The biosynthesis of the tropane alkaloid hyoscyamine in datura stramonium

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DECLARATION

The work contained in this thesis was carried out in the Department of Chemistry at the University of Durham between October 1995 and September 1998. All the work was carried out by the author, unless otherwise indicated. It has not been previously submitted for a degree at this or any other university.

ABSTRACT

The Biosynthesis of the Tropane Alkaloid Hyoscyamine in *Datura stramonium*

Chi W. Wong, B.Sc.

This thesis investigates the biosynthesis of the tropane alkaloid hyoscyamine in *Datura stramonium*.

The biosynthesis of the medicinally important tropane alkaloid hyoscyamine has been investigated for many years. However, the complete biosynthetic pathway is still to be unravelled. This thesis concentrates primarily on the last step of hyoscyamine biosynthesis – the rearrangement of littorine to hyoscyamine. In Chapter 1, an introduction to the biosynthesis of hyoscyamine and other related alkaloids is discussed. In Chapter 2, ^{18}O-labelling studies are utilised in order to probe the nature of the rearrangement. The results reveal that intriguingly that the rearrangement is indirect and perhaps involves an aldehyde intermediate, which requires to be reduced by a dehydrogenase to furnish hyoscyamine. In Chapter 3, aryl substituted fluorophenyllactates were used in order to probe the mechanism of the rearrangement of littorine to hyoscyamine. The working hypothesis suggests the involvement of a carbocation intermediate following literature reports on chemical and enzymatic models. The results reveal that the mechanism of the rearrangement is not as clear cut as predicted, and that perhaps binding affinities to the littorine synthase and littorine mutase affect the efficiency of the rearrangement. In Chapter 4, attention turned back to the tropane moiety of hyoscyamine. Its biosynthesis has been thoroughly investigated although there still remains the mystery as to how acetate units are condensed with the N-methylpyrrolinium salt to generate the bicyclic tropane ring.

To Mum and Dad

"One small step for man.....

One giant leap for mankind"

Neil Armstrong

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ABBREVIATIONS

ADC	Arginine decarboxylase
ATP	Adenosine triphosphate
br	Broad
B:	Base
CoA	Co-enzyme A
d	Doublet
dd	Doublet of doublets
D.	*Datura*
DAST	Diethylaminosulphur trifluoride
Diazald®	N-methyl-N-nitroso-*p*-toluenesulphonamide
EI	Electron impact
Fhyo	Fluorohyoscyamine
Flit	Fluorolittorine
Fpla	Fluorophenylactate
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
Hyo	Hyoscyamine
i.d.	Internal diameter
Lit	Littorine
m	Multiplet
M	Molecular ion
Me	Methyl
m.p.	Melting point
MPO	N-methylputrescine oxidase
MS	Mass spectroscopy
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
m/z	Mass/charge
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NMR	Nuclear magnetic resonance
ODC	Ornithine decarboxylase
OTMS	Oxytrimethylsilyl
PAL	Phenylalanine ammonia lyase

PCC	Pyridinium chlorochromate
Ph	Phenyl
Pi	Phosphate (inorganic)
PPi	Pyrophosphate (inorganic)
PMT	Putrescine methyltransferase
Ri	Root inducing
Rt	Retention time
s	Singlet
SIM	Single ion monitoring
sMMO	Soluble methane monooxygenase
S_N1	Nucleophilic substitution, unimolecular
S_N2	Nucleophilic substitution, bimolecular
t	Triplet
^tBu	Trimethylbutyl
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TMSCN	Trimethylsilylcyanide
TR I	Tropinone reductase I
TR II	Tropinone reductase II

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Chapter 1

1. Introduction

1.1 Forward

Over the years, man has discovered how plant extracts can be used to heal and kill. Folklore commonly refers to the healing properties of certain plant extracts, along with agents of death such as the curare poisons used to tip the hunting arrows of the South American natives.[1]

In recent times, man has isolated organic compounds from micro-organisms and plants that are useful in the battle against disease. The penicillin and tetracycline antibiotics are good examples. These natural compounds are known as secondary metabolites.

1.2 Primary and Secondary Metabolites

Primary metabolism is fundamental to all cells and begins from the biochemical enzyme catalysed reactions of CO_2 and photosynthesis to produce, for example, amino acids, acetyl-co-enzyme A, mevalonic acid, sugars, and nucleotides essential for survival.

Secondary metabolites are produced from precursors of the primary metabolism network. They differ from primary metabolites by virtue of their restricted distribution. They are mostly found in plants and microbes usually of specific genera, species or strains, and they are normally formed from specialised pathways from primary metabolites. Unlike primary metabolites, secondary metabolites are not generally essential to life although they are important to the organism that produces them. Although many aspects of their origin and function remain a mystery in most cases, some secondary metabolites, for example, those produced by plants, can be used to deter predators and thus assist survival in harsh environments.

1.3 Introduction to the Tropane Alkaloids

The tropane alkaloids L-hyoscyamine (**1**), atropine [(**2**) (a mixture of both L- and D-enantiomers of hyoscyamine)], and scopolamine [(**3**) (the 6,7-epoxide of L-hyoscyamine)] are pharmaceutically important anticholinergic compounds (fig. 1). These alkaloids are commonly found in the plant family Solanaceae, and *Atropa belladonna* (deadly nightshade), *Datura stramonium* (jimsonweed, thorn apple), and *Hyoscyamus niger* (henbane),[2] are three commercial sources.

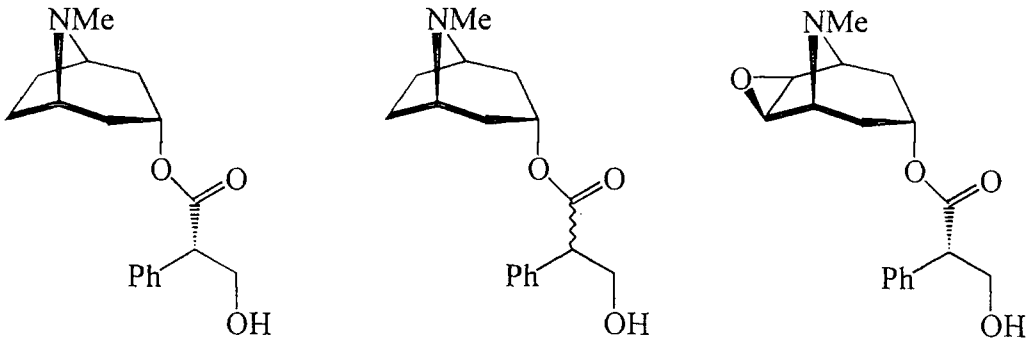

Figure 1 The tropane alkaloids hyoscyamine (**1**), atropine (**2**) and scopolamine (**3**) are commonly found in the Solanaceae.

Their use as poisons, hallucinogens and medicines have been widely adopted by ancient pharmacists and they have a long history in magic and witchcraft.[3,4] In ancient Greece, it was widely believed that people who inhaled the smoke from smouldering henbane became prophetic, like the priestesses of the Delphic oracle, who claimed to be regular users. It is known that the juice from a single berry from deadly nightshade squeezed onto food or drink would contain enough toxins to cause death. The juice from *Atropa belladonna* berries has been used to create a "doe-eyed beauty" look for Renaissance ladies by dilating their pupils – hence the name "belladonna". The main active constituent in these berries is atropine (**2**), which has been used in modern ophthalmology for the same purpose, since a fully dilated pupil allows easier examination of the retina.

Mandrake (*Mandragora officinarum*), is another member of the Solanaceae family that has scopolamine (**3**) as a major active constituent and has a wonderful folklore associated with its historical use. It has a Y-shaped root which has been associated with enhancement of fertility, since it appeared to resemble the human form (fig. 2).[5] The root was also supposed to possess aphrodisiac properties, and was associated with Aphrodite the goddess of love. This highly prized root was heavily sought after, especially in medieval Europe and herbalists invented ever more fanciful legends to deter their collection. Of particular note, was one of the terrible shriek that would been given from the mandrake as it was pulled from the ground. This sound was supposed to be so awful that the collector upon hearing it would die. To avoid this fate, collectors used dogs to uproot the mandrake. They tied a rope around the dog's neck and attached the other end to the plant while the collector blocked his ears with wax.

Figure 2 An ancient illustration from the 15th century, depicting the mandrake's resemblance to the human form and its association with the dog.

The seeds and berries of *D. stramonium* are particularly poisonous and contain hyoscyamine (**1**) and scopolamine (**3**) as their major constituents. In toxic doses, it ensured insensibility before painless death although in smaller doses, it was used as a sedative. It got its common name from the early settlers near Jamestown, Virginia,

USA, when they mistook it for spinach and nearly avoided death. The plant became known as Jamestown weed or jimsonweed and was used for a wide variety of medicinal purposes such as mania and epilepsy. In the nineteenth century, it was sold in the form of herbal cigarettes by the Spanish Cigarette Company, and was said to have brought relief to those suffering from bronchial asthma and other respiratory conditions. A modern equivalent of this drug, ipratropium bromide (**4**),[6] which is a synthetic structural analogue of atropine (**2**) was developed and is now widely used as an effective inhaled anti-asthmatic drug (fig. 3).

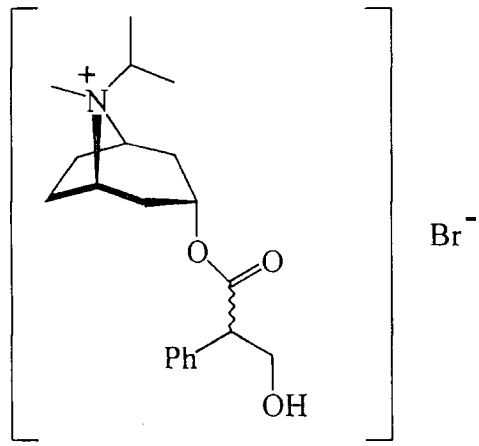

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Figure 3 Ipratropium bromide (**4**), a synthetic analogue of atropine (**2**) is used in modern medicine as an effective inhaled anti-asthmatic drug, and is marketed by the pharmaceutical company, Boehringer Ingelheim, under the proprietary name, Atrovent®.

Apart from their use in ophthalmology, atropine (**2**) and scopolamine (**3**) are also used in premedication prior to major surgery to dry bronchial and salivary secretions, which are increased by intubation and by some inhalational anaesthetics.[7] Scopolamine (**3**) is also used in the treatment of motion sickness and vertigo, although patients are usually advised that these types of drugs can have side effects such as drowsiness, a dry mouth, dizziness, blurred vision and difficulties with micturition.[7]

1.4 Techniques in Biosynthetic Studies

1.4.1 The use of transformed root cultures in biosynthetic studies

Much work has been carried out to understand how plants biosynthesise secondary metabolites. Researchers like Edward Leete,[8] who first started such investigations in the 1950's, were particularly fascinated by the biosynthesis of hyoscyamine (**1**) and related alkaloids in *Datura* plants. Before the development of root cultures, Leete fed isotopically labelled precursors to whole intact plants. However, these experiments required time and patience. Once fed, the plants were nurtured to maturity before any plant extract was isolated. Typically, low level incorporations were observed and experiments had to be repeated several times in order to draw satisfactory conclusions. Figure 4 shows an illustration of the plant, *D. stramonium*, which was used for tropane alkaloid biosynthetic studies in the 1950's.

Figure 4 *Datura stramonium* plant.

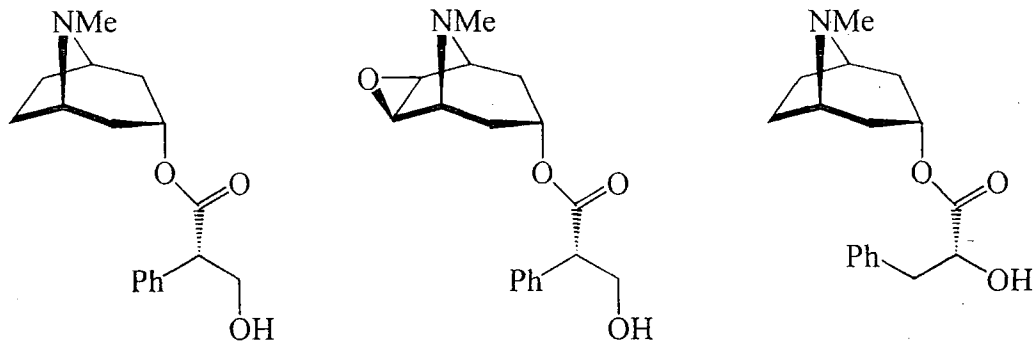
As further research in this area grew, it was discovered that the biosynthesis of these secondary metabolites occurred in the roots.[9] Later, advances in biotechnology developed fast growing "hairy root" cultures from these mature plants. These "hairy roots" are established by the infection of the natural plant pathogen *Agrobacterium rhizogenes*.[10,11] This microbe infects its host by transferring a piece of DNA known as a root inducing (Ri) plasmid into the plant cell nucleus. This transferred DNA expresses

the genes in the plant cell and induces it to behave as though it was a root cell. Thus, these fast growing transformed or transgenic "hairy roots" emerge from the site of inoculation and can be excised and cultured indefinitely once antibiotic treatment is used to kill any remaining bacteria. Figure 5 shows an illustration of a transformed root culture of *D. stramonium*, which was used in the biosynthetic experiments reported in this thesis.

Figure 5 Transformed root culture of *Datura stramonium*.

1.4.2 The transformed root cultures of *Datura stramonium*

Robins and co-workers[12,13] established a "hairy root" culture of *D. stramonium* by the infection of aseptic leaves with *Agrobacterium rhizogenes* LBA9402. After the removal of any remaining bacteria by antibiotics, the roots were maintained by subculturing into sterile Gamborg's B5 culture media[14] every 2-3 weeks. After screening numerous plants and lines of *D. stramonium*,[15] the root culture clone, designated D15/5, was used in biosynthetic studies as it produced the highest amount of alkaloid per fresh weight of plant root material. This clone biosynthesises hyoscyamine (**1**) as the major constituent, with littorine (**5**), the structural isomer of hyoscyamine, and scopolamine (**3**) as minor constituents and is particularly useful for the biosynthetic studies reported in this thesis (fig 6).[16]



1 3 5

Figure 6 The transformed root culture of *D. stramonium* (clone D15/5) produces hyoscyamine (**1**) as a major constituent along with littorine (**5**) and scopolamine (**3**) as minor constituents.

1.4.3 Radio- and stable isotope labelling as biosynthetic techniques

The main radioisotopes used in tracer experiments to investigate biosynthetic pathways are ^{14}C and ^{3}H. The use of radioisotopes is advantageous since it is a very sensitive technique and only trace amounts of radioisotope are needed. Detection by scintillation counting is straightforward and as ^{14}C and ^{3}H have half-lives of 5600 years and 12.26 years respectively, and any losses due to decay can be neglected. However, this technique does have its disadvantages. It is non-regiospecific and often requires the systematic chemical degradation of the investigated secondary metabolite, in order to find out where incorporation actually took place. Also, the use of radioisotopes is always accompanied by the dangers and problems of handling radioactive material.

The common stable isotopes used in biosynthetic studies of secondary metabolites are ^{13}C (natural abundance 1.1% of ^{12}C, nuclear spin = 0.5), ^{2}H (natural abundance 0.015%, nuclear spin = 1), ^{15}N (natural abundance 0.36%, nuclear spin = 0.5), ^{18}O (natural abundance 0.20%, nuclear spin = 0) and to a lesser extent, ^{19}F (natural abundance 100%, nuclear spin = 0.5).[17]

^{13}C, ^{2}H, ^{15}N And ^{19}F are all NMR-active and can be detected at an appropriate frequency. ^{18}O Is not NMR-active, but will influence the chemical shift of carbon signals in ^{13}C-NMR when the isotope is directly attached to the carbon atom. It does this by altering the distribution of electrons in the carbon-oxygen bond and thereby

effecting its carbon resonance.[18] Its presence can therefore be detected using high field ^{13}C-NMR techniques (Table 1).[19,20,21,22]

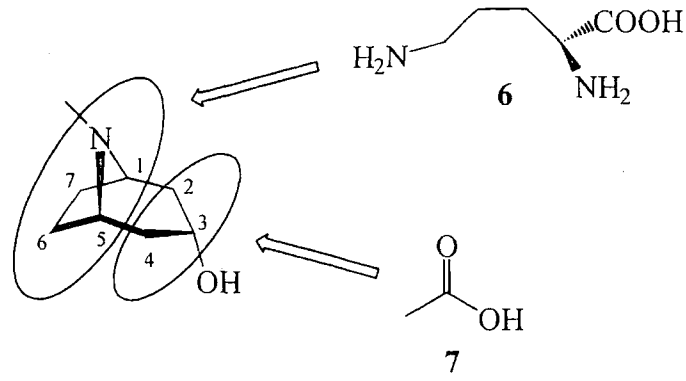
	Shift	Induced upfield shift
^{13}C		0
^{13}C-^{2}H	α-shift	~0.35ppm per ^{2}H
^{13}C-C-^{2}H	β-shift	~0.06ppm per ^{2}H
^{13}C-^{18}O	α-shift	~0.03ppm per ^{18}O
^{13}C-C-^{18}O	β-shift	Not easily detected

Table 1 The presence of ^{2}H and ^{18}O can be detected using high field ^{13}C-NMR techniques. A quantitative α- or β-shift is induced upfield from the enriched natural abundance signal. This value depends on the number of isotopes (^{2}H or ^{18}O) and its distance from the attached enriched carbon.

Of course, isotopes in sufficient abundance can be detected by MS (mass spectroscopy) and GC-MS (gas chromatography-mass spectroscopy) is particularly useful when the compound to be examined is present in a mixture.

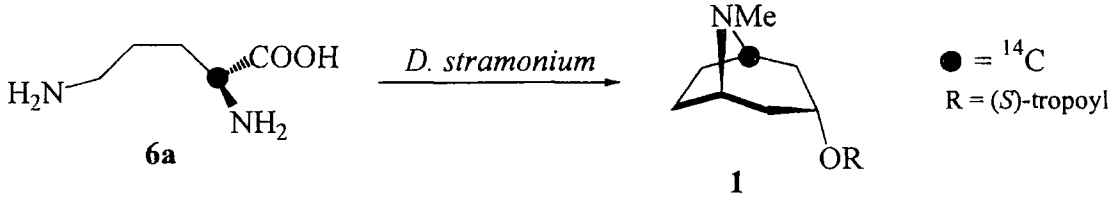
1.5 The Biosynthesis of Hyoscyamine

1.5.1 The biosynthesis of the tropane skeleton



Scheme 1 The tropane skeleton is derived from ornithine (**6**) and acetate (**7**).

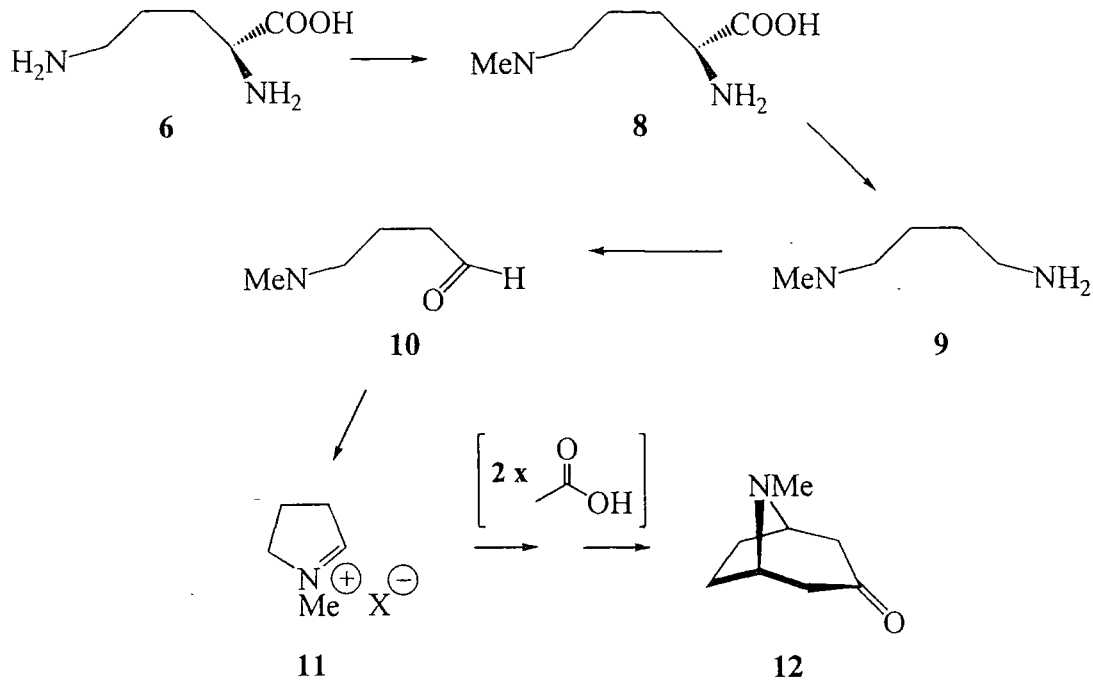
The tropane skeleton is derived from ornithine (**6**)[8,23] and acetate (**7**)[24,25] as shown in Scheme 1. Leete reported[8,23] in 1954 that [2-^{14}C]-ornithine (**6a**) was incorporated unsymmetrically into the tropane skeleton, labelling only at C-1 of hyoscyamine (**1**), as shown in Scheme 2.[26,27] Later, it was shown that ornithine was incorporated symmetrically in other plant alkaloid producing species.[28,29,30] It would seem plausible that in Leete's case, an unsymmetrical intermediate would be a viable precursor in the biosynthetic pathway to the tropane skeleton. Thus, δ-N-methylornithine (**8**), which is formed by the methylation of the δ-amino group of ornithine (**6**), was suggested as a relevant precursor.[31,32,33]



Scheme 2 A feeding experiment with [2-^{14}C]-ornithine (**6a**) to *D. stramonium* plants resulted in the unsymmetrical incorporation into C-1 of the tropane moiety of hyoscyamine (**1**).

Liebisch and co-workers[34] showed that N-methylputrescine (**9**) is an intermediate to the tropane alkaloids. This gave substance to Leete's proposal, since N-methylputrescine (**9**) could be easily formed by the decarboxylation of δ-N-methylornithine (**8**). N-Methylputrescine (**9**) was assumed to transaminate to 4-N-methylaminobutanal (**10**),

which would cyclise rapidly to the N-methylpyrrolinium salt (**11**). The system is then set up for the addition of acetate units and ring closure to form tropinone (**12**) as shown in Scheme 3.



Scheme 3 An early proposal for the biosynthetic pathway to tropinone (**12**), following the findings from a number of isotopically labelled feeding experiments.

Mizusaki and co-workers[35] reinforced this pathway by demonstrating that radiolabelled ornithine (**6**) was incorporated *in vivo* into 4-N-methylaminobutanal (**10**). This led to the proposal that the double bond of the N-methylpyrrolinium salt (**11**) would be able to tautomerise and thus lead to the symmetrical labelling of the tropane skeleton reported in some species (Scheme 4).

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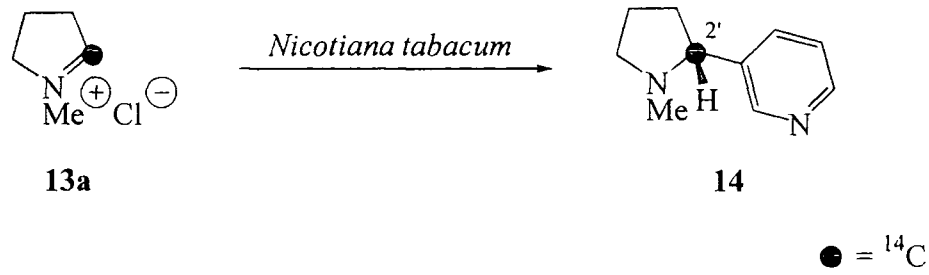
Scheme 4 Tautomerisation of the N-methylpyrrolinium salt (**11**) could explain the symmetrical incorporation of isotopically labelled precursors.

However, Leete's experiments failed to reveal this phenomenon *in vitro* or *in vivo* in the biosynthesis of nicotine (**14**) in *Nicotiana tabacum*, observing ^{14}C label only in the C-2′

of the pyrrolidine ring following a feeding experiment with N-methyl-[2-^{14}C]-pyrrolinium chloride (**13a**), as shown in Scheme 5.[36]



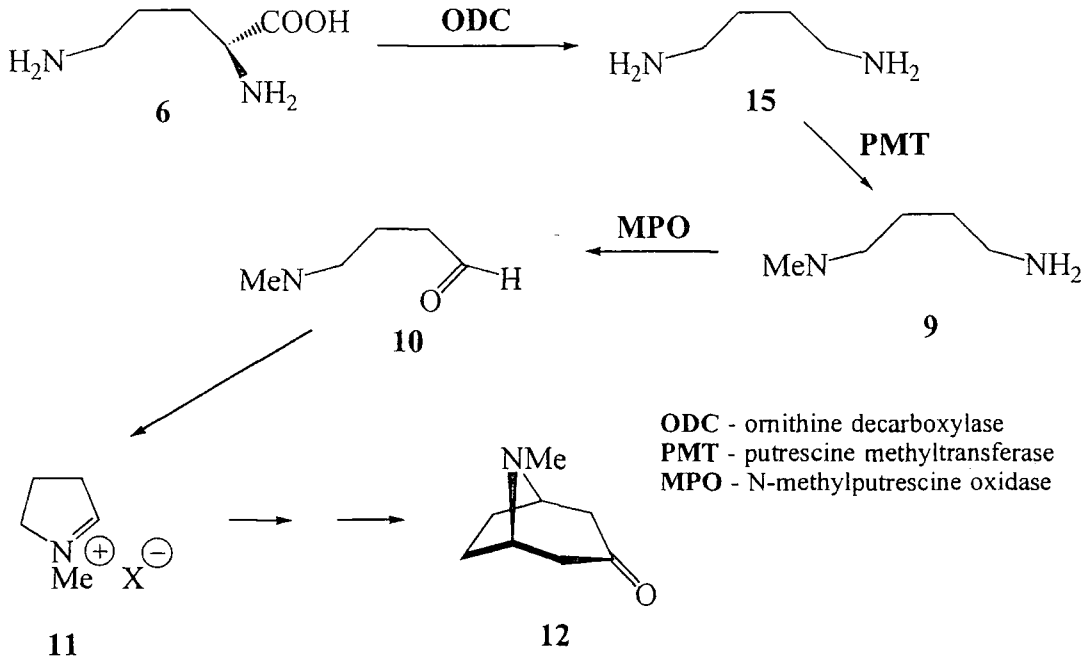
13a **14**

● = ^{14}C

Scheme 5 A feeding experiment with N-methyl-[2-^{14}C]-pyrrolinium chloride (**13a**) to *Nicotiana tabacum* revealed incorporation of ^{14}C label solely at C-2′ of nicotine (**14**).

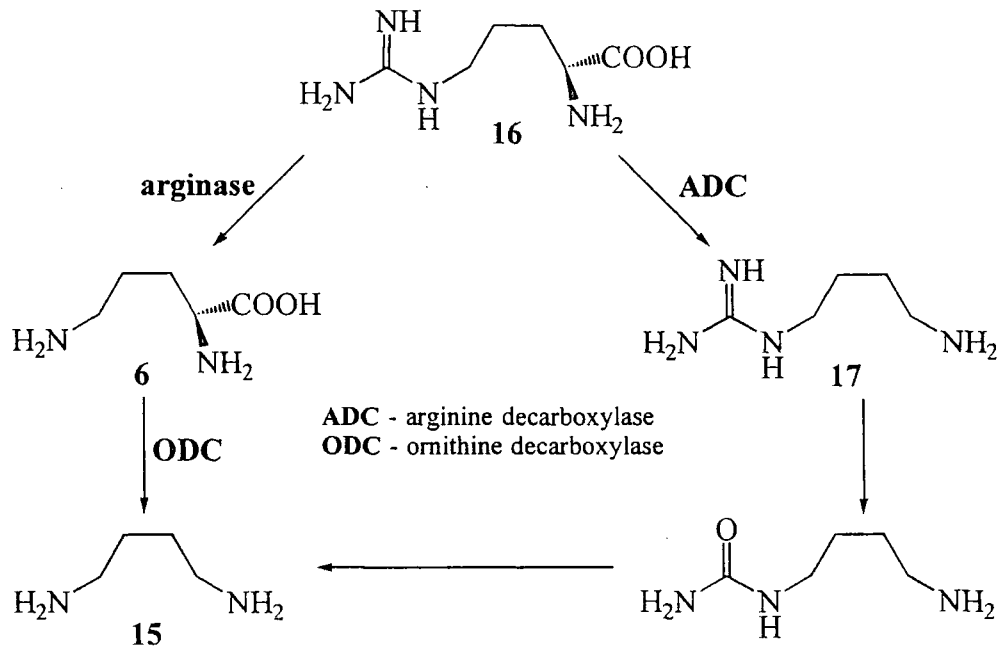
Putrescine (**15**) has been shown to become successfully incorporated into the tropane alkaloids,[37,38,39] but Leete suggested at the time that this result had been achieved through an aberrant biosynthetic pathway, since putrescine (**15**) is a symmetrical molecule. However, since the symmetrical incorporation of ornithine (**6**) observed in some species could not be accounted for by the tautomerisation of the N-methylpyrrolinium salt (**11**); the differentiation between C-2 and C-5 of ornithine (**6**) could be explained if free putrescine (**15**) was not an intermediate, but rather if it was enzyme bound in some way. Ornithine (**6**) would first be decarboxylated to form putrescine (**15**) followed by methylation to N-methylputrescine (**9**), yielding an unsymmetrical intermediate.[30] Thus, putrescine (**15**) became accepted as the true intermediate in the biosynthetic pathway (Scheme 6).

Through *in vivo* experiments, a series of enzymes was discovered, which verified the biosynthetic pathway to the N-methylpyrrolinium salt (**11**), and thus the tropane skeleton (Scheme 6).[40]



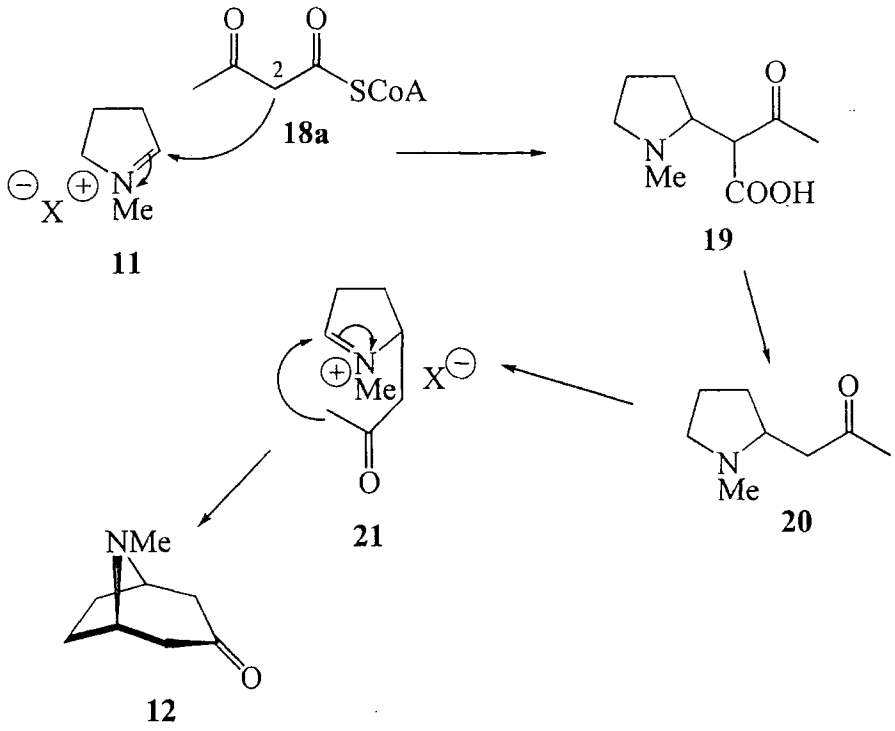
Scheme 6 The isolation and identification of various enzymes verified the biosynthetic pathway to tropinone (**12**).

In some species, arginine (**16**) is a precursor to putrescine (**15**) rather than ornithine (**6**). These species lack the enzyme arginase, which converts arginine (**16**) to ornithine (**6**). Instead, arginine (**16**) is decarboxylated by arginine decarboxylase to form agmantine (**17**), which is subsequently cleaved to yield putrescine (**15**), as shown in Scheme 7.[41]



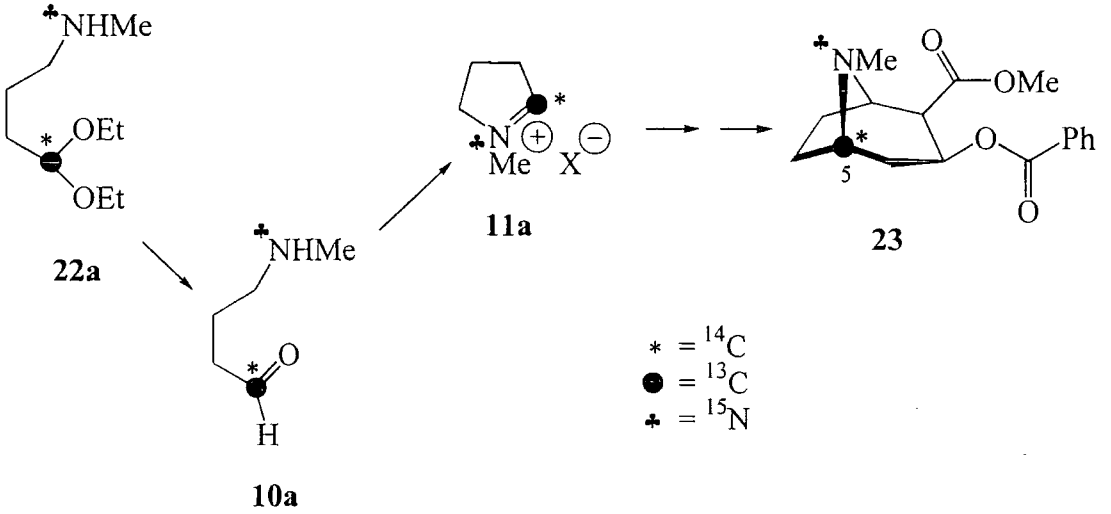
Scheme 7 The intermediacy of arginine (**16**) in some species was established by the discovery of arginine decarboxylase (ADC).

The remaining carbon atoms of the tropane skeleton are derived from acetate (**7**). Liebisch and co-workers[24] observed that acetoacetate (**18**) is efficiently incorporated into the tropane skeleton, and proposed that it is a true intermediate. The proposed scheme implicated acetoacetate (**18**) [or its activated CoA ester (**18a**)], which condenses with the N-methylpyrrolinium salt (**11**) to form α-carboxyhygrine (**19**). Hygrine (**20**) may be formed after subsequent decarboxylation, and further oxidation would generate dehydrohygrine (**21**). After cyclisation to tropinone (**12**), the skeleton is complete (Scheme 8). This scheme presented a plausible working hypothesis, since hygrine (**20**) had been reported as an intermediate in hyoscyamine biosynthesis,[24,42] although a role for hygrine has subsequently been disproven.[43]



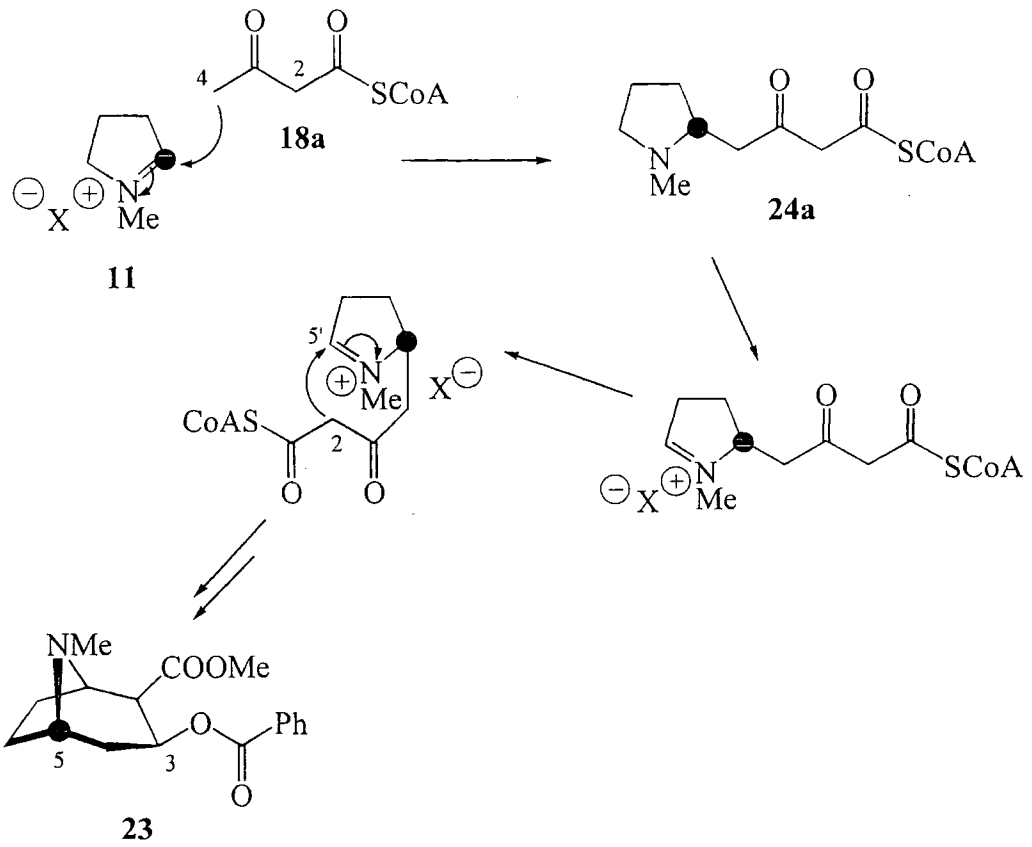
Scheme 8 An early working hypothesis for the formation of the bicyclic tropane ring.

An alternative biosynthetic pathway to the tropane skeleton came to light when [1-^{13}C,^{14}C,4-^{15}N]-4-N-methylaminobutanal diethylacetal (**22a**) was incorporated into cocaine (**23**).[44] In this experiment, it was considered that the diethylacetal (**22a**) would hydrolyse in the acidic plant tissues to give [1-^{13}C,^{14}C,4-^{15}N]-4-N-methylaminobutanal (**10a**). This would readily cyclise to generate the N-methylpyrrolinium salt (**11a**). However, it was perhaps surprising that incorporation was observed only at C-5 rather that at C-1, as shown in Scheme 9.



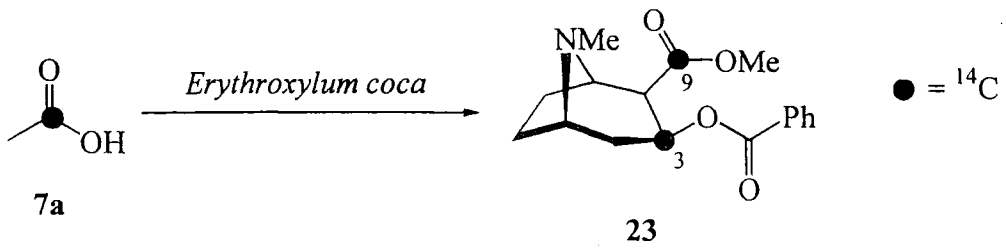
Scheme 9 The incorporation of [1-^{13}C,^{14}C,4-^{15}N]-4-N-methylaminobutanal diethylacetal (**22a**) into cocaine (**23**), *via* the N-methylpyrrolinium salt (**11a**).

In this case, if acetoacetate (**18**) [or its activated co-enzyme A ester (**18a**)] was a true intermediate, it should condense with the N-methylpyrrolinium salt (**11**) *via* C-4 rather than the observed position at C-2. However, this reaction appears to take advantage of the activated C-2 position of the N-methylpyrrolinium salt (**11**) and leaves C-2 of the product, 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-SCoA (**24a**) to condense with the C-5′ of the heterocycle, forming the bicyclic tropane ring system, as shown in Scheme 10.



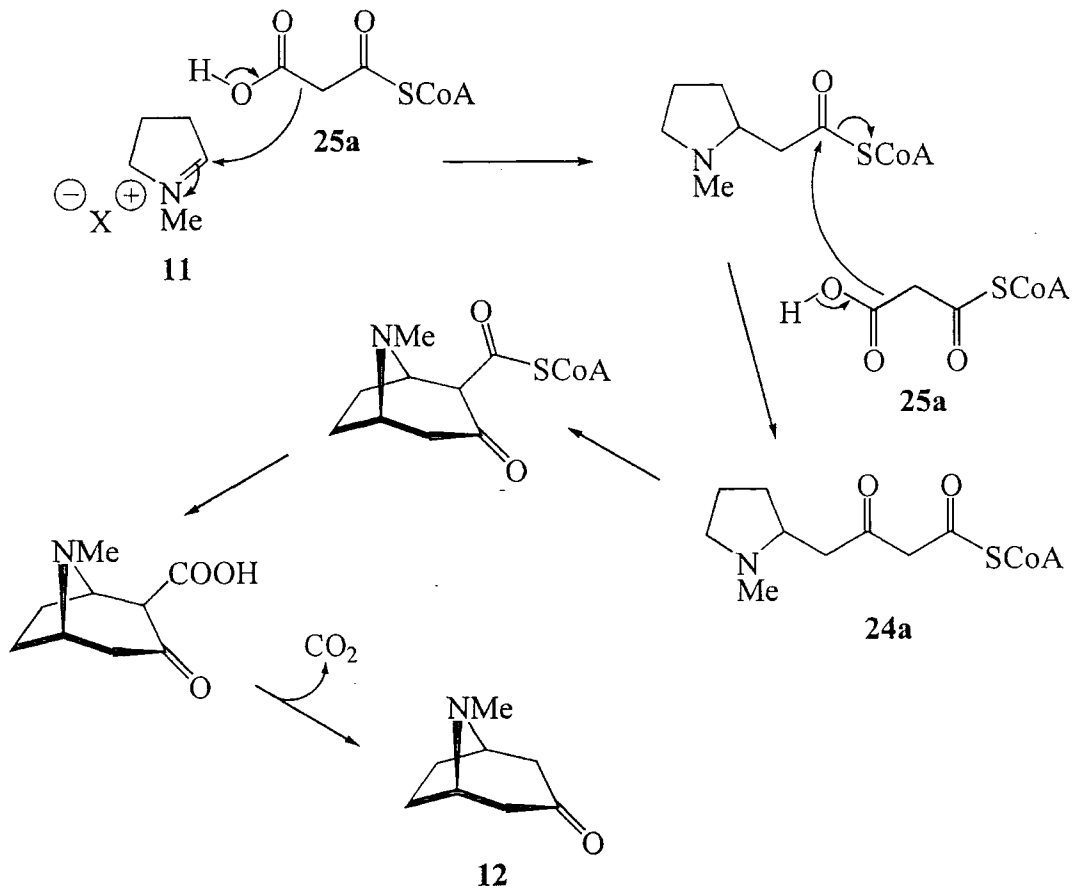
Scheme 10 A proposed scheme for the formation of the tropane ring of cocaine (**23**) *via* the condensation of acetoacetyl-SCoA (**18a**) with the N-methylpyrrolinium salt (**11**).

[1-^{14}C]-Acetate (**7a**) has been shown to become incorporated into C-3 and C-9 of cocaine (**23**) in the same manner as acetoacetate (**18**),[45] as shown in Scheme 11. This prompted a re-evaluation of the generally accepted acetoacetate pathway. Endo and co-workers[46] first revealed that the reaction between acetoacetate (**18**) and N-methylpyrrolinium salt (**11**) is a facile process and spontaneously occurs *in vitro*. Therefore, the suggestion that the *in vivo* process occurs *via* an alternative non-enzymatic pathway was proposed.[30,40]



Scheme 11 The incorporation of [1-^{14}C]-acetate (**7a**) into cocaine (**23**) in *Erythroxylum coca*.

Liebisch and co-workers earlier proposed an alternative route, which argued that acetate would become incorporated into the tropane skeleton *via* malonyl-CoA (**25a**), as shown in Scheme 12.[24]



Scheme 12 A proposed scheme for the formation of tropinone (**12**) *via* the successive condensation of two units of malonyl-CoA (**25a**) with the N-methylpyrrolinium salt (**11**).

Later, Hemscheidt and Spenser discredited acetoacetate as a potential intermediate to the tropane alkaloids.[47] They demonstrated that [1,2,3,4-$^{13}C_4$]-acetoacetate (**18b**) was not incorporated as an intact unit into the tropane skeleton of 6β-hydroxyhyoscyamine (**26**). Instead, they observed the same labelling pattern found in [1,2-$^{13}C_2$]-acetate (**7b**) feeding experiments to *D. stramonium* plants, and concluded that the isotopically labelled acetoacetate had been cleaved to acetate before incorporation, as shown in fig. 7.

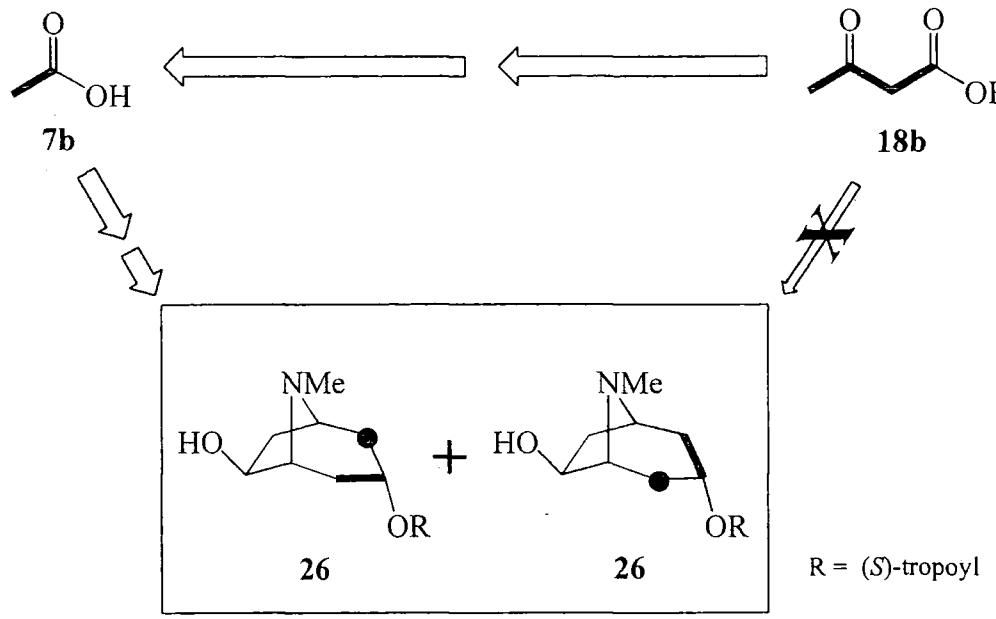


Figure 7 ^{13}C-Isotopic labelling pattern found in 6β-hydroxyhyoscyamine (**26**) after feeding sodium [1,2-$^{13}C_2$]-acetate (**7b**) and [1,2,3,4-$^{13}C_4$]-acetoacetate (**18b**) to *D. stramonium* roots or plants. [1,2,3,4-$^{13}C_4$]-Acetoacetate (**18b**) was cleaved to [1,2-$^{13}C_2$]-acetate (**7b**) before incorporation into 6β-hydroxyhyoscyamine (**26**).

Interestingly, these experiments revealed that both the bridgehead carbons (C-1 and C-5) of the tropane ring were equally labelled, and that feeding N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) resulted in the incorporation of deuterium at both of these sites, as shown in fig. 8.

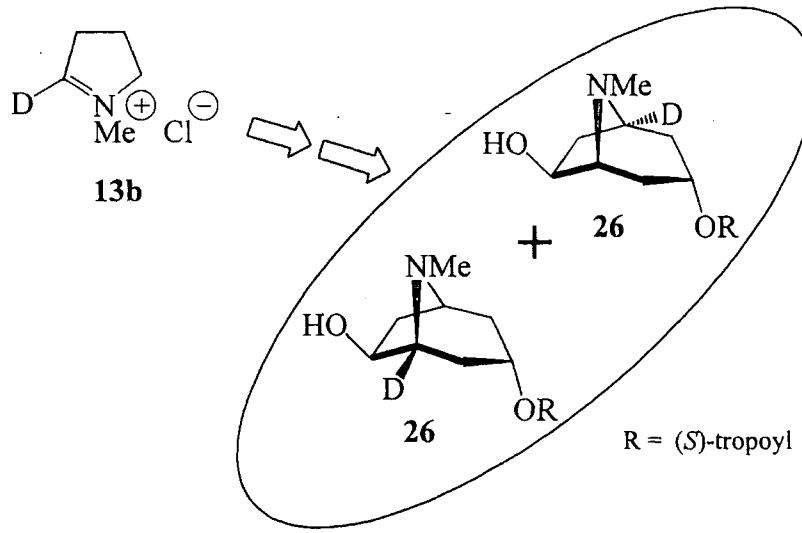


Figure 8 Deuterium was found to be incorporated into both bridgehead carbons of 6β-hydroxyhyoscyamine (**26**) after feeding N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) to *D. stramonium*.

This initiated a proposal that achiral N-methylpyrrolidine, or racemic hygrine, is involved in the biosynthesis of the tropane ring. A similar result was observed after feeding [2′,3′-$^{13}C_2$]-hygrine (**20a**) and ethyl [2,3-$^{13}C_2$]-N-methyl-2-pyrrolidinyl-3-oxobutanoate (**27a**) to *D. innoxia*, as shown in fig. 9. However, hygrine was shown to incorporate only at very low levels and this may be accounted for by an indirect incorporation *via* acetate.

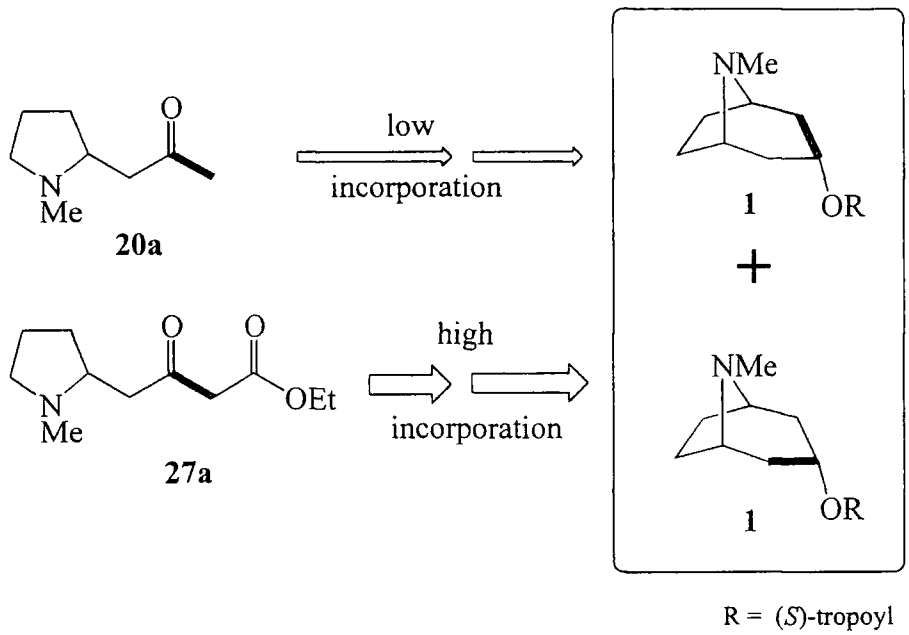
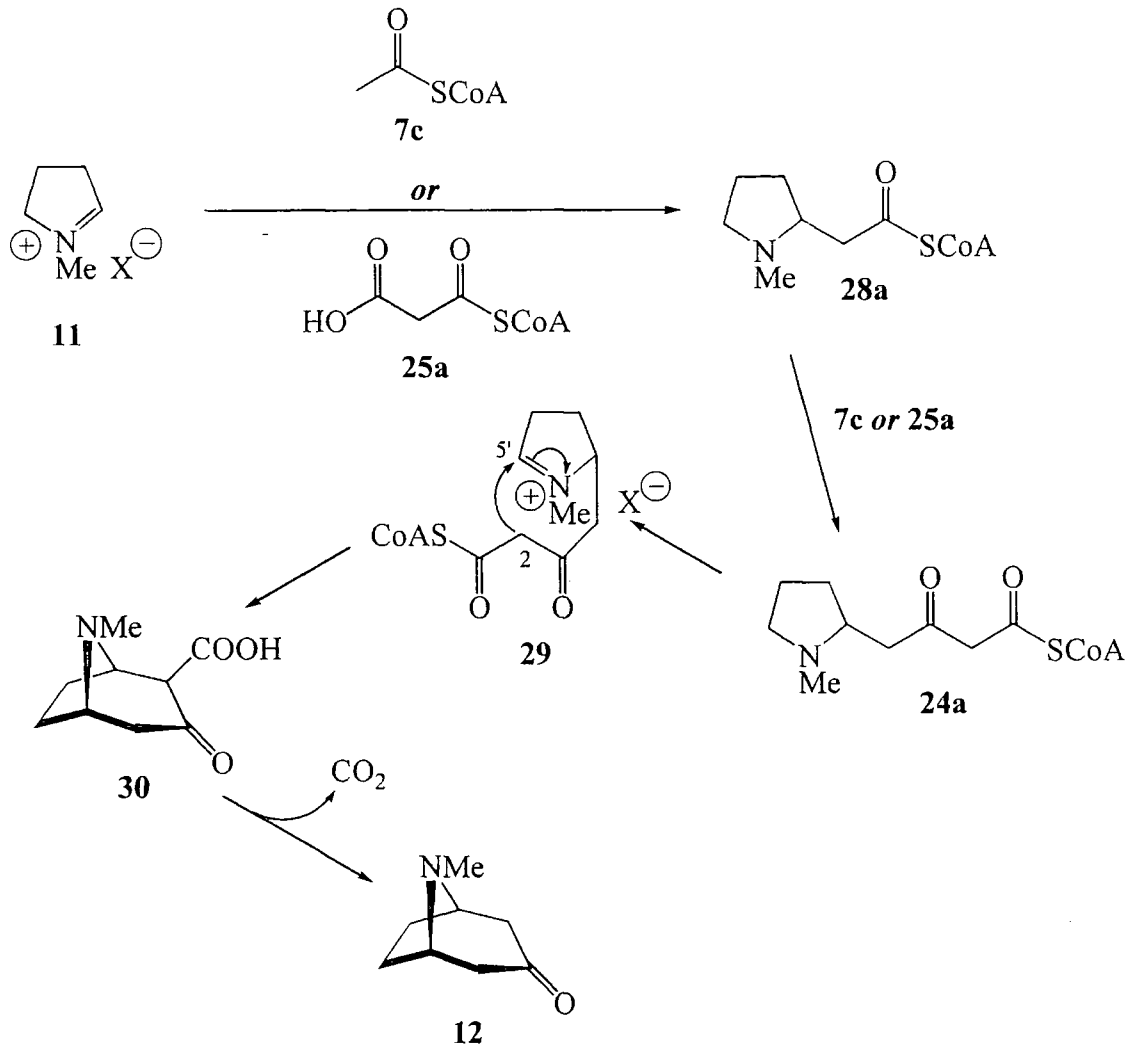


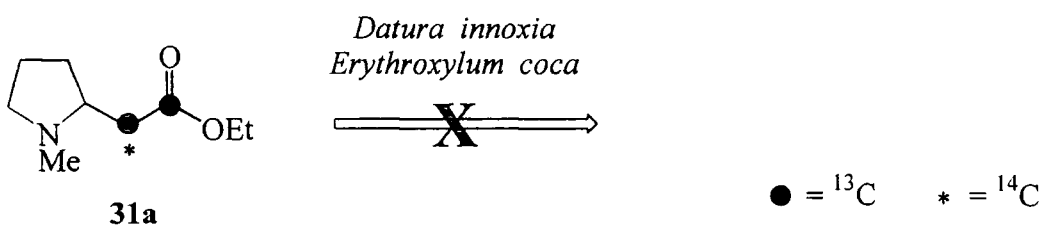
Figure 9 Feeding experiments with [2′,3′-$^{13}C_2$]-hygrine (**20a**) and ethyl [2,3-$^{13}C_2$]-N-methyl-2-pyrrolidinyl-3-oxobutanoate (**27a**) to *D. innoxia* revealed that the latter is the better precursor of the two substrates in hyoscyamine (**1**) biosynthesis.

These findings prompted a revision of the generally accepted pathway for the biosynthesis of the tropane skeleton. Starting from the N-methylpyrrolinium salt (**11**), two acetate units are added consecutively *via* acetyl co-enzyme A (**7c**), [or perhaps malonyl co-enzyme A (**25a**) followed by decarboxylation] to give firstly 2-(1-methyl-2-pyrrolidinyl)acetyl-CoA (**28a**) and then 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**). Following cyclisation through the iminium salt (**29**) to 2-carboxytropinone (**30**) and subsequent decarboxylation would give tropinone (**12**), a known precursor in the biosynthesis of the tropane alkaloids (Scheme 13).



Scheme 13 A proposed scheme for the formation of tropinone (**12**) *via* the successive condensation of two units of acetyl-CoA (**7c**) or malonyl-CoA (**25a**).

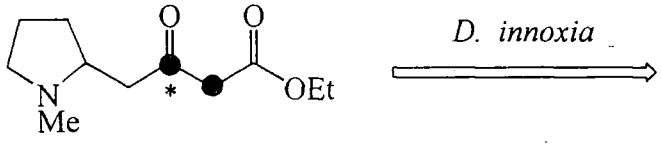
However, Leete and co-workers tested this new hypothesis by feeding a number of isotopically labelled 2-(1-methyl-2-pyrrolidinyl)acetates, such as ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate (**31a**) and other ester analogues to *D. innoxia* and *Erythroxylum coca* plants, and observed that ^{14}C incorporations were very low and hardly detectable by ^{13}C-NMR analysis (Scheme 14).[48]



Scheme 14 Ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate (**31a**) is not incorporated into the tropane alkaloids when administered to *D. innoxia* or *Erythroxylum coca*.

This negative result was mirrored in the biosynthesis of the tropane alkaloids of other plant species.[49,50] Nevertheless, 2-(1-methyl-2-pyrrolidinyl)acetate (**28**) could not be discounted as an intermediate as this type of consecutive addition of acetate units occurs widely in flavanoid[51] and polyketide[52] biosynthesis. The failure of such a labelled feeding experiment could be due to fact that this intermediate is transient and enzyme bound, and that the synthase does not utilise exogenous intermediate precursors.

Feeding experiments with ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**27b**) were more successful (Scheme 15).[49,50] High incorporations into cocaine (**23**), hyoscyamine (**1**) and scopolamine (**3**) in various species seem to indicate that this was a valid intermediate on the biosynthetic pathway.



27b

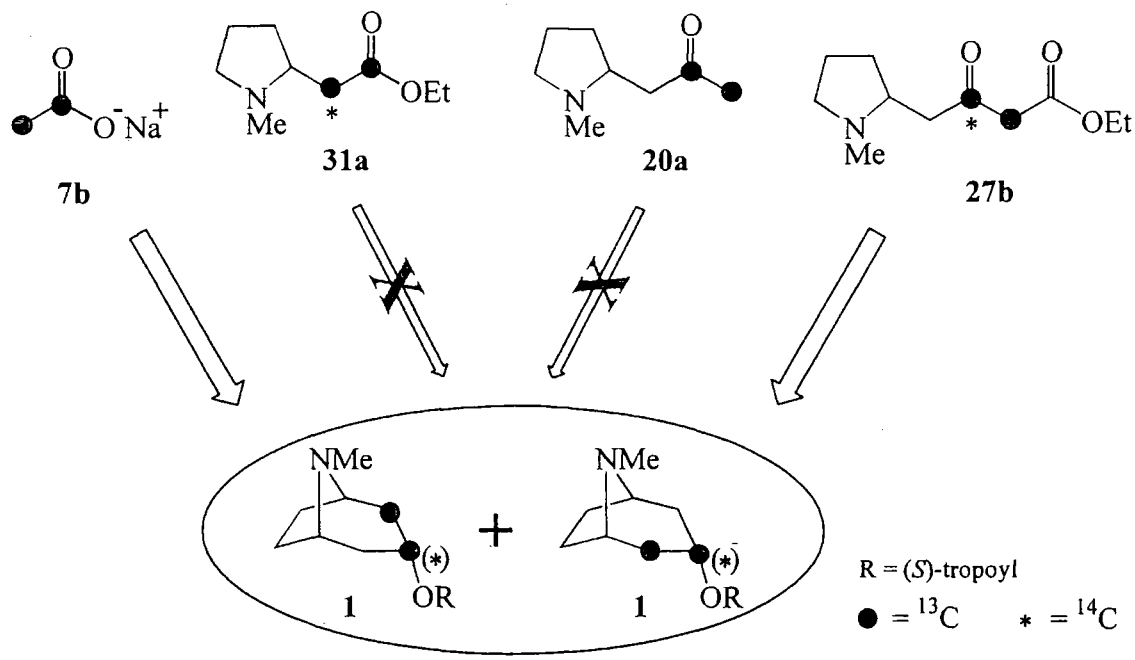
● = ^{13}C * = ^{14}C

Scheme 15 Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**27b**) is incorporated into the tropane alkaloids when administered to *D. innoxia*.

Interestingly however, there has been recent evidence for the incorporation of an intact 4-carbon unit, following a feeding experiment with sodium [1,2-$^{13}C_2$]-acetate.[43] A symmetrical incorporation into the C-2 and C-4 positions of hyoscyamine (**1**) was observed, but also the presence of a triply labelled species (C-2, C-3 and C-4), where both the C-2 and C-4 positions are equally labelled was present. Surprisingly, this finding suggests that the intermediate, 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate [(**24**), or its thioester (**24a**)], has no preference for the (*R*)-isomer over the (*S*)-isomer on cyclisation to form the tropane ring. Thus, these observations indicate that the acetate units have been incorporated as a single unit into some symmetrical precursor, rather than in a stepwise manner. This conflicts with previous findings by Hemscheidt and Spenser[47] working with *D. stramonium* who found the contrary, but it was suggested that perhaps they were unable to detect the low levels of the triply labelled species in their experiments. The discovery of the putative intermediate, 2-carboxytropinone (**30**) in *D. stramonium* root cultures supports the intermediacy of 4-(1-methyl-2-

pyrrolidinyl)-3-oxobutanoate (**24**), which will form 2-carboxytropinone (**30**) on cyclisation.[53]

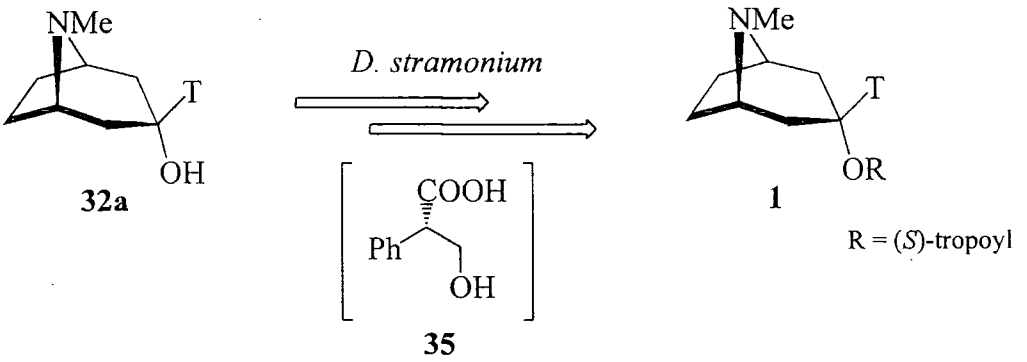
Recently, Robins and co-workers[43] have re-evaluated the intermediates between the N-methylpyrrolinium salt (**11**) and tropinone (**12**). Previous data had arisen from a variety of experiments using different techniques on a wide number of plant species. To clarify the biosynthetic pathway of the tropane alkaloids, a series of uniform experiments was carried out on transformed root cultures of *D. stramonium*. Feeding experiments with sodium [1,2-$^{13}C_2$]-acetate (**7b**), (*R,S*)-[2′,3′-$^{13}C_2$]-hygrine (**20a**), ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)-acetate (**31a**) and ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**27b**) revealed that 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**27b**) has a more important role than hygrine (**20a**) in the biosynthesis of the tropane ring system (Scheme 16).



Scheme 16 Feeding experiments with sodium [1,2-$^{13}C_2$]-acetate (**7b**), (*R,S*)-[2′,3′-$^{13}C_2$]-hygrine (**20a**), ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)-acetate (**31a**) and ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**27b**) to *D. stramonium* root cultures revealed that 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**27b**) and sodium [1,2-$^{13}C_2$]-acetate (**7b**) are incorporated into the tropane ring of hyoscyamine (**1**).

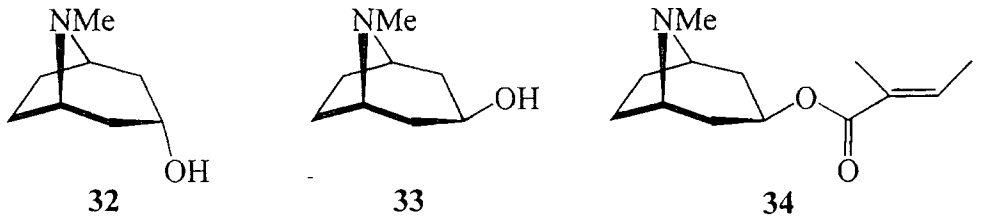
1.5.2 The biosynthesis of tropine ester alkaloids

Tropine (**32**) was initially envisaged as the intermediate to hyoscyamine (**1**). This is an intuitive conclusion that gained support from earlier feeding experiments. A labelling study using [3-^{3}H]-tropine (**32a**) indicated incorporation into hyoscyamine (**1**) without loss of tritium,[54] suggesting no interconversion between tropine (**32**) and tropinone (**12**) in the biosynthetic pathway (Scheme 17). Thus, tropinone (**12**) must be irreversibly reduced to tropine (**32**), which upon esterification with tropic acid (**35**) would generate hyoscyamine (**1**).



Scheme 17 The esterification of tropine (**32**) with (*S*)-tropic acid (**35**) was believed to be the last step in hyoscyamine biosynthesis for some time.

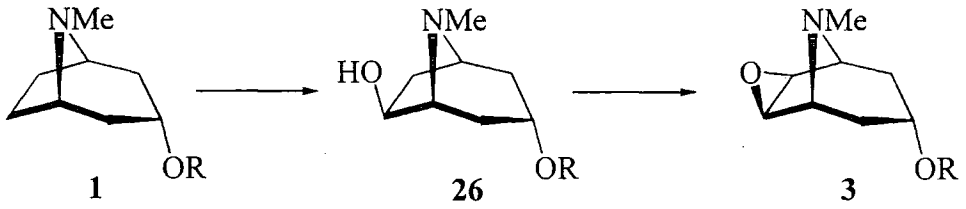
Tropinone reductase reduces tropinone (**12**) to tropine (**32**) prior to esterification. Two dehydrogenases have been identified and isolated from several sources, which reduce tropinone (**12**) to tropine (**32**) or its 3-β epimer, pseudotropine (**33**).[55] The *D. stramonium* tropinone dehydrogenases were found to require NADPH as the co-enzyme and inactive with NADH. However, each enzyme has a different pH dependency for activity, which would assume that they are different enzymes with different roles in plant metabolism. As expected, tropinone reductase I (TR I), which reduces tropinone to tropine, was found to be 5-fold more active than its partner, tropinone reductase II (TR II) in *D. stramonium* roots. This is consistent with the finding that hyoscyamine (**1**), which contains tropine (**32**), is the major alkaloid constituent in these roots. Pseudotropine (**33**) formed by tropinone reductase II is incorporated into various minor esters such as tigloidine (**34**) in *D. stramonium* (fig. 10). Recently, the enzyme responsible for tigloidine (**34**) biosynthesis, tigloyl-co-enzyme A:pseudotropine acyl transferase, has been isolated and characterised.[56]



32 33 34

Figure 10 The minor tropane alkaloid, tigloidine (**34**), is biosynthesised from pseudotropine (**33**), whereas tropine (**32**), is utilised in the biosynthesis of the major alkaloid, hyoscyamine, in *D. stramonium*.

Epoxidation across C-6 and C-7 of the tropane skeleton in scopolamine (**3**) occurs after hyoscyamine (**1**) formation.[57] Firstly, the tropane skeleton is hydroxylated to give 6β-hydroxyhyoscyamine (**26**) and is followed by ring closure to form the epoxide (Scheme 18). The enzyme that generates scopolamine (**3**) from hyoscyamine (**1**) requires oxygen, iron (II) and oxoglutarate.[58] However, despite many attempts, the hydroxylation and epoxidation reactions have not been separated and thus cannot be attributed to two separate enzymes since only one has been isolated. The consensus is that one protein is responsible for both reactions.[59,60]



1 **26** **3**

R = (*S*)-tropoyl

Scheme 18 The formation of scopolamine (**3**) from hyoscyamine (**1**) *via* 6β-hydroxyhyoscyamine (**26**) is mediated by one enzyme.

1.5.3 The elicitation of tropine biosynthesis by methyl jasmonate

Elicitors originating from the cell walls of fungi or plants have been shown to increase secondary metabolism in plant cell cultures.[61] Recently it was shown that the biosynthesis of alkaloids can be elicited with jasmonic acid and its esters.[62] Exogenous added methyl jasmonate was shown to elicit the accumulation of alkaloids in a wide

number of plant species.[63,64,65] Of particular interest was the reported 100% increase in concentration of hyoscyamine (**1**) in *D. stramonium* root cultures after treatment with very low concentrations (0.1μmol dm^{-3}) of methyl jasmonate.[65] Further work by O'Hagan and co-workers[66] revealed that a similar concentration of methyl jasmonate was shown to enhance levels of hyoscyamine (**1**) and littorine (**5**) by selectively eliciting the pathway to tropine (**32**) biosynthesis over that for phenyllactate (**51**), in *D. stramonium* root cultures (fig. 11). Although the concentration of phenyllactate (**51**) had reduced, it was reasoned that the demand for this precursor is exceeding its rate of synthesis.

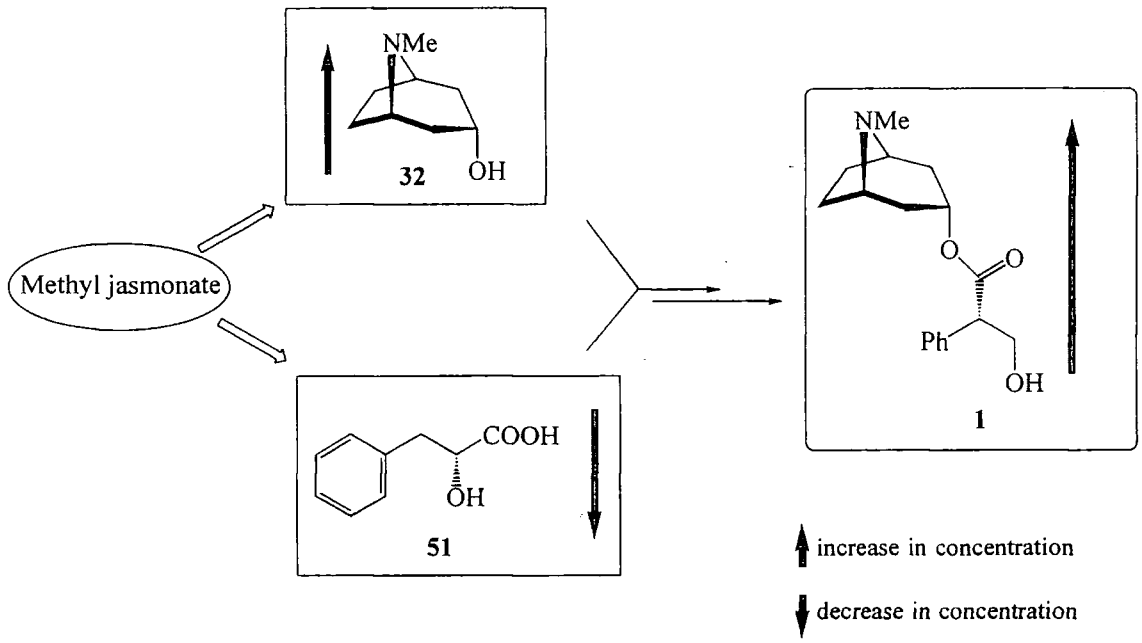
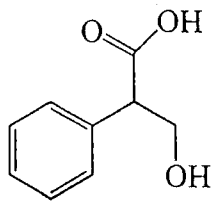


Figure 11 Methyl jasmonate enhances hyoscyamine (**1**) biosynthesis by selectively eliciting tropine (**32**) biosynthesis over that for phenyllactate (**51**) in *D. stramonium*.

This result was consistent with previous studies which have indicated that methyl jasmonate exerts its effect on alkaloid biosynthesis by increasing the alkaloid precursor pool and enhancing enzymes involved in alkaloid biosynthetic pathways.[67] These enzymes such as arginine decarboxylase[68,16] and putrescine methyltransferase,[68,16] are involved in tropine biosynthesis and link primary and secondary metabolic pathways.[67]

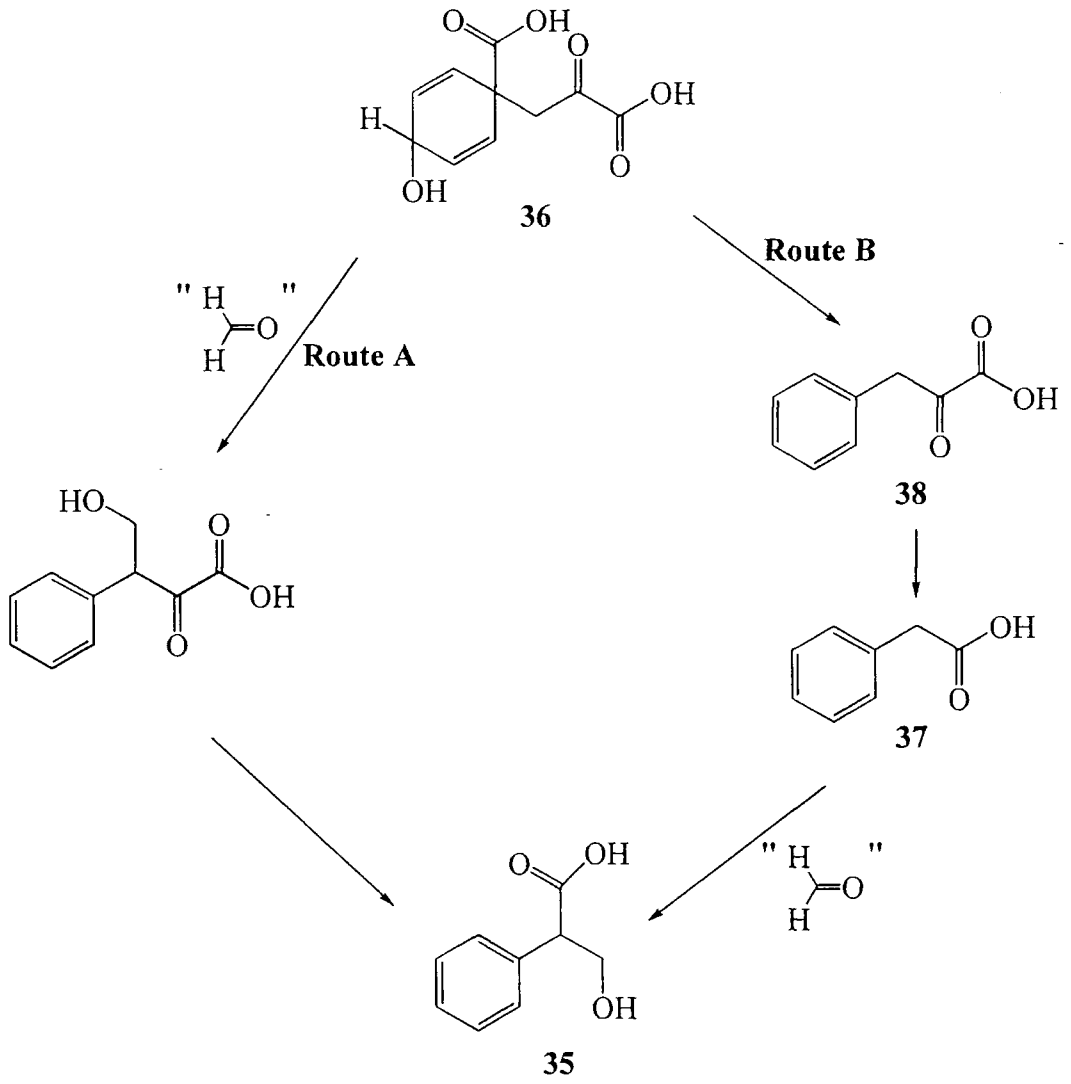
1.5.4 The biosynthesis of tropic acid



35

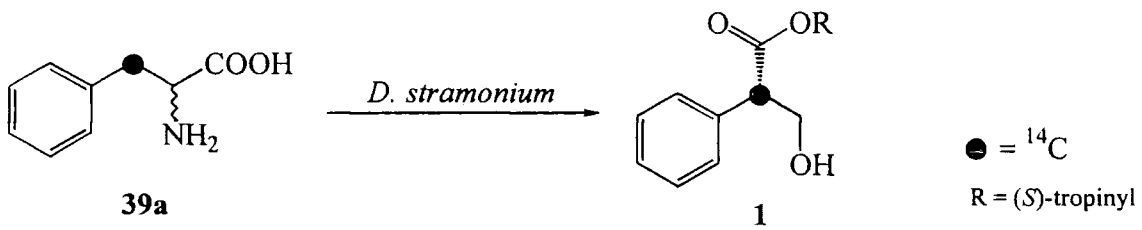
Figure 12 Tropic acid.

The biosynthesis of tropic acid (**35**) first attracted interest many years ago (fig. 12),[69,70] since it was considered that the biosynthesis of hyoscyamine (**1**) involved the esterification of tropine with (*S*)-tropic acid.[30] In the beginning, Wenkert proposed that tropic acid (**35**) may be derived from prephenic acid (**36**), which is established as an intermediate in the biosynthesis of many aromatic compounds.[71] Two biosynthetic routes were suggested, which differed in the timing of the introduction of the hydroxymethyl group. In the first case, it was proposed that formaldehyde or a biosynthetic equivalent would attack prephenic acid (**36**) itself (route A). In the other case, the same species would attack phenylacetic acid [(**37**) (route B)], which was formed from the degradation of prephenic acid (**36**) *via* phenylpyruvic acid (**38**), as shown in Scheme 19.

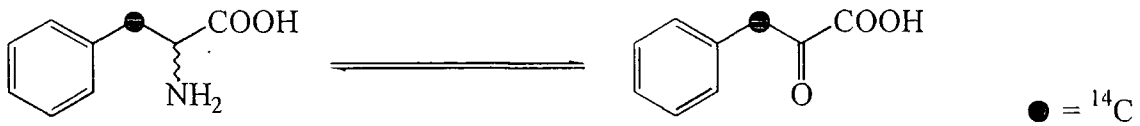


Scheme 19 An early proposal for the formation of tropic acid (**35**) from prephenic acid (**36**).[71]

Leete and co-workers fed DL-[3-^{14}C]-phenylalanine (**39a**) to *D. stramonium* plants to investigate phenylpyruvate (**38**) as a possible biosynthetic intermediate.[72] The incorporation of phenylalanine (**39a**) into C-2′ of hyoscyamine (**1**), as shown in Scheme 20, implicated phenylpyruvate (**38**), since both can interconvert *via* transamination (Scheme 21).



Scheme 20 DL-[3-^{14}C]-phenylalanine (**39a**) was incorporated into hyoscyamine (**1**) when administered to *D. stramonium* plants.

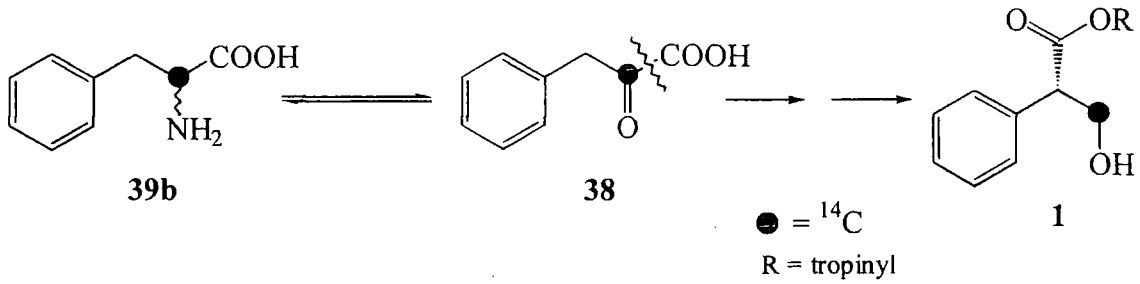


39a **38a**

Scheme 21 The feeding experiment with DL-[3-^{14}C]-phenylalanine (**39a**) to *D. stramonium* also implicated [3-^{14}C]-phenylpyruvate (**38a**) as they interconvert *in vivo*.

This result was later confirmed,[73] although attempts to trace the origins of the hydroxymethyl group remained a mystery since feeding experiments with [^{14}C]-formate[72] were unsuccessful. However, these findings failed to rule out formate as a precursor since it is a poor C-1 source in plants.

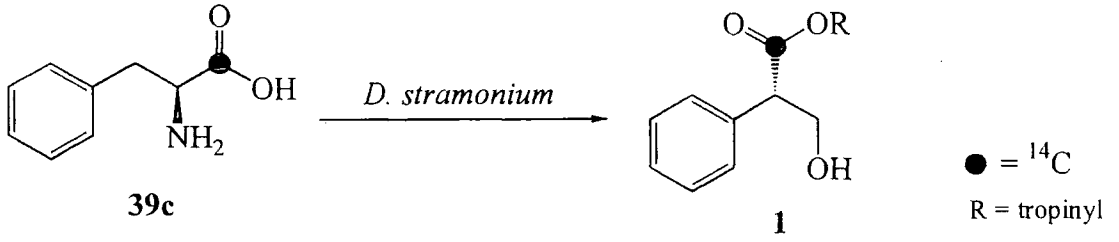
In a follow-up experiment, Leete and Louden[74] fed DL-[2-^{14}C]-phenylalanine (**39b**) expecting to observe incorporation into the C-1′ of hyoscyamine (**1**). However, the hydroxymethyl group at C-3′ of hyoscyamine (**1**) was found to be enriched. This led to the proposal that C-1 of phenylpyruvic acid (**38**) was lost during the biosynthesis of hyoscyamine (**1**), as shown in Scheme 22.



39b **38** **1**

Scheme 22 A feeding experiment with DL-[2-^{14}C]-phenylalanine (**39b**) to *D. stramonium* resulted in enrichment of C-3′ of hyoscyamine (**1**), suggesting that C-1 of phenylpyruvic acid (**38**) was lost during hyoscyamine biosynthesis.

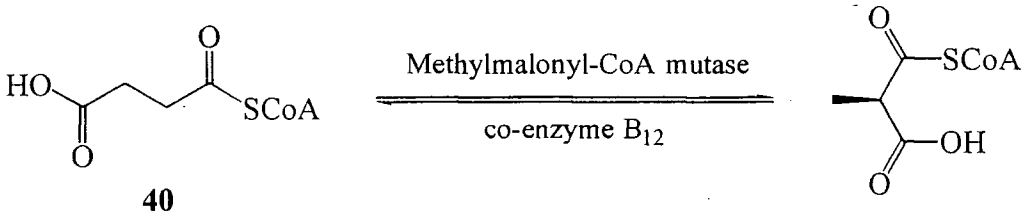
A feeding experiment then with L-[1-^{14}C]-phenylalanine (**39c**) resulted in the incorporation of label into C-1 of the tropic acid moiety of hyoscyamine (**1**), as shown in Scheme 23.[75,76] These results suggested that an isomerisation of the phenylpropanoid skeleton had occurred.



39c **1**

Scheme 23 A feeding experiment with L-[1-^{14}C]-phenylalanine (**39c**) to *D. stramonium* resulted in the incorporation of label into C-1 of the tropic acid moiety of hyoscyamine (**1**).

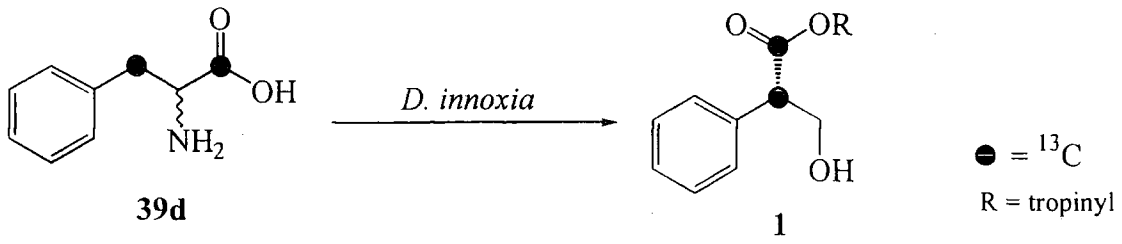
The contemporary discovery of the co-enzyme B_{12} mediated rearrangement of succinyl-CoA (**40**) to methylmalonyl-CoA (**41**),[77] prompted Leete to speculate that the rearrangement of the linear L-phenylalanine side chain to the isopropanoid tropate side chain may have a similar mechanism (Scheme 24).[76]



40 **41**

Scheme 24 The co-enzyme B_{12}-mediated methylmalonyl-CoA mutase rearrangement of succinyl-CoA (**40**) to methylmalonyl-CoA (**41**).

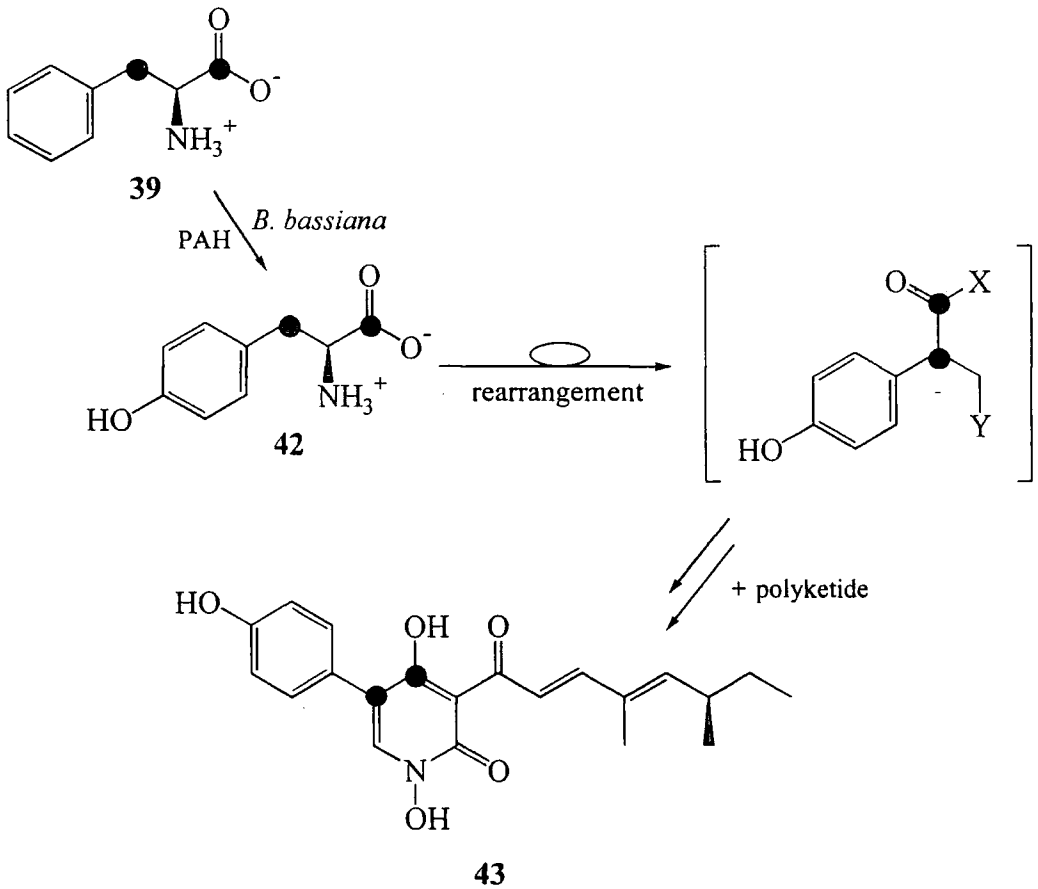
With the arrival of high field NMR spectroscopy, Leete was able to test this hypothesis with a definitive double labelling strategy. Leete and co-workers[78] fed DL-[1,3-$^{13}C_2$]-phenylalanine (**39d**) to *D. innoxia* plants, and observed that the ^{13}C enriched carbon atoms were coupled in the resultant ^{13}C-NMR spectrum of the isolated hyoscyamine (**1**), indicating that the isotopes had become contiguous as shown in Scheme 25. This experiment demonstrated unequivocally that tropic acid (**35**) was formed by the *intramolecular* rearrangement of L-phenylalanine (**39**), or a metabolite derived from it.



39d **1**

Scheme 25 A feeding experiment with DL-[1,3-$^{13}C_2$]-phenylalanine (**39d**) to *D. innoxia* plants revealed that an intramolecular rearrangement of the linear phenylpropanoid skeleton had occurred to form the tropate moiety of hyoscyamine (**1**).

This intriguing intramolecular rearrangement is unique in plants, although perhaps a similar type of process does occur during tenellin (**43**) biosynthesis in the fungus, *Beauveria bassiana* (Scheme 26).[79] The biosynthetic pathway towards tenellin (**43**) starts from L-phenylalanine (**39**), which is hydroxylated by a phenylalanine hydroxylase (PAH) to generate tyrosine (**42**). The phenylpropanoid skeleton of tyrosine (**42**) rearranges by an unknown process, but with a clear similarity to tropic acid biosynthesis, and it then combines with a polyketide fragment to generate tenellin (**43**).

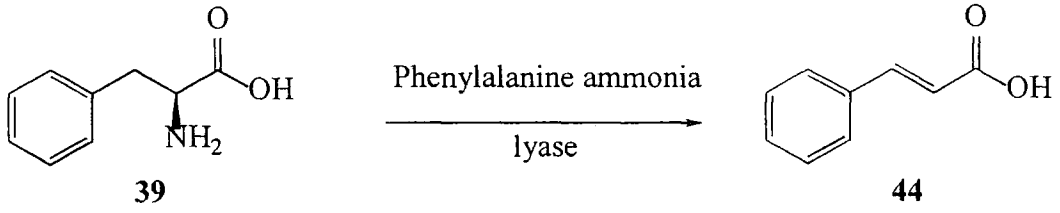


Scheme 26 The biosynthesis of fungal metabolite, tenellin (**43**), in *Beauveria bassiana* involves the intramolecular rearrangement of the phenylpropanoid skeleton of L- tyrosine (**42**).

1.5.5 The intermediates between L-phenylalanine and hyoscyamine

Once it was established that all of the carbon atoms of tropic acid (**35**) were derived from the intramolecular rearrangement of the L-phenylalanine side chain,[78] this stimulated further interest into identifying the biosynthetic intermediates between L-phenylalanine (**39**) and hyoscyamine (**1**).

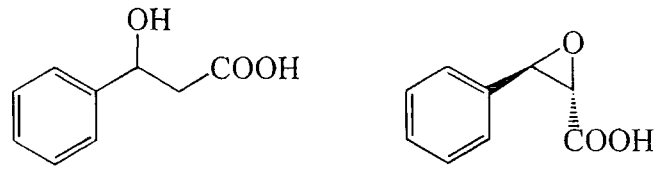
Cinnamic acid (**44**) was suggested as a closer intermediate to hyoscyamine (**1**) since it had been established as a key intermediate in the biosynthesis of many aromatic plant secondary metabolites.[80] Cinnamic acid (**44**) is formed from L-phenylalanine (**39**) by the action of phenylalanine ammonia lyase (PAL), as shown in Scheme 27. It is widespread in the biosynthesis of plant phenylpropanoids (C6-C3 compounds), and emerged as an attractive putative intermediate in tropic acid biosynthesis.[81]



39 **44**

Scheme 27 Cinnamic acid (**44**) is biosynthesised from L-phenylalanine (**39**) by the action phenylalanine ammonia lyase (PAL).

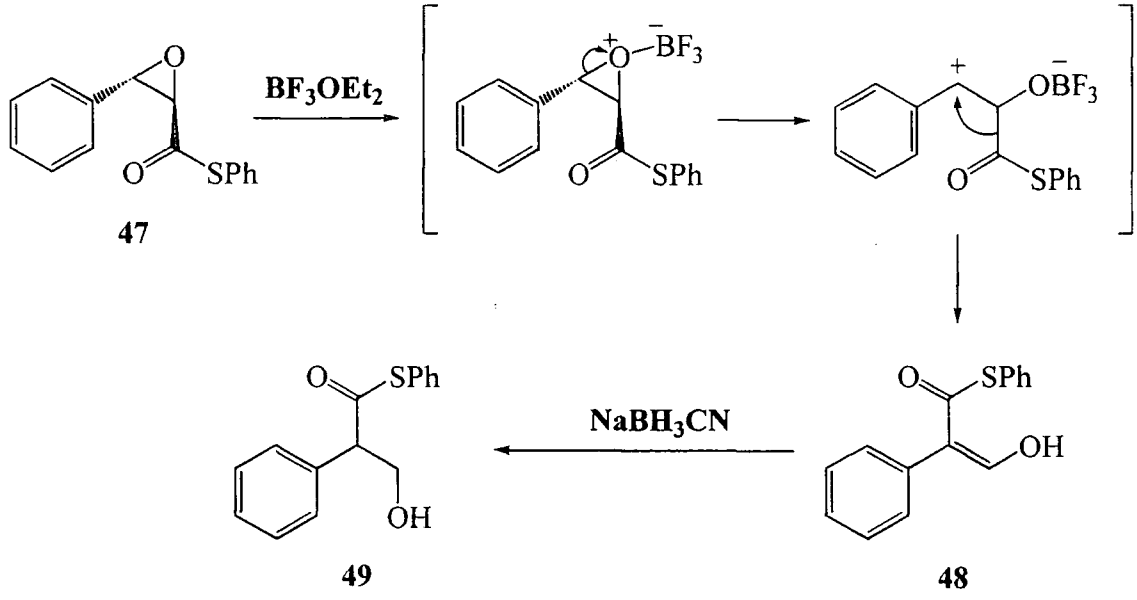
Nevertheless, Woolley[82] and Leete[83,84] both failed to observe the incorporation of radiolabelled cinnamic acids into tropane alkaloids. Feeding experiments with radiolabelled oxygenated derivatives of cinnamic acid including (*R*)- and (*S*)-3-hydroxyl-3-phenylpropionic acids (**45**)[83] and epoxycinnamic acid (**46**)[84] have all failed to provide positive results (fig. 13). Indeed, the ^{18}O results presented later in this thesis (Chapter 2, Section 2.2.3, page 62) prove definitively that cinnamic acid (**44**) is not a relevant intermediate.



45 46

Figure 13 The feeding of isotopically labelled (*R*)- and (*S*)-3-hydroxyl-3-phenylpropionic acids (**45**) and epoxycinnamic acid (**46**) to *Datura* all failed to become incorporated into hyoscyamine (**1**).

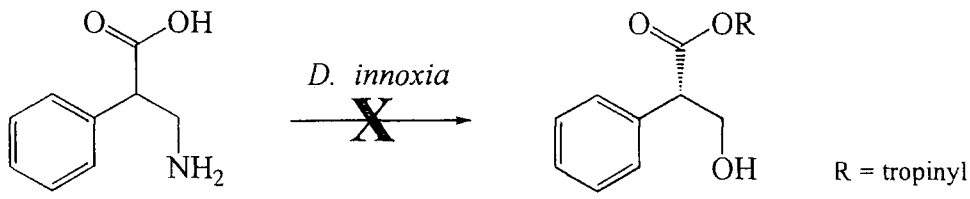
These experiments were prompted to the findings of Wemple,[85] who demonstrated that the phenyl thioester of epoxycinnamic acid (**47**) could be induced to rearrange *in vitro* by the addition of boron trifluoride etherate. The rearrangement proceeds by the migration of the carboxyl group from C-2 to C-3 to give α-formylphenylthioacetate (**48**), as shown in Scheme 28. The thioester of tropic acid (**49**) could then be formed by selective reduction with sodium cyanoborohydride.[86] There has been a report of cinnamic acid incorporation into tropane alkaloids.[87] However, this observation has since been disregarded as there was possible radiochemical contamination of a cinnamic acid derivative prior to the feeding experiment.[83]



47 49 48

Scheme 28 Wemple demonstrated the chemical rearrangement of the phenylpropanoid skeleton of the phenyl thioester of epoxycinnamic acid (**47**) to the thioester of tropic acid (**49**) using boron trifluoride etherate and sodium cyanoborohydride.[85]

2-Phenyl-3-aminopropionic acid (**50**) has also been investigated as a possible precursor to tropic acid (**35**), as it was envisaged that it could arise from the isomerisation of L-phenylalanine (**39**) by the putative mutase.[88] However, no incorporation of a ^{14}C-labelled sample of this amino acid was observed when fed to *D. innoxia* plants (Scheme 29).

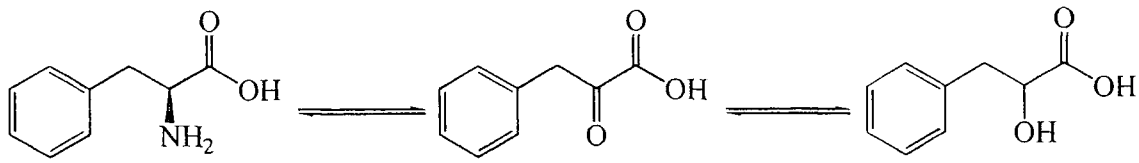


50 **1**

Scheme 29 A feeding experiment with isotopically labelled 2-phenyl-3-aminopropionic acid (**50**) to *D. innoxia* failed to incorporate into the tropic acid moiety of hyoscyamine (**1**).

The intermediacy of (*S*)-tropic acid (**35**) in hyoscyamine (**1**) biosynthesis has been generally accepted for many years. It was Stohs[89] who first reported the incorporation of [1-^{14}C]-tropic acid into hyoscyamine (**1**) when administered together with tropine (**32**) to suspension cultures of *D. stramonium*. However, it was intriguing that other researchers were not able to reproduce these findings later in the alkaloid producing root culture of *Duboisia lechhardtii*.[90] Robins and co-workers also revealed that when (*S*)-tropic acid (**35**) was administered to *D. stramonium* root cultures, the biosynthesis of hyoscyamine (**1**) was suppressed, and concluded that it is not an intermediate in the biosynthesis of the tropane alkaloids.[91] Also, it is noteworthy that no report has appeared describing the esterification of tropoyl-CoA with tropine (**32**), although the chemical synthesis of tropoyl-CoA has been established for some time.[92]

Attention then turned back to L-phenylalanine (**39**) and in particular, phenylpyruvic (**38**) and phenyllactic acids (**51**). Both were clearly candidate precursors since both can interconvert with L-phenylalanine (**39**) through transamination and reduction processes, as shown in Scheme 30.[93,94,95,96]



39 38 51

Scheme 30 L-phenylalanine (**39**), phenylpyruvic acid (**38**) and phenyllactic acid (**51**) can interconvert *via* transamination and reduction processes.

All of these acids have been successfully incorporated into tropane alkaloids in high and approximately equal levels, again indicative of their metabolic interconversion.[82,84,97,98,91] However, the feeding experiments were conducted on carbon labelled precursors rather than labelling with isotopes of hydrogen. Thus, it was impossible to distinguish which precursor was the closest in the biosynthetic pathway to hyoscyamine (**1**). However, it is interesting to note that phenyllactic acid (**51**) already possesses the hydroxyl group, which could contribute to the hydroxymethyl moiety of tropic acid (**35**) after a carbon skeletal rearrangement. Ever increasing evidence from past literature seemed to point to this conclusion. Evans and V. A. Woolley[99] in 1969 observed incorporations of fed DL-[1-^{14}C]- and [3-^{14}C]-labelled phenylalanines into littorine (**5**), [the tropine ester of (*R*)-phenyllactic acid (**51**)], hyoscyamine (**1**) and scopolamine (**3**). They observed incorporations decreasing in the order of littorine (**5**) > hyoscyamine (**1**) > scopolamine (**3**), which implied that phenyllactic acid (**51**) is a closer precursor than tropic acid (**35**) in the biosynthesis of tropane alkaloids (fig. 14).

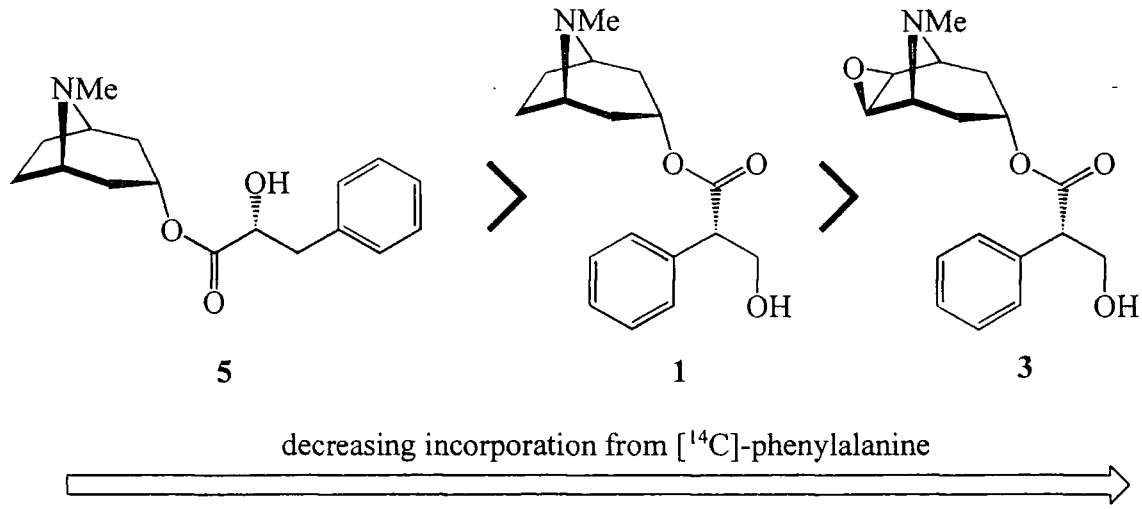
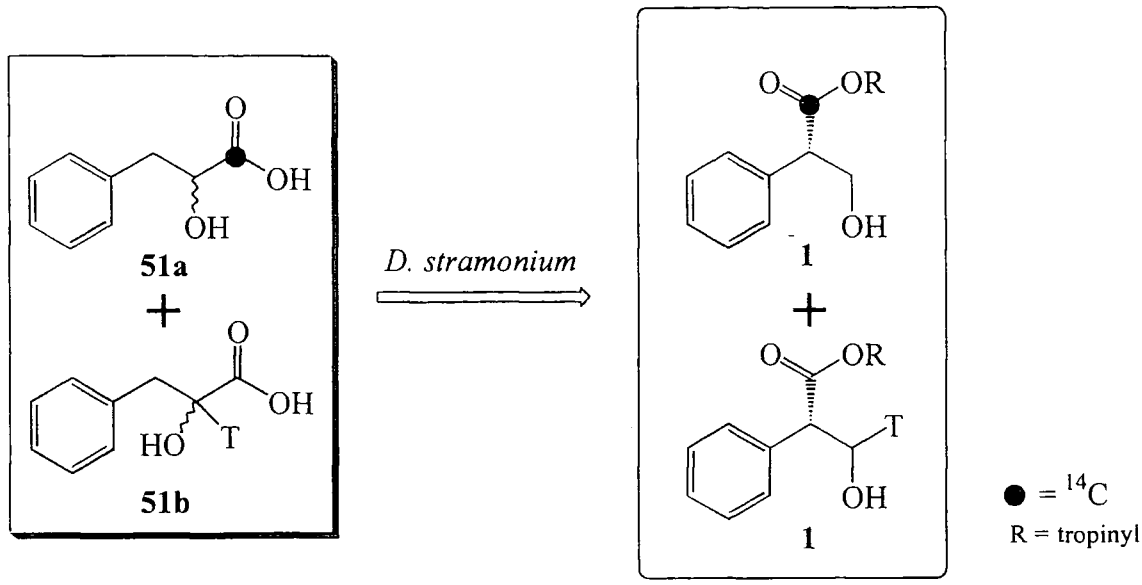


Figure 14 Feeding experiments with DL-[1-^{14}C]- and [3-^{14}C]-phenylalanines to *D. sanguinea* revealed that incorporations decreased in the order: littorine (**5**) > hyoscyamine (**1**) > scopolamine (**3**).

Kitamura and co-workers[100] have observed the *in vivo* interconversion of phenylalanine (**39**) and phenyllactic acid (**51**) into the tropane alkaloid producing root cultures of *Duboisia lechhardtii*. They used an ingenious isotope trapping technique, which involved the simultaneous feeding of [1-^{14}C]-phenylalanine and unlabelled phenyllactic acid to the root cultures. When the roots were harvested, the purified phenyllactic acid was found to be radioactive, thus "trapping" the fed radiolabelled phenylalanine as phenyllactic acid. Attempts to trap phenylpyruvic acid (**38**) and tropic acid (**35**) were unsuccessful. Although phenylpyruvic acid (**38**) is the bridging intermediate between the interconversion of phenylalanine (**39**) and phenyllactic acid (**51**), a failure to trap this intermediate could be due to the fact that the root cultures cannot use exogenously added phenylpyruvate (**38**).

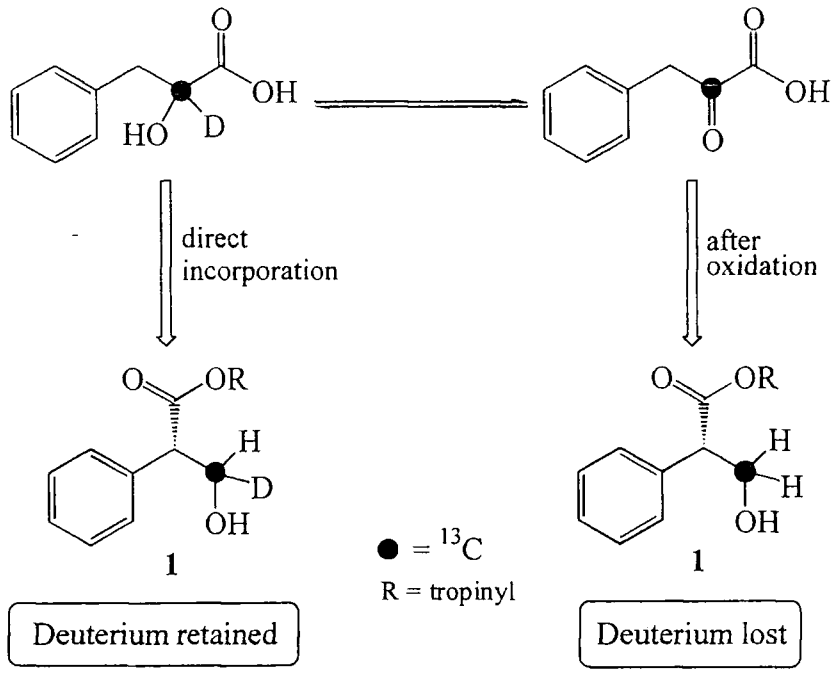
Competitive feeding experiments with [1-^{14}C]-phenyllactic acid (**51a**) and phenylpyruvic (**38**) acid, together, with unlabelled precursors, enabled Ansarin and Woolley to suggest that phenyllactic acid (**51**) is a closer precursor than phenylpyruvic acid (**38**) to the tropic acid moiety.[97] Subsequent feeding experiments with an admixture of (*R,S*)-[1-^{14}C]-phenyllactic acid (**51a**) and (*R,S*)-[2-^{3}H]-phenyllactic acid (**51b**) also strongly implicated phenyllactic acid (**51**).[101] They discovered that this dual radiolabelled acid mixture was incorporated into the tropate moiety of hyoscyamine (Scheme 31). The comparison of the ^{3}H:^{14}C ratio between the starting material and the isolated hyoscyamine revealed that they were similar. Although racemic radiolabelled acids were fed and the incorporations were typically low (~0.1%), this result was generally accepted and provided strong evidence which pointed to phenyllactic acid (**51**) as the closer precursor to hyoscyamine (**1**).



Scheme 31 The resultant incorporations into hyoscyamine (**1**) from the administered admixture of (*R,S*)-[1-^{14}C]-phenyllactic acid (**51a**) and (*R,S*)-[2-^{3}H]-phenyllactic acid (**51b**) to *D. stramonium*, provided strong evidence for the intermediacy of phenyllactic acid in hyoscyamine biosynthesis.

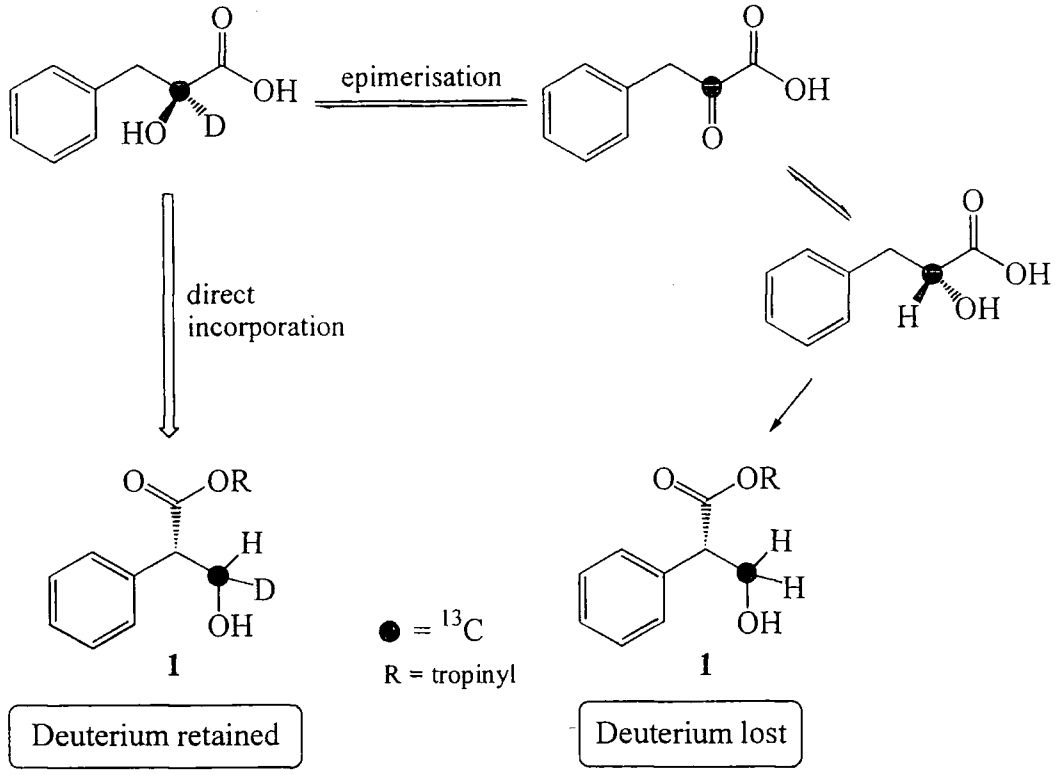
1.5.6 Stereochemical features of the rearrangement

To explore this issue in a discriminating way, O'Hagan and Chesters[102] devised a dual ^{13}C-^{2}H isotopic labelling strategy with phenyllactic acid (**51**). Deuterium was incorporated intact into the tropate moiety of hyoscyamine (**1**) from phenyllactate (**51**), demonstrating that phenyllactate (**51**) was the closer precursor than phenylpyruvate (**38**) to tropic acid (**35**). If phenylpyruvate was the closer precursor, deuterium would have been lost upon oxidation of phenyllactate to phenylpyruvate (Scheme 32). The experiment used ^{13}C-^{2}H at C-2 and the incorporation of ^{13}C-^{2}H into hyoscyamine was ~17%.



Scheme 32 Deuterium was incorporated intact into the tropate moiety of hyoscyamine (**1**) following a feeding experiment with [2-^{13}C,^{2}H]-phenyllactate to *D. stramonium*, demonstrating that phenyllactate (**51**) was the closer precursor than phenylpyruvate (**38**) to tropic acid (**35**).

In an extension of this work, O'Hagan and Chesters argued that that if retention of deuterium from the chiral centre of either [2-^{13}C,^{2}H]-(*R*)-D- or (*S*)-L-phenyllactic acid in the isolated tropic acid moiety of hyoscyamine (**1**) could be demonstrated, then it would clearly implicate that particular enantiomer as the true precursor (Scheme 33).[103]



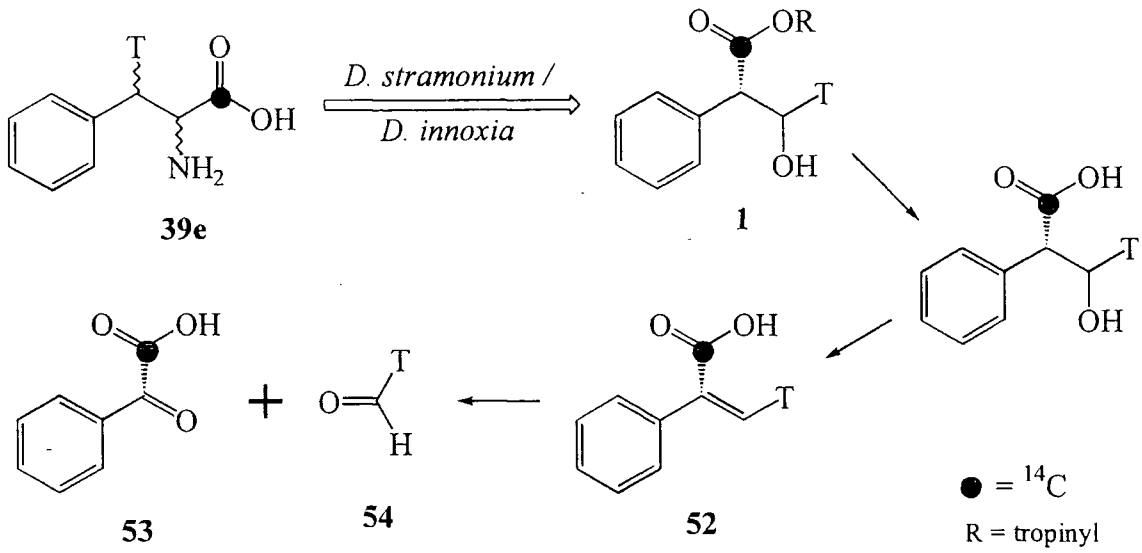
Scheme 33 Feeding experiments (*R*)-D-[2-^{13}C,^{2}H]- and (*S*)-L-[2-^{13}C,^{2}H]-phenyllactic acids to *D. stramonium* revealed that deuterium was retained at C-3′ of hyoscyamine (**1**) only with (*R*)-D-[2-^{13}C,^{2}H]-phenyllactate, thereby implicating the intermediacy of (*R*)-phenyllactate in hyoscyamine biosynthesis.

Accordingly, (*R*)-D-[2-^{13}C,^{2}H]- and (*S*)-L-[2-^{13}C,^{2}H]-phenyllactic acids were administered in separate experiments to transformed root cultures of *D. stramonium*.[103] By utilising high field ^{13}C-NMR with deuterium decoupling techniques, it was shown that the ^{13}C-^{2}H bond remained intact only in the (*R*)-D-phenyllactic acid experiments. This result was also confirmed by Ansarin and Woolley who fed (*R*)- and (*S*)-[1,3-$^{13}C_2$;1-^{14}C]-phenyllactates to whole plants.[104] (*S*)-Phenyllactate was only found to be incorporated in the roots at low levels (0.1%) relative to the (*R*)-isomer (0.9%). This difference was attributed to the interconversion of the (*S*)-isomer to the (*R*)-isomer *in vivo* through phenylpyruvate (**38**).

1.5.6.1 The unravelling of a stereochemical mystery in tropic acid biosynthesis

Leete first investigated the fate of the C-3 hydrogens of phenylalanine (**39**) in hyoscyamine (**1**) biosynthesis in 1984 by feeding a mixture of all four stereoisomers of [1-^{14}C,3-^{3}H]-phenylalanine (**39e**) to *D. stramonium* and *D. innoxia* plants.[105] The location of tritium at C-3′ of the tropic acid esters of hyoscyamine (**1**) and scopolamine

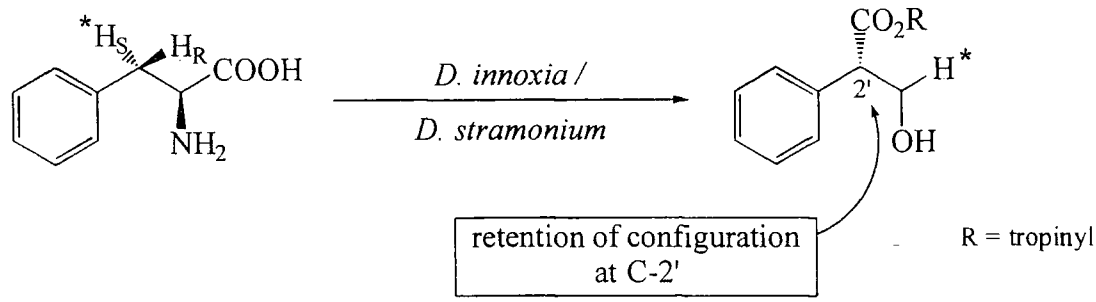
(**3**) was determined by subsequent serial degradation. This proceeded *via* hydrolysis of the esters to give the carboxylic acids, followed by dehydration to atropic acid (**52**). This was then further oxidatively cleaved to give benzoyl formic acid (**53**) and formaldehyde (**54**) as shown in Scheme 34.



Scheme 34 The administration of all four stereoisomers of [1-^{14}C,3-^{3}H]-phenylalanines (**39e**) to *D. stramonium* and *D. innoxia* plants revealed that tritium was located at C-3′ of the tropic acid moiety of isolated hyoscyamine (**1**) and scopolamine (**3**) after serial degradation. This prompted Leete to propose that a mutase is involved in mediating a 1,2 vicinal interchange process during the rearrangement.[105]

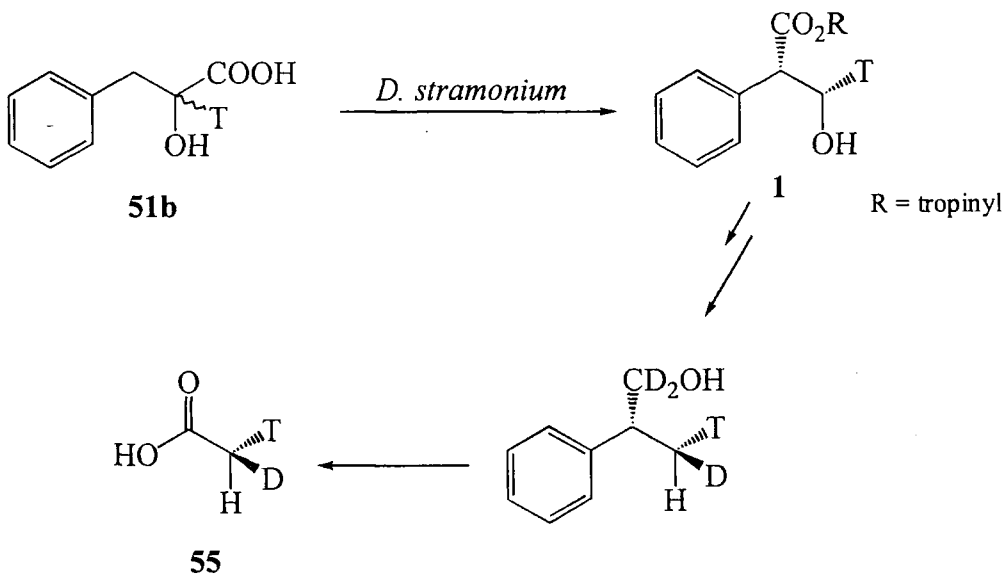
Leete observed the presence of tritium in the formaldehyde derivative, dimedone, suggesting its incorporation into C-3 of the tropic acid moiety of hyoscyamine (**1**). From this finding, it was proposed that hydrogen from C-3 of phenylalanine (**39**) was migrating in the opposite direction to the carboxyl group during the rearrangement. In other words, a mutase enzyme was responsible for a vicinal interchange process, which has potential similarities to the co-enzyme B_{12} mediated rearrangements.

In a follow-up study,[106] Leete synthesised and fed (*2S,3R*)-[1-^{14}C,3-^{3}H]-phenylalanine and (*2S,3S*)-[1-^{14}C,3-^{3}H]-phenylalanine to *D. stramonium* and *D. innoxia* plants. Analysis of the tropic acid moiety of hyoscyamine (**1**) and scopolamine (**3**) after barium hydroxide hydrolysis suggested that the 3-*pro-S* hydrogen of L-phenylalanine (**39**) had apparently migrated to C-3′ of the (*S*)-tropic acid moiety, and that the stereochemistry at C-2′ of the tropate ester was retained during the rearrangement (Scheme 35).



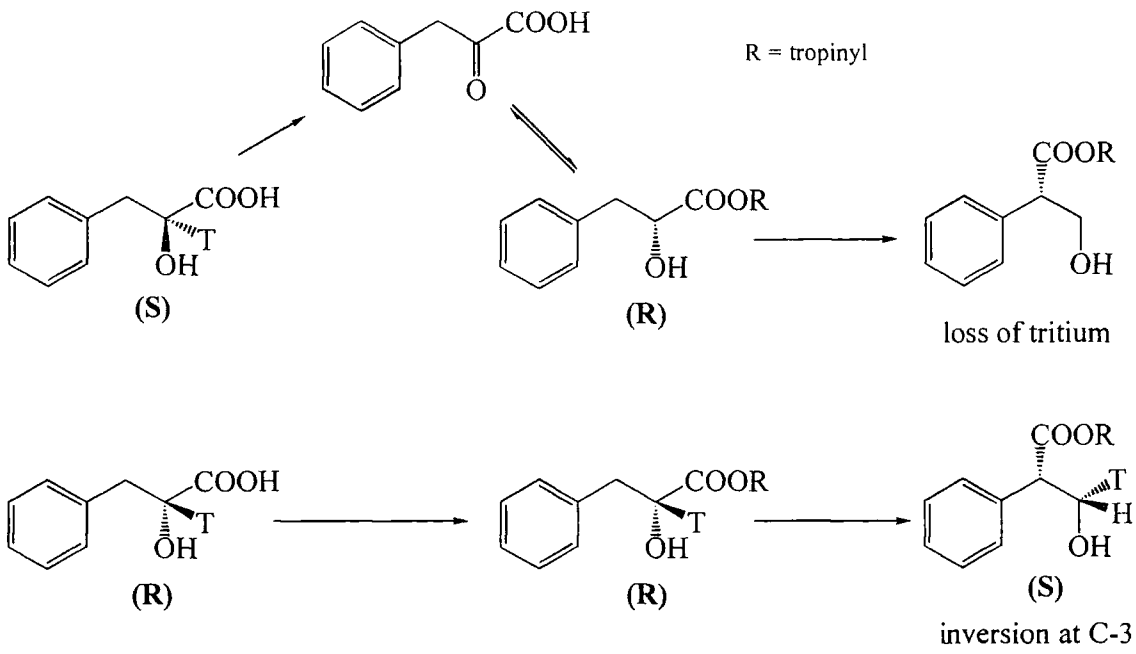
Scheme 35 The findings from the feeding experiments with (*2S,3R*)-[1-^{14}C,3-^{3}H]-phenylalanine and (*2S,3S*)-[1-^{14}C,3-^{3}H]-phenylalanine to *D. stramonium* and *D. innoxia* plants revealed that a 1,2 vicinal interchange process had occurred and that the stereochemistry at C-2′ of the tropate ester was retained during the rearrangement.

However, a later study by O'Hagan and co-workers gave no support to a vicinal interchange process, after examining the cryptic stereochemistry at C-3′ of the tropic acid moiety by utilising chiral methyl group methodology. This strategy involved the generation of a chiral methyl group using the three isotopes of hydrogen. Firstly tritium was introduced into the C-3′ pro-chiral centre of hyoscyamine (**1**) *via* a feeding experiment with [2-^{3}H]-phenyllactate (**51b**) in *D. stramonium* root cultures, as shown in Scheme 36. After isolation of the resultant hyoscyamine (**1**), deuterium was introduced chemically by displacement of the -OH group to give a chiral methyl moiety. This chiral methyl group was then excised from the molecule as chiral acetic acid (**55**) for analysis (Scheme 36).[107]



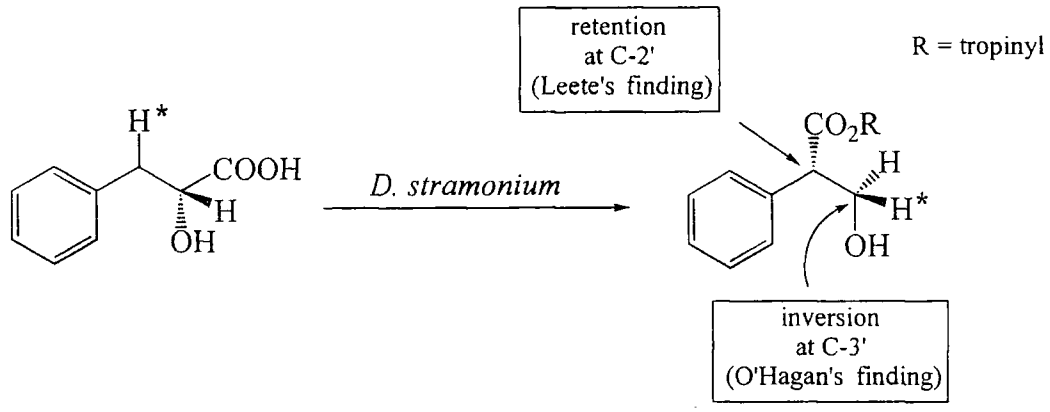
Scheme 36 Examination of the cryptic stereochemistry at C-3′ of the tropic acid moiety of hyoscyamine (**1**) by utilising chiral methyl group methodology.

This revealed that tritium had occupied the 3′-*pro-S* position of hyoscyamine (**1**). Therefore, the hydrogen atom that is delivered to this carbon during the rearrangement must occupy the 3′-*pro-R* site. Since it was known that (*R*)-D-phenyllactic acid and not (*S*)-L-phenyllactic acid is utilised in forming littorine (**5**), it was concluded that the C-3′-H bond of hyoscyamine (**1**) replaced the C-1′ – C-2′ bond of littorine (**5**) with inversion of configuration (Scheme 37).



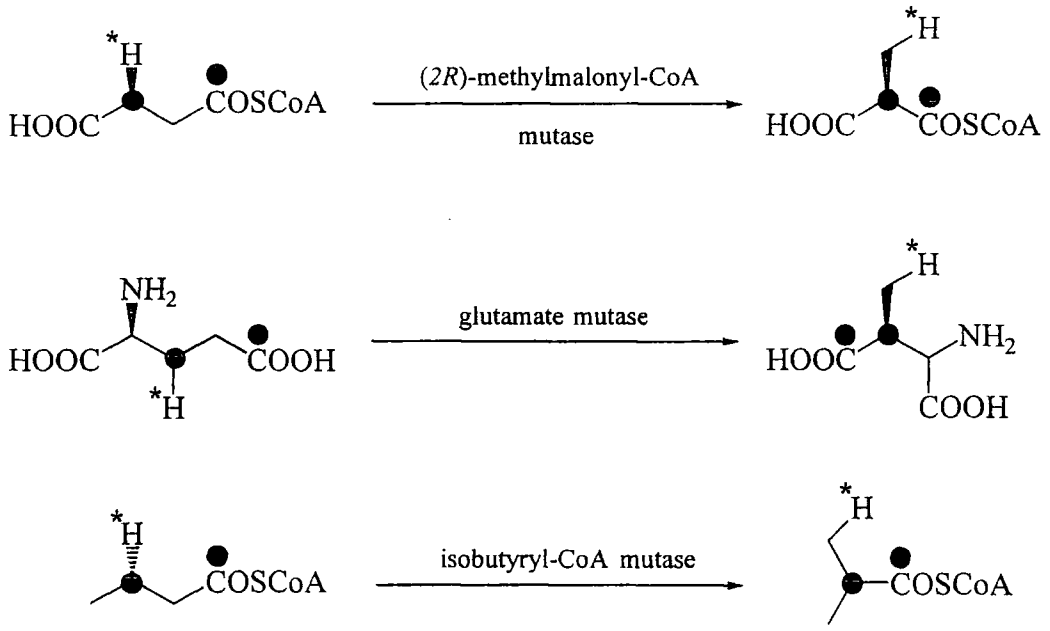
Scheme 37 By using chiral methyl group methodology to probe the cryptic stereochemistry at C-3′, the results revealed that the stereochemistry at C-3′ of the tropic acid moiety of hyoscyamine had inverted.

After establishing that the stereochemistry at C-3′ of tropic acid had inverted following the rearrangement, O'Hagan and co-workers re-examined Leete's proposal that there was a vicinal interchange process operating, i.e. a hydrogen atom (3-*pro-S*) from C-3 of phenylalanine [(**39**), and therefore littorine (**5**)] had migrated in the opposite direction of the carboxyl group to appear at C-3′ of hyoscyamine (**1**), resulting in retention of configuration at C-2′ of tropic acid (Scheme 38).



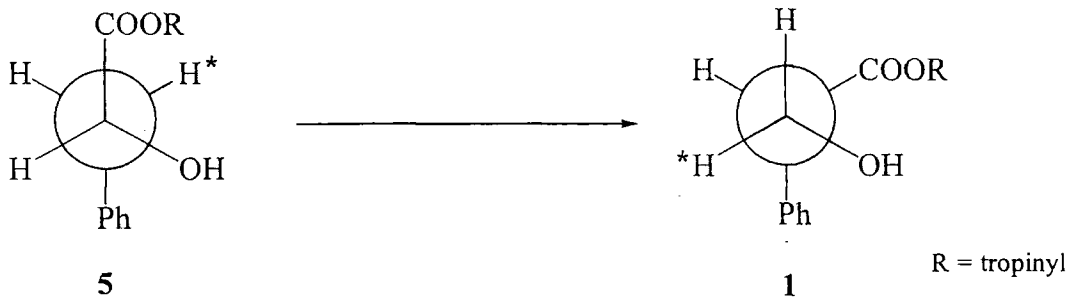
Scheme 38 A summary of Leete's and O'Hagan's findings for the stereochemical course of the rearrangement.

As discussed previously, the rearrangement of littorine (**5**) to hyoscyamine (**1**) has superficial similarities to the co-enzyme B_{12} mediated rearrangements such as methylmalonyl-CoA mutase,[108,109] isobutyryl-CoA mutase[110,111] and glutamate mutase.[112] All these rearrangements involve the vicinal interchange of a 1,2-migration of a carboxyl moiety with the subsequent 1,2-backmigration of a hydrogen atom (Scheme 39). However, the presence of co-enzyme B_{12} in plants have been rarely reported and Leete has failed to detect it in *Datura*,[106] although it is shown to be present in the herb comfrey (*Symphytum officinale*).[113]



Scheme 39 Some co-enzyme B_{12} mediated rearrangements.

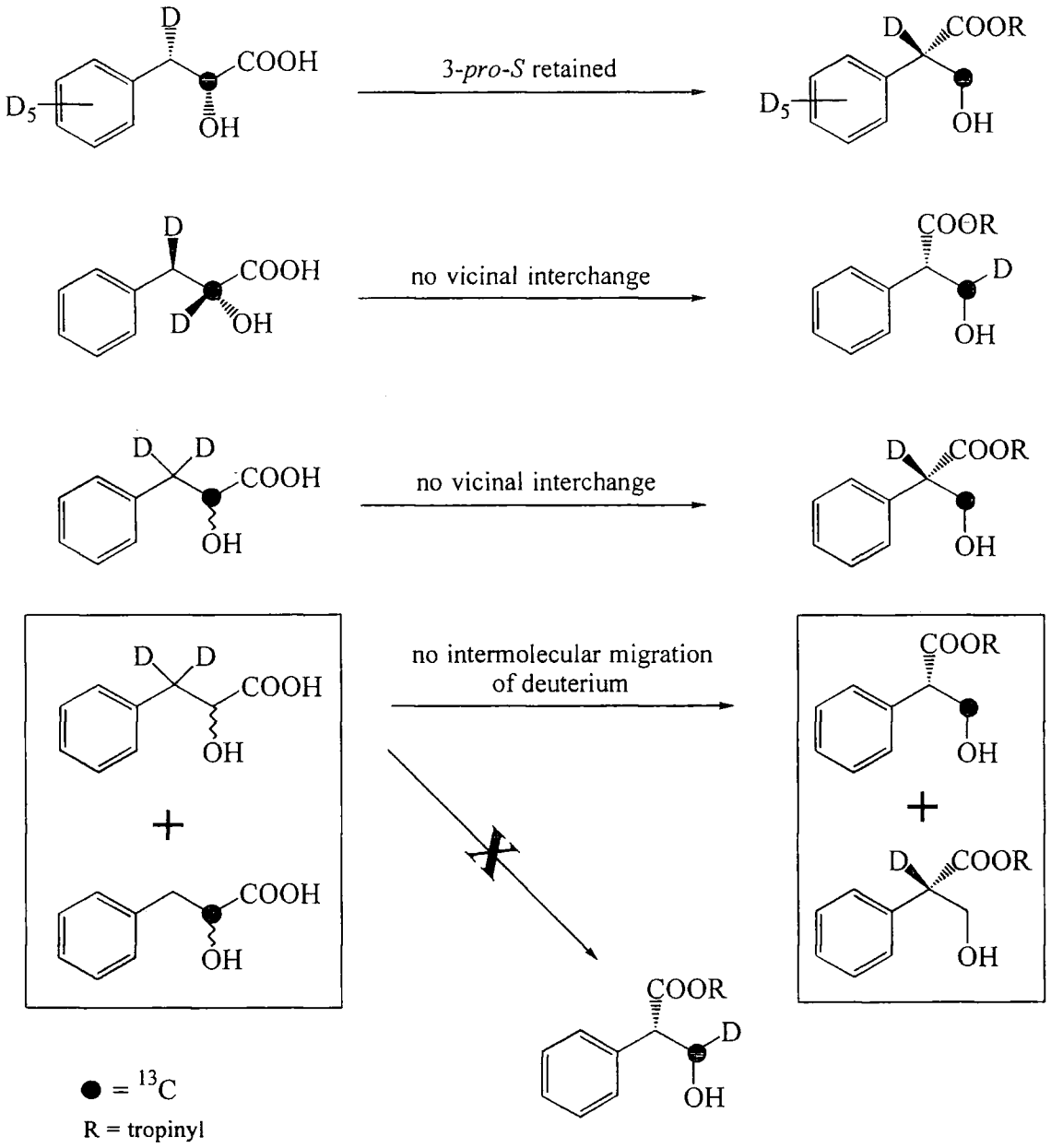
O'Hagan and co-workers suggested that if the 3-*pro-S* hydrogen of littorine (**5**) had been abstracted and delivered to C-3′ of tropic acid to invoke an inversion of stereochemistry, then the hydrogen atom must have been abstracted from one side of the molecule and returned to the other side (Scheme 40).



5 **1**

Scheme 40 A proposed 1,2 vicinal interchange process would have to been abstracted from one side of the molecule and returned to the other side.

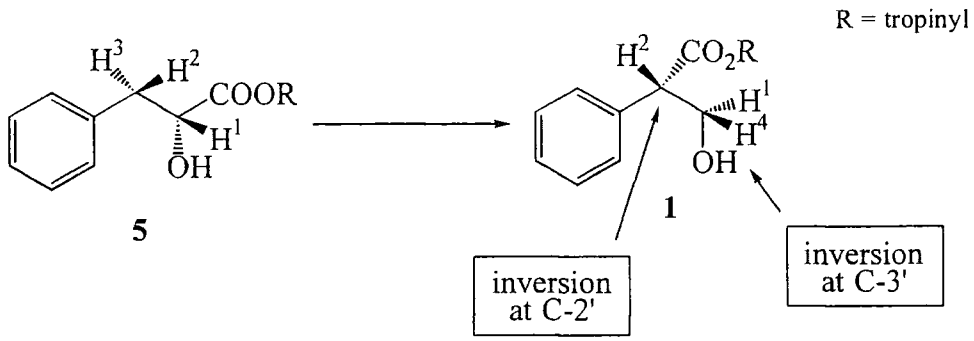
This seemed improbable since it would require either a substantial change in the conformation of the substrate or the active site of the enzyme. Therefore, a number of isotopically labelled (^{13}C and ^{2}H) phenyllactate precursors were prepared for feeding experiments to *D. stramonium* root cultures in order to assess if deuterium at C-3 undergoes migration following the rearrangement process (Scheme 41).[114]



Scheme 41 A number of isotopically labelled (^{13}C and ^{2}H) phenyllactate precursors were prepared for feeding experiments to *D. stramonium* root cultures. The results revealed that a 1,2 vicinal interchange process does not operate during the rearrangement.

The resulting incorporation patterns demonstrated unequivocally that the 3-*pro-R* hydrogen of phenyllactate (**51**), and therefore littorine (**5**)] was lost during the rearrangement process and that the 3-*pro-S* hydrogen was retained. Importantly, the results did not show any evidence of the redelivery of the hydrogen to C-3′ of the tropic acid moiety, leading to the conclusion that a vicinal interchange process does not occur.

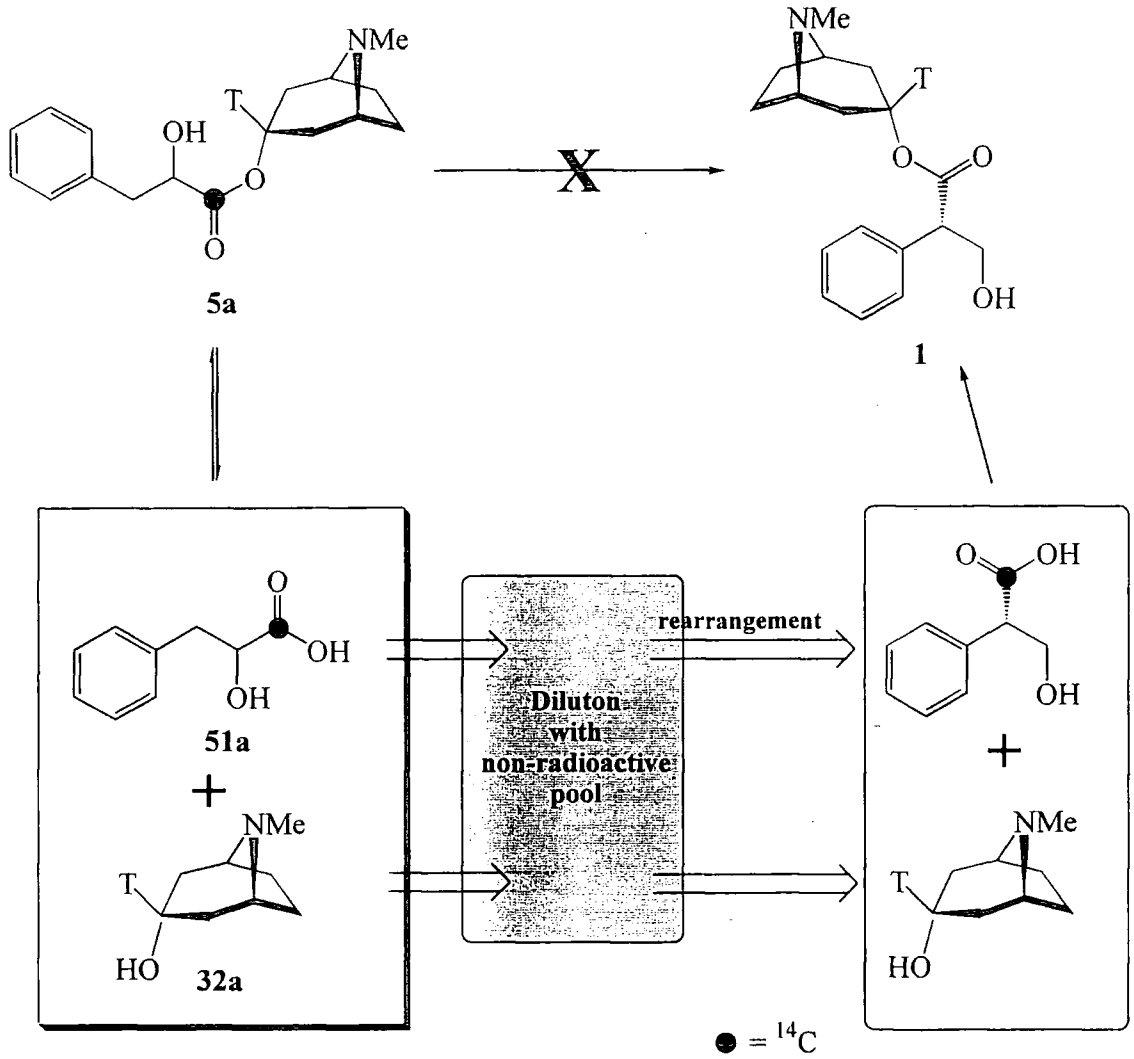
In summary, the current understanding for the rearrangement process involves an inversion of configuration at both migration termini, without a vicinal interchange process during the direct rearrangement of littorine (**5**) to hyoscyamine (**1**), as shown in Scheme 42. The origin of H^4 at C-3′ of hyoscyamine (**1**) following the rearrangement has not been investigated. However; if an aldehyde intermediate is involved in the rearrangement, as proposed in Chapter 2, Section 2.3, page 67, then H^4 could perhaps be delivered from an NADPH-mediated dehydrogenase.



Scheme 42 The rearrangement of littorine (**5**) to hyoscyamine (**1**) involves an inversion of configuration at both migration termini, without a vicinal interchange process.

1.5.7 Littorine – The ultimate intermediate to hyoscyamine

Littorine (**5**), the ester of tropine (**32**) and (*R*)-phenyllactate (**51**) was first isolated from *Anthocercis littorea*[115] and is widely found in tropane alkaloid plant producing species.[116] The role of littorine (**5**) in hyoscyamine (**1**) biosynthesis was first investigated by Leete in whole plants of *D. innoxia*.[84] By feeding [3β-^{3}H,1′-^{14}C]-littorine (**5a**), it was observed that the ^{3}H:^{14}C ratio (33:1) in the isolated hyoscyamine (**1**) was different to the ^{3}H:^{14}C ratio (6.75:1) in the administered starting material. From these results, Leete reasoned that the [3β-^{3}H,1′-^{14}C]-littorine (**5a**) had hydrolysed to [3β-^{3}H]-tropine (**32a**) and [1-^{14}C]-phenyllactic acid (**51a**), as shown in Scheme 43. After dilution with non-radioactive pools of tropine (**32**) and phenyllactic acid (**51**), these were re-esterified to form hyoscyamine (**1**) after phenyllactic acid (**51**) had rearranged into tropic acid (**35**). Thus, Leete concluded that littorine (**5**) did not appear to undergo direct rearrangement to hyoscyamine (**1**).

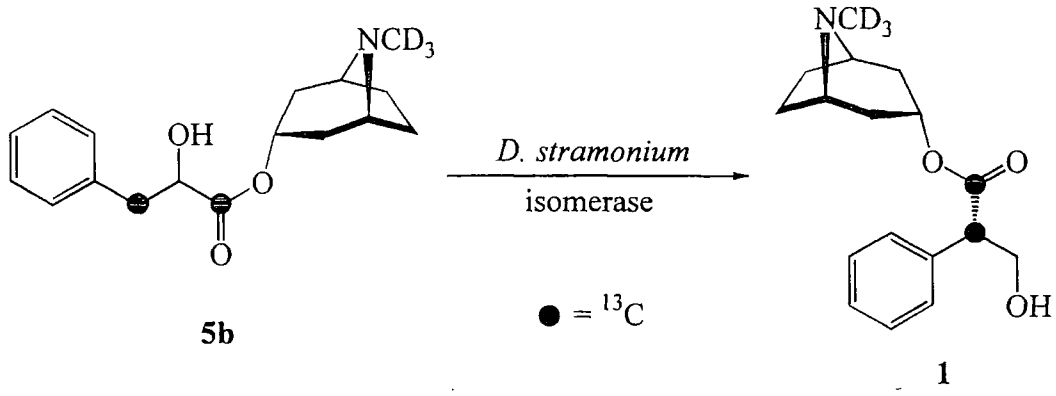


Scheme 43 The results from a feeding experiment with [3β-^{3}H,1′-^{14}C]-littorine (**5a**) to *D. innoxia*, prompted Leete to conclude that littorine (**5**) does not directly rearrange to hyoscyamine (**1**).

Since tropine esters have been shown to hydrolyse *in vivo*,[117,118] Leete proposed the possibility that the hydrolysis of littorine (**5**) is reversible. Hydrolysis of the labelled precursor, followed by re-esterification by a non-radioactive pool would mask any littorine rearranging directly by affecting the ^{3}H:^{14}C ratio. However, this proposal was subsequently discounted when the recovered littorine had the same ^{3}H:^{14}C ratio as the administered starting material. Further work on *D. stramonium* root cultures by Sauerwein and co-workers were the first to suggest littorine as the substrate for the rearrangement.[119] The incorporation of [1-^{13}C,2-^{2}H$_3$]-acetate into the acetate derived carbons of the tropane skeleton decreased in the order tropinone (**12**) > tropine (**32**) > littorine (**5**) > hyoscyamine (**1**) > 6β-hydroxyhyoscyamine (**26**) > scopolamine (**3**). This

range of enrichment values was interpreted as a direct indication of their position in the biosynthetic pathway as earlier intermediates would be more enriched than later ones.

Robins and co-workers later provided much more definitive evidence for the intermediacy of littorine (**5**).[120] They fed quintupally labelled littorine (**5b**) prepared from [*methyl*-$^{2}H_3$]-tropine (**32b**) and [1,3-$^{13}C_2$]-phenyllactic acid (**51c**) to transformed root cultures of *D. stramonium* (Scheme 44) and the presence of a M+5 mass enhancement (~5.5%) of hyoscyamine (**1**) was observed. It was reasoned that the level of enrichment was too high to arise from the hydrolysis of the administered isotopically enriched precursor and then recombination of labelled fragments, which would only account for 0.2%. Additional experiments with the inoculation of exogenously unlabelled phenyllactic acid (**51**) or tropine (**32**) managed to increase intact incorporations of [N-*methyl*-$^{2}H_3$,1,3-$^{13}C_2$]-hyoscyamine (**1**). Robins and co-workers therefore concluded that littorine (**5**) had undergone a direct rearrangement to hyoscyamine (**1**) and concluded that it is the ultimate precursor on the biosynthetic pathway to hyoscyamine (**1**).

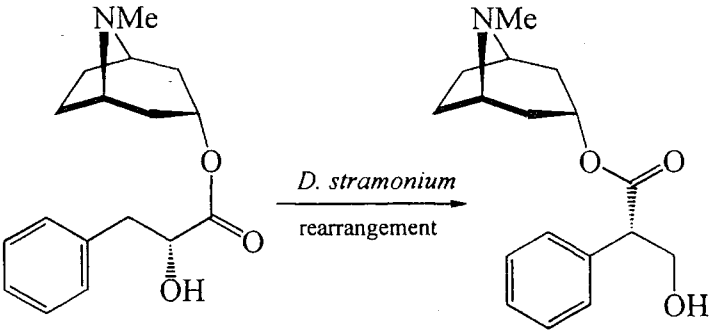


Scheme 44 The high level of incorporation of quintupally labelled littorine (**5b**) into hyoscyamine (**1**) following a feeding experiment to *D. stramonium*, strongly suggested that littorine (**5**) is the ultimate precursor on the biosynthetic pathway to hyoscyamine (**1**).

Chapter 2

2. ^{18}O Labelling in Hyoscyamine Biosynthesis

2.1 The Rearrangement of Littorine to Hyoscyamine

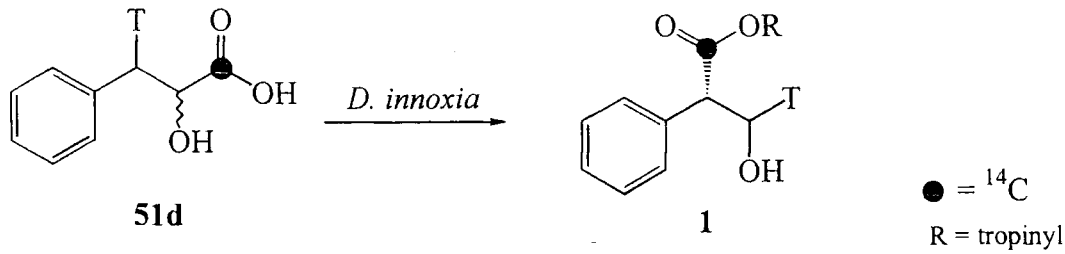


5 **1**

Scheme 45 The rearrangement of littorine (**5**) to hyoscyamine (**1**) has some similarities to co-enzyme B_{12} mediated isomerisations. However, recent studies have revealed that it is not co-enzyme B_{12} mediated, since a 1,2 vicinal interchange does not operate.[114]

The isomerisation of littorine (**5**) to hyoscyamine (**1**) shown in Scheme 45, has some similarities to co-enzyme B_{12} mediated mutases. In such co-enzyme B_{12} systems (around 12 are known),[121] a vicinal interchange process operates. A good example is (*R*)-methylmalonyl-CoA mutase,[108] which mediates the isomerisation of methylmalonyl-CoA to succinyl-CoA (Chapter 1, Section 1.5.4, Scheme 24, page 28).

As discussed in Chapter 1, early findings from a radiolabelled $^{3}H/^{14}C$ feeding study of [1-^{14}C,3-^{3}H]-phenyllactate (**51d**) have indicated that the hydrogen atom at C-3 of phenyllactate was removed and returned to C-3′ of hyoscyamine (**1**), as shown in Scheme 46.[105,106]



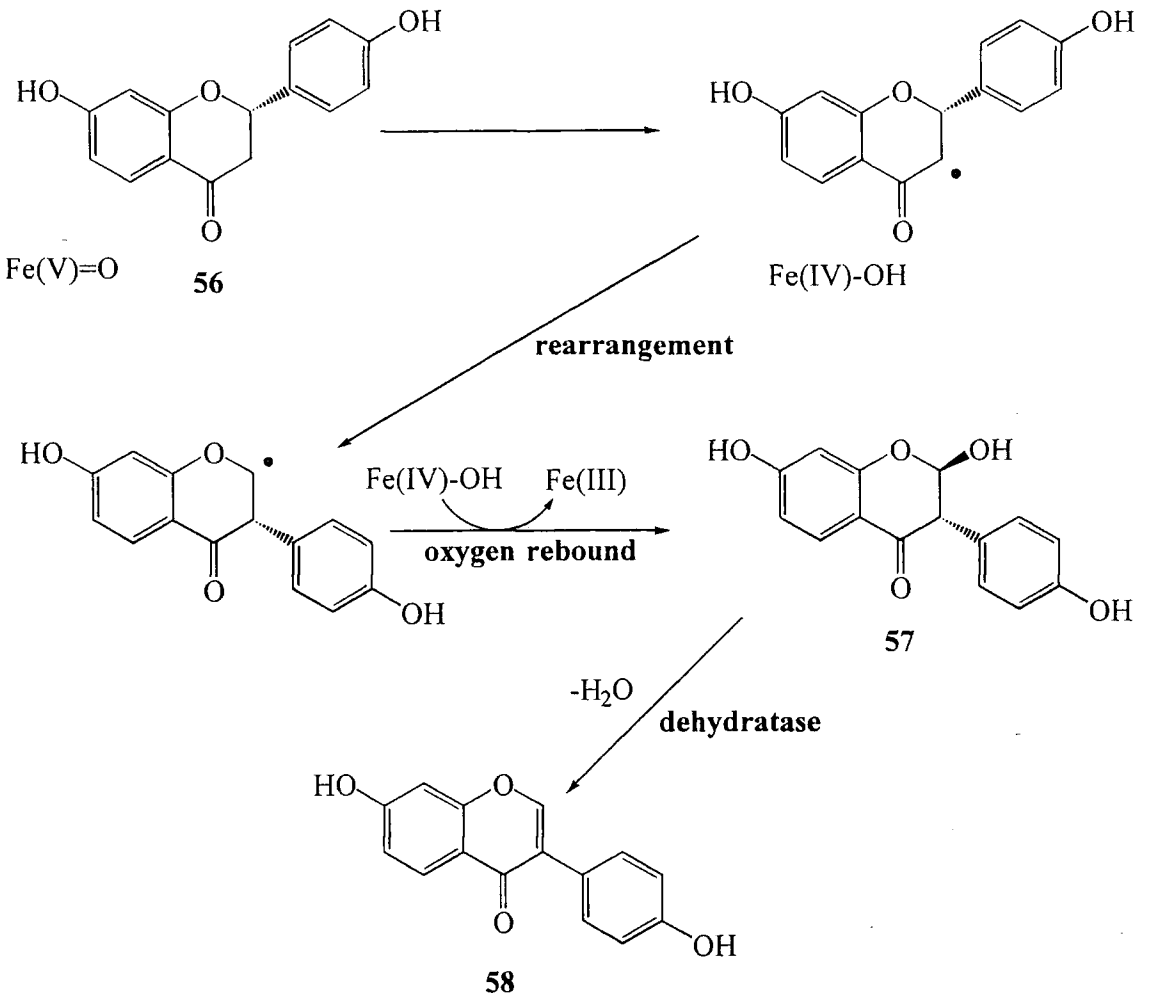
51d **1**

Scheme 46 The results from a feeding experiment with [1-^{14}C,3-^{3}H]-phenyllactate (**51d**) have indicated that a 1,2 vicinal interchange process operates during the rearrangement.

These results revealed an apparent 1,2 vicinal interchange process, which implicated a co-enzyme B_{12} type rearrangement. However, it must be noted that the radiochemical incorporations were very low (~0.1%). Later studies with stable isotopes[114] contradict these earlier conclusions, offering no evidence for a vicinal interchange process. In these stable isotope experiments, the 3-*pro-R* hydrogen of (*R*)-phenyllactate [and therefore littorine (**5**)] was removed, but it was not returned to C-3′ of hyoscyamine (**1**). The apparent lack of co-enzyme B_{12} observed in plants also supports these findings that co-enzyme B_{12} type process *is not* responsible for the rearrangement.

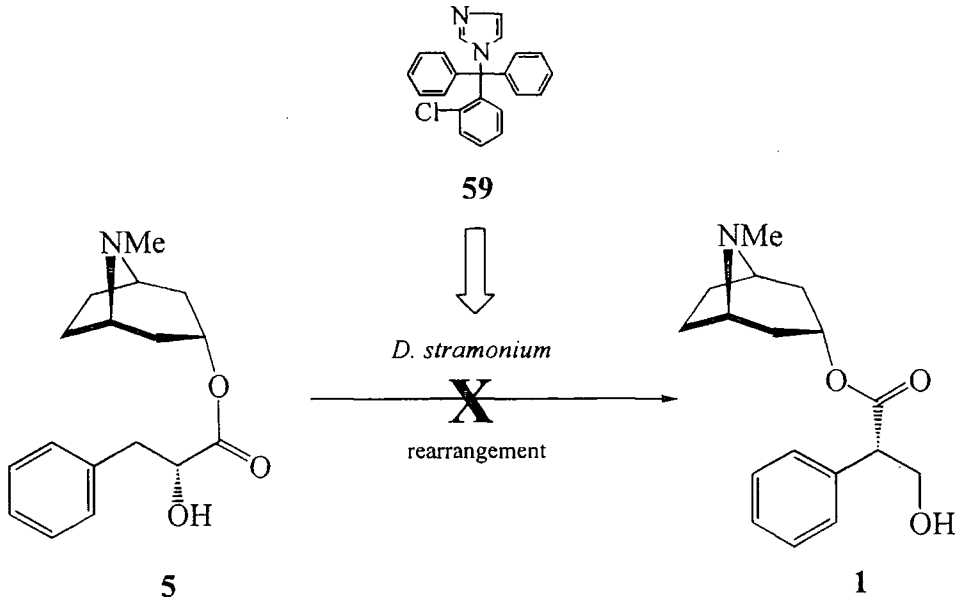
2.1.1 Iron-oxo enzyme chemistry

Iron-oxo enzymes are present in many plant systems and are responsible for generating radicals in biosynthetic pathways. An example of such a system is the iso-flavone synthase of *Pueraria lobata*. Sankawa was able to show in cell cultures that the conversion of liquiritigenin (**56**) to daidzein (**58**) is a cytochrome P-450 mediated process (Scheme 47).[122,123] Once a radical is formed, a vicinal aryl migration follows and the isomerised radical is quenched by an "oxygen rebound" process, *via* an hydroxyl radical from Fe(IV)-OH to generate 2,7,4′-trihydroxyisoflavone (**57**). In a cell free extract, Sankawa was able to characterise intermediate (**57**) and show that the new hydroxyl group was derived from molecular oxygen after a labelling experiment with $^{18}O_2$. Following the action of a dehydratase enzyme, the final iso-flavone product, daidzein (**58**) is produced.



Scheme 47 The conversion of liquiritigenin (**56**) to daidzein (**58**) is mediated by a cytochrome P-450 process in *Pueraria lobata*.

Recent work by Zabetakis and co-workers[124] in Durham, have shown that the P-450 inhibitor, clotrimazole (**59**), appears to inhibit the conversion of littorine (**5**) to hyoscyamine (**1**) in *D. stramonium* root cultures (Scheme 48). Clotrimazole (**59**) was also shown to neutralise the stimulatory effect of exogenously added littorine to *D. stramonium* and subsequently reduced the conversion of littorine (**5**) to hyoscyamine (**1**). However, clotrimazole (**59**) is a general phytotoxin and its role as a cytochrome P-450 inhibitor in the rearrangement of littorine (**5**) to hyoscyamine (**1**) must remain tentative at present.



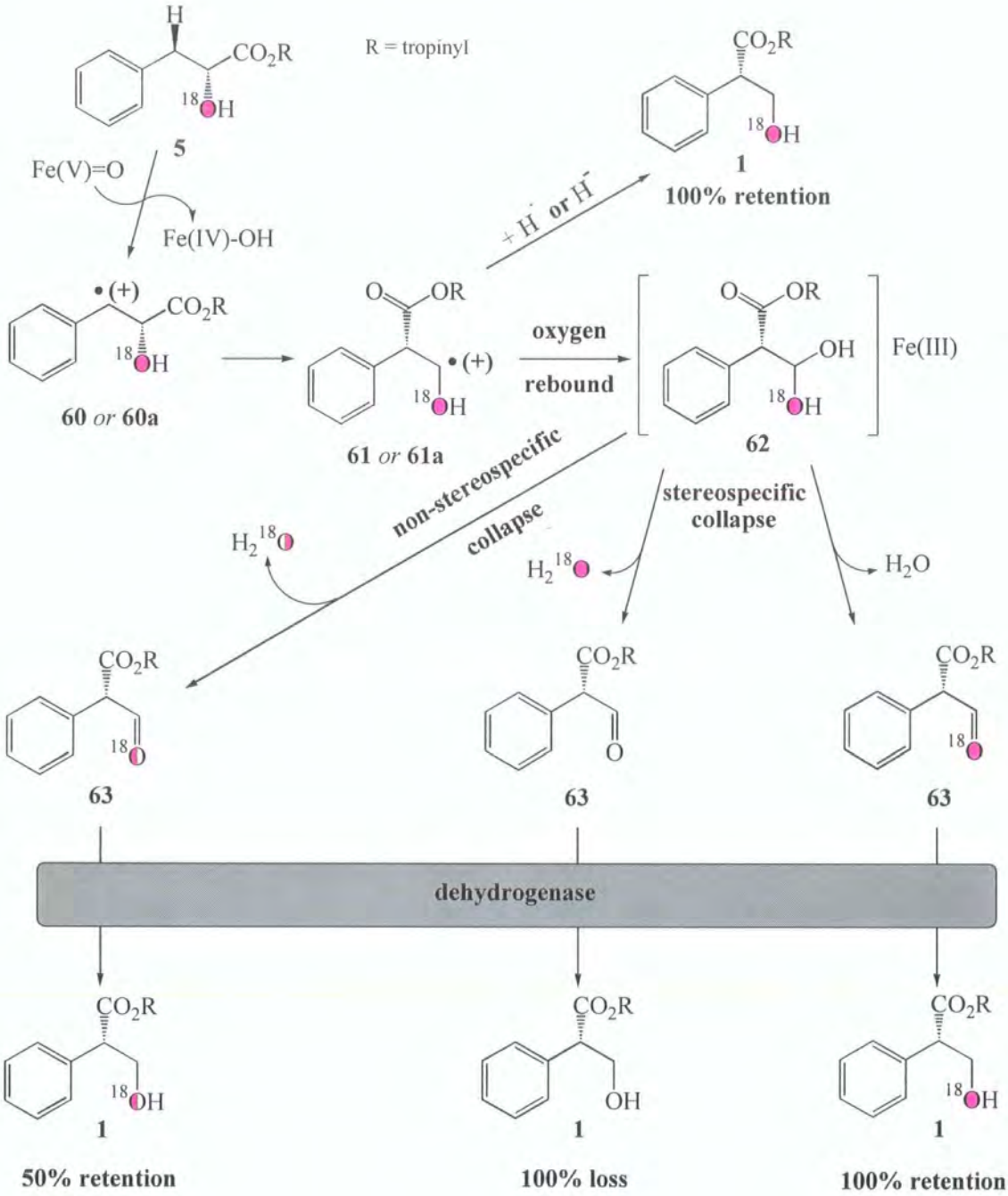
Scheme 48 The P-450 inhibitor, clotrimazole (**59**), appears to inhibit the conversion of littorine (**5**) to hyoscyamine (**1**) in *D. stramonium* root cultures.

2.2 ^{18}O Labelling Strategy

Following these findings and the literature reports discussed above, it appeared appropriate to examine the possibility of an oxygen rebound process operating during the rearrangement of littorine (**5**) to hyoscyamine (**1**).

Following abstraction of the 3-*pro-R* hydrogen of littorine (**5**) by the enzyme to leave a substrate radical (**60**) [(or carbocation (**60a**) in a 2 electron oxidation)], such a rearrangement would generate the isomeric product radical (**61**) [(or carbocation (**61a**)], shown in Scheme 49]. This product radical (or carbocation) may then be quenched in two ways. Firstly, direct quenching by a hydrogen radical (or hydride ion) would furnish the desired product, hyoscyamine (**1**). This would result in up to 100% retention of ^{18}O label from the rearrangement of littorine (**5**) to hyoscyamine (**1**). Secondly, an oxygen rebound process from Fe(IV)-OH could quench the radical to generate a geminal diol (**62**), which would collapse to give aldehyde (**63**). If a stereospecific collapse was to occur, either 100% retention or complete loss of the ^{18}O label would be observed. However, non-stereospecific collapse would give a statistical 50% retention of ^{18}O label. Once the aldehyde (**63**) is formed, it could then be reduced by a

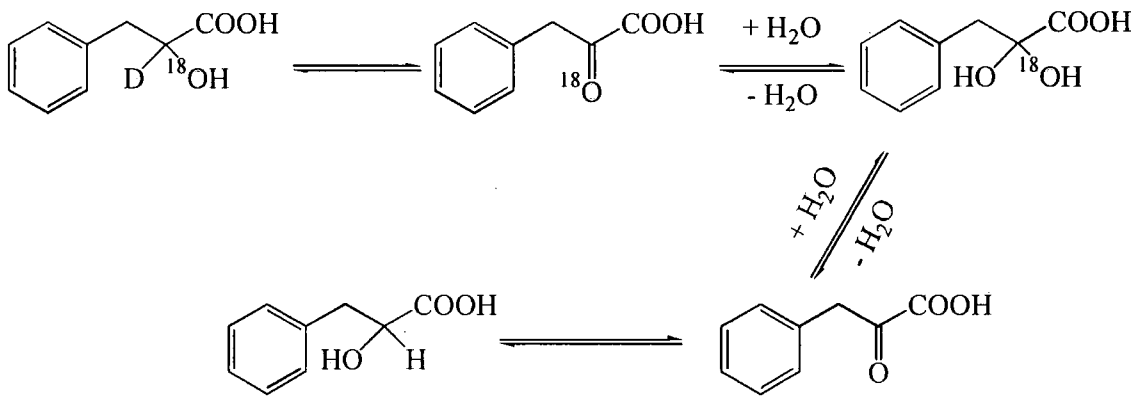
dehydrogenase to furnish hyoscyamine (**1**). These possibilities are summarised in Scheme 49.



Scheme 49 Anticipated possible outcomes for the incorporation of ^{18}O into hyoscyamine (**1**) following the rearrangement of littorine (**5**).

To pursue this investigation, a sample of phenyllactate labelled with ^{18}O at C-2 was required. This target was chosen since it has been shown that phenyllactate becomes efficiently incorporated into hyoscyamine. Such an experiment would label both littorine and hyoscyamine and allow isotope incorporations into both alkaloids to be compared.

Clearly, a preparation, which allows ^{18}O label to be placed at C-2 of phenyllactate, is required, however, it was decided also, to introduce deuterium at C-2. This label was included to act as a metabolic probe since a proportion of the phenyllactate molecules will interconvert with phenylpyruvate resulting in washout of the ^{18}O label with intracellular water (Scheme 50).



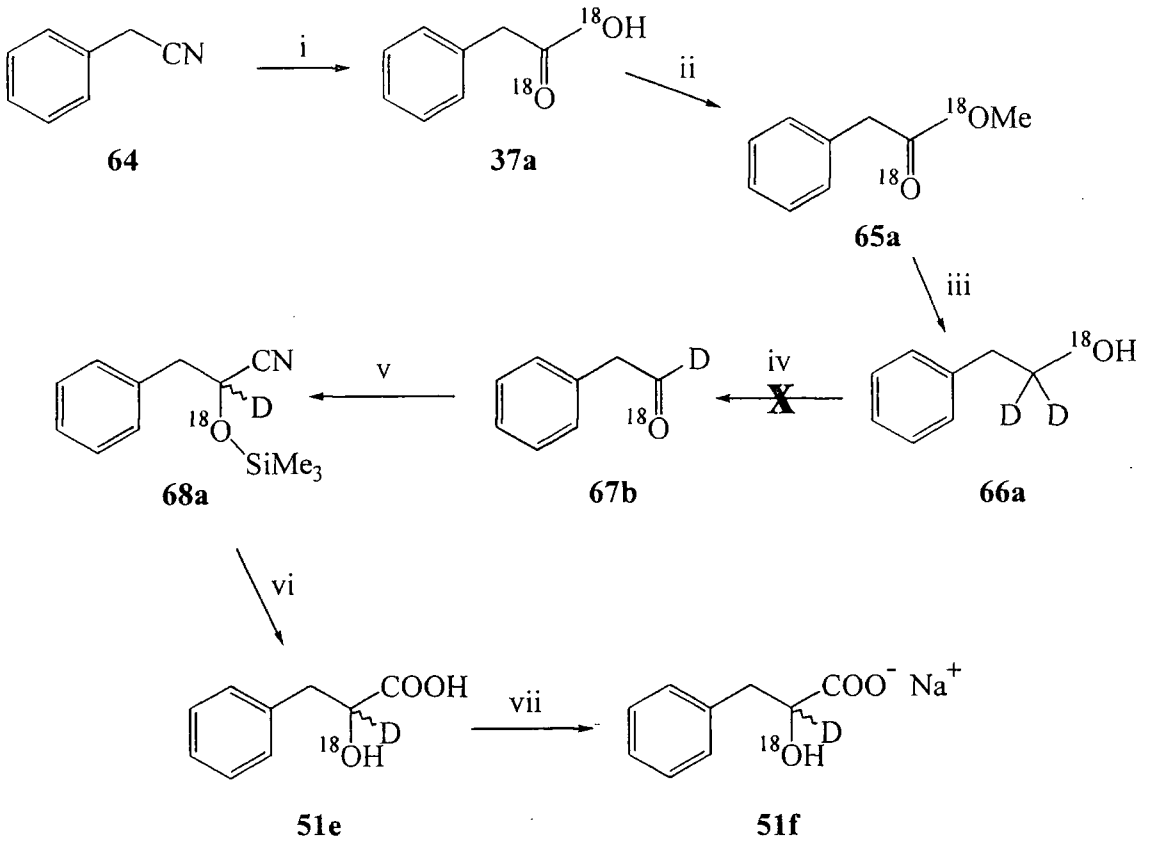
Scheme 50 Dually labelled phenyllactate with ^{2}H and ^{18}O was required for the feeding experiment to *D. stramonium* since phenyllactate molecules will interconvert with phenylpyruvate resulting in washout of the ^{18}O label with intracellular water.

Thus, in the experiment only those molecules of the alkaloids carrying both ^{2}H and ^{18}O will be considered in the final analysis, since any molecules of phenyllactate undergoing interconversion will lose deuterium before becoming esterified to form littorine. Thus it was necessary to prepare a sample of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**).

2.2.1 Preliminary attempt at the synthesis of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (51f)

At the outset, a strategy was devised to introduce ^{18}O by the hydrolysis of benzyl cyanide (**64**) with a limiting amount of expensive $H_2{}^{18}O$, to give [1-$^{18}O_2$]-phenylacetic

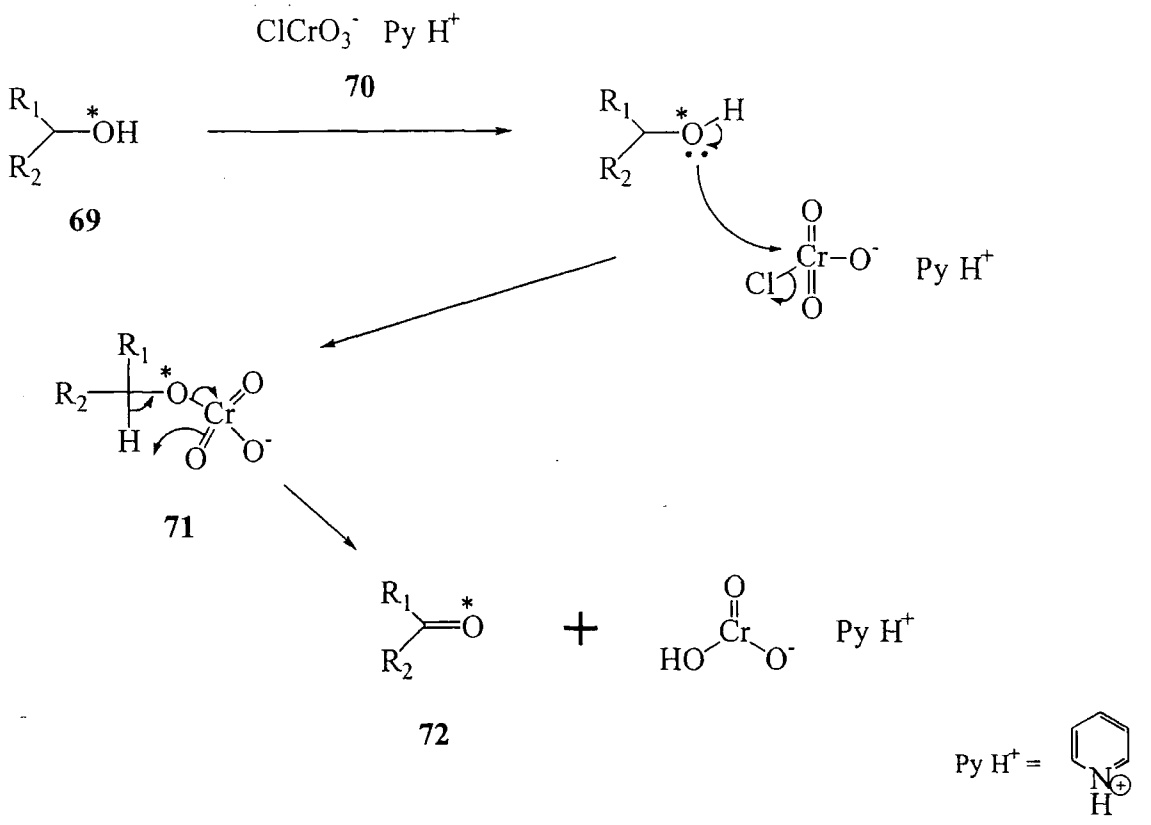
acid (**37a**), as shown in Scheme 51. Deuterium would then be introduced inexpensively *via* reduction of the carboxylic acid (**37a**) with lithium aluminium deuteride to generate 2-[1-$^{2}H_{2}$,^{18}O]-phenylethanol (**66a**). The methyl ester of [1-$^{18}O_{2}$]-phenylacetic acid (**65a**) was required, since previous preliminary experiments have shown[98,102] that the reduction with lithium aluminium hydride proceeds more smoothly with an ester rather than a carboxylic acid. Since 2-[1-$^{2}H_{2}$,^{18}O]-phenylethanol (**66a**) is one carbon short of that required, chain extension is necessary to form the linear phenylpropanoid chain of phenyllactate. This could be achieved *via* the nucleophilic attack of a cyanide anion on an electrophile such as phenylacetaldehyde to give a cyanohydrin. This route has its advantages since firstly, the oxidation step of 2-[1-$^{2}H_{2}$,^{18}O]-phenylethanol (**66a**) to give [1-^{2}H,^{18}O]-phenylacetaldehyde (**67b**) should retain both isotopic labels (^{2}H and ^{18}O); and secondly, the formation of a cyanohydrin would also retain these labels and prime the molecule for the hydrolysis of the nitrile group to give phenyllactic acid. Sodium phenyllactate could then be formed by the addition of base. Although, this route looked promising initially, careful attention was paid towards the reaction conditions. Previous work by Chesters and co-workers[102] established that pyridinium chlorochromate (PCC)[125] with ground molecular sieves gave the best results for the oxidation of 2-phenylethanol (**66**) to phenylacetaldehyde (**67**). Once [1-^{2}H,^{18}O]-phenylacetaldehyde (**67b**) was prepared using these reaction conditions, the formation of the cyanohydrin would be carried out under anhydrous conditions to avoid potential washout of the ^{18}O label. Kobayashi and co-workers[126] have successfully utilised trimethylsilylcyanide (TMSCN) in this way to generate silylated cyanohydrins and their methodology can be applied to our proposed route. Once the silylated cyanohydrin (**68a**) is prepared, the hydrolysis of the nitrile group would require acidic conditions to avoid loss of ^{18}O label and give [2-^{2}H,^{18}O]-phenyllactic acid (**51e**). At this point, the isotopic labels should be secure and the formation of sodium [2-^{2}H,^{18}O]-phenyllactate (**51f**) required for feeding would then be generated by the careful addition of base. However, the preliminary experiments to introduce the ^{18}O label in this way failed (Scheme 51). Such a discovery could only be made by GC-MS analysis of the final product, after a "hot" run.



i. $H_2{}^{18}O$, BuOK, ^{t}BuOH; ii. CH_2N_2; iii. $LiAlD_4$; iv. PCC, ground 3Å molecular sieves; v. TMSCN; vi. 50% $HCl_{(aq)}$; vii. $NaOH_{(aq)}$

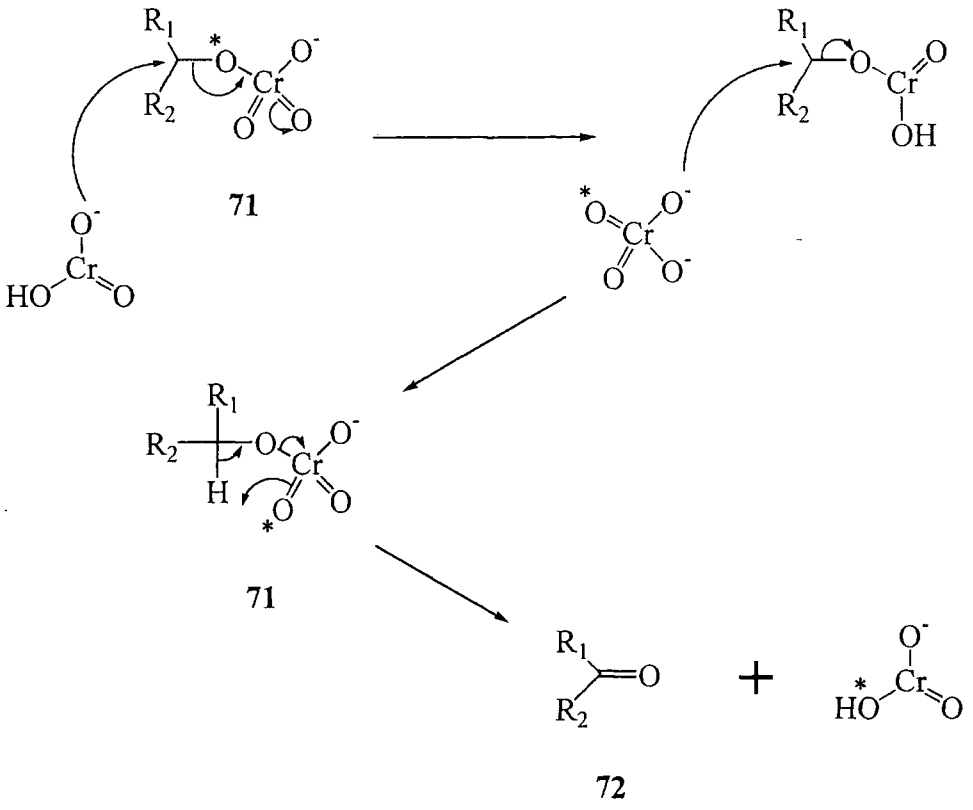
Scheme 51 Preliminary synthetic route to sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**).

It was clearly of interest to discover where in the reaction scheme ^{18}O label was lost. However, suspicion of the PCC (pyridinium chlorochromate) oxidation step arose by intuition (step iv, Scheme 51). Therefore, 2-[1-^{18}O]-phenylethanol was synthesised with $H_2{}^{18}O$ (~10% atom) and oxidised to [1-^{18}O]-phenylacetaldehyde using the same procedure as discussed below with PCC. GC-MS analysis revealed that the ^{18}O label from 2-[1-^{18}O]-phenylethanol (7.9%) was completely lost following oxidation to [1-^{18}O]-phenylacetaldehyde with PCC (**70**). This curious result somewhat contradicts the literature mechanism for the oxidation of alcohols with PCC (**70**). The accepted mechanism[127,128] involves a five atom concerted intermediate (**71**). This intermediate (**71**) then collapses in an elimination reaction to generate the product (**72**) with the original oxygen from the alcohol (**69**) retained, as shown in Scheme 52.



Scheme 52 The accepted mechanism for the oxidation of alcohols with PCC (**70**), results in the retention of the original hydroxyl oxygen atom.

Interestingly, there are no reported ^{18}O studies for this generally accepted chromate oxidation mechanism. Therefore, this finding is rather unique and perhaps could be explained by the operation of a "Ping-Pong" process, whereby other molecules of $HCrO_3^-$ [chromate (IV)] undergo nucleophilic substitution with the intermediate (**71**), whilst displacing CrO_4^{2-} in an equilibrium process. This would distribute the ^{18}O label though the chromate population (Scheme 53).



Scheme 53 Proposed mechanism for the loss of the original oxygen atom from the alcohol *via* a "Ping-Pong" process during oxidation with PCC, as discussed in the text.

In light of this finding, the introduction of ^{18}O label *via* a different method was pursued. It was envisaged that the isotope could be introduced into phenylacetaldehyde (**67**) *via* an exchange reaction with $H_2{}^{18}O$. Preliminary ^{18}O exchange experiments with phenylacetaldehyde (**67**) using again ~ 10% $H_2{}^{18}O$ were carried out under a number of conditions and the resultant GC-MS analyses of the phenylacetaldehyde (**67**) are presented in Table 2.

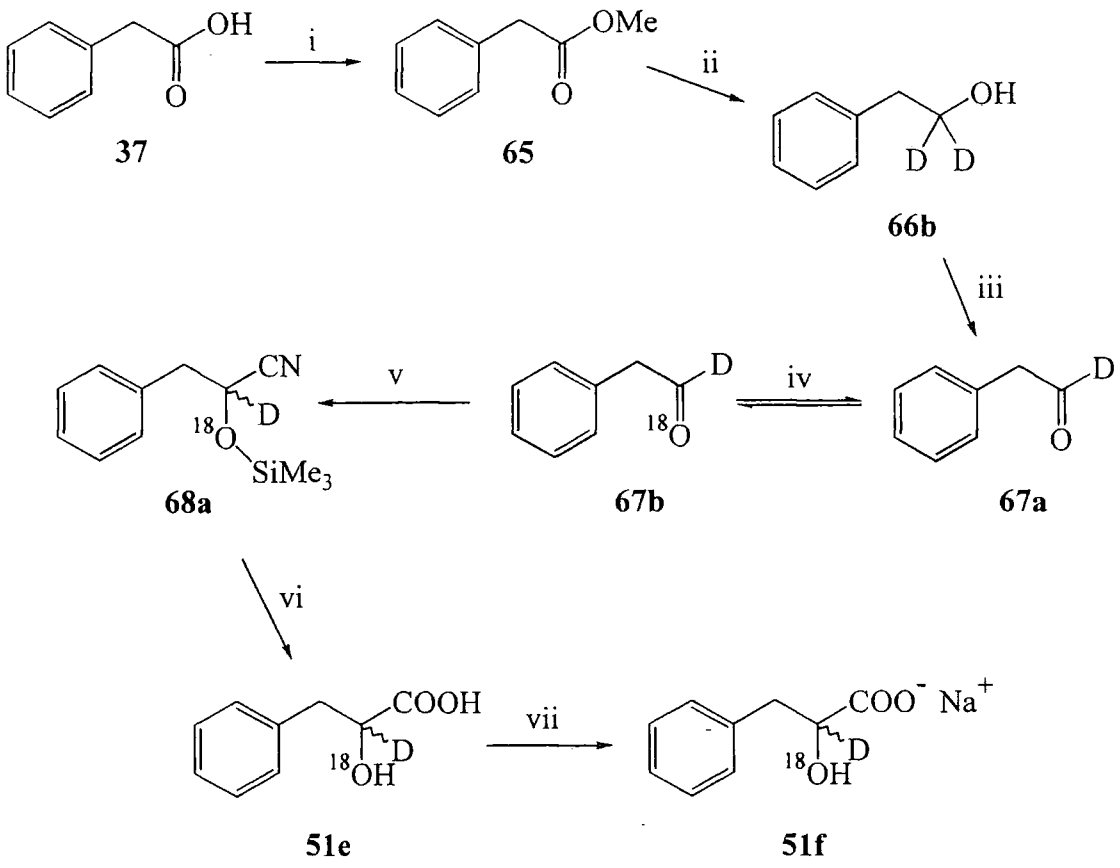
Phenylacetaldehyde	Reaction conditions		
	$H_2{}^{18}O$, 39eq., DCM, stir overnight	$H_2{}^{18}O$, 39eq., H^+, DCM, stir overnight	$H_2{}^{18}O$, 43eq., H^+, DCM, Carius tube, 120°C, 5 hours
M	92.1	92.2	91.2
M+1	3.8	4.4	3.8
M+2	3.9	3.3	5.2

Table 2 Comparison of incorporation levels into phenylacetaldehyde (**67**) following an exchange reaction with $H_2{}^{18}O$ (~10% atom) under three different reaction conditions. GC-MS analysis reveals that the incorporation of ^{18}O label (M+2) into phenylacetaldehyde (**67**) is most efficient under high temperature and pressure. Values in the table are adjusted after considering natural abundance.

The GC-MS results for the preliminary $H_2{}^{18}O$ (~ 10% atom) exchange reactions with phenylacetaldehyde (**67**) revealed that the reaction in the Carius tube was most effective and introduced 5.2% of ^{18}O label compared to 3.9% just from stirring, or from the catalytic addition of acid (3.3%), see Table 2. In the light of these observations, it was decided to introduce the ^{18}O label into [1-^{2}H]-phenylacetaldehyde (**67a**) by exchange in a Carius tube using $H_2{}^{18}O$ under acidic conditions as described below.

2.2.2 Synthesis of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (51f)

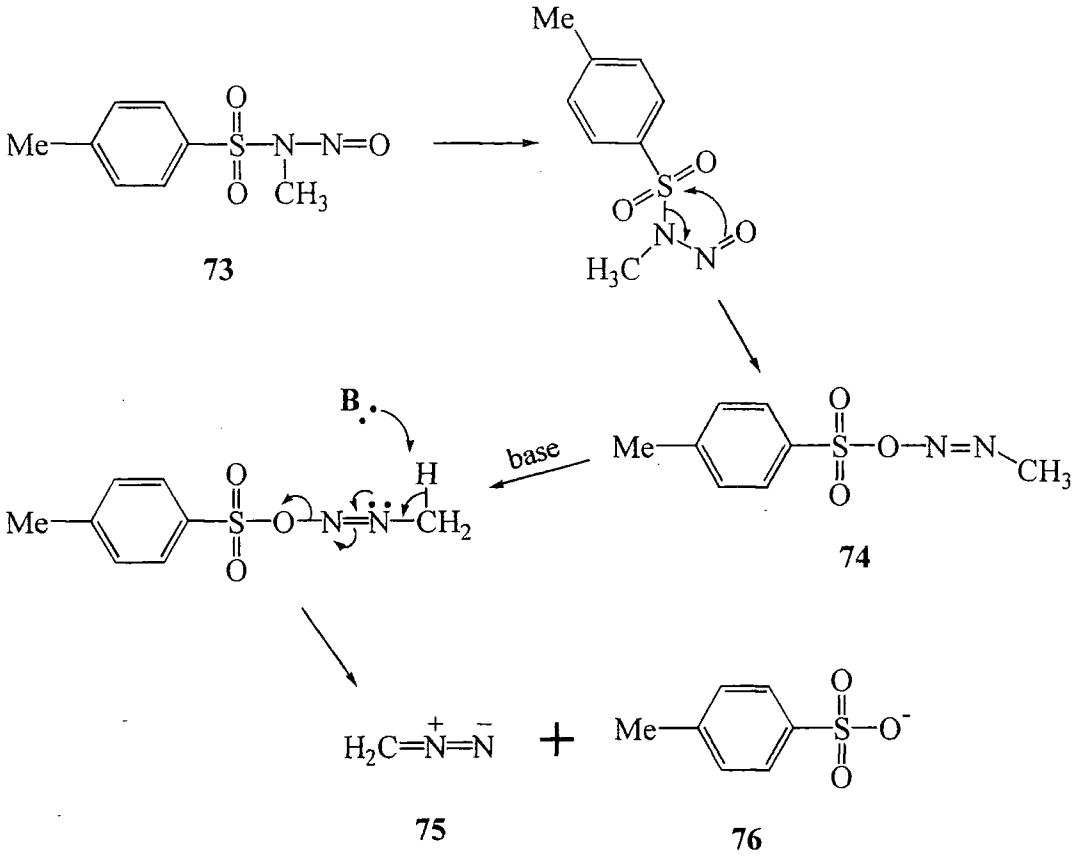
The optimised synthetic route which was developed for the synthesis of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**) is outlined in Scheme 54.



i. CH_2N_2; ii. $LiAlD_4$; iii. PCC, ground 3Å molecular sieves; iv. $H_2{}^{18}O$ / H^+; v. TMSCN; vi. 50% $HCl_{(aq)}$; vii. $NaOH_{(aq)}$

Scheme 54 The synthetic route to sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**).

The final strategy for the synthesis of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**) differs from the initial strategy (Scheme 51) only in the timing and the method for the introduction of the ^{18}O label. Accordingly, the starting reagent in the route is phenylacetic acid (**37**) rather than benzylcyanide (**64**). As the lithium aluminium hydride reduction proceeded more smoothly with an ester rather than a carboxylic acid, phenylacetic acid was converted to its methyl ester (**65**). Accordingly, phenylacetic acid (**37**) was treated with diazomethane (**75**), which was generated from the base-induced decomposition of Diazald® [N-methyl-N-nitroso-*p*-toluenesulphonamide (**73**)]. The resultant diazomethane is believed to be generated by the initial rearrangement of Diazald® to give intermediate (**74**).[129] Subsequent base mediated deprotonation results in decomposition of intermediate (**74**), generating gaseous diazomethane (**75**) and *p*-toluenesulphonate (**76**), as shown in Scheme 55.



Scheme 55 The mechanism of diazomethane (75) generation from the base-induced decomposition of Diazald® [N-methyl-N-nitroso-*p*-toluenesulphonamide (73)].

The evolved gaseous diazomethane (**75**) is dissolved by bubbling through ether and the solution added to a solution of phenylacetic acid (**37**) in ether. This gave methyl phenylacetate (**65**) in quantitative yield.

Lithium aluminium deuteride is a cheap source of deuterium isotope for synthetic applications. In order to introduce the deuterium isotope, methyl phenylacetate (**65**) was treated with an excess of lithium aluminium deuteride in dry ether. Careful quenching of any unreacted lithium aluminium deuteride from the reaction with wet ether was required during work-up. This step introduced the first of the isotopes, to give 2-[1-$^2H_2$]-phenylethanol (**66b**) in an excellent 99% yield.

2-[1-$^2H_2$]-Phenylethanol (**66b**) was required to be oxidised to [1-^2H]-phenylacetaldehyde (**67a**) in preparation for the exchange reaction with $H_2{}^{18}O$. Accordingly, 2-[1-$^2H_2$]-phenylethanol (**66b**) was oxidised with PCC in dichloromethane in order to generate an aldehyde group for the exchange reaction and further carbon

chain extension. Work-up involved the removal of the reduced chromate (IV) by filtering the reaction mixture through silica gel. A slight green colouration of the product was attributed to a minor chromate (III) impurity. This reaction generated a sample of [1-^{2}H]-phenylacetaldehyde (**67a**) in 85% yield and was immediately taken through to the next step without purification to avoid possible decomposition.

The crucial step in the sequence involved the introduction of the ^{18}O label by exchange with $H_2{}^{18}O$ at high temperature and pressure. [1-^{2}H]-Phenylacetaldehyde (**67a**) dissolved in tetrahydrofuran and acidified $H_2{}^{18}O$, was introduced into a Carius tube. The tube was evacuated, sealed and placed in an oven at 120°C for 5 hours. A small proportion of dry hydrogen chloride gas was introduced to catalyse the exchange process. Tetrahydrofuran was required to ensure an intimate mixture of both immiscible liquids. Following the reaction, dichloromethane was used to extract the crude product, [1-^{2}H,^{18}O]-phenylacetaldehyde (**67b**), from the tetrahydrofuran mixture in 98% yield. GC-MS data revealed that the resultant material was approximately 50% labelled with ^{18}O and 99% labelled with ^{2}H. This material was utilised immediately to avoid exchange and decomposition.

The extension of the carbon skeleton was initiated by the addition of TMSCN (trimethylsilylcyanide) to a solution of [1-^{2}H,^{18}O]-phenylacetaldehyde (**67b**) dissolved in dry dichloromethane. The reaction was carried out under anhydrous conditions to avoid potential washout of ^{18}O label through exchange with traces of water. Work-up involved the straightforward removal of excess solvent by lyophilisation. This gave DL-3-phenyl-[2-^{2}H,^{18}O]-2-(trimethylsilyloxy)-propionitrile (**68a**) as a yellow oil which was utilised immediately in the next synthetic step again for fear of decomposition.

The next step involved the hydrolysis of nitrile (**68a**) under acidic or basic conditions to generate [2-^{2}H,^{18}O]-phenyllactic acid (**51e**). Hydrolysis under acidic conditions was chosen to avoid potential loss of ^{18}O label through washout by formation of a geminal diol. In the event, DL-3-phenyl-[2-^{2}H,^{18}O]-2-(trimethylsilyloxy)-propionitrile (**68a**) was hydrolysed with 50% hydrochloric acid over 48 hours and gave the desired product, DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid (**51e**), which was recovered in 96% yield.

The sodium salt of DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid (**51e**) was required for the feeding experiments to *D. stramonium* root cultures. Accordingly, a solution for feeding

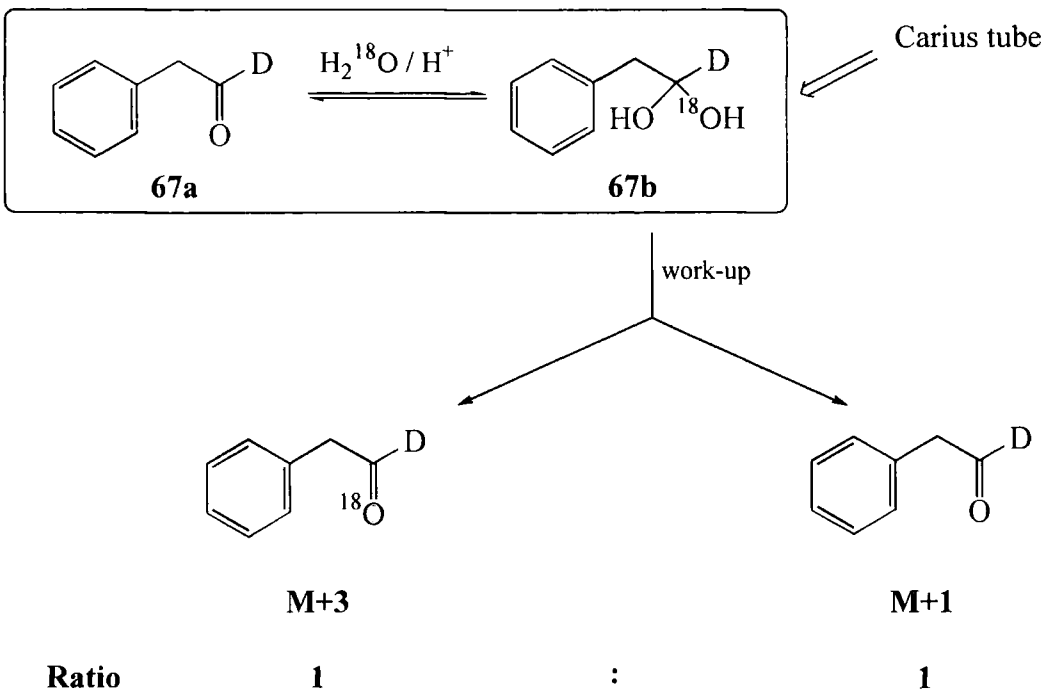
was prepared by dissolving DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid (**51e**) in water and adjusting to pH 8 with sodium hydroxide solution. This solution was partitioned between chloroform and water, and the salt was isolated upon freeze-drying of the aqueous layer in 54% yield. GC-MS analysis of the silyl derivative of a sample of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**) revealed that 95.7% of the ^{2}H label was incorporated but that only 46.7% of the ^{18}O isotope was introduced by exchange from $H_2{}^{18}O$. A rationale for this result is discussed below. In the event, this reaction scheme furnished a sample of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**), which gave a M+1/M+3 ratio of 1.05 as determined by GC-MS analysis (Table 3).

Ion	% incorporation
M	1.50
M+1	49.0
M+2	3.30
M+3	46.7
M+1/M+3 ratio	1.05

Table 3 GC-MS data for sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**) following derivatisation with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA). Values in the table are adjusted after considering natural abundance levels.

2.2.2.1 Explanations for the observed mass distribution of the synthesised precursor, sodium [2-^{18}O,^{2}H]-phenyllactate (51f)

The mass distribution of the synthesised precursor, sodium [2-^{18}O,^{2}H]-phenyllactate (**51f**) can be explained by a number of reasons. An initial rationale was to attribute it to a stable geminal diol intermediate generated under acidic conditions in the Carius tube. If that was the case, following work-up, the geminal diol would collapse to the aldehyde again and there would be a 50% chance of retaining the ^{18}O label. Naturally, the other 50% of molecules would lose ^{18}O and retain only the deuterium label (Scheme 56).



Scheme 56 To explain the ~50% incorporation of ^{18}O into sodium [2-^{18}O,^{2}H]-phenyllactate (**51f**) it was initially attributed to the exchange reaction of [1-^{2}H]-phenylacetaldehyde (**67a**) with $H_2{}^{18}O$. The formation of a stable geminal diol intermediate (**67b**), which is generated under acidic conditions in the Carius tube, would result in a 50% chance of retaining the ^{18}O label following work-up.

However, it appears more likely that the $H_2{}^{18}O$ used was not 98% enriched with ^{18}O as originally stated in the manufacturer's specification. In another synthesis reported in this thesis, where sodium [1-^{13}C,$^{18}O_2$]-acetate (Chapter 4, Section 4.3.3.1, page 134) was prepared, the same sample of $H_2{}^{18}O$ was used. MS analysis revealed also in that case that only ~50% of the molecules had been labelled with ^{18}O. This provided further evidence to suggest that the water obtained from the supplier was only ~50% enriched with ^{18}O.

2.2.3 Results from feeding sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (51f) to *Datura stramonium* root cultures

The sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**), (95.7%, ^{2}H; 46.7%, ^{18}O) prepared as discussed above was administered in aliquots to *D. stramonium* root cultures on day 5, 7 and 9 to a final concentration of 0.64mmol dm^{-3}. The roots were harvested and worked-

up after 11, 13, 15 and 17 days of growth. The alkaloids were then extracted and the extracts submitted for GC-MS analysis. Samples of each extract were dissolved in methanol and injected onto a chiral DB-17 column, which was temperature ramped to ensure good separation of the structural isomers, littorine (**5**) and hyoscyamine (**1**). The mass spectrometer was set to single ion monitoring (SIM) mode and targeted peaks containing the molecular ions in the GC spectrum for littorine (**5**) and hyoscyamine (**1**) [*m/z* 289 (M), 290 (M+1), 291 (M+2) and 292 (M+3)]. The resultant incorporation data after the feeding experiment with sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**), and after subtracting natural abundance levels, is shown in Table 4. Clear incorporations are found in ions 290 M+1 (^{2}H only) and 292 M+3 (^{2}H+^{18}O). The observed average incorporation level into littorine (**5**) and hyoscyamine (**1**) for *m/z* 291 (M+2) and 293 (M+4) were both negligible (< 0.1%) and are omitted from the table for clarity.

	Day 11		Day 13		Day 15		Day 17	
	littorine	**hyoscyamine**	**littorine**	**hyoscyamine**	**littorine**	**hyoscyamine**	**littorine**	**hyoscyamine**
M	83.39	90.58	86.49	90.73	84.39	89.00	87.93	90.01
M+1	9.19	6.48	7.48	6.53	8.50	7.58	6.74	6.78
M+3	7.24	3.15	5.88	2.95	6.92	3.61	5.24	3.35
M+1/M+3 ratio	1.27	2.06	1.27	2.21	1.23	2.10	1.29	2.02
^{18}O loss from littorine (5) to hyoscyamine (1)	25%		29%		28%		25%	

Table 4 GC-MS derived data for the alkaloids littorine (**5**) and hyoscyamine (**1**) on four different days after feeding (*R,S*)-sodium [2-^{18}O,2-^{2}H]-lactate to *D. stramonium* root cultures. The mass spectrometer was set to selected ion monitoring (SIM) mode, measuring ion currents at *m/z* 289 (M), 290 (M+1), 291 (M+2) and 292 (M+3). The data is presented after natural abundance levels have been subtracted by applying the "isotopic incorporation calculator", mentioned in the text.

2.2.3.1 The "isotopic incorporation calculator"

Determining accurate isotopic incorporation of an enriched compound can be a difficult process. Although the raw MS data provides the *m/z* distribution for a substance, it cannot be utilised directly without taking into account natural abundance isotope levels. Further complexity arises from the spread of enrichment for a particular compound to be analysed. For example, if a compound was partially enriched with ^{13}C, there should be a natural abundance distribution for these molecules (M+1 population) as well as the natural abundance distribution for the unlabelled molecules (M population). Thus, if a molecule is isotopically enriched in more than one position, then the calculations become increasingly complex. Therefore, the use of an "isotopic incorporation calculator", like the one described below, is required to compute these permutations.

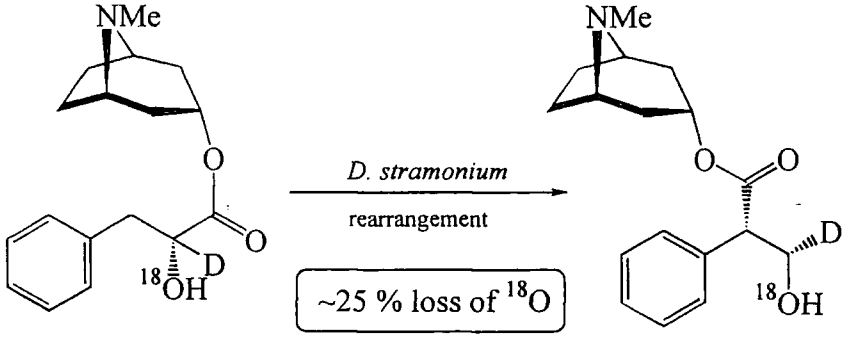
The "isotopic incorporation calculator" is composed of a series of formulae, which compute accurate natural abundance profiles to enriched ions and are laid down on a Microsoft Excel® spreadsheet. Once the percentage natural abundance values are entered into column B and the GC-MS peak areas entered into column C, then the two entries can be compared. Once this is done, the incorporation values are computed and displayed at the bottom of the spreadsheet. An example is shown in fig. 15 and shows the accurate isotopic incorporations into littorine (**5**) and hyoscyamine (**1**) from feeding sodium [2-^{2}H,^{18}O]-phenyllactate (**51f**) to *D. stramonium* root cultures on day 11 of growth. The percentage natural abundance levels for both hyoscyamine and littorine [*m/z* 289, (M)] have been entered under the field name "%-O-value" and the GC peak areas under the field name "value". Isotopic incorporations into hyoscyamine reveal that M+1 and M+3 ions are enriched with negligible incorporation (≤ 0.4%) for M+2 or M+4 (fig. 15). These results are presented in Table 4 and are discussed in detail below.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Fed 2-18O,2H-PLA-Na	Day 11	F-2H18OPLS-d11												
2															
3	HYOSCYAMINE	%-0-Value	Value	0-Value	Rest	1 x	Rest	2x	Rest	3x	Rest	4x	Rest	5x	Rest
4	289	81.86	13732893	13732893	0										
5	290	15.956	3174142	2191264	982878	982878	0								
6	291	1.966	400571	270029	130542	156831	-26290	-26290	0						
7	292	0.222	524039	30443	493596	18326	474269	-4196	478464	478464	0				
8	293	0.000	95815	0	95815	2179	93636	-517	94153	76345	17808	17808	0		
9	294	0.000	0	0	0	0	0	-58	58	9408	-9350	2841	-12191	-12191	0
10	295	0.000	0	0	0	0	0	0	0	1061	-1061	350	-1411	-1945	534
11						0		0		0		39		-240	
12								0		0		0		-27	
13										0		0		0	
14												0		0	
15														0	
16															
17		17928698	=	16224630	+	1161214	+	-31060	+	585278	+	21039	+	-14403	
18															
19															
20		Incorp.	0	80.51	%										
21			1	6.46	%										
22			2	-0.17	%										
23			3	3.15	%										
24			4	0.12	%										
25			5	-0.06	%	= 9.49	%								
26				100.00	%										
27															
28															
29	LITTORINE	%-0-Value	Value	0-Value	Rest	1 x	Rest	2x	Rest	3x	Rest	4x	Rest	5x	Rest
30	289	81.82	2522892	2522892	0										
31	290	15.761	675853	397632	278221	278221	0								
32	291	2.173	98763	54827	43956	43850	106	106	0						
33	292	0.147	228985	3713	225272	6046	219226	17	219209	219209	0				
34	293	0.000	47009	0	47009	409	46600	2	46597	34549	12048	12048	0		
35	294	0.000	0	0	0	0	0	0	0	4764	-4764	1899	-6663	-6663	0
36	295	0.000	0	0	0	0	0	0	0	323	-323	262	-584	-1050	466
37						0		0		0		18		-145	
38								0		0		0		-10	
39										0		0		0	
40												0		0	
41														0	
42															
43		3572919	=	2979084	+	328527	+	125	+	256845	+	14226	+	-7867	
44															
45															
46		Incorp.	0	83.38	%										
47			1	9.19	%										
48			2	0.00	%										
49			3	7.24	%										
50			4	0.40	%										
51			5	-0.22	%	= 16.62	%								
52				100.00	%										

Figure 15 Spreadsheet showing the computations using the "isotopic incorporation calculator" and shows accurate isotopic incorporations after considering natural abundance levels into littorine (**5**) and hyoscyamine (**1**) [*m/z* 289 (M)] from feeding sodium [2-^{2}H,^{18}O]-phenyllactate (**51f**) to *D. stramonium* root cultures after day 11 of growth.

2.3 Discussion

From the final results in Table 4, one can see that both littorine (**5**) and hyoscyamine (**1**) have enriched M+3 ions, indicating that both deuterium and ^{18}O had become incorporated into the alkaloids. However, it can also be deduced from the results that the level of incorporation for both of these metabolites was different. By comparing the relative ratios of M+1 (^{2}H only) to M+3 (^{2}H and ^{18}O) in the molecular ions of hyoscyamine (**1**) and littorine (**5**), one can calculate that ~71-75% of the ^{18}O label was retained or conversely, ~25-29% of the ^{18}O label was lost during the rearrangement of littorine (**5**) to hyoscyamine (**1**). By considering day 11 for example (see Table 4), the M+1 / M+3 ratio for littorine and hyoscyamine is 1.27 and 2.06 respectively. These values approximately equate to the fractions 56/44 and 67/33 respectively. These fractions offer a straightforward way to represent the proportion of molecules, which have only ^{2}H attached (numerator) or both ^{2}H and ^{18}O attached (denominator). By examining the M+3 (^{2}H and ^{18}O) littorine pool it can be calculated that 11 of the 44 "molecules" lose ^{18}O and contribute to the M+1 (^{2}H) hyoscyamine pool giving a numerator value of 67. Thus, approximately 25% of the dual labelled littorine (**5**) molecules with ^{2}H and ^{18}O lose their ^{18}O label during the rearrangement to hyoscyamine (**1**), as shown in Scheme 57.[130]

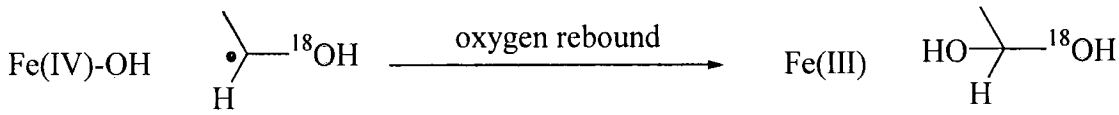


	5		**1**
M+1	**56**	**+ 11**	**67**
M+3	**44**	**- 11**	**33**
M+1 / M+3 ratio	1.27		2.06

Scheme 57 Calculating the loss of ^{18}O label following the rearrangement of littorine (**5**) to hyoscyamine (**1**) from the GC-MS results.

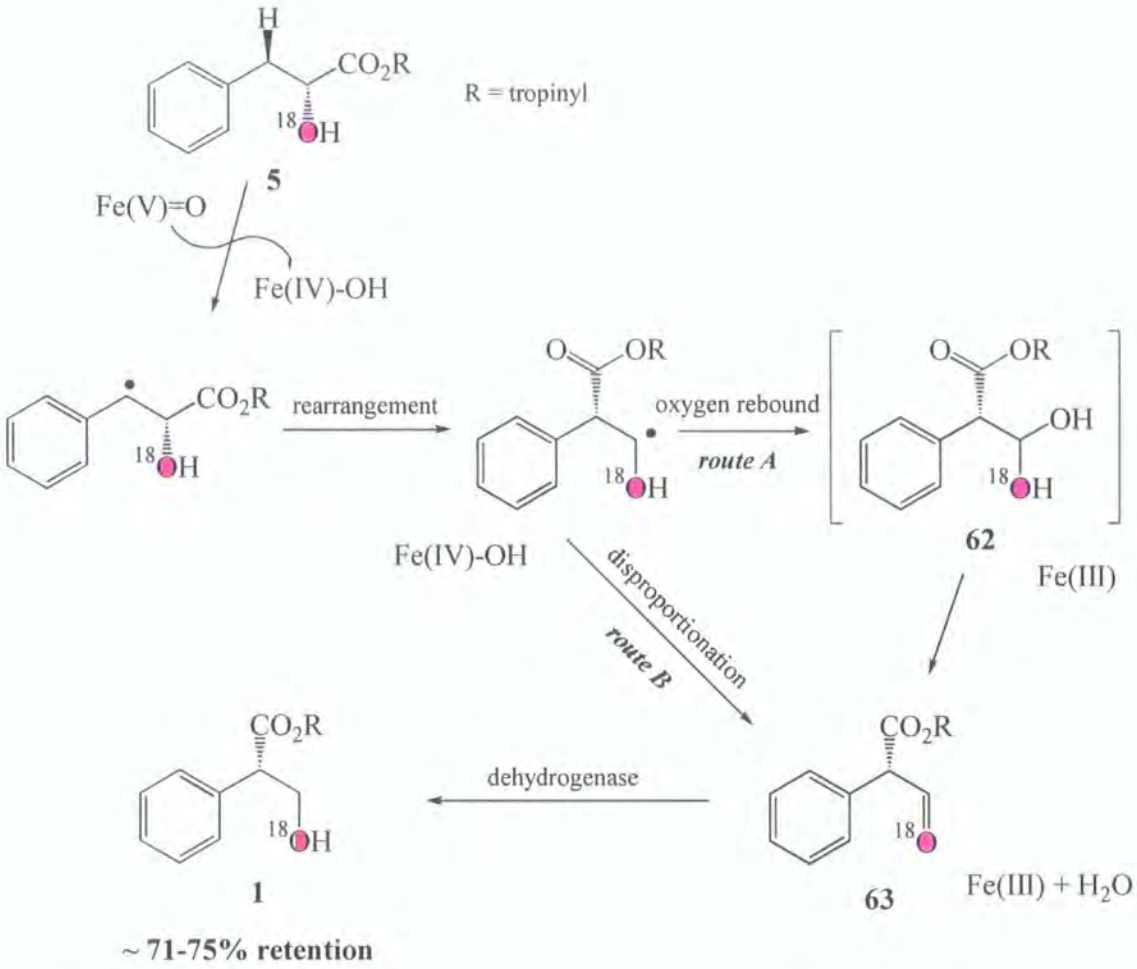
It is noteworthy that the calculated values for ^{18}O loss during the time course fall in between those previously hypothesised in Scheme 49 where either a) 100% retention, b) 50% loss / retention, or c) 100% loss was predicted. Nevertheless, this result can be rationalised within a number of mechanistic models.

Firstly, if the process begins by the abstraction of hydrogen atom by an iron-oxo enzyme to give a substrate radical. A carbon skeletal rearrangement will then give the product radical, which would be quenched by oxygen rebound (Scheme 58), furnishing a geminal diol intermediate (**62**), as shown in Scheme 59, route A).



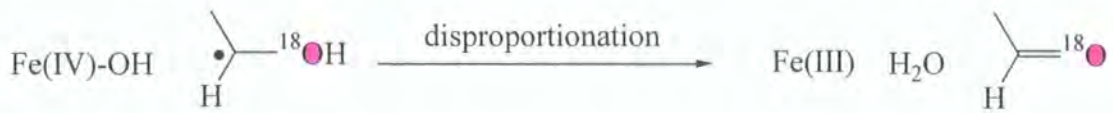
Scheme 58 An oxygen rebound process with the product radical and the putative Fe-(IV)-OH intermediate will generate a geminal diol intermediate. Subsequent stereospecific collapse of the geminal diol will either result in 100% retention or 100% loss of the ^{18}O label. A non-stereospecific collapse of the diol would give a 50% loss of ^{18}O label.

To account for the ~25-29% loss of ^{18}O label from the C-2′ oxygen of littorine (**5**), two mechanisms are now consistent with the observation: (a) The collapse of the geminal diol (**62**) to an aldehyde (**63**) prior to reduction by a dehydrogenase may only be partially stereospecific and favours the loss of unlabelled oxygen, or (b) the diol (**62**) collapse is fully stereospecific, resulting in full retention of the ^{18}O label, but loss of isotope is due to partial exchange of the aldehyde oxygen with the aqueous medium prior to a reduction (Scheme 59). A non-stereospecific collapse of the diol would give a 50% loss of ^{18}O label and this is inconsistent with the observed results.



Scheme 59 Two possible routes to account for the ~25-29% loss of ^{18}O label from the C-2′ oxygen of littorine (**5**) following the rearrangement to hyoscyamine (**1**).

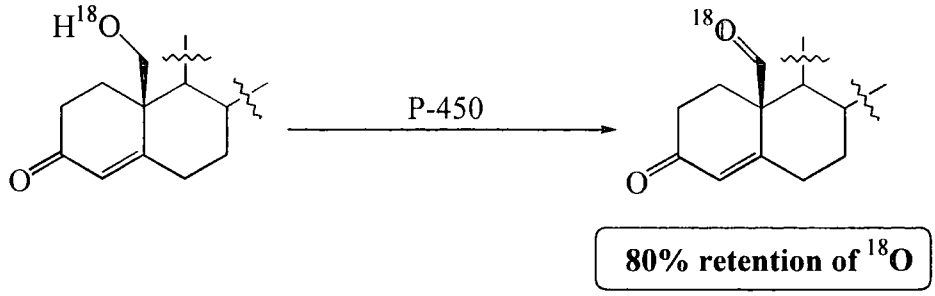
An alternative explanation of these results involves a disproportionation reaction (Scheme 60) with the product radical and the putative Fe-(IV)-OH intermediate, forming the aldehyde (**63**) *via* a more direct route and bypassing an oxygen rebound process (Scheme 59, route B).



Scheme 60 A disproportionation reaction with the product radical and the putative Fe-(IV)-OH intermediate results in the retention of the ^{18}O label on the resultant aldehyde.

This type of process has already been discussed by Akhtar and co-workers in the context of a P-450 mediated oxidation in oestrogen biosynthesis.[131,132] They observed

that a similar level of ^{18}O retention (80%) in the oxidation of a primary alcohol to an aldehyde as shown in Scheme 61.



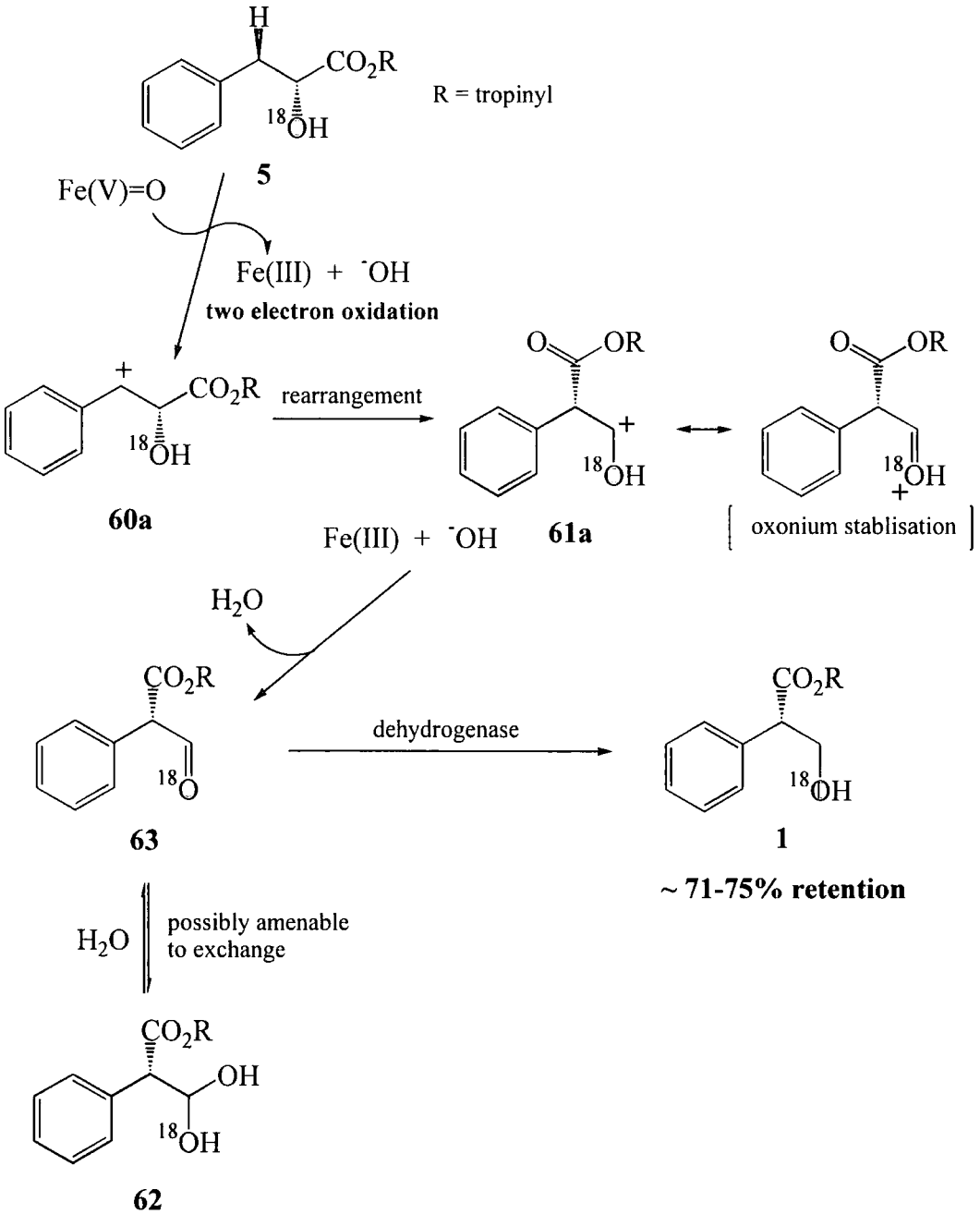
Scheme 61 In oestrogen biosynthesis, Akhtar and co-workers have also observed a similar level of ^{18}O retention following the P-450 mediated oxidation of the ^{18}O labelled primary alcohol to an aldehyde.[131]

The high retention of ^{18}O was explained either by a collapse of a geminal diol resulting from oxygen rebound, or by a disproportionation process. The loss of ~20% of ^{18}O label in the latter could be explained by the partial exchange of the aldehyde carbonyl with surrounding aqueous medium. The similarity of high ^{18}O retention between our results and Akhtar's findings are intriguing and by association perhaps suggest a common process.

Finally, another mechanism needs to be considered. Carbocation intermediates generated from iron-oxo systems are not generally regarded as commonplace in biosynthesis, but there is mounting evidence for them. For example, in the biosynthesis of prostacyclin and thromboxane,[133] where they are generated by closely related heme-thiolate enzymes. Also, products of carbocation rearrangement have been produced as minor side products in mechanistic probe reactions of P-450 enzyme hydroxylation chemistry (Chapter 3, Section 3.1.3, Scheme 72, page 82).[134] The enzymatic reaction of the mechanistic probe (**79**) generated primarily the unrearranged product following hydroxylation. However, a higher proportion of the isolated rearranged products (in a ratio of 6:1) was shown to undergo a cationic rearrangement compared to the radical mediated route.

A two electron oxidation of littorine (**5**) would generate a benzylic carbocation intermediate, which could undergo a carbon skeletal rearrangement (Scheme 62). The product carbocation would then collapse to an aldehyde (**63**) with no ^{18}O loss and

reduction by a dehydrogenase would furnish hyoscyamine (**1**). The attractive feature of this mechanism is that the substrate carbocation (**60a**) is predicted to rearrange to a more stable product carbocation (**61a**), which has oxonium ion stabilisation.[134,135] Such a process has been demonstrated chemically by Wemple[85] (Chapter 1, Section 1.5.5, Scheme 28, page 31). No loss of ^{18}O label should occur until the aldehyde intermediate (**63**). However, partial loss of ^{18}O label could be rationalised by exchange of the aldehyde carbonyl with the aqueous medium, as discussed above.



Scheme 62 The proposed hypothesis for the rearrangement of littorine (**5**) to hyoscyamine (**1**) involves a substrate benzylic carbocation intermediate (**60a**) and the intermediacy of the aldehyde (**63**).

Newcomb reported a very similar observation when studying methylcyclopropane ring opening reactions under both carbocation and radical conditions (Chapter 3, Section 3.1.2, Scheme 67, page 77).[135] Interestingly, the results from these models support a carbocation intermediate in the rearrangement of littorine (**5**) to hyoscyamine (**1**).

2.4 Conclusion

To conclude, our working hypothesis involves the activity of two enzymes, a mutase and a dehydrogenase. Experiments with the P-450 inhibitor, clotrimazole (**59**) perhaps suggest a role for a P-450 enzyme in the rearrangement.[124] Still, the mechanism of the rearrangement process is unclear and requires further study, and may have to await enzyme isolation.

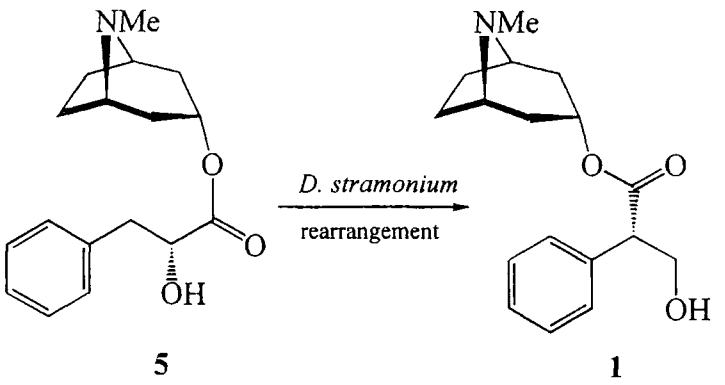
Very little is known about the enzymes involved on the biosynthetic pathway towards hyoscyamine. Previous literature claims have suggested that there are enzymes present which convert phenylalanine to phenylpyruvate and another which esterifies tropine with free tropic acid to give hyoscyamine, but none of these have been characterised yet. Later work by Doerk,[136] who isolated a phenylalanine transaminase from *Hyoscyamus albus* transformed root cultures only showed weak activity and poor kinetic properties. Efforts to convert phenylpyruvate (**38**) to phenyllactate (**51**) *in vitro* have proved to be largely inconclusive. Also, the putative CoA-thioligase for phenyllactate and phenyllactoyl-CoA:tropine acyltransferase activities have yet to be identified and isolated. However, there have been some notable successes,[40] for example, the extraction, isolation and purification of hyoscyamine 6β-hydroxylase[59,60] and the identification and isolation of two distinct tropinone reductases[55] from these plant tissues. Nevertheless, a full *in vitro* profile of these enzyme activities remains to be established to support the findings resulting from isotopic labelling experiments.

Chapter 3

3. The Biotransformation of Fluorinated Littorines

3.1 Investigating the Mechanism of the Rearrangement

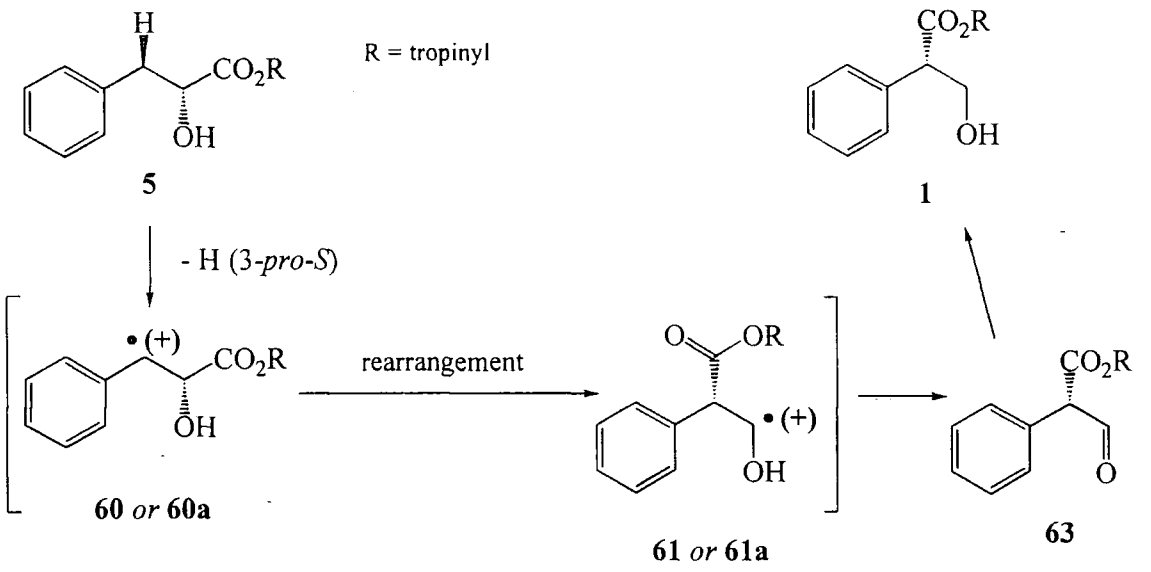
As covered in Chapter 1, the nature of the rearrangement of littorine (**5**) to hyoscyamine (**1**), as shown in Scheme 63, was first thought to be closely associated to co-enzyme B_{12} mediated rearrangements.[105,106] However, the absence of a vicinal interchange process has suggested otherwise.[114]



5 **1**

Scheme 63 The rearrangement of littorine (**5**) to hyoscyamine (**1**) involves the isomerisation of the phenylpropanoid skeleton of littorine (**5**). Recent studies have shown that the rearrangement is not co-enzyme B_{12} mediated, due to the absence of a vicinal interchange process.[114]

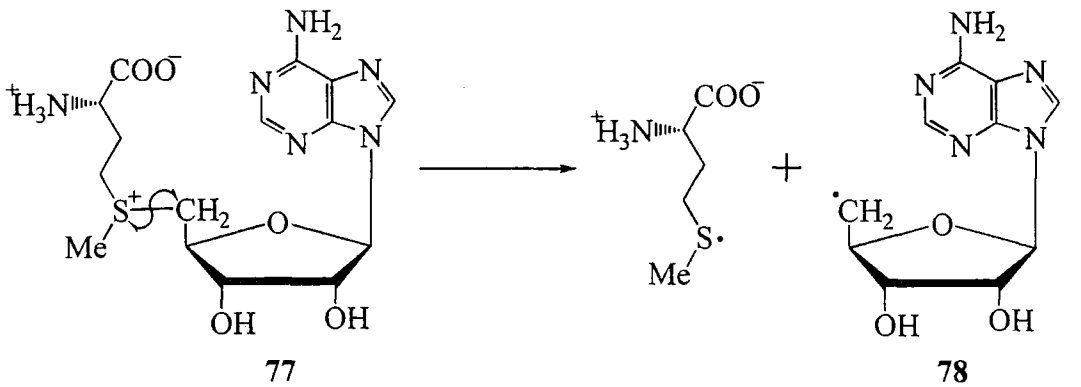
Clearly, the mechanism of the rearrangement has not been elucidated, but a number of factors are known which limit the mechanistic possibilities. Currently, our working hypothesis envisages the removal of the 3-*pro-S* hydrogen by the putative isomerase to generate either a radical (**60**) or carbocation (**60a**) at C-3. This species would then rearrange to form the product carbocation (**61a**) or radical (**61**) as shown in Scheme 64.



Scheme 64 A working hypothesis for the rearrangement of littorine (**5**) to hyoscyamine (**1**).

3.1.1 The "poor man's B_{12}"

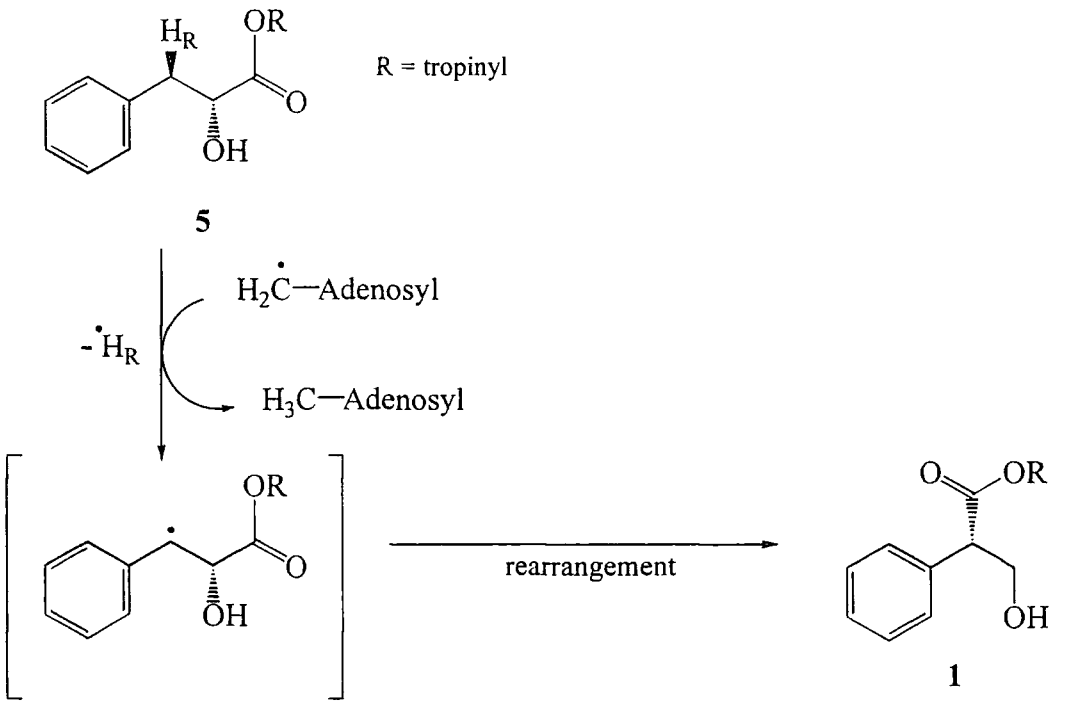
Few biochemical agents are known to abstract hydrogen atoms from non-activated positions. Apart from cytochrome P-450, nature uses the 5′-deoxyadenosyl radical (**78**) for this purpose. In animals and bacteria, co-enzyme B_{12} is the major source of this radical, although it has recently been reported that S-adenosylmethionine [(**77**), (SAM)] can also play a similar role to co-enzyme B_{12}. It has been shown that SAM mediated lysine 2,3-aminomutase from *Clostridia* catalyses the interconversion of L-lysine and L-β-lysine.[137] The enzyme contains iron-sulphur clusters and is activated by pyridoxal 5′-phosphate and the 5′-deoxyadenosyl radical (**78**) derived from S-adenosylmethionine (SAM). Thus, these findings led Rétey and other researchers to call SAM (**77**) the "poor man's B_{12}" (Scheme 65).[137]



Scheme 65 SAM (**77**) has been reported as a source of 5′-deoxyadenosyl radical (**78**) in *Clostridia*.[137]

Although SAM (**77**) has been shown to be the source of the 5′-deoxyadenosyl radical (**78**) for the last step in biotin synthesis in *Arabidopsis thaliana* plants,[138] it remains unlikely to be involved in the rearrangement process of littorine (**5**) to hyoscyamine (**1**) in *D. stramonium*, as no vicinal interchange process is observed.

However, recently, Rétey and co-workers[139] reported that S-adenosylmethionine (**77**) is the source of a 5′-deoxyadenosyl radical (**78**) in *D. stramonium*, which initiates the rearrangement of littorine (**5**) to hyoscyamine (**1**) in a similar manner to those analogous rearrangements catalysed by co-enzyme B_{12}-dependent enzymes (Scheme 66).



Scheme 66 Rétey and co-workers proposed that S-adenosylmethionine (**77**) is the source of a 5′-deoxyadenosyl radical (**78**) in *D. stramonium*, which initiates the rearrangement of littorine (**5**) to hyoscyamine (**1**).[139]

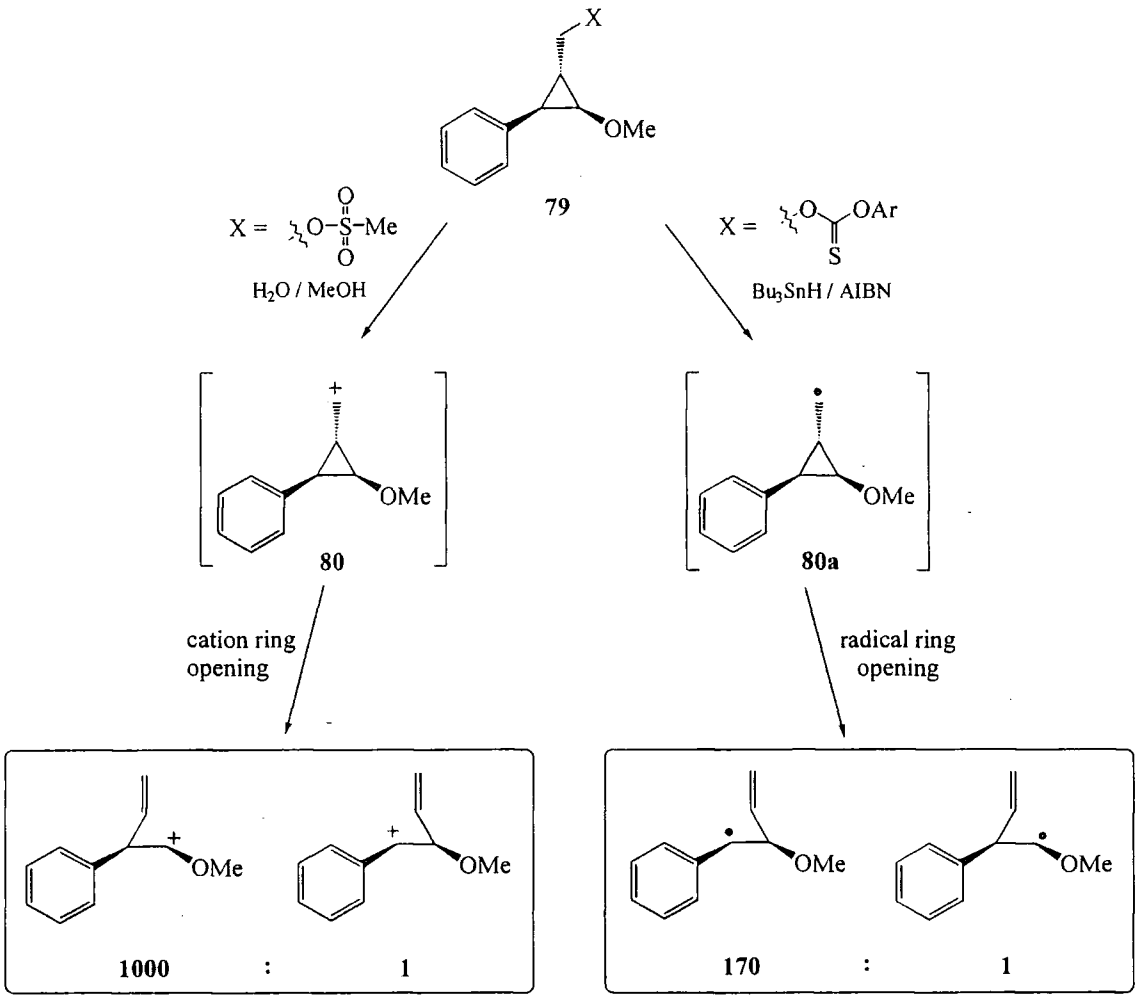
They reported a 10-20–fold enhancement for the conversion of littorine (**5**) to hyoscyamine (**1**) when SAM was added to cell-free extracts of *D. stramonium* roots. Also, in an effort to demonstrate the transfer of protons from the CH_2- group of the adenosyl radical, [2,8,5′-^{3}H]-SAM (65% ^{3}H in position 5′) was synthesised and added to cell-free extracts of *D. stramonium*. However, no incorporation of tritium into littorine (**5**) or hyoscyamine (**1**) was observed and this was attributed to washout by redox enzymes. The authors explained that the washout of the migrated H-atom could

also rationalise the reported 25-29% loss of ^{18}O from littorine (**5**) during the rearrangement,[130] if the resultant hyoscyamine (**1**) underwent reversible oxidation/reduction to the aldehyde (**63**), mediated by a putative dehydrogenase. The results are intriguing as they used L-littorine as the substrate for the rearranging enzyme, when it has been shown that the substrate for the rearrangement is the D-isomer. If SAM (**77**) was indeed the co-factor for the rearrangement, their results using [2,8,5′-^{3}H]-SAM, should have revealed the presence of tritium at C-3′ of hyoscyamine (**1**) *via* a vicinal interchange, but this was not observed. Also, their explanation for the loss of the migrated H-atom *via* washout by redox enzymes is not supported by the findings for the SAM mediated 2,3-aminomutase reaction.[140] In that case, the migrating hydrogen is not lost to the medium.

Their findings are at present inconclusive and remain to be confirmed. The increase in the conversion of littorine (**5**) to hyoscyamine (**1**) observed in their experiment could perhaps have resulted from the release of protein and membrane bound endogenous hyoscyamine pools, which were released into solution over time, following cellular disruption by sonication. This has been observed more recently in the Durham laboratory.

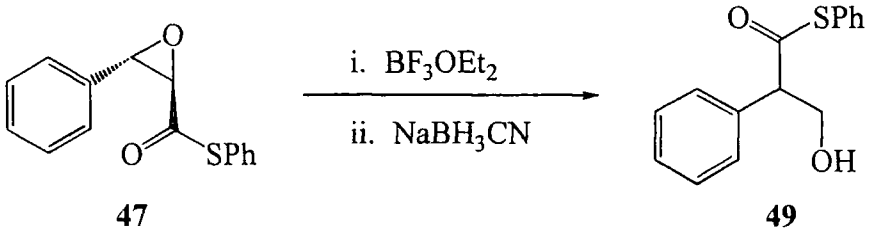
3.1.2 Carbocation or radical intermediate?

Newcomb and co-workers[135] have developed a probe (**79**) to distinguish between carbocation and radical mechanisms. Their probe (**79**) exploits the different reactivities of cyclopropylcarbinyl ring openings under both carbocation and radical conditions (Scheme 67). The stabilising substituents are aryl and oxygen and it is interesting that they closely model the putative intermediates proposed in the littorine (**5**) to hyoscyamine (**1**) rearrangement. They have demonstrated that a methylcyclopropane carbocation (**80**) opens preferably towards the oxygen in a ratio of 1000:1. This has clear similarities to the littorine (**5**) to hyoscyamine (**1**) rearrangement process in the forward direction. On the other hand, a methylcyclopropane radical (**80a**) opens preferentially towards the aryl ring in a ratio of 170:1. This is opposite to the direction of the rearrangement. These results provide some support for a carbocation process for the rearrangement of littorine (**5**) to hyoscyamine (**1**).



Scheme 67 The probe (79) has been developed to distinguish between carbocation and radical mechanisms. Interestingly, the methylcyclopropane carbocation (**80**) opens preferably towards the oxygen in a ratio of 1000:1, whereas the methylcyclopropane radical (**80a**) opens preferentially towards the aryl ring in a ratio of 170:1.[135]

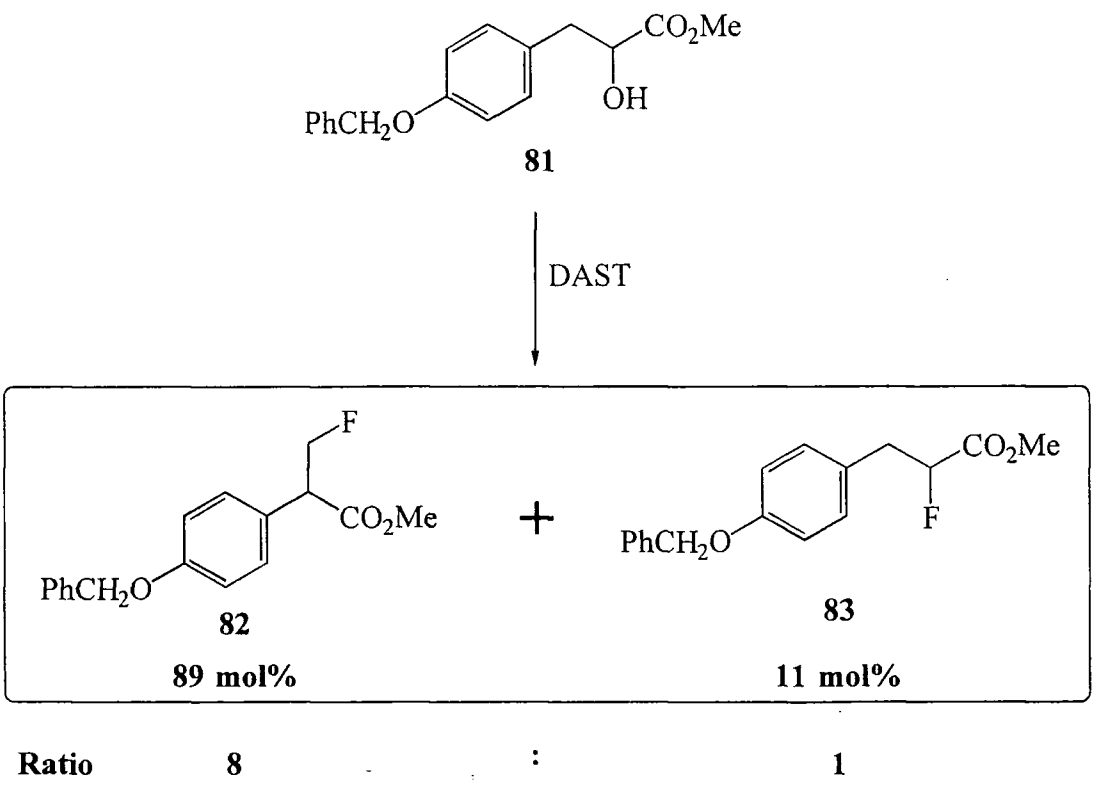
As discussed earlier, Wemple[85] has demonstrated an *in vitro* rearrangement of the phenyl thioester of epoxycinnamic acid (**47**) to generate the phenyl thioester of tropic acid (**49**), as shown in Scheme 68. This carbocation mediated process offers a very nice model for our current hypothesis for the *in vivo* process.



47 **49**

Scheme 68 The phenyl thioester of epoxycinnamic acid (**47**) has been chemically induced to rearrange to the phenyl thioester of tropic acid (**49**).

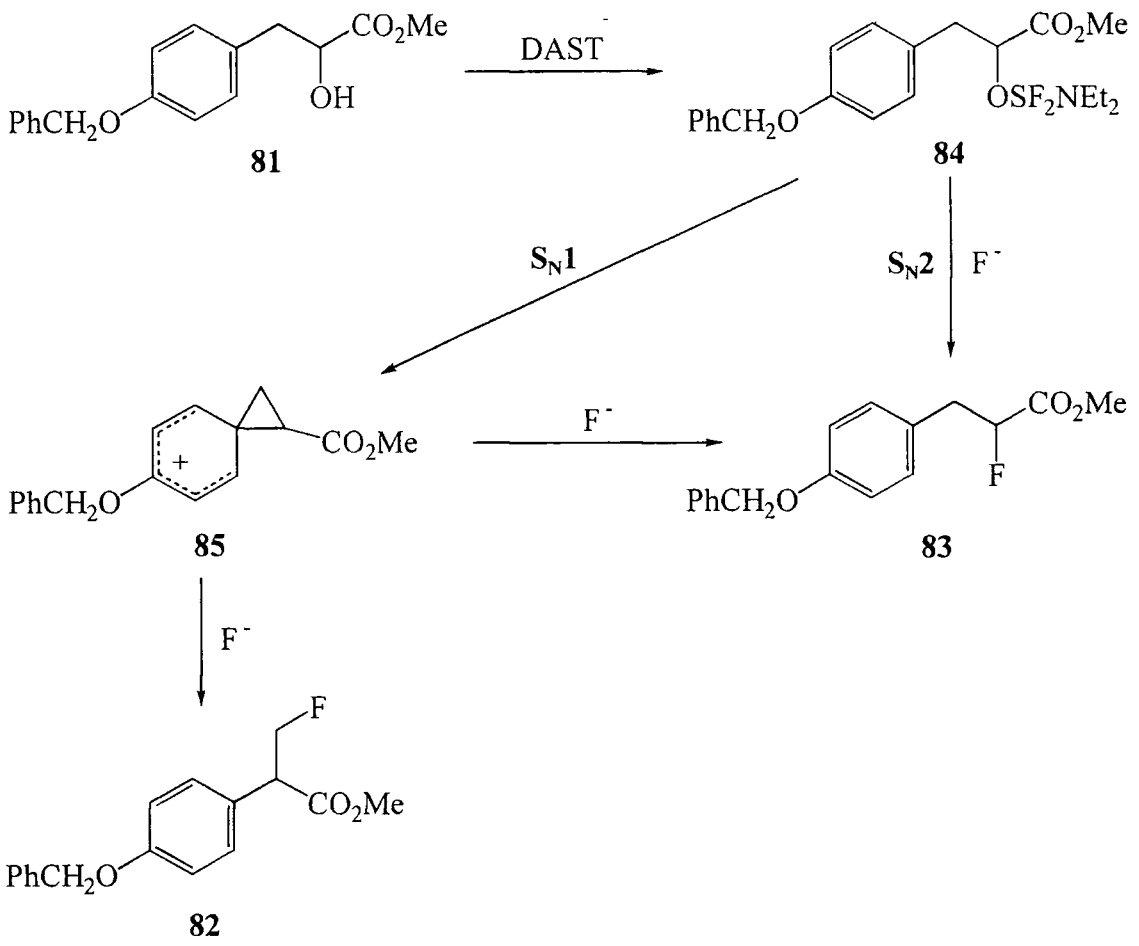
In a related, but mechanistically distinct process, Haigh and co-workers[141] have also demonstrated the rearrangement of a phenylpropanoid to a tropate skeleton following the treatment of 3-aryl-2-hydroxypropanoic esters with diethylaminosulphur trifluoride (DAST). When methyl 3-(*p*-benzyloxyphenyl)-2-hydroxypropanoate (**81**) was treated with DAST, a good yield was obtained (66%). However, on examination of the product, considerable amounts (89mol%) of rearranged 3-fluoro-2-(*p*-benzyloxyphenyl)propanoate (**82**) was obtained and the expected product, methyl 2-fluoro-3-(*p*-benzyloxyphenyl)propanoate (**83**) was only present to the extent of 11mol% (Scheme 69).



Scheme 69 The rearrangement of a phenylpropanoid to a tropate skeleton, following the treatment of methyl 3-(*p*-benzyloxyphenyl)-2-hydroxypropanoate (**81**) with diethylaminosulphur trifluoride (DAST) has been demonstrated. Interestingly, the rearranged product, 3-fluoro-2-(*p*-benzyloxyphenyl)propanoate (**82**) was obtained predominantly (in a ratio of 8:1) over the expected product, methyl 2-fluoro-3-(*p*-benzyloxyphenyl)propanoate (**83**).

The mechanism of DAST fluorination is thought to involve the formation of a covalent intermediate (**84**), which can react with fluoride ion by both S_N1 and S_N2 processes to give the rearranged (**82**) and unrearranged product (**83**).[142] The authors proposed that the 8:1 preference for the rearranged product (**82**) over the expected unrearranged product

(**83**) during the course of the reaction could be explained by the anchimeric assistance by the *para*-substituted aryl group in the S_N1 component of the reaction pathway to form the spiro-intermediate (**85**), see Scheme 70.



Scheme 70 The mechanism of DAST fluorination is thought to involve the formation of a covalent intermediate (**84**), which can react with fluoride ion by both S_N1 and S_N2 processes to give the rearranged product (**82**) and unrearranged product (**83**).

The extent of the rearrangement is dependent on the solvent and more interestingly, on the substitution pattern of the aryl ring. If the aryl substituent reduces the nucleophilicity of the aromatic ring, it also reduces the component of the anchimerically assisted pathway and leads to more of the direct substitution product (Table 5).

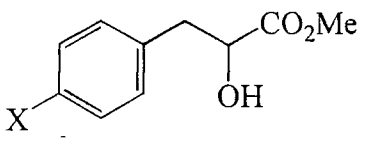
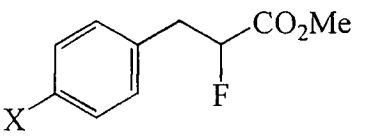
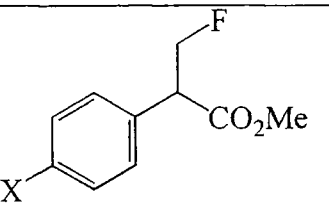
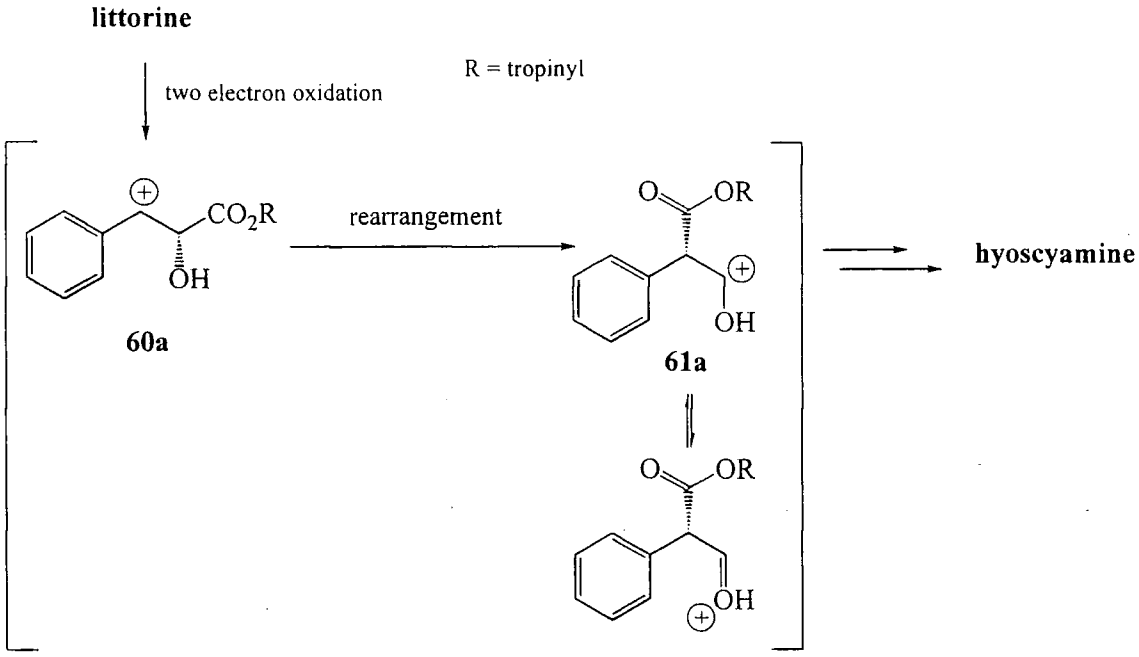
Reactant	Ratio of products after reaction with DAST		
			
X = *p*-OCH_2Ph	11	:	89
X = H	47	:	53
X = *p*-Cl	71	:	29
X = *p*-NO_2	100	:	0

Table 5 Comparison of the ratios of unrearranged and rearranged products following treatment of differently *para*-aryl substituted reactants with DAST.

Interestingly, in the case of the *para*-chloro aryl substituted reactant, the reaction with DAST proceeds with a higher proportion of the unrearranged product compared to the rearranged product in a ratio of 71:29 (Table 5). This suggests that the inductive effect of chlorine primarily overrides the conjugative mesomeric effects that could stabilise the proposed spiro-intermediate (**85**), which could generate a higher proportion of the rearranged product (Scheme 70).

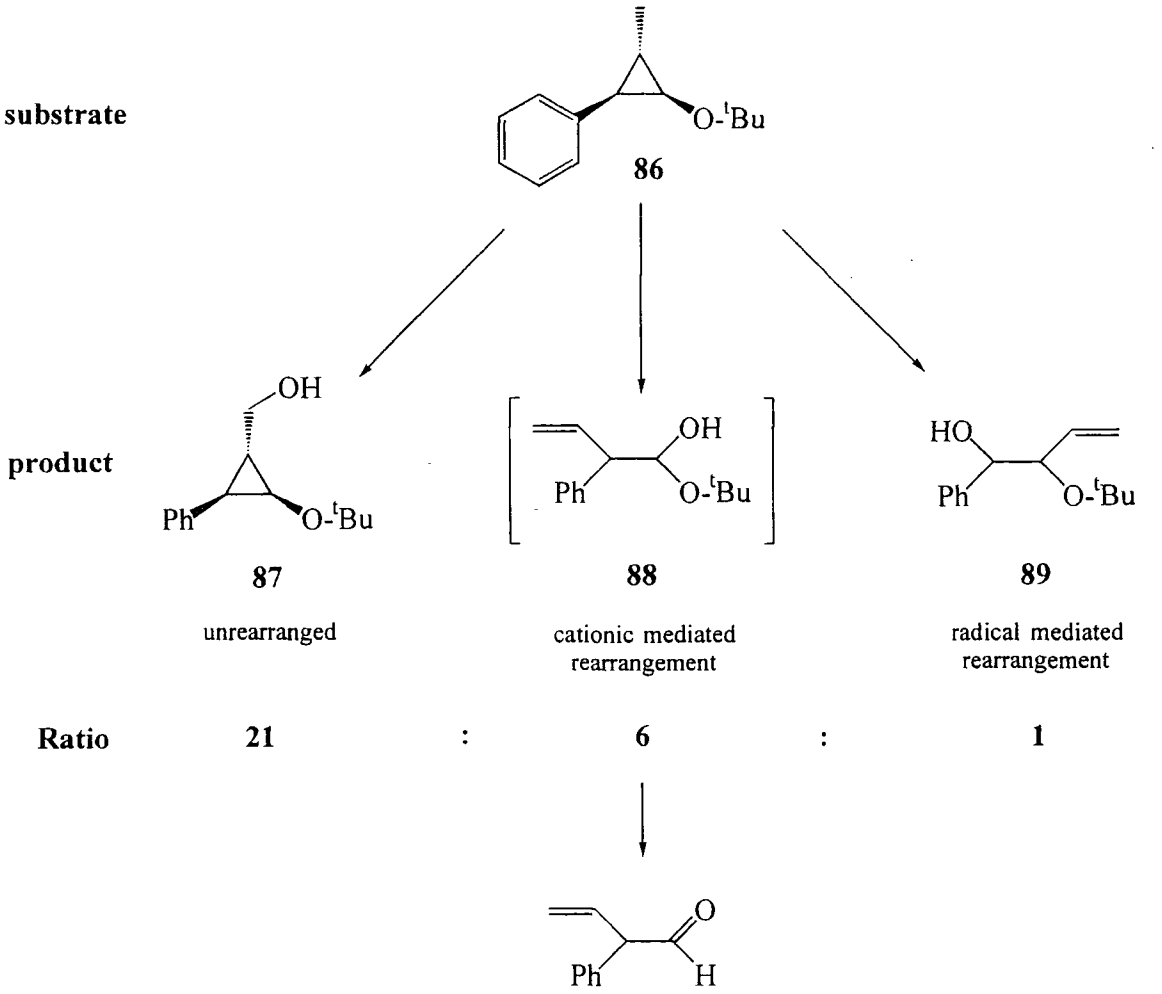
The Wemple model (Chapter 3, Section 3.1.2, Scheme 68, page 77) clearly supports a carbocation over a radical process for the rearrangement of littorine (**5**) to hyoscyamine (**1**). The attractive feature of a carbocation mechanism is that the substrate benzylic carbocation intermediate (**60a**) resulting from a two electron oxidation is predicted to rearrange to the more stable product oxonium ion intermediate (**61a**), as shown in Scheme 71. This intermediate (**61a**) would then require to be reduced, presumably by NADPH to generate hyoscyamine (**1**).



Scheme 71 Proposed hypothesis for the rearrangement of littorine to hyoscyamine *via* a substrate benzylic carbocation intermediate (**60a**).

3.1.3 A role for P-450 for the rearrangement?

Recently, Newcomb and co-workers[134] developed their mechanistic probe (**86**) to investigate the mechanism for the cytochrome P-450 catalysed hydroxylation reaction. Their results revealed that the enzymatic P-450 hydroxylation of the methyl group of the mechanistic probe (**86**) gave both unrearranged (**87**) and rearranged (**88** and **89**) hydroxylation products (Scheme 72).



Scheme 72 The mechanistic probe (**86**) was developed to investigate the mechanism for the cytochrome P-450 catalysed hydroxylation reaction. The enzymatic reaction generated the unrearranged product (**87**) predominantly following hydroxylation, and interestingly, a higher proportion of the isolated rearranged products (in a ratio of 6:1) had undergone a cationic rearrangement compared to the radical mediated route.

They observed that the enzymatic reaction generated the unrearranged product (**87**) predominantly following hydroxylation. Poignantly however, a higher proportion of the isolated rearranged products (in a ratio of 6:1) had undergone a cationic rearrangement compared to the radical mediated route (Scheme 72). Thus, these results clearly suggest that a P-450 process can generate carbocations and provide further evidence to implicate a carbocation intermediate for the rearrangement of littorine (**5**) to hyoscyamine (**1**), which is perhaps mediated by a cytochrome P-450 enzyme.

3.2 Fluorine and its uses in Bio-organic Chemistry

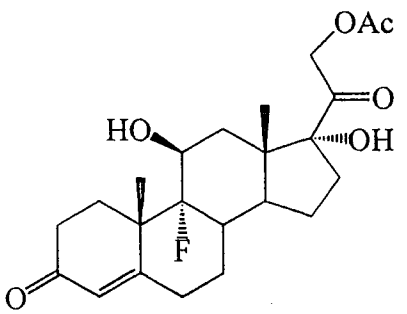
Fluorine has been widely used in bio-organic applications as a substitute for hydrogen.[143] Its size,[144] (van der Waals' radius 1.47Å) is comparable to hydrogen (van der Waals' radius 1.20Å), although its electronegativity is clearly far greater (Table 6).

	H	F
Electronic configuration	$1s^1$	$1s^2\ 2s^2\ 2p^5$
Electronegativity (Pauling)[145]	2.1	4.0
Van der Waals' radius (Å)	1.20	1.47

Table 6 Some electronic properties of hydrogen and fluorine

By replacing fluorine for hydrogen, the electronic profile of the molecule may be altered, although its steric profile remains largely unchanged. Therefore such analogues will generally bind well to target proteins, however in the case of enzymes, the electronic differences can now impede catalysis.

It was Fried[146] who first reported the use of selective fluorination to modify biological activity on reporting the synthesis of 9α-fluorohydrocortisone acetate [(**90**), fig. 16]. There are no significant steric changes, but fluorine at C-9 increases the acidity of the alcohol and promotes stronger hydrogen bonding. His work prompted other medicinal chemists and biochemists to use fluorine to modify a compound's biological activity.

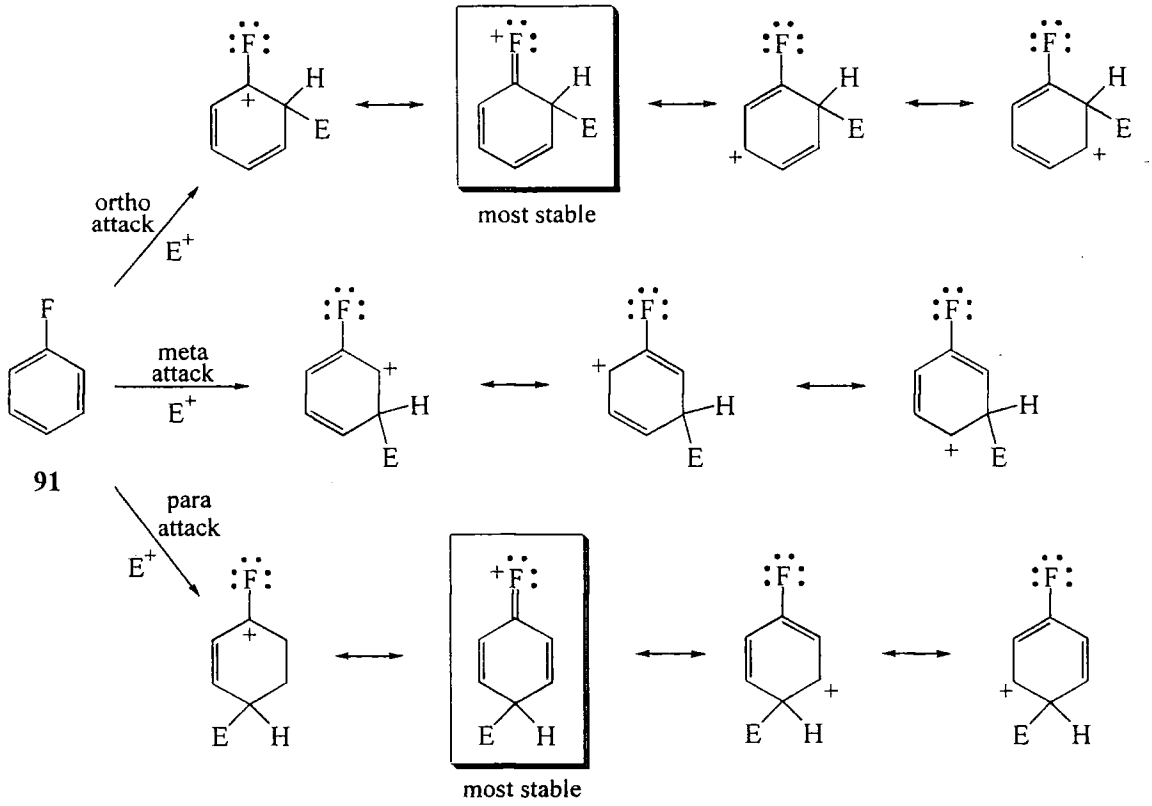


90

Figure 16 The synthetic corticosteroid, 9α-fluorohydrocortisone acetate (**90**) has a modified biological activity compared to its natural analogue by virtue of a fluorine substituent.

3.2.1 The effect of fluorine on the aromatic ring

The high electronegativity of fluorine and the observed electron-withdrawing power in aliphatic compounds suggests that aryl fluorine should be deactivating and perhaps *meta*-directing during electrophilic aromatic substitution. Fluorobenzene (**91**) is deactivated relative to benzene in such reactions but substitution occurs at the *ortho* and *para* positions. Classically, this is explained by resonance stabilisation of the Wheland intermediates and is made possible by donation of the fluorine lone pairs into the ring system (Scheme 73).[147]



Scheme 73 Electrophilic aromatic substitution of fluorobenzene (**91**) is preferable at the *ortho* and *para* positions, due to resonance stabilisation of the Wheland intermediates.

However, the resonance picture alone is inadequate to fully explain the observed data, since there is a striking preference for *para* substitution over *ortho* substitution in electrophilic aromatic substitution of fluorobenzene (89% *para*, 11% *ortho*).[148] The short C-F bond and similar size of the orbitals containing p and π electrons result in a maximal p-π interaction. The powerful inductive effect of fluorine acts most strongly at the *ortho* position relative to the *para* position. This results in the return of electron density by resonance, which overrides the small inductive effect at the distant *para*

position but is not large enough to reduce significantly electron withdrawal at the *ortho* position. Figure 17 shows the accepted patterns of electron flow in σ and π framework of fluorobenzene.[148]

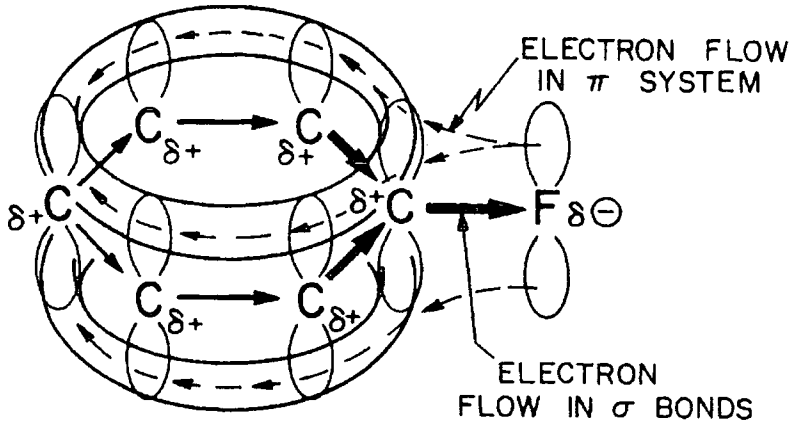


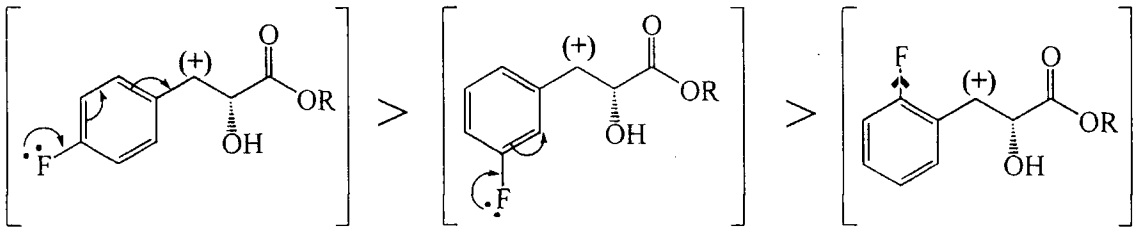
Figure 17 The high electronegativity of fluorine allows it to withdraw electrons from the σ framework and accumulate electron density on fluorine. However, this accumulation of charge is partly fed back into the π system by resonance, and must result from good orbital overlap of the p-orbitals on carbon and fluorine.

3.2.2 The use of fluorine as a mechanism probe

To investigate the mechanism of the rearrangement of littorine (**5**) to hyoscyamine (**1**) further, it was envisaged that if a fluorine substituent is attached to the phenyl ring, it would exert an effect on the putative benzylic carbocation required to initiate the rearrangement.

If fluorine is substituted for hydrogen on the phenyl ring, it would be anticipated to exert differing electronic effects at the benzylic carbon, depending on its position of substitution (*ortho*, *meta* or *para*). A 4-fluoro (*para*) substituent should stabilise the benzylic carbocation by means of electron resonance since inductive effects are small at this range. A 3-fluoro (*meta*) substituent is not anticipated to significantly stabilise the benzylic carbocation since electron resonance is not significant with *meta* substitution. Again, inductive effects are small and perhaps negligible at this position. A 2-fluoro (*ortho*) substituent should destabilise the benzylic carbocation since inductive destabilising effects are anticipated to override the electronic stabilising resonance effects. Thus, in summary, a fluorine substituent on the phenyl ring was anticipated, at

the outset, to stabilise a benzylic carbocation in the order 4-fluoro > 3-fluoro > 2-fluoro (fig. 18).

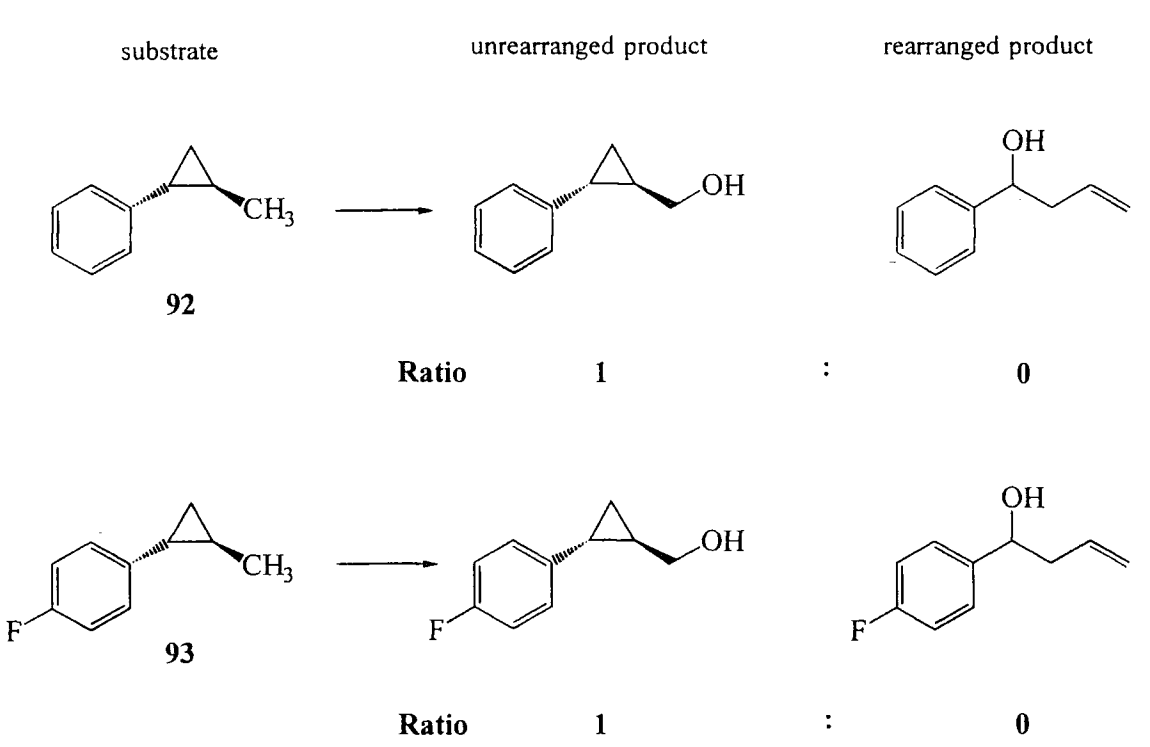


R = tropinyl

Figure 18 The anticipated stabilisation profile of the three aryl substituted fluorolittorines on a proposed benzylic carbocation intermediate.

By extension, if the proposed intermediate is carbocation in origin, then *ortho*-, *meta*-, and *para*-mono fluorinated littorines are anticipated to rearrange to the corresponding fluorohyoscyamines in the order 4F-littorine > 3F-littorine > 2F-littorine; providing that the fluorine substituent does not have an adverse effect on the binding of the analogue to the isomerase.

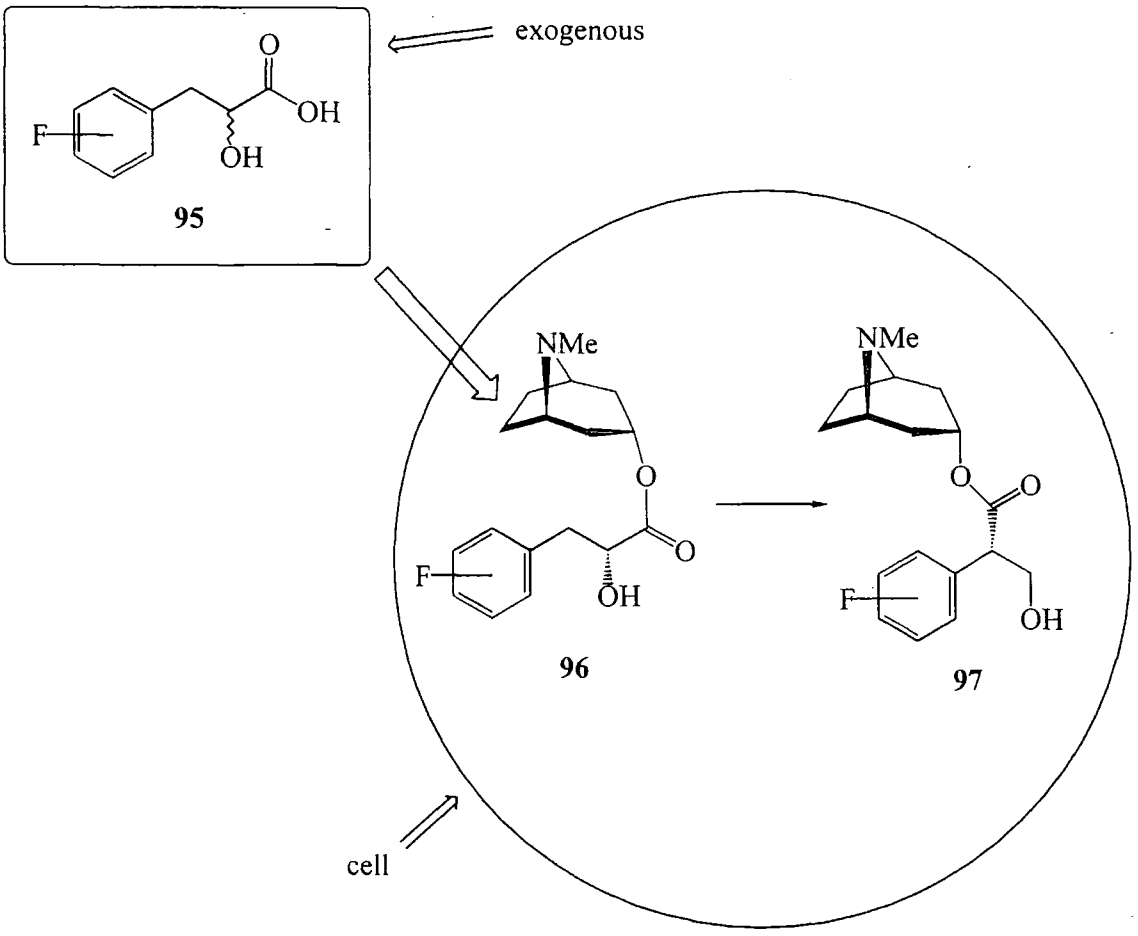
However, that said, very little is reported in the literature on the influence of an aryl fluorine substituent on the stability of the corresponding benzylic radical. Newcomb and co-workers[149] have utilised aryl fluorines in radical clock substrate probes, to investigate the oxidation process of soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus*. The fluorinated (**93**) and hydrocarbon (**92**) substrates behaved similarly. No rearranged products were generated, and an upper limit of 150 fs was calculated to be the lifetime of a putative radical species (Scheme 74). A lower conversion was observed for the fluorinated probe relative to the non-fluorinated probe, however, this was attributed to the lower solubility of the former, rather than any specific electronic effects.



Scheme 74 The oxidation process of soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* was investigated with radical clock substrate probes. The fluorinated (**93**) and hydrocarbon (**92**) substrates behaved similarly, and generated exclusively, the unrearranged product in both cases.

3.3 Introducing Fluorine into Tropane Alkaloids

To test our mechanistic probe, it was necessary to synthesise *ortho*, *meta* and *para* substituted fluorophenyllactates (**95**) for incubation studies with *D. stramonium*. It is known that phenyllactate (**51**) is esterified *in vivo* with tropine (**32**) to form littorine (**5**) prior to the rearrangement to hyoscyamine (**1**). It seemed appropriate therefore, to feed the fluorophenyllactates (**95**) rather than the fluorolittorines (**96**) for two reasons. Firstly, GC-MS analysis from the feeding experiments should reveal information concerning the ease of esterification *in vivo* of these fluorinated phenyllactates (**95**) to fluorolittorines (**96**). Secondly, by feeding at the fluorophenyllactate level in the biosynthetic pathway, a more accurate measurement of the *in vivo* conversions of fluorolittorine (**96**) to fluorohyoscyamine (**97**) would be obtained clearly from fluorohyoscyamine (**97**) : fluorolittorine (**96**) ratios (Scheme 75).

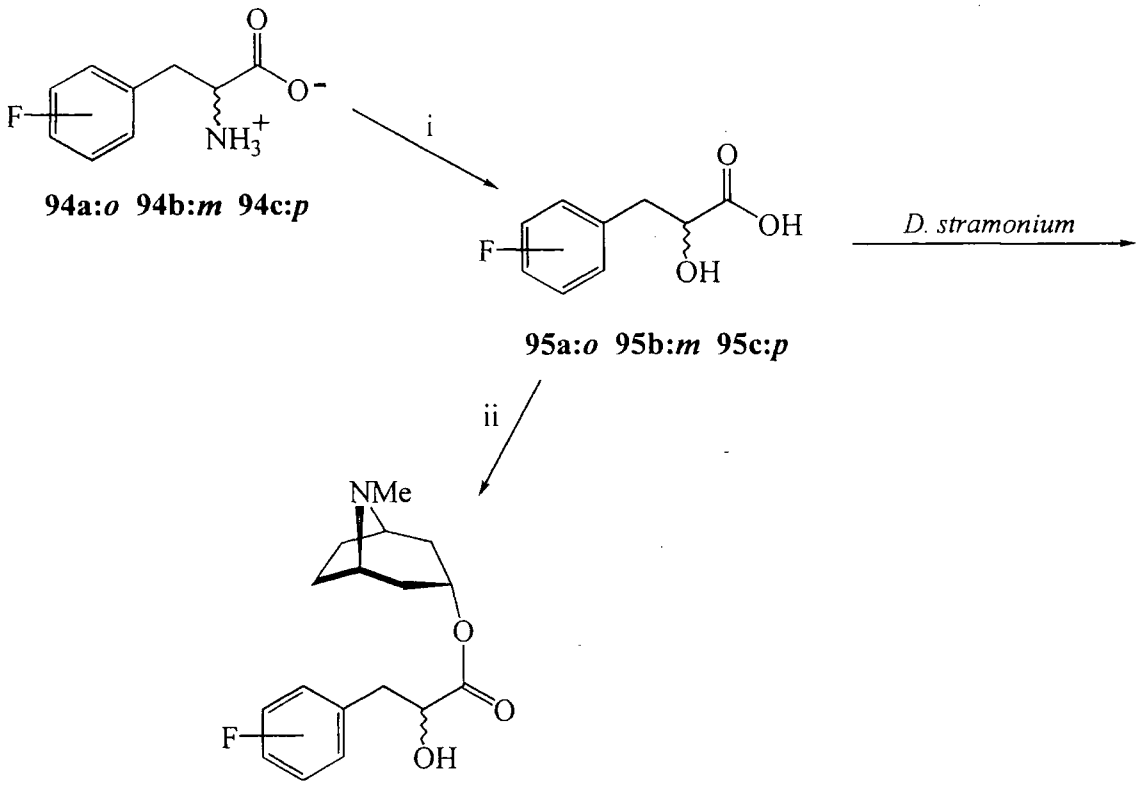


Scheme 75 Administered exogenous aryl substituted fluorophenyllactates (**95**) to *D. stramonium* will provide information regarding the esterification reaction with tropine to form fluorolittorines (**96**), and the *in vivo* biotransformation of fluorolittorine (**96**) to fluorohyoscyamine (**97**).

Accordingly, the synthesis of 2′-, 3′- and 4′-fluorophenyllactic acids (**95a**, **95b** and **95c**) became the subject of attention and emerged as the synthetic targets. It is anticipated that the GC-MS analysis of the isolated extracts from the feeding experiments with fluorophenyllactic acids (**95a**, **95b** and **95c**), will show two new peaks with the molecular ion (M) of *m/z* 307, corresponding to fluorolittorine (**96**) or fluorohyoscyamine (**97**) analogues. Thus, the corresponding fluorolittorines (**96a**, **96b** and **96c**) were also required for GC-MS reference compounds in order to assign one of the new peaks, and by a process of elimination, the other peak will be assigned to the fluorohyoscyamine analogue (**97**).

3.3.1 Synthesis of 2′-, 3′- and 4′-fluorophenyllactoyltropines [fluorolittorines (96a, 96b and 96c)]

The synthetic route to 2′-, 3′- and 4′-fluorolittorines (**96a**, **96b** and **96c**) is outlined in Scheme 76.



94a:*o* **94b:*m*** **94c:*p***

95a:*o* **95b:*m*** **95c:*p***

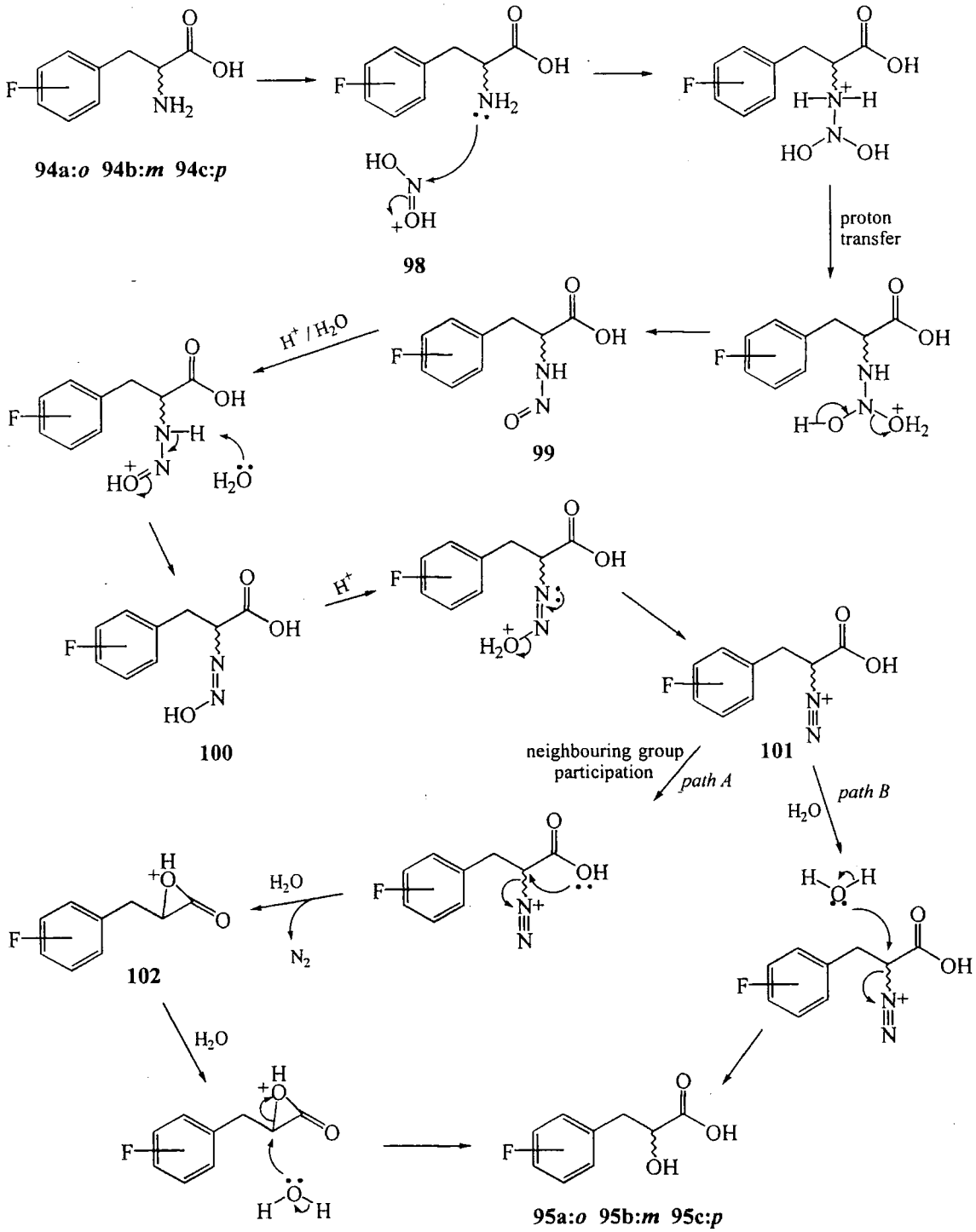
96a:*o* **96b:*m*** **96c:*p***

i. $NaNO_2$, HCl; ii. HCl

Scheme 76 The synthetic route to 2′-, 3′- and 4′-fluorolittorines (**96a**, **96b** and **96c**).

Starting from commercially available 2′-, 3′- and 4′-fluorophenyl-DL-alanines (**94a**, **94b** and **94c**), each isomer of fluorophenyllactate (**95**) was synthesised *via* a diazotisation reaction[150] with sodium nitrite in hydrochloric acid. The mechanism of the reaction (Scheme 77) proceeds firstly with the protonation in strong acidic conditions of nitrous acid, HONO, to generate the activated species [$HONOH^+$, (**98**)]. The amide of fluorophenyl-DL-alanine (**94**) then undergoes a nucleophilic substitution reaction with this species to generate the N-nitroso derivative of fluorophenyl-DL-alanine (**99**). Following further protonation and elimination of H_2O, a diazotic acid derivative (**100**) is formed. Elimination of another molecule of H_2O following protonation yields the diazonium ion (**101**). This unstable compound decomposes to release N_2 with the help of the neighbouring carboxylate group to generate the α-lactone [(**102**), Scheme 77,

path A]. Finally, nucleophilic attack by H_2O opens the α-lactone (**102**) to generate fluorophenyl-DL-lactic acid (**95**). All of the diazotisation reactions for the three isomers of phenyl-DL-alanine proceeded smoothly to generate the corresponding fluorophenyl-DL-lactic acids (**95a**, **95b** and **95c**) in yields ranging between 19-25%. The low yields are a consequence of an incomplete reaction and in all cases, the starting product was recovered from each reaction.



Scheme 77 The mechanism of the diazotisation reaction.

Although it is not significant in the synthetic route outlined in Scheme 76, it is noteworthy that the substitution of the amine by OH proceeds with predominant retention of configuration due to the intermediacy of the α-lactone (**102**). However, a small proportion of the product (**95**) has inverted stereochemistry at C-2.[151,152] This arises by direct substitution of the diazonium ion (**101**) by water (path B, Scheme 77).

In order to obtain samples of fluorolittorines (**96a**, **96b** and **96c**) for GC-MS standards, the respective fluorophenyllactic acids (**95a**, **95b** and **95c**) were treated with tropine (**32**) under acidic conditions, as described by Jowett and Pyman for the synthesis of littorine (**5**).[153] This reaction is unusually carried out in the solid phase without the presence of a solvent. It involves the coupling of fluorophenyllactic acid (**95**) and tropine (**32**) in an atmosphere of dry hydrogen chloride gas at a temperature of 130°C. This procedure resulted in a reddish brown melt which was worked-up to give each of the three isomers of fluorolittorine. Both 2′- and 3′-fluorolittorines required purification by buffered column chromatography using Hyflo®, following the method described by Evans and Partridge for littorine (**5**) purification.[154] This involved mixing a small volume of phosphate buffer (2ml) at pH 6.6 into a 10g sample of Hyflo®. This buffered Hyflo® was then transferred to a glass column and compacted down with a glass rod, prior to applying the sample and eluting with organic solvents. Silica gel and other similar products were not used to purify these alkaloids, as they stick to the solid phase. Although this crude purification procedure was successful for littorine (**5**), it was less so for the fluorinated analogues (**96**). In the event, 2′-fluorolittorine (**96a**) was isolated as a pale yellow oil in 14% yield and 3′-fluorolittorine (**96b**) as a pale yellow gum in 15% yield. Both of these compounds appeared to be clean by ^{1}H-NMR, but GC analysis (MSTFA derivatives) revealed that 2′-fluorolittorine (**96a**) was only 80% pure and 3′-fluorolittorine (**96b**) 93% pure. The sample of 4′-fluorolittorine (**96c**) was obtained as a pale white solid by crystallisation from chloroform in 57% yield and was found to be 84% pure after GC analysis of its MSTFA derivative. In all three cases, the impurity was the starting material, *ortho*, *meta* or *para*-fluorophenyl-DL-alanine (**94**).

3.3.2 Feeding fluorophenyl-DL-lactic acids (95a, 95b and 95c) to *Datura stramonium* root cultures

The fluorophenyllactates (**95a**, **95b** and **95c**) were administered to root cultures of *D. stramonium* at a final concentration of 0.1mmol dm^{-3} in a nutrient medium. This

concentration was selected on the basis of previous work by Zabetakis and co-workers.[124] That work demonstrated that when the root cultures of *D. stramonium* were supplemented with exogenous phenyllactate (**51**) at a concentration of 0.1mmol dm^{-3}, it stimulated littorine (**5**) levels by approximately 30%. Higher concentrations of phenyllactate (**51**) produced no significant increase in littorine (**5**) levels and this concentration was judged to be optimal for efficient bio-conversion.

Working in a laminar flow cabinet and applying aseptic techniques, solutions of 2′-, 3′- and 4′-fluorophenyl-DL-lactic acids (**95a**, **95b** and **95c**) in methanol were initially filter-sterilised through an irradiated sterile 0.2μm filter. Each sterile solution was then administered in separate experiments to six flasks of *D. stramonium* roots on day 7 of growth. The roots were then replaced on their rotary incubator to mature. Later, two flasks of roots from each experiment were harvested and freeze-dried after days 11, 14 and 17 of growth. The freeze-dried roots were worked-up to obtain a crude alkaloid extract, which was then submitted for GC-MS analysis.

3.3.3 Results from feeding 2′-, 3′- and 4′-fluorophenyl-DL-lactic acids (95a, 95b and 95c)

The GC-MS method involved dissolving a sample of the crude alkaloid extract in methanol and injecting it onto a chiral GC column (DB-17), which was temperature ramped from 65°C to 300°C to aid separation. A mass spectrometer was linked to the GC column and was set to single ion monitoring mode to identify littorine [(**5**), M^+ = 289], and hyoscyamine [(**1**), M^+ = 289], and the corresponding fluoro-analogues, fluorolittorine [(**96**), M^+ = 307] and fluorohyoscyamine [(**97**), M^+ = 307].

The chromatograms all had peaks which could be assigned for the elution of littorine (**5**) and hyoscyamine (**1**), (M^+ = 289, Rt = 47.7min and 48.3min respectively). However, in each case, two additional peaks with similar retention times were also present. Daughter ion mass analysis of these peaks revealed that both had molecular ions of M^+ = 307, which is characteristic of the monofluorinated analogues of littorine (**5**) and hyoscyamine (**1**), as shown in fig. 19.

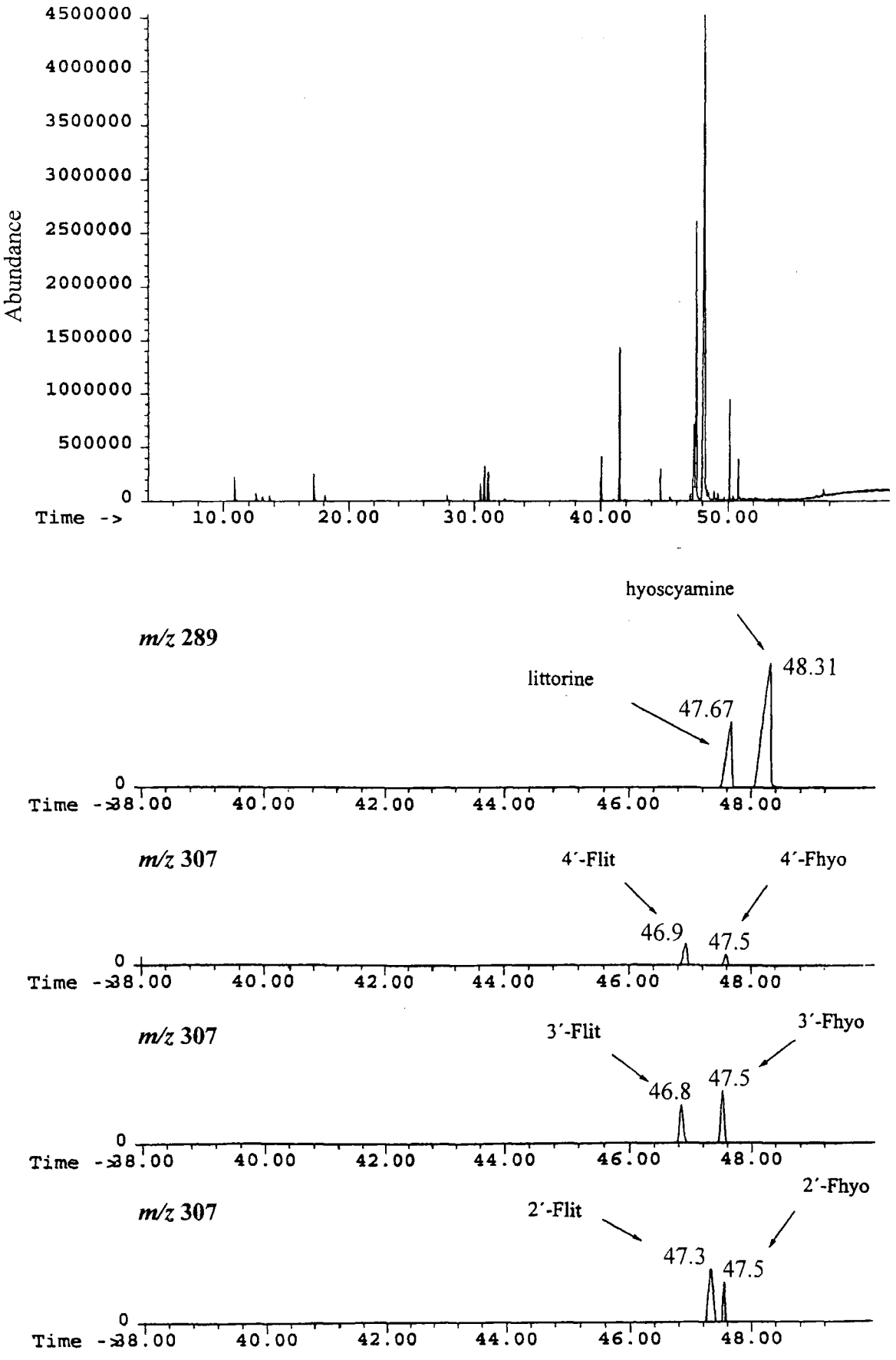


Figure 19 A typical GC and combined single ion monitoring (SIM) profile from the extracts isolated from feeding the three isomers of fluorophenyllactate (**95a**, **95b** and **95c**) to *D. stramonium* root cultures after 14 days of growth.

In each case, the peak with the earliest retention time (47.3min – 46.9min) containing a M^+ = 307 ion was identified as the fluorinated littorine analogue (**96**). This was confirmed by co-elution on GC and comparison of identical fragmentation patterns in GC-MS with the appropriate synthetic reference compound (Table 7). The peak with the later retention time was thus assigned to the fluorinated hyoscyamine analogue (**97**). Synthetic references were not available for the corresponding fluorohyoscyamines (**97**).

m/z	Compound	GC retention time of standard	average GC retention time of fluorohyoscyamine product
289	littorine (**5**)	47.5	
	hyoscyamine (**1**)	47.9	
307	2′-fluorolittorine (**96a**)	47.2	47.4
	3′-fluorolittorine (**96b**)	46.8	47.2
	4′-fluorolittorine (**96c**)	46.7	47.2

Table 7 GC-MS analysis of the alkaloid extracts obtained from the feeding experiments with the three isomers of fluorophenyllactic acid revealed the presence of two additional peaks (*m/z* 307) corresponding to their respective fluorolittorine and fluorohyoscyamine analogues. The earlier of the two peaks was identified as the fluorolittorine analogue *via* GC co-elution and MS comparison with a synthetic reference compound. The other peak was identified by a process of elimination, and was assigned to the fluorohyoscyamine analogue.

Fed precursor		Day 11	Day 14	Day 17	"average incorporation"	
					Flit	*Fhyo*
Control	hyo/lit	2.72	4.07	3.93		
2′-Fpla	hyo/lit	2.97 ± 0.101	3.08 ± 0.007	3.07 ± 0.026		
	Fhyo/Flit	0.09 ± 0.005	0.14 ± 0.001	0.08 ± 0.001		
	Flit/lit	0.26 ± 0.002	0.28 ± 0.030	0.20 ± 0.015	25%	
	Fhyo/hyo	0.01 ± 0.001	0.01 ± 0.001	0.01 ± 0.001		1%
3′-Fpla	hyo/lit	3.07 ± 0.025	3.12 ± 0.007	3.02 ± 0.021		
	Fhyo/Flit	1.15 ± 0.051	1.26 ± 0.027	1.60 ± 0.138		
	Flit/lit	0.13 ± 0.002	0.11 ± 0.001	0.09 ± 0.007	11%	
	Fhyo/hyo	0.05 ± 0.001	0.05 ± 0.001	0.05 ± 0.001		5%
4′-Fpla	hyo/lit	2.60 ± 0.001	2.73 ± 0.006	3.19 ± 0.057		
	Fhyo/Flit	0.40 ± 0.006	0.42 ± 0.006	0.62 ±0.021		
	Flit/lit	0.24 ± 0.001	0.23 ± 0.005	0.22 ± 0.006	23%	
	Fhyo/hyo	0.04 ± 0.001	0.04 ± 0.001	0.04 ± 0.002		4%

Table 8 Relative ratios of littorine (lit), hyoscyamine (hyo), 2′-, 3′-, 4′-fluorolittorines (Flit) and 2′-, 3′-, 4′-fluorohyoscyamines (Fhyo) after feeding experiments with 2′-, 3′-, 4′-fluorophenyllactates (Fpla) to *D. stramonium* root cultures. Errors refer to GC reproducibility from two injections.

The relative abundances of these compounds from each of the three experiments are shown in Table 8 and are calculated after considering natural abundance levels using the "isotopic incorporation calculator" as described in Chapter 2, Section 2.2.3.1, page 65. Values in the table are the ratios of two metabolites derived from GC integration values after feeding experiments worked up on days 11, 14 and 17.

Biotransformation	Day 11	Day 14	Day 17	Average efficiency
2′-Flit (**96a**) to 2′-Fhyo (**97a**)	3	4.5	2.6	3
3′-Flit (**96b**) to 3′-Fhyo (**97b**)	37	40	53	43
4′-Flit (**96c**) to 4′-Fhyo (**97c**)	15	15.3	19.4	17

Table 9 *In vivo* conversions (% values) of fluorolittorines (Flit) to fluorohyoscyamines (Fhyo) after biotransformations of 2′-, 3′-, and 4′-fluorophenyllactates (**95a**, **95b** and **95c**) in *D. stramonium* root cultures.

Table 9 shows the *in vivo* conversions (% values) of fluorolittorines (Flit) to fluorohyoscyamines (Fhyo) after biotransformations of fluorophenyllactates (**95a**, **95b** and **95c**) in *D. stramonium* root cultures in experiments worked up after days 11, 14 and 17. The conversions are percentage values relative to endogenous conversions of littorine (**5**) to hyoscyamine (**1**) in the same experiments. The data is calculated from Table 8 as Fhyo/Flit / hyo/lit x 100.

It is clear from examining the results that all three isomers of fluorophenyllactic acid (**95**) were esterified to their corresponding littorines (**96**) *in vivo*. Also, the GC-MS data reveals that these fluorolittorines (**96**) are biotransformed in varying degrees of efficiency to their corresponding fluorohyoscyamines (**97**). Both of these issues are discussed in more detail below.

3.4 Discussion

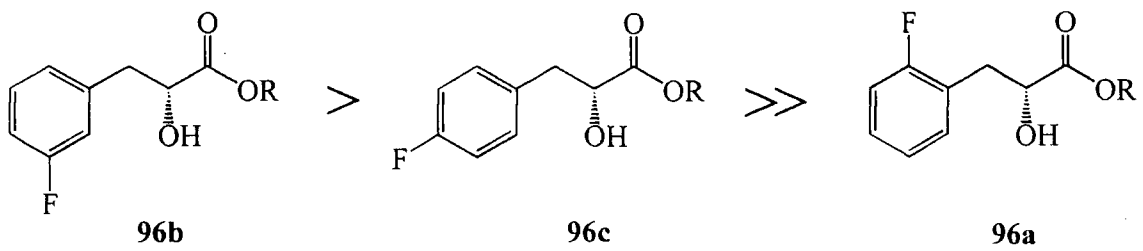
3.4.1 The biosynthesis of fluorolittorines *in vivo*

It is noteworthy that for all of the three fluorophenyllactates (**95a**, **95b** and **95c**) administered to root cultures of *D. stramonium*, both fluorolittorine (**96**) and fluorohyoscyamine (**97**) analogues were generated *in vivo*. The Flit/lit ratios in Table 8

give an approximate measure of "incorporation" of exogenous fluorophenyllactic acid (**95**) into the littorine (**5**) pool (lit+Flit). For 2′- (**95a**) and 4′-fluorophenyllactates (**95c**) the "average incorporation" levels are 25% and 23% respectively, whereas for 3′-fluorophenyllactate (**95b**), the "average incorporation" level is only 11%. This result would seem to indicate that by comparison, 3′-fluorophenyllactate (**95b**) is a poorer substrate for the esterification reaction to form the littorine analogue, compared to either the 2′- (**95a**) or 4′-fluorophenyllactates (**95c**). This could be due to differing binding affinities to the littorine synthase enzyme relative to the other two substrates during formation of 3′-fluorolittorine (**96b**). Alternatively however, the levels of 3′-fluorolittorine (**96b**) in the root extracts may be attenuated by the higher *in vivo* conversion of 3′-fluorolittorine (**96b**) to 3′-fluorohyoscyamine (**97b**) relative to the other two isomers of fluorolittorine (Table 9). This is discussed in more detail below.

3.4.2 The biotransformation of fluorolittorine to fluorohyoscyamine *in vivo*

The average "incorporations" of all fluorohyoscyamine isomers are low and range from 1% for the 2′-fluoro (**97a**), 4% for the 4′-fluoro (**97c**) and 5% for the 3′-fluoro (**97b**), as shown in Table 8. Nevertheless, it was still possible to determine which of the three fluorinated littorines is most efficiently isomerised *in vivo* to the corresponding fluorohyoscyamine. The Fhyo/Flit ratio was evaluated as a percentage of the endogenous hyo/lit ratio in each experiment. This was judged to offer a measure of the efficiency of conversion of fluorolittorine (**96**) to fluorohyoscyamine (**97**) for the three isomers and is shown in Table 9. The data reveal that the efficiency of biotransformation of the fluorolittorines (**96**) to fluorohyoscyamines (**97**) is in the order of 3′-fluorolittorine (**96b**) > 4′-fluorolittorine (**96c**) >> 2′-fluorolittorine (**96a**), as shown in fig. 20.



96b **96c** **96a**

R = tropinyl

Figure 20 The efficiency profile for the biotransformation of aryl substituted fluorolittorines to their corresponding fluorohyoscyamines *in vivo*.

In the cases of 3′- (**96b**), and 4′-fluorolittorine (**96c**), there is an apparent increase in the level of conversion from day 11 to day 17 [37% rising to 53% for 3′-fluoro (**96b**) and 15% rising to 19.4% for the 4′-fluoro isomer (**96c**)], whereas for 2′-fluorolittorine (**96a**), the conversion (3% on day 11 to 2.6% on day 17) is too low to be confident of attributing any significance to the data (Table 9).

The results reveal that the fluorinated littorines (**96**) were all substrates, but were not as efficiently converted to their respective fluorinated hyoscyamines (**97**) when compared to the endogenous conversion of littorine (**5**) to hyoscyamine (**1**). It is noteworthy that the *ortho* (**96a**) and *para* (**96c**) fluorinated substrates are biotransformed less well than the *meta* substrate (**96b**). This was not predicted and seems to indicate that the rearrangement process does not follow the predicted substituent effects for stabilisation of a benzylic carbocation. The very low conversion of 2′-fluorolittorine (**96a**) to 2′-fluorohyoscyamine (**97a**) can clearly be explained by the proximity of the fluorine atom to the benzylic reaction centre for the isomerisation, as the inductive effect of fluorine is greatest at this *ortho* position. However, the results do not reveal a progressive inductive effect as 3′-fluorolittorine (**96b**) proved to be a better substrate than 4′-fluorolittorine (**96c**) for the isomerisation. From classical studies on fluorine substituent effects, the intermediacy of a benzylic carbocation or radical is predicted to gain greater stability from fluorine at the *para* position over the *meta* position due to mesomeric conjugative effects overriding inductive effects. Perhaps significant differences in binding affinities to the isomerase exist and contribute to this biotransformation efficiency profile; and there is some evidence to suggest that this is the case for the binding of 3′-fluorophenyllactate (**95b**) to the littorine synthase. Undoubtedly, it will be necessary to assess the kinetics of these fluorinated substrates with a purified isomerase to obtain these answers, however, no such system is available at present.

Chapter 4

4. Biosynthetic Studies on the Tropane Ring

4.1 The Origin of the Tropane Ring

As discussed in Chapter 1, the tropane ring system of the medicinally important alkaloids hyoscyamine (**1**), scopolamine (**3**) and cocaine (**23**) is derived from ornithine (**6**),[8,23] acetate (**7**),[24,25] and L-methionine (**105**),[155,156] as summarised in fig. 21.

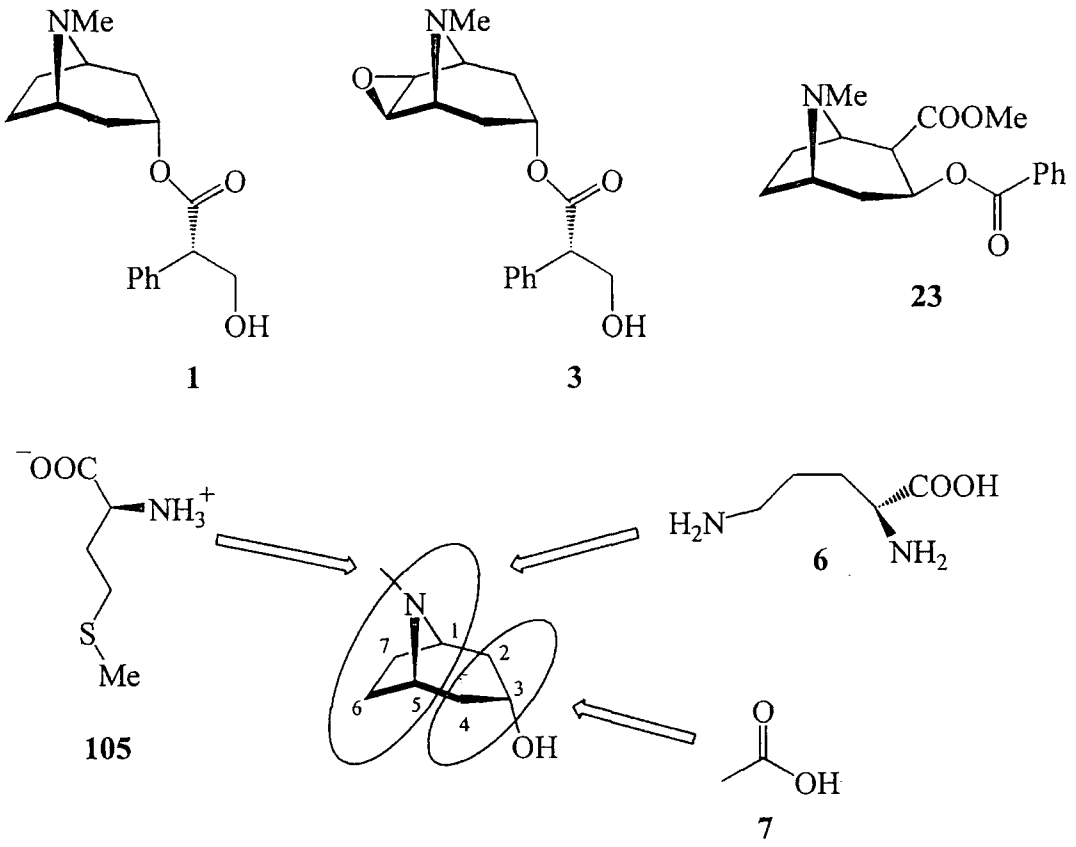
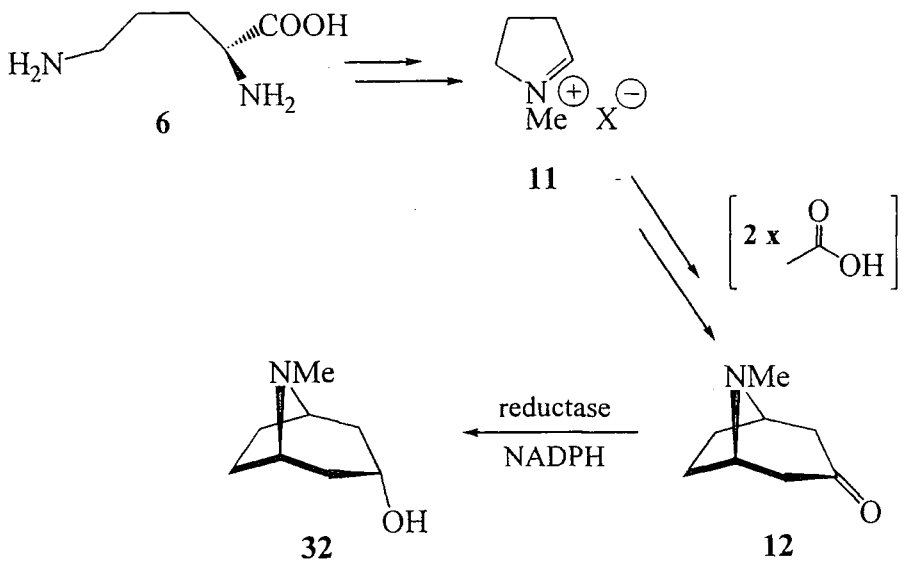


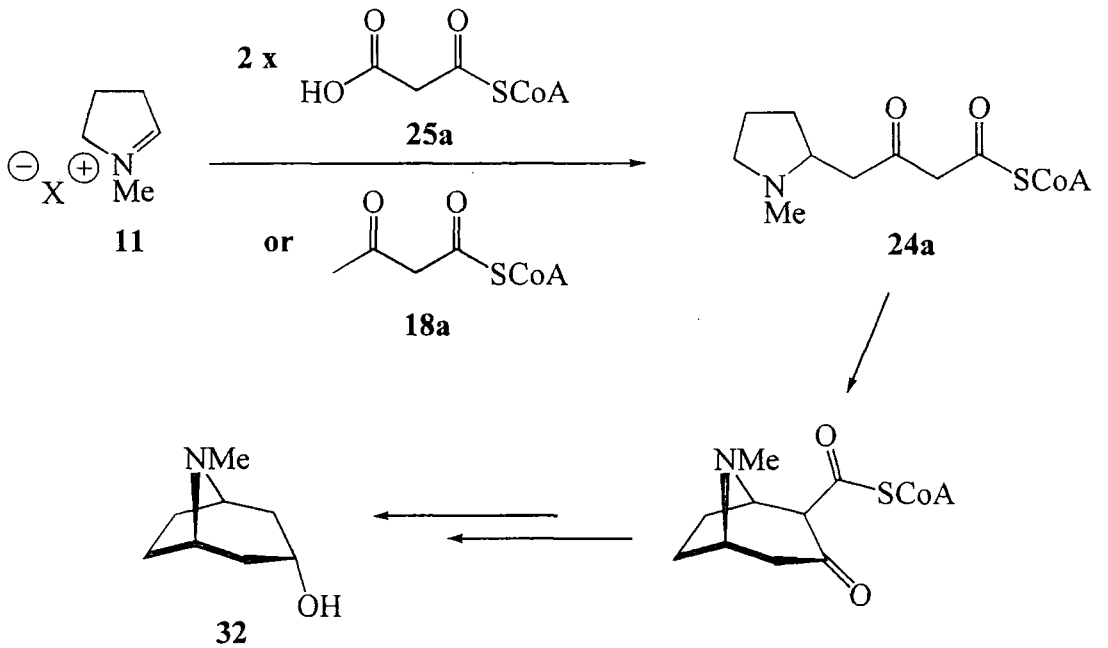
Figure 21 The origin of the tropane moiety of the medicinally important alkaloids, hyoscyamine (**1**), scopolamine (**3**) and cocaine (**23**).

The biosynthesis of tropine has been extensively investigated over the years (Scheme 78).[30] However, although the biosynthetic route from ornithine (**6**) to the N-methylpyrrolinium salt (**11**) has been established, the details of the pathway derived from acetate has yet to be confirmed.



Scheme 78 The biosynthetic pathway of the tropane ring has not been fully established, since the details of the acetate derived pathway remains to be confirmed.

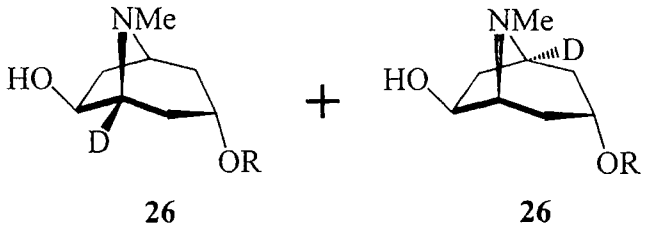
Recent studies have suggested that 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**) is a likely intermediate, which could be biosynthesised either from the successive condensation of two units of malonyl-CoA (**25a**) or from the condensation of just one acetoacetyl-SCoA unit (**18a**) with the N-methylpyrrolinium salt (**11**), as shown in Scheme 79.



Scheme 79 The current working hypothesis for the biosynthesis of tropine (**32**) from the N-methylpyrrolinium salt (**11**).

4.2 Studies of Deuterium Incorporation into the Tropane Ring

Hemscheidt and Spenser[47] were the first to use deuterium to probe the biosynthesis of the tropane ring. A feeding experiment with N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) to *D. stramonium* root cultures resulted in the incorporation of deuterium at both bridgehead sites in 6β-hydroxyhyoscyamine (**26**), as shown in fig. 22.

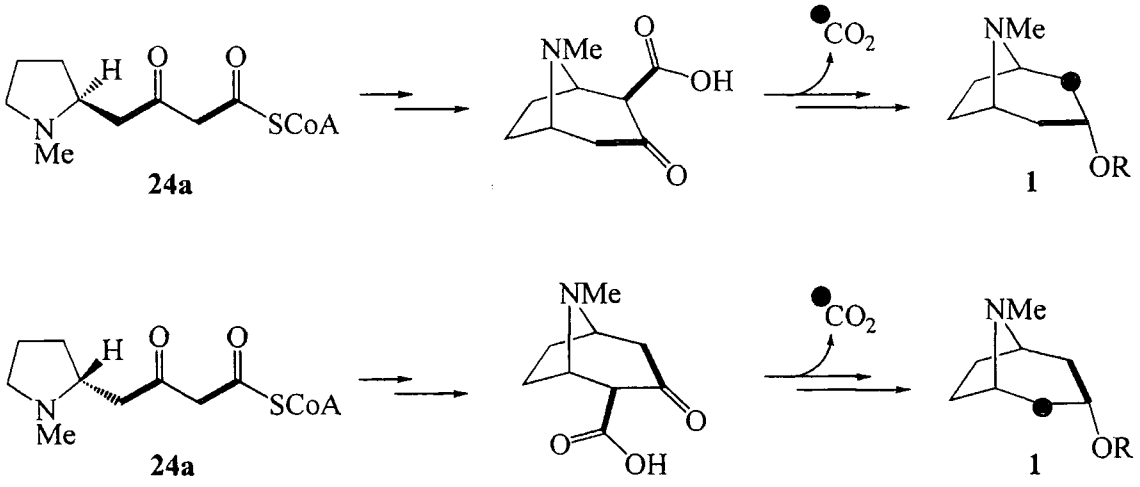


26 **26**

R = (*S*)-tropoyl

Figure 22 A feeding experiment with N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) to *D. stramonium* root cultures resulted in the incorporation of deuterium at both bridgehead sites in 6β-hydroxyhyoscyamine (**26**).

Surprisingly, the authors concluded from this result that both enantiomers of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**) are involved in the biosynthesis of hyoscyamine (Scheme 80).[47]



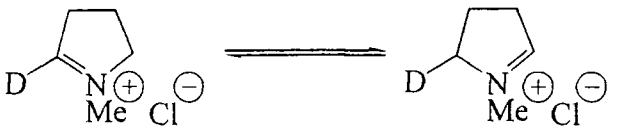
24a **1**

24a **1**

R = (*S*)-tropoyl

Scheme 80 To explain the symmetrical incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) and N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) into the tropane moiety of 6β-hydroxyhyoscyamine (**26**), Hemscheidt and Spenser proposed that both enantiomers of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**) are involved in the biosynthesis of hyoscyamine.

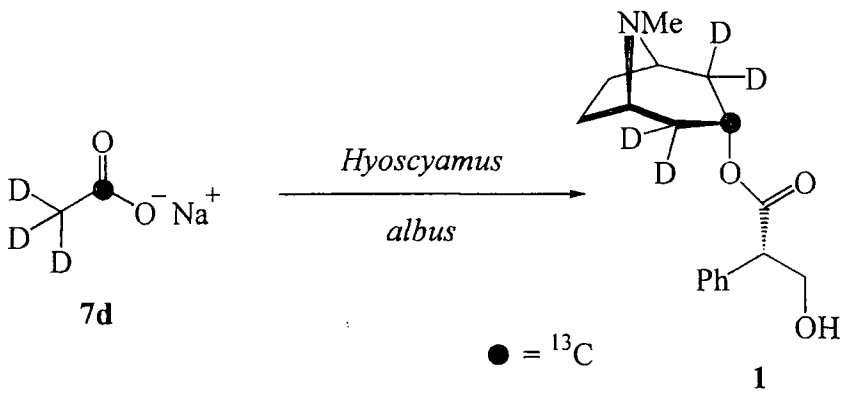
Perhaps however, the observed tautomerisation of N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) could give rise to the result (Scheme 81) and is a more likely explanation, although this was not considered in their discussion.



13b

Scheme 81 Tautomerisation of the administered N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) to *D. stramonium* could explain the observed symmetrical incorporation of deuterium at both bridgehead carbons of the tropane ring of 6β-hydroxyhyoscyamine (**26**).

Sauerwein and co-workers[119] fed sodium [1-^{13}C,2-^{2}H$_3$]-acetate (**7d**) to a hyoscyamine producing root culture of *Hyoscyamus albus* to investigate the biosynthesis of the tropane ring. They suggested that ^{13}C from the labelled acetate had become incorporated into C-3 of the tropane ring (Scheme 82).



7d **1**

Scheme 82 A feeding experiment with sodium [1-^{13}C,2-^{2}H$_3$]-acetate (**7d**) to *Hyoscyamus albus*, revealed that deuterium was incorporated into C-2 and/or C-4 of the tropane ring of hyoscyamine (**1**). However, the incorporation of deuterium is difficult to rationalise from the published ^{1}H-NMR data and remains controversial.

This result is consistent with conventional wisdom for the origin of C-3, however, their claim that deuterium was present at C-2 and C-4 of hyoscyamine remains controversial. It is clear from the published ^{13}C-NMR spectrum that the incorporation level at C-3 is ~3% (fig. 23, a). However, the authors also reproduced the ^{1}H-NMR spectrum of the

isolated alkaloid, and point to a diminution of the signal intensity assigned to the 2α and 4α protons by ~50% (fig. 24). This is difficult to rationalise with the ~3% incorporation from [1-^{13}C]-acetate, and is most probably a misinterpretation.

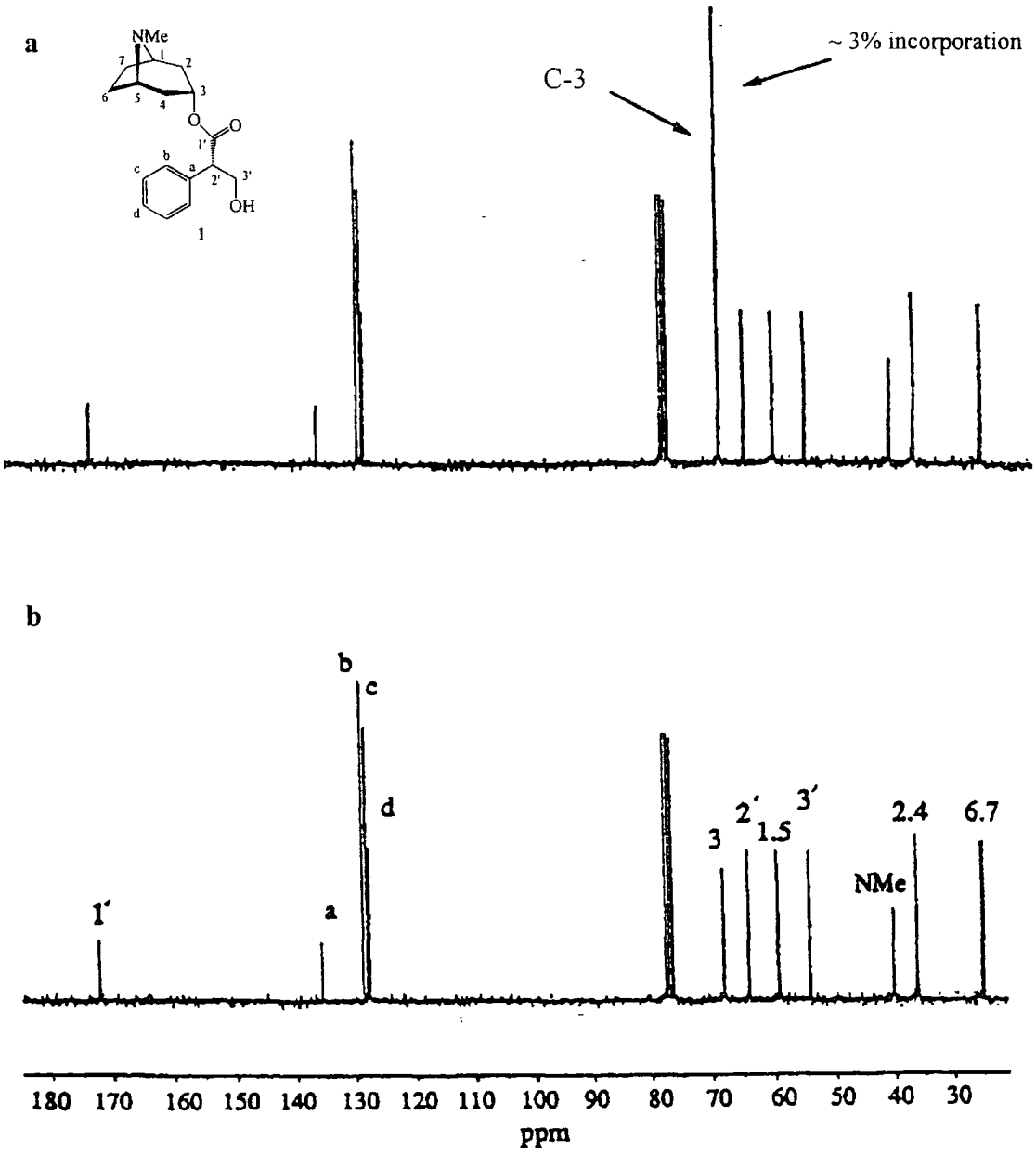


Figure 23 Incorporation of [1-^{13}C,2-^{2}H$_3$]-acetate (7d) in hyoscyamine (1) by hairy root cultures of *Hyoscyamus albus*. Numbers and letters indicate the carbon signals of hyoscyamine (1). Comparison of the ^{13}C-NMR spectrum (a) after feeding and (b) control reveals that C-3 of the tropane skeleton is enriched by ~3%.[119]

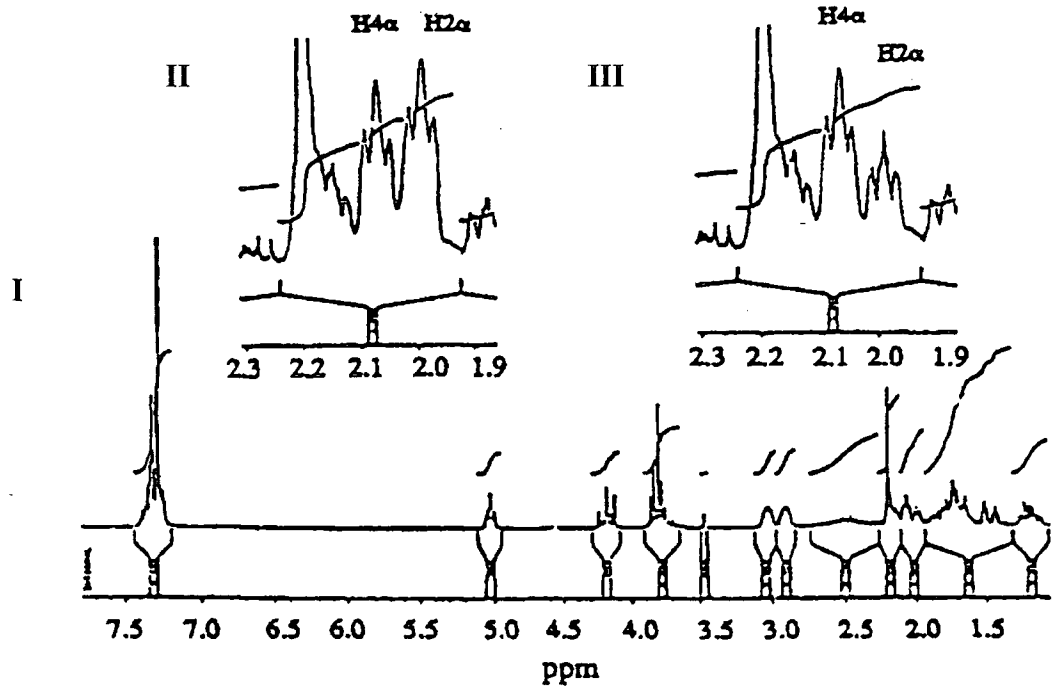


Figure 24 Incorporation of [1-^{13}C,2-^{2}H$_3$]-acetate (**7d**) in hyoscyamine (**1**) by hairy root cultures of *Hyoscyamus albus*. Comparison of ^{1}H-NMR spectrum of (II) control and (III) after feeding and reveals that H2α signal is diminished and is thus partially deuterated. (I) The complete ^{1}H-NMR spectrum after feeding.[119]

After comparing the diminished ^{1}H-NMR signals for H-2α and H-4α of hyoscyamine (**1**) with those in authentic samples, it was concluded that this arose from the incorporation of deuterium into the tropane ring through the administration of sodium [1-^{13}C,2-^{2}H$_3$]-acetate (**7d**). Since the diminution of the signals for H-2α and H-4α was not identical at both positions, it was suggested that labelled acetate was successively incorporated into the tropane ring (fig. 24). Thus concluding that the biosynthesis of the tropane ring from the N-methylpyrrolinium salt is a two step process, involving the sequential addition of two units of acetyl co-enzyme A (**7c**) rather than one of acetoacetyl-SCoA (**18a**). Their results also contradict those of Sankawa and co-workers[157] who reported that [1,2-^{13}C$_2$]-acetate (**7b**) was incorporated with equal efficiency at C-2 and C-4 in *Hyoscyamus albus*.

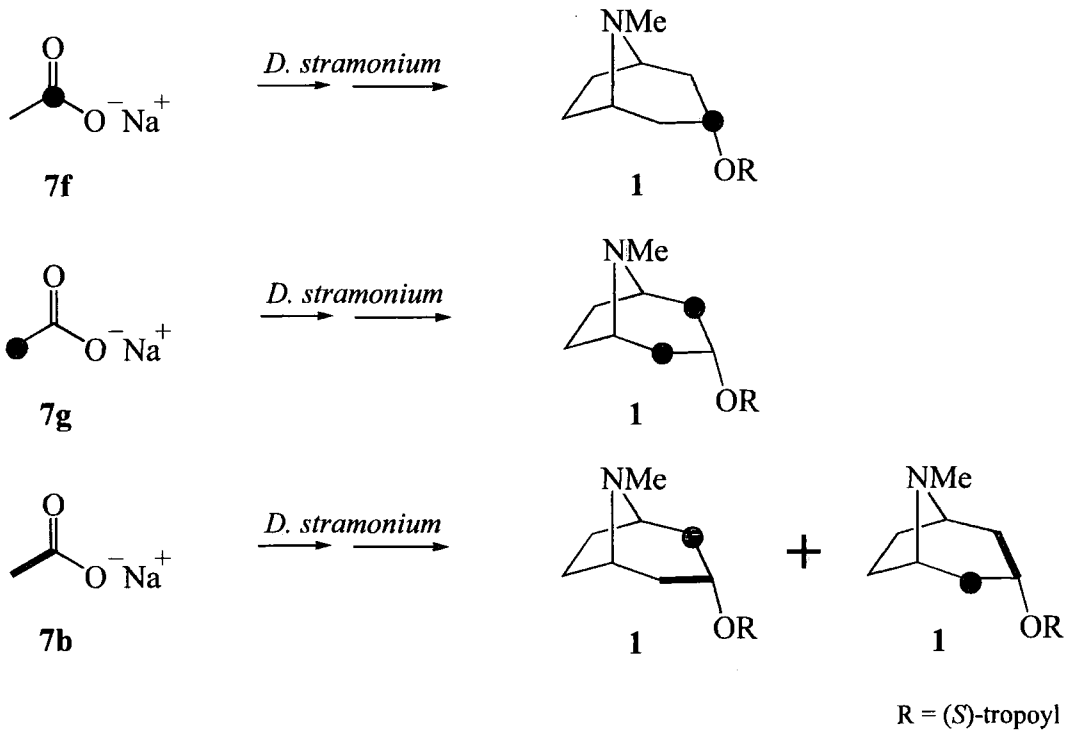
This experiment with sodium [1-^{13}C,2-^{2}H$_3$]-acetate (**7d**) clearly warranted a reinvestigation in order to clarify the findings of Sauerwein and co-workers.[119]

4.3 Stable Isotope Feeding Experiments to *Datura stramonium*

4.3.1 Biosynthetic studies with acetate

4.3.1.1 Preliminary feeding experiments of [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate

Prior to feeding sodium [2-^{13}C,2-$^{2}H_3$]-acetate (**7e**), preliminary experiments with [1-^{13}C]- (**7f**), [2-^{13}C]- (**7g**) and [1,2-$^{13}C_2$]-acetate (**7b**) were carried out in order to provide a feeding protocol. The results essentially reproduced the earlier findings of Robins[43] and Sankawa,[157] and a summary is presented in Scheme 83.



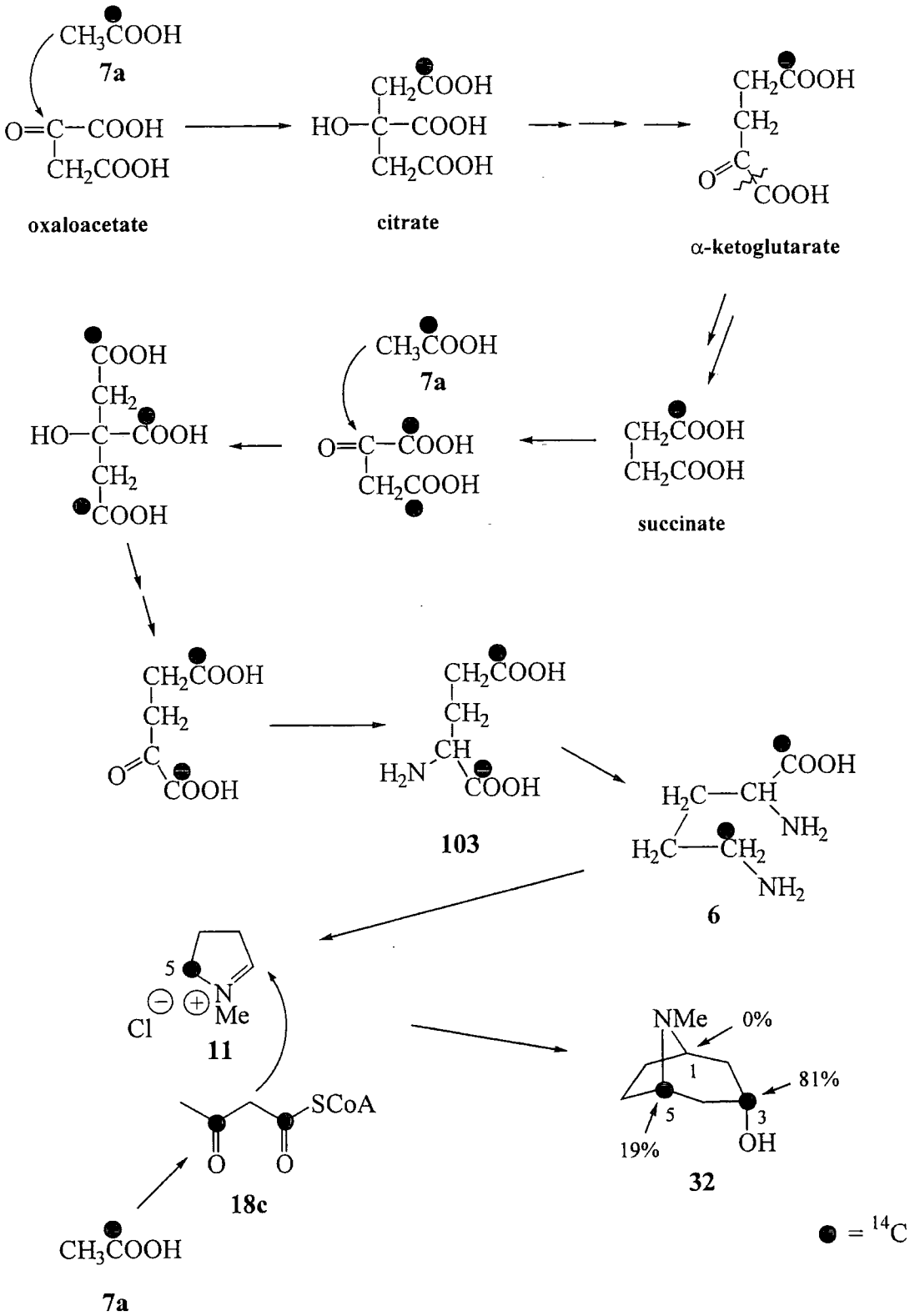
R = (*S*)-tropoyl

Scheme 83 Incorporation of [1-^{13}C]- (**7f**), [2-^{13}C]- (**7g**) and [1,2-$^{13}C_2$]-acetate (**7b**) into the acetate derived portion of the tropane ring of hyoscyamine (**1**).

4.3.1.2 Incorporation of [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetate into the tropane ring

This study revealed a high level of acetate incorporation into the ornithine derived moiety of the tropane ring. By examining the biosynthesis of this amino acid *via* the

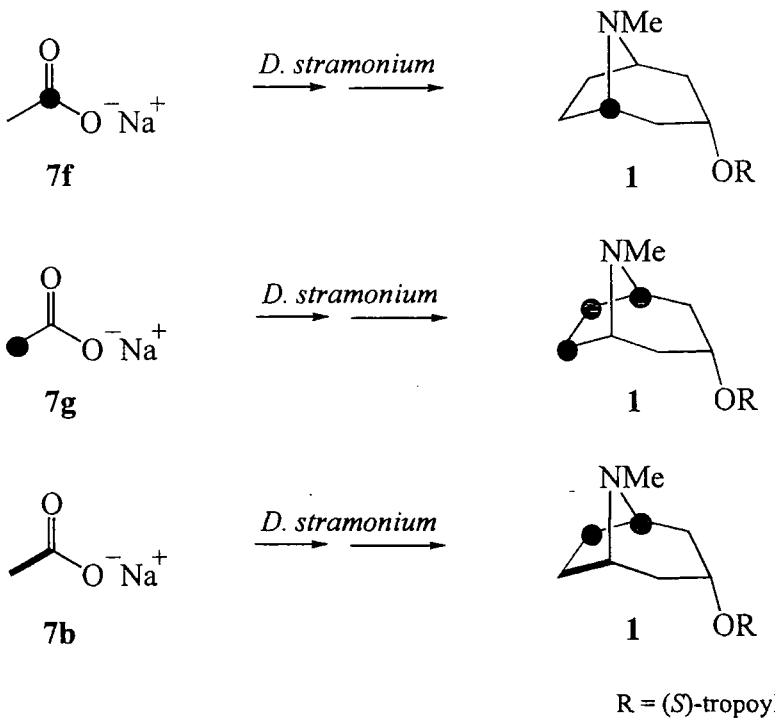
Krebs cycle, it can be rationalised that acetate will label all of the carbon atoms of the tropane ring as illustrated in Scheme 84.



Scheme 84 The incorporation of [1-^{14}C]-acetate (**7a**) into the tropine (**32**) *via* the Krebs cycle.

Bothner-By and co-workers[158] showed that the incorporation of [1-^{14}C]-acetate (**7a**) into the tropane ring, resulted in the enrichment of C-5 and C-3 (Scheme 84), and proposed that ornithine (**6**) derived from glutamate (**103**) would be labelled at C-1 and C-5 after two revolutions in the Krebs cycle. Decarboxylation will remove C-1 of ornithine prior to formation of the N-methylpyrrolinium salt (**11**), resulting in tropine (**32**) labelled at the C-5 bridgehead position. Labelling at C-3 of tropine (**32**) is observed from the incorporation of [1-^{14}C]-acetate (**7a**) {or [1,3-^{14}C]-acetoacetate (**18c**)}. It is noteworthy that the level of activity (81%) at C-3 is much higher than at C-5 (19%) since acetate (7) is biosynthetically closer to this part of the tropine molecule.

By following the biosynthesis of ornithine (**6**) from acetate (**7**) after consideration of the Krebs cycle, it can be rationalised that [1-^{13}C]-acetate (**7f**), [2-^{13}C]-acetate (**7g**) and [1,2-^{13}C$_2$]-acetate (**7b**) will label the ornithine derived portion of the tropane ring as shown in Scheme 85.



Scheme 85 An idealised incorporation of [1-^{13}C]-acetate (**7f**), [2-^{13}C]-acetate (**7g**) and [1,2-^{13}C$_2$]-acetate (**7b**) into the ornithine derived portion of the tropane skeleton of hyoscyamine (**1**).

4.3.1.3 Incorporation studies with sodium [1-^{13}C]-acetate (7f)

The ^{13}C-NMR spectrum of the isolated hyoscyamine from *D. stramonium* root cultures after feeding [1-^{13}C]-acetate (**7f**) at 9.3mmol dm^{-3}, revealed an enrichment of C-3 (66.7 ppm, ~20% incorporation) and C-5 (59.3 ppm or 59.2 ppm, ~10% incorporation) as expected (see fig. 25 and Table 10).

Tropane ring carbons	Chemical shift (ppm)	Approximate incorporation at each carbon position
C-1 and C-5	59.32 or 59.20	10%
C-3	66.72	20%

Table 10 Incorporation of sodium [1-^{13}C]-acetate (**7f**) into the tropane moiety of hyoscyamine (**1**).

Poignantly, C-1 (59.2 ppm or 59.3 ppm, ~10% incorporation) is also enriched at the same level as C-5. According to the Krebs cycle, C-1 should not be enriched since it is derived from C-2 of an acetate unit (see Scheme 85). This result suggests symmetrisation at some stage, although Leete demonstrated that this does not happen in the biosynthesis of nicotine in *Nicotiana tabacum*, it has not been reported in the biosynthesis of the tropane alkaloids in *Datura* plants.

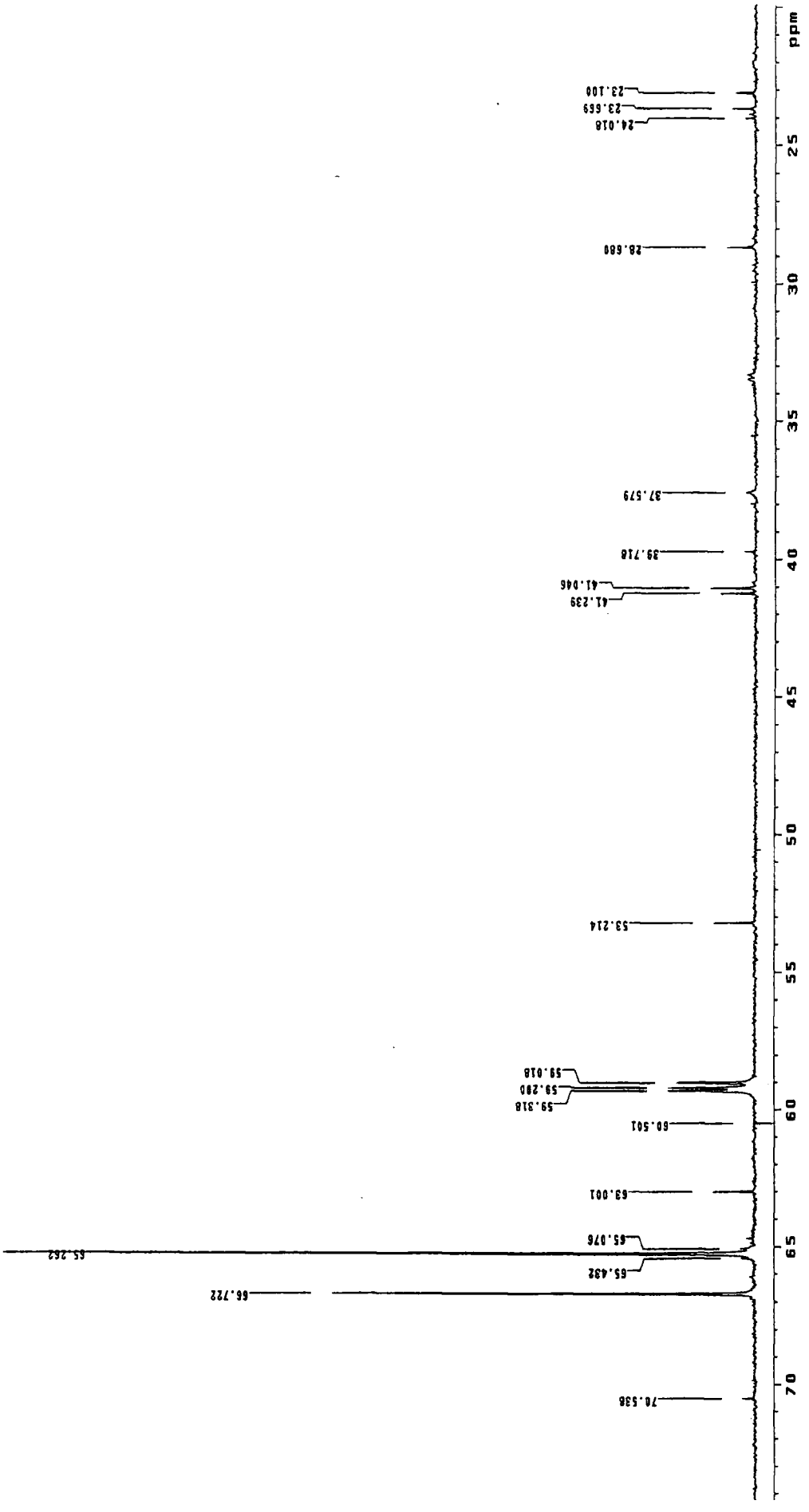
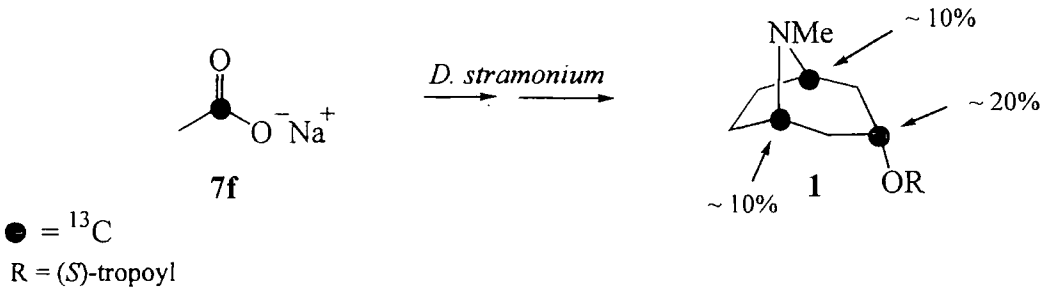


Figure 25 ^{13}C-NMR spectrum after feeding sodium [1-^{13}C]-acetate (**7f**) to transformed root cultures of *D. stramonium*.

Following the analysis of the ^{13}C-NMR spectrum after feeding sodium [1-^{13}C]-acetate (**7f**) to *D. stramonium* root cultures, a summary of the incorporation levels and positions into the tropane ring are shown in Scheme 86.



Scheme 86 Estimated incorporation levels above natural abundance into the tropane moiety of hyoscyamine (**1**) following a feeding experiment with sodium [1-^{13}C]-acetate (**7f**) to *D. stramonium* root cultures.

4.3.1.4 Incorporation studies with sodium [2-^{13}C]-acetate (7g)

The ^{13}C-NMR spectrum of the isolated hyoscyamine following a feeding experiment with [2-^{13}C]-acetate (**7g**) at 9.3mmol dm^{-3}, revealed enrichments of all carbons of the tropane ring except C-3 (Table 11 and fig. 26).

Tropane ring carbons	Chemical shift (ppm)	Approximate incorporation at each carbon position
C-1 and C-5	60.96 or 60.83	10%
C-2 and C-4	34.09 or 34.27	30%
C-6 and C-7	23.86 or 24.45	10%

Table 11 Incorporation of sodium [2-^{13}C]-acetate (**7g**) into the tropane moiety of hyoscyamine (**1**).

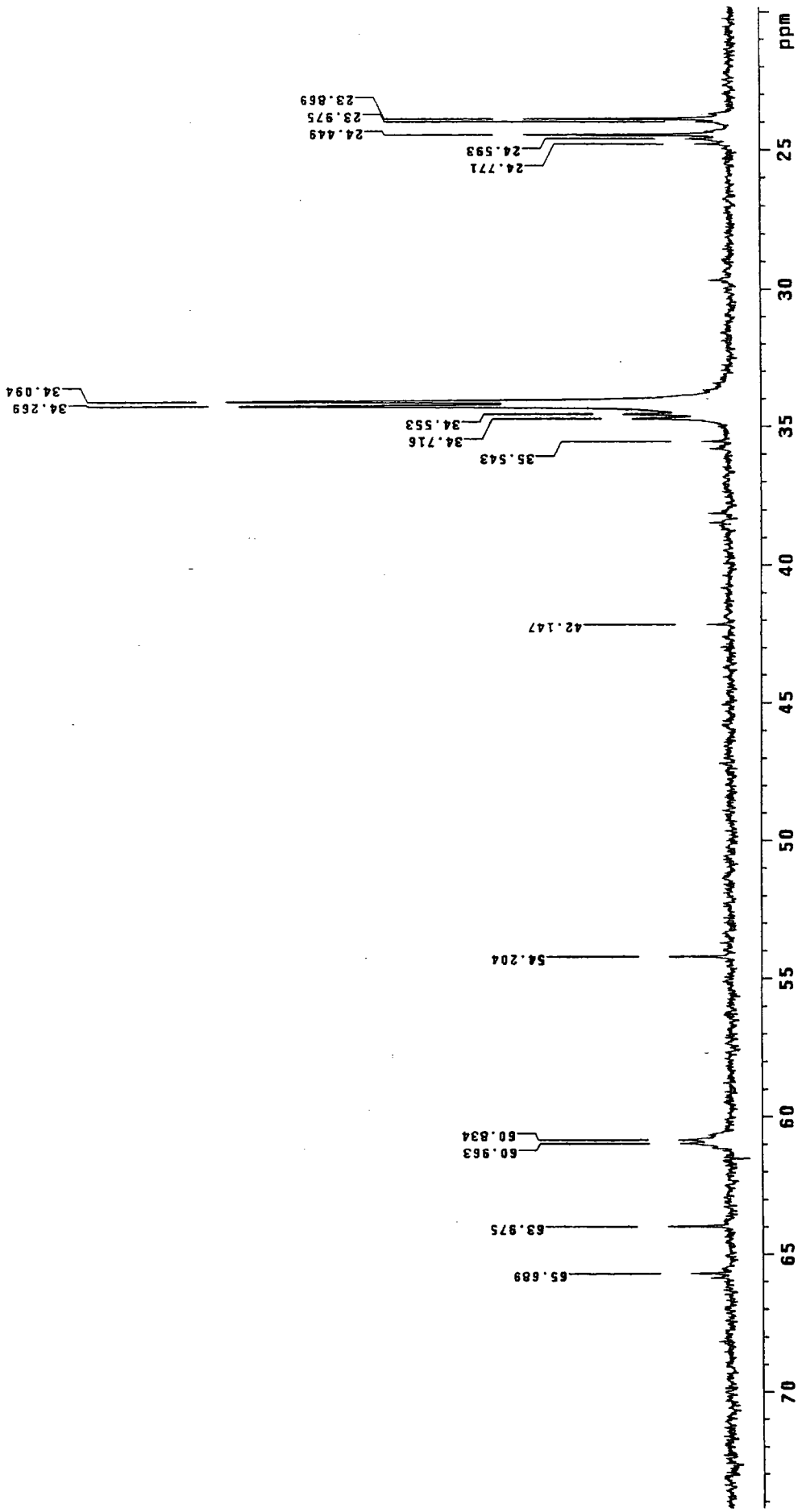


Figure 26 ^{13}C-NMR spectrum after feeding sodium [2-^{13}C]-acetate (**7g**) to transformed root cultures of *D. stramonium*.

Interestingly, closer examination of the C-6 and C-7 signals in the ^{13}C-NMR spectrum revealed the presence of carbon satellites (~4% enrichment) indicating that both carbons are coupling either to each other, or to an adjacent enriched carbon atom at C-1 and C-5 respectively. According to the Krebs cycle, both carbons (C-6 and C-7) and the adjacent C-1 are derived from separate acetate units (see Scheme 85). Thus, a small proportion (~4%) of tropine molecules derived from ornithine must have been biosynthesised from at least two exogenously added labelled acetate units.

This result had been observed previously in the feeding experiment with [1-^{13}C]-acetate (**7f**), but it is noteworthy that again C-5 is enriched at the same level as C-1. Following the Krebs cycle, C-5 should not be enriched if an unsymmetrical intermediate is generated (see Scheme 85). Furthermore, the resulting ^{13}C-NMR signals for C-1 and C-5 appear as a doublet of doublets, perhaps arising from the convergence of two triplets from each carbon each with an intensity ratio of 1:2:1 (fig. 27).

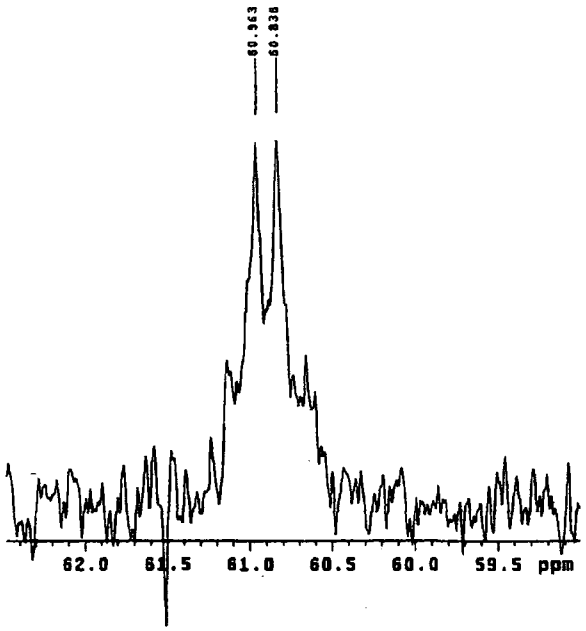


Figure 27 Expansion of ^{13}C-NMR spectrum showing a carbon signal (doublet of doublets) for both C-1 and C-5 after feeding [2-^{13}C]-acetate (**7g**) to *D. stramonium* root cultures.

The symmetrical appearance of the signal suggests that both C-1 and C-5 are similarly labelled with ^{13}C. The coupling constant (29.4 Hz) for both carbons reveal that C-1 and C-5 must be coupling to an adjacent enriched carbon atom. By considering the peak ratios from the observed signal, it can be estimated that approximately 50% of the

enriched carbon atoms at C-1 and C-5 are coupling to an adjacent carbon. Since no evidence of coupling or enrichment is observed at C-2 or C-4, C-1 and C-5 must be coupling to C-7 and C-6 respectively. An inspection of the enriched carbon signals for C-6 and C-7 support this analysis and reveal the presence of ^{13}C satellites with a ^{13}C-^{13}C coupling constant of 27.3 Hz (fig. 28).

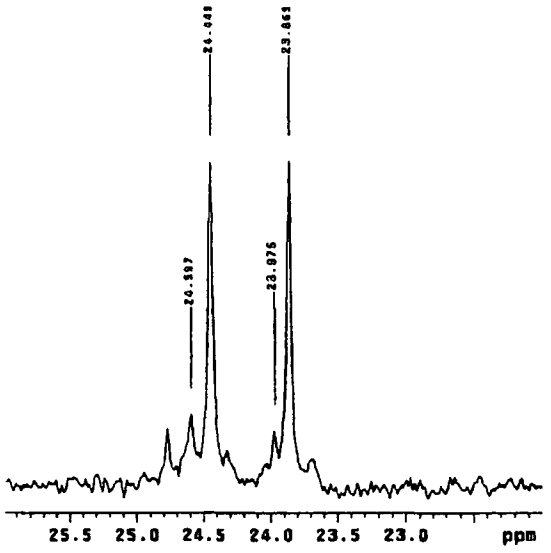
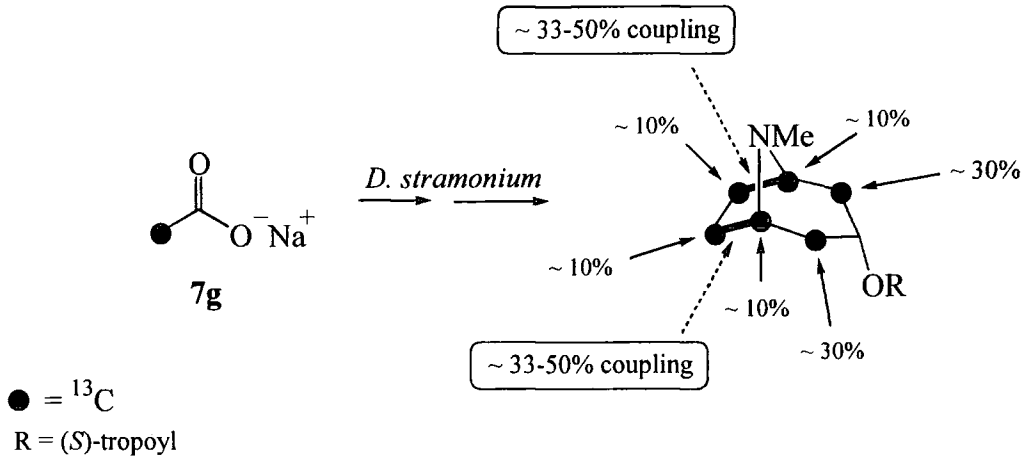


Figure 28 Expansion of ^{13}C-NMR spectrum showing carbon signals for both C-6 and C-7 after feeding sodium [2-^{13}C]-acetate (**7g**) to *D. stramonium* root cultures. Each carbon is enriched by approximately 10% above natural abundance, and show evidence of coupling (~33% of total enriched molecules) to an adjacent carbon on the tropane ring.

The estimated intensity ratio of 1:4:1 for these "triplets" confirms that approximately 33% of these enriched carbon atoms (C-6 and C-7) are coupling, presumably to C-5 and C-1 respectively. It is noteworthy too, that this value of 33% is close to 50%, the value obtained for the proportion of labelled atoms at C-1 and C-5 that are coupled to an adjacent carbon. Although the idealised pattern of incorporation for [2-^{13}C]-acetate [(**7g**), Scheme 85] would suggest that C-6 could potentially couple with C-7, it is not evident to any large extent in the resultant ^{13}C-NMR spectrum. If there was coupling between C-6 and C-7, the ^{13}C-^{13}C coupling satellites for C-6 and C-7 would show a greater percentage than 33% of the observed coupling.

Following the analysis of the ^{13}C-NMR spectrum after feeding sodium [2-^{13}C]-acetate (**7g**) to *D. stramonium* root cultures, a summary of the incorporation levels and positions into the tropane ring are shown in Scheme 87.



Scheme 87 Estimated incorporation levels above natural abundance into the tropane moiety of hyoscyamine following a feeding experiment with sodium [2-^{13}C]-acetate (**7g**) to *D. stramonium* root cultures.

4.1.3.5 Incorporation studies with sodium [1,2-$^{13}C_2$]-acetate (7b)

The ^{13}C-NMR spectrum of isolated hyoscyamine from the feeding experiment with sodium [1,2-$^{13}C_2$]-acetate (**7b**) revealed that all carbons of the tropane ring were enriched to various levels (see Table 12 and fig. 29). However, in general, the acetate derived carbons (C-2, C-3 and C-4) are enriched two fold higher (~20%) than the ornithine derived carbons [(C-1, C-5, C-6 and C-7), ~10%].

Tropane ring carbon	Chemical shift (ppm)	Approximate incorporation at each carbon position
C-3	65.45	20%
C-1 and C-5	61.28 or 61.15	10%
C-2 and C-4	34.21 or 34.03	20%
C-6 and C-7	24.36 or 23.75	10%

Table 12 Incorporation of sodium [1,2-$^{13}C_2$]-acetate (**7b**) into the tropane moiety of hyoscyamine (**1**).

This is clearly consistent with our understanding of the origin of the tropane ring and the feeding experiments with [1-^{13}C]- (**7f**) and [2-^{13}C]-acetate (**7g**). Interestingly the ^{13}C-NMR spectrum revealed that each carbon of the tropane ring was coupled to an adjacent isotopically enriched carbon atom. Once again, a proportion of tropine molecules must have been biosynthesised from ornithine (**6**), which has been generated from at least two exogenously added labelled acetate units.

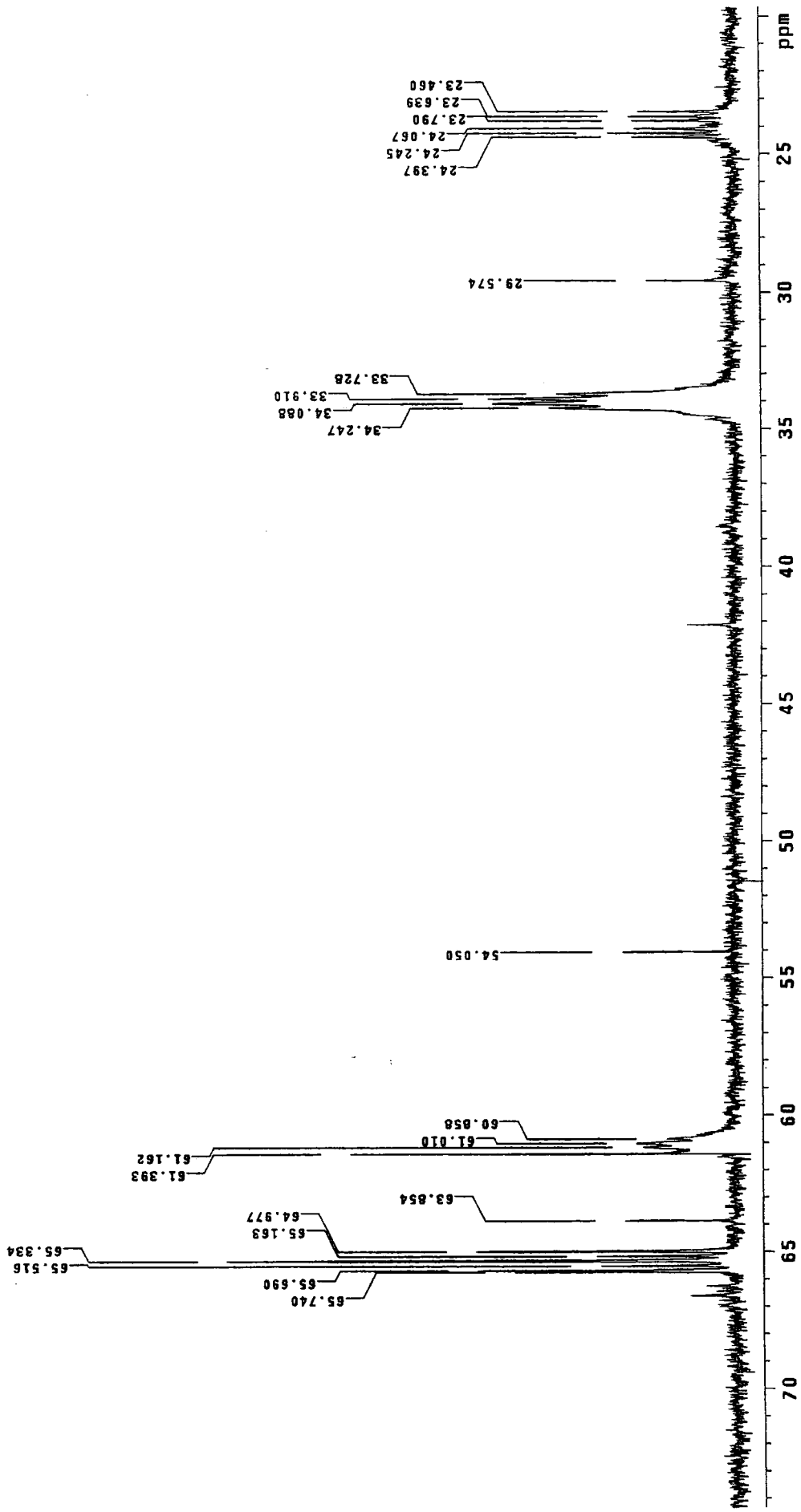


Figure 29 ^{13}C-NMR spectrum after feeding sodium [1,2-$^{13}C_2$]-acetate (**7b**) to transformed root cultures of *D. stramonium*.

By deconstructing the observed ^{13}C-^{13}C coupling patterns for each carbon atom, it can be established how [1,2-$^{13}C_2$]-acetate (**7b**) is incorporated into the tropane ring. The ^{13}C-NMR signal corresponding to C-3 emerges as a quintet (fig. 30).

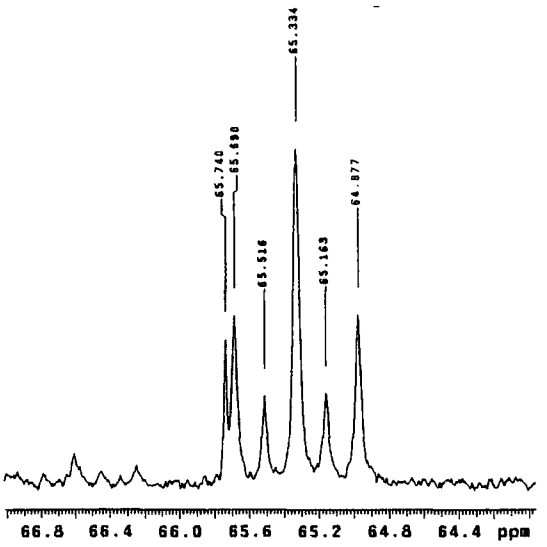
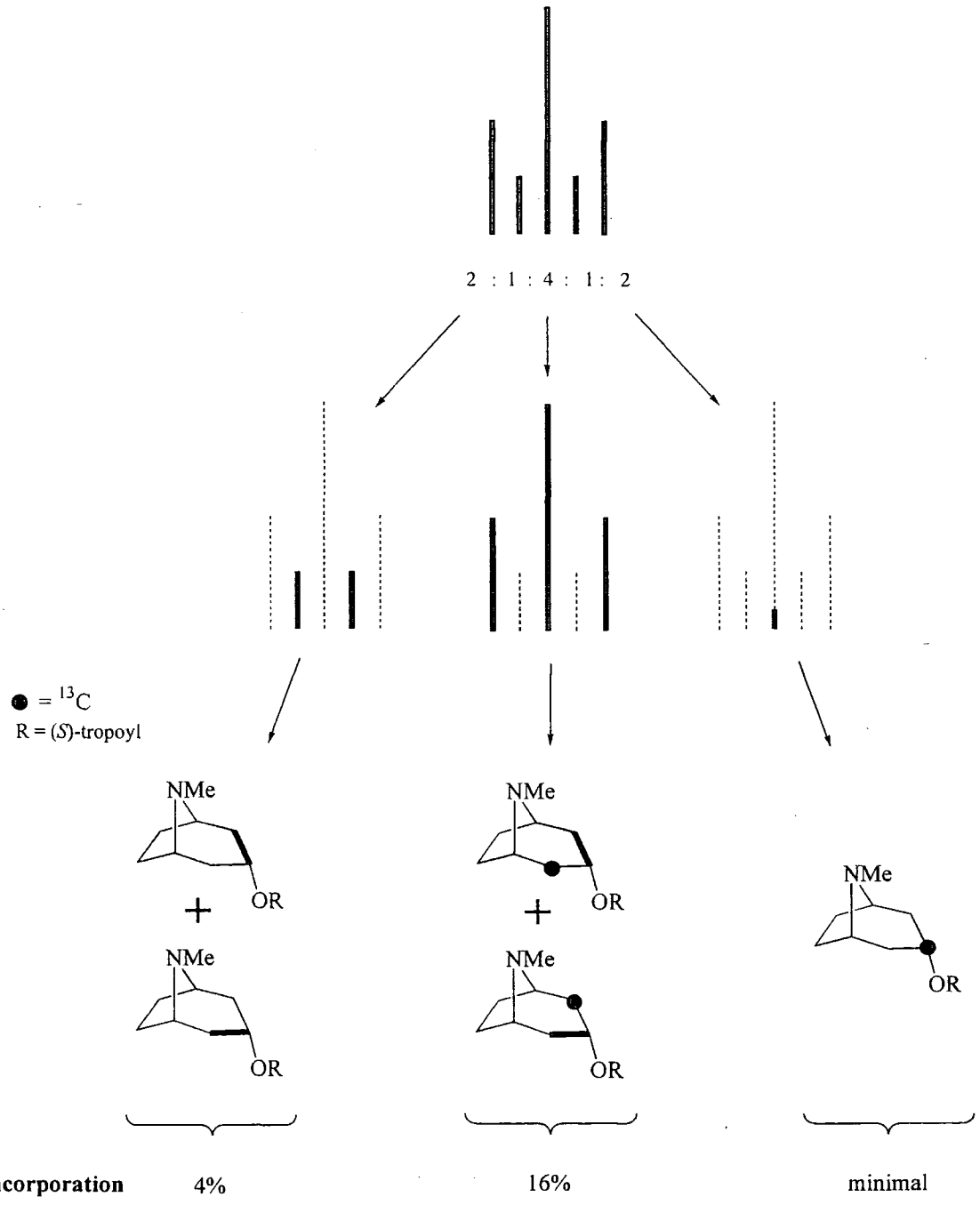


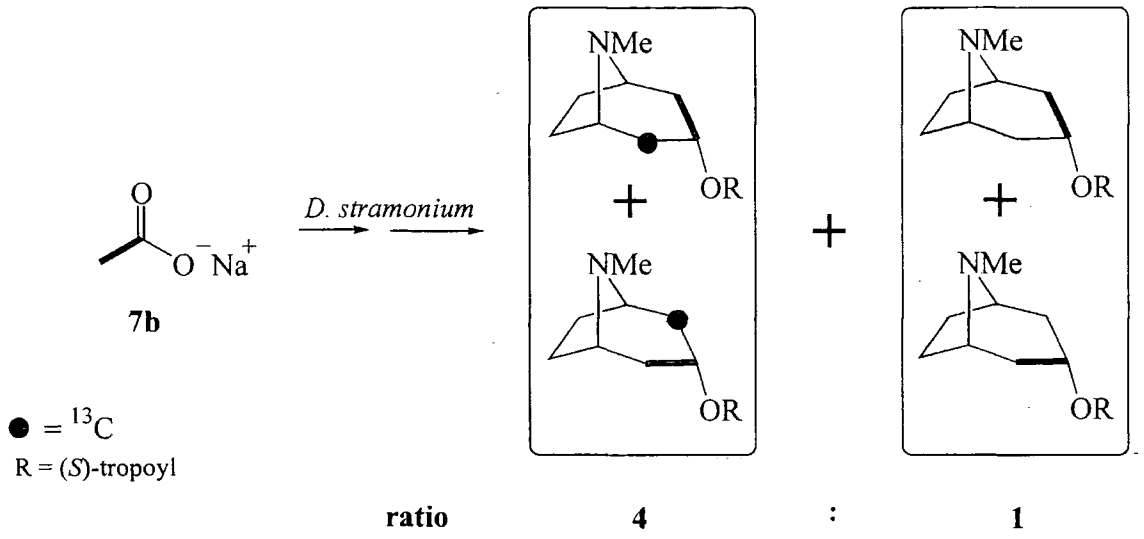
Figure 30 Expansion of ^{13}C-NMR spectrum showing a quintet-like carbon signal for C-3 of hyoscyamine (1) after feeding sodium [1,2-$^{13}C_2$]-acetate (7b) to *D. stramonium* root cultures. The signal at 65.74ppm is probably due to an enriched impurity or poor peak resolution.

Carbon-3 is enriched by approximately 20% above natural abundance, and shows evidence of coupling to both adjacent carbons (C-2 and C-4). The intact incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into C-3 and an adjacent carbon (C-2 or C-4) gives rise to a doublet with a C-C coupling constant of 35.5 Hz. Simultaneous labelling of the other adjacent carbon gives rise to another C-C coupling in the satellite signals. The values of the two coupling constants are almost identical due to the similarity of the environment of C-2 and C-4 and thus results in a quintet-like signal. Interestingly, this signal has an intensity ratio of 2:1:4:1:2, which equates to ~20% of the total enriched carbons at C-3 are coupling with C-2 or C-4, along with the other 80% also coupling simultaneously to the other adjacent carbon (Scheme 88). Any ^{13}C label incorporated solely at C-3 must contribute to the central enriched peak of the quintet signal and by assessment of the intensity ratio, this is minimal.



Scheme 88 A deconstruction of the observed quintet-like signal (with a ratio of 2:1:4:1:2) for C-3 of the tropane ring of hyoscyamine, following a feeding experiment with [1,2-$^{13}C_2$]-acetate (**7b**) to *D. stramonium*.

Thus, in summary, these signal patterns suggest that [1,2-$^{13}C_2$]-acetate (**7b**) is incorporated intact into C-2 and C-3, as well as C-3 and C-4. However, it is noteworthy that the triply ^{13}C-labelled tropane skeleton (C-2 – C-4) of hyoscyamine (**1**), exists predominantly over the doubly labelled skeleton (C-2 – C-3 and C-3 – C-4) in a ratio of 4:1 (Scheme 89).



Scheme 89 A summary for the incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into C-3 of the tropane ring of hyoscyamine.

The ^{13}C-NMR signals for C-2 and C-4 of hyoscyamine appear as a quartet, which arises from the sum of two triplet signals, each with a ratio of 2:1:2 (fig. 31).

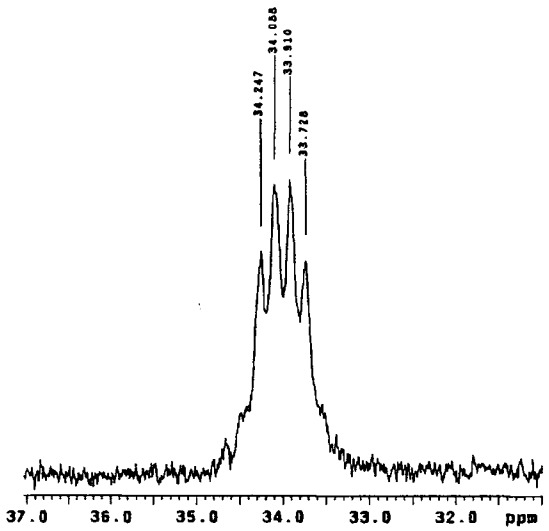
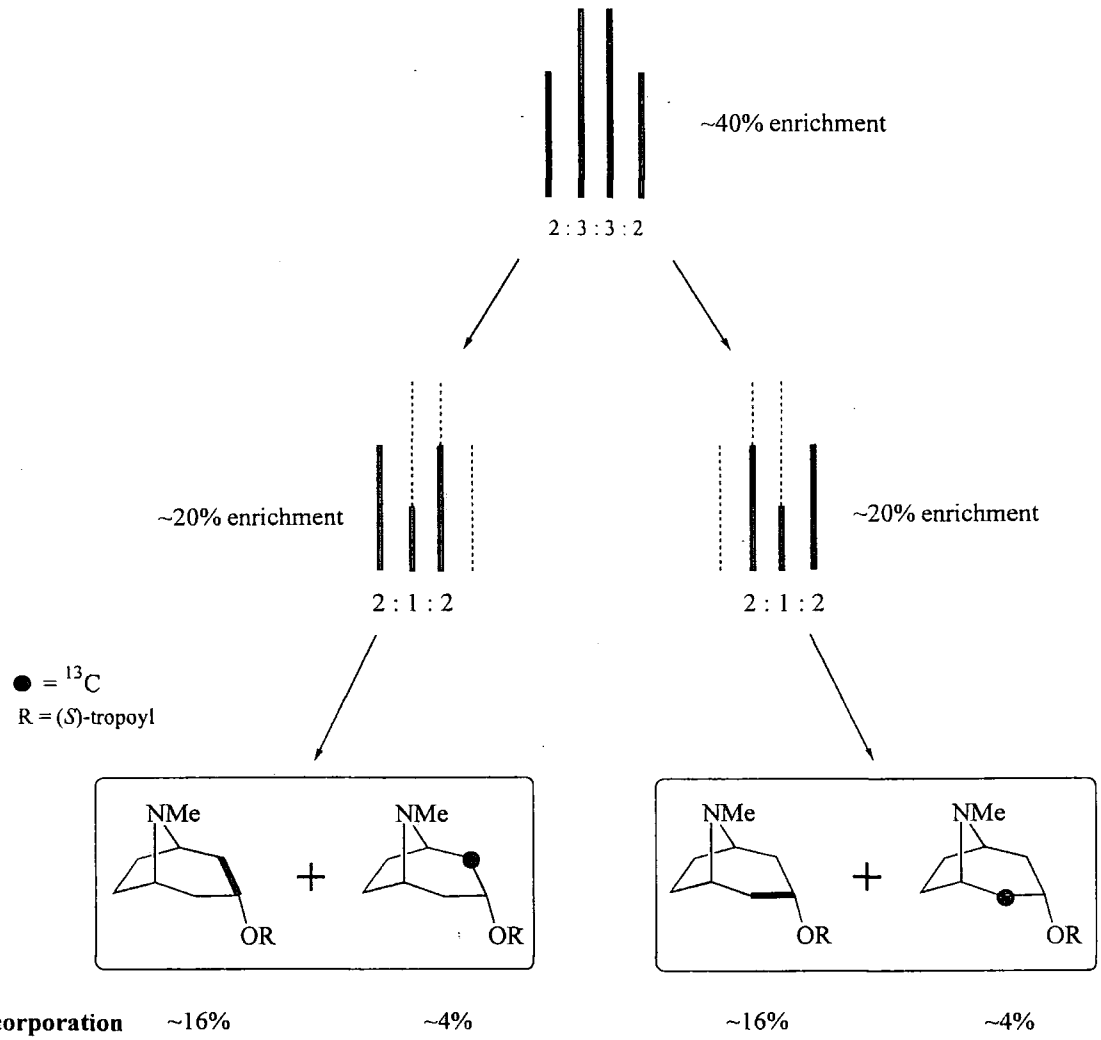


Figure 31 Expansion of ^{13}C-NMR spectrum showing a quartet carbon signal for C-2 and C-4 of hyoscyamine after feeding sodium [1,2-$^{13}C_2$]-acetate (**7b**) to *D. stramonium* root cultures.

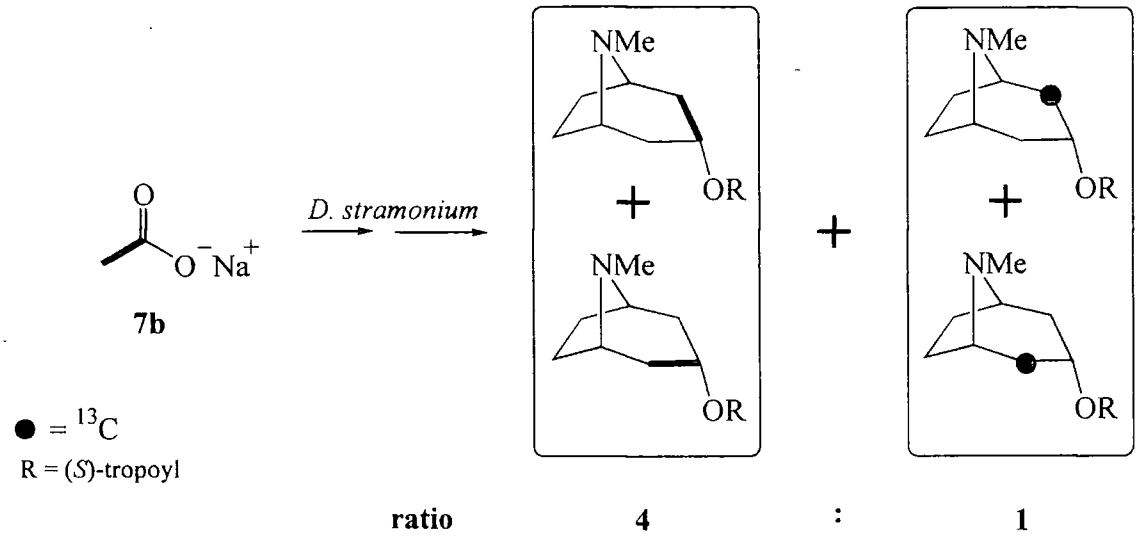
The high incorporation level (~20%) for each carbon (C-2 and C-4) and similar C-C coupling constants (33.9 Hz and 36.2 Hz) indicate that both are coupling to adjacent carbons. The estimated ratio of 2:1:2 for the satellite and single label, indicates that approximately 80% of the ^{13}C label at C-2 or C-4 is coupling presumably to C-3, whereas the remaining 20% of the total ^{13}C-enrichment is located solely at C-2 or C-4 (Scheme 90).



Scheme 90 A deconstruction of the observed quartet signal (with a ratio of 2:3:3:2) for C-2 and C-4 of the tropane ring of hyoscyamine, following a feeding experiment with [1,2-$^{13}C_2$]-acetate (**7b**) to *D. stramonium*.

Thus, in summary, the ^{13}C-NMR quartet for C-2 and C-4 suggests that [1,2-$^{13}C_2$]-acetate (**7b**) is incorporated intact into C-2 and C-3, as well as C-3 and C-4 in a symmetrical manner. Also, it is noteworthy that this intact incorporation exists

predominantly over the singly labelled carbons of the tropane skeleton (C-2 or C-4) in a ratio of 4:1 (Scheme 91).



Scheme 91 A summary for the incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into C-2 and C-4 of the tropane ring of hyoscyamine.

Although these results agree with Sankawa's findings for the incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into hyoscyamine (**1**),[157] it is noteworthy that the incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into the ornithine derived moiety of the tropane skeleton (C-1, C-5, C-6 and C-7) was not discussed, presumably because the author's focus was on the biosynthesis of the acetate derived moiety (C-2, C-3 and C-4) of the tropane ring.

Again, incorporation of ^{13}C from [1,2-$^{13}C_2$]-acetate (**7b**) is observed also at C-1 and C-5 at a lower level (~10% for each carbon) than for the acetate derived moiety (C-2 – C-4) of the tropane ring (~20% for each carbon). The ^{13}C-NMR spectrum reveals a quartet-like signal for C-1 and C-5, which arises from the convergence of two triplets with similar C-C coupling constants (38.5 Hz and 30.6 Hz) and in a ratio of 1:1:1 (fig. 32).

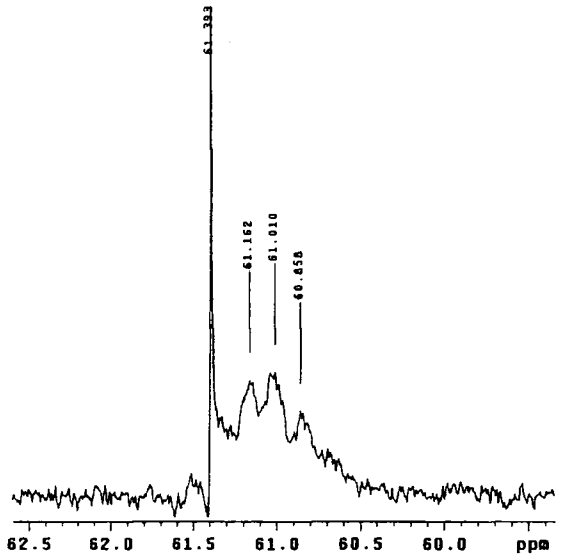
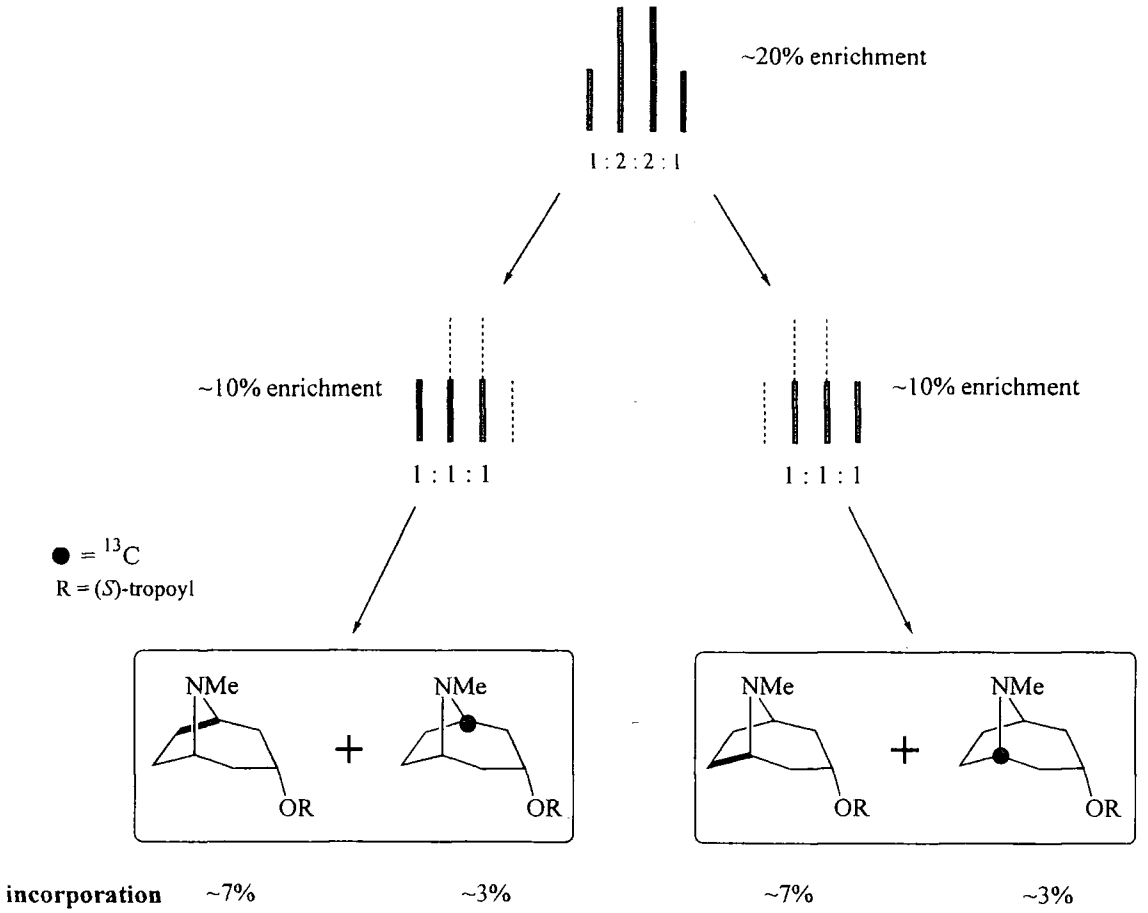


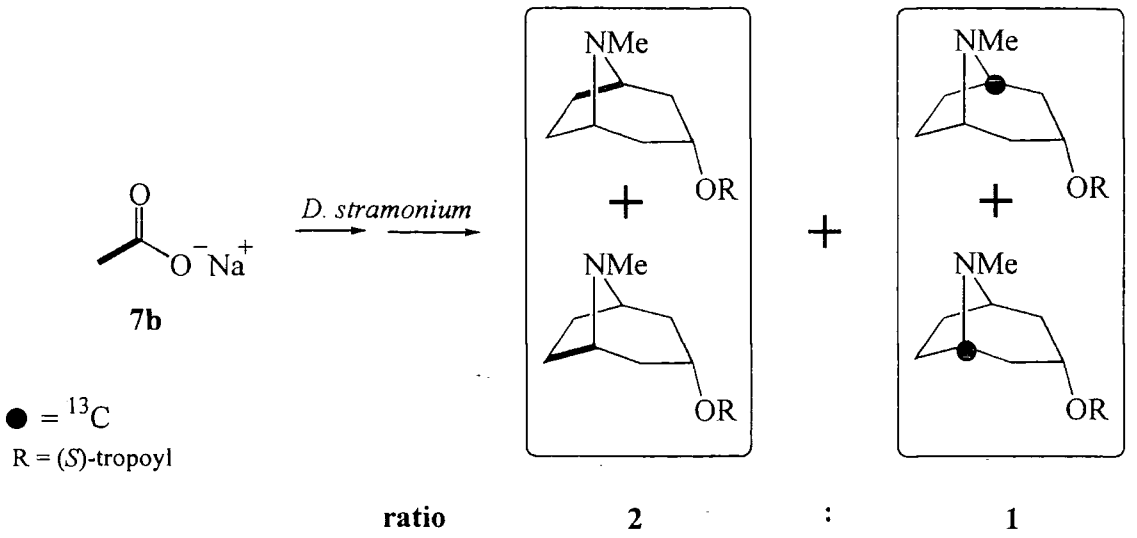
Figure 32 Expansion of ^{13}C-NMR spectrum showing a quartet-like carbon signal for C-1 and C-5 of hyoscyamine (**1**) after feeding sodium [1,2-$^{13}C_2$]-acetate (**7b**) to *D. stramonium* root cultures. The signal at 61.39ppm is probably due to an enriched impurity in the sample.

The triplet signal for each carbon indicates that ~33% of the ^{13}C label has labelled C-1 and C-5 and that the majority (~66%) of these enriched bridgehead carbons are coupling to adjacent carbons on the tropane ring (Scheme 92). Presumably, these bridgehead carbons (C-1 and C-5) are more likely to couple to C-6 and C-7 since they are all derived from the ornithine moiety of the tropane ring.



Scheme 92 A deconstruction of the observed quartet signal (with a ratio of 1:2:2:1) for C-1 and C-5 of the tropane ring of hyoscyamine, following a feeding experiment with [1,2-$^{13}C_2$]-acetate (**7b**) to *D. stramonium*.

In summary, the ^{13}C-NMR quartet signal for the bridgehead carbons C-1 and C-5 of the tropane ring suggest that a symmetrical intact incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into C-1 and C-7, as well as C-5 and C-6 had occurred. Also, it is noteworthy that this intact incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) exists predominantly over the singly labelled bridgehead carbons (C-1 or C-5) in a ratio of 2:1 (Scheme 93).



Scheme 93 A summary for the incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into C-1 and C-5 of the tropane ring of hyoscyamine.

Consistent with this analysis, the signals for C-6 and C-7 in the ^{13}C-NMR spectrum show that these positions are enriched (~10% for each carbon), see fig. 33.

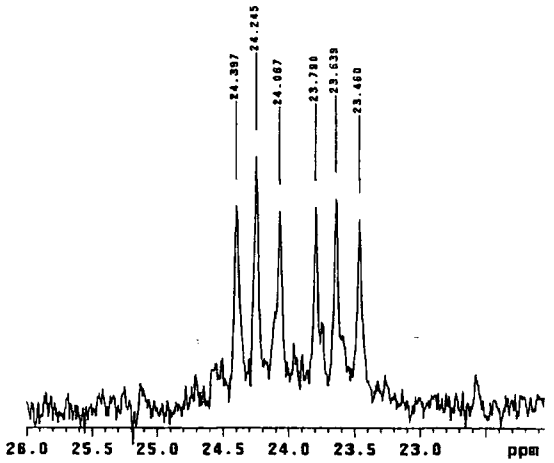
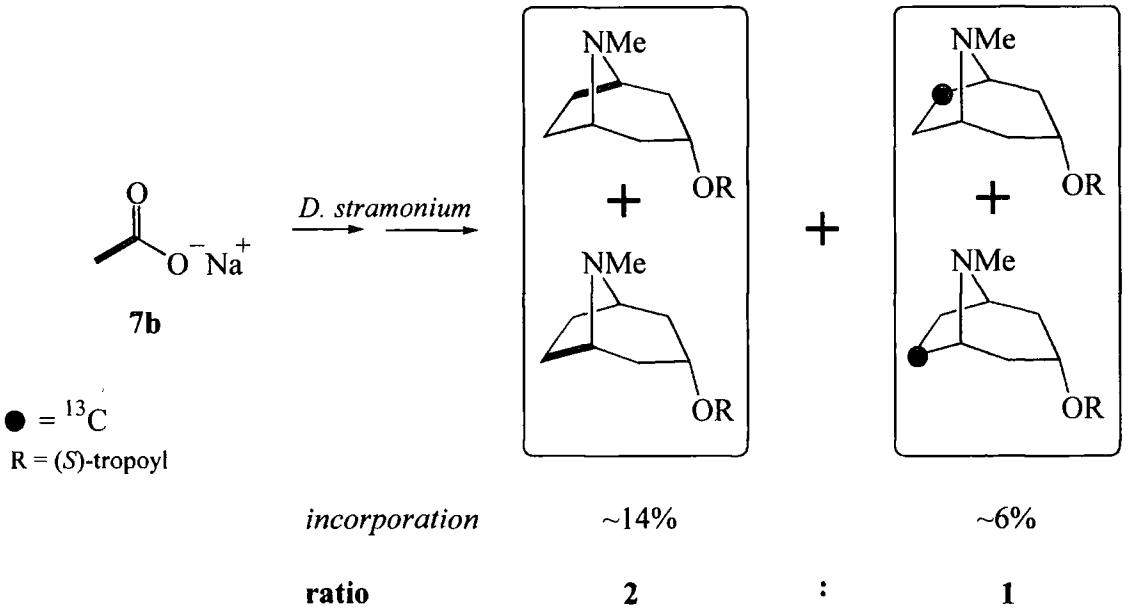


Figure 33 Expansion of ^{13}C-NMR spectrum showing triplet carbon signals for C-6 and C-7 of hyoscyamine (**1**) after feeding sodium [1,2-$^{13}C_2$]-acetate (**7b**) to *D. stramonium* root cultures.

Both ^{13}C-NMR signals for C-6 and C-7 appear as well resolved triplets with an intensity ratio of 1:1:1. The coupling constant for both C-6 and C-7 of 33.2 Hz is consistent with a C-C coupling interaction with another adjacent enriched carbon. By considering the signal intensities of both carbons, it can be deduced that 66% of the labelled atoms are

coupled presumably to C-1 and C-5, which are also ornithine derived. Thus, it can be calculated that ~6% of the ^{13}C enrichment from [1,2-$^{13}C_2$]-acetate (**7b**) has labelled solely C-6 or C-7 and that ~14% is incorporated intact into C-6 and C-5, as well as C-7 and C-1 (Scheme 94). Also, it is noteworthy that this analysis agrees with the earlier findings for the bridgehead carbons (C-1 and C-5), since both incorporation values and ratios for double and single labels are identical.

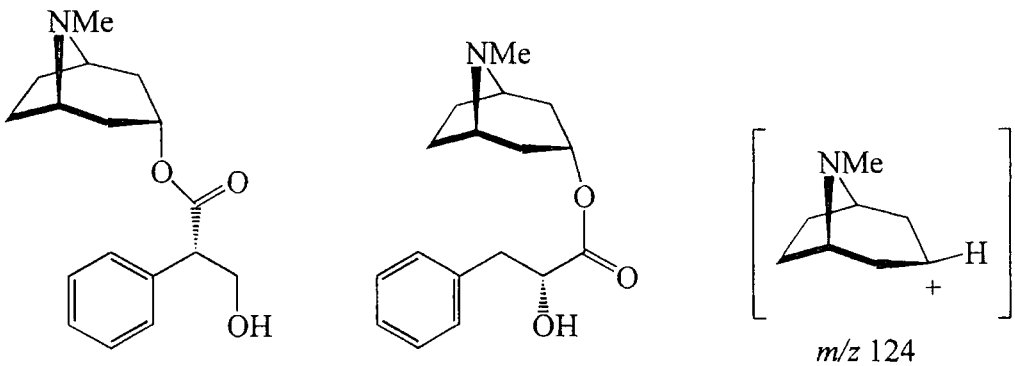


Scheme 94 A summary for the incorporation of [1,2-$^{13}C_2$]-acetate (**7b**) into C-6 and C-7 of the tropane ring of hyoscyamine.

4.1.3.6 Incorporation studies with sodium [2-^{13}C, 2-$^{2}H_3$]-acetate (7e)

Sodium [2-^{13}C,2-$^{2}H_3$]-acetate (**7e**) was pulse fed to transformed root cultures of *D. stramonium* on days 5, 7 and 9 to a final concentration of 18.0mmol dm^{-3} in the medium. The alkaloids were extracted after 17 days of growth. Analysis of the alkaloid extract by ^{13}C-NMR after purification revealed that all carbons of the tropane ring were enriched except for C-3. Closer inspection of the signals corresponding to C-2 and C-4 in the ^{13}C-NMR spectrum did not reveal any upfield shifted signals associated with deuterium coupling. In order to increase the sensitivity of the analysis, deuterium and proton decoupling was applied to the ^{13}C-NMR spectrum. However, the resulting $^{13}C\{^{1}H,^{2}H\}$-NMR spectrum was no different to the $^{13}C\{^{1}H\}$-NMR spectrum and did not indicate any deuterium coupling at these sites. The NMR data do not support the

presence of deuterium at C-2 or C-4 of the tropane skeleton. However, GC-MS analysis of the resultant hyoscyamine (**1**) did reveal that the tropane ring was significantly enriched with deuterium (Table 13), which is probably located at C-6 and C-7. Both littorine (**5**) and hyoscyamine (**1**) were enriched from M+1 to M+6, although the majority of the ions detected were M+1 [~24% for littorine (**5**) and ~13% for hyoscyamine (**1**)] and M+2 [~26% for littorine (**5**) and ~13% for hyoscyamine (**1**)]. It is interesting to note that the ion distribution of the tropyl fragment [(**104**) (*m/z* 124)] for littorine (**5**) and hyoscyamine (**1**) respectively, almost identically matches the ion distribution of the parent alkaloids (see fig. 34 and Table 13). Thus, the incorporation of sodium [2-^{13}C,2-^{2}H$_3$]-acetate (**7e**) must have occurred solely into the tropane skeleton of littorine (**5**) and hyoscyamine (**1**).



m/z 289 **1**

m/z 289 **5**

m/z 124 **104**

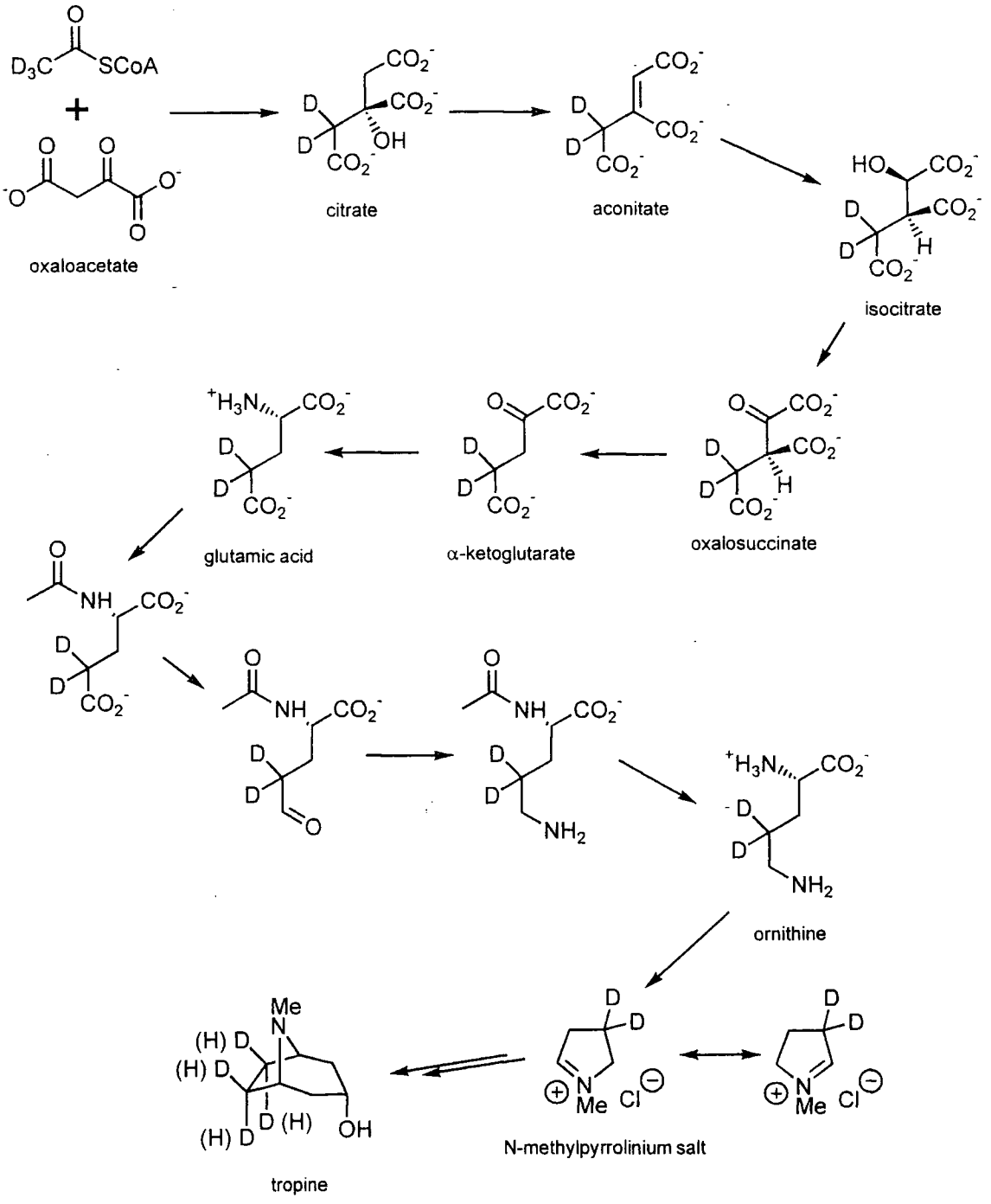
Figure 34 The tropyl fragment [(**104**), *m/z* 124] of both hyoscyamine [(**1**), *m/z* 289] and littorine [(**5**), *m/z* 289] was found to be exclusively enriched following a feeding experiment with sodium [2-^{13}C,2-^{2}H$_3$]-acetate (**7e**) to *D. stramonium*.

	m/z	Littorine (5)	Hyoscyamine (1)
M	*289*	31.93	64.20
M+1	*290*	23.76	12.78
M+2	*291*	25.71	13.28
M+3	*292*	8.67	4.38
M+4	*293*	4.68	2.33
M+5	*294*	3.72	2.12
M+6	*295*	1.27	0.80
		Tropyl fragment (**104**)	
		Littorine (5)	**Hyoscyamine (1)**
M	*124*	37.59	66.81
M+1	*125*	21.72	9.66
M+2	*126*	23.23	13.74
M+3	*127*	7.07	3.65
M+4	*128*	4.45	2.53
M+5	*129*	4.20	2.43
M+6	*130*	1.20	0.65

Table 13 Incorporation (% values) into littorine [(**5**), *m/z* 289 (M)], hyoscyamine [(**1**), *m/z* 289 (M)] and their respective tropyl fragments [(**104**), *m/z* 124 (M)] after a feeding experiment with sodium [2-^{13}C,2-$^{2}H_3$]-acetate (**7e**) to transformed *D. stramonium* root cultures. Values in the table are calculated after considering natural abundance incorporations.

Although the presence of deuterium in the tropane skeleton has been established by GC-MS data, its location on the tropane ring remains inconclusive. More recent work in our laboratories (Dr. Rosa Duran-Patron, 1999)[159] has demonstrated that sodium [2-$^{2}H_3$]-acetate (**7h**) fed at the lower final concentration of 6 mmol dm^{-3} in the medium is also incorporated into hyoscyamine. GC-MS data have revealed in that case, the presence of only M+1 (3.8%) and M+2 (4.2%) ions, which correspond to the incorporation of deuterium into the tropane ring and further ^{2}H-NMR analysis has revealed that

deuterium was located only at C-6 and/or C-7. Again, there was no evidence to support incorporation of deuterium into C-2 and C-4. Also, the relatively high percentage of M+1 ion (3.8%), must be attributable to some deuterium washout during the biosynthesis of the tropane ring. These findings can be rationalised by considering how deuterium from [2-$^2H_3$]-acetyl-CoA (**7i**) is incorporated into the tropane ring *via* the Krebs cycle (Scheme 95).



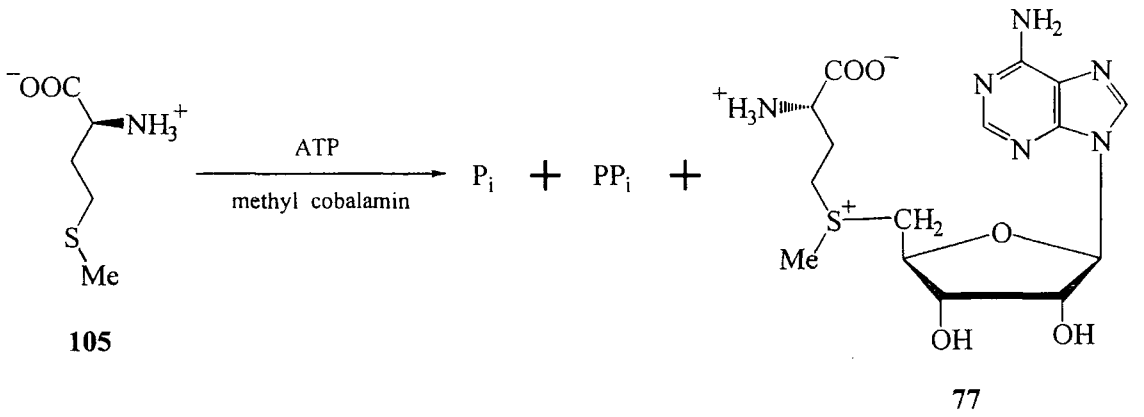
Scheme 95 The incorporation of deuterium from [2-$^2H_3$]-acetyl-CoA (7i) into C-6 and C-7 (*via* tautomerisation of the N-methylpyrrolinium salt) of tropine *via* the Krebs cycle.

It is noteworthy that by following the Krebs cycle, C-6 is the only site that should be labelled with deuterium. However, our [^{13}C]-acetate labelling experiments have now shown that C-6 and C-7 become equivalent during the biosynthesis suggesting again tautomerisation of the N-methylpyrrolinium salt (**11**).

4.3.2 Biosynthetic studies with L-methionine

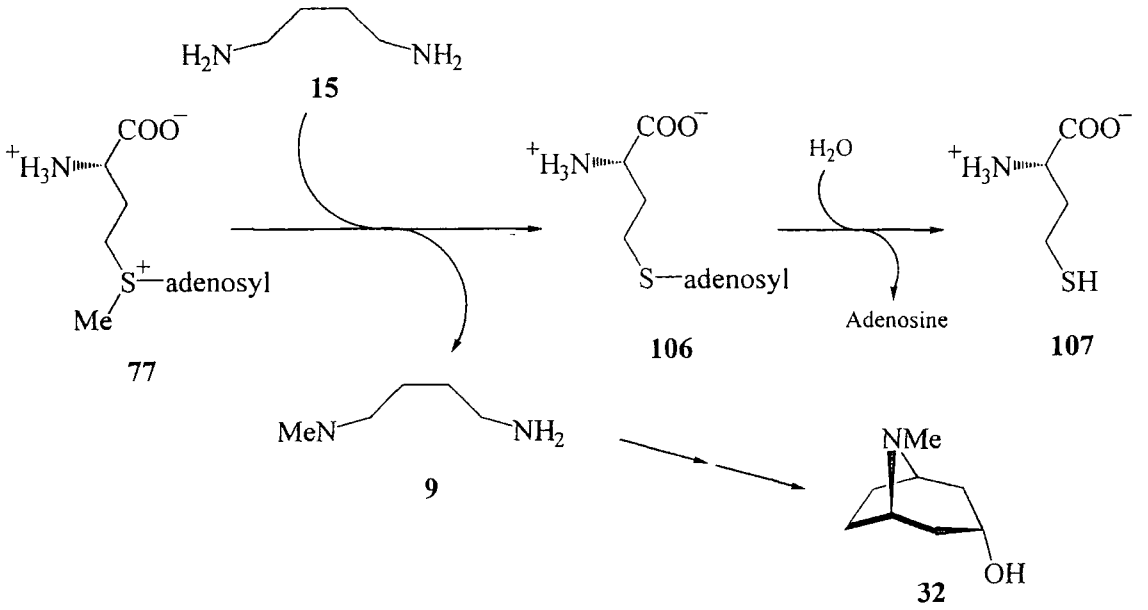
4.3.2.1 S-Adenosylmethionine (SAM)

The origin of the N-methyl group of tropine is derived by an S-adenosylmethionine dependent N-methyl transferase.[155] S-Adenosylmethionine (77) is synthesised by the transfer of an adenosyl group from ATP to the sulphur atom of L-methionine (**105**) in a methyl cobalamin mediated process (Scheme 96). The methyl group is now activated to nucleophilic attack and can be transferred to an acceptor molecule.[160]



Scheme 96 The generation of S-adenosylmethionine (77) from L-methionine (**105**) expends ATP in a methyl cobalamin mediated process.

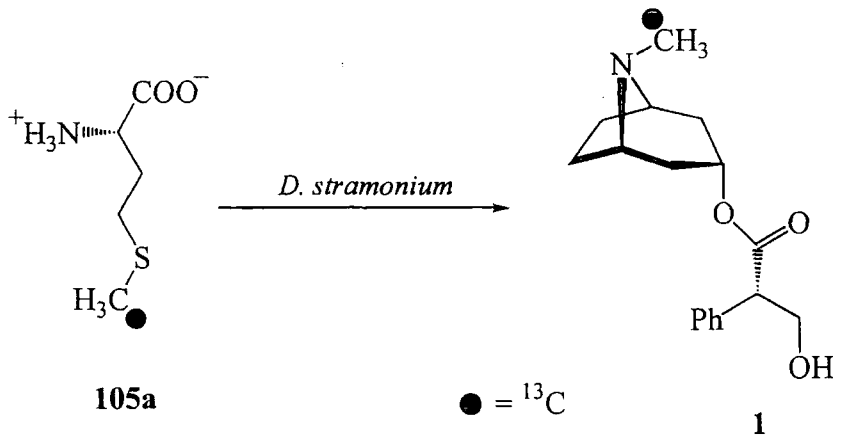
In the case of tropine (**32**) biosynthesis, S-adenosylhomocysteine (**106**) is formed when the methyl group is transferred to putrescine (**15**) to generate N-methylputrescine (**9**), an intermediate in tropine (**32**) biosynthesis (Scheme 97). Adenosine is regenerated after hydrolysis of S-adenosylhomocysteine (**106**) to homocysteine (**107**) thereby facilitating the biosynthesis of further ATP molecules.



Scheme 97 The N-methyl group of tropine (**32**) is derived from a SAM (**77**) mediated methylation of putrescine (**15**) to N-methylputrescine (**9**).

4.3.2.2 Preliminary feeding experiment with L-[^{13}C-*methyl*]-methionine (105a)

A preliminary feeding experiment with L-[^{13}C-*methyl*]-methionine (**105a**) was carried out with *D. stramonium* root cultures prior to feeding L-[^{13}C, $^{2}H_3$-*methyl*]-methionine (**105b**). ^{13}C-NMR analysis revealed that the N-methyl group of the isolated hyoscyamine (**1**) had become highly enriched (~30%) with ^{13}C (Scheme 98).



Scheme 98 A feeding experiment with L-[^{13}C-*methyl*]-methionine (**105a**) to *D. stramonium* resulted in the enrichment of the N-methyl group of the tropane ring of hyoscyamine (**1**).

4.3.2.3 Incorporation studies with L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (105b)

The ^{13}C-NMR spectrum of the isolated hyoscyamine (**1**) after a feeding experiment with L-[^{13}C,$^{2}H_3$-*methyl*]-methionine [(**105b**), ^{13}C, 99%; $^{2}H_3$, 99%] revealed the presence of an enriched signal upfield from the natural abundance signal for the –NMe group at 38.52ppm (fig. 35). This result clearly demonstrated that the [^{13}C,$^{2}H_3$]-methyl group from L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (**105b**) has become incorporated *via* S-adenosyl-L-[^{13}C,$^{2}H_3$-*methyl*]-methionine into the tropane skeleton. On application of deuterium and proton decoupling, the resultant $^{13}C\{^{1}H,^{2}H\}$-NMR spectrum collapsed the original multiplet and revealed two enriched peaks at 38.0ppm and 37.7ppm (fig. 36, c). The former peak is only enriched by approximately 3%, whilst the latter is enriched by ~20%. This difference of ~0.3ppm between the two peaks indicates that a small proportion [~10%, (calculated from 3% / 20%+3%)] of the labelled methyl group had lost one deuterium atom. The difference spectrum of $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ further illustrated this by revealing a perturbation in the spectrum at 38.0ppm (fig. 36, a). Thus, the [^{13}C,$^{2}H_3$]-methyl group from L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (**105b**) has become incorporated into the tropane ring with approximately 10% washout of one deuterium atom. The rationale for the observed washout of deuterium is discussed in the conclusions. Of course, it must be considered that the observed peak at 38.0ppm following deuterium and proton decoupling could have arisen from an enriched ^{13}C and ^{2}H minor impurity in the sample. However, if this was the case, then it would seem highly coincidental that this observed peak at 38.0ppm is ~0.6ppm upfield from the natural abundance signal at 38.5ppm, which corresponds to a $-NCD_2H$ group.

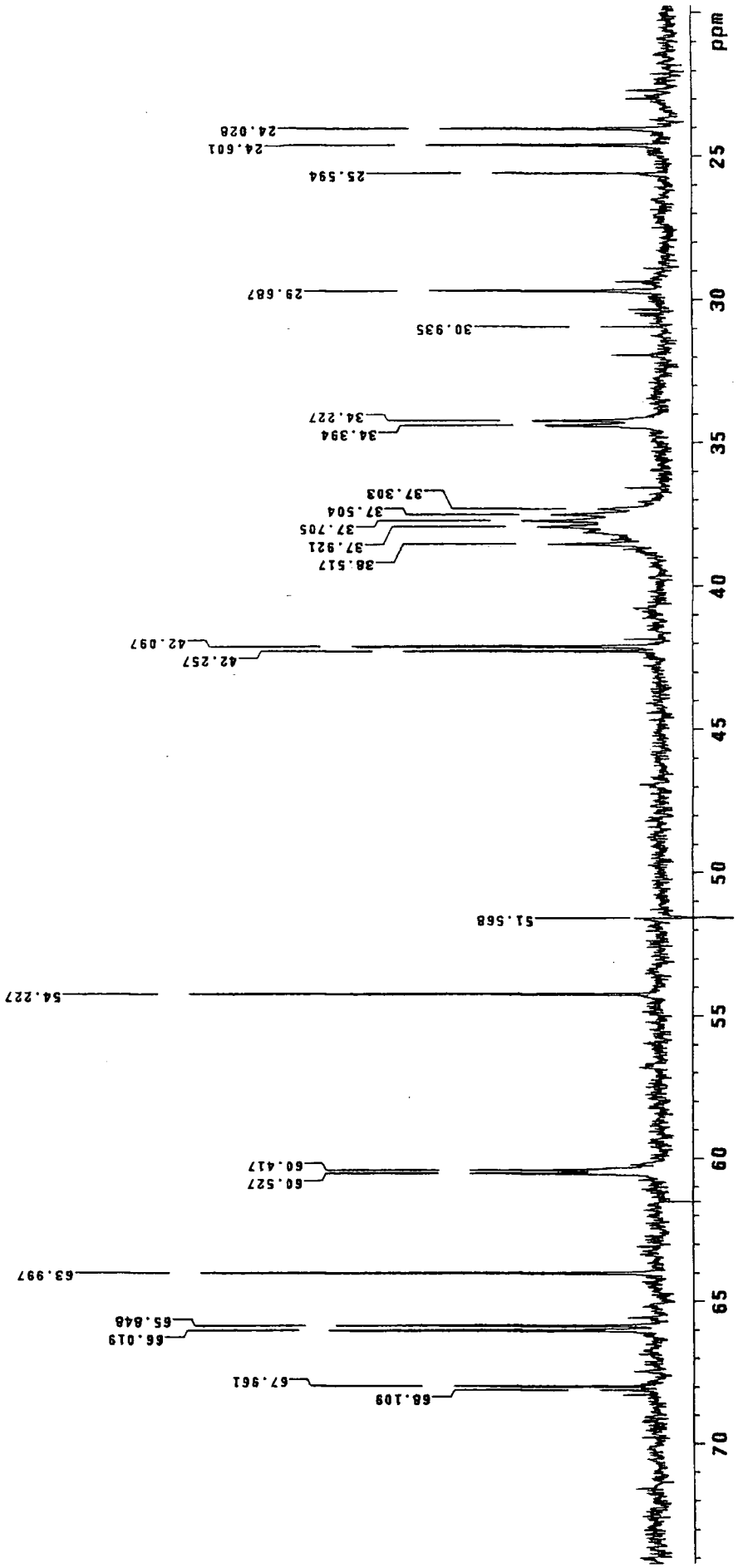


Figure 35 ^{13}C-NMR spectrum after feeding L-[^{13}C,^{2}H$_3$-*methyl*]-methionine (**105b**) to transformed root cultures of *D. stramonium*.

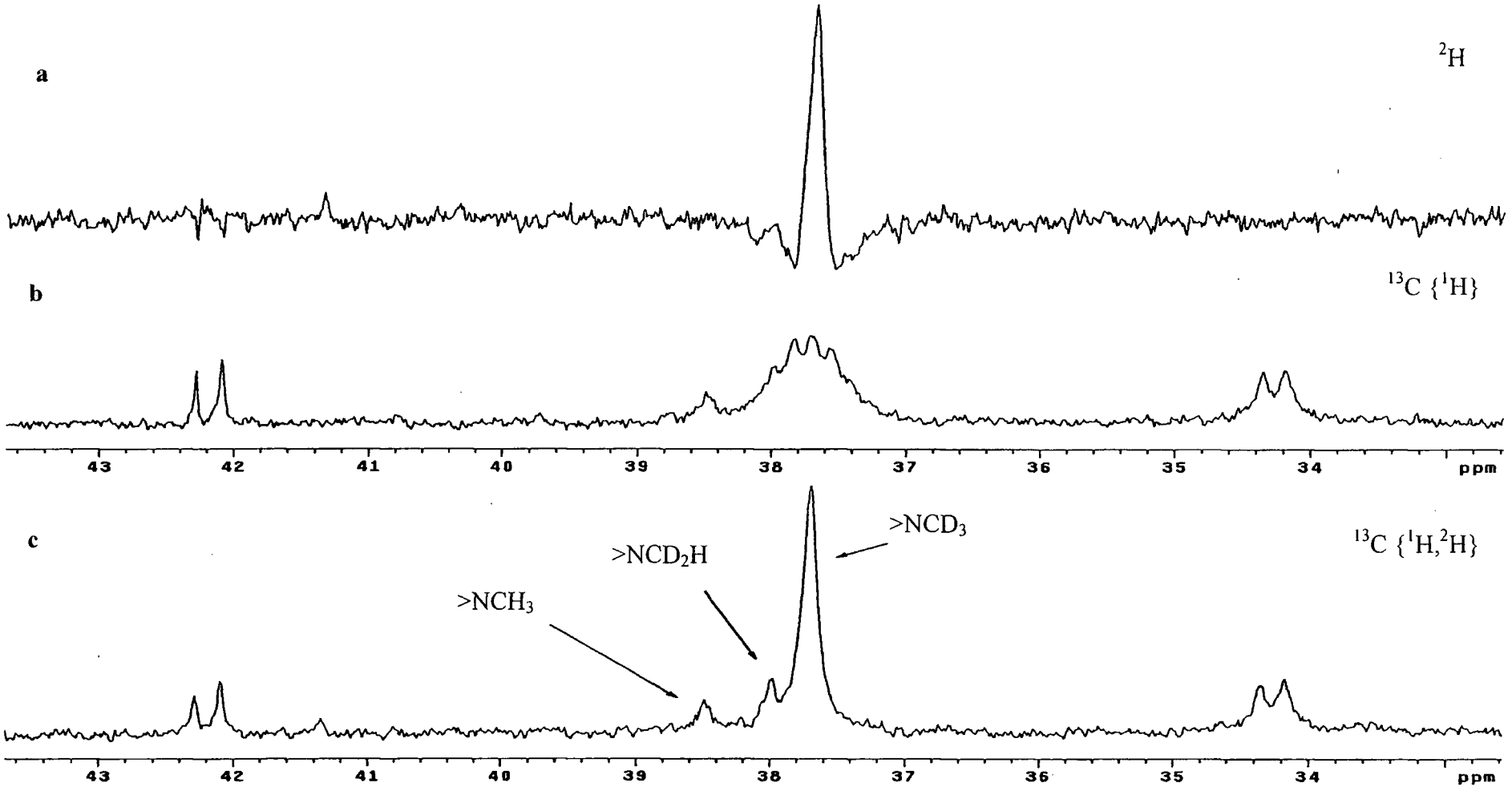
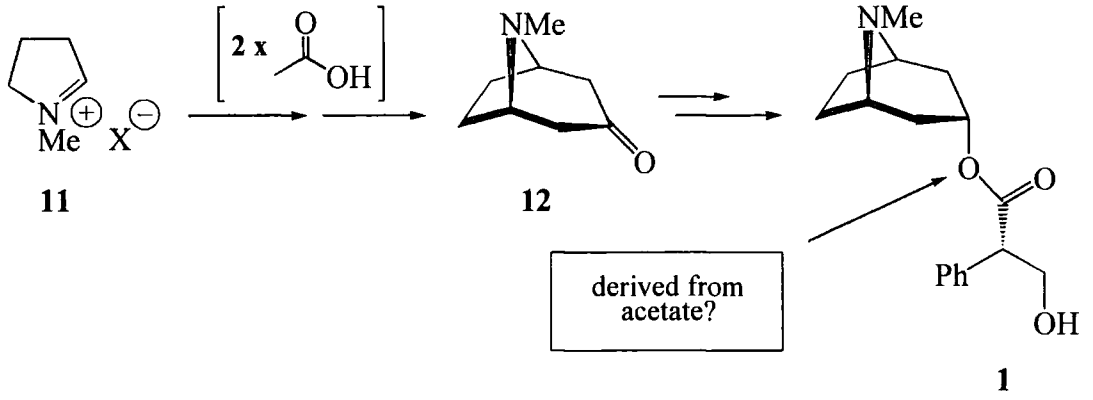


Figure 36 (a) Difference spectrum after recording (b) $^{13}C\{^{1}H\}$- and (c) $^{13}C\{^{1}H,^{2}H\}$-NMR spectra, following a feeding experiment with L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (**105b**) to transformed root cultures of *D. stramonium* (University of Edinburgh).

4.3.3 Biosynthetic ^{18}O labelling study on the tropane ring

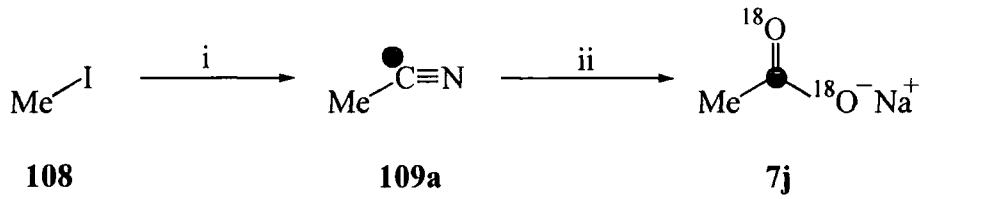
To date, no ^{18}O labelling studies have been reported to investigate the origin of the oxygen atom of the tropane ring and it seemed appropriate to investigate the retention of ^{18}O from acetate. Accordingly, sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**) was prepared and fed to transformed root cultures of *D. stramonium* to establish whether the tropic acid ester oxygen of hyoscyamine (**1**) is retained from acetate (Scheme 99).



Scheme 99 Investigating whether the oxygen atom of the tropane ring of hyoscyamine (**1**) is derived from acetate.

4.3.3.1 Synthesis of sodium [1-^{13}C,$^{18}O_2$]-acetate [97% ^{13}C, 49% $^{18}O_2$, (7j)]

The synthetic scheme established by Cane and co-workers[161] was followed for the preparation of sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**), starting from methyl iodide (**108**) as outlined in Scheme 100.



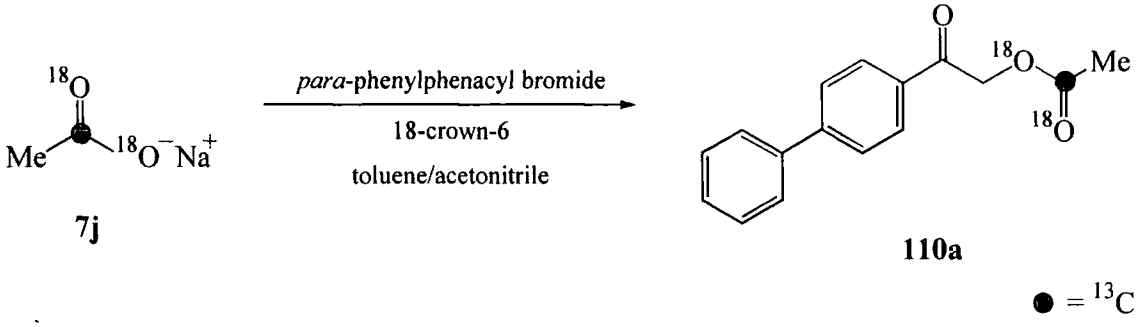
i. $K^{13}CN$; ii. $H_2{}^{18}O$, ^tBuOK

Scheme 100 Synthetic scheme for sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**) from methyl iodide (**108**).

[1-^{13}C]-Acetonitrile (**109a**) was generated by the nucleophilic substitution of methyl iodide (**108**) with potassium [^{13}C]-cyanide in methanol. The resultant [1-^{13}C]-acetonitrile (**109a**) was distilled from the mixture and hydrolysed under basic conditions with $H_2{}^{18}O$ to give the potassium salt of [1-^{13}C,$^{18}O_2$]-acetate. Purification of the product was achieved by converting the salt to the free carboxylic acid by acidification and subsequent lyophilisation. Sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**) was then generated in 84% yield after conversion of the free acid to its sodium salt with sodium hydroxide solution, followed by freeze-drying.

4.3.3.2 Analysis of the isotope composition of sodium [1-^{13}C,$^{18}O_2$]-acetate (7j)

In order to determine the isotopic composition of the synthesised sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**), it was necessary to prepare a derivative of the acetate for MS analysis. *Para*-phenylphenacyl was chosen as it was previously described by Cane and co-workers in their study.[161] The derivatisation route is outlined in Scheme 101.



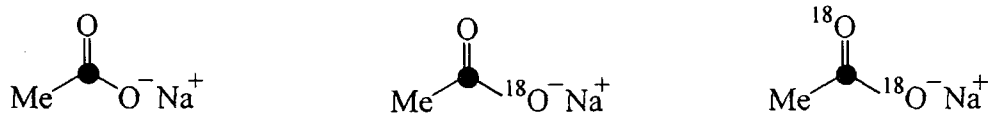
Scheme 101 Synthetic scheme for the *para*-phenylphenacyl derivative (**110a**) of sodium [1-^{13}C,$^{18}O_2$]-acetate (7j).

Accordingly, sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**) was reacted with *para*-phenylphenacyl bromide in the presence of 18-crown-6 in a toluene/acetonitrile solvent mixture. After the reaction was complete, purification was carried out on silica gel. In the event, *para*-phenylphenacyl-[1-^{13}C,$^{18}O_2$]-acetate (**110a**) was generated in 12% yield. The resultant MS data is presented in Table 14.

ion	*m/z*	*para*-phenylphenacyl acetate (110a)
M	254	0
M+1	255	24.3
M+3	257	46.8
M+5	259	26.1

Table 14 Isotopic distribution (% values) of synthesised *para*-phenylphenacyl-[1-^{13}C,$^{18}O_2$]-acetate [(**110a**), *m/z* 254 (M)]. Values in the table are calculated after considering natural abundance incorporations.

It can be seen from the MS analysis that the synthesised *para*-phenylphenacyl acetate (**110a**) is highly enriched. No presence of the unlabelled molecular ion [*m/z* 254 (M^+)] was observed. The MS results reveal that the product is enriched over a range with ^{13}C (M+1, 24.3%), ^{13}C + ^{18}O (M+3, 46.8%) and ^{13}C + 2 x ^{18}O (M+5, 26.1%). These enrichments have an approximate ratio of 1:2:1 (fig. 37), and infer that the product, *para*-phenylphenacyl-[1-^{13}C,$^{18}O_2$]-acetate (**110a**) is 97.2% enriched with ^{13}C but only 49.9% enriched with ^{18}O. It is concluded that the $H_2{}^{18}O$ used in the synthetic procedure was only ~50% enriched with ^{18}O and not 98% as stated on the manufacturer's label. This finding also agrees with our earlier experiments using the same sample of $H_2{}^{18}O$, where the results revealed that the desired product, sodium [2-^2H,^{18}O]-phenyllactate (**51f**), was found also to be ~50% enriched with ^{18}O (Chapter 2, Section 2.2.2, Table 3, page 61).



	24.30%		46.83%		26.09%
Ratio	**1**	**:**	**2**	**:**	**1**

Figure 37 The isotopic distribution of ^{13}C and ^{18}O in sodium [1-^{13}C,$^{18}O_2$]-acetate (7j).

4.3.3.3 Incorporation studies with sodium [1-^{13}C,$^{18}O_2$]-acetate (7j)

The prepared sodium [1-^{13}C,$^{18}O_2$]-acetate [(**7j**), 97% ^{13}C, 50% ^{18}O] was pulse fed to transformed root cultures of *D. stramonium* on days 5, 7 and 9 to a final concentration of 4.43mmol dm^{-3} in the medium. After 17 days of growth, the alkaloids were extracted in the usual manner. ^{13}C-NMR revealed a clear enrichment at C-3 (~8%) of hyoscyamine, however, there was no obvious ^{18}O induced upfield signals at 65ppm. GC-MS analysis of the crude alkaloid extract only revealed the presence of an M+1 ion, indicating that both littorine (**5**) and hyoscyamine (**1**) were enriched with ^{13}C. However, no M+3 ion was detected in either alkaloid, and thus no indication that the ^{18}O label had become incorporated into the tropane ring system (Table 15).

ion	*m/z*	Littorine	Hyoscyamine
M	289	94.3	93.9
M+1	290	6.2	6.7
M+2	291	0	0
M+3	292	0	0

Table 15 Incorporations (% values) into littorine [(**5**), *m/z* 289 (M)] and hyoscyamine [(**1**), *m/z* 289 (M)] after feeding experiment with sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**) to transformed root cultures of *D. stramonium* after 17 days. Values in the table are calculated after considering natural abundance incorporations.

Of course, in view of the relatively low level of ^{13}C incorporation (6.7%) into hyoscyamine, a significant exchange of the oxygen with water from the medium may have lowered the ^{18}O incorporation level below a detectable threshold.

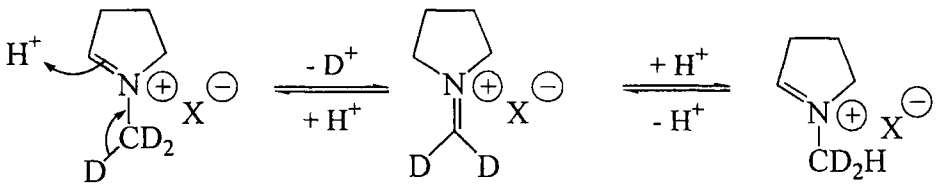
4.4 Conclusions

Interestingly, our experimental NMR data have revealed an approximate 2:1 ratio for the incorporation of [^{13}C]-acetate into the C-2 – C-4 portion of the tropane ring versus the ornithine derived moiety (C-5, C-6, C-7 and C-1). Thus, these [^{13}C]-acetate experiments have shown that the tropane skeleton is derived from both ornithine (**6**) and acetate (**7**), which is consistent with previous data.

The finding from the feeding experiment with sodium [2-^{13}C,2-$^{2}H_3$]-acetate (**7e**) and current work in Durham laboratories contradict early findings[119] for the presence of deuterium at C-2 and/or C-4 of the tropane moiety of hyoscyamine (**1**) following a feeding experiment with [1-^{13}C,2-$^{2}H_3$]-acetate (**7d**). Instead, there is evidence for deuterium retention at C-6 and/or C-7. The presence of deuterium at C-6 and/or C-7 can be rationalised by the biosynthesis of ornithine (**6**) from [2-$^{2}H_3$]-acetate (**7h**) *via* the Krebs cycle. However, since deuterium is detected both at C-6 and C-7, this suggests that tautomerisation occurs at the N-methylpyrrolinium salt (**11**), prior to the formation of the bicyclic tropane ring.

The observed ~10% washout of deuterium in the N-methyl group after feeding L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (**105b**), can be rationalised by at least two different ways (Scheme 102). The first (A), involves the loss of deuterium from the N-methylpyrrolinium salt (**11**) by exchange, whereas in the second (B), would involve loss of deuterium from SAM (**77**). However, since there has never previously been a report of exchange in this way from SAM, the observed exchange of deuterium is more likely to have arisen by exchange at the N-methylpyrrolinium salt (**11**).

A



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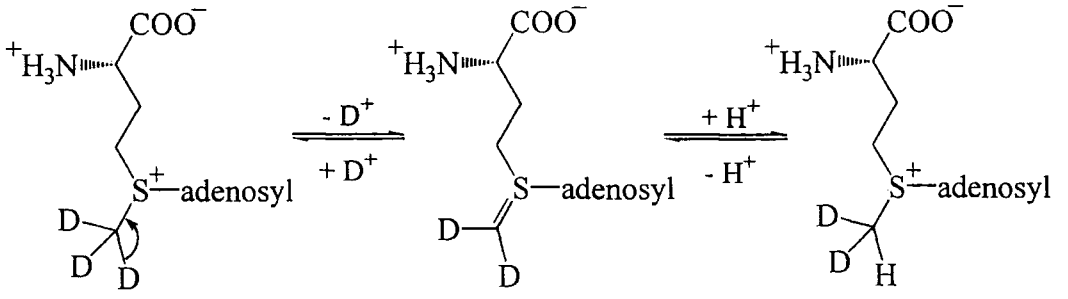
B



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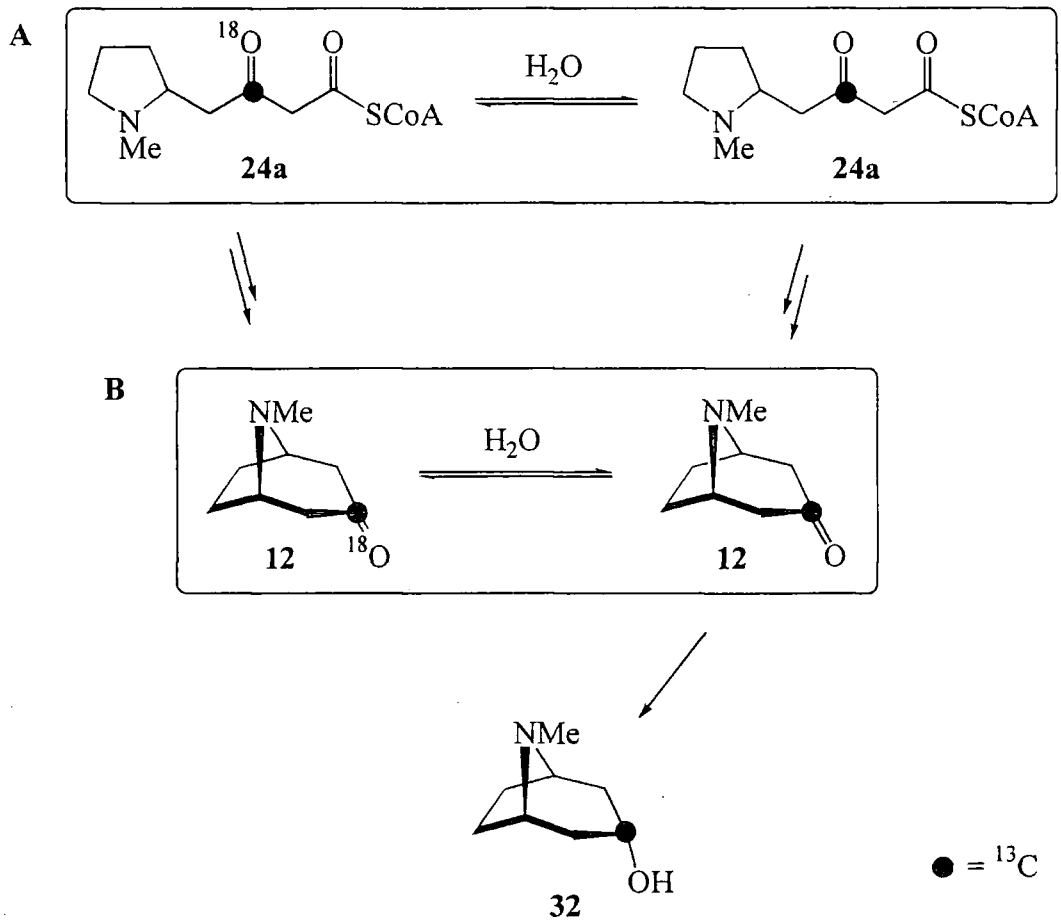
Scheme 102 Two possible routes for the observed 10% washout of deuterium from the N-methyl group of hyoscyamine. (A) Washout from the N-methylpyrrolinium salt (**11**) and (B) Washout from SAM (77), prior to the transfer of the methyl group to ornithine.

The feeding experiment with sodium [1-^{13}C,$^{18}O_2$]-acetate (**7j**) revealed that no ^{18}O label was incorporated into the tropine oxygen at C-3 of hyoscyamine (**1**). This result could have potentially arisen *via* exchange of the labelled oxygen with water from the medium prior to the formation of littorine (**5**). This proposed exchange process *via* an acetal intermediate could occur during the biosynthesis of tropine in two ways. Firstly, exchange could occur prior to the formation of the tropane skeleton, perhaps through the proposed 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**) intermediate (Scheme 103). Of course, even after ring closure, exchange could still occur at tropinone (**12**) prior to reduction to form tropine (**32**).



Scheme 103 Two possible routes for the observed washout of ^{18}O-label from tropine (**32**) following the feeding experiment with [1-^{13}C,$^{18}O_2$]-acetate (**7j**) to *D. stramonium* root cultures. (A) *via* the proposed 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**) intermediate and (B) *via* tropinone (**12**).

The intermediacy of the proposed 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA intermediate (**24a**) between the N-methylpyrrolinium salt (**11**) and tropinone (**12**) during tropine (**32**) biosynthesis could explain the absence of deuterium at C-2 and/or C-4 following the [2-^{13}C,2-^{2}H$_3$]-acetate (**7e**) feeding experiments to *D. stramonium*. The hydrogen atoms at C-2 and C-4 of this intermediate (**24a**) are highly enolisable and any deuterium labelled at these sites will be lost to the medium prior to ring closure to form the bicyclic tropane ring (fig. 38).

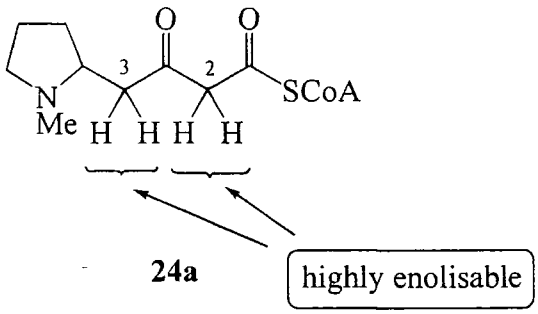


Figure 38 The hydrogen atoms at C-2 and C-4 of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutyl-CoA (**24a**) are highly enolisable, and could explain the lack of deuterium observed at C-2 and C-4 of the tropane moiety of hyoscyamine (**1**).

Finally, the ^{13}C-NMR analysis from the feeding experiments with [1-^{13}C]- (**7f**), [2-^{13}C]- (**7g**), and [1,2-^{13}C$_2$]-acetate (**7b**) reveal symmetrical incorporations into the bridgehead carbon positions of the tropane moiety of the resultant isolated hyoscyamine (**1**) in *D. stramonium*. This result was not expected as Leete has shown that [2-^{14}C]-ornithine (**6a**) specifically labels the tropane moiety of hyoscyamine at C-1.[26,27] Leete's finding was confirmed in a later feeding experiment to *D. metel*,[162] which led Leete to conclude that a symmetrical intermediate is not involved in the biosynthesis of tropine (**32**). However, there have been reports of symmetrical labelling of the bridgehead carbons of the tropane ring, following feeding experiments with DL-[2-^{14}C]-ornithine (**6a**) to *Nicotiana tabacum*[163,164] and DL-[5-^{14}C]-ornithine (**6b**) to *Duboisia lechhardtii*[165], *Erythroxylum coca*,[29] and *Hyoscyamus albus*.[166] This prompted Leete to suggest that biosynthesis of the tropane moiety of hyoscyamine (**1**) and other related alkaloids is species-dependent.[28]

The tautomerisation of the N-methylpyrrolinium salt (**11**) was investigated as possible explanation for the observed symmetrical incorporation in a number of plant species.

However, a feeding experiment with N-methyl-[2-^{14}C]-pyrrolinium chloride (**13a**) to *Nicotiana tabacum*[36] revealed that the isotopic label was solely incorporated into C-2′ of the pyrrolidine ring of the isolated nicotine (Chapter 1, Section 1.5.1, Scheme 5, page 11). Thus, confirming that tautomerisation of the N-methylpyrrolinium salt does not occur in the biosynthesis of nicotine (**14**). However, a more recent report by Hemscheidt and Spenser[47] revealed that the administration of N-methyl-[2-^{2}H]-pyrrolinium chloride (**13b**) to *D. stramonium* (Chapter 1, Section 1.5.1, Figure 8, page 17), resulted in the incorporation of deuterium at both bridgehead carbons of the tropane moiety of 6β-hydroxyhyoscyamine (**26**). These findings curiously contradict Leete's earlier reports of unsymmetrical labelling of C-1 and C-5 of the tropane ring. Interestingly, however, the findings reported in this thesis, following the administration of [^{13}C]-acetates to *D. stramonium* support the tautomerisation of the N-methylpyrrolinium salt (**11**). Also, it is noteworthy that if tautomerisation of the iminium salt does occur, then this could explain the observed symmetrical labelling of the acetate derived moiety of the tropane ring. Of course, this observation could also arise from the condensation of an acetate derived symmetrical intermediate with the N-methylpyrrolinium salt (**11**), prior to ring closure to form the bicyclic tropane ring. Therefore, in order to delineate both these possibilities, further investigations are required.

Chapter 5

5. Experimental

5.1 General

NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer; (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz), Varian XL-200; (^{1}H at 200.057 MHz), Varian VXR 400(S); (^{1}H at 399.95 MHz, ^{13}C at 100.577 MHz and ^{19}F at 376.347 MHz). $^{13}C\{^{2}H, ^{1}H\}$ experiments were carried out using a 600MHz spectrometer (Varian VXR-600, ^{13}C at 150.869 MHz, University of Edinburgh). ^{13}C chemical shifts are quoted relative to TMS ($\delta = 0$). IR spectra were recorded on a Perkin Elmer F.T. 1720X or 1600 spectrometer. Low resolution mass spectra were recorded on VG Analytical 7070E organic mass spectrometer. Melting points were determined using a digital Gallenkamp melting point apparatus and are uncorrected. All chemicals were used as supplied from manufacturers. Solvents were routinely dried and distilled prior to use: tetrahydrofuran and ether (sodium benzophenone, under nitrogen), dichloromethane and triethylamine (calcium hydride), ethanol and methanol (dry magnesium turnings, iodine). Reactions requiring anhydrous conditions were carried out under a nitrogen atmosphere. Flash chromatography was carried out using Fluka silica gel-60 (35-70mm) or Sorbsil-C60-H (40-60mm) unless otherwise stated. Transformed root cultures of *Datura stramonium* were maintained on an Infors HT (Bottmingen, Germany) ITE rotary planar incubator. Ether refers to diethyl ether and DCM refers to dichloromethane.

5.2 Production, Isolation and Analysis of Hyoscyamine from Transformed Root Cultures of *Datura stramonium*

Preparation of culture medium[16]

Gamborg's B5 salts® [(1.85g) (Sigma Chem. Co.)] and sucrose (15g) were dissolved in distilled water to a volume of 500ml and adjusted to pH 5.8 ± 0.2 using 0.1M NaOH. Aliquots (50ml) were transferred to ten conical flasks (250ml). The flasks were tightly covered with triple layered aluminium foil lids (15cm x 15cm) and the foils secured with masking tape. The flasks were then autoclaved at 121°C for 20 minutes at 15lbs/in^2, cooled and then placed in a rotary planar incubator operating at a speed of 91rpm and a temperature of 28 °C. The flasks were left for several days to ensure that they were sterile prior to subculturing.

Growth of *Datura stramonium* root cultures[16]

Samples of transformed root cultures of *Datura stramonium* (~0.5g) were aseptically transferred after ~14 days of growth, in a laminar airflow cabinet, to a previously prepared 250ml conical flask of sterile culture medium (50ml). The flask was tightly covered under aseptic conditions with new double layered aluminium foil lids (15cm x 15cm), (previously sterilised at 200°C for at least 24 hours), and replaced in rotating planar incubator (28°C, 91rpm).

Alkaloid extraction procedure and analysis[167]

The roots were harvested after 17 days and freeze-dried. The freeze-dried roots were then ground with acid washed sand (~0.2g) and extracted into H_2SO_4 (10ml per 0.5g of roots, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and was then filtered through Hydromatrix® (Varian) and the alkaloids were eluted with chloroform : methanol (20:1). The eluent was evaporated to a brown oil on a rotary evaporator and the extract was then purified by preparative TLC on silica gel plates {(25cm x 25cm, 0.2mm) [chloroform : diethylamine (9:1)], R_f = 0.8}, and visualised using Dragendorff's reagent, (Sigma Chemical Co.) to give hyoscyamine and a trace of littorine. $\delta C(CDCl_3)$[78,168] 172.2 (C-1′), 135.7 (C-1′′), 128.8

(C-2′′, C-6′′), 128.1 (C-3′′, C-5′′), 127.6 (C-4′′), 68.1 (C-3), 64.1 (C-3′), 59.53 (C-1 or C-5), 59.44 (C-1 or C-5), 54.4 C-2′), 40.3 (NMe), 36.35 (C-2 or C-4), 36.15 (C-2 or C-4), 25.34 (C-6 or C-7), 24.84 (C-6 or C-7).

GC-MS Analysis (Queen's University, Belfast, N. Ireland)

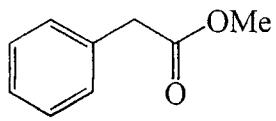
The system used was a Hewlett Packard (Palo Alto, CA) 5890 gas chromatograph directly linked to a Hewlett Packard 5970 Mass Selective Detector (MSD), controlled by a Hewlett Packard computer using ChemStation software. The gas chromatograph was equipped with a Hewlett Packard 7673A autosampler and fitted with a SGE fused silica capillary column (25m × 0.22mm i.d. with a 0.25μm Cydex-B phase). The injector port and the transfer line temperatures were maintained at 250°C. The column head pressure was set at 15psi using helium as the carrier gas. The oven was temperature programmed at 100°C for 1 min, ramped at 10°C/min to 250°C and held at this temperature for 1 min. The samples were dissolved in $CHCl_3$ and a 1 μl aliquot was injected in the split mode onto the gas chromatograph employing a split ratio of 30:1. The MSD was operated in the selected ion monitoring (SIM) mode measuring ion currents at *m/z* 289, 290, 291, 292, 293 and 294.

GCMS Analysis (University of Nantes, Nantes, France)[169]

The system used was a Hewlett Packard (Palo Alto, CA) 5890 series II gas chromatograph directly linked to a Hewlett Packard 5971A Mass Selective Detector (MSD), controlled by a Hewlett Packard computer using ChemStation software. The gas chromatograph was fitted with a DB-17 fused silica capillary column [J&W Scientific, Folson, USA, 30m x 0.32mm i.d. with a 0.23μm (50%-phenyl)-methylpolysiloxane film)]. Chromatography was performed at a flow rate of 1.5ml min^{-1} He with an initial temperature of 65 °C and a ramp of 6 °C min^{-1} to 300 °C.

5.3 Synthesis Procedures

Preparation of methyl phenylacetate (65)[102]



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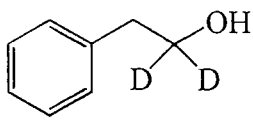
Phenylacetic acid (1.02g, 7.51mmol) was stirred with an excess of diazomethane generated from Diazald® (Aldrich, 2.14g, 10mmol) in ether (60ml) and potassium hydroxide (0.8g, 14.3mmol) dissolved in ethanol (20ml) for 1 hour. The reaction was quenched with a few drops of glacial acetic acid until the residual yellow colour of diazomethane disappeared. The solution was dried over $MgSO_4$, filtered and evaporated under reduced pressure to give methyl phenylacetate (1.13g, 7.51mmol, 100%); δH($CDCl_3$) 7.26-7.44 (m, 5H, Ar-H), 3.70 (s, 3H, OMe), 3.64 (s, 2H, -CH_2); δC($CDCl_3$) 171.9 (C-1), 133.8 (C-1′), 129.1 (C-2′, C-6′), 128.4 (C-3′, C-5′), 127.0 (C-4′), 51.9 (OMe), 41.0 (C-2).

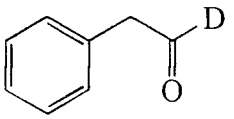
Preparation of 2-[1-$^2H_2$]-phenylethanol (66b)[102]



66b

A solution of methyl phenylacetate (1.13g, 7.51mmol), in dry ether (10ml) was added dropwise to a stirred suspension of $LiAlD_4$ (1.58g, 37.6mmol) in dry ether (40ml), under N_2. The mixture was then heated under reflux for 2 hours. The reaction was quenched by the addition of wet ether (30ml), dried over $MgSO_4$, filtered and evaporated under reduced pressure to give the product as a yellow oil, (921mg, 7.42mmol, 99%); δH($CDCl_3$) 7.22-7.35 (m, 5H, Ar-H), 2.85 (s, 2H, -CH_2), 2.20-2.60 (br, 1H, OH); δC($CDCl_3$) 139.2 (C-1′), 129.6 (C-2′, C-6′), 129.1 (C-3′, C-5′), 126.9 (C-4′), 63.4 (pentet, $^1J_{CD}$ = 21.9 Hz, C-1), 39.5 (C-2).

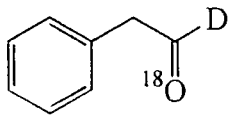
Preparation of [1-^{2}H]-phenylacetaldehyde (67a)[170]



67a

A solution of 2-[1-$^2H_2$]-phenylethanol (498mg, 4.01mmol), in dry DCM (10ml) was added dropwise to a stirring suspension of pyridinium chlorochromate (1.8g, 8.4mmol, 2eq.), dry powdered 3Å molecular sieves (35g, Sigma, 1/16" pellets, ground) and dry DCM (50ml). The mixture was stirred vigorously for 2 hours under N_2. The black suspension was filtered to remove solids and the filtrate was then re-filtered through a sintered glass funnel (20cm diameter) under reduced pressure, containing a 15cm depth of silica gel, while washing liberally with DCM (approx. 400ml). The combined washings were then evaporated under reduced pressure to give the product as a green oil (413mg, 3.41mmol, 85%), which was immediately used without characterisation. Previous selected spectroscopic data from an unlabelled synthesis; δH($CDCl_3$) 9.78 (t, ^3J = 2.8 Hz, 1H, -CHO), 7.25-7.40 (m, 5H, Ar-H), 3.69 (d, ^3J = 2.6 Hz, 2H, -CH_2). *m/z* (EI+) 120 (M^+, 12.39%), 91 (M^+-29, 100%)

Preparation of [1-^{2}H,^{18}O]-phenylacetaldehyde [^{2}H, 91%; ^{18}O, 43% (67b)]



67b

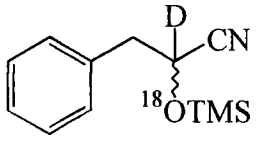
Previously prepared [1-^{2}H]-phenylacetaldehyde (305mg, 2.52mmol) was dissolved in THF (1ml) and transferred to a Carius tube. $H_2{}^{18}O$ (1ml, 98%) was acidified under N_2 with dry HCl and quickly added to the Carius tube, washing with dry THF (2ml). The tube contents were frozen in liquid N_2 and the tube was evacuated, sealed and placed in an oven at 120°C for 5 hours. After cooling, to room temperature, the Carius tube was frozen in liquid N_2 and opened. The contents were allowed to thaw to room temperature and extracted into DCM (3 x 20ml). The organics were collected and evaporated under reduced pressure to yield a pale green oil (305mg, 2.48mmol, 98%). GC-MS data is shown in Table 15. Previous selected spectroscopic data from a [1-^{2}H] labelled

synthesis; δH($CDCl_3$) 7.25-7.40 (m, 5H, Ar-H), 3.69 (s, 2H, -CH_2). *m/z* (EI+) 121 (M^+, 15.35%), 91 (M^+-30, 100%)

	m/z (EI+)	Ion	% incorporation
M	120	$(C_6H_5CHO)^+$	0
M+1	121	$(C_6H_5C^2HO)^+$	47.67
M+2	122		5.16
M+3	123	$(C_6H_5C^2H^{18}O)^+$	43.07

Table 15 Ion distribution and incorporation levels of [1-^{2}H,^{18}O]-phenylacetaldehyde [(**67b**), *m/z* 120, (M^+)] after GC-MS analysis. Values in the table are adjusted after considering natural abundance and demonstrate an isotope content of ^{2}H at 91% and ^{18}O at 43%.

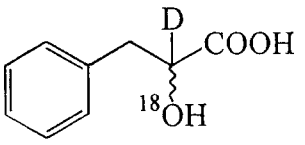
Preparation of DL-3-phenyl-[2-^{2}H,^{18}O]-2-(trimethylsilyloxy)-propionitrile (68a)[126]



68a

Trimethylsilylcyanide (0.33ml, 3.22mmol) was added dropwise to a cooled, stirred solution of [1-^{2}H,^{18}O]-phenylacetaldehyde (305mg, 2.48mmol) in DCM (2ml) with Et_3N (35μl, 10mol%) at 0°C. The reaction was stirred for 2 hours under N_2. The excess solvent was removed by lyophilisation under reduced pressure to give the product as a clear oil (397mg, 1.78mmol, 72%). This material was used immediately without further characterisation. Previous selected spectroscopic data from an unlabelled synthesis; δH($CDCl_3$) 7.20-7.40 (m, 5H, Ar-H), 4.60 (dd, ^3J = 6.5 Hz, 1H, -CH), 3.09 (d, ^3J = 6.7 Hz, 2H, -CH_2); δC($CDCl_3$) 133.9 (C-1′), 129.6 (C-2′, C-6′), 128.8 (C-3′, C-5′), 127.7 (C-4′), 119.4 (C-1), 62.1 (C-2), 41.1 (C-3).

Preparation of DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid [^{2}H, 99%; ^{18}O, 49% (51e)]



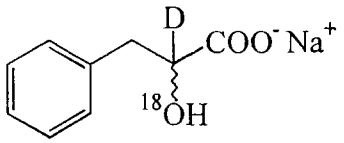
51e

A solution of 50% HCl (20ml) was added to DL-3-phenyl-[2-^{2}H,^{18}O]-2-(trimethylsilyloxy)-propionitrile and the mixture heated under reflux for 48 hours. The solution was allowed to cool, and the product was extracted into ether (3 x 30ml). The organic extracts were then combined and evaporated under reduced pressure to give the product as a green oil (289mg, 1.71mmol, 96%); δH($CDCl_3$) 7.23-7.44 (m, 5H, Ar-H), 3.19 (d, ^{2}J = 14.2 Hz, 1H, -CHH), 2.98 (d, ^{2}J = 13.9, 1H, -CHH). δC($CDCl_3$) 177.8 (C-1), 136.0 (C-1′), 129.4 (C-2′, C-6′), 128.4 (C-3′, C-5′), 126.9 (C-4′), 71.8 (t, $^{1}J_{13C2H}$ = 21.8 Hz, C-2), 39.9 (C-3). GC-MS data is shown in Table 16.

	m/z (EI+)	Ion	% incorporation
M	193	$(C_6H_5CHOTMS)^+$	1.70
M+1	194	$(C_6H_5C^2HOTMS)^+$	52.61
M+2	195		3.96
M+3	196	$(C_6H_5C^2H^{18}OTMS)^+$	49.43

Table 16 Ion distribution and incorporation levels of DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid [(**51e**), *m/z* 193 (M^+)]. A sample (1mg) of DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid (**51e**) was derivatised with N-methyl-N-trimethylsilyl-trifluoroacetamide [(MSTFA) 200μl)] by heating at 95°C for 25min prior to GC-MS analysis. Values in the table are adjusted after considering natural abundance levels and show an isotope content of ^{2}H at 99% and ^{18}O at 49% in the sample.

Preparation of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate [^{2}H, 96%; ^{18}O, 47% (51f)]



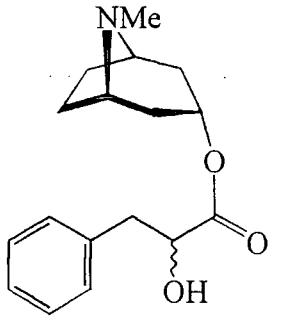
51f

A solution of DL-3-phenyl-[2-^{2}H,^{18}O]-lactic acid (289mg, 1.71mmol) in $CHCl_3$ (10ml) was covered with water (10ml). The product was then extracted into the aqueous layer and was adjusted to pH 8 with aqueous NaOH. The aqueous layer was separated and evaporated under reduced pressure to afford sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate as a white amorphous solid (178mg, 0.93mmol, 54%); δH(D_2O) 7.20-7.30 (m, 5H, Ar-H), 2.96 (d, ^{2}J = 13.9 Hz, 1H, -CHH), 2.73 (d, ^{2}J = 14.0 Hz, 1H, -CHH). δC(D_2O) 185.0 (C-1), 141.2 (C-1′), 133.8 (C-2′, C-6′), 132.9 (C-3′, C-5′), 131.0 (C-4′), 72.8 (t, $^{1}J_{^{13}C^{2}H}$ = 22.4 Hz, C-2), 39.9 (C-3). GC-MS data is shown in Table 17.

	m/z (EI+)	Ion	% incorporation
M	193	$(C_6H_5CHOTMS)^+$	1.50
M+1	194	$(C_6H_5C^{2}HOTMS)^+$	48.95
M+2	195		3.28
M+3	196	$(C_6H_5C^{2}H^{18}OTMS)^+$	46.65
M+4	197		1.54

Table 17 Ion distribution and incorporation levels of sodium DL-3-phenyl-[2-^{2}H,^{18}O]-lactate [(**51f**), *m/z* 193 (M^+)]. A sample (1mg) of DL-3-phenyl-[2-^{2}H,^{18}O]-lactate (**51f**) was derivatised with N-methyl-N-trimethylsilyl-trifluoroacetamide [(MSTFA) 200µl)] by heating at 95°C for 25min prior to GCMS analysis. Values in the table are adjusted after considering natural abundance levels and show an isotope content of ^{2}H at 96% and ^{18}O at 47% in the sample.

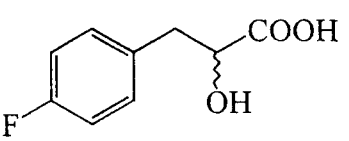
Synthesis of (*RS*)-phenyllactoyltropine [littorine (5)][120]



5

Phenyllactic acid (0.56g, 3.3mmol), previously dried over phosphorus pentoxide was mixed intimately in the solid phase with tropine (4.3g, 3.0mmol), itself previously dried for 24 hours over sodium hydroxide pellets. The mixture was heated to 130°C and a current of dry HCl gas was passed over periodically for 5 hours. After this treatment, the reaction mixture was allowed to cool to room temperature. The product was dissolved as completely as possible in 0.05M H_2SO_4 (10ml) and was filtered. The filtrate was basified with 10% aqueous ammonium hydroxide and extracted into chloroform (4 x 15ml), dried over $MgSO_4$ and evaporated under reduced pressure to yield the product as a pale yellow gum (0.72g, 75%); δH($CDCl_3$) 7.16-7.26 (m, 5H, Ar-H), 4.99 (t, ^3J = 5.2 Hz, 1H, H-3_e), 4.32 (dd, ^3J = 4.8 Hz, ^3J = 6.8 Hz, 1H, -CH), 3.06 (dd, ^3J = 4.8 Hz, ^2J = 14.0 Hz, 1H, -CHH), 3.00-3.04 (br, 2H, H-1, H-5), 2.91 (dd, ^3J = 7.2 Hz, ^2J = 14.0 Hz, 1H, -CHH), 2.50-2.80 (br, 1H, -OH), 2.20 (s, 3H, NMe), 2.04-2.12 (m, 2H, H-2_e, H-4_e), 1.89-1.96 (m, 2H, H-6_e, H-7_e), 1.68-1.73 (m, 2H, H-7_a, H-6_a), 1.58 (t, ^2J = 12.8 Hz, 2H, H-4_a, H-2_a). δC($CDCl_3$) 173.3 (C-1′), 136.3 (C-1′′), 129.4 (C-2′′, C-6′′), 128.5 (C-3′′, C-5′′), 126.9 (C-4′′), 71.4 (C-2′), 69.2 (C-3), 59.6 (C-1, C-5), 40.3 (NMe), 40.5 (C-3′), 36.56 (C-2 or C-4), 36.45 (C-2 or C-4), 25.48 (C-6 or C-7), 25.44 (C-6 or C-7).

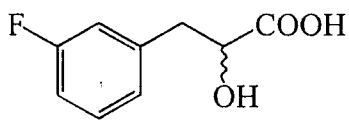
Preparation of 4′-fluorophenyl-DL-lactic acid (95c)



95c

Concentrated HCl (6.9ml, 3eq.) was added to a suspension of 4′-fluorophenyl-DL-alanine (4.98g, 27.2mmol) in water (260ml), and the reaction mixture stirred until dissolved. The solution was then cooled to 0°C and $NaNO_2$ (3.90g, 56.5mmol, 2eq.) was added portion-wise over a period of 4 hours and the reaction mixture kept at this temperature. The reaction was then allowed to rise to ambient temperature overnight, and stirred at this temperature for a further 48 hours. The solution was concentrated under reduced pressure and extracted with ether (3x150ml). The combined organic extracts were dried over $MgSO_4$, filtered and evaporated under reduced pressure to yield a yellow oil. Crystallisation from chloroform gave 4′-fluorophenyl-DL-lactic acid as a white crystalline solid (1.25g, 25%). M.p. 100.5-101.5°C. ν_{max} 3417, 2950, 1702, 1600, 1507, 1445, 1417, 1363, 1228 cm^{-1}. GC (MSTFA), 99.5%. δH(CD_3OD) 7.26 (dd, $^4J_{HF}$ = 5.6 Hz, $^3J_{HH}$ = 8.0 Hz, 2H, H-2′, H-6′), 6.98 (t, $^3J_{HF, HH}$ = 8.8 Hz, 2H, H-3′, H-5′), 4.30 (dd, ^3J = 4.4 Hz, ^3J = 8.0 Hz, 1H, -C<u>H</u>), 3.07 (dd, ^3J = 4.4 Hz, ^2J = 14.0 Hz, 1H, -C<u>H</u>H), 2.88 (dd, ^3J = 8.0 Hz, ^2J = 14.0 Hz, 1H, -CH<u>H</u>). δC(CD_3OD) 177.0 (C-1), 72.6 (C-2), 40.6 (C-3), 163.2 (d, $^1J_{CF}$ = 242.6 Hz, C-4′′), 134.8 (d, $^4J_{CF}$ = 2.9 Hz, C-1′′), 132.3 (d, $^3J_{CF}$ = 8.0 Hz, C-2′′, C-6′′), 115.7 (d, $^2J_{CF}$ = 21.3 Hz, C-3′′, C-5′′). δF(CD_3OD) -119.6 (m, 1F, Ar-F). Anal. Calcd. for $C_9H_9F_1O_3$: C, 58.70; H, 4.93. Found: C, 58.47; H, 4.87. *m/z* (EI+) 184 (M^+, 6.55%), 166 (M^+-18, 20.97%), 109 (M^+-75, 100%). Calcd. mass for $C_9H_9F_1O_3$: 184.053573. Found: 184.053398.

<u>Preparation of 3′-fluorophenyl-DL-lactic acid (95b)</u>

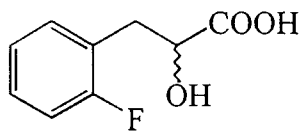


95b

The above procedure for 4′-fluorophenyl-DL-lactic acid was repeated using 3′-fluorophenyl-DL-alanine (4.83g, 26.4mmol) to yield a yellow oil. Crystallisation from chloroform gave 3′-fluorophenyl-DL-lactic acid as a white crystalline solid (1.21g, 25%). M.p. 106-107°C. ν_{max} 3428, 2940, 1722, 1585, 1486, 1450, 1429, 1324, 1240 cm^{-1}. GC (MSTFA), 98.7%. δH(CD_3OD) 7.27 (dt, $^3J_{HH}$ = 7.6 Hz, $^4J_{HF}$ = 6.8 Hz, 1H, H-5′), 7.07 (d, $^3J_{HH}$ = 7.6 Hz, 1H, H-6′), 7.02 (d, $^3J_{HH}$ = 10.0 Hz, 1H, H-2′), 6.93 (t, $^3J_{HF, HH}$ = 8.8 Hz, 1H, H-4′), 4.34 (dd, ^3J = 4.4 Hz, ^3J = 8.0 Hz, 1H, -C<u>H</u>), 3.10 (dd, ^3J = 4.0 Hz, ^2J = 14.0 Hz, 1H, -C<u>H</u>H), 2.91 (dd, ^3J = 8.0 Hz, ^2J = 14.0 Hz, 1H, -CH<u>H</u>). δC(CD_3OD)

176.8 (C-1), 72.4 (C-2), 41.2 (C-3), 164.1 (d, $^{1}J_{CF}$ = 243.8 Hz, C-3′′), 141.7 (d, $^{3}J_{CF}$ = 7.3 Hz, C-1′′), 130.8 (d, $^{3}J_{CF}$ = 8.0 Hz, C-5′′), 126.5 (d, $^{4}J_{CF}$ = 2.6 Hz, C-6′′), 117.3 (d, $^{2}J_{CF}$ = 21.3 Hz, C-2′′ or C-4′′), 114.2 (d, $^{2}J_{CF}$ = 22.4 Hz, C-4′′ or C-2′′). δF(CD_3OD) -116.6 (m, 1F, Ar-F). Anal. Calcd. for $C_9H_9F_1O_3$: C, 58.70; H, 4.93. Found: C, 58.32; H, 4.85. *m/z* (EI+) 184 (M^+, 3.37%), 166 (M^+-18, 32.26%), 109 (M^+-75, 100%). Calcd. mass for $C_9H_9F_1O_3$: 184.053573. Found: 184.053243.

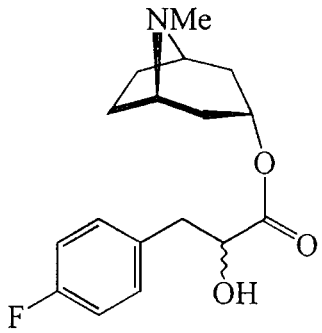
Preparation of 2′-fluorophenyl-DL-lactic acid (95a)



95a

The above procedure for 4′-fluorophenyl-DL-lactic acid was repeated using 2′-fluorophenyl-DL-alanine (5.09g, 27.8mmol) to yield a yellow oil. Crystallisation from chloroform gave 2′-fluorophenyl-DL-lactic acid as a white crystalline solid (0.957g, 19%). M.p. 80-81°C. ν_{max} 3416, 2950, 1703, 1604, 1507, 1450, 1418, 1362, 1230 cm^{-1}. GC (MSTFA), 97.3%. δH(CD_3OD) 7.29-7.32 (m, 1H, Ar-H), 7.20-7.24 (m, 1H, Ar-H), 7.02-7.09 (m, 2H, Ar-H), 4.34 (dd, ^{3}J = 4.4 Hz, ^{3}J = 7.6 Hz, 1H, -C<u>H</u>), 3.17 (dd, ^{3}J = 3.2 Hz, ^{2}J = 14.0 Hz, 1H, -C<u>H</u>H), 2.91 (dd, ^{3}J = 8.4 Hz, ^{2}J = 13.2 Hz, 1H, -CH<u>H</u>). δC(CD_3OD) 176.9 (C-1), 71.6 (C-2), 34.9 (C-3), 162.8 (d, $^{1}J_{CF}$ = 244.1 Hz, C-2′′), 133.1 (d, $^{3}J_{CF}$ = 4.2 Hz, C-6′′ or C-4′′), 129.5 (d, $^{3}J_{CF}$ = 8.0 Hz, C-4′′ or C-6′′), 125.8 (d, $^{2}J_{CF}$ = 15.7 Hz, C-1′′), 125.0 (d, $^{4}J_{CF}$ = 3.0 Hz, C-5′′), 116.0 (d, $^{2}J_{CF}$ = 22.1 Hz, C-3′′). δF(CD_3OD) -120.4 (m, 1F, Ar-F). Anal. Calcd. for $C_9H_9F_1O_3$: C, 58.70; H, 4.93. Found: C, 58.63; H, 4.88. *m/z* (EI+) 184 (M^+, 2.47%), 166 (M^+-18, 23.19%), 109 (M^+-75, 100%). Calcd. mass for $C_9H_9F_1O_3$: 184.053573. Found: 184.053840.

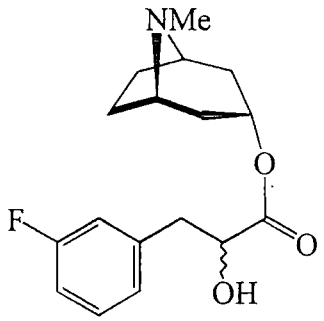
Preparation of DL-4′-fluorophenyllactoyltropine [4′-fluorolittorine, (96c)]



96c

4′-Fluorophenyl-DL-lactic acid (390mg, 2.1mmol), previously dried over phosphorus pentoxide was mixed intimately in the solid phase with tropine (270mg, 1.9mmol), itself previously dried for 24 hours over sodium hydroxide pellets. The mixture was heated to 130°C and a current of dry HCl gas was passed over periodically for 5 hours. After this treatment, the reaction mixture was allowed to cool to room temperature. The product was dissolved as completely as possible in 0.05M H_2SO_4 (10ml) and filtered. The filtrate was basified with 10% aqueous ammonium hydroxide solution and was extracted into chloroform (4 x 15ml), dried over $MgSO_4$ and concentrated under reduced pressure to give a white solid (367mg, 57%). M.p. 77-79°C. ν_{max} 2944, 1730, 1601, 1508, 1448, 1418, 1218 cm^{-1}. GC (MSTFA), 4′-fluorolittorine (84.5%), 4′-fluorophenyl-DL-lactic acid (15.5%). δH($CDCl_3$) 7.20 (dd, $^4J_{HF}$ = 5.6 Hz, $^3J_{HH}$ = 8.8 Hz, 2H, H-2′′, H-6′′), 6.99 (t, $^3J_{HF, HH}$ = 8.8 Hz, 2H, H-3′′, H-5′′), 5.06 (t, ^3J = 5.2 Hz, 1H, $H\text{-}3_e$), 4.36 (dd, ^3J = 4.8 Hz, ^3J = 7.2 Hz, 1H, -CH), 3.10 (dd, ^3J = 4.8 Hz, ^2J = 14.0 Hz, 1H, -CHH), 3.10-3.18 (br, 2H, H-1, H-5), 2.95 (dd, ^3J = 6.8 Hz, ^2J = 14.0 Hz, 1H, -CHH), 2.30 (s, 3H, NMe), 2.12-2.26 (m, 2H, $H\text{-}2_e$, $H\text{-}4_e$), 1.96-2.04 (m, 2H, $H\text{-}6_e$, $H\text{-}7_e$), 1.74-1.85 (m, 2H, $H\text{-}7_a$, $H\text{-}6_a$), 1.67 (t, ^2J = 14.0 Hz, 2H, $H\text{-}4_a$, $H\text{-}2_a$). δC($CDCl_3$) 173.2 (C-1′), 162.0 (d, $^1J_{CF}$ = 245.3 Hz, C-4′′), 132.0 (d, $^4J_{CF}$ = 3.4 Hz, C-1′′), 130.9 (d, $^3J_{CF}$ = 7.9 Hz, C-2′′, C-6′′), 115.3 (d, $^2J_{CF}$ = 21.3 Hz, C-3′′, C-5′′), 71.3 (C-2′ or C-3), 69.3 (C-3 or C-2′), 59.7 (C-1, C-5), 40.3 (NMe or C-3′), 39.6 (C-3′ or NMe), 36.4 (C-2, C-4), 25.4 (C-6, C-7). δF($CDCl_3$) -116.4 (m, 1F, Ar-F). *m/z* (EI+) 307 (M^+, 25.02%), 140 (M^+-167, 15.06%), 124 (M^+-183, 100%). Calcd. mass for $C_{17}H_{22}F_1NO_3$: 307.158372. Found: 307.158504.

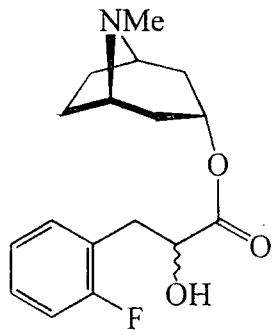
Preparation of DL-3′-fluorophenyllactoyltropine [3′-fluorolittorine, (96b)]



96b

The above preparation for 4′-fluorolittorine was repeated using previously prepared 3′-fluorophenyl-DL-lactic acid (201mg, 1.1mmol) and tropine (155mg, 1.1mmol). 3′-Fluorolittorine (51mg, 15%) was obtained as a pale yellow gum after purification on Hyflo® (10g), containing phosphate buffer (2ml, 0.5mol dm^{-3}) at pH 6.6 and eluting with successive volumes (50ml) of light petroleum, ether and chloroform.[154] ν_{max} 2948, 1740, 1620, 1592, 1492, 1452, 1254 cm^{-1}. GC (MSTFA), 3′-fluorolittorine (92.5%), 3′-fluorophenyl-DL-lactic acid (7.5%). δH($CDCl_3$) 7.22-7.29 (m, 1H, Ar-H), 6.90-7.02 (m, 3H, Ar-H), 5.05 (t, ^3J = 4.8 Hz, 1H, H-3_e), 4.37 (dd, ^3J = 4.4 Hz, ^3J = 6.8 Hz, 1H, -C<u>H</u>), 3.11 (dd, ^3J = 4.4 Hz, ^2J = 14.4 Hz, 1H, -C<u>H</u>H), 3.11-3.16 (br, 2H, H-1, H-5), 2.96 (dd, ^3J = 7.2 Hz, ^2J = 14.0 Hz, 1H, -CH<u>H</u>), 2.29 (s, 3H, NMe), 2.14-2.24 (m, 2H, H-2_e, H-4_e), 1.98-2.04 (m, 2H, H-6_e, H-7_e), 1.79 (d, J = 8.0 Hz, 2H, H-7_a, H-6_a), 1.66 (t, ^2J = 16.4 Hz, 2H, H-4_a, H-2_a). δC($CDCl_3$) 173.4 (C-1′), 163.0 (d, $^1J_{CF}$ = 245.7 Hz, C-3′′), 139.2 (d, $^3J_{CF}$ = 7.5 Hz, C-1′′), 130.1 (d, $^3J_{CF}$ = 8.3 Hz, C-5′′), 125.3 (d, $^4J_{CF}$ = 2.3 Hz, C-6′′), 116.6 (d, $^2J_{CF}$ = 21.0 Hz, C-2′′ or C-4′′), 114.0 (d, $^2J_{CF}$ = 20.9 Hz, C-4′′ or C-2′′), 71.4 (C-2′ or C-3), 69.5 (C-3 or C-2′), 60.0 (C-1, C-5), 40.5 (NMe or C-3′), 40.4 (C-3′ or NMe), 36.6 (C-2 or C-4), 36.5 (C-4 or C-2), 25.7 (C-6, C-7). δF($CDCl_3$) -113.8 (m, 1F, Ar-F). *m/z* (EI+) 307 (M^+, 19.62%), 140 (M^+-167, 13.92%), 124 (M^+-183, 100%). Calcd. mass for $C_{17}H_{22}F_1NO_3$: 307.158372. Found: 307.158538.

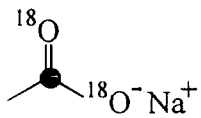
Preparation of DL-2′-fluorophenyllactoyltropine [2′-fluorolittorine, (96a)]



96a

The above preparation for 4′-fluorolittorine was repeated using previously prepared 2′-fluorophenyl-DL-lactic acid (180mg, 0.98mmol) and tropine (138mg, 0.98mmol). 2′-Fluorolittorine (42mg, 14%) was obtained as a pale yellow oil after purification on Hyflo® (10g), containing phosphate buffer (2ml, 0.5mol dm^{-3}) at pH 6.6 and eluting with successive volumes (50ml) of light petroleum, ether and chloroform).[154] ν_{max} 2923, 1739, 1587, 1493, 1461, 1377, 1260 cm^{-1}. GC (MSTFA), 2′-fluorolittorine (80%), 2′-fluorophenyl-DL-lactic acid (20%). δH($CDCl_3$) 7.20-7.30 (m, 2H, Ar-H), 7.00-7.11 (m, 2H, Ar-H), 5.01-5.10 (br, 1H, H-3_e), 4.40 (dd, ^3J = 5.2 Hz, ^3J = 7.2 Hz, 1H, -CH), 3.17 (dd, ^3J = 4.8 Hz, ^2J = 14.0 Hz, 1H, -CHH), 3.16-3.24 (br, 2H, H-1, H-5), 3.01 (dd, ^3J = 7.2 Hz, ^2J = 13.6 Hz, 1H, -CHH), 2.33 (s, 3H, NMe), 2.20-2.32 (m, 2H, H-2_e, H-4_e), 1.96-2.08 (m, 2H, H-6_e, H-7_e), 1.78-1.92 (m, 2H, H-7_a, H-6_a), 1.75 (d, ^2J = 14.4 Hz, 1H, H-2_a), 1.65 (d, ^2J = 14.4 Hz, 1H, H-4_a). δC($CDCl_3$) 173.4 (C-1′), 161.2 (d, $^1J_{CF}$ = 245.3 Hz, C-2′′), 131.9 (d, $^3J_{CF}$ = 4.2 Hz, C-6′′ or C-4′′), 128.8 (d, $^3J_{CF}$ = 8.4 Hz, C-4′′ or C-6′′), 124.1 (d, $^4J_{CF}$ = 3.1 Hz, C-5′′), 123.4 (d, $^2J_{CF}$ = 15.6 Hz, C-1′′), 115.3 (d, $^2J_{CF}$ = 22.1 Hz, C-3′′), 70.3 (C-2′or C-3), 68.9 (C-3 or C-2′), 59.9 (C-1, C-5), 40.0 (NMe), 36.1 (C-2 or C-4), 35.8 (C-4 or C-2), 34.1 (C-3′), 25.3 (C-6 or C-7), 25.1 (C-7 or C-6). δF($CDCl_3$) -118.2 (m, 1F, Ar-F). *m/z* (EI+) 307 (M^+, 23.67%), 140 (M^+-167, 12.47%), 124 (M^+-183, 100%). Calcd. mass for $C_{17}H_{22}F_1NO_3$: 307.158372. Found: 307.159058.

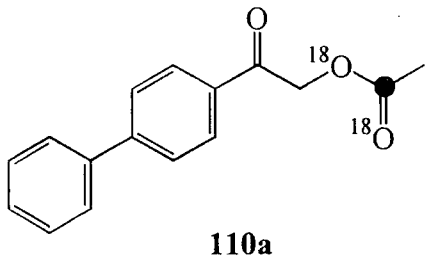
Preparation of sodium [1-^{13}C,$^{18}O_2$]-acetate [^{13}C, 100%, ^{18}O, 47%; $^{18}O_2$, 26% (7j)][161]



7j

Iodomethane (870mg, 6.13mmol) and $K^{13}CN$ (405mg, 6.13mmol) were heated under reflux in anhydrous methanol (6ml) for 2 hours and then the solution was distilled leaving a solid residue. A solution of potassium t-butoxide in t-butanol (8ml, 0.77mmol dm^{-3}) was added to the distillate, followed by $H_2{}^{18}O$ (0.3ml, 98atom%, 2.5eq.) and the mixture was then heated under reflux for another 72 hours. The solution was then evaporated under reduced pressure and the residue dissolved in water (5ml), acidified to pH 1 (50% H_2SO_4) and lyophilised. The lyophilisate was taken to pH 9 (10% NaOH) and was then freeze-dried to afford sodium [1-^{13}C,$^{18}O_2$]-acetate (449mg, 5.16mmol, 84%); δH(D_2O) 1.86 (d, $^2J_{13CH}$ = 5.9 Hz, 3H, -CH_3). δC(D_2O) 181.51 [s, C-1, ($^{13}C^{16}O_2$)], shoulder at 181.46 [s, C-1, ($^{13}C^{18}O_2$)], 23.3 (d, ^1J = 52.3 Hz, C-2). Accurate isotopic composition was determined after preparation of the para-phenylphenacyl derivative as described below.

Preparation of 4-phenylphenacyl-[1-^{13}C,$^{18}O_2$]-acetate [^{13}C, 100%, ^{18}O, 47%; $^{18}O_2$, 26% (110a)][161]



110a

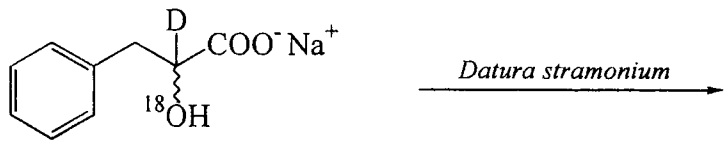
A sample of sodium [1-^{13}C,$^{18}O_2$]-acetate (28mg, 0.32mmol), was added to a solution of 4-phenylphenacylbromide (103mg, 0.37mmol, 1.2eq.) and 18-crown-6 (12mg) in a toluene/acetonitrile solvent mixture (8ml, 1:1). The reaction was heated under reflux for 24 hours, cooled to room temperature and was then evaporated under reduced pressure to leave a solid residue. Purification by column chromatography over silica gel using toluene as the eluent, gave the desired product as a white solid (10mg, 0.04mmol, 12%). δH($CDCl_3$) 8.00 (d, ^3J = 8.1 Hz, 2H, Ar-H), 7.71 (d, ^3J = 8.1 Hz, 2H, Ar-H), 7.63 (d, ^3J = 8.7 Hz, 2H, Ar-H), 7.38-7.52 (m, 3H, Ar-H), 5.38 (d, $^3J_{13CH}$ = 4.5 Hz, 2H, -CH_2), 2.25 (d, $^2J_{13CH}$ = 6.9 Hz, 3H, -CH_3). GC-MS data is shown in Table 18.

	m/z (EI+)	Ion	% incorporation
M	254	$(PhC_6H_4COCH_2OCOCH_3)^+$	0
M+1	255	$(PhC_6H_4COCH_2O^{13}COCH_3)^+$	24.28
M+2	256		1.78
M+3	257	$(PhC_6H_4COCH_2O^{13}C^{18}OCH_3)^+$	46.80
M+4	258		0.99
M+5	259	$(PhC_6H_4COCH_2{}^{18}O^{13}C^{18}OCH_3)^+$	26.08
M+6	260		0.07

Table 18 Ion distribution and incorporation levels of 4-phenylphenacyl-[1-^{13}C,$^{18}O_2$]-acetate [(**110a**), *m/z* 254 (M^+)] after GC-MS analysis. Values in the table are adjusted after considering natural abundance and demonstrate an isotope content of ^{13}C, 100%; ^{18}O, 47% and $^{18}O_2$, 26% in the sample.

5.4 Feeding Experiment Procedures

Feeding sodium DL-[2-^{2}H,^{18}O]-phenyllactate (51f) to *Datura stramonium* root cultures

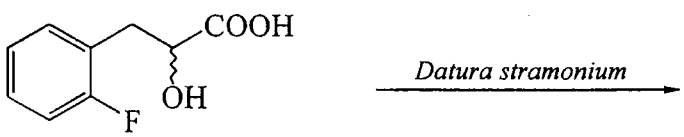


51f

To eight subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a previously prepared sterile solution of sodium DL-[2-^{2}H,^{18}O]-phenyllactate (11.3mmol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 0.64mmol dm^{-3} in the medium. Two flasks of roots were harvested and freeze-dried after days 11, 13, 15 and 17.

The freeze-dried roots (d11, 0.6g; d13, 0.6g; d15, 0.7g; d17, 0.6g) were ground with washed acid sand and extracted into H_2SO_4 (15ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (d11, 6mg; d13, 10mg; d15, 12mg; d17, 9mg) which was submitted for GC-MS analysis.

Feeding 2′-fluorophenyl-DL-lactic acid (95a) to *Datura stramonium* root cultures

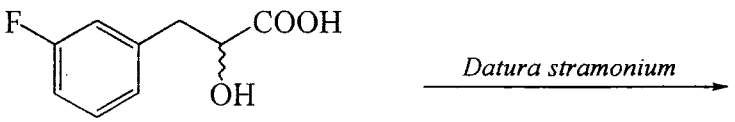


95a

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution (1ml) of 2′-fluorophenyl-DL-lactic acid (54.3mmol dm^{-3}) dissolved in MeOH was fed on day 7 to a final concentration of 0.1mmol dm^{-3} in the medium. Two flasks of roots were harvested and freeze-dried after days 11, 14 and 17.

The freeze-dried roots (d11, 0.6g; d14, 0.6g; d17, 0.6g) were ground with acid washed sand and extracted into H_2SO_4 (10ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated under reduced pressure to a brown oil (d11, 7mg; d14, 8mg; d17, 10mg) which was submitted for GC-MS analysis.

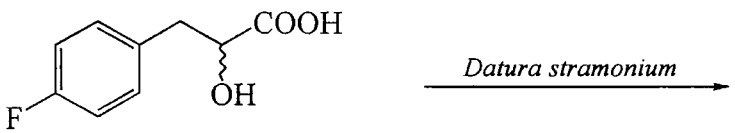
Feeding 3′-fluorophenyl-DL-lactic acid (95b) to *Datura stramonium* root cultures



95b

The above procedure for 2′-fluorophenyl-DL-lactic acid was repeated in parallel for 3′-fluorophenyl-DL-lactic acid. The freeze-dried roots obtained (d11, 0.5g; d14, 0.6g; d17, 0.5g) yielded a brown oil (d11, 14mg; d14, 8mg; d17, 7mg) which was submitted for GC-MS analysis.

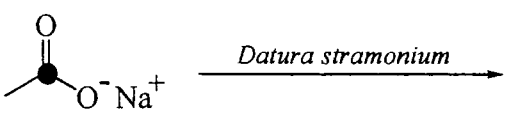
Feeding 4′-fluorophenyl-DL-lactic acid (95c) to *Datura stramonium* root cultures



95c

The above procedure for 2′-fluorophenyl-DL-lactic acid was repeated in parallel for 4′-fluorophenyl-DL-lactic acid. The freeze-dried roots obtained (d11, 0.5g; d14, 0.6g; d17, 0.6g) yielded a brown oil (d11, 10mg; d14, 9mg; d17, 10mg) which was submitted for GC-MS analysis.

Feeding sodium [1-^{13}C]-acetate (7f) to *Datura stramonium* root cultures

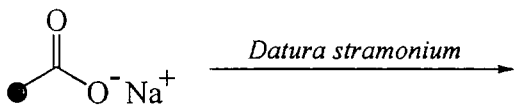


7f

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of sodium [1-^{13}C]-acetate (0.125mol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 9.29mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (0.8g) were ground with washed acid sand and extracted into H_2SO_4 (20ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (22mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (4mg).

Feeding sodium [2-^{13}C]-acetate (7g) to *Datura stramonium* root cultures

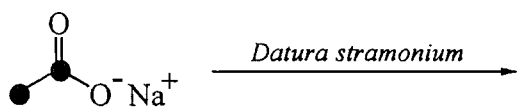


7g

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of sodium [2-^{13}C]-acetate (0.125mol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 9.29mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (1.3g) were ground with washed acid sand and extracted into H_2SO_4 (30ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (25mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (6mg).

Feeding sodium [1,2-$^{13}C_2$]-acetate (7b) to *Datura stramonium* root cultures (I)

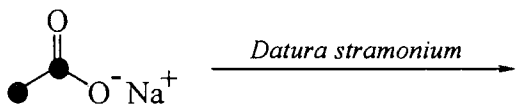


7b

To six subcultured flasks each containing an initial inoculum (0.5g) of fresh mass of roots in culture medium (50ml), a sterile solution of sodium [1,2-$^{13}C_2$]-acetate (0.248mol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 18.4mmol dm^{-3} in the medium. The roots were harvested and freeze dried after 17 days.

The freeze dried roots (0.6g) were ground with acid washed sand and extracted into H_2SO_4 (15ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (10mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (3mg).

Feeding sodium [1,2-$^{13}C_2$]-acetate (7b) to *Datura stramonium* root cultures (II)

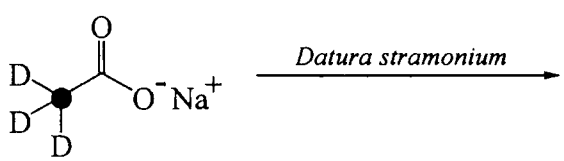


17

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of sodium [1,2-$^{13}C_2$]-acetate (62.0mmol dm^{-3}) diluted with unlabelled sodium acetate (63.4mmol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 9.29mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (1.8g) were ground with washed acid sand and extracted into H_2SO_4 (40ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (36mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (3mg).

Feeding sodium [2-^{13}C,$^{2}H_3$]-acetate (7e) to *Datura stramonium* root cultures

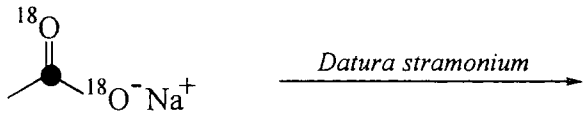


7e

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of sodium [2-^{13}C,$^{2}H_3$]-acetate (0.243mol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 18.0mmol dm^{-3} in the medium. The roots were harvested and freeze dried after 17 days.

The freeze dried roots (0.4g) were ground with washed acid sand and extracted into H_2SO_4 (10ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (7mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (5mg), which was submitted for ^{13}C-NMR and GC-MS analysis.

Feeding sodium [1-^{13}C,$^{18}O_2$]-acetate (7j) to *Datura stramonium* root cultures

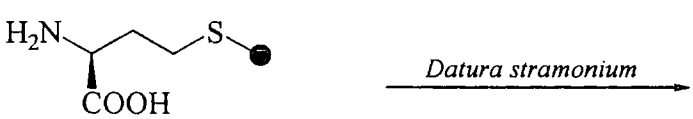


7j

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a previously prepared sterile solution of sodium [1-^{13}C,$^{18}O_2$]-acetate (59.8mmol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 4.43mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (1.8g) were ground with washed acid sand and extracted into H_2SO_4 (40ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (30mg) which was submitted for GC-MS analysis (see Chapter 4).

Feeding L-[^{13}C-*methyl*]- methionine (105a) to *Datura stramonium* root cultures

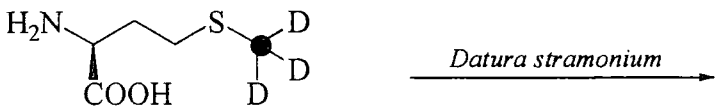


105a

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of L-[^{13}C-*methyl*]-methionine (27.7mmol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 2.05mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (1.0g) were ground with washed acid sand and extracted into H_2SO_4 (20ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (13mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (1mg).

Feeding L-[^{13}C,^{2}H$_3$-*methyl*]-methionine (105b) to *Datura stramonium* root cultures (I)

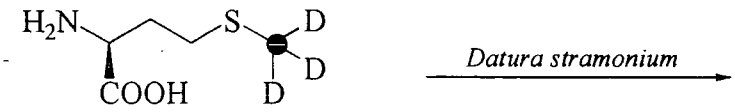


105b

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of L-[^{13}C,^{2}H$_3$-*methyl*]-methionine (40.5mmol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 3.00mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (1.5g) were ground with washed acid sand and extracted into H_2SO_4 (30ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (30mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (4mg).

Feeding L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (105b) to *Datura stramonium* root cultures (II)



105b

To six subcultured flasks each containing an initial inoculum of 0.5g fresh mass of roots in culture medium (50ml), a sterile solution of L-[^{13}C,$^{2}H_3$-*methyl*]-methionine (27.2mmol dm^{-3}) was pulse fed on days 5, 7 and 9 to a final concentration of 2.01mmol dm^{-3} in the medium. The roots were harvested and freeze-dried after 17 days.

The freeze-dried roots (1.2g) were ground with washed acid sand and extracted into H_2SO_4 (25ml, 0.05mol dm^{-3}) by stirring for 20 minutes. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$-MeOH (20:1). The eluent was evaporated to a brown oil (15mg) which was purified by preparative TLC ($CHCl_3$-Et_2NH, 9:1) to give hyoscyamine (1mg).

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Appendix

Research conferences attended

1-10-1995 – 30-9-1996

8th December, 1995
RSC Perkin Regional Meeting,
University of Durham, Durham, England

18th December, 1995
RSC Postgraduate Symposium (Perkin Division – Bio-organic Chemistry),
University of Southampton, Southampton, England

2nd April, 1996
North East Graduate Symposium,
University of Sunderland, Sunderland, England

12-15th September, 1996
RSC Highland Meeting in Bio-organic Chemistry,
Crieff, Perthshire, Scotland

1-10-1996 – 30-9-1997

16th December, 1996
RSC Postgraduate Symposium (Perkin Division – Bio-organic Chemistry),
University of Liverpool, Liverpool, England

17th December, 1997
26th RSC Scottish Regional Meeting, Perkin Division,
University of Strathclyde, Strathclyde, Scotland
(Poster presentation)

1-10-1997 – 30-9-1998

22nd December, 1997
Zeneca Postgraduate Poster Symposium,
University of Durham, Durham, England
(Poster presentation)

7th April, 1997
North East Universities Postgraduate Symposium
University of Newcastle, Newcastle, England

6-9th April, 1998
RSC National Congress 1998 and Young Researchers' meeting,
University of Durham, Durham, England
(Poster presentation)

15-18th May, 1998
32nd European Symposium on Bio-organic Chemistry (ESBOC),
Gregynog, Powys, Wales

29th August – 2nd September, 1998
12th European Symposium on Fluorine Chemistry,
Freie Universität Berlin, Berlin, Germany
(Poster presentation)

Colloquia, lectures and seminars attended from invited speakers

1-10-1995 – 30- 9-1996

18th October, 1995

Prof. A. Alexakis, University of Pierre and Madame Curie, Paris, France

"Synthetic and analytical uses of chiral diamines"

1st November, 1995

Prof. W. Motherwell, University College London, London, England

"New reactions for organic synthesis"

8th November, 1995

Dr. D. Craig, Imperial College London, London, England

"New strategies for the assembly of heterocyclic systems"

15th November, 1995

Dr. A. Sella, University College London, London, England

"Chemistry of the Lanthanides with polypyrazoylborate systems"

17th November, 1995

Prof. D. Bergbrieter, Texas A&M, USA

"Design of smart catalysts, substrates and surfaces from simple polymers"

10th January, 1996

Dr. B. Henderson, Waikato University, New Zealand

"Electrospray mass spectrometry – a new sporting technique"

24th January, 1996

Dr. A. Armstrong, University of Nottingham, Nottingham, England

"Alkene oxidation and natural product synthesis"

14th February, 1996

Dr. J. Rohr, University of Göttingen, Göttingen, Germany

"Goals and aspects of biosynthetic studies on low molecular weight natural products"

28th February, 1996

Prof. E. W. Randall, Queen Mary and Westfield College, London, England

"New perspectives in NMR imaging"

6th March, 1996

Dr. R. Whitby, University of Southampton, Southampton, England

"New approaches to chiral catalysts: Induction of planar and metal centred asymmetry"

12th March, 1996

Prof. V. Balzani, University of Bologna, Bologna, Italy

"Supramolecular photochemistry" – RSC endowed lecture

13th March, 1996

Prof. D. Garner, Manchester University, Manchester, England

"Mushrooming in chemistry"

1-10-1996 – 30-9-1997

16th October, 1996

Prof. Ojima, State University of New York at Stony Brook, USA

"Silylformylation and silylcarbocyclisations in organic synthesis"

22nd October, 1996

Prof. B. J. Tighe, University of Aston, Birmingham, England

"Making polymers for biomedical application – can we meet Nature's challenge?"

23rd October, 1996

Prof. H. Ringsdorf, Johannes Gutenburg-Universität, Mainz, Germany

"Function based on organisation"

29th October, 1996

Prof. D. M. Knight, Department of Philosophy, University of Durham, Durham, England

"The purpose of experiment – a look at Davy and Faraday"

6th November, 1996

Dr. K. Reid, University of Nottingham, Nottingham, England

"Probing dynamical processes with photoelectrons"

12th November, 1996

Prof. R. J. Young, Manchester Materials Centre, University of Manchester Institute of Science and Technology, Manchester, England

"New materials – fact or fantasy?"

13th November, 1996

Dr. G. Resnati, University of Milan, Milan, Italy

"Perfluorinated oxaziridines: Mild yet powerful oxidising agents"

18th November, 1996

Prof. G. A. Olah, University of Southern California, USA

"Crossing conventional lines in my chemistry of the elements"

20th November, 1996

Prof. J. Earnshaw, Department of Physics, Queen's University, Belfast, N. Ireland

"Surface light scattering: Ripples and relaxation"

27th November, 1996

Dr. R. Templer, Imperial College, London, England

"Molecular tubes and sponges"

3rd December, 1996

Prof. D. Phillips, Imperial College, London, England

"A little light relief"

11th December, 1996

Dr. C. Richards, University of Cardiff, Cardiff, Wales

"Stereochemical games with metallocenes"

15th January, 1997

Dr. V. K. Aggarwal, University of Sheffield, Sheffield, England

"Sulphur mediated asymmetric synthesis"

16th January, 1997

Dr. S. Brooker, University of Otago, New Zealand

"Macrocycles: Exciting yet controlled thiolate co-ordination chemistry"

12th February, 1997

Dr. G-J. Boons, University of Birmingham, Birmingham, England

"New developments in carbohydrate chemistry"

26th February, 1997

Dr. T. Ryan, University of Manchester Institute of Science and Technology, Manchester, England

"Making hairpins from rings and chains"

4th March, 1997

Prof. C. W. Rees, Imperial College, London, England

"Some very heterocyclic chemistry"

5th March, 1997

Dr. J. Staunton, Cambridge University, Cambridge, England

"Tinkering with biosynthesis – towards a new generation of antibiotics"

1-10-1997 – 30-9-1998

2nd October, 1997

Dr. T. Umemoto, Daikin Corporation, Japan

"Power-variable electrophilic trifluoromethylating agents"

23rd October, 1997

Prof. M. Bryce, University of Durham, Durham, England

"New tetrathiafulvalene and macromolecular chemistry: Controlling the electronic properties of organic solids" - Inaugural lecture

28th October, 1997

Prof. A. P. de Silva, Queen's University, Belfast, N. Ireland

"Luminescent signalling systems"

29th October, 1997

Prof. B. Peacock, University of Glasgow, Glasgow, Scotland

"Probing chirality with circular dichroism"

5th November, 1997

Dr. M. Hii, Oxford University, Oxford, England

"Studies of the Heck reaction"

20th November, 1997

Dr. L. Spiccia, Monash University, Melbourne, Australia

"Polynuclear metal complexes"

26th November, 1997

Prof. R. Richards, University of Durham, Durham, England

"A random walk in polymer science" - Inaugural lecture

2nd December, 1997

Dr. C. J. Ludman, University of Durham, Durham, England

"Explosions"

3rd December, 1997

Prof. A. P. Davis, Trinity College Dublin, Dublin, Ireland

"Steroid frameworks for supramolecular chemistry"

10th December, 1997

Sir G. Higginson, former Prof. of engineering in Durham and retired Vice-chancellor of Southampton University

"1981 and all that"

10th December, 1997

Prof. M. Page, University of Huddersfield, Huddersfield, England

"The mechanism and inhibition of beta-lactamases"

20th January, 1998

Prof. J. Brooke, University of Lancaster, Lancaster, England

"What's in a formula? Some chemical controversies of the 19th century"

21st January, 1998

Dr. T. Fawcett, Biology Department, University of Durham, Durham, England

"The Effects of Cellular Environment on Metabolic Pathways"

28th January, 1998

Dr. S. Rannard, Courtaulds Coatings, Coventry, England

"The synthesis of dendrimers using highly selective chemical reactions"

11th February, 1998

Prof. J. A. Murphy, Strathclyde University, Strathclyde, Scotland

"Synthetic adventures in radical chemistry. TTF as a catalyst in radical reactions"

17th February, 1998

Dr. S. Topham, ICI Chemicals and Polymers, Billingham, England

"Perception of environmental risk – The River Tees, two different views"

18th February, 1998

Prof. G. Hancock, Oxford University, Oxford, England

"Surprises in the photochemistry of tropospheric ozone"

24th February, 1998

Prof. R. Ramage, University of Edinburgh, Edinburgh, Scotland

"The synthesis and folding of proteins"

18th March, 1998

Dr. J. Evans, Oxford University, Oxford, England

"Materials which contract on heating – from shrinking ceramics to bullet proof vests"

5th May, 1998

Prof. A. Eschenmoser, ETH Zurich, Switzerland.

"Why RNA?"

Papers Published

"Tropic acid biosynthesis: the incorporation of (*RS*)-phenyl[2-^{18}O,2-^{2}H]lactate into littorine and hyoscyamine in *Datura stramonium*", C. W. Wong, J. T. G. Hamilton, D. O'Hagan and R. J. Robins, *Chem. Commun.*, 1998, 1045

"Fluorinated tropane alkaloids generated by directed biosynthesis in transformed root cultures of *Datura stramonium*", D. O'Hagan, R. J. Robins, M. Wilson, C. W. Wong, M. Berry and I. Zabetakis, *J. Chem. Soc., Perkin Trans. 1*, 1999, 2117

"Biosynthesis of novel alkaloids 1. Metabolism of N-alkyldiamines and N-alkylnortropinones by transformed root cultures of *Nicotiana* and *Brugmansia*", H. D. Boswell, B. Dräger, J. Eagles, C. McClintock, A. Parr, A. Portsteffen, D. J. Robins, R. J. Robins, N. J. Walton and C. W. Wong, *Phytochemistry*, 1999, **52**, 855

"The biosynthetic relationship between littorine and hyoscyamine in *Datura stramonium*", I. Zabetakis, C. W. Wong, R. Edwards and D. O'Hagan, in *Plant Biotechnology and In Vitro Biology in the 21st Century; Proceedings of the IXth International Congress of the International Association of Plant Tissue Culture and Biotechnology, Jerusalem, Israel, 14-19 June 1998*, A. Altman, M. Ziv and S. Izhar (Eds.), Kluwer Academic Publishers, Dordrecht, 1999, pp. 347-350