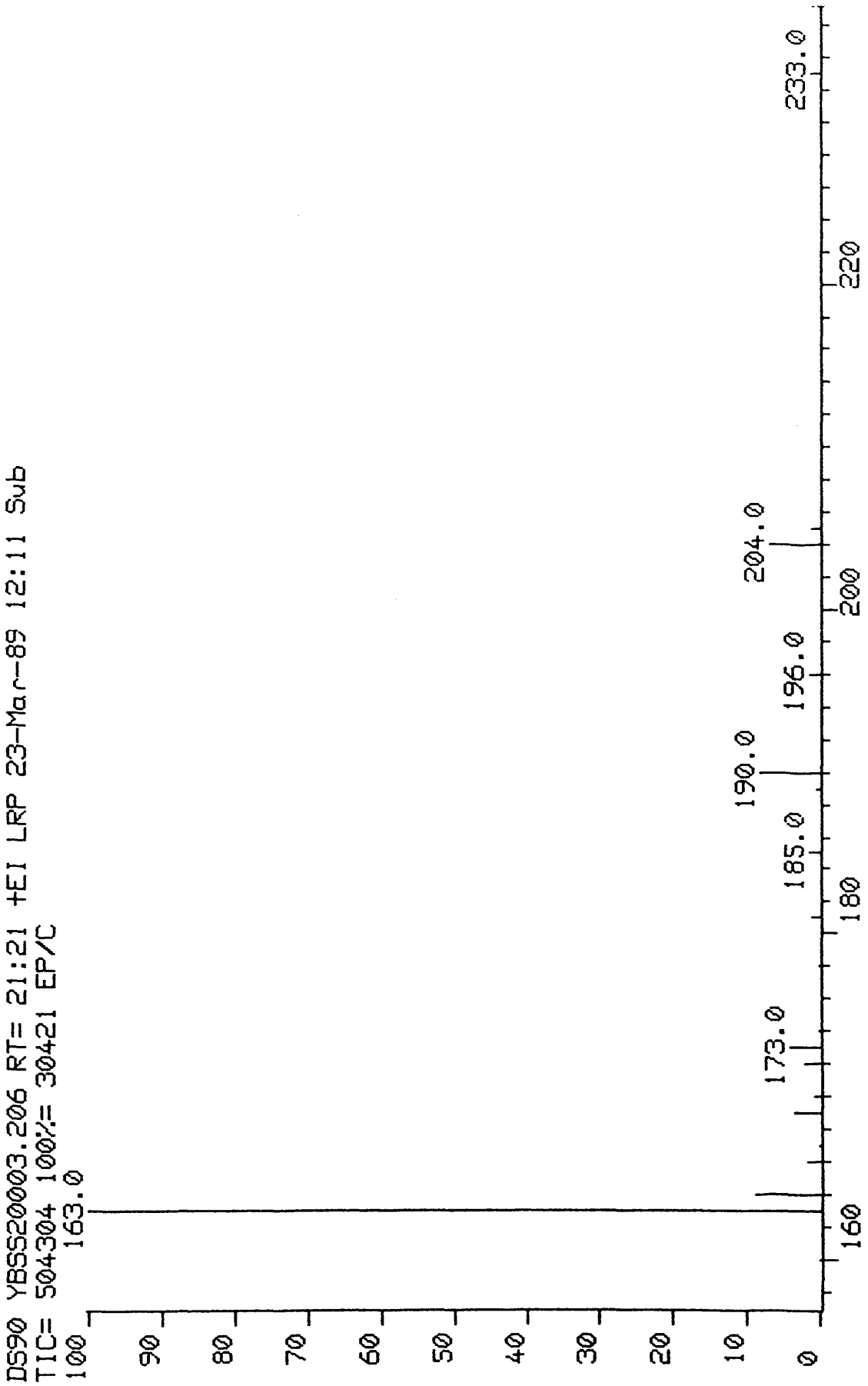

DS90 YBSS20003.206 RT= 21:21 +EI LRP 23-Mar-89 12:11 Sub
TIC= 504304 100%= 30421 EP/C
163.0
173.0
185.0
190.0
196.0
204.0
233.0
100
90
80
70
60
50
40
30
20
10
0
160
180
200
220

Figure 43

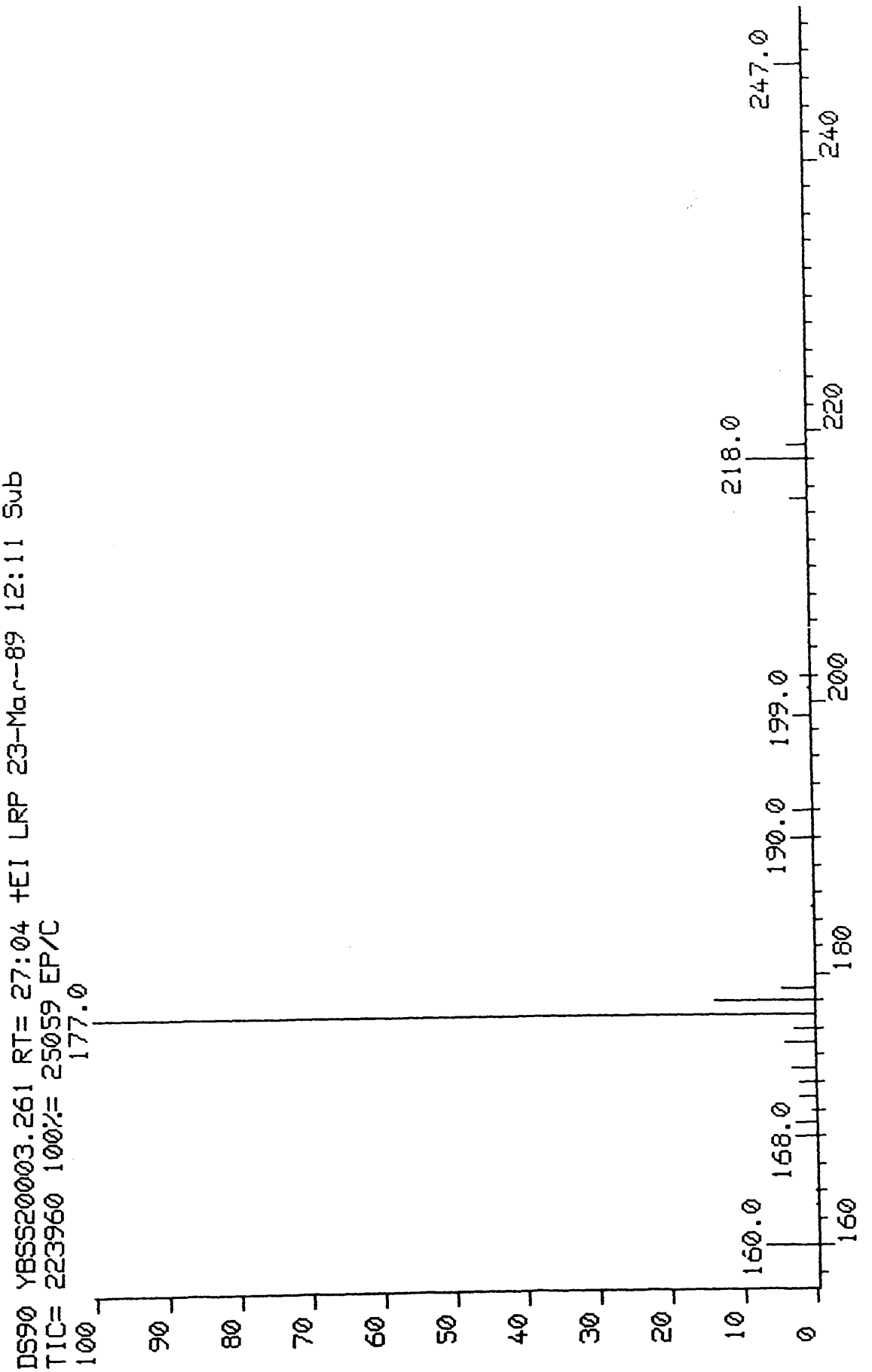
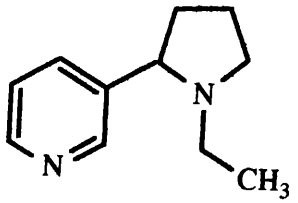

DS90 YBSS20003.261 RT= 27:04 +EI LRP 23-Mar-89 12:11 Sub
TIC= 223960 100%= 25059 EP/C
177.0
218.0
199.0
190.0
168.0
160.0
247.0
240
220
200
180
160
100
90
80
70
60
50
40
30
20
10
0

Figure 44

fragment with a m/z of 98.0951 amu which corresponds to a calculated value of 98.0970 amu for the N-ethylpyrrolinium ion (197).

In conclusion, the administration of N-ethylputrescine (186) dihydrochloride to N.rustica transformed root cultures produces an analogue of (-)-nicotine (16), namely N-ethylnornicotine (195). In addition this alkaloid analogue is not produced at the expense of (-)-nicotine (16) biosynthesis. This strongly suggests that N-ethylputrescine (186) is a reasonable substrate for diamine oxidase. N-ethylnornicotine has recently been isolated from burley tobacco plants[145].

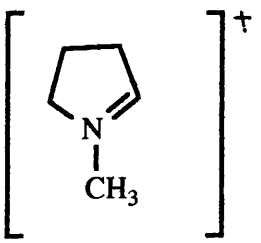
Various attempts were made to find a synthesis of N-alkylputrescines from which adequate quantities could be used in a wide range of feeding experiments. Firstly, the above synthesis was followed to synthesise N-ethyl-N-(3-cyanopropyl)benzylamine (194). Reduction of the cyano group of (194) was attempted with borane-dimethylsulphide complex (BMS). Analysis of the crude reaction mixture showed mainly starting material with a trace of another product. It was thought that under our reaction conditions (1.1 equivalents of BMS) complexation of boron with the tertiary nitrogen of N-ethyl-N-(3-cyanopropyl)benzylamine (194) blocked the desired reduction. In a second experiment the quantity of BMS used was increased to 2.2 equivalents (relative to the cyano compound). Analysis by t.l.c. showed that only a trace of starting material was retained. The crude reaction mixture was subjected to purification



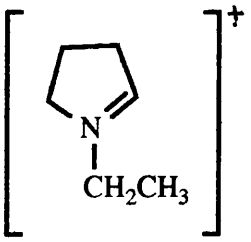
(195)

on neutral alumina with a chloroform-methanol solvent gradient. However, inadequate separation was achieved. Subsequent changes in the solvent gradient used were unable to separate the desired product. The crude reaction mixture was therefore used in an attempted debenzylation reaction in which it was subjected to transfer hydrogenation in the presence of 10% palladium on charcoal and ammonium formate. The reaction was followed by t.l.c. No cleavage of the benzyl group was observed over six hours.

Synthesis of the N-alkylputrescines was attempted by adapting a published procedure by Golding and co-workers[145]. The carbamate of 4-aminobutan-1-ol (198) was synthesised using benzyl chloroformate (199). This was converted into the azide by hydrazoic acid (HN_3) in a Mitsunobu-type reaction. An adaption of the procedure of Golding and co-workers[145] was then carried out. An internal Wittig reaction was attempted between N-benzyloxycarbonyl-4-aminobutan-1-azide (200) and acetaldehyde (201). No reaction was observed. In a second experiment the reaction was followed by t.l.c. No reaction was observed over a six hour period. This attempted synthesis can be seen in Figure 45. Subsequent reduction of the imine by sodium borohydride and removal of the benzyl carbonate group was therefore not attempted.



(196)



(197)

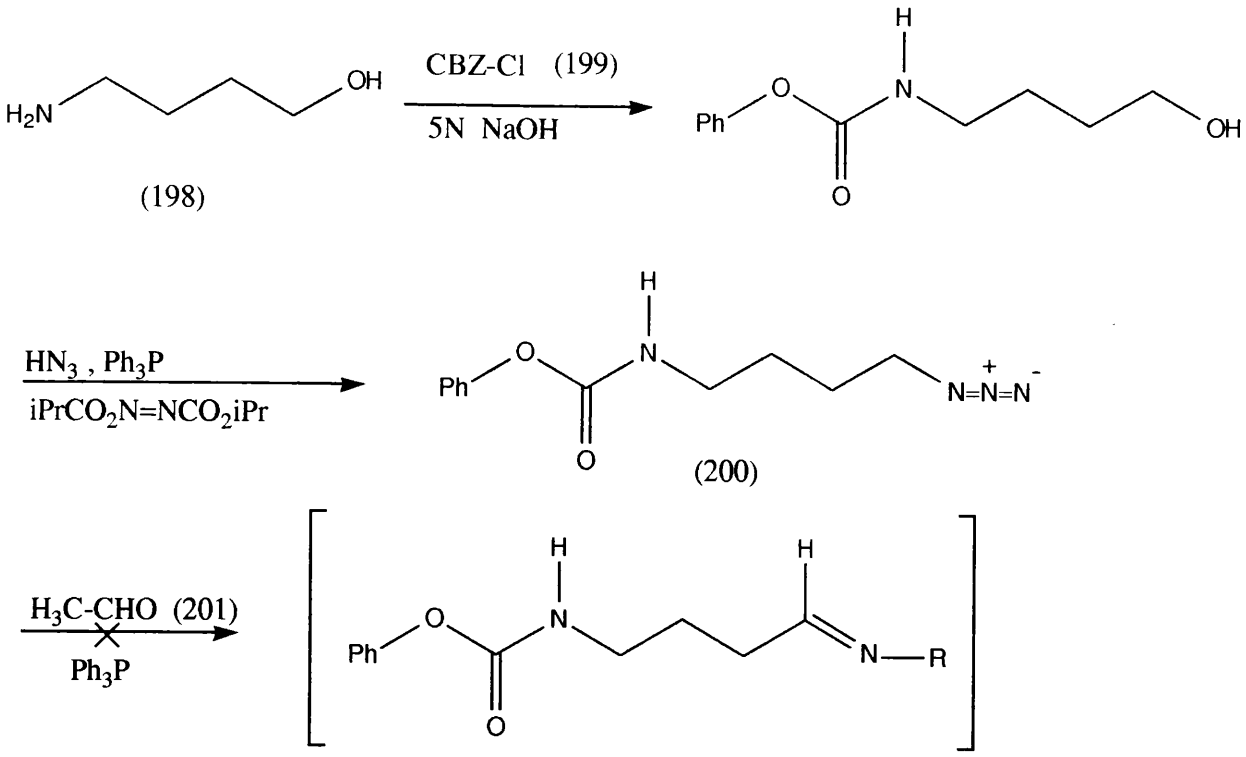


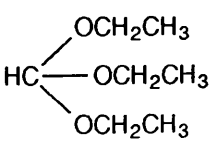
Figure 45

Another synthesis of the N-alkylputrescines was attempted using known reactions. The first part of this synthesis involved the formation of 4-phthalimidobutyronitrile (202) from N-(3-bromopropyl)phthalimide (203) and sodium cyanide. Purification was undertaken by flash chromatogrphy with a pet.ether (40-60^{o})-ethyl acetate solvent gradient. The yield of this reaction was 69%. Alkylation of the nitrile group was attempted with triethyloxonium tetrafluouroborate

(204). Isolation and analysis of the white solid retrieved showed only starting material. No evidence of product formation was observed. In a subsequent experiment the course of reaction was observed by t.l.c.

Over a period of 48 hours no product formation was observed. A publication by Borch[147] showed that diethoxy carbonium tetrafluoroborate (205) was a suitable reagent for alkylation of the cyano group. This was synthesised from triethyl orthoformate (206) and boron trifluoride etherate.

The addition of diethoxycarbonium tetrafluoroborate (205) to 4-phthalimidobutyronitrile (202) heated under reflux yielded a yellow oil which was immediately reduced with sodium borohydride. T.l.c. of the crude oil showed only a trace of the starting material with one major new spot. Isolation of the crude product after reduction with sodium borohydride yielded a white solid in low yield. The resultant ^{1}H n.m.r spectrum of this crude product showed that reduction of the phthalimido group had occurred. A publication search yielded a paper by Osby and co-workers[148] in which sodium borohydride was used to cleave a phthalimide group. It was decided to try to reduce the imine selectively in the presence of the phthalimide group. The reduction of the imine, without the reduction of the phthalimide group, was attempted under high pressure hydrogenation conditions. The procedure of Burke et al.[148] was followed. Isolation and subsequent analysis by t.l.c. showed only the presence of the imino compound. It was decided to abandon this general synthesis. The routes described above can be seen in **Figure 46.**



(206)

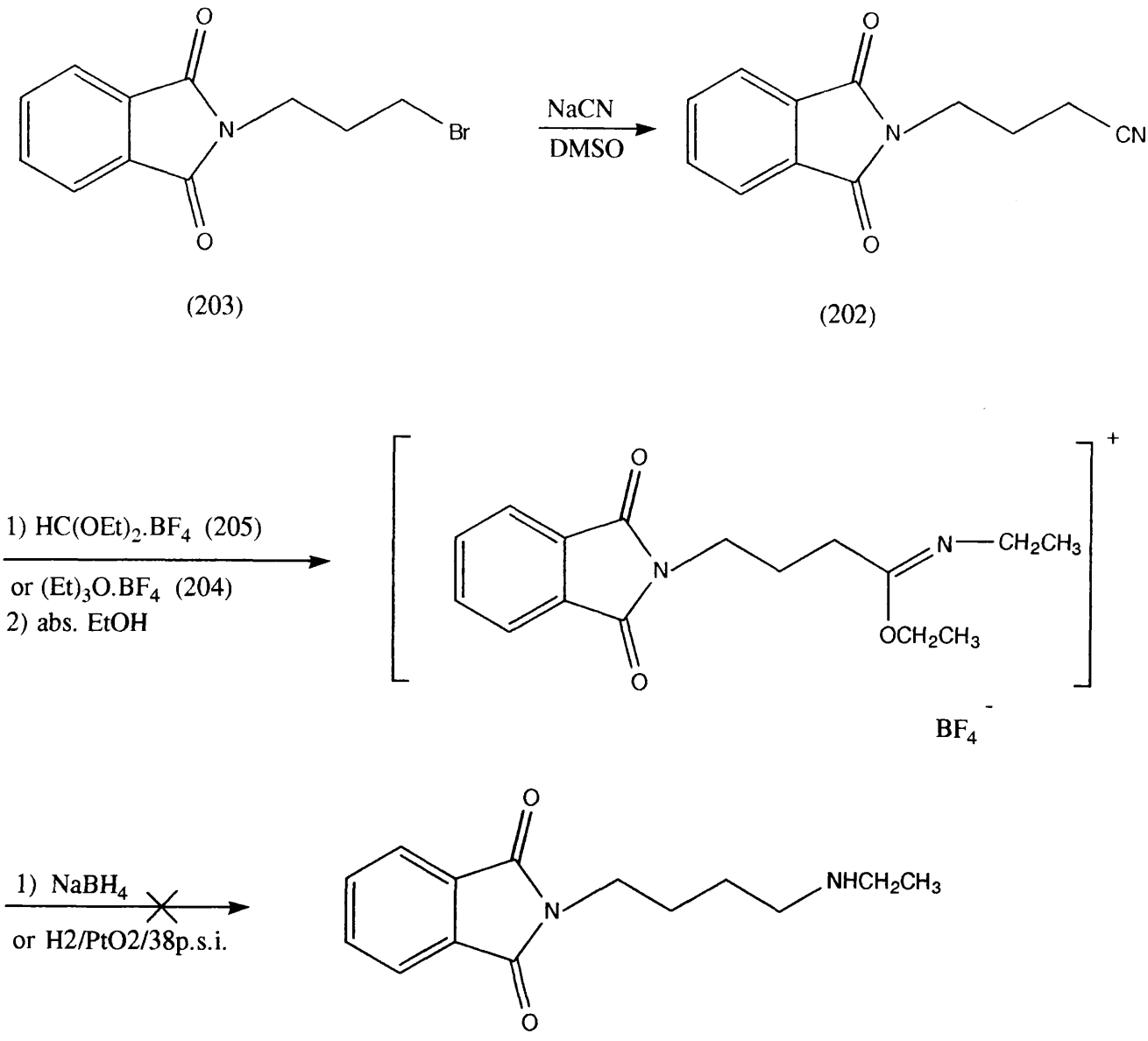
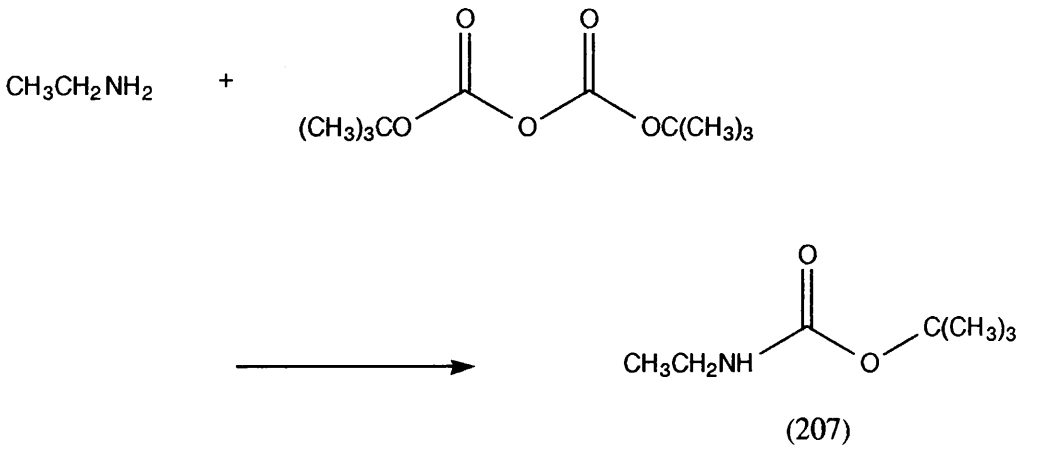
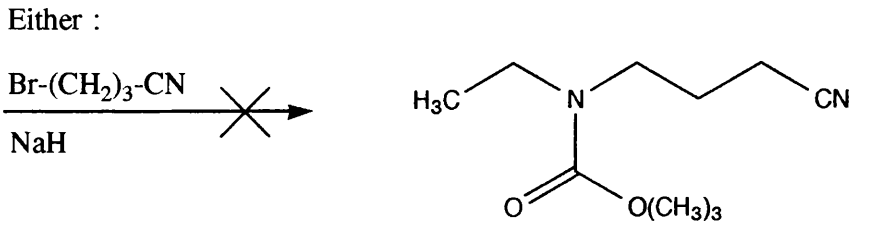


Figure 46

A final synthesis of the N-alkylputrescines was attempted. A publication of Croce and co-workers[149] reported monoalkylation of primary amines using the N-t-butoxycarbonyl residue as a protecting group. Synthesis of N-t-butoxycarbonylethylamine (207) was carried

out using the conditions of Croce and co-workers[149] in a relatively high yield. The coupling reaction between N-t-butoxycarbonylethylamine (207) and 4-bromobutyronitrile (190) was attempted using sodium hydride as the base. However, only starting materials were recovered. Various modifications to the prescribed procedure were made but only starting material was ever recovered. The use of 4-bromo-N-t-butoxycarbonylbutylamine (208) as a coupling reagent did not yield any desired product, only starting material (Figure 47). A possible reason for no product formation may be that the negative charge generated by the reaction of sodium hydride and N-t-butoxycarbonylethylamine (207) is delocalised through the carbonyl group which on work-up is re-protonated (Figure 48). However, no experimental evidence is available to substantiate this proposal.





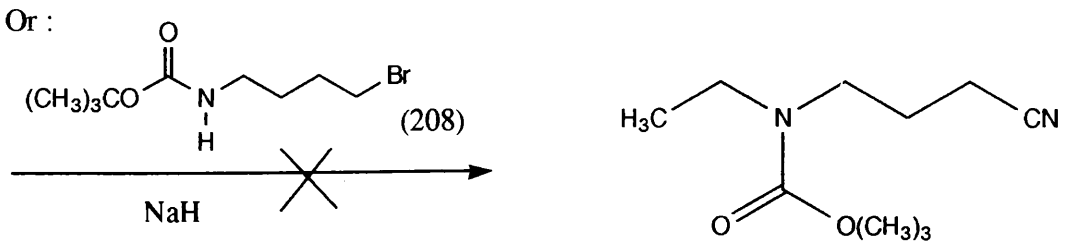


Figure 47

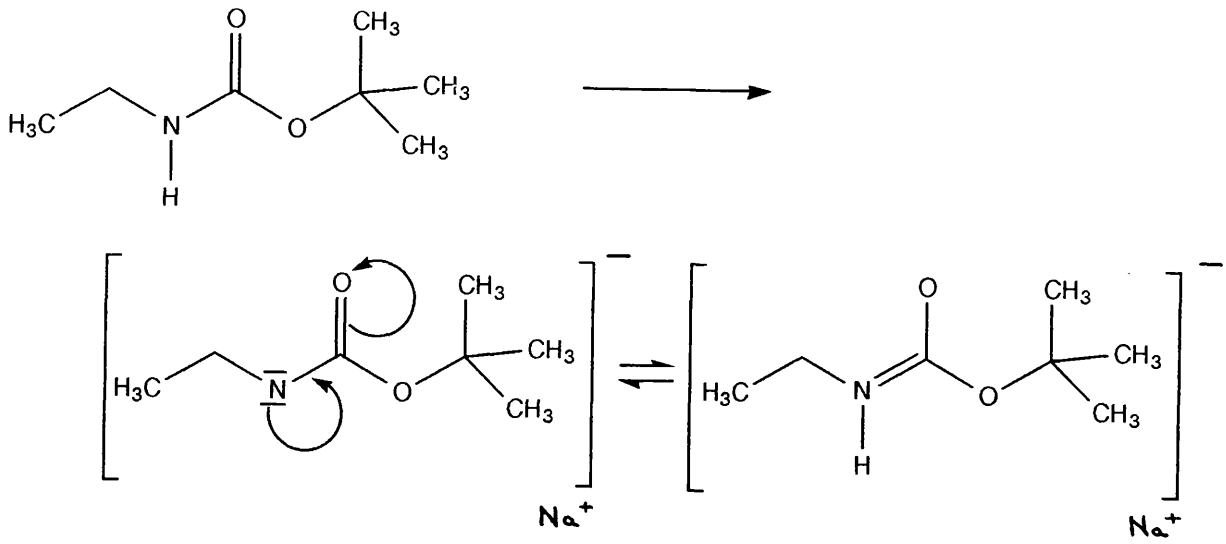
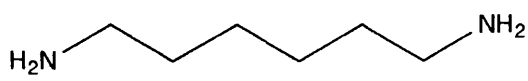


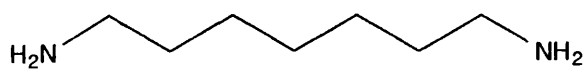
Figure 48

8.3 The synthesis and feeding of deuterium labelled polyamines to Nicotinana transformed root cultures

In order to study the selectivity of the enzyme systems involved in Nicotiana transformed root cultures it was decided to synthesise deuterium labelled 1,6-diaminohexane (209) and 1,7-diaminoheptane (210).



(209)



(210)

The substitution of the α-protons of 1,4-dicyanobutane (211) by $^{2}H_{2}O$ in the presence of DBU yielded [1,1,4,4-$^{2}H_{4}$]-1,4-dicyanobutane (212). The corresponding [1,1,5,5-$^{2}H_{4}$]-1,5-dicyanopentane (213) was prepared under the same conditions from 1,5-dicyanopentane (214). In both cases the reduction of the nitrile groups was undertaken with a borane-dimethyl sulphide complex. The resultant deuterium labelled diamine dihydrochlorides were isolated in high yields with deuterium incorporations of ca.95% (as estimated from the ^{1}H n.m.r. spectra of [2,2,5,5-$^{2}H_{4}$]-1,6-diaminohexane dihydrochloride (215) and [2,2,6,6-$^{2}H_{4}$]-1,7-diaminoheptane dihydrochloride (216)). The above reactions can be seen in Figures 49 and 50. The synthesis of

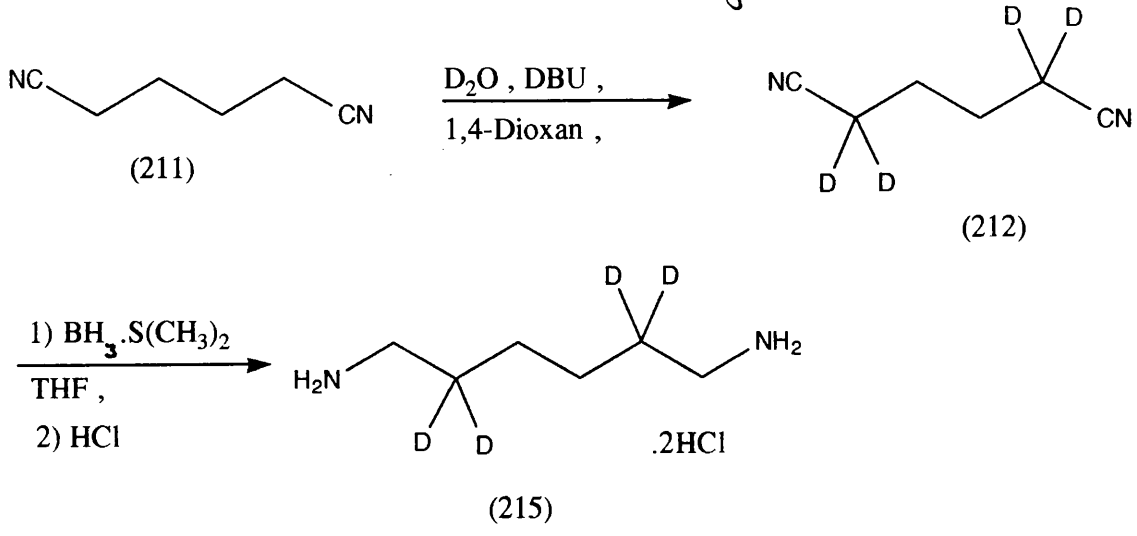


Figure 49

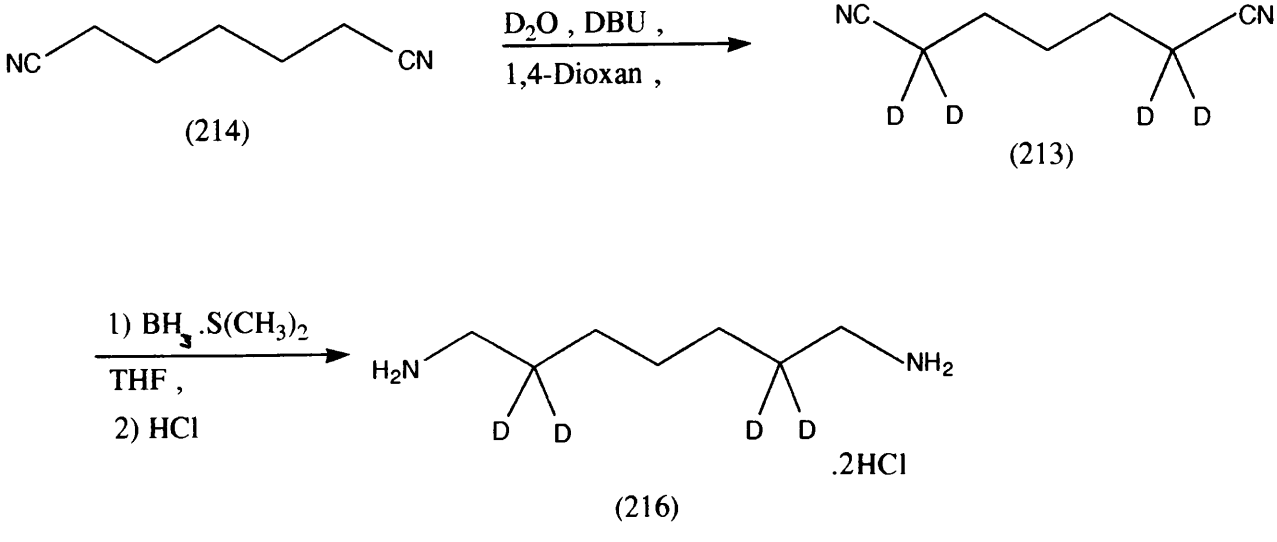


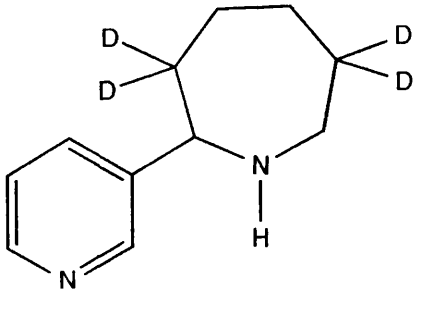
Figure 50

[2,2,5,5-$^2H_4$]-1,6-diaminohexane dihydrochloride (215) and [2,2,6,6-$^2H_4$]-1,7-diaminoheptane dihydrochloride (216) was analogous to that of [2,2,3,3-$^2H_4$]-1,4-diaminobutane dihydrochloride.

It was decided to feed [2,2,5,5-$^2H_4$]-1,6-diaminohexane dihydrochloride (215) and [2,2,6,6-$^2H_4$]-1,7-diaminoheptane dihydrochloride (216) to Nicotiana transformed root cultures.

The administration of [2,2,5,5-$^2H_4$]-1,6-diaminohexane dihydrochloride (215) was carried out at a concentration of 1.0 mM and the compound was fed to Nicotiana rustica transformed root cultures three days after sub-culture. The flasks of transformed root cultures were harvested after a further eleven days. The crude alkaloid extract was obtained after an acid-base extraction procedure. T.l.c. was initially used to analyse the crude alkaloid extract with an extract obtained from a control experiment as a reference. Visualisation of the alkaloids with the Dragendorff reagent showed the presence of an additional alkaloid analogue in the precursor fed extract.

The crude alkaloid extract was submitted for ^2H n.m.r. spectroscopy. The ^2H n.m.r spectrum showed two peaks at δ1.45 and 2.24 respectively. It is suggested that the new alkaloid alkalogue has the structure (217).



(217)

Time constraints did not allow this proposal to be substantiated either by isolation of the pure compound or by other analytical or spectroscopic techniques.

The administration of [2,2,6,6-$^{2}H_{4}$]-1,7-diaminoheptane dihydrochloride (216) was carried out at 1.0 mM four days after sub-culture of the transformed root culture. The Nicotiana rustica transformed root cultures were harvested after a further eleven days. The crude alkaloid extract was obtained by an acid-base extraction procedure. Initially, analysis was by the comparison of the crude alkaloid extract with that obtained in a control experiment. Visualisation of the alkaloids by the Dragendorff reagent[135] failed to show any new alkaloid analogues. Subsequent analysis of the crude alkaloid extract by g.c. and h.p.l.c. techniques gave conflicting results. The g.c. trace failed to show any new alkaloid analogues but the h.p.l.c. trace showed a small peak with a retention time of 13.9 minutes. It was decided to obtain further analytical information before making any proposals. The crude alkaloid extract was submitted for ^{2}H n.m.r. spectroscopic analysis. A broad singlet was seen at δ1.41 which initially suggested the production of a new alkaloid analogue. However, the ^{2}H n.m.r. spectrum of [2,2,6,6-$^{2}H_{4}$]-1,7-diaminoheptane dihydrochloride (216) shows a singlet at δ1.48. Further analysis of the crude alkaloid extract by g.c.-m.s. and h.p.l.c.-m.s. was not completed in time for inclusion in this thesis. This is required in order to make any proposals on the conversion of [2,2,6,6-$^{2}H_{4}$]-1,7-diaminoheptane dihydrochloride (216) into alkaloid analogues.

The medium from the N.rustica transformed root cultures was subjected to an acid-base extraction procedure and the extract obtained was examined by h.p.l.c. This showed a new peak with a retention time of 20.2 minutes but further analysis was not obtained due to time constraints.

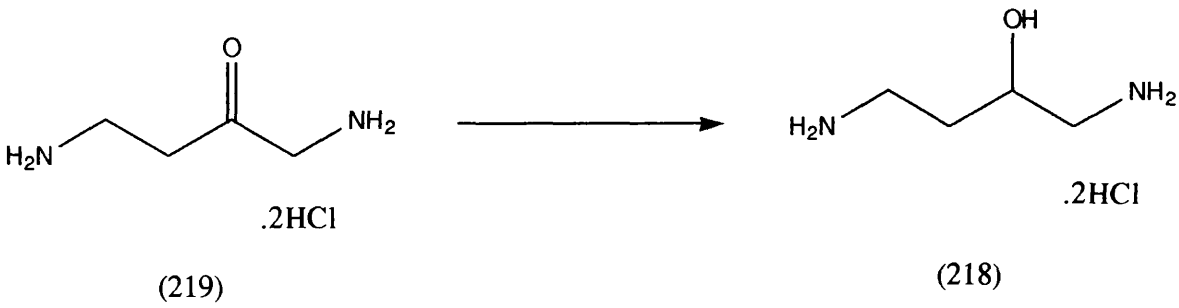
When the h.p.l.c. trace of the crude alkaloid extract obtained from the transformed roots was examined a substantial change in the ratio of (-)-nicotine (16) to anatabine (19) was observed. This ratio is usually 4:1 in control flasks of N.rustica but was found to be 1:4, respectively, in flasks fed with [2,2,6,6-$^{2}H_{4}$]-1,7-diaminoheptane dihydrochloride (216).

It is possible that [2,2,6,6-$^{2}H_{4}$]-1,7-diaminoheptane (216) acts as a competitive inhibitor of diamine oxidase in N.rustica transformed root cultures.

However, further experimental evidence is required to substantiate this proposal. Initial enzyme studies[150] with partially purified diamine oxidase have shown that 1,7-diaminoheptane (210) is a better binder than either putrescine or cadaverine. However, the rate of oxidation of 1,7-diaminoheptane (210) is less than putrescine and cadaverine.

8.4 The attempted synthesis of (+)- and (-)-1,4-diamino-2-hydroxy butane dihydrochloride

A publication by Macholan[151] provided a method to synthesise (±)-1,4-diamino-2-hydroxybutane dihydrochloride (218) from 1,4-diamino-2-butanone dihydrochloride (219). This was considered an excellent starting point for this work. Hydrogenation of 1,4-diamino-2-butanone dihydrochloride (219) over Adams catalyst (PtO_2) gave a high yield of dihydrochloride (218) (Figure 51).

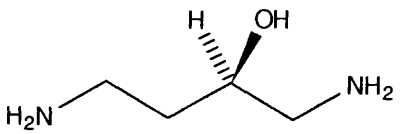


(219) (218)

Figure 51

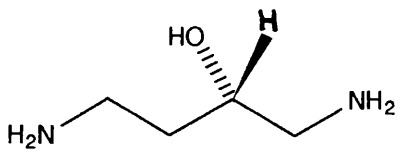
The administration of (±)-1,4-diamino-2-hydroxybutane dihydrochloride (218) to flasks of a Datura stramonium transformed root culture was carried out four days after sub-culture and feeding at a concentration of 1.0 mM was continued for a further fourteen days. In addition control flasks of the Datura stramonium transformed root culture were established. In both cases a standard acid-base extraction procedure was followed. The crude alkaloid extracts were subjected to g.c. and h.p.l.c. analysis. It was suggested that (±)-1,4-diamino-2-hydroxybutane dihydrochloride (218) may be marginally toxic to the transformed root culture although it must be stressed that no definitive experimental evidence is available to substantiate this proposal. The above proposal was made as the weight of roots obtained (from both the control and administered flasks) was similar but the weight of alkaloids obtained after an acid-base extraction procedure was diminished in the treated transformed root cultures. The crude alkaloid extract obtained after the feeding of (±)-1,4-diamino-2-hydroxybutane dihydrochloride (218) did not show the production of any new alkaloid analogues in the g.c. and h.p.l.c. traces. Therefore, although this compound may have some effect on the production of tropane alkaloids it was decided to conclude this aspect of the work.

Synthesis of (R)- and (S)-1,4-diamino-2-hydroxybutane dihydrochloride, (220) and (221) respectively, was continued in order for these compounds to be used in enzymic studies (see Section 8.7).



.2HCl

(220)



.2HCl

(221)

Esterfication of (S)-malic acid (222) to produce diethyl (S)-(-)malate (223) proceeded with a yield of 93%. Protection of the secondary hydroxyl group with 2-methoxyethoxymethyl chloride (224) was carried out using the procedure of Corey and co-workers[152] in a 60% yield. Reduction of the resultant diethyl (S)-2-(2-methoxyethoxymethylhydroxy)butane-1,4-dioate (225) with DIBAL-H at -50^{o}C yielded (S)-2-(2-methoxyethoxymethylhydroxy)butane-1,2,4-triol (226) which was subjected to the Golding procedure[145] outlined previously. Isolation

yielded a light brown solid which was shown by t.l.c. to be a single product. Subsequent analysis by ^{1}H n.m.r. spectroscopy showed the product to be (S)-2-hydroxyputrescine (227). During the final step deprotection had occured. However the yield of this final step was 5% making a large scale preparation of (S)-2-hydroxyputrescine (227) unrealistic. The above synthesis can be seen in **Figure 52.**

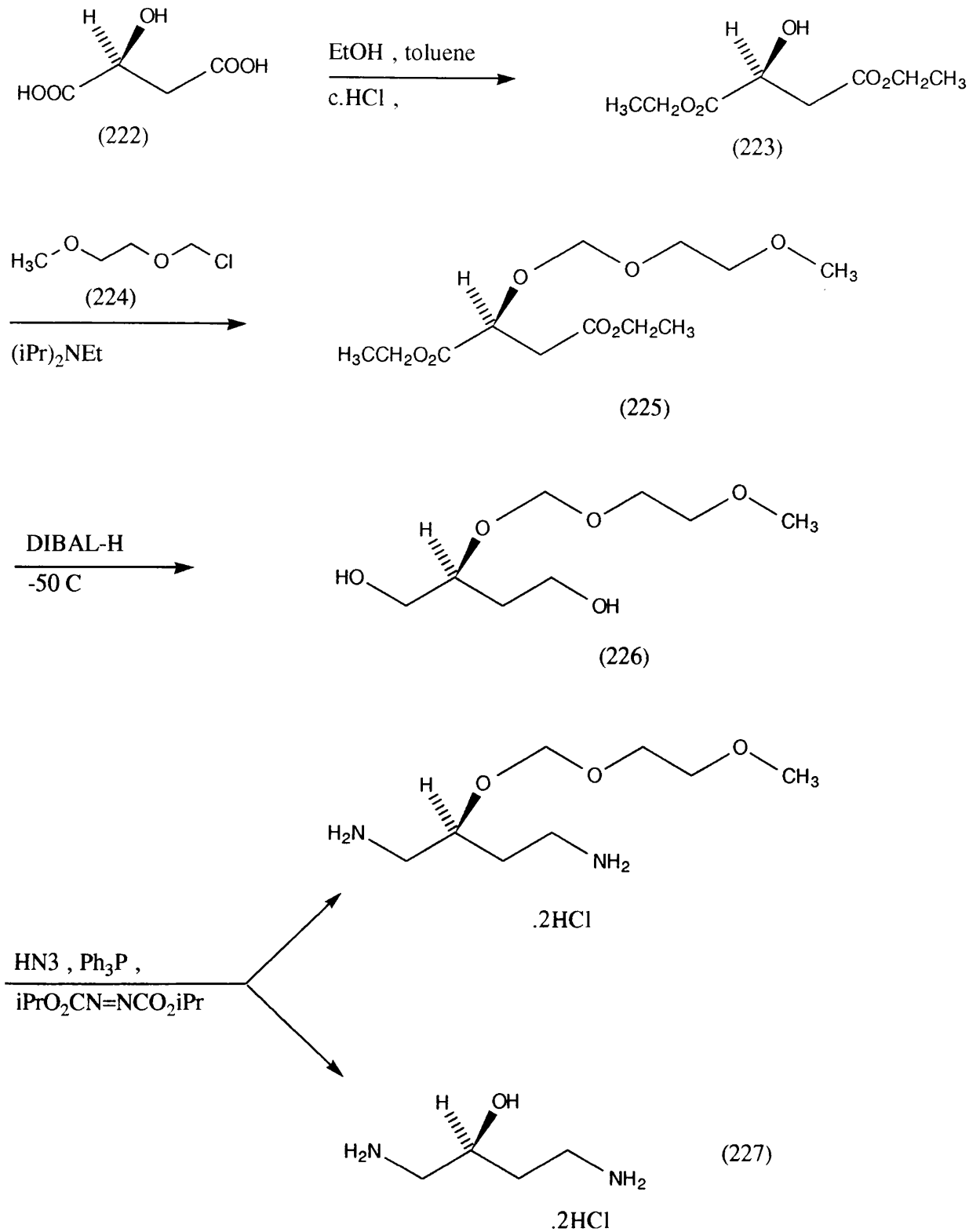
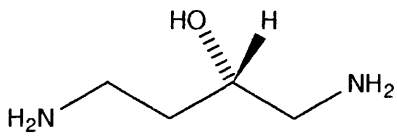

H
OH
HOOC
COOH
(222)
EtOH , toluene
c.HCl ,
H
OH
H3CCH2O2C
CO2CH2CH3
(223)
H3C
O
O
Cl
(224)
(iPr)2NEt
H
O
O
O
CH3
H3CCH2O2C
CO2CH2CH3
(225)
DIBAL-H
-50 C
H
O
O
O
CH3
HO
OH
(226)
H
O
O
O
CH3
H2N
NH2
.2HCl
HN3 , Ph3P ,
iPrO2CN=NCO2iPr
H
OH
H2N
NH2
(227)
.2HCl

Figure 52

Consequently the synthesis of (R)-2-hydroxyputrescine (228) by this synthesis was not attempted due to the low yield of the final step.



(228)

In a second synthesis a direct reduction of (S)-malic acid (222) with BH_3.THF was attempted. A clear thick oil was recovered after isolation which gave the correct analytical data. However the yield of the reduction was 17% making the overall synthesis unrealistic. The following step, i.e the Golding reaction[145], was not attempted as a sufficient quantity of (S)-2-hydroxyputrescine (227) was available for enzymic studies.

No further synthesis of the (R)- and (S)-2-hydroxyputrescines, (227) and (228), were attempted.

8.5 The attempted synthesis of 2-haloputrescines

Adaption of a procedure by Lowe and Potter[153] was used to prepare diethyl (R)-fluorosuccinate (229) by the action of diethylaminosulphur trifluoride (230) on diethyl (S)-(-)-malate (223). Total inversion in this reaction has been proved by Lowe and Potter[153]. Subsequent reduction was carried out with DIBAL-H at -50°C to yield a yellowish oil. The crude oil gave the correct n.m.r. spectrum for (R)-2-fluorobutan-1,4-diol (231). Purification by distillation resulted in a black tar being formed due to overheating of the sample. Time constraints did not allow this reaction to be repeated. The suggested reactions can be seen in Figure 53.

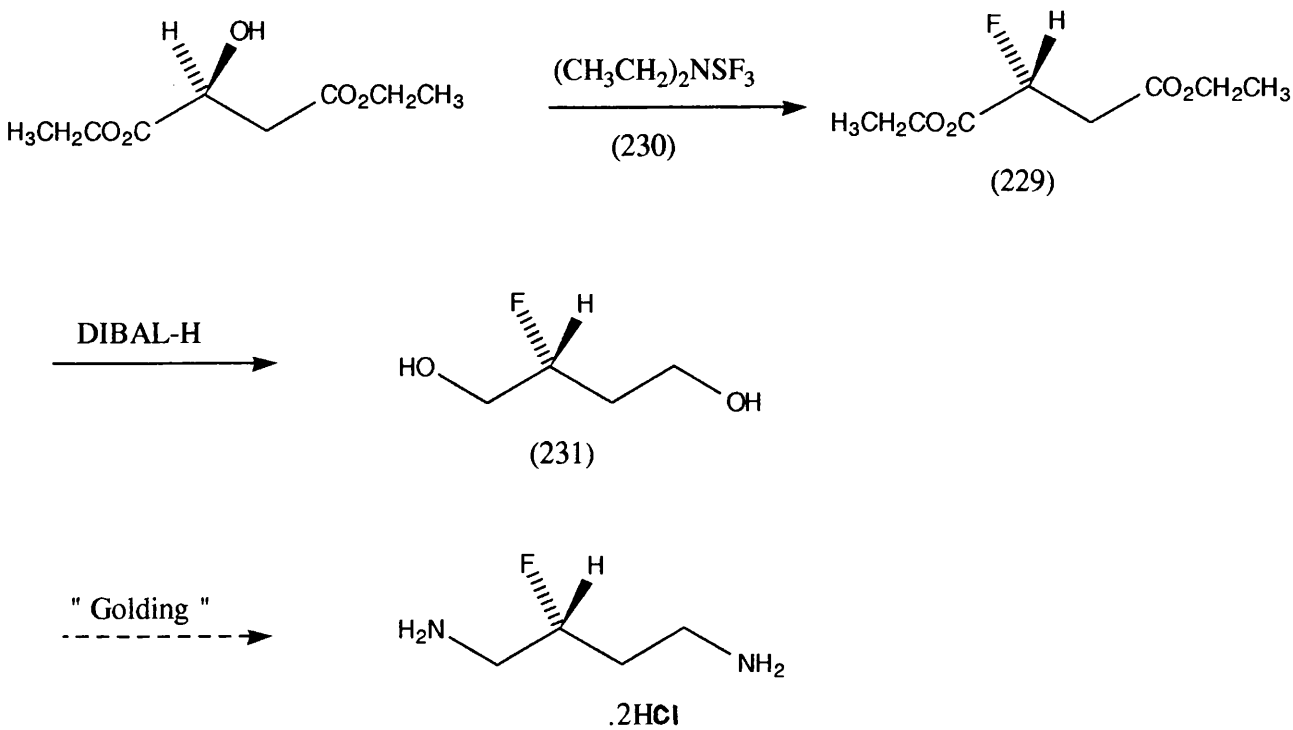
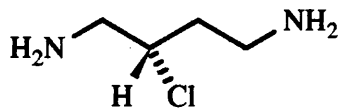
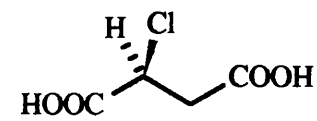


Figure 53

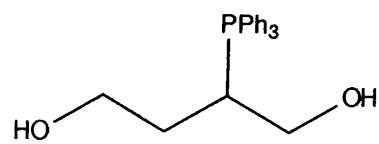
The synthesis of (S)-2-chloro-1,4-diaminobutane dihydrochloride (232) was attempted using (S)-2-chlorobutane-1,4-dioic acid (233) as the starting material. This was prepared in an analogous manner to that used in the synthesis of (R)-[2-^{2}H]putrescine dihydrochloride (158). This reaction scheme (Figure 19) was followed in order to synthesise (S)-2-chlorobutane-1,4-diol (162). The synthesis of (S)-2-chloro-1,4-diaminobutane (232) was continued using the procedure outlined by Golding and co-workers[145]. A thick clear oil was isolated which solidified to an amorphous solid on standing. A ^{1}H n.m.r. spectrum of this oil suggested the following structure (234).



(232)



(233)



(234)

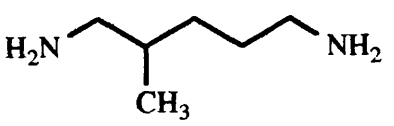
However, no other analytical data was readily available before the product started to decompose. Time constraints did not allow further analysis of this product or modifications to the procedure to be made.

8.6 Feeding experiments with 1,4-diamino-2-butanone dihydrochloride, 2-methyl-1,5-diaminopentane dihydrochloride and 8-thiabicyclo[3.2.1]-cyclooctan-3-one

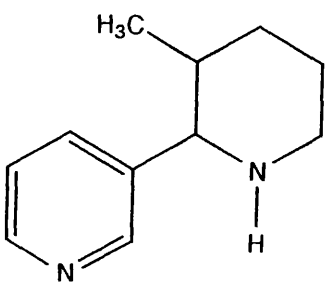
The first two products are readily available from the Aldrich Chemical Company.

The administration of 1,4-diamino-2-butanone dihydrochloride (219) to Nicotiana rustica transformed root cultures was carried out at a concentration of 1.0 mM four days after sub-culture. During the next two days the roots turned black and appeared to stop root growth. Half of the transformed root cultures were removed to be analysed. An acid-base extraction procedure was followed and the extract was submitted for g.c. and h.p.l.c. analysis. This showed the usual distribution of alkaloids. However, it must be noted that the weight of this extract was extremely low when compared to control samples and the ratio of the alkaloids was altered. The ratio of anatabine (19) to (-)-nicotine (16) was 3:1 whereas in the control samples it was 1:4, respectively. The remaining transformed root cultures were left for a further two days. New root growth was observed and the feeding experiment was continued to the end of the time period prescribed at the outset of the experiment. After extraction and analysis the distribution and ratio of the alkaloids in the flasks removed after two days and in the flasks removed after fourteen days were similar. 1,4-Diamino-2-butanone dihydrochloride has been sent for anti-fungal testing.

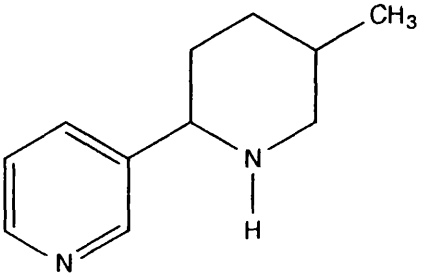
The dihydrochloride salt of 1,5-diamino-2-methylpentane (235) was prepared by the saturation of an ethereal solution of the free base by HCl gas. Administration of 1,5-diamino-2-methylpentane (235) dihydrochloride to N.rustica transformed root cultures was carried out at a concentration of 0.6 mM five days after sub-culture. After a further fourteen days the transformed roots were harvested. Extraction was carried out by the usual procedure. The crude alkaloid extract was subjected to g.c. and h.p.l.c. analysis. The g.c. traces shows two major peaks with retention times of 13.8 and 22.2 minutes. The peak at 13.8 minutes corresponds to (-)-nicotine (16) as shown by a reference trace. However, the peak at 22.2 minutes does not correspond to any of the normal tobacco alkaloids. An initial proposal is that this new alkaloid analogue is either 2'-methylanabasine (236) or 5'-methylanabasine (237). The h.p.l.c. trace in addition to the peaks associated with the normal tobacco alkaloids has a peak with a retention time of 12.9 minutes. In order to determine the m/z of the new alkaloid the sample was submitted for g.c.-m.s. and h.p.l.c.-m.s. analysis (Norwich). However, at the time of writing this thesis these results were not available. It was decided to try to purify the crude alkaloid extract by preparative t.l.c. in order to determine the structure of the new alkaloid analogue. However, during repeated purification attempts it was not possible to remove an impurity which was thought to be (-)-nicotine (16). This was because of the closeness of the compounds on t.l.c.



(235)



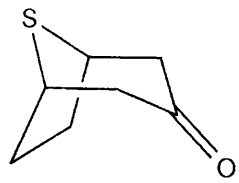
(236)



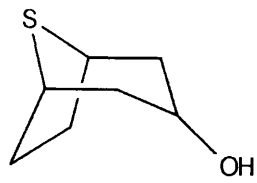
(237)

In order to determine the structure of this alkaloid it is necessary to find new purification methods and to complete the m.s. analysis.

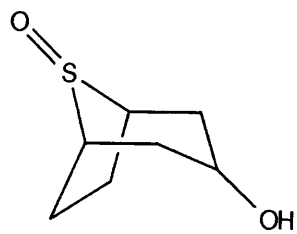
In a final experiment an analogue of tropinone (32) namely 8-thiacyclo[3.2.1]cyclooctan-3-one (238) was administered to transformed root cultures of a Datura candida x Datura aurea hybrid at 1.2 mM. This compound was supplied by Dr P. McCabe (University of Glasgow). It was fed four days after sub-culutre and the roots were harvested after a further fourteen days. No loss in root weights or normal alkaloid distribution was observed when compared to a control transformed root culture. The roots were subjected to an acid-base extraction procedure. The crude extract was analysed by g.c. techniques. The g.c. trace showed in addition to the normal alkaloids a peak with a retention time of 11.5 minutes. Further examination of this peak by g.c.-m.s. yielded further information. This major new peak showed two peaks in the spectrum which are characteristic of a sulphur containing compound. The two peaks in question have accurate mass values of 140.0824 and 144.0618 amu in a ratio of 24:1 respectively. This compound is proposed to be 8-thiabicyclo[3.2.1]octan-3-ol (239). No definition of the sterochemistry at C-3 was possible. In addition a smaller peak was observed. It had an accurate mass of 160.0513 amu and a molecular formula of $C_{12}H_{12}SO_2$. This was proposed to be the sulphoxide (240) of the alcohol of the starting material (239). In order to check if this oxidation occurred as a m.s. artefact it was decided to look at the mass spectrum of the starting material. No evidence of the sulphoxide was found. This suggests that the oxidation takes place in the biological system.



(238)



(239)



(240)

In conclusion, further experimental evidence is required to confirm these proposals. In addition the purification of these products (236) or (237), (239) and (240) and subsequent spectral analysis is a necessary goal.

8.7 The action of diamine oxidases on various diamines

Whenever possible the supply of various diamine dihydrochlorides for testing with pea seedling diamine oxidase and hog kidney diamine oxidase has taken place. These have been tested by Miss A. Equi who is studying for a University of Glasgow Ph.D. sponsored by the S.E.R.C. As a general overview interesting results have been found. The first compounds tested were N-methylputrescine (23), N-ethylputrescine (186) and N-propylputrescine (187). These were supplied as their dihydrochloride salts.

In a publication by Frydman and co-workers[154] it was stated that N-methylputrescine (23) is a poor substrate for diamine oxidase. This was considered a strange result by us as it is well known that N-methylputrescine (23) is a precursor of (-)-hyoscyamine (14) and (-)-scopolamine (15).[31,32]. Subsequent enzymic studies by Miss Equi have show that N-methylputrescine (23) is as efficient as putrescine as a substrate for diamine oxidase. The oxidation of N-ethylputrescine (186) by diamine oxidase was also shown to proceed at a reasonable rate.

Work concerning other diamines supplied in this work is not yet complete. It will be presented in a subsequent Ph.D thesis by Miss Equi. The enzymic studies with N-alkylputrescines have been published:

(i) A Cooper, A M Equi, S K Ner, A B Watson and D J Robins
Tetrahedron, 1989, 30, 5167

(ii) A M Equi, A M Brown, A Cooper, S K Ner, A B Watson and D J Robins
Tetrahedron, 1991, 47, 507

CHAPTER NINE

EXPERIMENTAL

All melting points were measured on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured with an Optical activity Limited AA10 Polarimeter. Infra red spectra were obtained on a Perkin Elmer 590 spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R32 spectrometer opertaing at 90 MHz (δ_H), a Bruker WP200-SY spectrometer operating at 200 MHz (δ_H), 50 MHz (δ_C) and 30.72 MHz (δ_D), or a JEOL GX400 spectrometer operating at 399.65 MHz (δ_H) and 100.4 MHz (δ_C). unless otherwise stated all spectra were recorded for solutions in deuteriochloroform with tetramethylsilane as an internal standard. Mass spectra were obtained with either A.E.I. MS12 or 902 spectrometers. Gas liquid chromatography spectra were recorded using a silica capillary column coated with DB1 with phosphorus and nitrogen detection. High performance liquid chromatography spectra were recorded using a μBondapack C_{18} reverse phase column with u.v. detection at 230 nm and a running buffer of H_2O (450 parts), acetonitrile (50 parts) acetic acid (5 parts) and tetrahydrofuran (2 parts). For tobacco alkaloids this running buffer was H_2O (450 parts), acetonitrile (12 parts) acetic acid (3 parts) and tetrahydrofuran (1 part) which was adjusted to pH4 with sodium hydroxide. Gas chromatography-mass spectra were obtained using a Carlos Erba 4167/Kratos MS80RFA integrated system. High performance liquid chromatography-mass spectra were obtained usina a Kratos MS90RFA in conjunction with the above mentioned h.p.l.c. systems.

T.l.c. was carried out on Kieselgel G plates of 0.25 mm thickness unless otherwise indicated with various developing mixtures. Tropane alkaloids were developed with ethyl acetate - isopropanol - 25% ammonia (45:35:10). Tobacco alkaloids were developed with toluene - acetone - methanol - 25% ammonia (4:4.5:1:0.5). The alkaloids were detected with the Dragendorff reagent (Murnier modification) [135]. [1,4-^{14}C] Putrescine dihydrochloride was purchased from Amersham International. Radioactivity was measured with a Philips PW4700 Liquid Scintillation Counter using toluene - methanol scintillation solutions. Sufficient counts were accumulated to give a standard error of less than 1% for each determination. Radioactive samples were counted in duplicate. A Panax thin-layer scanner RTLS-1A was used for radioscanning of t.l.c. plates. Tetrahydrofuran (THF) was dried by distillation from potassium hydroxide and then from sodium-benzophenone under nitrogen prior to use. Dimethylsulphoxide (DMSO) was dried by distillation from calcium hydride under nitrogen prior to use. Anhydrous ethanol was dried by distillation from magnesium turnings and catalytic iodine under nitrogen prior to use. Dichloromethane was dried by distillation from calcium hydride under nitrogen prior to use. Ethanol-free chloroform was prepared by passing chloroform through a column of basic aluminimum oxide. Organic solutions were dried over either anhydrous magnesium sulphate or sodium sulphate and solvents were removed under reduced pressure below 50^{o}C.

Hairy root cultures of Datura candida x Datura aurea were established after sterilisation of seeds of a Datura candida x Datura surea plant hybrid with 10% (v/v) Domestos bleach (Lever Brothers, Kingston-upon-Thames, UK for 30 minutes. The seeds were washed and germinated on 1% (w/v) agar containg Gamborg's B5 salts (Flow Laboratories, Irvine, UK). Seedlings that were 2-3 days old were wounded on the stem with a hypodermic needle containing a suspension of Agrobacterium rhizogenes LBA 9402. Roots appeared from the wounds in the seedlings within 7-14 days. The mergent roots were excised when they had grown to a length of approximately 10 mm and placed in Gamborg's B5 liquid medium (8 ml) with 3% (w/v) sucrose and 0.5 $mg.ml^{-1}$ of ampicillin sulphate (Sigma, Poole, UK). Rapidly grown roots were passaged into 50 ml fresh medium in 250 ml Erlenmeyer flasks with between 0.2 and 0.5 g being transferred. Serial sub-culture led to an omittance of ampicillin sulphate (after eight sub-cultures). Roots were then maintained by sub-culture every two or three weeks. All precursor solutions were filter sterilised before administration.

Hairy root cultures of Nicotiana rustica and Nicotiana tabacum were prepared in an analogous manner with all precursor solutions filtered sterilised prior to use.

After harvesting, the hairy root cultures were macerated in methanol. The methanol extracts were combined and concentrated in vacuo to yield light brown residues. These residues, from hairy root cultures which produce either tropane alkaloids or tobacco alkaloids, were dissolved in 1M hydrochloric acid solution (100-150 ml). The acidic solutions were washed with dichlormethane (6 x 75 ml).

The acidic solution was then basified to pH9 with concentrated ammonia and extracted with chloroform (4 x 100 ml). The combined organic extracts were dried (Na_2SO_4), filtered and concentrated <u>in vacuo</u> to give the crude alkaloids.

The alkaloids were seperated by preparative t.l.c. using the t.l.c. systems described previously and the alkaloids were visualised with u.v. light (254 nm) and Dragendorff's reagent. The alkaloids were removed from the silica using methanol.

Isolated (-)-hyoscyamine (14) gave the following analytical data. R_f (silica, ethyl acetate - isopropanol - 25% ammonia, (45:35:10), ninhydrin spray) 0.40; δ_H 2.94 (H-1), 2.02 ($H\text{-}2_{ax.}$), 1.60 ($H\text{-}2_{eq.}$), 4.94 (H-3), 1.94 ($H\text{-}4_{ax.}$), 1.39 ($H\text{-}4_{eq.}$), 2.83 (H-5), 1.60 ($H\text{-}6_{ax.}$), 1.12 ($H\text{-}6_{eq.}$), 1.78 ($H\text{-}7_{ax.}$), 1.66 ($H\text{-}7_{eq.}$), 2.11 (CH_3-N), 3.69 (H-2'), 4.09, 3.74 (H-3') 7.19, 7.22, 7.26 (aromatics).
For all experiments between 5 - 20 mg were isolated.

Isolated (-)-scopolamine (15) gave the following analytical data. R_f (silica, ethyl acetate - isopropanol - 25% ammonia, (45:35:10), ninhydrin spray) 0.61; δ_H 2.88 (H-1), 2.01 ($H\text{-}2_{ax.}$), 1.49 ($H\text{-}2_{eq.}$), 4.92 (H-3), 1.93 ($H\text{-}4_{ax.}$), 1.25 ($H\text{-}4_{eq.}$), 3.00 (H-5), 3.31 (H-6), 2.66 (H-7), 2.36 (CH_3-N), 3.65 (H-2'), 3.71, 4.06 (H-3'), 7.15, 7.23, 7.27 (aromatics).
For all experiments between 3 - 17 mg were isolated.

Isolated (-)-anabasine (17) gave the following analytical data. Rf (silica, toluene - acetone - methanol - 25% ammonia, (4:4.5:1:0.5), ninhydrin spray) 0.42; δ_H 3.62 (H-2'), 1.52, 1.75 (H_2-3'), 1.45, 1.86 (H_2-4'), 1.49 1.63 (H_2-5'), 2.76 (H-6'$_{ax.}$), 3.15 (H-6'$_{eq.}$), 8.55 (H-2), 7.71 (H-4), 7.20 (H-5), 8.43 (H-6). In experiments it was estimated from h.p.l.c. analysis of isolated crude extracts that 5-6 mg of (-)-anabasine (17) was present.

Experimental to Chapter Five

[2,2,3,3-$^2H_4$]Succinonitrile

Deuterium oxide (35 ml, 99.3% atom ^2H), 1,4-dioxane (10 ml) and DBU (364 mg, 4.56 mmol) were added to succinonitrile (3.64g, 45.6 mmol) in a 100 r.b. flask. The mixture was heated and stirred at reflux for 24 hours. The reaction mixture was cooled and the solvent was removed *in vacuo* to yield on amorphous white solid which solidified on standing. (Hydrochloric acid (1M) was added to dissolve the amorphous white solid and to make the resulting solution acidic. The acidic solution was extracted with chloroform (5 x 25 ml), dried (Na_2SO_4), filtered, and the solvent was removed *in vacuo* to yield an amorphous white solid. This solid was recrystallised from hot benzene to yield a white crystalline material.

75.3%; R_f ($CHCl_3$)0.25; ν_{max} ($CHCl_3$) 3010, 2250 and 2160 cm^{-1}; δ_C 14.1 (C-2),C-3, quintet, $J_{^{13}C-^{1}H}$=21.1 Hz) and 116.4 (C-1,C-4); δ_D 2.72 (s) ($^2H_4$ content of *ca*. 94%); *m/z* 84 (M^+, 24.7%), 82, 56 (100%), 54 and 42; (Found: M^+ 84.0618 $C_4{}^2H_4N_2$ requires 84.0625).

[2,2,3,3-$^{2}H_4$]-1,4-Diaminobutane dihydrochloride

[2,2,3,3-$^{2}H_4$]Succinonitrile (1.1g, 13.1 mmol) was added to a suspension of platinum oxide (165mg, 15% by weight) in glacial acetic acid (45 ml). The mixture was stirred under hydrogen at atmospheric pressure at 20°C for 35 h until no more hydrogen was taken up. The reaction mixture was filtered through a pad of Florisil and the filtrate was evapourated to dryness under reduced pressure to yield an amorphous clear solid. The residue was dissolved in 1M hydrochloric acid and the solution was evaporated to dryness. The resulting light brown solid was recrystallised from aqueous ethanol to yield white needles which were dried in vacuo for 24 hours, 65.2%; R_f (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0.54; ν max (KBr disc) 3040, 2000, 1600 and 1500 cm^{-1}; $\delta_H(^{2}H_2O)$2.98(4H, br s); $\delta_C(H_2O$,dioxane)24.3 (C-2,C-3,quintet, $J_{^{13}C-^{1}H}$ = 17.6Hz) and 39.6 (C-1, C-4); $\delta_D(H_2O)$1.54(s)($^{2}H_4$ content of ca 90.0%). The N-phenylamino-thiocarbonyl derivative was prepared, m/z 362, 269, 240, 93(100); (Found:M^+362.1540. $C_{18}H_{18}{}^{2}H_4N_4S_2$ requires 362.1537).

(R)-[2-^{2}H]-1,4-Diaminobutane dihydrochloride

The synthesis of (R)-[2-^{2}H]butane-1,4-diol followed the published procedure[127]. The synthesis of (R)-[2-^{2}H]-1,4-diaminobutane dihydrochloride used an adaption of the procedure of Golding and co-workers[157].

Hydrazoic Acid (HN_3)

In a 500ml 3-necked r.b. flask fitted with a dropping funnel, alcohol thermometer, overhead stirrer and gas outlet tube a paste was prepared from sodium azide (32.5g, 0.5 moles) and water (32.5 ml). To this paste was added benzene (200 ml). The mixture was cooled below 10°C. Concentrated sulphuric acid (13.3 ml, 0.25 mole) was added keeping the temperature of the mixture below 10°C. The mixture was stirred and kept below 10°C for a further hour. The mixture was then cooled to 0°C and the organic layer was decanted, dried (Na_2SO_4) and filtered. The concentration of hydrazoic acid was determined by titration against 1M NaOH solution with phenolphthalein as the indicator.

(R)-[2-^{2}H]-1,4-Diaminobutane dihydrochloride

A solution of hydrazoic acid in benzene (0.8M, 4.5 ml, 3.6mmol) was added to (R)-[2-^{2}H]butane-1,4-diol (137mg, 1.5mmol) in dry THF (1ml). Then a solution of di-isopropylazodicarboxylate (667mg, 3.3mmol, 2.2 equivalents) in dry THF (1 ml) was added with stirring.

To this mixture was added Ph_3P (1.73g, 6.6 mmol, 4.4 equivalents) in dry THF (10 ml). The reaction temperature of the mixture was maintained at approximately 40°C during this addition. The reaction mixture was stirred for one hour at room temperature then heated to 50°C for three hours. Water (0.3ml) was added and the solution was heated at 50°C for a further three hours.

The solvents were removed in vacuo. The residue was partitioned between 1M HCL (10 ml) and CH_2Cl_2 (10 ml). The aqueous layer was evaporated in vacuo to yield a light brown solid which was crystallised from aqueous ethanol, 58%;R_f (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0.66;ν max (KBr disc) 3040, 1600 and 1500 cm^{-1}; $\delta_H(^2H_2O)$1.63 (3H,m), 2.94 (4H, m); $\delta_C(H_2O$,dioxane)24.41(C-2,t,J_{C-H}=19.6Hz), 24.65 (C-3), 39.67 (C-4), 39.71 (C-1); $\delta_D(H_2O)$1.58(s, ^2H content of ca 100%); m.p = > 300°C. Correct microanalysis data could not be obtained.

(S)-[2-^{2}H]-1,4-Diaminobutane dihydrochloride

The synthesis of (S)-[2-^{2}H]butane-1,4-diol followed the published procedure[127]. The synthesis of (S)-[2-^{2}H]-1,4-diaminobutane dihydrochloride followed the procedure outlined for (R)-[2-^{2}H]-1,4-diaminobutane dihydrochloride, 56%; Rf (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0.66; ν max (KBr disc) 3040, 1600, 1500 cm^{-1}; $\delta_H(^2H_2O)$1.63(3H,m), 2.94(4H,m); $\delta c(H_2O$,dioxane)24.4 (C-2t, J_{C-H}=19.5 Hz), 24.65 (C-3), 39.67 (C-4), 39.70 (C-1); $\delta_D(H_2O)$ 1.58 (s,^2H content of ca 98%); m.p.300°C. Correct microanalysis data could not be obtained.

Experimental to Chapter six

(R)- and (S)-[1-^{2}H]-1,4-Diaminobutane dihydrochloride

The synthesis of (R)-and (S)-[1-^{2}H]-1,4-diaminobutane dihydrochlorides followed the general procedure of Richards and Spenser[132] with minor changes.

(R)-[1-^{2}H]-1,4-Diaminobutane dihydrochloride

Anhydrous sodium carbonate (100 mg) was added to a solution of perdeuterioacetic acid (Aldrich, 99.7 atom % ^{2}H) in $^{2}H_2O$ (0.2M, 30 ml) under an argon atmosphere to obtain a buffer pH of 5.1. The solution was stirred under argon for one hour. After one hour L-ornithine monohydrochloride (Aldrich)(250 mg, 1.5mmol) [which had been stripped of exchangeable potons by repeated solution in $^{2}H_2O$ and evaporation to dryness (4 times)], and L-ornithine decarboxylase (Sigma,5 units were added).

The solution was incubated at 37 $\pm$ 1^{o}C for 72 hours with shaking. The mixture was acidifed with 4M HCl (10 ml) and heated at reflux for 30 minutes. The solution was filtered through a bed of celite 535 after cooling. The filtrate was evaporated in vacuo to yield a yellow solid which was dissolved in aqueous sodium hydroxide (2.5M, 10 ml) and extracted with n-butanol (10 x 5 ml). The combined extracts were acidified with 4M HCl and solvent was removed in vacuo to yield (R)-[1-^{2}H]-1,4-diaminobutane dihydrochloride. This was recrystallised from 50% aqueous ethanol with the addition of a few drops of acetone to yield white crystals, 58%; R_f(cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0:62; ν_{max}(KBr disc) 3040, 3010, 1650cm^{-1}; δ_H($^{2}H_2O$)1.72 (4H,m), 3.00 (3H,m); δ_C(H_2O,dioxane) 25.0(C-3), 25.1(C-2), 39.8 (C-1,t,J_{C-H}=21.9Hz), 40.1 (C-4); δ_D(H_2O) 2.87 (s, ^{2}H content of ca 95%); m.p.>300^{o}C. Correct micro-analysis data could not be obtained.

Diethyl 2-acetamido-2-(3-phthalimidopropyl)malonate

This was prepared by Dr. M. Rodgers by a published procedure.[136]

DL-[2-^{2}H]Ornithine monohydrochloride

Diethyl 2-acetamido-2-(3-phthalimidopropyl)malonate (784mg, 2mmol) was suspended in a solution of $^{2}H_2O$ containing ^{2}HCl (38% w/w in $^{2}H_2O$, 10 ml) in a 50ml r.b. flask fitted with a reflux condenser and a drying tube. The mixture was heated at reflux for 18 hours, cooled to 0°C and diluted with water (10 ml). Phthalic acid, which precipitated was filtered off and washed with water (2 x 1 ml). The combined fractions were concentrated in vacuo to yield a yellow residue which was repeatedly dissolved in water to remove exchangeable deuterium (4 times). The residue, crude ornithine dihydrochloride, was dissolved in hot 95% aqueous ethanol (8 ml). The solution was cooled to room temperature, filtered through a pad of celite 535, and pyridine was added (600 mg). DL-[2-^{2}H]Ornithine monohydrochloride which precipated was recrystallised from 50% aqueous ethanol with a few drops of acetone, 58.4%; Rf (cellulose, isopropanol-conc.ammonia (5:3).ninhydrin spray) 0.54; ν_{max} (KBr disc) 3100, 1630, 1580, 1480; $\delta_H(^{2}H_2O)$1.72 (4H,m), 2.89 (2H,t,J=7.66Hz); $\delta_C(^{2}H_2O$,dioxane) 23.5 (C-4), 28.0 (C-3), 39.7 (C-5), 54.4 (C-2,t,J_{C-H}=22.6 Hz), 174.6 (C-1); $\delta_D(H_2O)$ 3.62 (s,^{2}H content of ca 95%); m.p. 235-237°C (lit.value, 235-237°C). A sample of the monohydrochloride was converted into the 'free' amino acid,[158] m/z 133.0 (M^+,3.4%), 117.5 and 89.6(100%) (Found : M^+33.0965. $C_5H_{11}{}^{2}HN_2O_2$ requires M, 133.0961).

(S)-[1-^{2}H]-1,4-Diaminobutane dihydrochloride

Anhydrous analar sodium carbonate (100 mg) was added to aqueous acetic acid (0.2M,30ml) while stirring under argon to obtain a buffer pH of 5.1. The solution was stirred under argon for one hour and the pH was re-checked. Then DL-[2-^{2}H]ornithine monohydrochloride (500 mg, 3mmol)[which had previously been stripped of exchangeable protons by immersion in $^{2}H_{2}O$ (4 times) and evaporation in vacuo], and L-ornithine decarboxylase (5 units) were added.

The solution was incubated at 37±1^{o}C for 72 hours with shaking. The mixture was acidified with 4M HCl (10 ml) and heated at reflux for 30 minutes. The solution was filtered through a bed of celite 535 after cooling. The filtrate was concentrated in vacuo to leave a yellow solid which was dissolved in aqueous sodium hydroxide (2.5M, 10 ml) and extracted with n-butanol (10 x 5 ml). The extracts were combined and HCl gas was bubbled through until the solvent was saturated. A white solid which precipitated was filtered and was recrystallised from 95% aqueous ethanol to yield white crystals, 42.3%, R_{f} (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0.62; Further spectroscopic analysis was identical to that of (R)-[1-^{2}H]-1,4,-diaminobutane dihydrochloride. No correct micro-analytical data were obtained.

Experimental to Chapter Seven

The synthesis of (R)- and (S)-[1-^{2}H]cadaverine dihydrochlorides was carried out by Dr A M Brown using known procedures[132].

Experimental to Chapter Eight

N-Methyl-1,4-diaminobutane dihydrochloride

The synthesis of N-methyl-1,4-diaminobutane dihydrochloride followed the publication of Frydman and co-workers[144].

N-Methyl-N-(3-cyanopropyl)benzylamine

The synthesis of N-methyl-N-(3-cyanopropyl)benzylamine was carried out by the published procedure[144]. In addition the crude product was purified by flash chromatography on Kieselgel 60 (230-400 mesh) using a $CHCl_3$/MeOH solvent gradient to yield a clear oil in 81% yield. It gave the published spectroscopic data and R_f (silica, chloroform-methanol (50:50), iodine stained) 0.61; m/z 188 (3.4%), 134 (27.8%), 91 (100%); (Found: M^+ 188.1314. $C_{12}H_{16}N_2$ requires M, 188.1314).

N-Methyl-1,4-diaminobutane dihydrochloride

The synthesis of Frydman and co-workers[144] was followed in a yield of 12% and the product gave the following data in addition to those published R_f (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0.88; m.p 175-6^{o}C (lit value 170^{o}C).

N-Ethyl-N-(3-cyanopropyl)benzylamine

The published procedure was again followed[144]. The crude product was purified using the conditions outlined previously, 84.5%; R_f (silica, chloroform-methanol (50:50), iodine stained) 0.65; m/z 202 (2.1%), 148 (30.4%), 119 (67.1%), 91 (100%); (Found : M^+ 202.1467. $C_{13}H_{18}N_2$ requires M, 202.1470).

N-Ethyl-1,4-diaminobutane dihydrochloride

N-Ethyl-N-(3-cyanopropyl)benzylamine (200 mg, 1 mol) was dissolved in absolute ethanol (30 ml) and 36% HCl (1 ml). It was reduced with hydrogen over 10% palladium on charcoal (1.2 g) at atmospheric pressure for 17 hours. The solution was filtered through a pad of celite 535, and the solvent was removed in vacuo to yield a light brown residue which was crystallised from anhydrous methanol to yield white crystals, 5.3%; R_f (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin spray) 0.73; ν_{max} (KBr disc) 3400, 2805, 1625, 1590 cm^{-1}; δ_H (^2H_2O) 1.55 (3H,t,J=7.0Hz), 2.00 (4H,m), 3.31 (6H,m); δ_c (H_2O,dioxane) 23.48 (C-4), 24.90 (C-3), 33.70 (C-2), 34.42 (CH_3); 39.61 (C-1), 40.21 ($N-CH_2$); m.p 220-1°C (lit value, [144]218-220°C). No correct microanalysis data were obtained.

N-Benzylidenepropylamine and N-propyl-N-(3-cyanopropyl)benzylamine

The synthesis of Frydman and co-workers was again followed[144]. The N-propyl-N-(3-cyanopropyl)benzylamine was purified in an analogous manner to that described previously, 74%; R_f (silica, chloroform-methanol (50:50), iodine stained) 0.73; m/z 187 (9.2%), 162 (22.4%), 91 (100%); (Found M^+ : 216.1630. $C_{14}H_{20}N_2$ requires M,216.1626).

N-Propyl-1,4-diaminobutane dihydrochloride

Hydrogentaion at atmospheric pressure was used on the cyanopropyl compound to yield white crystals of N-propyl-1,4-diaminobutane dihydrochloride, 4.1%; R_f (cellulose, isopropanol-conc.ammonia (5:3) ninhydrin spray) 0.56; ν_{max} (KBr disc) 3400, 2805, 1625, 1590 cm^{-1}; δ_H (^2H_2O) 1.34 (3H,t,J=7.2Hz), 2.10 (6H,m), 3.30 (6H,m); m.p 270°C (lit.value,[144] 272°C). No correct micranalysis data could be obtained.

First attempted synthesis of N-ethyl-1,4-diaminobutane dihydrochloride

N-Ethyl-N-benzyl-1,4-diaminobutane

N-ethyl-N-(3-cyanopropyl)benzylamine (2.02g, 10 mmol) and dry THF (1 ml) were heated to reflux under a nitrogen atmosphere. Boron-dimethysulphide complex in THF (11 ml, 2M, 22 mmol) was added slowly over 5 min. Dimethylsulphide was allowed to distil from the mixture. The residue was left for 15 min then it was cooled to room

temperature and 6M HCl (12 ml, 27 mmol) was addded dropwise (hydrogen gas was evolved). The reaction mixture was heated under reflux for 30 min. The resultant clear solution was cooled to $0^{o}C$ and NaOH (2.7g, 54mmol) was added. The aqueous phase was saturated with K_2CO_3 and the diamine was extracted with Et_2O (3x10ml). The organic extracts were dried (K_2CO_3), filtered and the solvent was removed in vacuo. Attempted purification of the resultant oil was carried out by flash chromatography on neutral aluminium oxide with a chloroform-methanol solvent gradient. No separation was achieved.

N-Ethyl-1,4-diaminobutane

N-Ethyl-N-benzyl-1,-4-diaminobutane (crude) (292 mg, 1.44mmol), ammonium formate (432mg, 7mmol), 10% palladium on charcoal (500 mg) and methanol (30ml) were mixed and heated at reflux while the reaction was followed by t.l.c. No change of the benzyl group was indicated over 8h. The catalyst was removed by filtration through a pad of celite 535 and the solvent was removed by distillation to yield a clear oil which was shown to be starting material.

Second attempted synthesis of N-ethyl-1,4-diaminobutane dihydrochloride

N-Benzyloxycarbonyl-4-aminobutan-1-azide

The procedure of Golding and co-workers[145] was carried out to yield a product with the correct spectroscopic data.

Attempted synthesis of N-benzyloxycarbonyl-N'-ethyl-1,4-diaminobutane dihydrochloride

Triphenylphosphine (1.2g, 4.6 mmol) was added to N-benzyloxycarbonyl-4-aminobutan-1-azide (1.0g, 40mmol) in dry Et_2O (15 ml). The mixture was stirred under nitrogen for one hour at ambient temperature. Acetaldehyde (352 mg, 8 mmol) was added and the reaction mixture was stirred for a further 5 min. The solvent was removed to yield a yellow oil which was dissolved in dry ethanol (20ml). Sodium borohydride (0.45g, 12mmol) was added slowly and the resultant mixture was sitrred at room temperature overnight. 1M Hydrochloric acid (5 ml) was added dropwise. The solvents were removed and the resultant white solid was dissolved in 1M hydrochloric acid (20ml). The acidic solution was washed with dichloromethane (2x20ml). The combined organic layers were extracted with 1M hydrochloric acid (4x20ml). The pH of the combined aqueous layers was altered to 14 with 4M sodium hydroxide solution. The aqueous layers were extracted with dichloromethane (10x30ml) to yield a yellow oil which was shown to contain the starting azide.

Third attempted synthesis of N-ethyl-1,4-diaminobutane dihydrochloride 4-phthalimidobutanenitrile

Sodium cyanide (490mg, 10mmol) and sodium iodide (150mg, 1mmol) were dissolved in dry DMSO (40 ml) and heated to 90°C under a nitrogen atmosphere. N-(3-Bromopropyl)phthalimide (Aldrich)(2.44g, 9mmol) in DMSO (10ml) was added with stirring. The mixture was maintained

at 85-90°C for 3 hours. The solution was allowed to cool to room temperature overnight and Et_2O (120 ml) was then added. The mixture was washed with H_2O (6x25ml) and saturated brine solution (2x25ml). The combined aqueous fractions were extracted with Et_2O (2x25ml). The combined aqueous fractions were extracted with Et_2O (2x25ml). The combined organic fractions were dried (Na_2SO_4), filtered and the solvent removed in vacuo to yield a white solid which was purified by flash chromatography on Kieselgel 60 (230-400 mesh) using a pet.ether (b.p. 40-60°C)-ethyl acetate solvent gradient, 69%; R_f(silica, $CHCl_3$) 0.23; ν_{max} 2225, 1775 and 1715 cm^{-1}; δ_H 2.02 (2H, dt, J_{AB} = 6.6Hz, J_{CD} = 6.6Hz), 2.39 (2H t, J = 7.9 Hz), 3.76 (2H, t, J = 6.7 Hz), 7.76 (4H,m); δ_c 14.9 (C-3), 24.6 (C-2), 36.4 (C-4), 118.7 (CN), 123.2 (CH(Ar)), 131.6 (C(Ar)), 134.1 (C-4), 118.7 (CN), 123.2 (CH(Ar)), 131.6 (C(Ar)), 134.1 (CH(Ar)), 166.0 (C = 0); m/z 214 (12%), 161 (21%), 160 (100%); (Found: M^+ 214.0739; C, 66.97; H, 4.61; N 12.89. $C_{12}H_{10}N_2O_2$ requires M, 214.0742; C, 67.27; H,4.71; N, 13.08%).

Diethyloxycarbonium tetrafluoroborate[159]

This was prepared by adapting the procedure of the above reference. Boron-trfluoride etherate (15 ml, 0.12 mol) in dry CH_2Cl_2 (12ml) was cooled to -30°C under a nitrogen atmosphere. Triethyl orthoformate (16.5ml, 100mmol) was added and the mixture was cooled to -50°C. The mixture was filtered at -40°C and the solid residue was washed with CH_2Cl_2-Et_2O (20 ml, 1:1). The organic solution was again filtered at -40°C. The solvent was removed in vacuo to yield a hygroscopic orange solid which melted between 0-10°C, 65%; δ_H (30°C) 1.60 (6H, t, J_{AB} = 6.3Hz), 5.05 (4H,m), 9.05 (1H,s). No other chracterisation data were obtained due to the instability of the product.

N-Ethyl-N'-phthalimido-1,4-diaminobutane

A solution of 4-phthalimidobutanenitrile (473 mg, 2.25mmol) in dry CH_2Cl_2 (5ml) was added to a solution of diethyloxycarbonium tetrafluoroborate (780mg, 4.5mmol) in dry CH_2Cl_2 (5ml) under a nitrogen atmosphere. The resultant solution was heated at reflux for 19 hours then cooled to $0^{\circ}C$. Absolute ethanol (0.5ml) was added. The solvents were removed in vacuo. The amorphous residue was dissolved in methanol (7ml). Sodium borohydride (500mg, 13.5mmol) was added in small portions at $0^{\circ}C$. After stirring at $0^{\circ}C$ for one hour, the solution was brought to pH 1 with 6M HCl. The methanol was removed in vacuo. The residue was dissolved in H_2O (5ml) and the solution was adjusted to pH 10 with 6M NaOH, saturated with NaCl, and extracted with Et_2O (4x10ml). The combined organic extracts were dried ($MgSO_4$), filtered and solvent removed by distillation to yield a white solid. Analysis of this white solid by t.l.c and ^1H n.m.r. spectroscopy showed the presence of starting material and the imine produced during the course of the reaction.

Hydrogenation of the imine synthesised above

The imine (288mg, 1mmol) was added to EtOAc (40ml) containing Adams' catalyst (PtO_2)(29mg, 10% by weight). The solution as hydrogenated at an initial pressure of 3 atmospheres for 12 hours. The solution was filtered through a pad of celite 535 and the solvent was removed in vacuo to yield a pale yellow oil. Analysis by t.l.c and ^1H n.m.r spectroscopy showed no reduction had taken place.

Fourth attempted synthesis of N-ethyl-1,4-diaminobutane dihydrochloride-N-t-Butoxycabonylethylamine

A solution of ethylamine (450mg, 10mmol) in dry THF (10ml) was added to di-t-butyl dicarbonate (2.4g, 11mmol) in dry THF (15ml) under a nitrogen atmosphere. The mixture was stirred at 25°C for 16 hours. The solvent was removed in vacuo to yield a white solid which was re-crystallised from $CHCl_3$, 77%; R_f(silica, ether)0.74; ν_{max} 3450, 1710 and 1510cm^{-1}; δ_H 1.05 (3H, t, J = 9.4Hz), 1.38 (9H,s), 3.09 (2H, 9, J = 9.2Hz), 4.60 (1H, br s); δ_c 15.2 (C-1), 27.3, 27.9, 28.3 (tertiary CH_3), 35.3 (CH_2N), 78.8 (quaternary C), 155.8 (C=O); m.p = 40-2°C; m/z 145 (0.1%), 57 (100%), 44 (14.1); (Found: M^+45.1114. $C_7H_{15}NO_2$ requires M, 145.1103).

N-t-Butoxycarbonyl-N-(3-cyanopropyl)ethylamine

To a stirred suspension of sodium hydride (80% in oil, 106mg, 3.5mmol) in dry THF (12ml) was added N-t-butoxycarbonylethylamine (508mg, 3.5mmol). The mixture was stirred for 30 minutes and 4-bromobutanenitrile (518mg, 3.5mmol) was added. The reaction mixture was heated at 60°C for 12 hours before cooling to room temperature. The reaction mixture was filtered through a pad of celite 535 which was washed with Et_2O (2x25ml). The solvent was removed in vacuo to yield a yellow oil. Analysis by t.l.c. showed this yellow oil to be a mixture of starting materials.

4-Bromo-N-t-butoxycarbonylbutylamine

N-t-Butoxycarbonyl-4-aminobutan-1-ol (275 mg, 1.45 mmol) in dry pentane (2 ml) was cooled to 0°C. Phosphorous tribromide (69 µl, 0.725 mmol) was added dropwise. After 30 minutes MeOH (1.25 ml) was added. The reaction mixture was washed with H_2O (5 ml), 10% $NaHCO_3$ (5 ml) and saturated brine solution (5 ml). The organic solution was dried (Na_2SO_4), filtered, and solvent was removed in vacuo to yield a yellow oil which was purified by flash column chromatography on Kieselgel 60 (230 - 400 mesh) using a Et_2O/EtOAc solvent gradient, 45%; R_f(silica, diethyl ether, iodine stain) 0.21; ν_{max} 3450, 1710 cm^{-1}; δ_H 1.25 (1H, br s), 1.45 (9H, s), 1.75 (4H, m), 3.15 (2H, t, J = 8Hz), 3.5 (2H, t, J + 8Hz); m/z 252 (4.8%), 171 (23.2%), 57 (100%); (found: M^+ 252.1669 $C_9 H_{18} NO_2 Br$ requires M, 252.1663).

[2,2,5,5-$^2H_4$]-1,6-Diaminotexane dihydrochloride and
[2,2,6,6,-$^2H_4$]-1,7-diaminoheptane dihydrochloride
[1,1,4,4-$^2H_4$]-1,4-Dicyanobutane

^2H_2O (35 ml, 99.8 atom % ^2H), dioxane (10 ml) and DBU (694 mg, 4.56 mmol) were added to 1,4-dicyanobutane (4.93 g, 45.6 mmol). The mixture was heated at reflux with stirring for 24 hours. The mixture was evaporated to dryness in vacuo. ^2H_2O (35 ml), dioxane (10 ml) and DBU (694mg, 4.56mmol) were again added to the residue which was heated at reflux for a further 24 hours. The solvent was removed in vacuo and 1M HCl was added to the residue (until acidic) and the solution wash extracted with $CHCl_3$ (5 x 25 ml).

The organic extracts was dried (Na_2SO_4), filtered, and the solvent was removed in vacuo to yield a clear oil which was distilled, b.p. 295°C; 58.3%; R_f (silica, chloroform, iodine stain) 0.41; ν_{max} 2245 cm^{-1}; δ_H 1.85 (s); δ_c 15.9 (C-2,C-5 quintet, $J_{\beta C-^2H}$ = 20.7 Hz, triplet, J_{C-H} = 20.7 Hz) 23.3 (C-3, C-4), 118.7 (C-1, C-6); δ_D 2.33 (s) ($^2H_4$ content of ca 94%); m/z 112 (1.4%), 70 (100%), 56 (55.2%), 42 (36.8); (found: M^+ 112.0941 $C_6H_4{}^2H_4N_2$ requires 112.0939).

[2,2,5,5-$^2H_4$]-1,6-Diaminohexane dihydrochloride

A solution of (1,1,4,4-$^2H_4$)-1,4-dicyanobutane (1.1 g, 10 mmol) in dry THF (1 ml) was heated to reflux under a nitrogen atmosphere. Borone-dimethylsulphide complex (11 ml, 2 M, 22 mmol) was added dropwise over 5 minutes. The dimethyl sulphide was allowed to distil from the reaction mixture. After 30 minutes the distillation of dimethyl sulphide was complete. The reaction mixture was cooled to room temperature and 6M HCl (12 ml, 72 mmol) was added dropwise (hydrogen was evolved). The reaction mixture was heated at reflux for 30 minutes. The clear solution was cooled to 0°C and NaOH (2.7 g, 54 mmol) was added. The aqueous phase was saturated with K_2CO_3 and extracted with Et_2O (3 x 10 ml). The organic extracts were combined and HCl gas was bubbled through until saturation of the etheral extracts was achieved. A white solid precipitated and was recrystallised from 50% aqueous ethanol, 72.3%; R_f (cellulose, isopropanol-conc. ammonia (5:3), ninhydrin spray) 0.26; ν_{max} (KBr disc) 3000, 1600 and 1500 cm^{-1}; δ_H (^2H_2O) 1.35 (4H,s), 2.94 (4H, s); δ_c 25.6 (C-3, C-4), 26.5 (C-2, C-5, quintet, $J_{\beta C-^1H}$ = 18.8 Hz), 40.1 (C-1, C-6); δ_D (^2H_2O) 1.53 ($^2H_4$ content of ca 94.2%).

The product was derivatised as its N-phenylaminothiocarbonyl derivative, m/z 390 (0.2%), 297, 238, (100%); (Found: M^+ 390.1857. $C_{30}H_{22}{}^2H_4N_4S_2$ requires M, 390.1850).

[1,1,5,5-$^2H_4$]-1,5-Dicyanopentane

The exhchange process outline above was followed with 1,5-dicyanopentane to give the title product b.p. = 175-6°C/14 mmHg 53.4%; R_f (silica, chloroform, iodine stain) 0.58; ν_{max} 2250 cm^{-1}; δ_H 1.55 (6H, m); δ_C 16.6 (C-2,C-6 quintet, $J_{^{13}C-^2H}$ = 20.6 Hz, triplet, $J_{^{13}C-^2H}$ = 20.7 Hz) 24.4 (C-4), 27.5 (C-3, C-5), 119.5 (C-1, C-7); δ_D 2.37 (s) ($^2H_4$ content of ca 95.3%); m/z 126 (0.8%), 85, 84, 70, 57 (100%); (Found: M^+ 126.1082 $C_7H_6{}^2H_4N_2$ requires 126.1095).

[2,2,6,6-$^2H_4$]-1,7-Diaminoheptane dihydrochloride

The borane reduction outlined above was again followed to give the title product 89%; R_f (cellulose, isopropanol-conc. ammonia (5:3), ninhydrin spray), 0.82; ν_{max} 3040, 1600 and 1500 cm^{-1}; δ_H 1.27 (6H, s), 2.88 (4H, m); δ_C 26.3 (C-4), 27.0 (C-2, C-6, quintet, $J_{^{13}C-^2H}$ = 19.3 Hz), 40.5 (C-3, C-5); 48.6 (C-1, C-7); δ_D (^2H_2O) 1.48 ($^2H_4$ content of ca 90.2%). The diamine was converted into its N-phenylaminothiocarbonyl drivative, m/z 404 (0.2%), 311, 251 (100%); (Found: M^+ 404.2009 $C_{21}H_{24}N_4S_2$ requires M, 404.2006).

(±)-1,4-Diamino-2-hydroxybutane dihydrochloride

The general synthesis of Macholan [151] was followed in good yield and the product gove the correct analytical data.

Diethyl (S)-(-)-malate

(S)-(-)-Malic acid (8 g, 60 mmoles) has dissolved in a rapidly stirred solution of EtOH (25 ml). Toluene (16 ml), conc.HCl (0.2 ml) and a further aliquot of EtOH (10 ml) were added to the mixture. The reaction mixture was heated at 40°C for 8 hours. The azeotropic mixture of EtOH, toluene and water was distilled off under reduced pressure below 50°C. Toluene (16 ml) and EtOH (25 ml) were added to the residue and the solution was heated at 40°C for a further 8 hours. The solvent was removed in vacuo and the residue was distilled, b.p. 120°C/0.3 mm Hg; 92.7%; R_f (silica, chloroform, iodine stain) 0.3; ν_{max} 3480, 1740 cm^{-1}; δ_H 1.20 (6H, q, J = 7 Hz), 2.76 (2H,dd,J_a = 4.7 Hz, J_b = 5.9 Hz), 3.36 (1H, br s), 4.16 (4H, dq, J_{AB} = 7.2 Hz, J_{CD} = 7.2 Hz), 4.43 (1H,dd,J_{AB} = 5.8 Hz, J_{CD} = 5.8 Hz); δ_c 13.9 (CH_3), 39.6 (CH_2), 60.8 (CH_2-O), 61.8 (CH_2-O), 67.2 (quaternary C), 170.4 (C = O), 173.2 (C = O); m/z 190 (8.6%), 117 (96%), 71 (100%); (Found: M^+ 190.0846 $C_8H_{14}O_5$ requires M, 190.0841).

Diethy (S)-2-(2-methoxyethoxymethylhydroxy)butane-1,4-dioate

Hunig's base (3.87 g, 29.5 mmol) was added to diethyl (S)-(-)malate (3.75 g, 20 mmol) in dry CH_2Cl_2 (20 ml) under a nitrogen atmosphere. 2-Methoxyethoxymethyl chloride (3.68 g, 29.5 mmol) was added slowly at 0°C. The solution was allowed to warm to room temperature and the reaction was followed by t.l.c. When the reaction was complete the solution was washed with 1M HCl, water, saturated aqueous $NaHCO_3$ and a further aliquot of water.

The organic solution was dried (Na_2SO_4), filtered and solvent was removed in vacuo to yield an oil which was purified by flash chromatography on Kieselgel 60 using a pet.ether (40 - 60°C)-EtOAC solvent gradient, 62%; R_f (silica, ethyl acetate, iodine) 0.66; ν_{max} 2820, 1735, 1070 cm^{-1}; δ_H 1.14 - 1.24 (6H, dt, J_{AB} = 7.16 Hz, J_{CD} = 7.16 Hz), 2.71 (2H,d,J = 6.29 Hz), 3.30 (3H, s), 3.46 (2H, m), 4.09 (4H,dq,J_{AB} = 7.11 Hz, J_{CD} = 6.95 Hz); 4.49 (1H, t, J = 6.43 Hz), 4.74 (2H, m); δ_c 13.9(CH_3), 37.7 ($\underline{C}H_2$-CO), 58.8 (CH_3-O), 60.7 ($\underline{C}H_2CH_3$), 61.1 ($\underline{C}H_2$-CH_3), 67.3 ($\underline{C}H_2$-OCH_3), 71.4 ($\underline{C}H_2$-CH_2OCH_3), 71.6 ($\underline{C}$H-CO), 95.1(O-$\underline{C}H_2$-O), 169.8 (C=O); m/z 278 (0.2%), 145, 117 (100%); (Found: M^+ 278.1362 $C_{12}H_{22}O_7$ requires M, 278.1366).

(S)-2-(2-Methoxyethoxymethylhydroxy)butan-1,2,4-triol

A solution of diethyl (S)-2-(2-methoxyethoxymethylhydroxy)butan-1,4-dioate (695 mg, 2.5 mmol) in dry toluene (6.5 ml) was cooled to -30°C under a nitrogen atmosphere. With stirring, DIBAL-H (7.5 ml, 1.5 M in toluene) was added. The mixture was stirred for 30 minutes at 30°C and then allowed to warm to 0°C. Ethyl acetate (0.5 ml) was added, and the mixture was poured onto a suspension of celite 535 (3 g) in acetone (8 ml). Methanol (3 ml) was added cautiously with vigorous stirring until a gel formed. The gel was left for 1 hour then water (6 ml) has added to break up the gel. The mixture was filtered, washed with methanol (3 x 10 ml) and water (2 x 10 ml), and solvent was then removed in vacuo.

The crude oil was purified by distillation, b.p. 175°C/0.2 mm Hg; 53% ν_{max} 3460, 2820, 1040 cm^{-1}; δ_H 1.75 (2H, m), 3.05 (2H, s), 3.35 (3H,s), 3.45 - 3.85 (9H, m), 4.80 (2H, m); δ_c 35.6 ($\underline{C}H_2$-C-O), 59.0 (CH_3), 59.3 ($\underline{C}H_2$-OH), 65.4 ($\underline{C}H_2$-OH), 65.4 ($\underline{C}H_2$-OH), 68.1 ($\underline{C}H_2$-OCH_3), 72.8 (CH_2-O), 77.5 (CH), 96.2 (O$\underline{C}H_2$O).

(S)-2-Hydroxy-1,4-diaminobutane dihydrochloride

A solution of hydrazoic acid in benzene (0.9M, 4.4 ml, 4 mmol) was added to (S)-2-(2-methoxyethoxymethylhydroxy)butan-1,2,4-triol (324 mg, 1.67 mmol) in dry THF (1 ml). Then a solution of di-isopropylazadicarboxylate (740 mg, 3.67 mmol, 2.2 equivalents) in dry THF was added with stirring.

To this mixture was added Ph_3P (1.92 g, 7.3 mmol, 4.9 equivalents) in dry THF (10 ml). The reaction temperature of this mixture was maintained at approximately 40°C during this addition. The reaction mixture was stirred for 1 hour at room temperature then heated to 50°C for 3 hours. Water (0.3 ml) was added and the solution heated at 50°C for a further 3 hours.

The solvents were removed in vacuo. The residue was partitioned between 1M HCl (10 ml) and CH_2Cl_2 (10 ml). The aqueous layer was concentrated in vacuo to yield a light brown solid which was crystallised from aqueous ethanol, 5%; R_f (cellulose, isopropanol-conc.ammonia (5:3), ninhydrin) 0.27; ν_{max} 3440, 3030, 1600, 1040 cm^{-1}; δ_H 1.70 - 1.90 (2H, m) 2.86 - 3.17 (4H, m), 3.97 (1H, m); δ_c 31.43 (CH_2), 36.80 (CH_2), 44.5 (CH_2), 65.7 (CH - OH).

(S)-2-Butane-1,2,4-triol

(S)-Malic acid (2.68g, 20 mmol) in dry THF (20ml) was heated to reflux under a nitrogen atmosphere. BH_3.THF (1M, 41ml, 41mmol) was added dropwise and heating was continued for a further 18 hours. The reaction mixture was cooled to room temperature then water (20 ml) was added. The aqueous layer was saturated with K_2CO_3 (ca 4g). Et_2O (10ml) was added to the reaction mixture. The reaction mixture was extracted with Et_2O (4x20ml). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated in vacuo to yield a thick oil which was purified by distillation, b.p 112°C/0.1mmHg; 17%; ν_{max} 3460, 1040 cm^{-1}; δ_H 1.65 (4H, m), 3.45 (2H, m), 3.60 (3H, s), and 4.10-4.50 (1H, m).

Diethyl(R)-fluourosuccinate

To a stirred solution of diethylaminosulphur trifluouride (DAST) (1.0g, 6.2 mmol) in dry ethanol-free chloroform 95ml) cooled to 0°C was added dropwise over 15 minutes a solution of diethyl (2S)-malate (1.0g, 6.2 mmol) in dry ethanol-free chloroform (5ml). The mixture was allowed to reach ambient temperature and water (10ml) was added slowly. The organic layer was separated, washed with satured $NaHCO_3$ solution and saturated brine solution. The organic layer was dried (Na_2SO_4), filtered and concentrated in vacuo to yield a clear oil which was purified by distillation, b.p 150°C/1.5mmHg; 90.2%; R_f (silica, chloroform-methanol (50:50), iodine stain) 0.86; ν_{max} 1740, 1370cm^{-1}; δ_H 1.23 (6H, dt, J_{AB} = 7.13Hz), 2.72-3.05 (2H,m), 4.17 (4H, dq, J=7.17Hz), 5.08 and 5.33 (1H,ddd, $^2J_{HF}$ = 47.34Hz, $^2J_{H-H}$=6.34Hz,

$^{3}J_{H-H}$=5.08Hz); δ_c 3.8 (CH_3), 37.1 (CH_2, d, $^{2}J_{C-F}$ = 23.04Hz), 61.1 (CH_2), 61.8 (CH_2), 84.7 (CH, d, J_{C-F} = 186.4 Hz), 167.9 (C=O,d,$^{4}J_{CF}$=10.2Hz), 167.7 (C=O,d,$^{3}J_{C-F}$ = 15.8Hz); δ_F 1919.6 (1F,ddd,$^{2}J_{H-F}$=35.9Hz, $^{3}J_{H-F}$=22.5Hz, $^{3}J_{H-F}$ = 24.1 Hz); m/z 192 (7.4%), 147, 127, 119, 29 (100%); (Found: M^+, 192.0799.$C_8H_{13}O_4F$ requires M, 192.0798).

Attempted synthesis of(R)-2-fluorobutan-1,4-diol

A solution of diethyl (R)-fluorosuccinate (480mg, 2.5mmol) in dry toluene (6.5ml) was cooled to -30°C under a nitrogen atmosphere. With stirring, DIBAL-H (7.5ml, 1.5M in toluene) was added. The mixture was stirred for 30 minutes at 30°C and was then allowed to warm to 0°C Ethyl acetate (0.5ml) was added, and the mixture was poured onto a suspension of celite 535 (3g) in acetone (8ml). Methanol (3ml) was added cautiously with vigorous stirring until a gel formed. The gel was left for one hour then water (6ml) was added to break up the gel. The mixture was filtered, washed with methanol (3x10ml), and water (2x10ml), and the solvent was removed in vacuo. Distillation was attempted but resulted in a black tar.

Attempted synthesis of(S)-2-chloro-1,4-diaminobutane dihydrochloride

The general synthesis of Golding and co-workers[157] was followed in this attempted synthesis using (S)-2-chlorobutane-1,4-diol as the alcohol to yield the proposed phosphorus containing compound.

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