STUDIES ON THE BIOSYNTHESIS OF THE TROPANE ALKALOID HYOSCYAMINE IN *DATURA STRAMONIUM*

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DECLARATIONS:

(i) I, Stephen Patterson, hereby certify that this thesis, which is approximately 42,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Stephen Patterson

(ii) I was admitted as a research student in October 1999 and as a candidate for the degree of PhD in Organic Chemistry in September 2000; the higher study for which this is a record was carried out in the University of Durham* between October 1999 and August 2000 and University of St. Andrews* between September 2000 and June 2002.

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ABSTRACT

Studies on the Biosynthesis of the Tropane Alkaloid Hyoscyamine in Datura stramonium

Stephen Patterson B.Sc. (Hons) *Dunelm*

This thesis investigates the biosynthesis of the two moieties of the tropane alkaloid hyoscyamine, both the ester moiety tropic acid and tropine, a structural motif present in many tropane alkaloids. Chapter 2 describes experiments performed using transformed root cultures of *D. stramonium* aimed at determining the biosynthetic precursors of the tropane ring. Feeding experiments with carbon-13 labelled acetates have confirmed that tropine is biosynthesised from ornithine *via* the symmetrical intermediate putrescine and that C2,3,4 of tropine are derived from an acetate derived precursor. A feeding experiment with sodium [$^{18}O_2$]acetate did not result in incorporation of oxygen label into tropine, the implications of which are discussed. The investigations described in chapter 3 are concerned with elucidating the mechanism of the isomerisation of littorine to hyoscyamine, the final transformation in tropic acid biosynthesis. To this deuterium labelled phenyllactic acids were synthesised including (*RS*)-[2,3,3-$^{2}H_3$]-phenyllactate, which upon feeding to *D. stramonium* root cultures produced hyoscyamine containing *two* deuterium atoms. This result demonstrates that a deuterium atom is lost during the transformation, inconsistent with a proposed vicinal interchange mechanism for the isomerisation. Additionally incubation of synthetic (*RS*)-[1'-^{13}C,3',3'-$^{2}H_2$]-hyoscyamine showed that hyoscyamine is stable to oxidation at C3', discounting a proposed mechanism of deuterium loss from feeding labelled phenyllactic acids. Chapter 4 describes attempts to prepare cell free extracts of *D. stramonium* containing alkaloid biosynthetic activities. However, no success was achieved in this part of the research programme. The work contained in chapter 3 has been published in the form of 2 research papers; Patterson & O'Hagan (2002) *J. Labelled Compd. and Radiopharm.*, Patterson & O'Hagan (2002) *Phytochemistry*.

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Thanks must also go to Dr Rosa Duron-Patron for passing on her knowledge of subculturing the organisms used in the study and to Dr Richard Robins from whom the original *Datura stramonium* strain was obtained.
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List of Abbreviations

ATP	adenosine 5'-triphosphate
aux	auxin production
BioB	biotin synthase
CoA	coenzyme A
COSY	correlation spectroscopy
DCC	1,3-dicyclohexylcarbodiimide
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
E-64	*N*-(trans-Epoxysuccinyl)-$_L$-leucine 4-guanidinobutylamide; trans-3-Carboxyoxirane-2-carbonyl-$_L$-leucylagmatine; *trans*-Epoxysuccinyl-$_L$-leucylamido(4-guanidino)butane; $_L$-*trans*-3-Carboxyoxiran-2-carbonyl-$_L$-leucylagmatine
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GC	gas chromatography
GC-CIMS	gas chromatography-chemical ionisation mass spectrometry
GC-EIMS	gas chromatography-electron ionisation mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GLC	gas liquid chromatography
HETCOR	hetero-nuclear correlation
HPLC	high performance liquid chromatography
LAM	lysine 2,3-aminomutase
LDA	lithium diisopropylamide
LiHMDS	lithium hexamethyldisilazane
Me	methyl
MS	mass spectrometry
MSTFA	*N*-methyl-*N*-trimethylsilyl trifluoroacetamide

NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
n-BuLi	n-butyl lithium
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
ODC	ornithine decarboxylase
PCC	pyridium chlorochromate
PLP	pyridoxal phosphate
PMSF	phenylmethanesulfonyl fluoride
PMT	putrescine-*N*-methyltransferase
PVPP	poly(vinylpolypyrrolidone)
Ri	root inducing
Rol	root loci
Rt	reaction time
RT	room temperature
SAM	*S*-Adenosyl methionine
SIM	selective ion monitoring
TCA	tricarboxylic acid
T-DNA	transfer DNA
TFA	trifluoroacetic acid
THF	tetrahydrofuran
t.l.c	thin layer chromatography
TMS	trimethylsilyl
TR-I/II	tropinopne reductase-I/II
u.v	ultraviolet

Chapter 1

Introduction

1 INTRODUCTION

The study of natural products has been of interest to the organic chemist and biologist for over a century. Total synthesis as a method of structural determination helped drive the development of organic chemistry at the turn of the 20th century, and still today much research into synthetic methodology is conducted with the same aim. The complex synthetic problems arise due to the almost limitless variety in structure of the natural products, which can be difficult to elucidate even with modern analytical techniques.

Chemists also have an interest in understanding how nature biosynthesises natural products. Early 20th century chemists could only hypothesise the 'building blocks' of natural products through structural relations.[1,2] Now with the advent of molecular biology and protein chemistry complex biosynthetic systems can be understood at the genetic, enzymological and chemical levels.

1.1 Metabolism and Natural Products

Metabolism is defined as the integrated network of biochemical reactions in a living organism.[3] It is through a combination of catabolic processes; the degradation of complex molecules, and anabolism; the synthesis of complex molecules from simple precursors that organisms derive the molecular resources for natural product biosynthesis.

1.1.1 Primary and Secondary Metabolism

Metabolism and the resultant metabolites are often sub-divided into two categories, primary and secondary. Primary metabolites are those that are central to metabolism and thus are essential for the survival of an organism. Due to their core role the primary metabolites are common to large numbers of, if not all organisms.[4] However, despite their ubiquity the biosynthetic pathways responsible for the production of primary metabolites are not necessarily the same in different organisms. Examples of primary

metabolites include proteins, nucleic acids, carbohydrates, lipids and the photosynthetic pigments of higher plants.

Secondary metabolites are defined as compounds that are not essential for the survival of the organisms that produce them, but improve their biological fitness.[5] Unlike the primary metabolites a specific secondary product is generally produced by a small number of closely related species within the same family or genera. Some secondary metabolites are rarer still being the product of a single species or strain.[4] The line between primary and secondary metabolism is not always clear-cut, some metabolites are not involved in an essential life process, e.g. the structural components of plant cell walls, however, their distribution is not restricted being common to all higher plants.

1.1.2 Biosynthetic Pathways

All biosynthetic processes are inter-linked through a complex series of metabolic pathways. The starting point for these pathways is glucose (**1**); obtained from gluconeogenesis, photosynthesis, dietary uptake or the breakdown of polysaccharides. Through glycolysis, glucose (**1**) is converted into other small molecules e.g. glyceraldehyde-3-phosphate (**2**), phosphoenol pyruvate (**3**) and pyruvate (**4**), which provide the intermediates for core metabolic pathways. Other anabolic and catabolic processes also provide intermediates for core metabolism e.g. fatty acid oxidation yields acetyl CoA (**5**), a molecule central to many biosynthetic pathways (Figure 1.1).

All biosynthetic pathways, both primary and secondary, derive their precursors from intermediates or products of core metabolism e.g. glycolysis[6] (Figure 1.1). The secondary metabolites are formed along specialised pathways derived from primary metabolites.[4]

1.1.3 Function of Secondary Metabolites

As discussed the products of secondary metabolism are not essential for the life of an organism. However, the capacity to biosynthesise these compounds has evolved and been retained by most bacterial, fungal and plant species. Clearly, the production of secondary metabolites must confer a selective advantage upon the producing organism,

enough to compensate for the metabolically expensive production of the metabolites and their dedicated enzyme pathways.

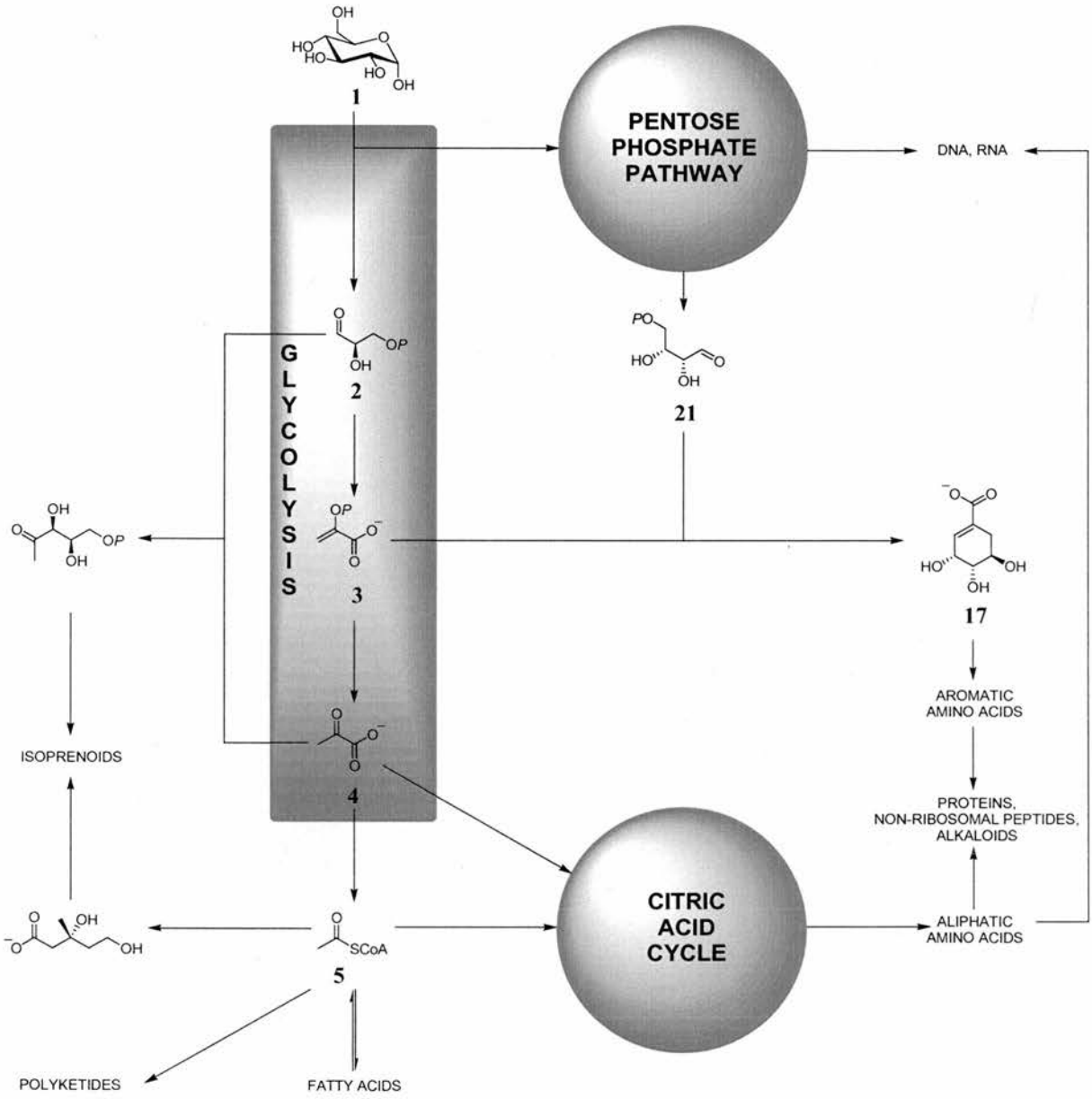


Figure 1.1: The links between primary and secondary biosynthetic pathways The core metabolic pathways are highlighted in blue. Plants and microorganisms also possess the glyoxalate cycle, which provides many of the same intermediates as the citric acid cycle.

Whilst their exact functions in most cases are unknown a number of those produced by plants have been extensively studied and their biological roles determined. Plants utilise specific secondary metabolites to perform a structural role, e.g. suberin and cutin are vital constituents of gas and water impermeable layers and lignin provides mechanical strength to plant cell walls. Secondary metabolites also mediate interactions

between plants and other species, a phenomenon known as chemical ecology.[7] Many plants utilise secondary products as a defence against herbivory, the metabolites produced can be highly toxic, e.g. fluoroacetate produced by *Dichapetalum cymosum*. Other metabolites are both toxic and bitter tasting to deter feeding e.g. caffeine (**6**) produced by the coffee plant *Coffea arabica*.[7] Some plant secondary products act as defensive agents by mimicking insect hormones, e.g. the balsam fir *Abies balsamea* produces juvabione, a compound that mimics insect juvenile hormone and so prevents larval metamorphosis, ultimately resulting in death of the larvae.[8]

Attractive stimuli produced by secondary plant products include the floral volatiles and pigments, which both play an important role in attracting pollinators to plants. The odours produced by the volatile metabolites are specific to the pollinating species, e.g. linalool produced by *Daphne mezereum* attracts the *Colletes* bee. Common pigments include the carotenoids e.g. lycopene providing a red coloration to *Rosa canina* amongst others.[7]

1.1.4 Production of secondary metabolites

In general, secondary metabolites are not continually produced; rather their biosynthetic pathways are tightly regulated by elicitation, controlled by environmental stimuli or the developmental stage of the organism (if multicellular).

If nutrient levels become limiting an organism (or culture) can suspend growth (or replication), and reorganise its metabolism resulting in the initiation of secondary metabolite production.[9] In microbial cultures this is known as the stationary phase. Similarly exogenous chemical stimuli can 'trigger' the production of secondary metabolites in organisms or culture.

In some plants mechanical damage or insect feeding can also elicit the production of secondary metabolites, e.g. the levels of hyoscyamine (**7**) and related alkaloids have been shown to increase by 160% in *Atropa acuminata* upon mechanical damage and slug predation.[7] In multi-cellular organisms the production and accumulation of secondary metabolites can be tissue specific, e.g. salicylaldehyde and other plant antifeedants commonly occur in the leaves or buds of the plant where predation is greatest.[7]

1.2 The Secondary Metabolites

Secondary metabolites are grouped according to common biosynthetic origins. Additionally many metabolites contain structural elements from more than one class of metabolite and are termed 'mixed metabolites'. There are four main classes of secondary metabolites, the polyketides, the isoprenoids, the alkaloids and the shikimic acid derived metabolites. In addition there are metabolites derived from amino acids and the fatty acid metabolites. Despite the relatively small number of groups secondary products display a staggering diversity of structure (Figure 1.2, Figure 1.4). A complete description of the properties and biosynthesis of all the natural product classes is beyond the scope of this introduction. Instead only the shikimic acid metabolites (section 1.2.1) and the alkaloids (section 1.2.2) will be elaborated.

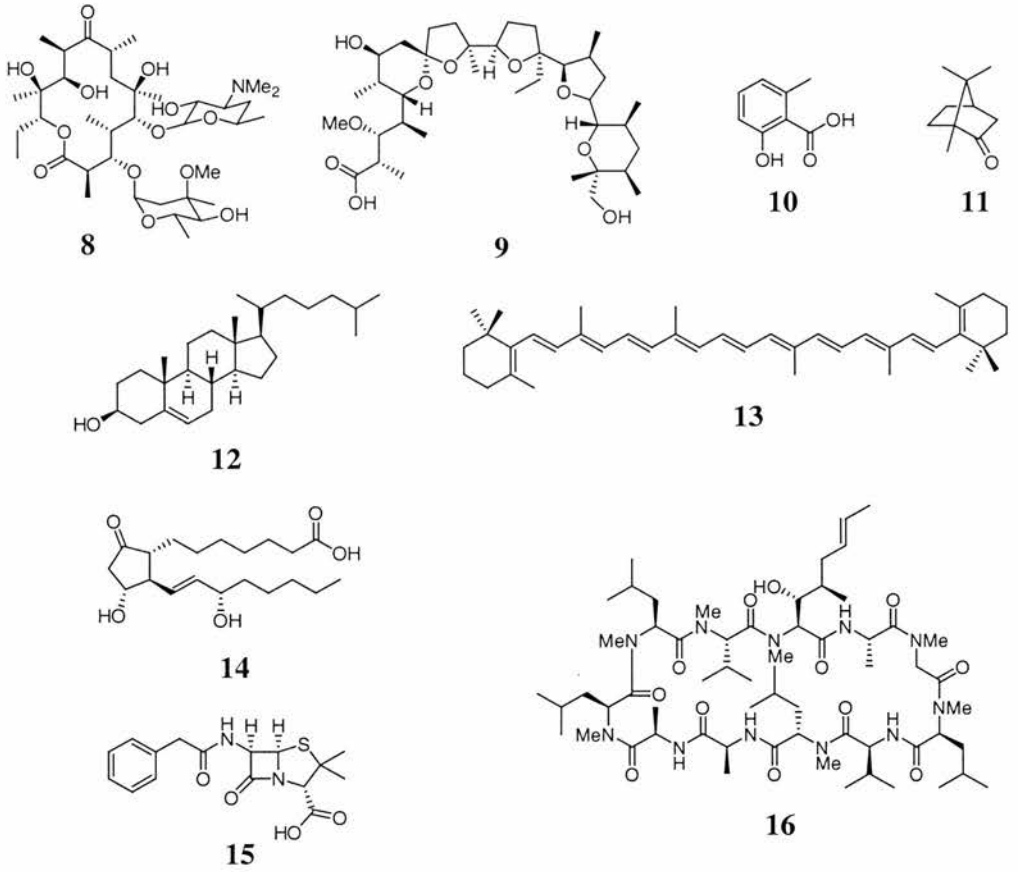


Figure 1.2: Structural variety of the natural products. Above are shown the polyketide metabolites erythromycin A (**8**), monensin A (**9**) and 6-methylsalicylic acid (**10**), the isoprenoids camphor (**11**), cholesterol (**12**) and β-carotene (**13**), the fatty acid metabolite prostaglandin E1 (**14**), the amino acid metabolite penicillin G (**15**) and cyclosporin B (**16**) a non-ribosomal peptide.

1.2.1 Shikimic acid metabolites

In plants and microorganisms shikimic acid (**17**) is the biosynthetic precursor to virtually all aromatic compounds including the aromatic amino acids, phenylalanine (**18**), tyrosine (**19**) and tryptophan (**20**) (Figure 1.3).[10] The shikimic acid pathway is not present in higher animals and they must obtain the essential intermediates through their diet, the exception to which is tyrosine (**19**) which animals can biosynthesise directly from phenylalanine (**18**).

Shikimic acid (**17**) is biosynthesised in several steps from phosphoenolpyruvate (**3**) and erythrose-4-phosphate (**21**). It is then converted into chorismic acid (**22**), again in several enzymatic steps including the addition of another molecule of phosphoenolpyruvate (**3**).[23] Chorismic acid (**22**) is the precursor for the aromatic acids and aromatic amino acids, which are in turn intermediates on several biosynthetic pathways in both primary and secondary metabolism (Figure 1.3).

Phenylalanine (**18**) and tyrosine (**19**) are the biosynthetic precursors of the phenylpropanoids, a group of plant metabolites containing a phenyl ring with a C_3 side chain.[56] Some classes of phenylpropanoid e.g. the flavonoids have a second aromatic ring, which is biosynthesised from three units of malonyl CoA (**23**), and several metabolites e.g. lignin are composed of polymerised phenylpropane units.

As we shall see later phenylalanine (**18**) is also a precursor of tropic acid (**24**) the ester moiety of some tropane alkaloids (section 1.6.1).

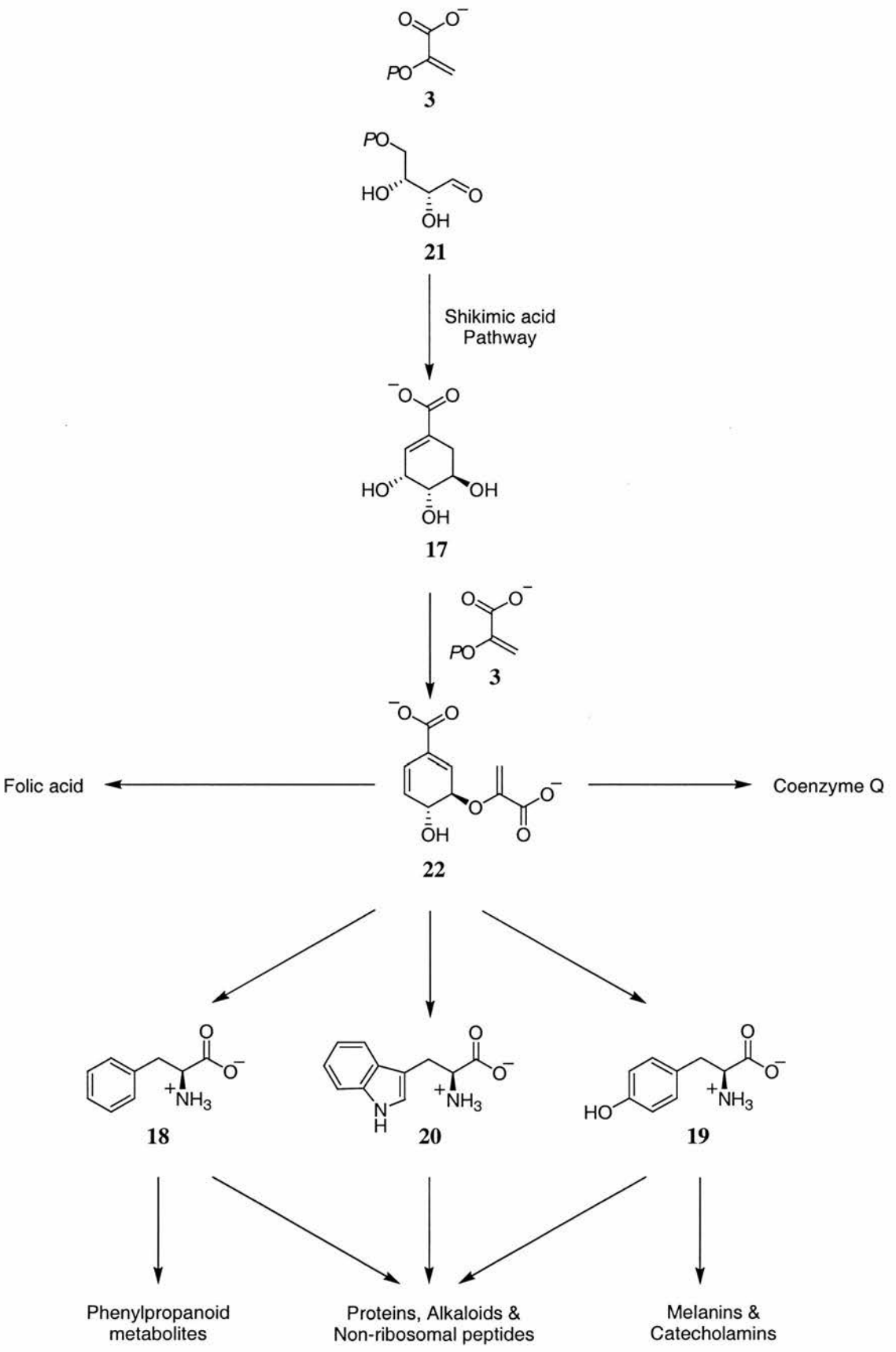
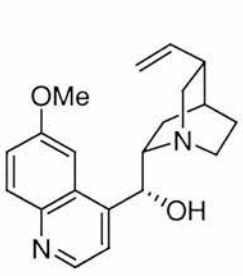


Figure 1.3: Shikimic acid (**17**) the biosynthetic precursor to the amino acids phenylalanine (**18**), tyrosine (**19**) and tryptophan (**20**). Each arrow represents several biosynthetic steps.

1.2.2 Alkaloids

Traditionally the term alkaloid was applied to members of a class of plant natural products of a basic nature, and is derived from the name 'vegetable alkali', which was first applied to these substances.[11,23] A more precise definition of an alkaloid is a non-peptidic, non-nucleosidic compound containing one or more nitrogen atoms as constituents of heterocycles.[23,56] There are also a number of compounds which do not contain their nitrogen as part of a heterocycle e.g. mescaline (**25**), although it is more correct to classify these as biological amines, or protoalkaloids,[12] they are commonly classified as true alkaloids.

More than 10,000 alkaloid structures are known,[56] the vast majority of which are produced by plant species. The alkaloids are sub-divided into several classes relating to the nitrogen heterocycle that they contain (Figure 1.4). These nitrogen heterocycles are biosynthesised from amino acid precursors, the remainder of the alkaloid structure being derived from various small molecules. However, the biosynthetic pathways to the different classes of alkaloids have little in common and it is beyond the scope of this thesis to describe each individually. The biosynthesis of the tropane ring system from the amino acid ornithine (**26**) is described in detail in section 1.5.1.1.



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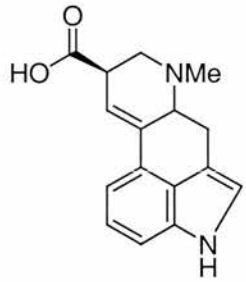

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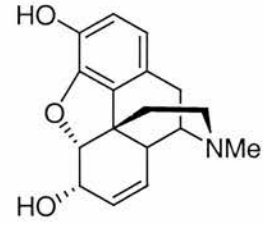

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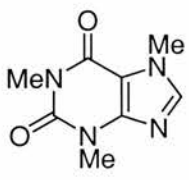

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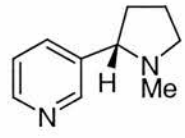

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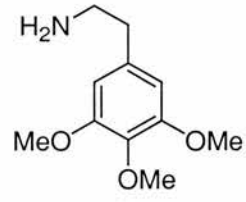

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Figure 1.4: The alkaloids quinine (**27**), lysergic acid (**28**), morphine (**29**), caffeine (**6**), nicotine (**30**), and mescaline (**25**).

1.3 Investigating Biosynthesis

After the structure of a metabolite has been deduced through total synthesis, crystallographic or spectroscopic analysis it becomes of interest to determine the biosynthetic route to the metabolite. Biosynthetic studies on secondary metabolites can lead to the discovery of novel enzyme activities, and a complete understanding of a biosynthetic pathway allows the production of novel metabolites by directed biosynthesis. At the start of the 20th century it was impossible to elucidate a biosynthetic pathway, only hypotheses could be formulated from observation of structural relationships and known chemical reactivities.[1] Now with developments in NMR and isotopic labelling studies, protein chemistry and molecular biology it is possible for metabolic pathways to be elucidated in the finest detail.

1.3.1 Use of Isotopes

Over the last fifty years our understanding of metabolic pathways has greatly increased with the introduction of isotopic 'feeding' experiments; the administration of an isotopically labelled compound to a growing organism or culture. The isotopes used fall into two categories; radioactive and stable. Early studies generally utilised radioactive isotopes, however, more recently stable isotope techniques have developed as the methodology of choice.

1.3.1.1 Radioactive isotopes

The radioisotopes, which were commonly used in biosynthetic studies are tritium (hydrogen-3), carbon-14, and to a lesser extent phosphorus-32 and sulfur-35 (Table 1.1). They are used in 'tracer' or 'feeding' experiments, whereby a putative precursor compound is synthesised containing a radioisotope, administered to a growing organism or culture, and the metabolite(s) of interest isolated and analysed for radioactivity. The radiation emitted (β^-) from the fission of radioactive nuclei can be detected and quantified by the use of a scintillation counter.

Radioisotope	Radiation emitted	Half life
^{3}H	β	12.33 years
^{14}C	β	5730 years
^{32}P	β	14.28 days
^{35}S	β	87.4 days

Table 1.1: Selected properties of radioisotopes commonly used in biological experiments.[13]

Photosynthesis was one of the first biosynthetic pathways to be investigated with the aid of radioisotope feeding experiments.[14] In a series of experiments[14,15,16,17] Ruben and co-workers exposed green plants and *Chlorella* to an atmosphere containing [^{11}C]carbon dioxide, followed by isolation of amino acids, pigments, sugars, organic acids *etc.* and individually determining their radioactivity levels. However, carbon-11 has a short half-life (22 minutes), which limited its usefulness in biosynthetic investigations.

The discovery and post-war production of carbon-14 in large quantities gave chemists a more convenient tool for investigating biosynthesis. Carbon-14 has a half-life of 5730 years (Table 1.1), which allows organic synthesis of more complex biosynthetic precursors, and extensive chromatographic separation of extracted metabolites, effectively without loss of specific activity. For example Calvin utilised [^{14}C]carbon dioxide to elucidate the photosynthesis cycle which is named after him. The use of chromatography was important to identify the radioactive products of his feeding experiments.

In addition to determining whether an isotopic label is incorporated into a metabolite, it is also important that the position(s) at which the molecule is labelled is elucidated (regiospecificity). For molecules labelled with carbon-14 this requires a well-characterised, stepwise degradation procedure. Generally the degradation products (or derivatives of) are recrystallised to constant radioactivity and then individually analysed for levels of radioactivity and the position of the label in the original molecule deduced. It may be necessary to degrade the molecule to a single carbon entity in order to determine the position of label with complete certainty. The process is exemplified by the chemical degradation of tropic acid (**24**) (Figure 1.5). For the analysis of common

primary metabolites e.g. glucose (**1**) it is also possible to perform degradation with microorganisms followed by chemical procedures and analysis.[18] Similar degradation procedures are required when using isotopes other than carbon in feeding experiments.

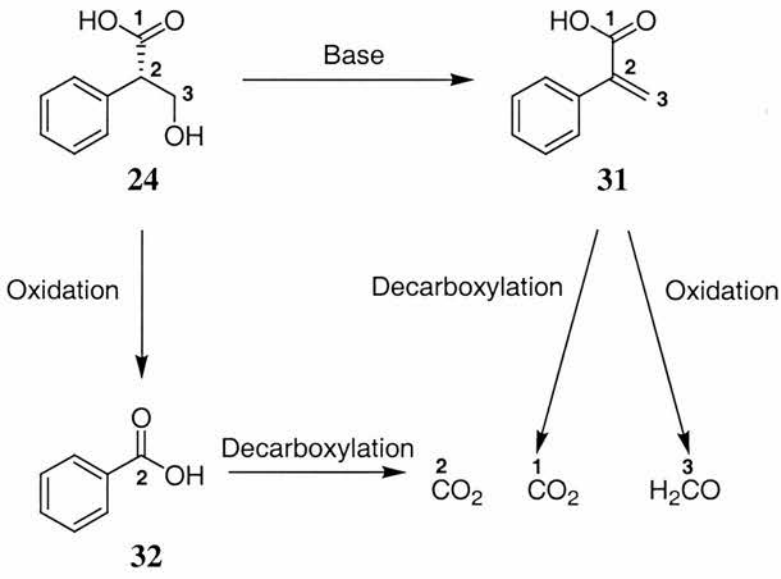


Figure 1.5: The chemical degradation of tropic acid (**24**) to allow the separate analysis of each aliphatic carbon.[19] Treatment of tropic acid (**24**) with strong base produces atropic acid (**31**), which can be decarboxylated to give C-1 separate from all other carbon as carbon dioxide. In addition, periodate oxidation of atropic acid (**31**) liberates C-3 as formaldehyde. Finally C-2 was isolated as carbon dioxide by permanganate oxidation of tropic acid (**24**) to give benzoic acid (**32**), which is in turn decarboxylated.[19]

Tritium can be detected by ^{3}H-NMR spectroscopy (Table 1.2) in addition to scintillation counting. The chemical shifts and coupling constants for tritium are similar to those of the equivalent ^{1}H-NMR spectrum, allowing the position of incorporation to be assigned by reference to the ^{1}H-NMR spectrum of the molecule of interest.[20] However, in practice ^{3}H-NMR is rarely used as a tool for biosynthetic investigation. Experiments using both ^{3}H and ^{14}C labelled compounds have proved important in the history of biosynthetic experiments. In these experiments it is also possible to determine the position of incorporation of tritium by treating the compound of interest with base, a reduction in the ^{3}H:^{14}C ratio indicating that the tritium is present at an acidic position.

Whilst radioisotopes proved a useful tool in the investigation of biosynthetic pathways, there are also several disadvantages associated with their use. Foremost their radioactive nature and long half lives make them hazardous to use; laboratory work with radioisotopes must be carried out with great care, by trained researchers, and in specially designated areas.[13] Additionally, legislative controls make working with radioisotopes problematic. Furthermore, as NMR spectroscopic methodology has become more advanced the need for extensive degradation experiments to determine the structures of isolated metabolites is no longer necessary. Consequently the use of radioisotopes has largely been replaced with the use of stable isotopes (see section 1.3.1.2).

The use of radiolabelled compounds is still useful for a number of research applications. The very low level of incorporation required for detection has led to their continued use in identifying intermediates which accumulate at low levels in biosynthetic pathways, and tritium is essential in the use of chiral methyl group methodology (see section 1.6.2).

1.3.1.2 Stable isotopes

The stable isotopes commonly used in biosynthetic studies are deuterium (hydrogen-2), carbon-13, nitrogen-15 and oxygen-18. Feeding experiments with stable isotope containing compounds are carried out in a similar manner to those containing a radioisotope. However, the feeding concentrations are higher (not trace) and the methods of detecting the label in the isolated metabolite rely on spectroscopy. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the two principal methods for the detection of stable isotopes in metabolites.

1.3.1.2.1 Mass spectrometry

The presence of isotopic nuclei can be detected by mass spectrometric analysis of the metabolite of interest. Simply, if an isotope label is present within the sample this will result in an increased *m/z* ratio of the molecular ion (and possibly molecular fragments) proportional to the mass difference between isotopes and the number of heavy isotopes in the sample. MS methods are also quantitative; the percentage level of isotope incorporation being calculated by comparison of the spectra from natural abundance

standards. However, whilst mass spectrometry allows assignment of the isotope label to a particular molecule or molecular fragment, the precise position(s) of the isotope label within the sample molecule cannot always be achieved, analysis may also be complicated if there are multiple positions of incorporation in the metabolite. If the isotope label is incorporated concurrently at multiple positions in the metabolite then the increase in *m/z* of the molecular ion will correspond to the multiple enrichments. However, if isotope label is incorporated at several individual positions, non-concurrently then observation of the molecular ion's *m/z* will not indicate the multiple incorporation sites. However, the *m/z* of individual molecular ions may still enable detection of multiple incorporation sites. Non-concurrent positions of incorporation can however be detected by NMR analysis (see below).

1.3.1.2.2 NMR spectroscopy

Advances in instrumentation and the introduction of sensitive Fourier Transform techniques have resulted in NMR spectroscopy becoming a viable technique to study the incorporation of stable isotopes. Deuterium, tritium, carbon-13 and nitrogen-15 are all directly observable by NMR (Table 1.2) whereas, the incorporation of oxygen-18 (and other nuclei) can be detected indirectly by heavy atom induced shifts in ^{13}C-NMR analysis.

Nuclei	Natural abundance (%)	Relative sensitivity (^{1}H = 1.00)	Spin (*I*)
^{2}H	1.5×10^{-2}	9.65×10^{-3}	1
^{3}H	0	1.21	½
^{13}C	1.11	1.59×10^{-2}	½
^{15}N	0.37	1.04×10^{-3}	½

Table 1.2: The magnetic properties of selected isotopic nuclei.[21]

Feeding experiments with ^{13}C labelled precursors can lead to isotopic enrichment in metabolites where the resultant ^{13}C-NMR spectra contain enhanced peak heights relative to spectra derived from natural abundance samples. The exact position(s) of incorporation can also be determined without complex degradation studies, as an enriched peak(s) can be assigned to a single carbon in the metabolite, providing that a

full assignment of the ^{13}C-NMR spectra is available. As ^{13}C-NMR has become an integral part of structural elucidation a full spectral assignment has invariably been deduced.

There are two methods for quantifying the level of incorporation of carbon-13; direct measurement of the increase in peak area of resonances in the ^{13}C-NMR spectrum, or indirectly, by measuring the increased intensity of satellites in the ^{1}H-NMR spectrum resulting from ^{13}C-^{1}H spin-spin coupling. The former is preferred as it does not exclude quaternary carbons, and individual carbon signals are more readily resolvable than peaks in ^{1}H-NMR spectra.[22]

In natural abundance ^{13}C-NMR spectra the probability of ^{13}C-^{13}C spin-spin coupling from adjacent carbon-13 nuclei is low (0.01%). This allows the incorporation of intact carbon-carbon bonds to be investigated by conducting feeding experiments with doubly labelled precursors, e.g. [1,2-$^{13}C_2$]acetate (**33a**). Detection of sets of doublets with identical *J* values in such an experiment indicates the incorporation of an intact bond, providing that there is no adjacent incorporation of two labelled precursors within the same molecule. Adjacent incorporation can be prevented by dilution strategies by adding unlabelled carrier in feeding experiments.

The incorporation of other isotopes can also be detected indirectly by ^{13}C-NMR spectroscopy. The presence of isotope (^{2}H, ^{15}N, or ^{18}O) at one or two bonds distance results in heavy isotope induced ^{13}C-NMR shifts. A one bond shift, or α shift, is greater in magnitude than a two bond, or β shift. These upfield shifts are also cumulative. [23,24] Carbon-deuterium spin-spin coupling is also detectable in ^{13}C $\{^{1}H\}$ spectra, the carbon resonance being split into a 1:1:1 triplet, multiple deuterium atoms producing more complex multiplets.[25]

Deuterium incorporation can also be observed directly by ^{2}H-NMR spectroscopy.[20] The chemical shifts in ^{2}H-NMR spectra are approximate to those in a ^{1}H-NMR spectrum. However, ^{2}H resonances have extensive line-broadening due to the quadrapolar nucleus resulting in poorly resolved spectra, which may complicate interpretation. The low natural abundance of deuterium (Table 1.2) allows very low levels of isotope enrichment to be detected and quantified by integration of peak areas.

^{15}N-NMR has rarely been employed in biosynthetic investigations, it has however, been utilised in the study of tropane alkaloid biosynthesis.[26,27,28] As the natural abundance of ^{15}N is low, feeding experiments with ^{15}N labelled precursors can lead to isotopic enrichment in metabolites where the resultant ^{15}N-NMR spectra contain an observable enhancement in peak heights relative to natural abundance spectra, in a similar manner to experiments performed with ^{13}C enriched material.

Analysing complex mixtures of metabolites for the presence of isotopic label has been simplified by the development of techniques that couple chromatographic separation with analysis. The most commonly used techniques being gas chromatography-mass spectrometery (GC-MS) and high performance liquid chromatography-mass spectrometery (HPLC-MS). More recently high performance liquid chromatography-nuclear magnetic resonance (HPLC-NMR) has become a viable method for detecting the presence of isotope label through examination of ^{1}H-NMR spectra.

1.3.2 Plant experimental systems

Early investigations of plant secondary metabolism utilised whole plants as experimental systems. There are many difficulties associated with using whole plants for biosynthetic studies; most problematic being their slow growth rates, low yields of metabolites and the low levels of isotope incorporation in isolated metabolites.

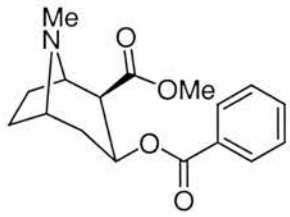
Within the last thirty years the use of deregulated cell suspension cultures and more recently plant organ cultures has greatly aided biosynthetic investigations. Plant tissue cultures have many experimental advantages over whole plants including fast growth rates, the ability to cultivate cells on a large scale, the capability of developing cell lines with high yields of metabolites, and the possibility of investigating and isolating biosynthetic enzymes.[29]

1.4 The Tropane Alkaloids

The tropane alkaloids are a group of secondary metabolites produced in the plant families Convolvulaceae, Dioscoraceae, Erythroxylaceae and Solonaceae.[30] Within the solonaceae (potato family) they are characterisic of the genera *Datura*, *Brugmansia*, and *Duboisia*.[31] Structurally they all contain the bicyclic amine tropane ring (nortropane, **34**) (Figure 1.6), many occurring as esters of tropine (3-hydroxy-8-aza-8-methyl-[3,2,1]-bicyclooctane, **35**). Common examples include cocaine (**36**) produced by *Erythroxylum coca*, hyoscyamine (**7**) and scopolamine (**37**) produced (not exclusively) by *Datura stramonium* (Jimson weed) and *Hyoscyamus niger* (Henbane) (Figure 1.7).

34

Figure 1.6: Nortropane (**34**) the core bicyclic heterocycle of the tropane alkaloids.



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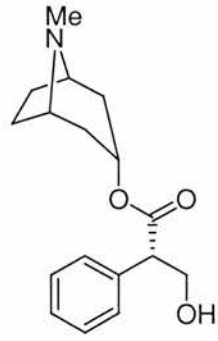

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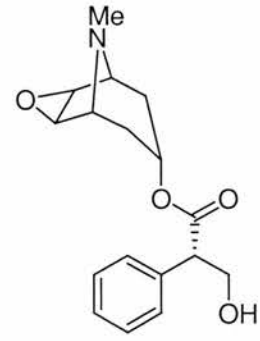

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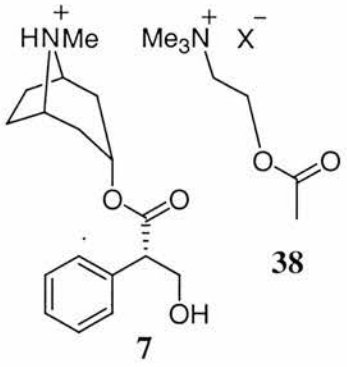
Figure 1.7: Structures of the tropane alkaloids cocaine (**36**), hyoscyamine (**7**), and scopolamine (**37**).

1.4.1 Bioactivity of the tropane alkaloids

Some tropane alkaloids are known to stimulate a variety of physiological responses upon mammalian organisms. Particularly well studied are the bioactive effects of hyoscyamine (**7**) (or as its racemate atropine), scopolamine (**37**) and cocaine (**36**).

Hyoscyamine (**7**) and scopolamine (**37**) are potent mydriatic (pupil dilatory) drugs, the former as its racemate was until recently used in ophthalmology. The addition of a 1% solution of hyoscyamine (**7**) to the human eye causes pupil dilation within 30 minutes and maximal effect after one hour, the effect is prolonged and can last for several days. Hyoscyamine (**37**) exerts its mydriatic effect by blocking the stimulatory effect of acetylcholine (**38**) upon the sphincter muscle responsible for contraction of the pupil.[32]

Hyoscyamine (**7**) is able to bind to muscarinic acetylcholine receptors due to its structural similarity to acetylcholine (**38**). Hyoscyamine (**7**) binds to the receptor, but is unable to switch it on due to differential binding, which induces different conformational changes upon the receptor. [33]



38 **7**

Figure 1.8: Comparison of the structures of hyoscyamine (**7**) and acetylcholine (**38**).[33] Note hyoscyamine (**7**) is protonated at physiological pH.

The tropane alkaloids exert a variety of other effects upon mammalian organisms including, inhibition of glandular secretions, relaxation of smooth muscle, vasodilatation, and a reduction in gastric secretion and motility. Scopolamine (**37**) has been widely used as a pre-medication to dry up mucous secretions prior to general anaesthetic. In addition they are known to promote hallucinations, unconsciousness and in higher doses are fatal.[34]

1.4.2 Historical uses of tropane alkaloids

The physical and psychological effects that tropane alkaloids exert upon humans (section 1.4.1) have led to their wide use in ritualistic practices and early medical procedures.

Extracts of tropane alkaloid producing plants have been used extensively in magical rituals throughout the world. Aztec priests imbibed drinks containing extracts of *Datura meteloides* in the belief that it enabled them to communicate with spirits[35] and extracts of *Datura* species are believed to have influenced the Delphian oracle.[36] Alkaloid containing plant extracts combined with fats and oils produced 'witches salves', which were applied to the skin. The psychoactivity of the tropane alkaloids may explain witches' beliefs that they could fly upon their broomsticks.[37]

The anaesthetic properties of mandrake (*Mandragora*) extracts have been known for 2000 years. The first century AD physician Dioscorides wrote much on mandrake including,

"For a man sleeps in the same fashion, as when he ate it, sensible of nothing for three or four hours, from the time that it is brought to him. And physicians also, use this, when they are about to cut, or cauterise."

Dioscorides was not alone in his writings, a review of Greek and Latin surgical literature demonstrates that mandrake was the principal anaesthetic of that time.[38] *Mandragora* species are known to produce tropane alkaloids, which are probably in part responsible for their extracts anaesthetic properties. Atropine's mydriatic effect was utilised by Renaissance ladies who would drop extract of deadly nightshade (*Atropa belladonna*) into their eyes to give them the 'doe-eyed' beauty appearance.[37]

1.4.3 Structural elucidation

Richard Willstätter is credited with the first total synthesis and therefore structural elucidation of tropine (**35**).[39,40,41,42,43] The final synthetic step of his synthesis (Figure 1.9) involves the reduction of tropinone (**39**) to tropine (**35**), a reaction that mimics its biosynthesis (see section 1.5.1.3). The penultimate step of the synthesis is the oxidation of pseudotropine (ψ-tropine, **40**), which like tropine (**35**) is also the core alcohol of many tropane alkaloids. Willstätter also performed the total syntheses of cocaine (36) and structures related to tropine (**35**).[44,45,46] Willstätter went on to win the Nobel Prize for Chemistry in 1915 in part for his work on the tropane alkaloids.

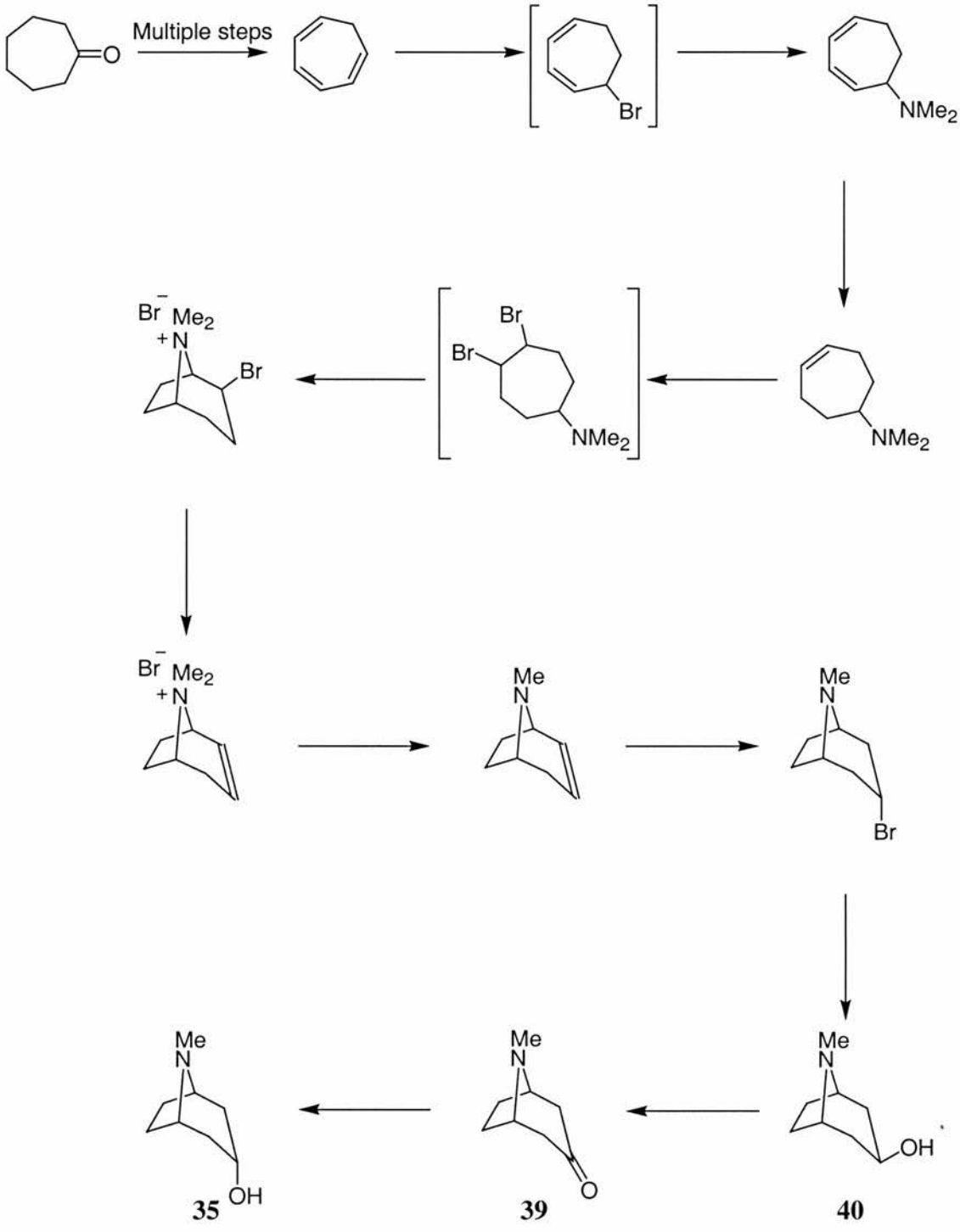


Figure 1.9: Willstätter's first synthesis of tropine (**35**).[39,40,41,42,43,47]

1.4.4 Structural Diversity

The tropanes are a diverse subclass of alkaloid with over 200 naturally occurring structures reported.[48] Most tropane alkaloids are esters of either tropine (**35**), psuedotropine (**40**), or other di- and trihydroxylated derivatives of nortropane (**34**),

additionally unesterified tropanes, tricyclic pyronotropanes and glycoside derivatives have been isolated (Figure 1.10).[49,50] The calystegines, a group of recently discovered tri-, tetra- and pentahydroxylated tropanes (Figure 1.11) share a common biosynthetic origin with the tropane alkaloids, as demonstrated by the incorporation of label into calystegines from [^{15}N]tropinone administered to *Calystegia sepium*.[51] A small number of structurally complex dimeric and trimeric tropane alkaloids with a central cyclobutane ring moiety have been reported (Figure 1.12), the cyclobutane ring could arise perhaps from a photochemical 2+2 cycloaddition either during or as the final step of their biosynthesis.[31,49]

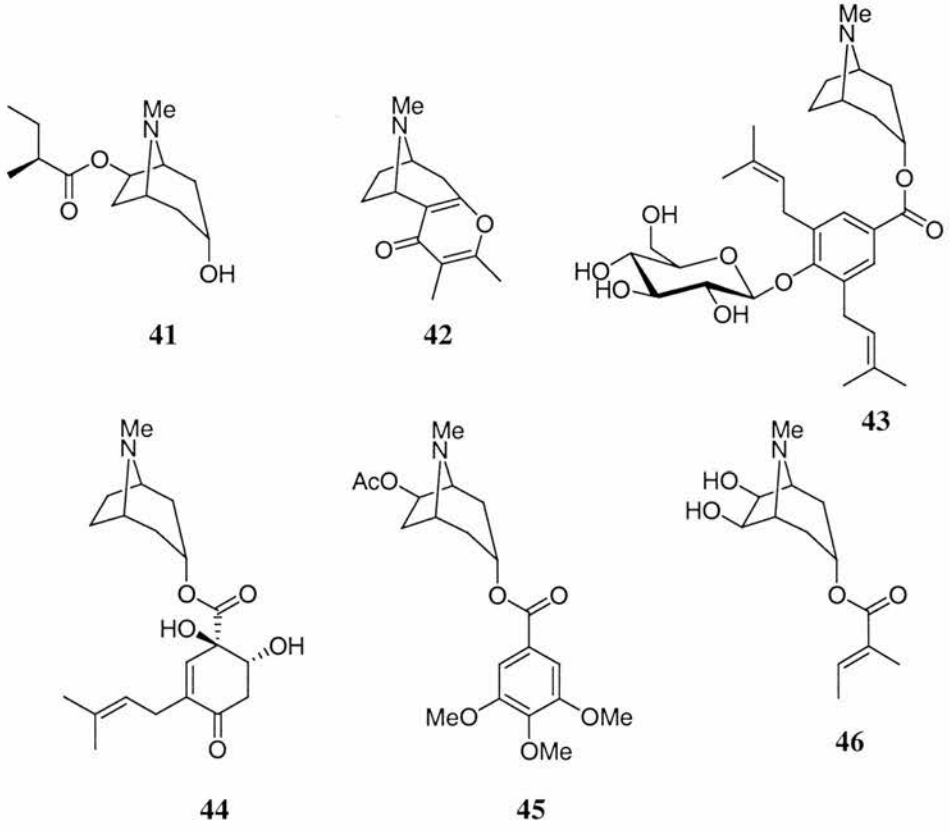


Figure 1.10: The tropane alkaloids 6β-[2-methylbutanoyloxy]tropan-3α-ol[52] (**41**), darlingine[53] (**42**), merresectine B (**43**), consiculoyl 3α-tropanol[54] (**44**), erythrozeylanine A[55] (**45**), and meteloidine[49] (**46**).

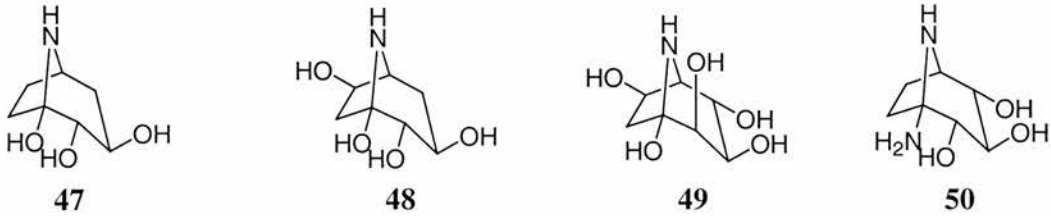
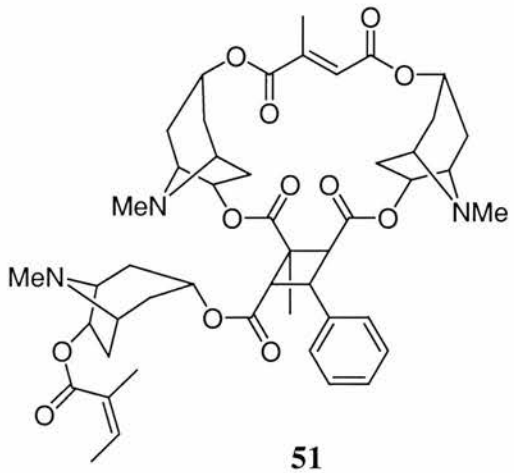


Figure 1.11: Examples of the calystegines, calystegine A_3 (**47**), calystegine B_1 (**48**), calystegine C_2 (**49**) and calystegine N_1 (**50**).



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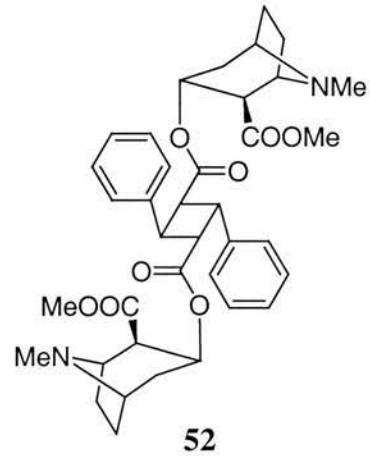

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Figure 1.12: Examples of the cyclobutane ring containing tropane alkaloids grahamine (**51**) and α-truxilline (**52**).

1.4.5 Root cultures for investigating tropane alkaloid biosynthesis

Differentiated root cultures producing a number of secondary product classes including alkaloids, terpenoids and flavonoids have been established. A number of these, notably tropane alkaloid producing lines such as *D. stramonium*, have provided useful experimental systems for biosynthetic investigation (section 2.1).[29]

Plant biosynthesis research has been advanced by the development of transformed root cultures, which possess improved stability in culture. Additionally as transformed roots have no requirement for exogenous phytohormones the cultures are easier to maintain. Transformed hairy root cultures are produced by genetic modification using the pathogenic plant bacterium *Agrobacterium rhizogenes*. Upon infection of a wounded plant *A. rhizogenes* inserts a short length of T-DNA from its Ri (root inducing) plasmid into the genome of the plant.[56,57] The inserted T-DNA genes include two sets of genes responsible for root induction; the *aux* (auxin production) genes, and the *rol* (root *loci*) genes, in addition there are genes responsible for opine production. The genetic transformation results in the growth and proliferation of roots at the point of infection, these roots can subsequently be excised and grown to create independent cultures.

1.5 Biosynthesis of the Tropane alkaloids

1.5.1 The tropane ring

The biosynthesis of the tropane ring can be considered in two parts; (i) the amino acid derived fragment constituting the five membered C_4N ring containing carbon atoms 1,5,6,7 and (ii) the acetate derived C_3 fragment comprising of the remaining carbon atoms 2,3 and 4.

Sir Robert Robinson was one of the first to formulate a biosynthetic hypothesis for the origin of the tropane ring. In 1917 Robinson published his classic 'one-pot' total synthesis of tropinone (**39**) from succindialdehyde (**53**), methylamine (**54**), and acetonedicarboxylate (**55**) (Figure 1.13).[58] Robinson devised his synthesis by formulating synthetic equivalents for the products of four bond disconnections of tropinone (**39**) (Figure 1.13), although it should be noted that Robinson was not the first to propose these disconnections.[2] Upon attempting the synthesis Robinson discovered that acetonedicarboxylate (**55**) was a better synthetic equivalent for acetone than acetone itself.

In a subsequent publication with regard to his synthesis Robinson wrote, "on account of its simplicity, is probably the method employed by the plant" suggesting his chemical synthesis was akin to tropinone's (**39**) biosynthesis.[59] Robinson stated that 'phytochemical syntheses' must involve reactions that parallel organic syntheses performed under reaction conditions approximate to those within a plant. His synthesis meets this criterion, being performed in aqueous solution at neutral pH and at ambient temperature.

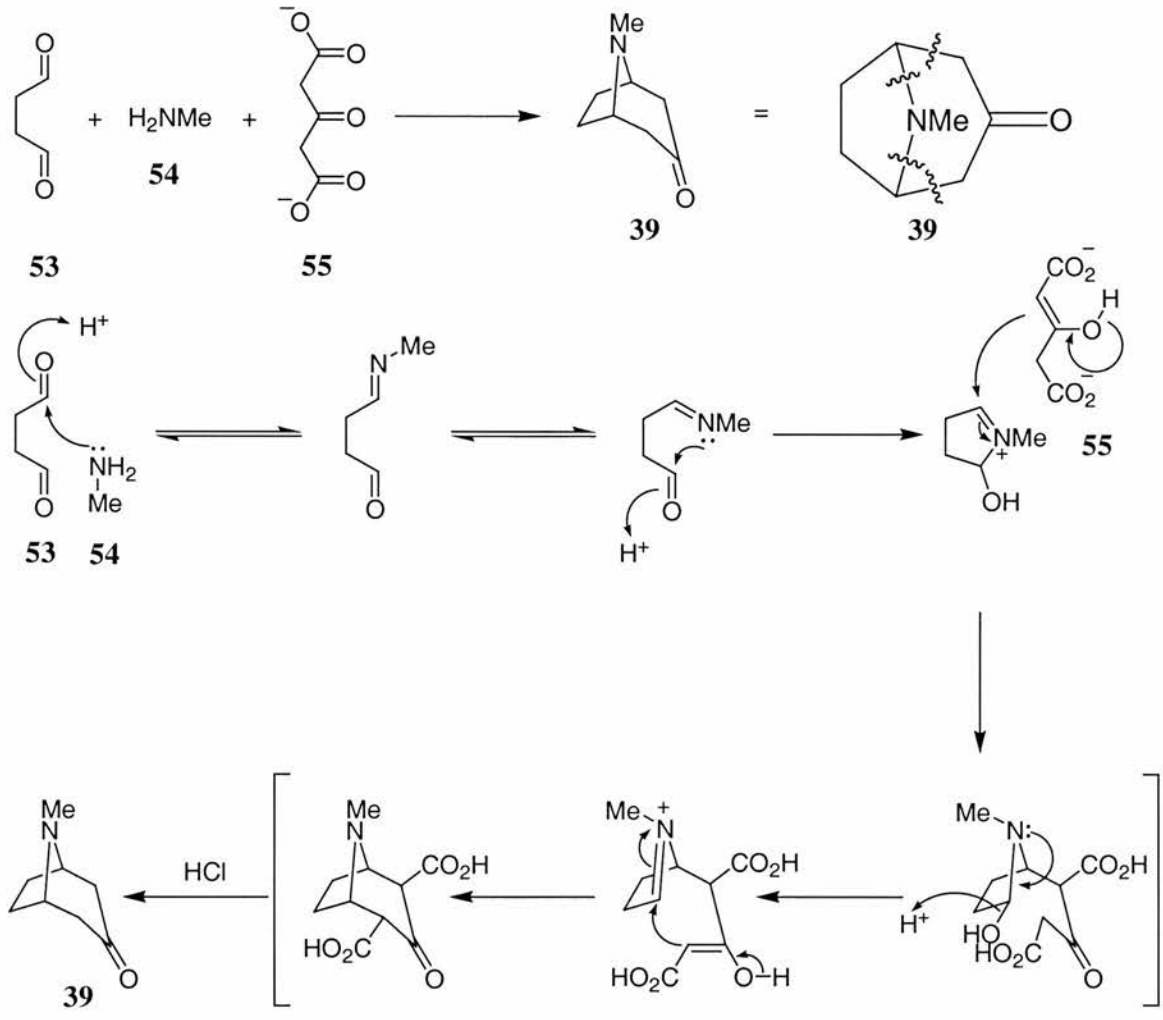


Figure 1.13: Robinson's one-pot synthesis of tropinone (**39**) from succindialdehyde (**53**), methylamine (**54**) and acetonedicarboxylate (**55**).[58,60] The four bond disconnections he made when formulating the synthesis are also shown.

1.5.1.1 The amino acid derived fragment

1.5.1.1.1 Early isotope feeding experiments

Before the advent of isotopic feeding experiments there were two leading hypotheses regarding the biosynthesis of the tropane ring system. The first was that of Robinson outlined above (section 1.5.1.1), which he elaborated upon by speculating that the amino acid ornithine (**26**) could be a biosynthetic precursor of succindialdehyde (**53**), and therefore the C_4N fragment of the tropane ring.[1,59,61] The second hypothesis published by Mortimer[62] and based in part upon Dawson's theories of alkaloid biogenesis[63] proposed tryptophan (**20**) as the biosynthetic precursor of the entire tropane ring *via* known biosynthetic intermediates to nicotinic acid[64] (Figure 1.14).

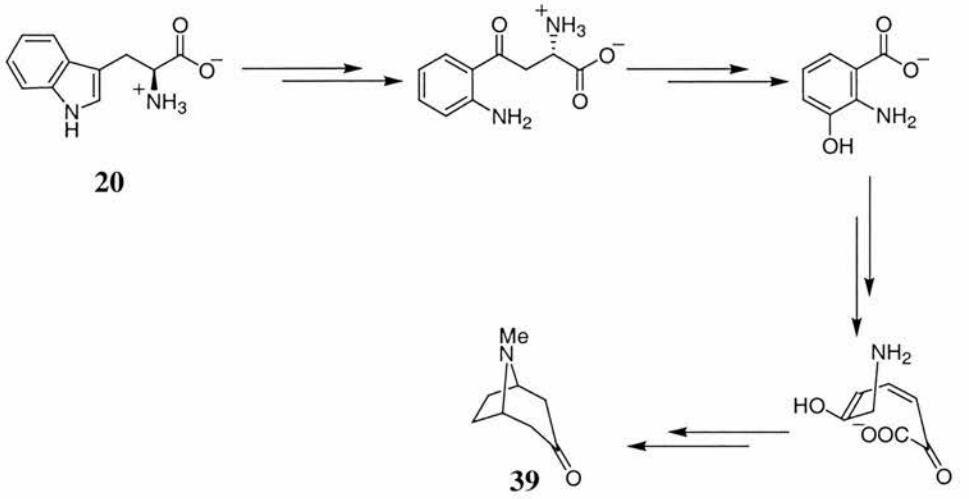


Figure 1.14: Mortimer's proposed biosynthesis of tropinone (**39**) from tryptophan (**20**).

The Robinson hypothesis was tested by feeding (*RS*)-[2-^{14}C]ornithine (**26a**) to mature *D. stramonium* plants. The resultant isolated hyoscyamine (**7a**) was found to contain radio-label in tropine (**35**) upon ester hydrolysis. Further chemical degradation demonstrated that the radioactivity was located at one or both of the bridgehead carbons (C-1 and C-5),[65] thus supporting Robinson's hypothesis. This result was in agreement with an earlier experiment that had shown that feeding ornithine (**26**) to *A. belladonna* leaves increased their alkaloid content to a greater extent than other amino acids.[66]

Leete repeated the feeding experiment with (*RS*)-[2-^{14}C]ornithine (**26a**),[67] but instead utilised an alternative degradation scheme for tropine (**35**) developed by Bothner-By and co-workers,[68] which allowed the two bridgehead carbons to be differentiated. Leete discovered that label from (*RS*)-[2-^{14}C]ornithine (**26a**) was retained exclusively at C-1 of hyoscyamine (**7a**) (Figure 1.15) and that isolated scopolamine (**37**) was also radioactive.

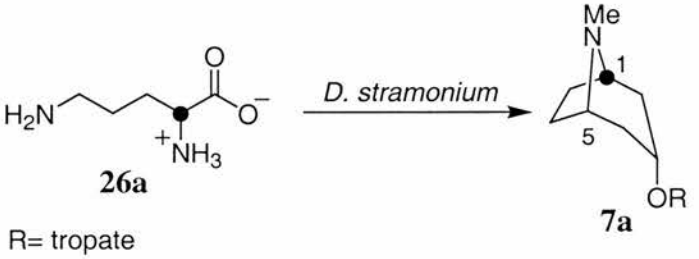


Figure 1.15: Incorporation of (*RS*)-[2-^{14}C]ornithine (**26a**) into hyoscyamine (**7a**) in *D. stramonium* plants.[65,67]

The asymmetric incorporation of label into hyoscyamine (**7**) was independently confirmed by feeding sodium [1-^{14}C]acetate (**33b**) to root cultures of *D. stramonium*.[68] The biosynthesis of ornithine (**26**) from acetate (**33**) is well understood (see section 2.4). [1-^{14}C]Acetate (**33b**) produces [5-^{14}C]ornithine (**26b**), the label from which is incorporated exclusively at C-5 of hyoscyamine (**7b**) (Figure 1.16).

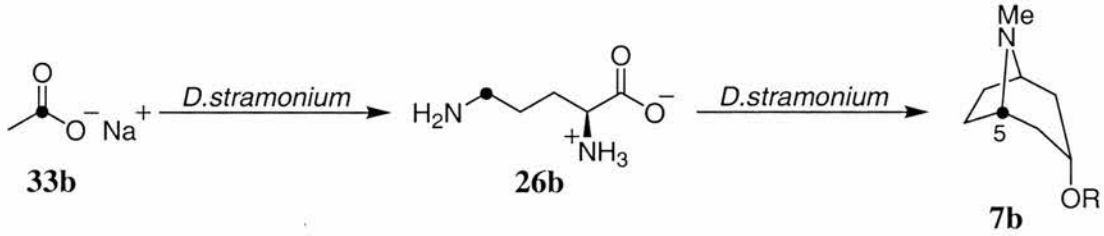


Figure 1.16: Incorporation of sodium [1-^{14}C]acetate (**33b**) into hyoscyamine (**7b**) in root cultures of *D. stramonium*.[68]

1.5.1.1.2 The biosynthesis of the C_4N pyrrolidine ring of tropane

Transformation from ornithine (**26**) to the C_4N fragment of the tropanes requires in some order a methylation, a decarboxylation and a cyclisation. The apparent asymmetric incorporation of ornithine (**26**) into the tropane ring (Figure 1.15) precludes the involvement of an intermediate with C_{2v} symmetry in the biosynthetic pathway. However, feeding experiments with [1, 4-$^{14}C_2$]putrescine (**56a**) in *D. metel*[69,70,71] and *D. stramonium*[70,71,72] produced radioactive tropane alkaloids. Chemical degredation of the isolated hyoscyamines (**7c**) revealed that the radioactivity was located in the pyrrolidine ring, most probably at C-1 and C-5 (Figure 1.17).

Figure 1.17: Incorporation of [1, 4-$^{14}C_2$]putrescine (**56a**) into hyoscyamine (**7c**) in *Datura* species.[69,70,71,72]

Several hypothesese have been proposed to explain the apparent inconsistencies between the ornithine (**26**) and putrescine (**56**) feeding experiments. Initially it was proposed[69] that although putrescine (**56**) can act a precursor of the tropane ring when administered to root cultures of *Datura* species, it is not a natural intermediate in the biosynthesis of the tropane alkaloids. The unsymmetrical incorporation of (*RS*)-[2-^{14}C]ornithine (**26a**) into hyoscyamine (**7a**) (Figure 1.15) was also rationalised[73] by proposing that ornithine (**26**) was first methylated to produce δ-*N*-methylornithine, decarboxylation of which would give *N*-methylputrescine (**57**). Subsequent oxidation and cyclisation reactions would furnish a salt of *N*-methylpyrrolinium (**58**). Importantly, all of the intermediates in this proposed sequence are unsymmetrical. The intermediate role of *N*-methylputrescine (**57**) had been previously proven by feeding [*methyl*-^{14}C, ^{15}N]*N*-methylputrescine *D. metel* rootlets.[74] The resultant hyoscyamine was found to be radioactive. Further degradation experiments determined the location of radiolabel to be the *N*-methyl group of hyoscyamine and combustion spectrometry showed that the nitrogen-15 was also incorporated at the *N*-methyl group. Radiolabelled (*RS*)-δ-*N*-methylornithine was also found to incorporate into hyoscyamine (**7**) in *D. stramonium*[75] and *A. belladonna*[76] plants, supporting its hypothetical role as an intermediate. However, whilst δ-*N*-methylornithine has been identified as a natural constituent of *A. belladonna*,[77] it has not been detected in *Datura* species. Therefore, the decarboxylation of δ-*N*-methylornithine to *N*-methylputrescine (**57**) and subsequent incorporation into tropine (**35**) is considered an unnatural process, which does not ordinarily occur *in vivo*.[78]

Leete proposed[78] a biosynthetic pathway from ornithine (**26**) to tropinone (**39**) (Figure 1.18). The first step on the pathway is the decarboxylation of ornithine (**26**) to give the polyamine putrescine (**56**), subsequent *N*-methylation of putrescine (**56**) gives *N*-methylputrescine (**57**) the first dedicated metabolite of tropane skeleton biosynthesis. Oxidative transamination of the primary amine of **57** yields 4-(methylamino)butanal (**59**) and the cyclisation product of this amino aldehyde is a salt of *N*-methylpyrrolinium (**58**). The intermediate role of 4-(methylamino)butanal (**59**) and *N*-methylpyrrolinium (**58**) has been demonstrated by isotopic feeding experiments (section 2.7.1). Finally condensation of *N*-methylpyrrolinium (**58**) with an acetate-derived metabolite would provide tropinone (**39**).

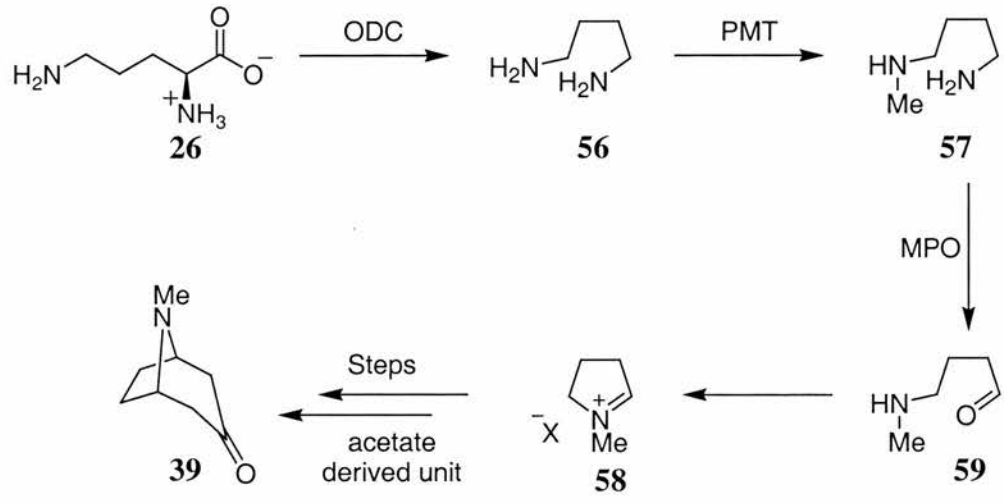


Figure 1.18: Biosynthesis of tropinone (**39**) from ornithine (**26**) as proposed by Leete.[78]

More recent investigations of the enzyme activities involved in the biosynthesis of *N*-methylputrescine (**57**) support Leete's hypothesis (Figure 1.18). In a series of experiments,[79] performed with cell free extracts of transformed root cultures of *D. stramonium* and *A. belladonna*; ornithine δ–*N*-methyltransferase and δ–*N*-methylornithine decarboxylase activities were undetectable. However, putrescine-*N*-methyltransferase (PMT, EC 2.1.1.53) and ornithine decarboxylase (ODC, EC 4.1.1.17) activities were observed, consistent with a previous biochemical study in *H. albus*[80] and the observations from isotopic feeding experiments.[69,70,71,72,74] PMT from *D. stramonium* has been partially purified and characterised. *S*-Adenosyl methionine (SAM, **60**) has been shown to act as the methyl donor for PMT.[81] This result was confirmed by administering [$^2H_3$-*methyl*]methionine to transfomed root cultures of *D. stramonium*,[82] the resultant hyoscyamine incorporating the deuterium label.

The demonstration that putrescine (**56**) a molecule with C_{2v} symmetry is an intermediate to the C_4N fragment of tropine (**35**) is inconsistent with the reported unsymmetrical incorporation of label from ornithine (**26**) into tropine (**35**). To account for this Leete proposed[78] an intermediate role for enzyme bound putrescine (**56**), in which the two primary amines are rendered non-equivalent by covalent bonding to pyridoxal phosphate (**61**, PLP) (Figure 1.19). The role of a symmetrical intermediate is further disscussed in chapter 2.4.1.

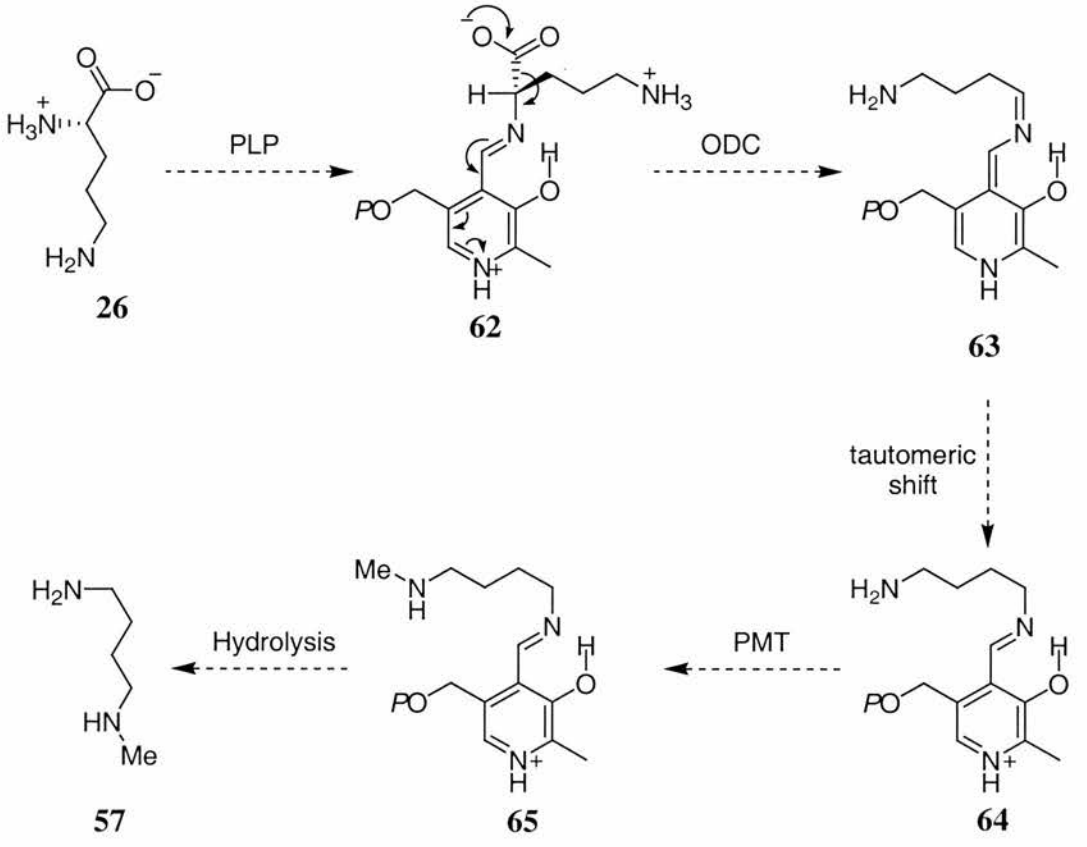


Figure 1.19: Proposed biosynthetic route to *N*-methylputrescine (**57**) *via* PLP bound intermediates (**62**, **63**, **64**, **65**).[78]

1.5.1.1.3 Ornithine or arginine?

The amino acids ornithine (**26**) and arginine (**66**) are metabolically interconvertable through the urea cycle (Figure 1.20). Therefore, administering labelled ornithine (**26**) to a plant system will result in the *in vivo* synthesis of labelled arginine (**66**), which could be the more direct precursor of tropinone (**39**). Similarly the incorporation of radiolabel from (*L*)-[2, 3, 3-$^3H_3$]arginine into hyoscyamine (**7**)[83] could occur *via* ornithine (**26**).

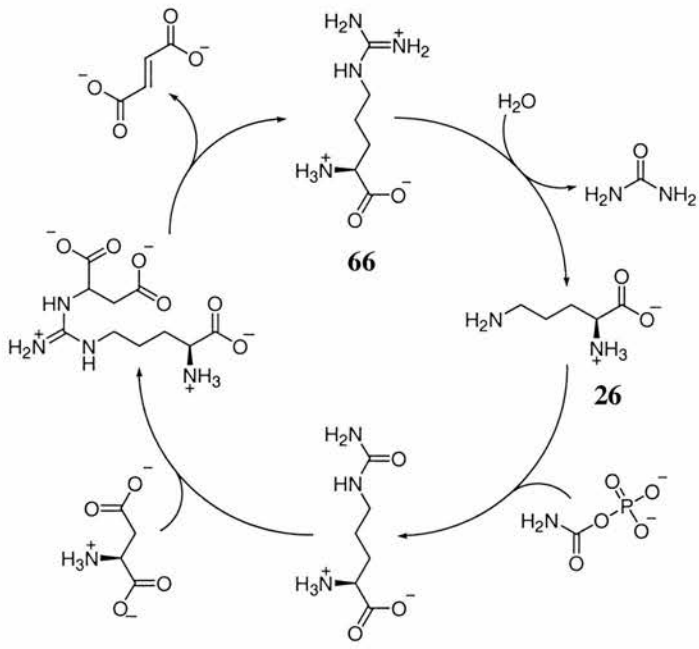


Figure 1.20: The urea cycle, showing the interconversion of ornithine (**26**) and arginine (**66**).[84]

Work in *Datura* plant tissue cultures[79] has shown that the specific inhibition of arginine decarboxylase (ADC, EC 4.1.1.19) using (*RS*)-2-difluoromethylarginine reduces the production of hyoscyamine (**7**) by 80%, whilst inhibition of ODC with (*RS*)-2-difluoromethylornithine did not significantly reduce hyoscyamine (**7**) levels. These results suggest that arginine (**66**) is the more direct precursor of tropinone (**39**). This conclusion was further tested by feeding [U-^{14}C]agmatine, the decarboxylation product of arginine (**66**) to *D. stramonium*,[79] the hyoscyamine isolated from this experiment was found to be radioactive. As agmatine is not interconvertable with ornithine (**26**), agmatine and therefore arginine (**66**) must be direct precursors to the tropane ring.

1.5.1.2 The acetate derived fragment

Robinson's biosynthetic hypothesis[59] suggested acetone, acetate (**33**) or acetoacetate (**67**) as putative precursors to C-2,3,4 of tropinone (**39**). The role of acetate (**33**) as a precursor to the C_3 fragmant of the tropane alkaloids was tested by feeding [1-^{14}C]acetate (**33b**) and [2-^{14}C]acetate (**33c**) to excised roots of *D. metel*.[85] Radiolabel was found to incorporate into the isolated alkaloids, and subsequent degradation of hyoscyamine (**7**) demonstrated that the label was predominantly incorporated into the C_3 bridge. Additionally C-3 was only labelled by feeding [1-^{14}C]acetate (**33b**), whereas

[2-^{14}C]acetate (**33c**) didn't label this position. Further investigations, which involved feeding [3-^{14}C]acetoacetate and [2,2,2-$^{3}H_3$]acetate established the former as the more direct precursor to the tropane ring.[86] These results implicated the known alkaloid hygrine (**68**) as a biosynthetic intermediate (Figure 1.21) and this hypothesis was supported by the apparent incorporation of radiolabel from (*RS*)-[*N-methyl*-^{14}C]hygrine into hyoscyamine (**7**) in *D. stramonium*.[87] However, a more recent feeding experiment with (*RS*)-[2, 3-$^{13}C_2$]hygrine resulted in a very low level of incorporation of label into scopolamine (**37**) and hyoscyamine (**7**)[88] and as a result hygrine (**68**) is not considered to be an intermediate of tropane biosynthesis (Figure 1.21).

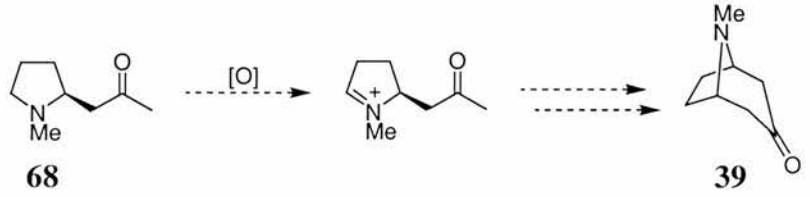


Figure 1.21: Hygrine (**68**) is not a precursor to tropinone (**39**).

The most advanced labelled precursor that has been successfully incorporated into hyoscyamine (**7**) is (*RS*)-ethyl [2, 3-$^{13}C_2$]4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69a**, R=OEt).[88,89] It is presumed that the ethyl ester is hydrolysed *in vivo*, and that the true substrate for incorporation is the free acid or a co-enzyme A ester. Interestingly both studies reported the incorporation of label at C-2, C-3 *and* at C-3, C-4 (Figure 1.22). Unusually this pattern of incorporation requires that both enantiomers of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69**) be transformed to tropinone (**39**) in subsequent enzymatic steps (Figure 1.22) as suggested by Hemscheidt.[90] However, there is little precident for such a non-stereospecific enzymatic pathway and this observation awaits a satisfactory explanation.

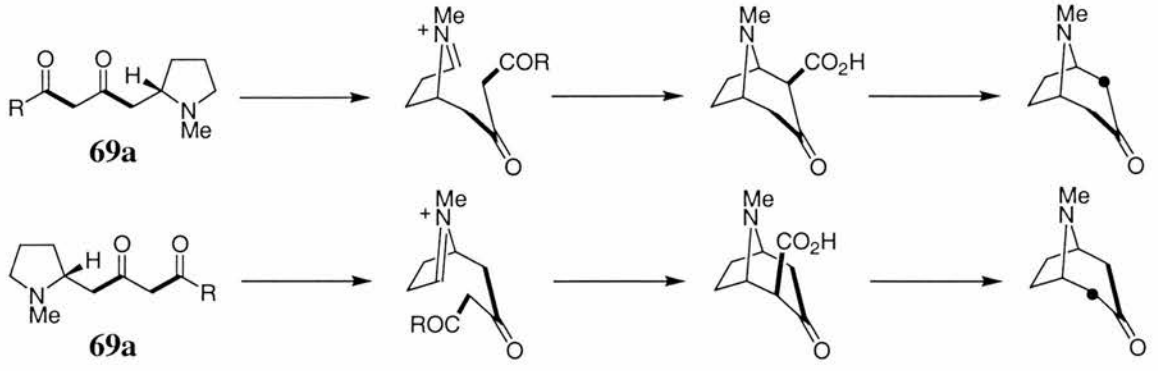


Figure 1.22: Incorporation of (*RS*)-ethyl [2, 3-$^{13}C_2$]4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69a**, R=OEt) into the tropane ring in *D. innoxia* and *D. stramonium*. The utilisation of both enantiomers is proposed to explain the incorporation pattern.

The mechanism for the formation of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**68**, R=O$^-$) or its ester is unresolved. Clearly **68** can arise from addition of C-4 of acetoacetate (**67**, R=O$^-$) to *N*-methylpyrrolinium (**58**) (Figure 1.23, path A). This hypothesis was tested by feeding ethyl [1,2,3,4-$^{13}C_4$]acetoacetate (**67a**) to *D. stramonium*.[91] In the event the observed labelling pattern in the tropane ring was identical to that obtained when [1, 2-$^{13}C_2$]acetate (**33a**) was administered. This suggests that acetoacetate (**67**) is not incorporated as an intact unit, but rather a retro-Claisen type reaction occurred *in vivo* to give two molecules of double labelled acetate (**33a**), which were subsequently incorporated. Alternatively, Leete proposed that *N*-methylpyrrolinium (**58**) could act as a 'starter unit' for a polyketide type process (Figure 1.23, path B),[92] i.e. the sequential condensation of acetyl (**5**) or malonyl CoA (**23**) esters.[25] Leete's proposal was examined by feeding (*RS*)-[1, 2-$^{13}C_2$]2-(1-methyl-2-pyrrolidinyl)-acetate (**70a**, R=O$^-$),[89] the resultant alkaloid extract was subjected to GC-MS analysis showing large amounts of unmetabolised precursor (**70a**, R=O$^-$) and no incorporation of label into hyoscyamine (**7**). Therefore the proposed step-wise condensation of acetyl/malonyl units with *N*-methylpyrrolinium (**58**) is not supported by experimental observations.

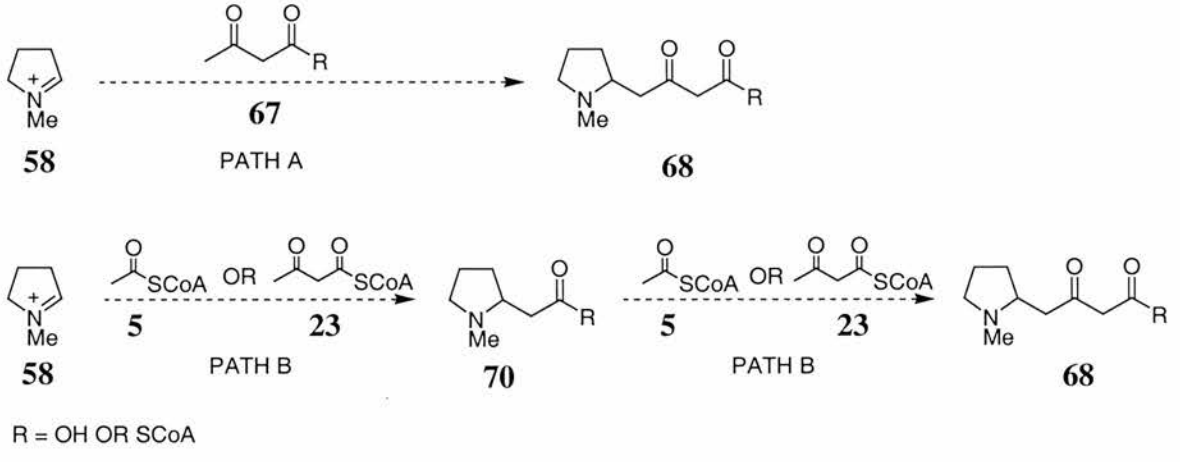


Figure 1.23: Proposed biosynthetic routes to 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**70**, R=O$^-$) from N-methylpyrrolinium (**58**).

A further discussion of the biosynthesis of the C_3 fragment of the tropane ring is given in chapter 2.

1.5.1.3 Reduction of tropinone

Tropinone (**39**) was established as a precursor of the tropane alkaloids by administering [*methyl*-^{14}C]tropinone to *D. innoxia* plants. The label was efficiently incorporated into the basic moeities of scopolamine (**37**) and hyoscyamine (**7**).[93] Two separate tropinone reductase enzymes have been isolated and purified from transformed root cultures of *D. stramonium*. GC analysis of the reaction products of both enzymes revealed that one, tropinone reductase-I (TR-I) transformed tropinone (**39**) into tropine (**35**) and the other, tropinone reductase-II (TR-II) produced pseudotropine (**40**) (Figure 1.24).[94] Both enzymes were found to have a requirement for NADPH. Tropinone reductase activities have been identified in several tropane alkaloid producing species,[95,96,97] and the protein structure of TR-II has subsequently been solved by X-ray analysis.[98]

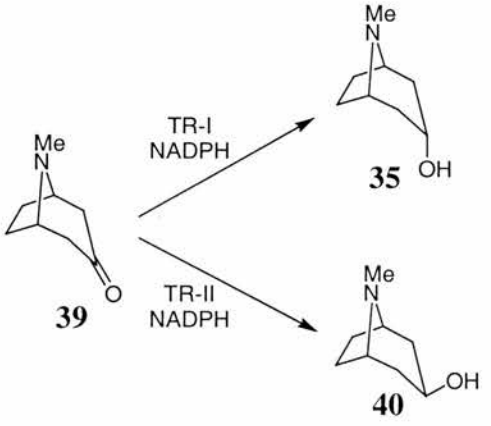


Figure 1.24: The stereospecific reduction of tropinone (**39**) by TR-I to form tropine (**35**) and TR-II to form psuedotropine (**40**).[94]

1.5.1.4 Biosynthesis of scopolamine and other tropane derivatives

There are numerous alkaloids in which the tropane ring is hydroxylated (Figure 1.10, Figure 1.11). The mechanism and enzymology of tropane ring oxidation has been determined in most detail for the alkaloid scopolamine (**37**), and is described below as an example. The tropane alkaloid scopolamine (**37**, hyoscine), the 6,7-β-epoxide of hyoscyamine (**7**) (Figure 1.7) was first isolated in 1881 from *Hyoscyamus muticus*.[99] Scopolamine (**37**) is accumulated by many of the same plant species that produce hyoscyamine (**7**) and in some cases at higher concentrations.[100,101]

Fodor and co-workers are credited with the first total syntheses of scopine (**71**) and from it scopolamine (**37**);[102] the key step in their synthesis was the epoxidation of 6,7-dehydroacetyltropine (**72**, 3-α-acetoxy-trop-6-ene) to acetyl scopine (**73**) by treatment

with trifluoroperacetic acid (Figure 1.25, route A). They then went on to synthesise scopolamine (**37**) from the direct epoxidation of 6,7-dehydrohyoscyamine (**74**)[103] (Figure 1.25, route B).

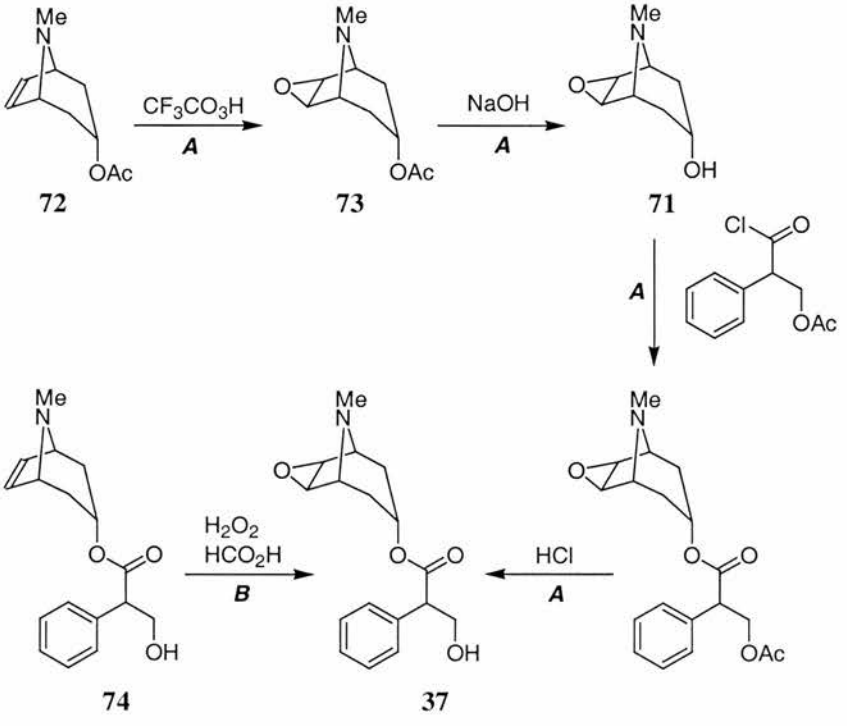
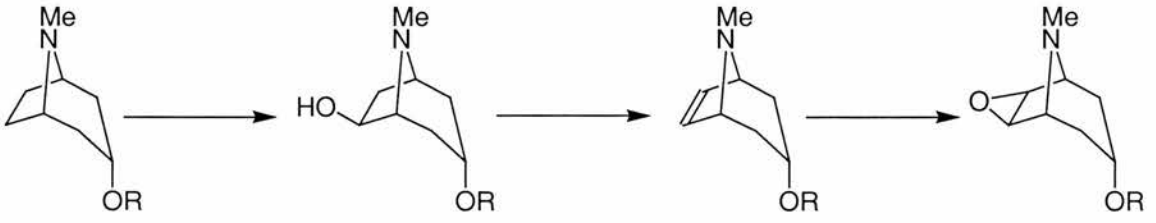


Figure 1.25: The first total synthesis of scopolamine (**37**).[102]

It was already reported[104] that alkaloid free excised *Datura ferox* scions had the ability to convert hyoscyamine (**7**) into scopolamine (**37**), and from his synthesis (Figure 1.25, route B) Fodor was led to propose not unreasonably that the biosynthesis could occur *via* an analogous enzymatic epoxidation of 6,7-dehydrohyoscyamine (**74**).[105] Fodor and co-workers tested his hypothesis by feeding *D. ferox* scions with 6,7-dehydrohyoscyamine (**74**).[106] Consistant with this hypothesis scopolamine (**37**) was detected in the scions. However, when excised scions were administered with hyoscyamine (**7**) no 6,7-dehydrohyoscyamine (**74**) was detected, so its role as a true *in vivo* intermediate could not be confirmed.

The proposal was modified suggesting that scopolamine (**37**) was generated from hyoscyamine (**7**) *via* 6β-hydroxyhyoscyamine (**75**) and 6,7-dehydrohyoscyamine (**74**) (Figure 1.26).[106] The discovery of 6β-hydroxyhyoscyamine (**75**) as an intermediate product in *D. ferox* scions[107,108] supported this hypothesis. In the event a feeding experiment with [*N-methyl*-^{14}C, 6β, 7β-$^{3}H_2$]tropine resulted in complete loss of tritium,

suggesting that the formation of 6,7-dehydrohyoscyamine (**74**) from 6β-hydroxyhyoscyamine (**75**) involves a *cis*-dehydration.[109]



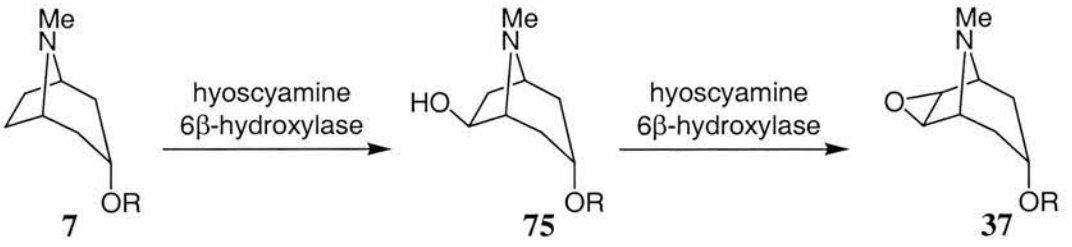
7 **75** **74** **37**

R=tropate

Figure 1.26: Fodor and co-workers' proposed biosynthesis of scopolamine (**37**).[106]

Hyoscyamine 6β-hydroxylase (E.C.1.14.11.11) the enzyme responsible for the conversion of hyoscyamine (**7**) to 6β-hydroxyhyoscyamine (**75**) was first identified and characterised by Hashimoto and Yamada in cell free extracts of *Hyoscyamus niger* root cultures.[110] The enzyme was shown to have an absolute requirement for (*S*)-hyoscyamine (**7**), Fe^{2+} ions, molecular oxygen and α-ketoglutarate (**76**, 2-oxoglutaric acid), which is oxidised and decarboxylated to form succinic acid (**77**) during the hydroxylation reaction. Hyoscyamine 6β-hydroxylase is therefore an α-ketoglutarate-dependent dioxygenase.

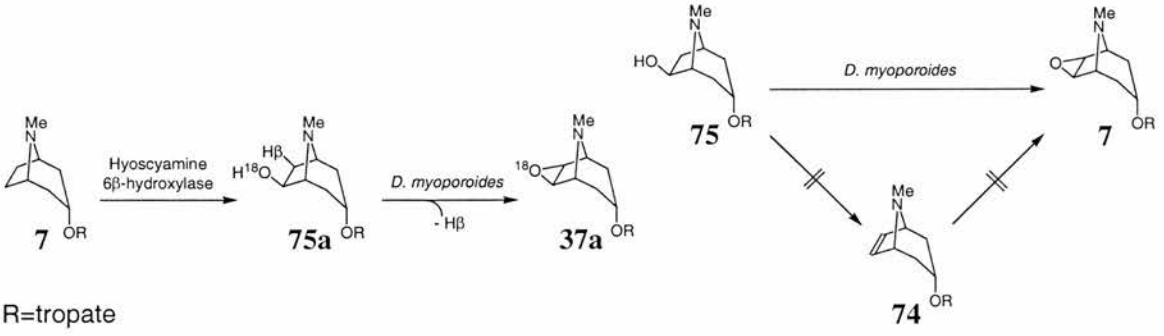
Further purification of hyoscyamine 6β-hydroxylase (310 fold) using ammonium sulfate fractionation and three column chromatography steps, revealed that the fractions possessing hydroxylase activity were also capable of epoxidising 6β-hydroxyhyoscyamine (**75**) to scopolamine (**37**).[111] Furthermore the cofactor requirements of the two activities were found to be identical, which led the authors to propose that a single, bifunctional enzyme catalyses the conversion of hyoscyamine (**7**) to scopolamine (**37**) in two separate steps (Figure 1.27). Purified hyoscyamine 6β-hydroxylase was also found to epoxidise 6,7-dehydrohyoscyamine (**74**) to scopolamine (**37**) consistent with the biosynthetic scheme proposed by Fodor and co-workers (Figure 1.26).



R=tropate

Figure 1.27: The biosynthesis of scopolamine (**37**) in two steps from hyoscyamine (**7**) *via* 6β-hydroxyhyoscyamine (**75**). Both steps are catalysed by the enzyme hyoscyamine 6β-hydroxylase.

In a further experiment Yamada and co-workers used partially purified hyoscyamine 6β-hydroxylase enzyme in a biotransformation reaction with hyoscyamine (**7**) and oxygen-18 gas to produce [6-^{18}O]6β-hydroxyhyoscyamine (**75a**) (82.5% atom ^{18}O), which was fed to *Duboisia myoporoides* shoot cultures deficient in hyoscyamine (**7**) production.[112] The resultant scopolamine (**37a**) was found to be 84.6% atom ^{18}O by GC-MS analysis, therefore the 6β-hydroxy oxygen is retained during the biosynthesis of scopolamine (**37**). Also the experiment revealed that 6β-hydroxyhyoscyamine (**75**) is not dehydrated to 6,7-dehydrohyoscyamine (**74**) prior to epoxidation (Figure 1.28) as speculated by Fodor and co-workers (Figure 1.26). Incubating [7β-^{2}H]hyoscyamine with hyoscyamine 6β-hydroxylase preparations from *H. niger* root cultures confirmed that the 7β-hydrogen is lost during the epoxidation.[113]



R=tropate

Figure 1.28: Incorporation of [6-^{18}O]6β-hydroxyhyoscyamine (**74a**) into scopolamine (**7a**) in *Duboisia myoporoides*. The incorporation of oxygen-18 label into scopolamine (**7a**) is inconsistant with the proposed intermediate role of 6,7-dehydrohyoscyamine (**74**).

To test the bifunctional enzyme hypothesis, hyoscyamine 6β-hydroxylase was purified to homogeneity from *H. niger*[114] and monoclonal antibodies raised against the pure protein.[115] One antibody was subsequently tested for its ability to inhibit the hydroxylation and epoxidation activities of partially purified hyoscyamine 6β-hydroxylase, and found to inhibit both to an equal extent, indicating that a common epitope is shared by both the hydroxylase and the epoxidase.[116]

The hyoscyamine 6β-hydroxylase gene has been cloned[117] and overexpressed in *E. coli*.[118] Hyoscyamine (**7**) incubated with the transformed *E. coli* cultures was completely converted into scopolamine (**37**) after 1 week, definitive proof that a single enzyme is responsible for the hydroxylation and epoxidation activities as initially proposed by Hashimoto and Yamada (Figure 1.27).

1.5.2 Esterifying acids of tropines

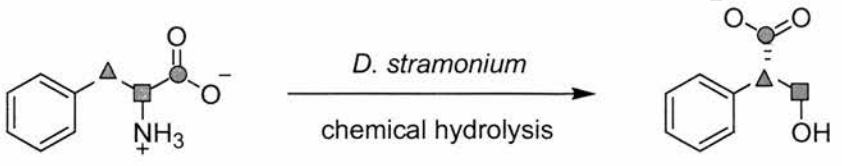
The majority of tropane alkaloids exist as esters of tropine (**35**) or other hydroxylated derivatives of nortropane (**34**). There are numerous esterifying acids, which are not necessarily structurally or biosynthetically related. These range from common metabolic acids e.g. acetic to more complex structures (Figure 1.7, Figure 1.10 and Figure 1.12). With the exception of phenyllactic (**78**) and tropic acid (**24**) (see section 1.6) little experimental evidence as to the biosynthetic origin of the esterifying acids is known.

1.6 Biosynthesis of Tropic acid

The biosynthesis of tropic acid (**24**), the ester moiety of the tropane alkaloids hyoscyamine (**7**) and scopolamine (**37**) has been the subject of extensive and early biosynthetic investigations. Again Robinson formulated a biosynthetic hypothesis, recognising that tropic acid (**24**) could be a phenylpropanoid (or as he termed C_6-C_3 complex) metabolite (section 1.2.1), the 'triose' fragment occurring as a branched rather than linear chain, perhaps arising from a 'special rearrangement'.[119]

1.6.1 Biosynthetic precursors of tropic acid

Again it was Leete who used isotopic feeding experiments to test the various hypotheses for the biogenesis of tropic acid (**24**). The role of phenylalanine (**18**) as a precursor of tropic acid (**24**) was first demonstrated by feeding (*RS*)-[3-^{14}C]phenylalanine (**18a**) to whole plants of *D. stramonium*. The incorporation was found to be uniquely at C-2 of tropic acid (**24a**), as revealed by hydrolysis of the resultant alkaloids and further chemical degradation.[120] A further feeding experiment employing (*RS*)-[2-^{14}C]phenylalanine (**18b**) resulted in tropic acid (**24b**) containing label at C-3,[121] and similarly, administering (L)-[1-^{14}C]phenylalanine (**18c**) to whole plants of *D. stramonium* resulted in tropic acid (**24c**) carrying label at C-1.[122,123] The incorporation of the entire side chain of phenylalanine (**18**) (Figure 1.29) discounted phenylacetic acid (a known metabolite of phenylalanine (**18**)) as a precursor to tropic acid (**24**) and led Leete to hypothesise that an intramolecular rearrangement reaction was operating during the biosynthesis of tropic acid (**24**).



18a △ =^{14}C
18b □ =^{14}C
18c ○ =^{14}C

24a △ =^{14}C
24b □ =^{14}C
24c ○ =^{14}C

R=tropine

Figure 1.29: Summary of the incorporation of label from carbon-14 labelled phenylalanines (**18a,b,c**) into tropic acid (**24a,b,c**).

Leete tested his intramolecular rearrangement hypothesis by administering (*RS*)-[1,3-$^{13}C_2$]phenylalanine (**18d**) to *D. innoxia* plants.[124] Significantly the resultant hyoscyamine (**7d**) displayed $^1J_{CC}$ spin-spin coupling consistent with an intramolecular rearrangement of phenylalanine (**18**) and a vicinal migration of the C-1 carboxyl group (Figure 1.30). Further mechanistic investigations on this rearrangement are described below (section 1.6.4).

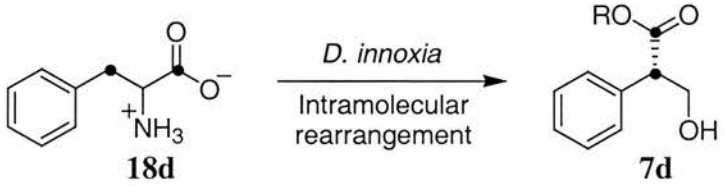


Figure 1.30: Incorporation of ^{13}C label from (*RS*)-[1,3-$^{13}C_2$]phenylalanine (**18d**) into hyoscyamine (**7d**).

It is possible to rationalise the incorporation of phenylalanine (**18**) into tropic acid (**24**) *via* a series of known enzymatic transformation reactions (Figure 1.31). Phenylalanine (**18**) could first undergo a transamination reaction to yield phenylpyruvic acid (**79**), the reverse reaction of that which occurs during the biosynthesis of phenylalanine (**18**) in bacteria.[84] The action of a dehydrogenase enzyme upon phenylpyruvic acid (**79**) would produce phenyllactic acid (**78**), isomerisation of which, by an unknown process would yield tropic acid (**24**).[125]

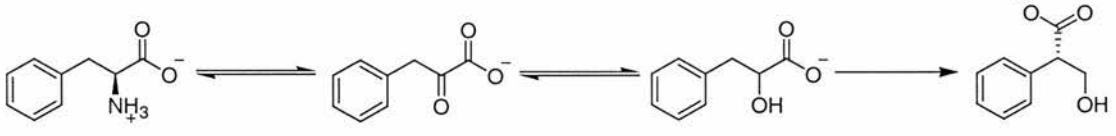


Figure 1.31: Proposed biosynthesis of tropic acid (**24**) from phenylalanine (**18**).

The proposed biosynthesis was tested by separately feeding [2-^{14}C]phenylpyruvic acid and [1-^{14}C]phenyllactic acid to *D. stramonium*.[126,127] The isolated hyoscyamine (**7**) and scopolamine (**34**) were found to retain radiolabel, suggesting their role as biosynthetic precursors of tropic acid (**24**). When (*RS*)-[1,3-$^{13}C_2$]phenyllactic acid was administered to transformed cultures of *D. stramonium*[128] the isolated hyoscyamine (**7d**) retained isotope label and possessed ^{13}C-^{13}C spin-spin coupling, confirming both

phenyllactic acid's role as an intermediate of tropic acid (**24**) and the intramolecular rearrangement first demonstrated by Leete.[124]

Proof that phenyllactic acid (**78**) (or an ester of) and not phenylpyruvic acid (**79**) is the substrate for the the intramolecular rearrangement was obtained by administering (*RS*)-[2-^{3}H]phenyllactic acid (**78a**) to *D. stramonium* plants.[126] The isolated hyoscyamine was hydrolysed and the resultant tropic acid found to retain the isotope at C-3, indicating that phenyllactic acid (**78**) is not first oxidised prior to isomerisation. This result was confirmed with stable isotopes by feeding (*RS*)-[2-^{13}C, 2-^{2}H]phenyllactate (**78b**) to transformed root cultures of *D. stramonium*. GC-MS analysis and comparison of $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ NMR data from the isolated alkaloids demonstrated the intact incorporation of the C-2-H bond from phenyllactic acid (**78**) to tropic acid (**24**).[129]

Subsequent experiments[130] administering (*R*)-[2-^{13}C, ^{2}H]phenyllactic acid and (*S*) [2-^{13}C, ^{2}H]phenyllactic acid separately to transformed root cultures of *D. stramonium* demonstrated that the deuterium was only incorporated into the tropate moiety of hyoscyamine (**7**) in the case of the (*R*) enantiomer. Significantly the carbon-13 label was efficiently incorporated into hyoscyamine (**7**) from both enantiomers (albeit at a lower level for the (*S*) enantiomer) indicating that the (*S*) enantiomer can be racemised to (*R*) phenyllactic acid (**78**) *via* phenylpyruvic acid (**79**).

1.6.2 Stereochemistry of the rearrangement of littorine to hyoscyamine

In order to determine the stereochemical course of the rearrangement at C-3` of tropic acid (**24**) (*RS*)-[2-^{3}H]phenyllactic acid (**78a**) was incubated with root cultures of *D. stramonium*.[131] The resultant hyoscyamine (**7e**) was converted to chiral acetic acid (**33d**) in a five step reaction sequence (Figure 1.32). The resultant chiral methyl group was enzymatically assayed and found to have the *R* stereochemistry. It was therefore concluded that the tritium must have occupied the 3-*pro-S* site in the hyoscyamine (**7e**) isolated from the feeding experiment. Consequently as (*R*)-phenyllactic acid (**78**) is the biosynthetic precursor to the tropate moiety there must have been an *inversion of configuration* at that stereogenic centre during the isomerisation.

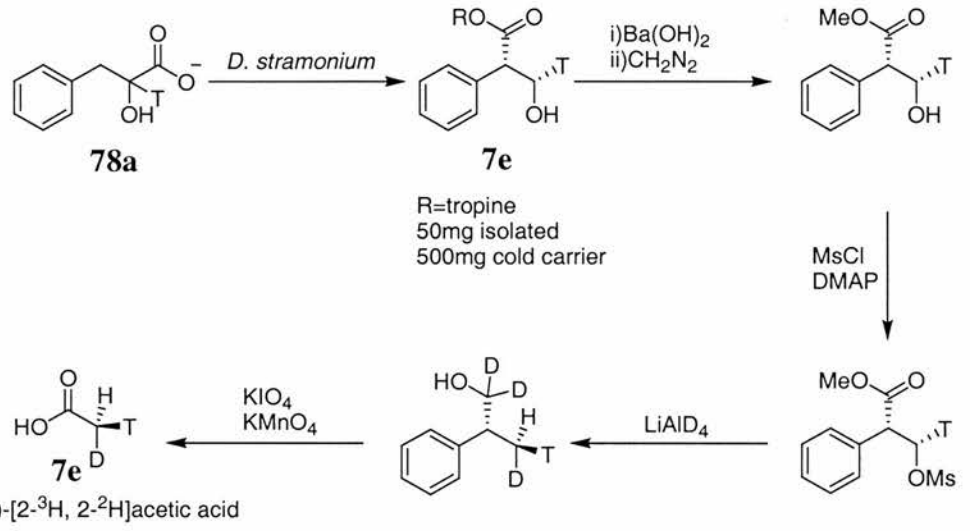


Figure 1.32: Feeding (*RS*)-[2-^{3}H]phenyllactic acid (**78a**) to *D. stramonium* and the sebsequent chemical conversions.[131]

To investigate the stereochemical course of the isomerisation at C-3 of phenyllactic acid (**78**) / C-2 of tropic acid (**24**), (2*R*,3*S*)-[2-^{13}C, 3-^{2}H, *phenyl*-^{2}H$_5$]phenyllactic acid (**78c**) and (2*R*,3*R*)-[2-^{13}C, 2,3-^{2}H$_2$]phenyllactic acid (**78d**) were prepared and fed to transformed root cultures of *D. stramonium* (Figure 1.33)[132]. The ^{13}C-NMR spectra of the resultant hyoscyamines (**7f, 7g**) revealed that only the 3-*pro-S* deuterium atom is retained during the transformation. As the tropate moiety had the (*S*) stereochemistry the transformation must occur with an inversion of stereochemistry. Overall the studies concluded that both migration centres undergo inversion of stereochemistry during the rearrangement reaction, thus correcting the conclusions of an earlier stereochemical study with radioisotopes.[133] Interestingly these results are consistent with a very early report by Haslam *et al.*[134]

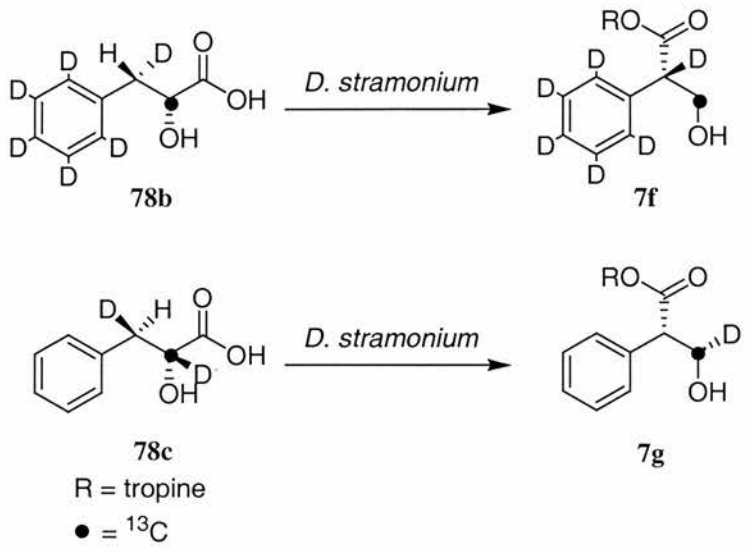


Figure 1.33: Results of feeding (2*R*,3*S*)-[2-^{13}C, 3-^{2}H, *phenyl*-$^{2}H_5$]phenyllactic acid (**78c**) and (2*R*,3*R*)-[2-^{13}C, 2,3-$^{2}H_2$]phenyllactic acid (**78d**) to root cultures of *D. stramonium*.[132]

1.6.3 Identification of the substrate for the rearrangement reaction

Feeding experiments in transformed root cultures of *D. stramonium* with (*RS*)-[1,3-$^{13}C_2$]phenyllactic acid either alone or in combination with (*RS*)-tropic acid (**24**) demonstrated that tropic acid (**24**) is unable to reduce the incorporation of label into hyoscyamine (**7**).[135] These experiments suggest that free tropic acid (**24**) is not an intermediate in the biosynthesis of hyoscyamine (**7**), *i.e.* direct esterification of tropic acid (**24**) wth tropine (**35**) does not occur. This result is also consistent with the low levels of isotope incorporation into hyoscyamine (**7**) from feeding [^{14}C]tropic acid.[125] It was suggested that littorine (**80**), an alkaloid that commonly co-occurs with hyoscyamine (**7**),[136] could be the substrate for the rearrangement of the phenyllactoyl moiety to the tropate moiety. This hypothesis was tested by administering (*RS*)-[*methyl*-$^{2}H_3$, 1′,3′-$^{13}C_2$]littorine (**80a**) to root cultures of *D. stramonium* (Figure 1.34).[137] GC-MS analysis of the resultant hyoscyamine (**7h**) detected a M+5 enrichment that was not reduced in intensity by the addition of unlabelled tropine (**35**) or phenyllactic acid (**78**), proving that ester hydrolysis prior to rearrangement and esterification does not occur. It was concluded that littorine (**80**) is the true substrate for the rearrangement of the phenyllactate moiety.

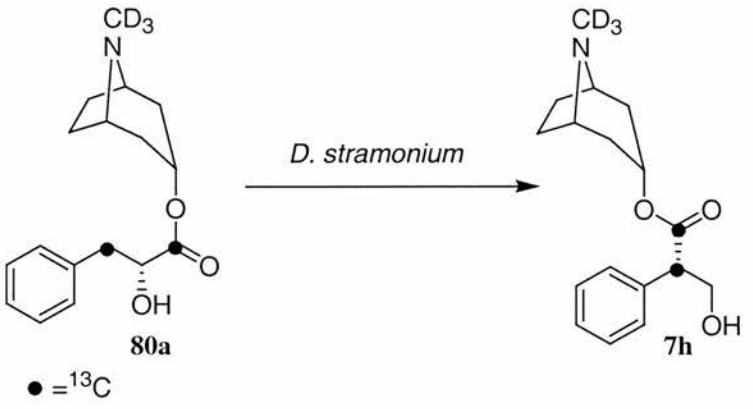


Figure 1.34: The intact incorporation of (*R*)-[*methyl*-$^{2}H_3$, 1′,3′-$^{13}C_2$]littorine (**80a**) into hyoscyamine (**7h**) in root cultures of *D. stramonium*.

1.6.4 Mechanism of rearrangement of phenylalanine to tropic acid

An insight to the possible mechanism of this carbon skeletal rearrangement was obtained in an early study by feeding (2*RS*, 3*RS*)-[1-^{14}C, 3-^{3}H]phenylalanine (**18e**) to whole plants of *D. stramonium* and *D. innoxia*. This initial study reported that the tritium from C-3 of phenylalanine (**18e**) had migrated to C-3′ of hyoscyamine (**7i**) (Figure 1.35).[138] A further study, feeding (2*S*, 3*S*)-[1-^{14}C, 3-^{3}H]phenylalanine apparantly confirmed the 'back migration' of tritium, and assigned the carboxyl group migration as occurring with a retention of configuration. However, incorporations in this study were low (2%).[133] Leete[133] noted the similarity of this transformation to that of the B_{12} mediated methylmalonyl-CoA mutase, which involves a 1,2 migration of a carboxyl group and a hydrogen, a vicinal interchange process (Figure 1.36).[139] However, no co-enzyme B_{12} dependent enzymes have been identified in plants to date, and an examination of *D. stramonium* cell free extract did not detect any cobalt.[133] In addition a more recent stereochemical study[132] using stable isotope methodology failed to detect the back migration of a deuterium atom, ruling out a vicinal interchange process and the involvement of co-enzyme B_{12} (Figure 1.37).

Figure 1.35: The apparent incorporation of (2*RS*, 3*RS*)-[1-^{14}C, 3-^{3}H]phenylalanine (**18e**) into hyoscyamine (**7i**) suggesting the operation of a vicinal interchange process.[138] However, this process was not supported by stable isotope experiments.[132]

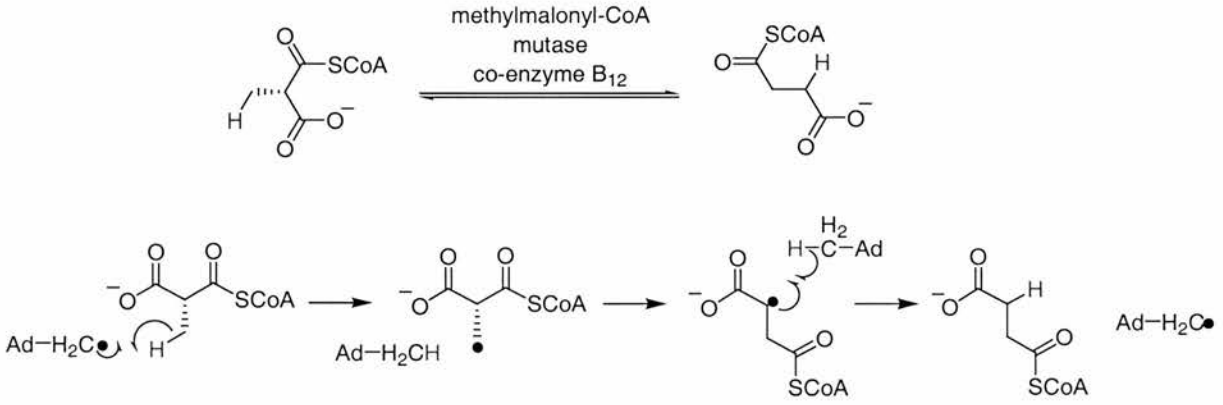


Figure 1.36: The minimal mechanism of methylmalonyl-CoA mutase.

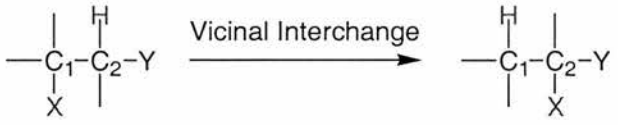


Figure 1.37: The general form of co-enzyme B_{12} dependent rearrangement reactions.[140]

In view of the B_{12} revision, a radical mediated transformation still offered an attractive mechanistic hypothesis for the enzymatic rearrangement. It has been reported that plants can utilise iron-oxo species to generate radicals. For example *Pueraria lobata* expresses a cytochrome P-450 dependent isoflavone synthase that catalyses the conversion of liquiritigenin (**81**) to daidzein (**82**) *via* radical intermediates (Figure 1.38).[141,142] A key feature of this transformation and other two-step radical processes, in which an iron-oxo species abstracts a hydrogen radical, is the subsequent 'quenching' of the carbon radical by reaction with an iron bound hydroxyl radical,[140] a mechanism known as oxygen rebound.[143] An analogous mechanism for the rearrangement of the

phenyllactate moiety to tropate *via* a two-step radical process, followed by an oxygen rebound process, collapse of the resulting *gem*-diol (**83**) to an aldehyde (**84**) and subsequent reduction by a dehydrogenase has been proposed (Figure 1.39).[125] Clotrimazole, a cytochrome P-450 inhibitor has been shown to inhibit the conversion of littorine (**80**) to hyoscyamine (**7**) when incubated in the media of transformed root cultures of *D. stramonium*, indirectly supporting the hypothetical role of a P-450 dependent isomerase.[144]

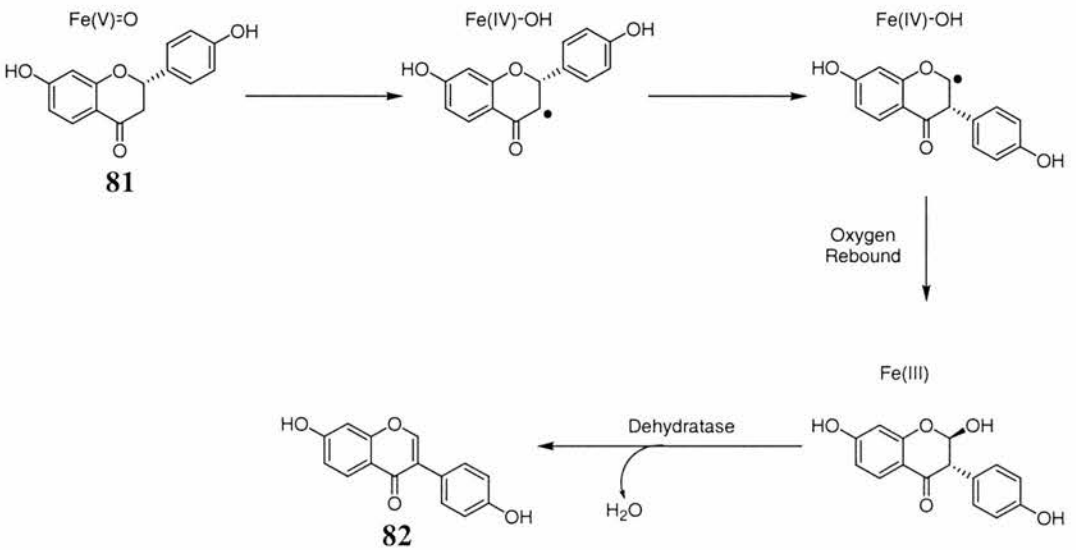


Figure 1.38: The two-step radical reaction sequence and subsequent dehydration that transform liquiritigenin (**81**) to daidzein (**82**) in *P. lobata*.[141,142]

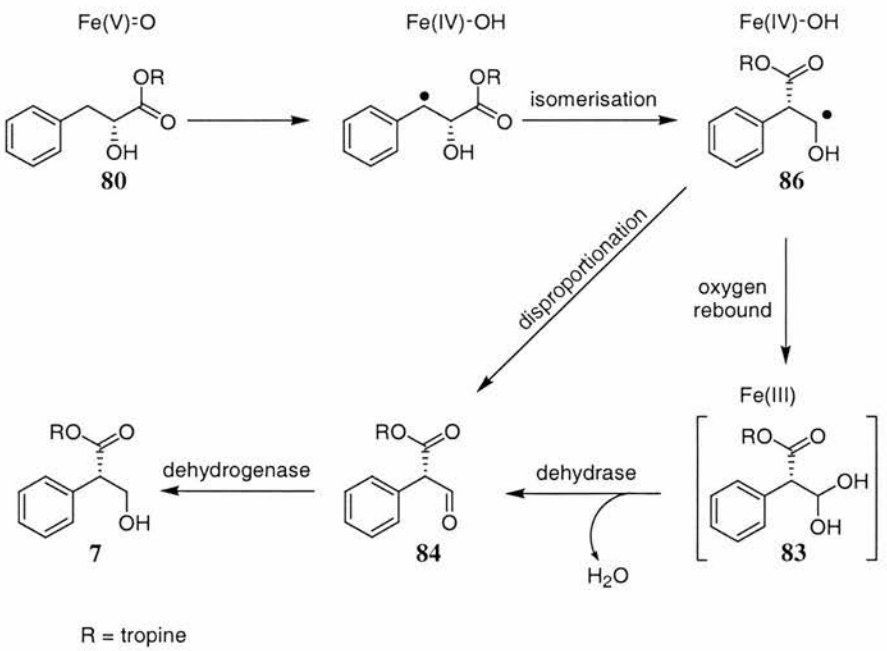


Figure 1.39: Proposed cytochrome P-450 dependent mechanism for the rearrangement of littorine (**80**) to hyoscyamine (**7**) *via* radical intermediates.[125]

In order to investigate the putative role of an oxygen rebound process (*RS*)-[2-^{2}H, ^{18}O]phenyllactic acid (**78e**) was fed to root cultures of *D. stramonium*. [145] GC-MS analysis of the resultant hyoscyamine (**7j**) demonstrated that 25-29% of the oxygen-18 isotope was lost during the rearrangement (Figure 3.4). The proposed oxygen rebound process (Figure 1.39) requires the intermediacy of an aldehyde (**84**), exchange of the aldehyde oxygen in aqueous conditions could account for the loss of isotope. In addition a partially stereospecific dehydration of the *gem*-diol (**83**) could lead to loss of isotope label. Similarly Arigoni and co-workers observed a 12% loss of oxygen-18 label when investigating the stereochemical course of the diol dehydrase involved in the formation of propionaldehyde (**85**) (Figure 1.40).[146] Alternatively, product radical (**86**) could be converted directly to the aldehyde (**84**) by disproportionation *via* hydrogen abstraction from the hydroxyl group, thus retaining the hydroxyl oxygen in the resultant aldehyde (**84**) (Figure 1.39). Such a mechanism has been proposed to occur during the biosynthesis of oestrogen,[147] a 20% loss of oxygen-18 label was observed during isotope feeding experiments in this system (Figure 1.41),[148] a similar figure to the feeding experiment in *D. stramonium*.[145]

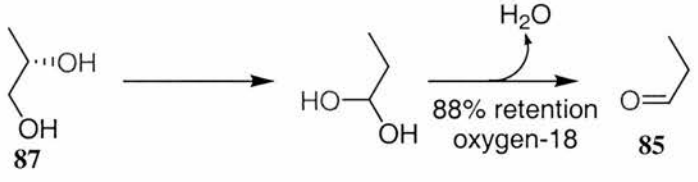


Figure 1.40: Loss of oxygen-18 label during the biosynthesis of propionaldehyde (**85**) from (*2S*)-propanediol (**87**).[146]

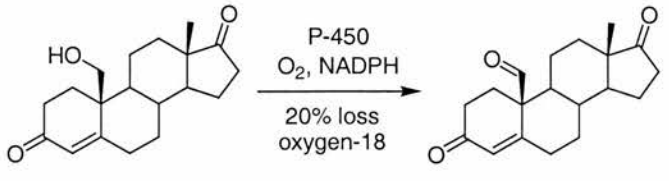


Figure 1.41: Retention of oxygen-18 label during the biosynthesis of oestrogen.[147,148]

Alternatively the rearrangement could occur *via* carbocation intermediates. Such a mechanism would result in retention of the hydroxyl oxygen from phenyllactic acid (**78**), although again an aldehyde intermediate (**84**) is implicated (Figure 1.42).[125] The

hemethiolate ('P-450') mediated generation of carbocations by two electron oxidation has been proposed to operate during the biosynthesis of prostaglandin and thromboxane,[149] although this mechanism has less precedent than a radical process.

Cationic intermediates have been reported to occur as side products during the cytochrome P-450 oxidation of some substrates.[150] Radical and carbocation ring opening reactions with substituted methylcyclopropane ring model systems support the proposed role of a carbocation intermediate.[150,151] In model system (**88**) the substituents are methoxy and phenyl. It was found that a methylcyclopropane carbocation ring opens towards oxygen as per the proposed mechanism and the methylcyclopropane radical ring opens in the opposite direction towards the aryl substituent (Figure 1.43). Therefore these models support a carbocation mechanism rather than a radical process.

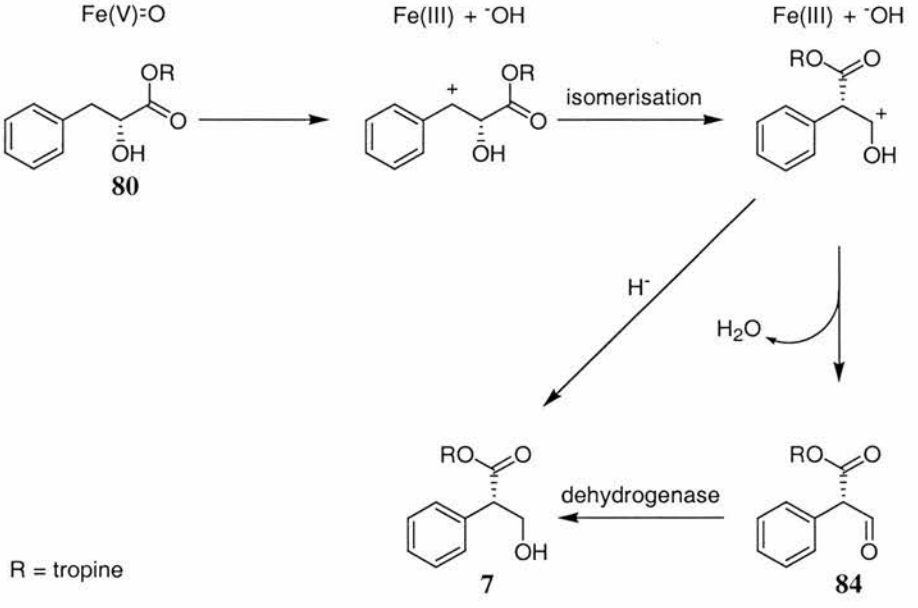


Figure 1.42: Proposed cytochrome P-450 dependent mechanism for the rearrangement of littorine (**80**) to hyoscyamine (**7**) *via* carbocation intermediates.[125,152]

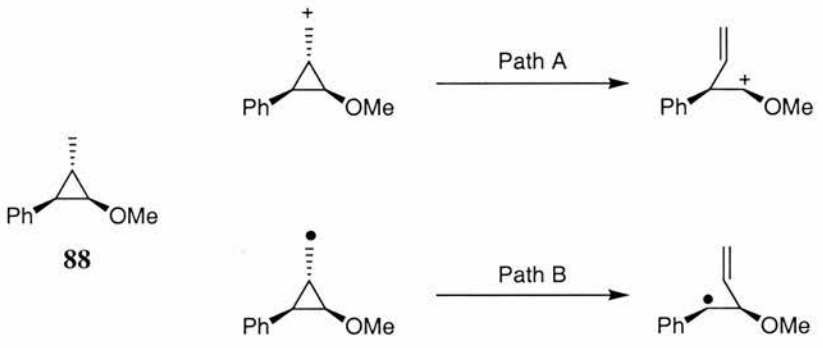


Figure 1.43: Cationic (path A) and radical (path B) ring openings of methylcyclopropanes.[150,151]

This experimental evidence led O'Hagan and Robins[125] to propose a carbocation mechanism for the isomerisation of littorine (**80**) to hyoscyamine (**7**) involving two enzyme activities (a mutase and a dehydrogenase).

1.6.5 A role for *S*-adenosyl methionine?

Quite unexpectedly there was a report of a *S*-adenosyl-L-methionine (**60**, SAM) stimulated cell free extract that catalysed the transformation of littorine (**80**) to hyoscyamine (**7**).[153] This study observed that addition of SAM (**60**) stimulated the rearrangement 10-20 fold as determined by GC in an extract of *D. stramonium*. SAM (**62**) has been shown to catalyse rearrangement reactions in an analogous manner to co-enzyme B_{12}, e.g. lysine 2,3-aminomutase (figure 3.6).[154,155] Further discussion of the putative role of SAM (**62**) will be given in chapter three.

1.7 Chapter summary

The biosynthesis of the tropane alkaloid hyoscamine (**7**) has been investigated in detail, however, a number of important issues remain to be resolved. Firstly the immediate biosynthetic precursor of the C_3 acetate derived fragment of the tropane ring is unknown. Secondly although the biosynthetic precursors to tropic acid (**24**) have been determined the mechanistic detail of the transformation of littorine (**80**) to hyoscyamine (**7**) remains unknown, as is any associated enzyme(s) responsible for the rearrangement.

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Chapter 2
Biosynthesis of the Tropane Ring

2 BIOSYNTHESIS OF THE TROPANE RING

This chapter describes experiments designed to investigate the biosynthesis of the tropane ring moiety of hyoscyamine (**7**). Transformed root cultures of *D. stramonium* are introduced, as are the methods of alkaloid isolation and analysis, including a GC-MS method for separation and analysis of littorine (**80**) and hyoscyamine (**7**). A number of labelled precursors of the tropane ring, both synthetic and commercial have been administered to the root cultures. The biosynthesis of the tropane skeleton is discussed in light of these feeding experiments.

2.1 Datura stramonium L.

2.1.1 Introduction

D. stramonium is an annual, terrestrial herb possessing white flowers and spiny capsules. The spiny capsules containing black seeds give rise to the common name, thorn apple. The plant is distributed throughout North and South America, Europe and Asia although its origin is uncertain.[1] Hyoscyamine (**7**) and scopolamine (**37**) are the major alkaloids of *D. stramonium* making it an ideal plant system to study the biosynthesis of the tropane alkaloids. Prior to the development of transformed root cultures (section 2.1.2) whole plants of *D. stramonium* (Figure 2.1) were frequently used as a plant model for biosynthetic investigations.[2,3] The putative precursors were administered to the plant *via* the 'wick method'[2] or by addition to the hydroponic growth solution.[3]

2.1.2 *D. stramonium* root cultures

Transformed root cultures of *D. stramonium* have been engineered. These cultures possess many advantages over whole plants for use in biosynthetic experiments (section 1.3.2). One line of tissue culture cells, D15/5, has been shown to possess good growth characteristics[4] and has been used extensively for biosynthetic and biochemical studies.[5,6] This cell line produces hyoscyamine (**7**) as its dominant alkaloid, with scopolamine (**37**) co-produced at trace levels.[7] The production of a single tropane

alkaloid at high levels is advantageous, as the majority of isotopic label from administered substrates is incorporated into a single metabolite, which can be isolated in sufficient quantity to perform NMR analysis. Accordingly *D. stramonium* D15/5 root cultures were used as the plant system for this research programme.

Figure 2.1: Whole plant of *Datura stramonium*.

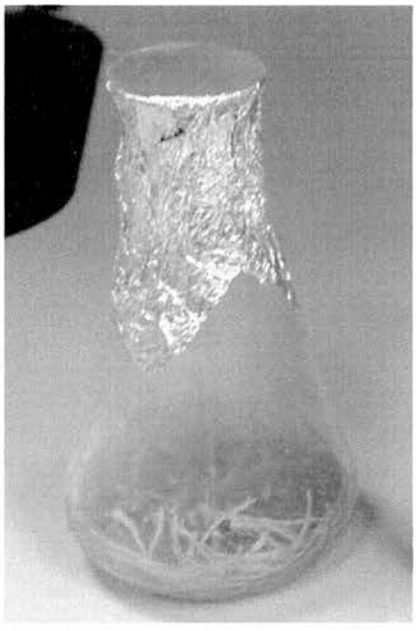


Figure 2.2: Transformed root culture of *Datura stramonium* D15/5.

2.2 Maintenance and growth of transformed root cultures of *D. stramonium*.

D. stramonium D15/5 obtained from the laboratory of R.J. Robins (CNRS, Université de Nantes) had been used and maintained in our laboratory for a number of years prior to the study. The cultures used in biosynthetic experiments and for the continuation of the cell line were grown in identical media. This chemically defined minimal liquid media consisted of sucrose (30 gL^{-1}) as the sole carbon source and Gamborg's B-5 basal medium[8] powder (Sigma) (3.2 gL^{-1}), which provided all the other essential nutrients. The media was made up with distilled water and the pH adjusted to 6.8 using dilute sodium hydroxide. Aliquots (50 mL) of this media were dispensed into Erlenmeyer flasks (250 mL), which were covered with three foil lids and sterilised by autoclaving. When possible the culture media was stored for a week prior to use to ensure that no contaminating organisms were present.

To inoculate a flask, root tissue (500 mg) was removed from a 2-3 week old culture and transferred to a fresh flask of media under aseptic conditions. The root cultures were incubated at 27°C on an orbital shaker (100 rpm), under constant illumination. The cell line was maintained by continual subculture every 2-3 weeks in the manner described above.

2.2.1 Production of Alkaloids

The temporal production of tropane alkaloids has previously been studied in transformed root cultures of *D. stramonium* D15/5.[7] The production of hyoscyamine (**7**) was found to be greatest between days 7-17 after subculture and the amount of hyoscyamine (**7**) maximal in the tissue on day 22 (Figure 2.3). Accordingly when conducting a feeding experiment (section 2.3.1) compounds were administered to the roots on day 6 or 7 after subculture and the alkaloids isolated (section 2.3.2) on day 17 or 18.

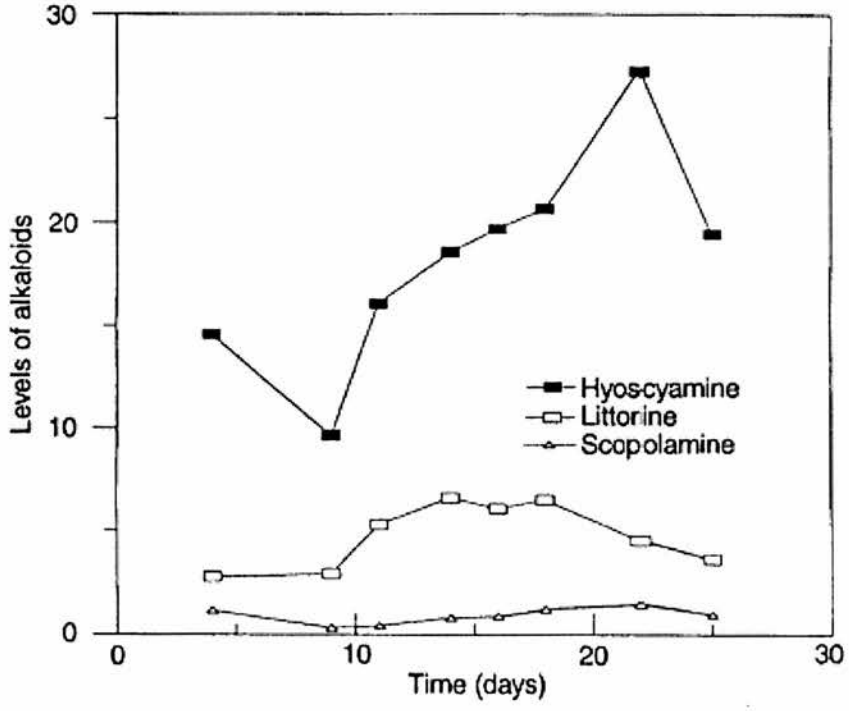


Figure 2.3: The temporal accumulation of littorine (**80**), hyoscyamine (**7**) and scopolamine (**37**) in root cultures of *D. stramonium*. Alkaloid levels are given in μmol per gram dry weight.[7]

2.3 Studying tropane alkaloid biosynthesis

2.3.1 Feeding experiments

Putative intermediates to the tropane alkaloids were administered to the root cultures in solution (water or ethanol) *via* a sterile filter to remove contaminating micro organisms. Compounds were administered on day 6 or 7 after feeding prior to the period of maximal alkaloid production (section 2.2.1), to facilitate the most efficient incorporation of isotope into hyoscyamine (**7**). The feeding experiments were worked up on day 17 or 18 after subculture; beyond this period hyoscyamine (**7**) is degraded in the root tissue.

2.3.2 Extraction and purification of hyoscyamine from *D. stramonium*

Hyoscyamine (**7**) was extracted from transformed root cultures of *D. stramonium* by the method of Robins and co-workers.[4] Accordingly the roots were collected by filtration, the residual media washed off with distilled water and the root mass dried between tissue paper. The roots were then quickly frozen in liquid nitrogen and lyophilised. The dried roots were ground with sand and suspended in dilute sulfuric acid with stirring for 20 minutes. The solution was then basified and loaded onto Hydromatrix (Varian) columns. The large surface area of the Hydromatrix produces efficient liquid/liquid emulsion free extractions between the aqueous and organic extraction solvent. The columns were eluted with chloroform/methanol to yield the crude alkaloid fraction. This crude alkaloid sample could be analysed directly by GC (section 2.3.3.2), or further purified by preparative t.l.c prior to ^{13}C-NMR analysis (section 2.3.3.1). Alkaloids were visualised on the t.l.c. plate under u.v. light, or by staining with Dragendorff's spray reagent (Sigma), an iodide based stain.

2.3.3 Analysis of hyoscyamine

Two principal methods of analysing isolated hyoscyamine (**7**) were employed during the research programme. ^{13}C-NMR spectroscopy was used to determine the regiospecific incorporation of carbon-13 isotope from administered substrates (section 2.3.3.1). GC-MS allowed isotope incorporations to be determined by detection of

molecular ion populations with an increased mass (section 2.3.3.2). A HPLC method for separation of the isomeric alkaloids was also developed (section 2.3.3.3).

2.3.3.1 NMR analysis

In order to determine the regiospecific incorporation of isotopic label from feeding experiments with carbon-13 enriched substrates it was necessary to have a complete assignment of the ^{13}C-NMR spectrum of hyoscyamine (**7**). A complete ^{13}C-NMR assignment of hyoscyamine (**7**) has been reported,[9] achieved through a combination of ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR NMR experiments. These experiments were unable to differentiate between diastereotopic carbons 1 and 5, 2 and 3, or 6 and 7 due to the high level of symmetry of the tropane ring (Table 2.1). The ^{13}C-NMR spectrum of hyoscyamine (**7**) with full assignment is shown in Figure 2.4 and Table 2.1.

Position	δ_C	Position	δ_C
C-1, C-5	61.0, 61.1	C-2´	56.2
C-2, C-4	36.5, 36.7	C-3´	64.6
C-3	68.5	a	137.4
C-6, C-7	25.8, 26.1	b	129.9
NMe	40.2	c	129.2
C-1´	173.2	d	128.7

Table 2.1: ^{13}C-NMR assignments for hyoscyamine (**7**).[9] The structure of hyoscyamine (**7**) with the carbon atoms numbered is shown in Figure 2.4.

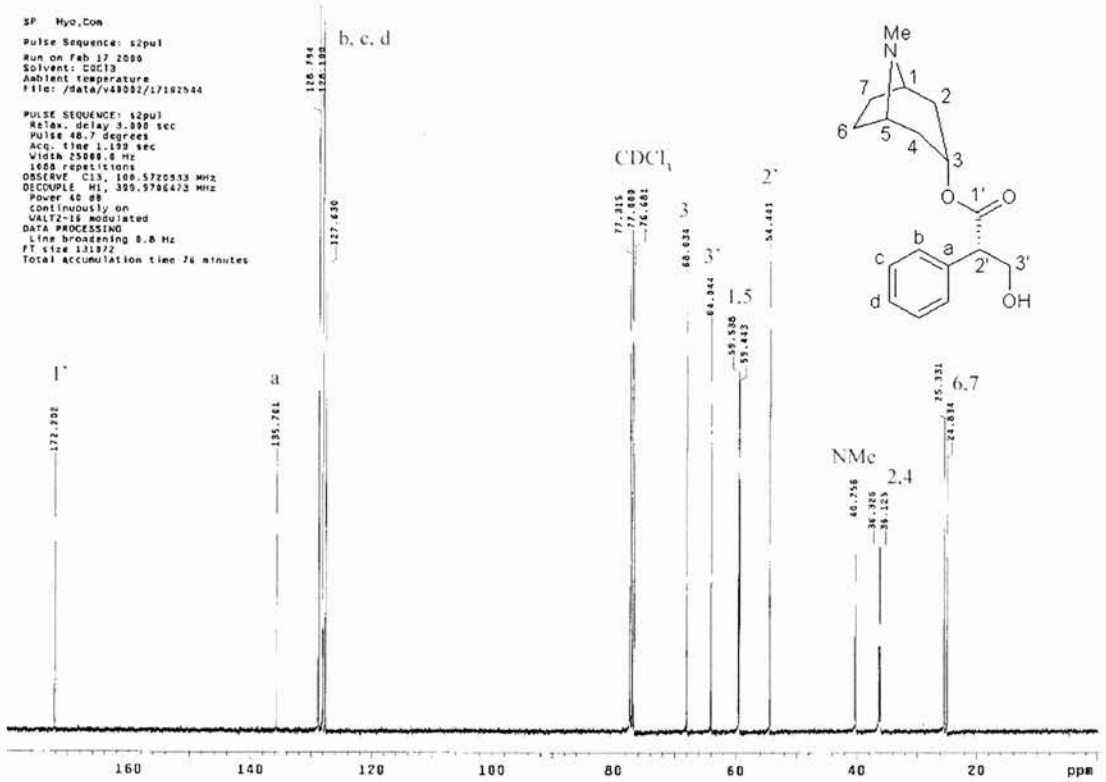


Figure 2.4: ^{13}C-NMR spectrum of hyoscyamine (**7**) ($CDCl_3$, 100 MHz).

2.3.3.2 GC-MS analysis of alkaloids from *D. stramonium*.

Although GC-MS analysis does not readily indicate regiospecific isotope incorporations (section 1.3.1.2.1), the technique is useful as it allows detection of isotope incorporations at a lower level than NMR experiments (~0.4%), and the analysis of product mixtures is possible, facilitating simultaneous analysis of littorine (**80**) and hyoscyamine (**7**) where desirable.

2.3.3.2.1 Synthesis of littorine

Unlike hyoscyamine (**7**), littorine (**80**) is not commercially available. Therefore it was necessary to prepare a synthetic sample of littorine (**80**) in order to develop a chromatographic method to separate littorine (**80**) from its isomer hyoscyamine (**7**). Littorine (**80**) had previously been synthesised by acid catalysed esterification of tropine (**35**) with (*R*)-phenyllactic acid (**78**) (Figure 2.5).[10] Accordingly tropine (**35**) and (*R*)-phenyllactic acid (**78**) were heated at 145°C and a stream of dry HCl gas passed intermittently over the mixture. ^{1}H-NMR analysis of the crude reaction product after workup showed a mixture of littorine (**80**) and cinnamoyl tropine (**89**) (olefinic ^{1}H-

NMR resonances were visible), the latter formed by the elimination of water from littorine (**80**) due to the high reaction temperature and acidic conditions (Figure 2.7). Column chromatography over silica yielded littorine (**80**) with a good recovery (60-80%).

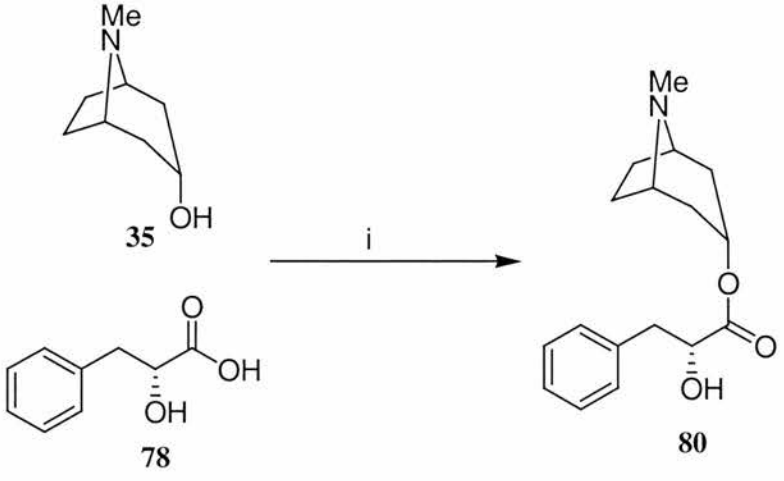


Figure 2.5: Synthesis of littorine (**80**) from tropine (**35**) and (*R*)-phenyllactic acid (**78**). Reagents and conditions: i, HCl (g), 145°C, 3.5 hrs.

2.3.3.2.2 GC analysis of littorine and hyoscyamine

A sensitive GC method for separating and assaying the levels of the tropane alkaloids had previously been developed within the laboratory.[7] For this method trimethylsilyl ethers of the alkaloids were prepared by use of the commercially available derivatising reagent *N*-methyl-*N*-trimethylsilyl trifluoroacetamide[11] (**90**, MSTFA) (Fluka) (Figure 2.6). The derivatisation was also found to prevent the artefactual formation of cinnamoyl tropine (**89**) from littorine (**80**) during the analysis (Figure 2.7). The method has a short run time (20 minutes), therefore it is suited to the repeated analyses required for calculating accurate incorporation of label into the alkaloids.

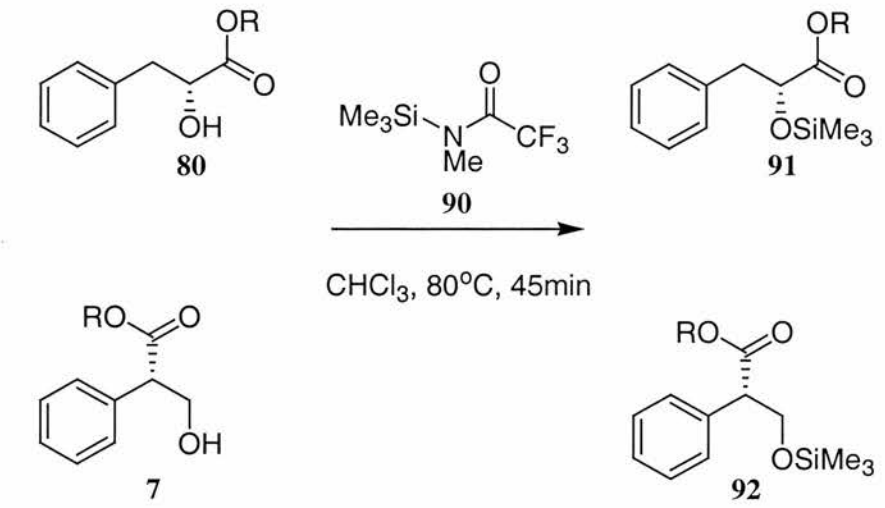


Figure 2.6: Synthesis of trimethylsilyl ether derivatives (**91**, **92**) of the tropane alkaloids littorine (**80**) and hyoscyamine (**7**) by treatment with MSTFA (**90**), for GC-MS analysis.

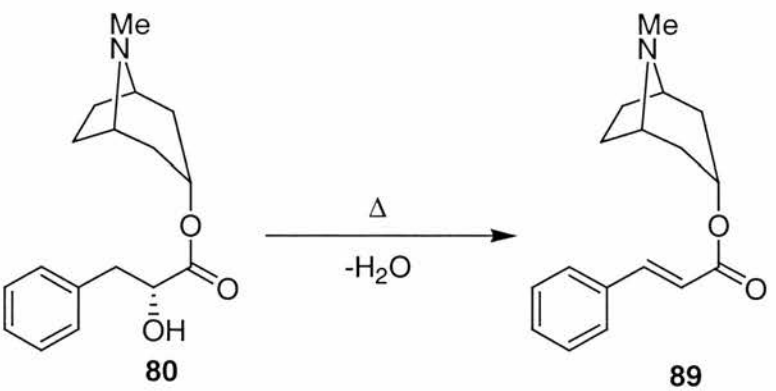


Figure 2.7: Formation of cinnamoyl tropine (**89**) from dehydration of littorine (**80**).

A sample of synthetic littorine (**80**) and commercial hyoscyamine (**7**) was analysed using a chiral SGE capillary column (fused silica, cydex-B). The oven heating profile contained a shallow temperature gradient (3°C min^{-1}) over the elution period of the two isomeric alkaloids to aid separation (Figure 2.8). This method was unable to separate enantiomers in racemic mixtures of the alkaloids although separation of hyoscyamine (**7**) and littorine (**80**) was routinely achieved. The same GC method was applied to a crude alkaloid extract from *D. stramonium* root cultures and found to be reliable for alkaloid separations.

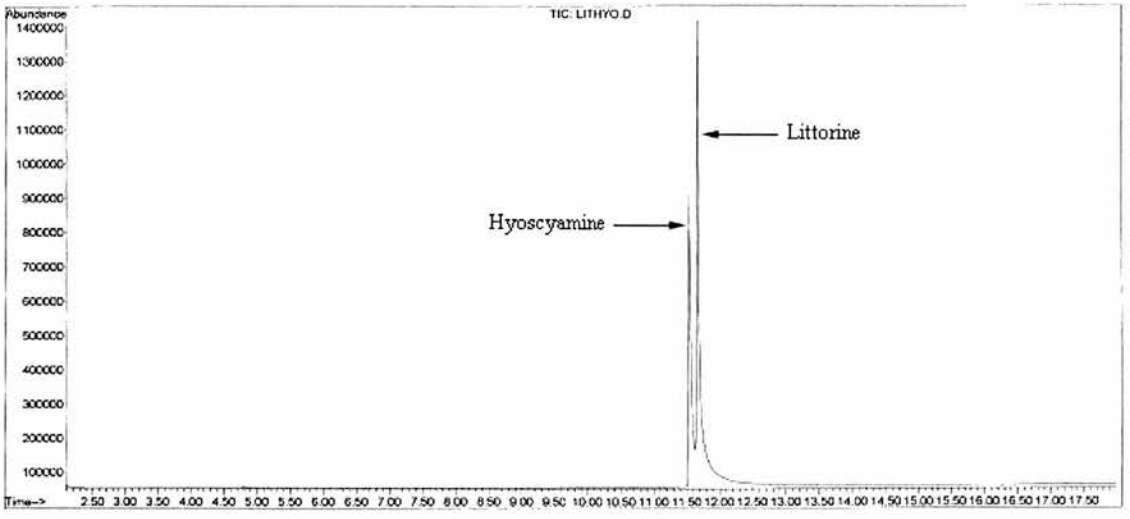


Figure 2.8: GC-MS total ion chromatogram of littorine (**80**) and hyoscyamine (**7**).

The trimethylsilyl derivatised alkaloids (**91**, **92**) gave low percentage abundance molecular ion (*m/z* 361) in GC-EIMS. Similarly GC-CIMS analysis using methane as the reagent gas gave low percentage abundance for the molecular ion. However, the abundance of the molecular ion was sufficient for calculating mass enrichments due to isotope incorporation. Mass analysis was performed in selective ion monitoring (SIM) mode, measuring the ion currents for *m/z* 361 up to that appropriate for the feeding experiment being analysed. Mass enrichments were calculated by determining the abundances of the molecular ion and the M+1, M+2 ions etc. and correcting for the natural abundance levels of these ions by comparison with unlabelled standards. All analyses were performed ten times and the mean enrichment calculated. With this methodology it is possible to have confidence in low enrichment levels (~0.4-0.5%) provided that the standard deviation of the data is low. Performing analysis in SIM mode reduces the size of the computer data files collected, which was essential given the requirement for ten repetitions of each analysis.

2.3.3.3 HPLC analysis of hyoscyamine and littorine

In addition to GC, HPLC was explored as a technique to separate littorine (**80**) and hyoscyamine (**7**). Initial analyses using a C_{18} reverse phase column with a solvent gradient running from 100:0 to 0:100 water : acetonitrile containing 0.01% TFA

resolved the alkaloids by approximately 30 seconds. The alkaloids were detected by measuring the absorbance at 260 nm. The identity of the chromatogram peaks was determined unambiguously by individual analysis of samples of hyoscyamine (**7**) and synthetic littorine (**80**). The separation was improved by using an isocratic elution profile of 80:20 water : acetonitrile (+0.01% TFA) (Figure 2.9). This optimised method resolved the alkaloids by almost two minutes. This HPLC method was also effective when scaled up using a semi-preparative reverse phase column.

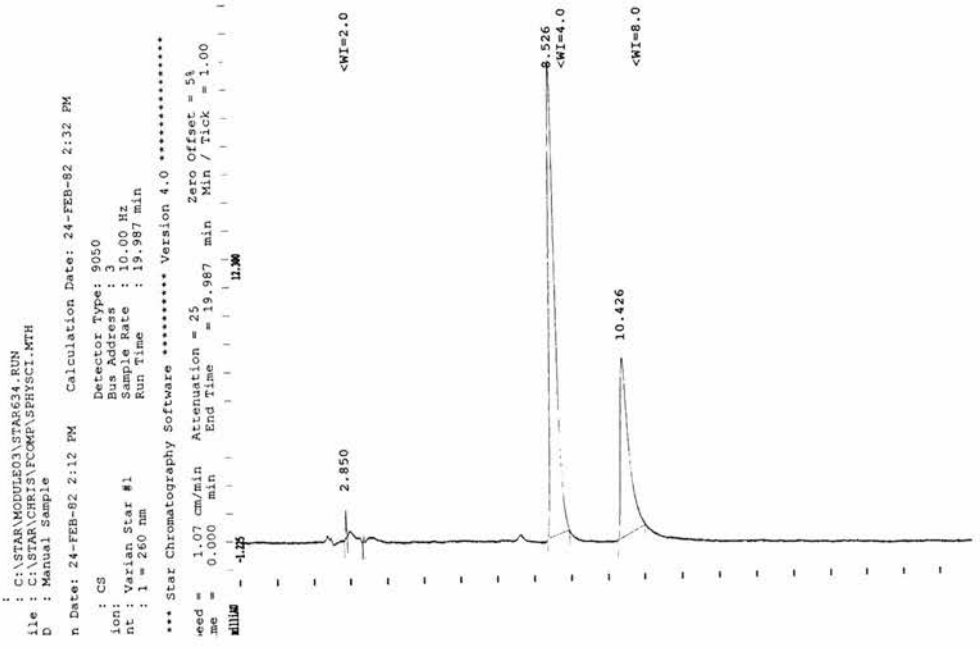


Figure 2.9: HPLC chromatogram of hyoscyamine (**7**) (RT=8.5) and littorine (**80**) (RT=10.4).

Despite the superior separation achieved by HPLC analysis relative to GC and the ability to analyse samples directly without derivatisation the methodology was not employed further in the research programme. However, when the HPLC method was applied to a crude alkaloid extract from *D. stramonium* root cultures it was found not to separate the alkaloids from other components of the extract. Additionally, the auto sampler facility attached to the GC-MS instrument made GC a more convenient source of analysis for the multiple samples produced during the investigations. Mass analysis was an absolute requirement to interpret the majority of feeding experiments conducted during the study. Therefore, GC-MS became the analytical technique of choice for the research programme.

2.4 Feeding experiments with carbon-13 labelled acetates

Supplementation of carbon-13 labelled sodium acetates to root cultures of *D. stramonium* allowed the simultaneous investigation of both the biosynthesis of the amino acid derived C_4N fragment and the acetate derived C_3 fragment of the tropane alkaloids.

2.4.1 Previous feeding experiments

A number of studies have investigated the biosynthesis of the tropane skeleton by the feeding of labelled acetate precursors.[12] It has long been established that the C_3 bridge of the tropane ring is derived from acetate (**33**).[13] An early feeding experiment also demonstrated the incorporation of acetate (**33**) into the C_4N fragment.[14]

Acetate (**33**) enters the TCA cycle *via* its co-enzyme A thioester acetyl-CoA (**5**), which condenses with oxaloacetate (**93**) to produce citric acid (**94**) (Figure 2.10). Citric acid (**94**) is then transformed by the action of three enzymes into α-ketoglutarate (**76**), which serves as a precursor to the amino acid glutamate (**95**), which in turn is a precursor of ornithine (**26**). Through these biosynthetic pathways C-1 and C-2 of acetate (**33**) label C-5 and C-4 of ornithine (**26**) respectively. Ornithine (**26**) as postulated by Robinson is a proven precursor of the C_4N fragment (section 1.5.1.1.1). In this way acetate (**33**) serves as an ornithine (**26**) surrogate in feeding experiments.

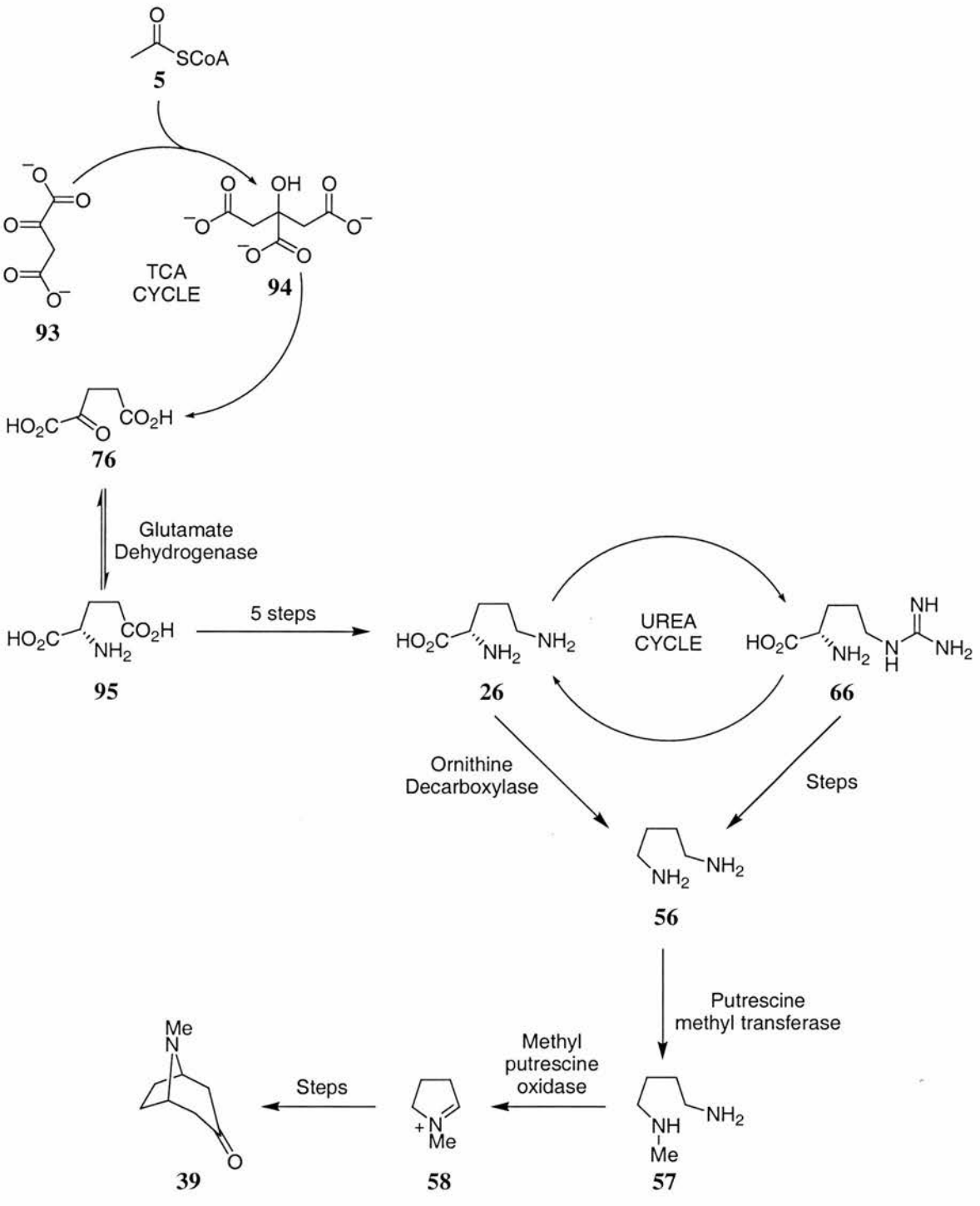


Figure 2.10: The biosynthetic transformations from acetyl-CoA (**5**) to the tropane ring.

Feeding experiments with isotopomers of acetate (**33**) have drawn conflicting conclusions as to the role of a symmetrical intermediate in the biosynthesis of the C_4N fragment of the tropane ring.[12] The more recent studies[9,15,16] employing stable isotope methodology have demonstrated a symmetrical pattern of incorporation into C_4N fragment of the tropane ring system (Figure 2.11), whilst an earlier investigation[14] using radiotracer techniques concluded that no free symmetrical intermediate could be

implicated in the pathway (Figure 1.16). A review[12] of feeding experiments investigating the biosynthesis of the C_4N fragment revealed that with only a single exception[17] all experiments that utilised stable isotope methodology have reported a symmetrical incorporation of isotope into tropine (**35**). However, those employing radiotracer techniques have resulted in unsymmetrical incorporation of label into tropine (**35**). Initially these inconsistencies were rationalised as variations between species.[18] However, there are now reports with conflicting results within the same species (*D. stramonium*).[9,14,15] This suggests that the methodology employed may be the cause of the conflicting incorporation patterns into tropine (**35**).

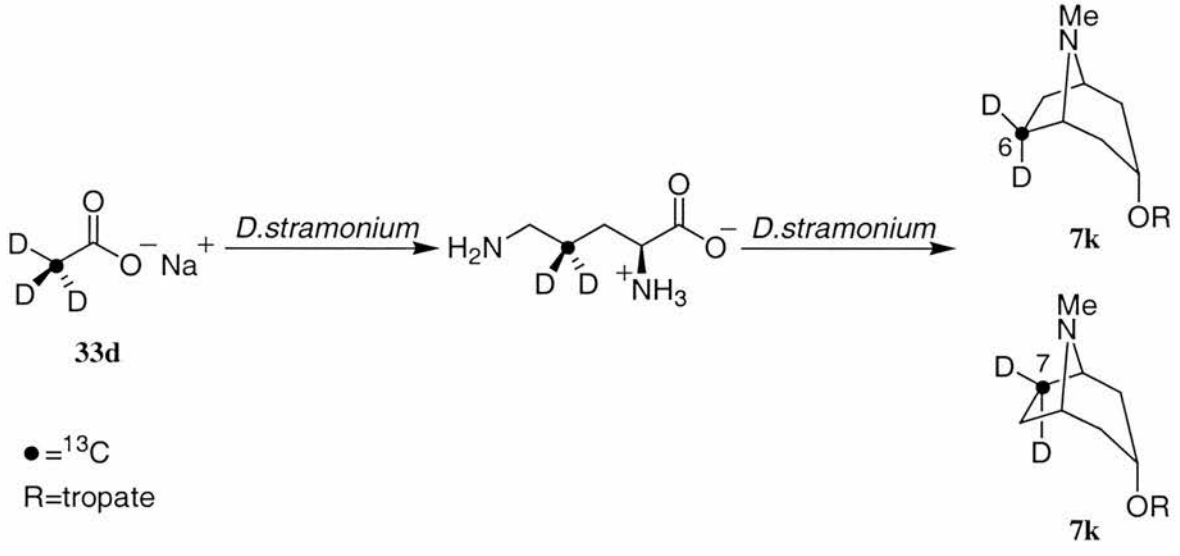


Figure 2.11: The symmetrical incorporation of sodium [2-^{13}C, $^{2}H_3$]acetate (**33d**) into C-6 and C-7 of hyoscyamine (**7k**) in root cultures of *D. stramonium*.[9]

It has been speculatively proposed[12] that these inconsistencies are the result of administering stable isotopomers of acetate (**33**) at high concentrations, which results in an aberrant metabolic flux through the biosynthetic pathway. An increased metabolic flux could result in the accumulation of an intermediate *in vivo*, allowing it to racemise prior to further metabolism. This would result in the unsymmetrical incorporation of label observed in stable isotope feeding experiments. Radiotracer experiments, which administer compounds in microgram quantities, do not affect the natural *in vivo* metabolic flux of a biosynthetic pathway. Therefore, no racemisation of the intermediate occurs, consistent with the observed symmetrical incorporation pattern.

However, although no fault can be found with the methodology of the early radio labelling feeding experiments, investigation with stable isotopes has previously proven to be a more rigorous approach. For example, experiments with stable isotopomers of phenyllactic acid (**78**) have corrected earlier investigations into the stereochemical course of the isomerisation of littorine (**80**) to hyoscyamine (**7**) (section 1.6.4). Therefore, the conclusions from experiments employing stable isotope methodology, i.e. the involvement of a symmetrical intermediate, are more secure.

2.4.2 Feeding of sodium [1-^{13}C]acetate to *D. stramonium*.

Sodium [1-^{13}C]acetate (**33e**) was administered to nine transformed root cultures of *D. stramonium* at a final concentration of 8mmol dm^{-3} in the medium. Hyoscyamine (**7l**) was isolated, purified and subjected to ^{13}C-NMR analysis. The resultant ^{13}C-NMR spectrum (Figure 2.12) showed an approximate 2% enrichment of the peaks corresponding to C-1 and C-5, and a high (15%) enrichment of the peak corresponding to C-3 of the tropane ring (Figure 2.13). The percentage enrichment values were calculated by observing the increased peak heights of these resonances relative to that of C-1′, which is not expected to readily incorporate label from acetate (**33**).

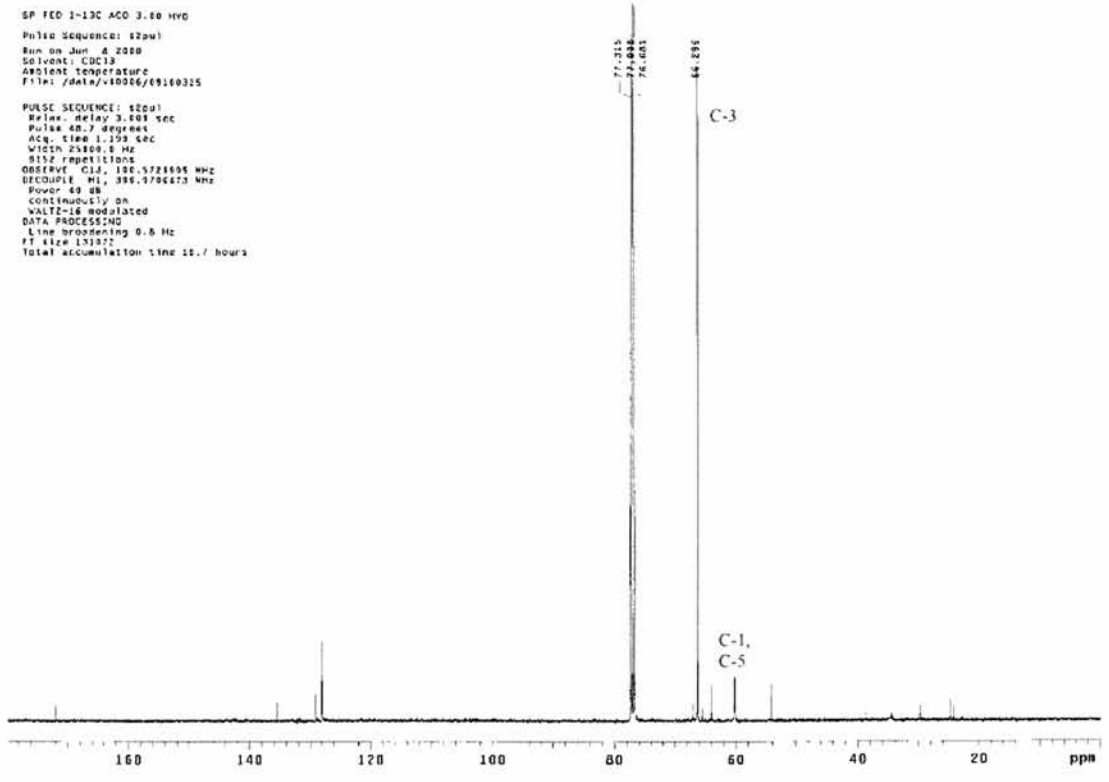
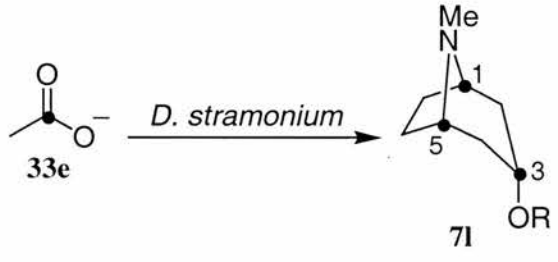


Figure 2.12: The ^{13}C-NMR spectrum of hyoscyamine (**7l**) isolated from *D.stramonium* root cultures fed sodium [1-^{13}C]acetate (**33e**). The enriched peaks corresponding to C-1, C-3 and C-5 are labelled.

The positions and levels of incorporation are consistent with those observed previously from the feeding of acetate (**33**) carrying carbon-13 label at C-1 into transformed root cultures,[9,19] but are inconsistent with the asymmetric incorporation reported when sodium [1-^{14}C]acetate (**33b**) was fed to root cultures of *D. stramonium* (figure 1.16).[14] The differential levels of incorporation can be rationalised by considering the number of

metabolic steps from acetyl CoA (**5**) to different positions in the tropane ring. The level of enrichment into C-1 and C-5 is relatively low (2%), as the label has to be carried through several metabolic steps (Figure 2.10). Additionally, most of these metabolic steps supply intermediates to pathways other than the biosynthesis of the tropane ring. Due to the supply of intermediates into other pathways the label is successively 'diluted'. The level of enrichment into C-3 is relatively high (15%) and from this it can be concluded that the number of biosynthetic steps from acetate (**33**) to the C_3 fragment of the tropane ring is fewer than that for the C_4N fragment, and/or a greater number of the steps are committed to the biosynthesis of the tropane ring.



33e **7l**

R=tropate

Figure 2.13: Summary of the incorporation of sodium [1-^{13}C]acetate (**33e**) into the tropane ring of hyoscyamine (**7l**).

2.4.3 Feeding of sodium [2-^{13}C]-acetate to *D. stramonium*.

Sodium [2-^{13}C]acetate (**33f**) was administered to nine transformed *D. stramonium* root cultures at a final concentration of 8mmol dm^{-3}. The isolated hyoscyamine (**7m**) was analysed by ^{13}C-NMR spectroscopy. The resultant spectrum (Figure 2.14) shows a 6% and 30% enrichment of the peaks corresponding to C-6, C-7 and C-2, C-4 respectively (Figure 2.15). Enrichment levels were calculated as described for hyoscyamine (**7l**) from the feeding experiment with sodium [1-^{13}C]acetate (**33e**) (section 2.4.2).

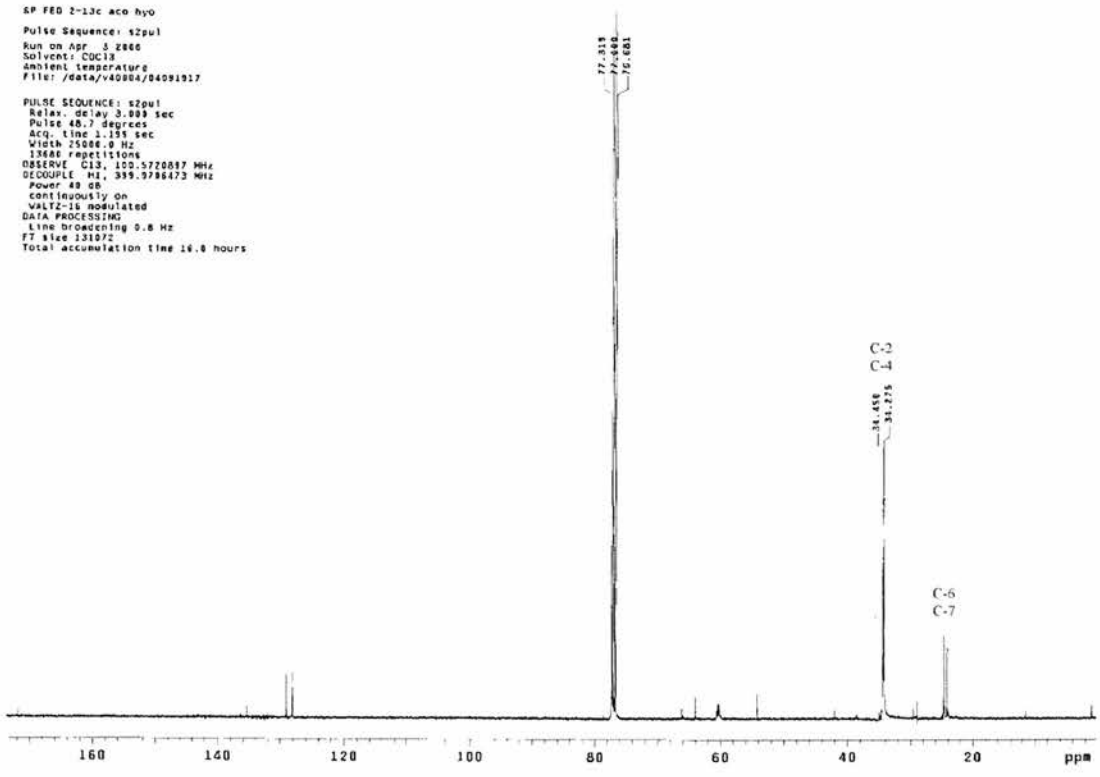


Figure 2.14: The ^{13}C-NMR spectrum of hyoscyamine (**7m**) isolated from *D.stramonium* root cultures fed sodium [2-^{13}C]acetate (**33f**). The enriched peaks corresponding to C-2, C-4, C-6 and C-7 are labelled.

The positions of carbon-13 enrichment into the tropane ring are consistent with C-2 of acetate (**33**) enriching C-4 of ornithine (**26**), which is subsequently incorporated into hyoscyamine (**7**) *via* known biosynthetic steps (Figure 2.10). The differential levels of incorporation between the C_4N fragment and the C_3 bridge of tropine (**35**) are consistent with those for the incorporation of sodium [1-^{13}C]acetate (**33e**) outlined above (see section 2.4.2), but the enrichment levels are higher in this experiment. Again this pattern of incorporation suggests that a symmetrical intermediate (putrescine (**56**)) is involved in the biosynthesis as a free non-enzyme bound molecule.

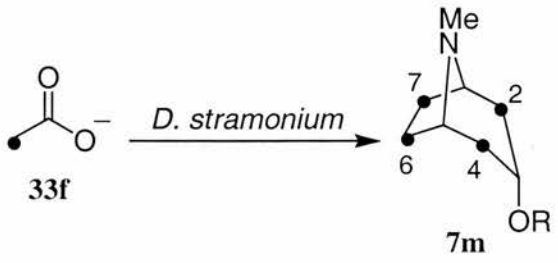


Figure 2.15: Incorporation of sodium [2-^{13}C]acetate (**33f**) into the tropane ring of hyoscyamine (**7m**).

In addition to enriched peaks resulting from the incorporation of a carbon-13 from sodium [2-^{13}C]acetate (**33f**) the ^{13}C-NMR spectrum of the resultant hyoscyamine (**7m**) also shows extensive ^{13}C-^{13}C spin-spin coupling, resulting from the incorporation of two adjacent carbon-13 atoms into the tropane ring. As this coupling cannot arise from the intact incorporation of a ^{13}C-^{13}C bond from the fed [2-^{13}C]acetate (**33f**), two isotope labels must become contiguous in a biosynthetic precursor between acetyl CoA (**5**) and the tropane ring.

Examination of the peaks corresponding to C-1 and C-5 (Figure 2.16) reveals a complex multiplet. This multiplet (six signals) arises from the C-1 and C-5 natural abundance resonance signals, plus a pair of doublets. These doublets are clearly the result of ^{13}C-^{13}C spin-spin coupling between C-1/C-5 and an adjacent carbon-13 atom. The $^{1}J_{CC}$ coupling constants for the peaks are 35 Hz.

The total integrals of the coupled peaks are similar to that of the natural abundance signals. This can only be rationalised if approximately 50% of the hyoscyamine (**7m**) molecules in the sample contain carbon-13 at C-2, C-7 and C-4, C-6. The combined level of enrichment at either pair of these positions is 36% (6% + 30%) is in close agreement to the estimated level (50%). The coupling constants for the different intact ^{13}C-^{13}C bonds must be approximately equal, as only a single doublet is visible for C-1/C-5.

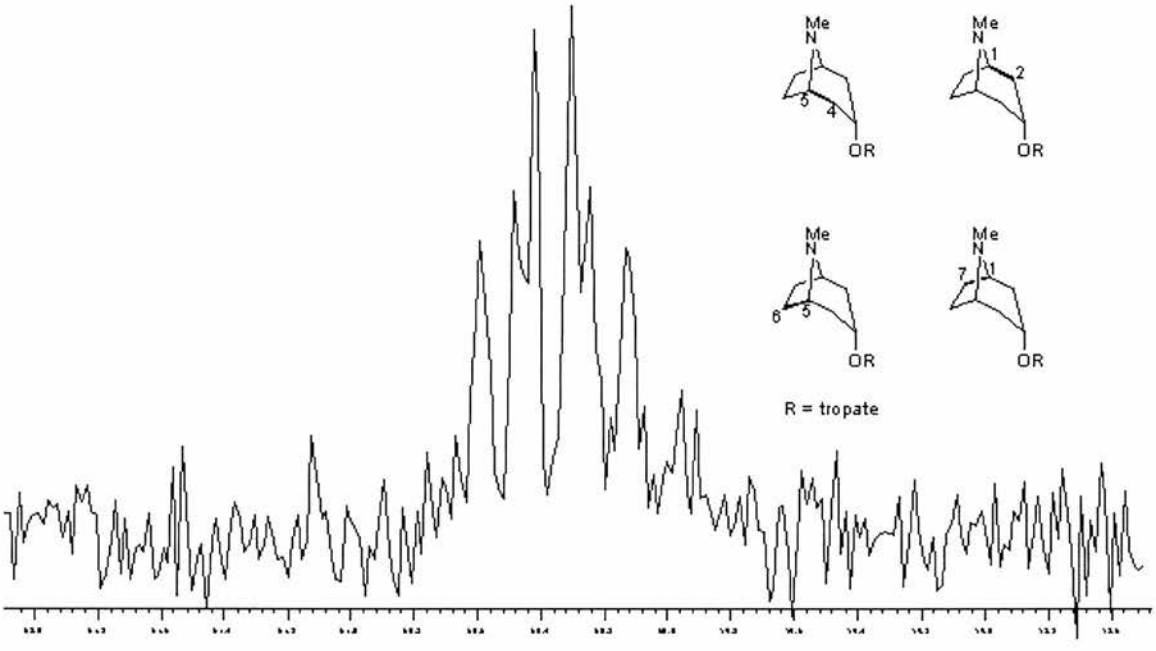


Figure 2.16: ^{13}C-NMR of hyoscyamine (**7m**) extracted from root cultures fed sodium [2-^{13}C]acetate (**33f**), showing the resonances for C-1 and C-5.

Additionally, there are also small satellites visible around the signals corresponding to C-6 and C-7 in the ^{13}C-NMR spectrum (Figure 2.17). Close examination reveals two distinguishable doublets around each resonance, both doublets have similar coupling constants (~26 Hz). As there are two sets of doublet satellites, there is a requirement for carbon-13 label to be present at the bridgehead carbon adjacent to the enriched C-6/C-7 signal *and* for an intact ^{13}C-^{13}C bond to be incorporated between C-6 and C-7.

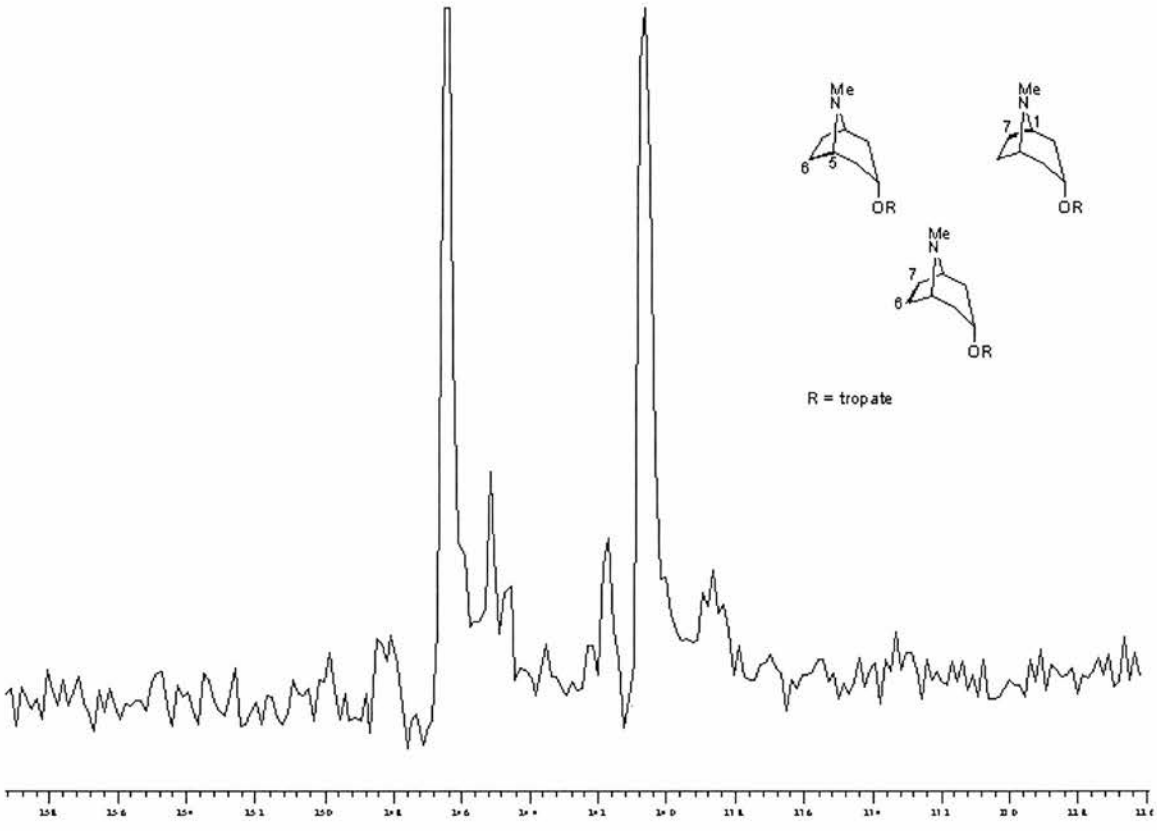


Figure 2.17: ^{13}C-NMR of hyoscyamine (**7m**) extracted from root cultures fed sodium [2-^{13}C]acetate (**33f**), showing the resonances for C-6 and C-7.

This contiguous incorporation of carbon-13 label can be rationalised if isotope label from [2-^{13}C]acetate (**33f**) is carried through more than one 'turn' of the TCA cycle (Figure 2.18). Incorporation of an intact ^{13}C-^{13}C bond between C-6 and C-7 can arise if two [2-^{13}C]acetate (**33f**) molecules enter the TCA cycle in successive 'turns' then leave the cycle as α-ketoglutarate (**76a**), and ultimately incorporate into tropine (**35a**) (Figure 2.18). To incorporate an intact ^{13}C-^{13}C bond between C-1/C-7 or C-2/C-6 of tropine (**35b**) two molecules of [2-^{13}C]acetate (**33f**) must enter the TCA cycle in successive 'turns' as described above, followed by the incorporation of a 'cold' molecule of acetyl CoA (**5**) before leaving as α-ketoglutarate (**76b**) (Figure 2.18).

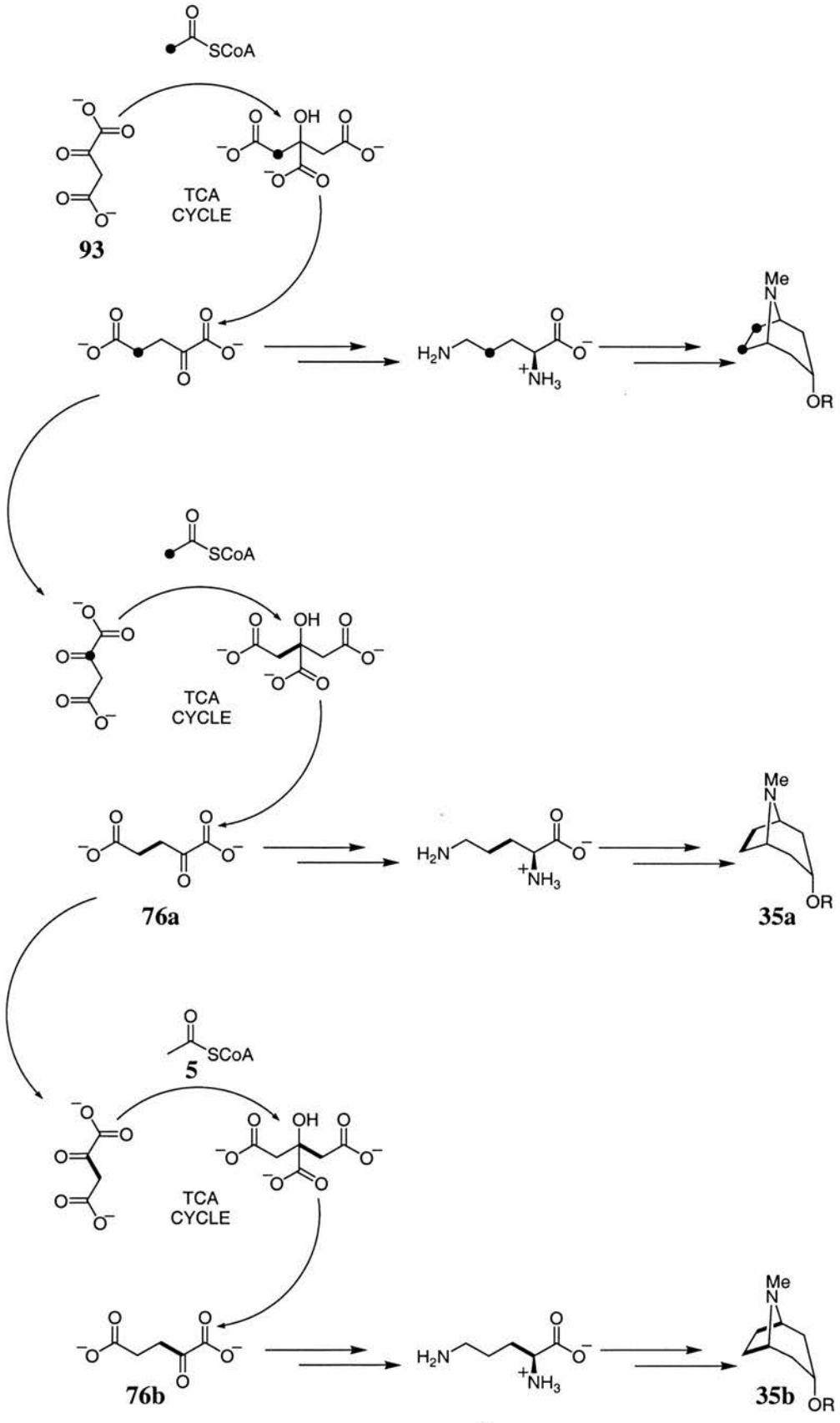


Figure 2.18: Incorporation of sodium [2-^{13}C]acetate (**33f**) into the tropane ring *via* multiple passes through the TCA cycle.

2.4.4 Feeding of sodium [1, 2-$^{13}C_2$]acetate to *D. stramonium*.

Initial feeding experiments with sodium [1,2-^{13}C]acetate (**33a**) were conducted with the same concentration (8 mmol dm^{-3}) as the feeding experiments with singularly labelled acetate (**33e**, **33f**). However, the resultant hyoscyamine produced a ^{13}C-NMR spectrum with extensive ^{13}C-^{13}C spin-spin coupling. Interpretation of this spectrum proved difficult and it was decided to repeat the experiment administering 'cold' sodium acetate (**33**) together with sodium [1,2-$^{13}C_2$]acetate (**33a**). Thus, a mixture of sodium [1, 2-^{13}C]acetate (**33a**) and sodium acetate (**33**) was fed to nine root cultures of *D. stramonium* to a final concentration of 2 mmol dm^{-3} and 6 mmol dm^{-3} respectively. The isolated hyoscyamine (**7n**) was analysed again by ^{13}C-NMR (Figure 2.19), which revealed spin-spin coupling for every carbon resonance of the tropane ring (Figure 2.20). The resultant incorporation patterns can be rationalised by a combination of the incorporation patterns from the individual feeding of sodium [1-^{13}C] (**33e**) and [2-^{13}C]acetates (**33f**).

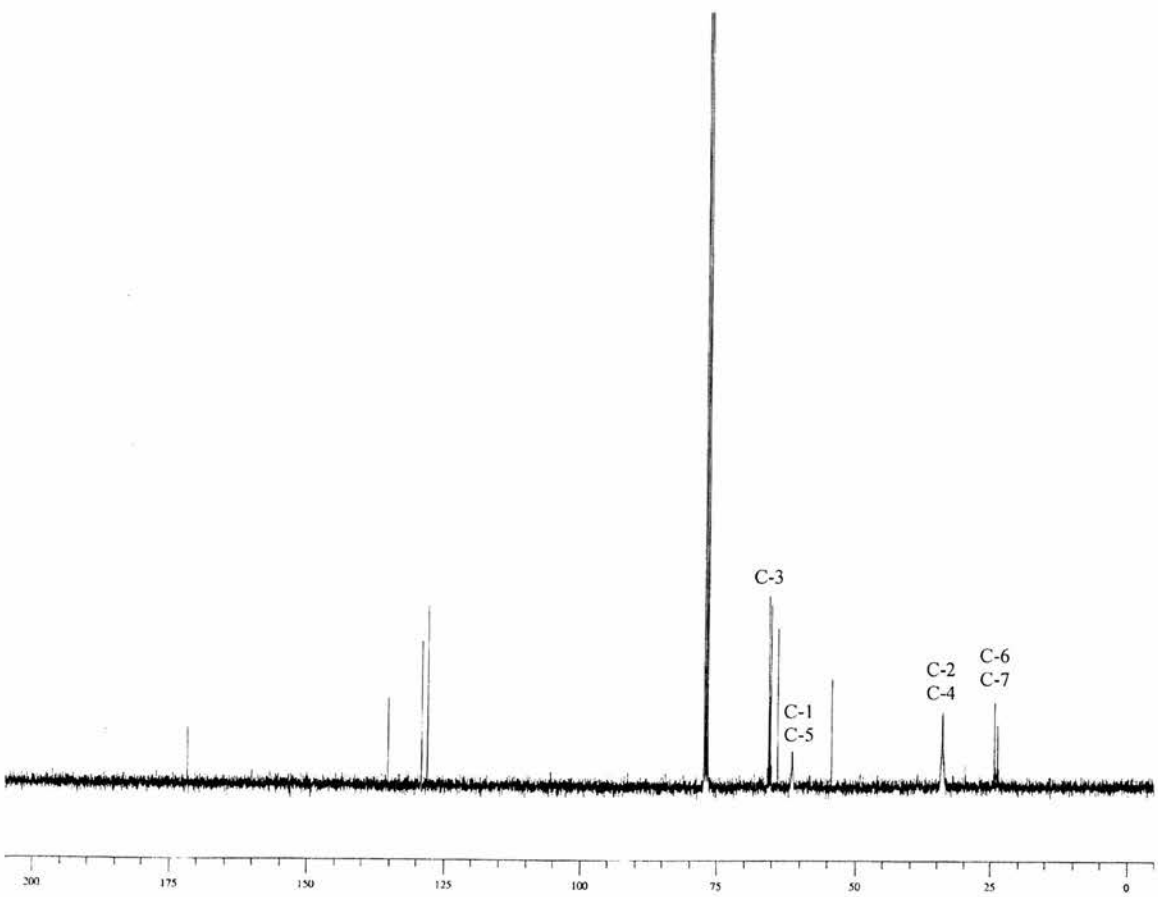
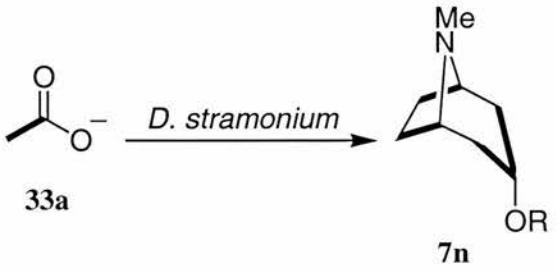


Figure 2.19: ^{13}C-NMR of hyoscyamine (**7n**) isolated from root cultures after feeding sodium [1,2-$^{13}C_2$]acetate (**33a**). The enriched peaks corresponding to C-1 through to C-7 are identified.



33a **7n**

R=tropate

Figure 2.20: Incorporation of sodium [1,2-$^{13}C_2$]acetate (**33a**) into the tropane ring of hyoscyamine (**7n**).

Feeding double-labelled [1,2-$^{13}C_2$]acetate (**33a**) allows the incorporation of an intact ^{13}C-^{13}C bond into the tropane ring to be investigated. The preferred biosynthetic scheme to the C_4N fragment from acetate (*via* a symmetrical intermediate) predicts the incorporation of two intact bonds between C-1 and C-7 and between C-5 and C-6. Examination of the peaks corresponding to C-6 and C-7 (Figure 2.21) show both to be present as triplets, consisting of the natural abundance signals and a pair of doublets ($^1J_{CC}$=33 Hz) resulting from the incorporations of a single intact bond between C-5 and C-6 and between C-1 and C-7 respectively. Comparison of the integrals from the natural abundance and coupled peaks allows the level of enrichment to be calculated as 0.5%. This is consistent with the incorporation levels observed after feeding [1-^{13}C] (**33e**) and [2-^{13}C]acetate (**33f**) given that 'cold' acetate (**33**) was co-administered, 'diluting' the labelled acetate pool to 25% of the level in the other feeding experiments.

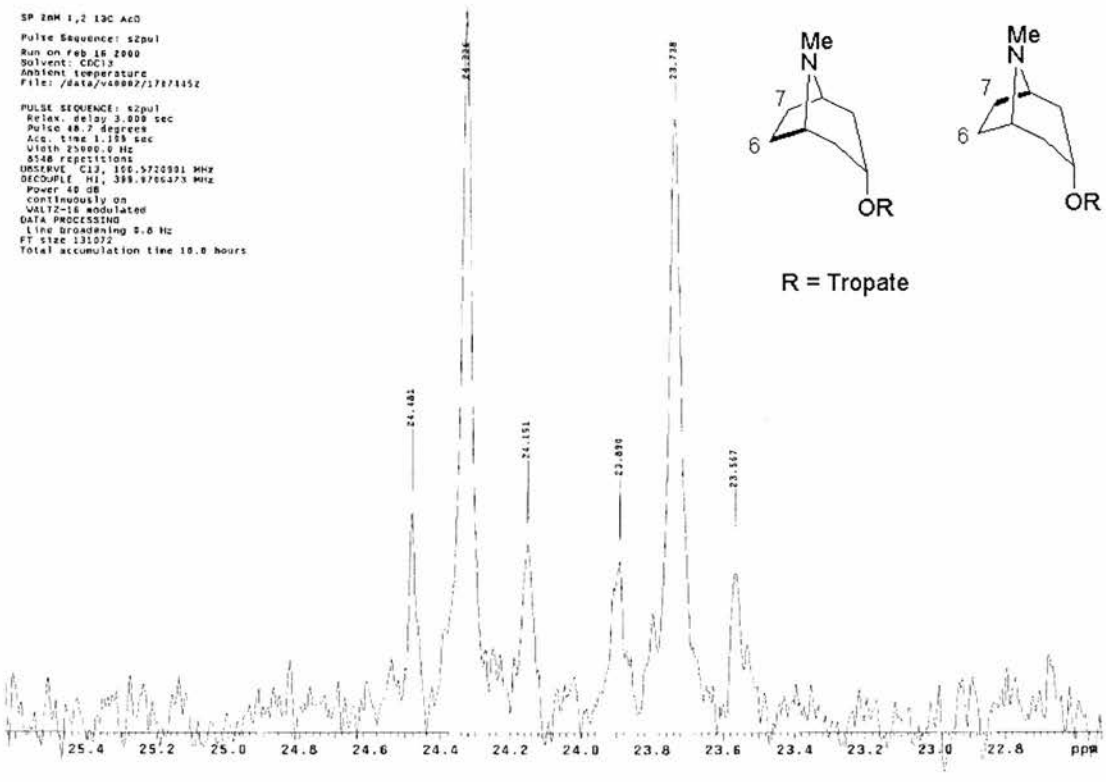


Figure 2.21: ^{13}C-NMR of hyoscyamine fed sodium [1, 2-$^{13}C_2$]acetate (**33a**), showing the signals for C-6 and C-7. $^{1}J_{CC}$=33 Hz

Detailed analysis of the resonance corresponding to C-3 (Figure 2.22) shows an unusual mutiplet pattern consisting of five peaks. The central peak of the multiplet (δ_C 65.4) corresponds to the natural abundance peak. The two largest peaks of the multiplet (δ_C 65.6 and 65.3, $^{1}J_{CC}$=36 Hz) are an enriched doublet resulting from the expected incorporation of an intact ^{13}C-^{13}C bond between C-3 and C-2 or C-4. Additionally, there are two low abundance satellites (δ_C 65.8 and 65.1). These can be rationalised by incorporation of carbon-13 into all positions in the C_3 bridge. Three adjacent carbon-13 atoms would give a doublet of doublets splitting pattern. However, due to the high level of symmetry in the tropane ring, the two ^{13}C-^{13}C coupling constants are identical ($^{1}J_{CC}$=36 Hz) and this results in a 1:2:1 abundance triplet. This triplet contributes the two low abundance satellites and enriches the central natural abundance resonance.

Comparison of the integrals from the natural abundance and coupled peaks allows the level of enrichment to be calculated; the incorporation of a single intact ^{13}C-^{13}C bond is 4.5% and for three contiguous ^{13}C atoms it is approximately 0.8%. This is consistent with the incorporation levels observed into the C_3 fragment after feeding [1-^{13}C] (**33e**)

and [2-^{13}C]acetate (**33f**) given that 'cold' acetate (**33**) was co-administered with [1, 2-$^{13}C_2$]acetate (**33a**).

The incorporation of three contiguous carbon labels is not unexpected given the high levels of enrichment into C-2, C-3, C-4 observed from the feeding experiments with singularly labelled acetate. This multiplet pattern is identical to that observed from a previous feeding experiments with sodium [1,2-$^{13}C_2$]acetate (**33a**) to *D. stramonium*[15] and *H. albus*.[16] A diagrammatic representation of the coupling pattern is given in Figure 2.22. As three contiguous carbon labels are incorporated there must also be incorporation of a single carbon-13 into C-2 or C-4, the intact bond being broken during the biosynthesis of the tropane ring. This gives a total of six enrichment patterns into the C_3 bridge (Figure 2.23).

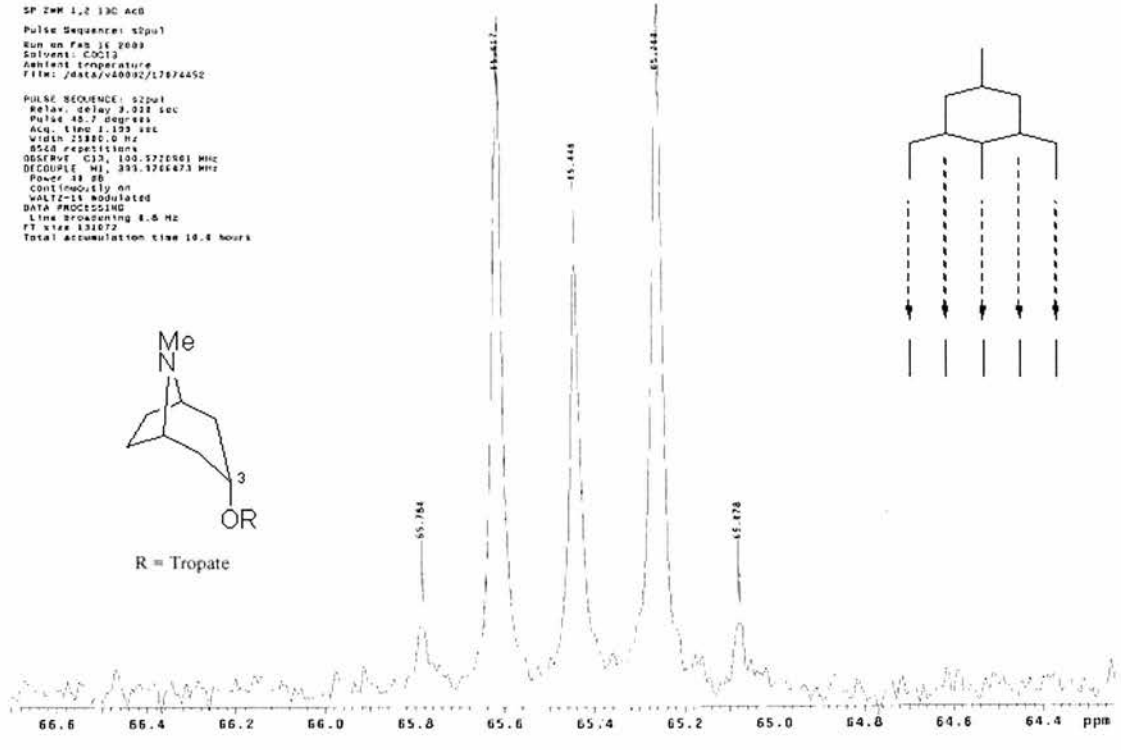


Figure 2.22: ^{13}C-NMR of hyoscyamine fed sodium [1, 2-$^{13}C_2$]acetate (**33a**), showing the resultant multiplet for C-3. A diagrammatic representation of the splitting pattern is shown.

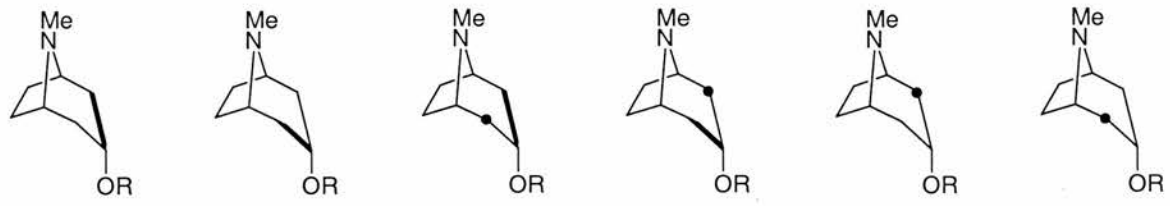


Figure 2.23: The six incorporation patterns from feeding sodium [1,2-$^{13}C_2$]acetate (**33a**) into C-2, C-3 and C-4 of hyoscyamine (**7n**).

2.4.5 Conclusions from feeding carbon-13 labelled acetates

2.4.5.1 The C_4N fragment

This series of feeding experiments support the role of a symmetrical intermediate in the biosynthesis of the C_4N fragment. There was no evidence of unsymmetrical enrichment into the tropane ring, inconsistent with previous feeding experiments with stable isotope labelled substrates (section 2.4.1). This suggests that putrescine (**56**) does not remain enzyme bound after decarboxylation of ornithine (**26**) (Figure 1.19) as previously suggested.[18] Instead 'free' putrescine (**56**) is implicated in the pathway to the tropane ring.

2.4.5.2 The C_3 bridge

As expected acetate (**33**) has been confirmed as a biosynthetic precursor of the C_3 bridge. Additionally, C-2, C-3 and C-4 of hyoscyamine (**7**) were found to incorporate label from acetate (**33**) in a symmetrical pattern, suggesting a role for an intermediate with C_{2v} symmetry in the biosynthetic pathway of this fragment. The results from this feeding experiment are consistent with the proposed role of 1,3-acetonedicarboxylic acid (**96**) as a precursor of the acetate derived fragment of the tropane ring (section 2.5.1).

2.5 Biosynthesis of the acetate derived fragment

2.5.1 A role for 1,3-acetonedicarboxylic acid?

It has been proposed that 1,3-acetonedicarboxylic acid (**96**, 3-oxoglutaric acid) (or its bis co-enzyme A ester) is the biosynthetic precursor to the acetate-derived fragment of the tropane ring.[12,20]

This hypothesis was formed in light of investigations into the biosynthesis of C_3 bridges in the alkaloid lycopodine (**97**).[21,22] Feeding [1, 2-$^{13}C_2$]acetate (**33a**) to whole plants of *Lycopodium tristachyum*[22] revealed an incorporation pattern into the C_3 bridges of lycopodine (**97**) identical to that observed in the C_3 fragment of the tropane ring resulting from an identical feeding experiment in *D. stramonium* (Figure 2.24).[23] This labelling pattern required acetate incorporation to occur *via* an intermediate with C_{2v} symmetry.

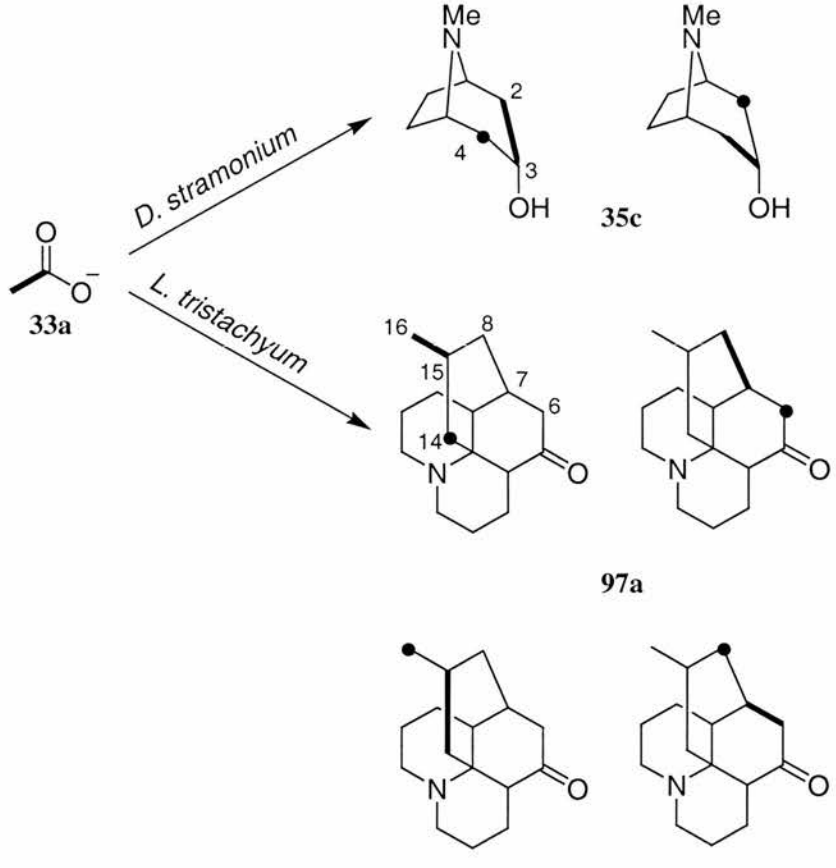
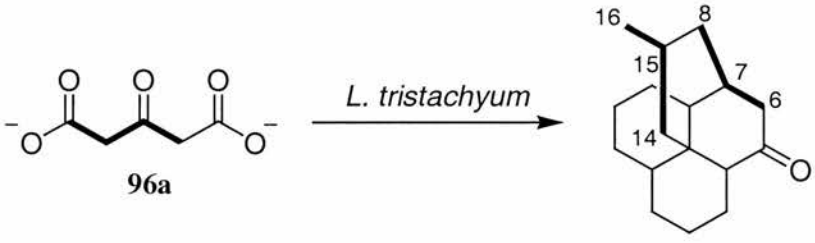


Figure 2.24: Comparison of incorporation patterns of [1,2-$^{13}C_2$]acetate (**33a**) into tropine (**35c**) and lycopodine (**97a**).

An additional feeding experiment[22] with [1, 2-$^{13}C_2$, $^{2}H_3$]acetate gave no detectable incorporation of deuterium into the C_3 bridges of lycopodine (**97**).[22] This implicates an intermediate in which the acetate derived protons are readily exchangeable due to enolisation. 1,3-Acetonedicarboxylic acid (**96**) fulfils this requirement and satisfies the requirement for an intermediate possessing C_{2v} symmetry. A subsequent feeding experiment with sodium [2-^{13}C, $^{2}H_3$]acetate (**33d**) in transformed root cultures of *D. stramonium* failed to detect incorporation of deuterium into C-2 and C-4 of hyoscyamine (**7k**) (Figure 2.11).[9] Clearly this result mirrors that of lycopodine (**97**) and is also consistent with the role of a highly enolisable biosynthetic intermediate to the C_3 fragment.

This hypothesis was tested for lycopodine (**97**) by administering [1,2,3,4-$^{13}C_4$]1,3-acetonedicarboxylate (**96a**) to *L. tristachyum* (Figure 2.24).[21] The resultant lycopodine (**97a**) was found to incorporate carbon-13 label into both of its C_3 fragments, demonstrating clearly that 1,3-acetonedicarboxylate (**96**) is the biosynthetic precursor to the C_3 fragments of lycopodine (**97**). This experiment and the observations above provide compelling evidence that 1,3-acetonedicarboxylate (**96**) may likewise be the precursor to C-2,3,4 of tropine (**35**).



96a **97a**

Figure 2.25: Incorporation of [1,2,3,4-$^{13}C_4$]1,3-acetonedicarboxylate (**96a**) into lycopodine (**97a**) in whole plants of *L. tristachyum*.[21]

The observed incorporation of [1,2-$^{13}C_2$]acetate (**33a**) into the tropane ring can be rationalised if 1,3-acetonedicarboxylic acid (**96a**) is biosynthesised as outlined in Figure 2.26.[12] An alternative biosynthetic route to 1,3-acetonedicarboxylic acid (**96**) *via* oxidative decarboxylation of citric acid (**94**) is not supported by the observed incorporation pattern of label from acetate (**33**) into the tropane ring.

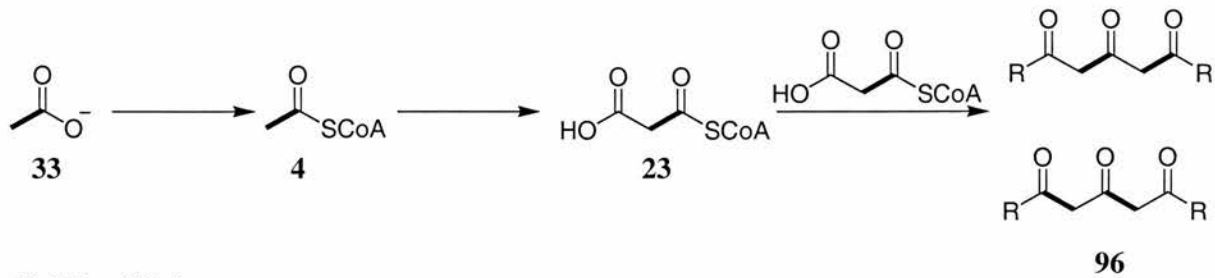


Figure 2.26: Proposed biosynthesis of 1,3-acetonedicarboxylic acid (**96**).[12,22]

Biomimetic studies have demonstrated that tropinone (**39**) can be generated non-enzymatically by the reaction of 1,3-acetonedicarboxylate (**96**) and *N*-methylpyrrolinium (**58**). Therefore, the potential for an aberrant non-enzymatic formation of tropinone (**39**) resulting in a 'non-natural' incorporation of label into hyoscyamine (**7**) perhaps makes the role of **96** as a precursor to the tropane ring difficult to unequivocally prove experimentally.

The proposed biosynthetic scheme to tropinone (**39**) from 1,3-acetonedicarboxylate (**96**) is shown in Figure 2.27.

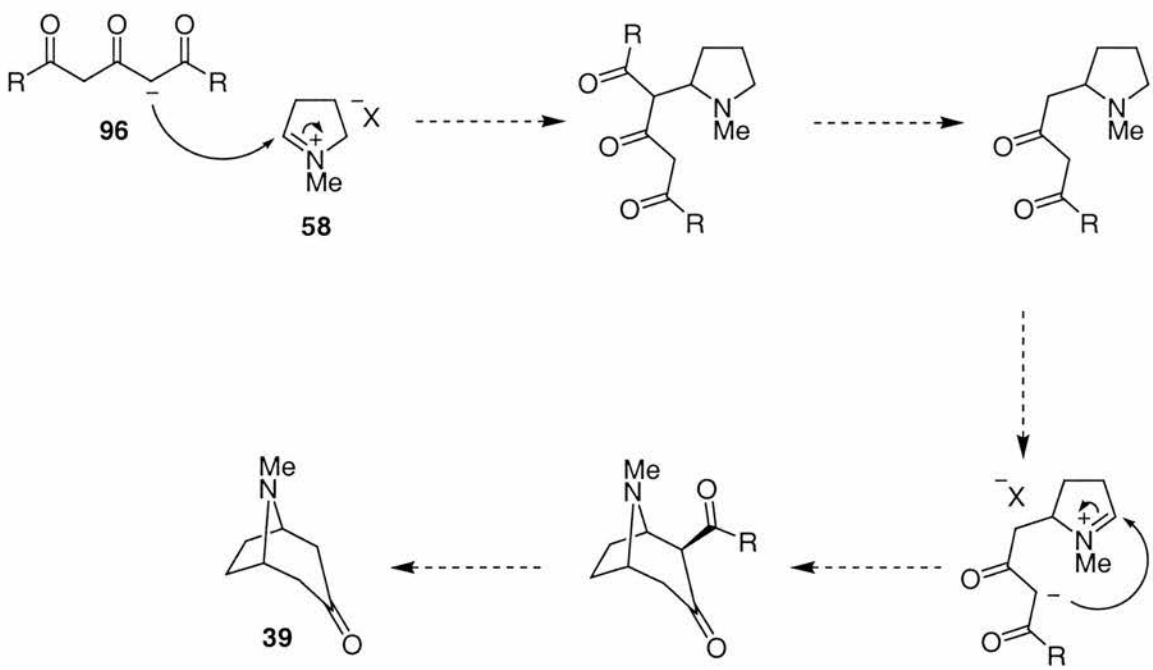
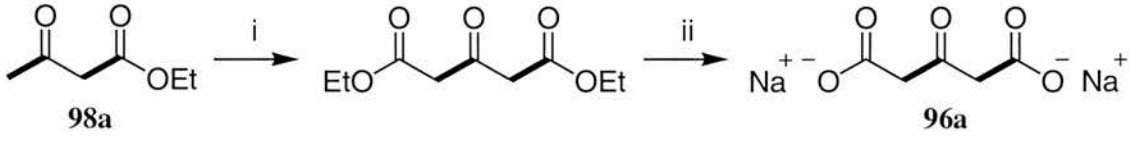


Figure 2.27: Proposed biosynthesis of the C_3 bridge of tropinone (**39**).

2.5.2 Synthesis of 1,3-acetonedicarboxylate

In order to explore a potential role for 1,3-acetonedicarboxylic acid (**96**) as a precursor of the tropane ring system it was necessary to prepare a labelled sample of the substrate. As 1,3-acetonedicarboxylic acid (**96**) readily enolises all of the protons are susceptible to exchange in aqueous media, therefore a carbon-13 labelled sample was required as a substrate for a feeding experiment.

In the biosynthetic investigation on lycopodine (**97**) sodium [1,2,3,4-$^{13}C_4$]1,3-acetonedicarboxylate (**96a**) was prepared in two steps[21] from commercially available ethyl [1,2,3,4-$^{13}C_4$]acetoacetate (**98a**) (Figure 2.28) using a modification of a published procedure (Winkel *et al*).[24]



98a **96a**

Figure 2.28: Synthesis of sodium [1,2,3,4-$^{13}C_4$]1,3-acetonedicarboxylate (**96a**) from ethyl [1,2,3,4-$^{13}C_4$]acetoacetate (**98a**).[21] Reagents: i, n-BuLi, LiTMP, diethyl carbonate; ii, alkaline hydrolysis.

It was judged unnecessary for the substrate for the feeding experiment to contain four carbon-13 atoms, as only three carbon atoms (C-2, C-3, C-4) of 1,3-acetonedicarboxylic acid (**96**) are expected to be incorporated into the tropane ring (Figure 2.27). Therefore, [2, 4-$^{13}C_2$]1,3-acetonedicarboxylic acid (**96b**) became the target molecule. [2, 4-$^{13}C_2$]1,3-Acetonedicarboxylic acid (**96b**) can be prepared by the same synthetic route as [1,2,3,4-$^{13}C_4$]1,3-acetonedicarboxylate (**96a**) described above (Figure 2.28). However, ethyl [2, 4-$^{13}C_2$]acetoacetate (**98a**) is expensive (£200 for 500mg). Therefore, it was decided instead to attempt the synthesis of dimethyl 1,3-acetonedicarboxylate (**99**) in three steps from sodium acetate (**33**) by the procedure of Winkel,[24] followed by ester hydrolysis to yield sodium 1,3-acetonedicarboxylate (**96**) (Figure 2.29). Performing this synthesis using sodium [2-^{13}C]acetate (**33f**) would provide the target molecule (**96b**) *via* a more economical synthetic route.

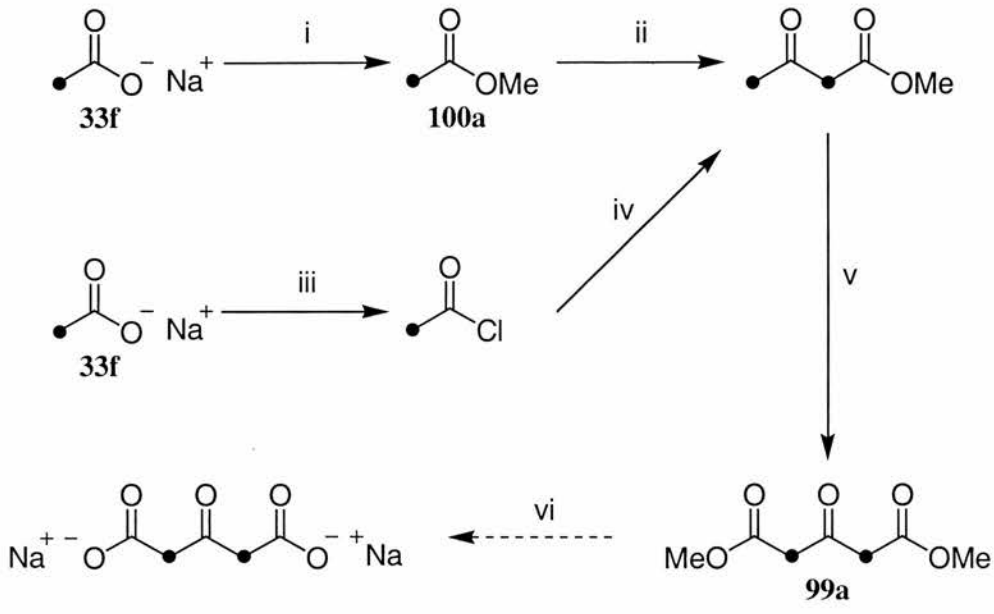


Figure 2.29: Synthetic routes to dimethyl [2, 4-$^{13}C_2$]1,3-acetonedicarboxylate (**99a**) from sodium [2-^{13}C]acetate (**33f**).[24] Reagents: i, dimethyl sulfate; ii, NaH; iii, BzCl; iv, LDA, methyl [2-^{13}C]acetate (**100a**); v, LiHMDS, dimethyl carbonate; vi, alkaline hydrolysis.

Therefore, the initial goal of the project was to prepare an unlabelled sample of 1,3-acetonedicarboxylate (**96**), to assess the suitability of the synthetic route for performing a labelled synthesis. Accordingly, sodium acetate (**33**) was treated with neat dimethyl sulfate in a micro-distillation apparatus and the product ester (**100**) recovered by distillation of the crude reaction mixture. Claisen condensation of the resultant methyl acetate (**100**) using sodium hydride as the base gave methyl acetoacetate (**101**) in a moderate yield.

Winkel *et al*[24] also report an alternative synthesis of methyl acetoacetate (**101**), by treatment of methyl acetate (**100**) with LDA followed by quenching of the resultant lithium enolate with acetyl chloride (**102**). This synthetic route requires the preparation of [2-^{13}C]acetyl chloride (**102a**). A synthetic route amenable to the preparation of [2-^{13}C]acetyl chloride (**102a**) from sodium [2-^{13}C]acetate (**33f**) is described by Winkel *et al* (Figure 2.29).[24] Accordingly, a suspension of sodium acetate (**33**) in benzoyl chloride was heated and acetyl chloride (**102**) recovered from the mixture by distillation. Treatment of methyl acetate (**100**) with LDA in THF followed by reaction with acetyl chloride (**102**) and aqueous workup furnished methyl acetoacetate (**101**). However, this reaction similarly gave a moderate yield of product.

Dimethyl 1,3-acetonedicarboxylate (**99**) was prepared by generating the dianion of methyl acetoacetate (**101**) by treatment with LiHMDS (3 equivalents) followed by reaction with dimethyl carbonate and acidic workup. However, the reaction yield was unsatisfactory (20%) and significantly lower than that reported by Winkel *et al* (65%). A literature investigation into the synthesis of β,δ-diketo esters from methyl acetoacetate (**101**) similarly reported low reaction yields (30-40%).[25] In this study the low yields were attributed to proton transfer from the β,δ-diketo ester product to the dianion of methyl acetoacetate (**101**).

The low reaction yields obtained made this synthetic route unsuitable for the synthesis of [2,4-$^{13}C_2$]1,3-acetonedicarboxylate (**96b**).

Due to the synthetic difficulties encountered with the preparation of 1,3-acetonedicarboxylate (**96**) and the possibility of non-enzymatic incorporation of **96** into the tropane ring it was decided not to further pursue this objective of the research programme. Therefore, the proposed biosynthetic role of 1,3-acetonedicarboxylate (**96**) as a precursor to the tropane ring remains to be proven directly by an isotopic feeding experimental.

2.6 Origin of the tropane oxygen

Despite extensive investigations into the origin of the carbon skeleton (sections 1.5.1 and 2.4.1) and nitrogen atom[26] of the tropane ring, the origin of the C-3 oxygen atom of tropine (**35**) has remained unreported. Therefore, it became of interest to explore this unresolved issue.

2.6.1 Previous work

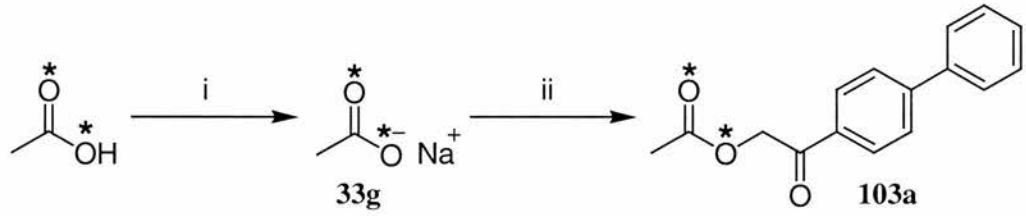
It is known that C-3 of tropine (**35**) incorporates label from [1-^{13}C]acetate (**33e**) at high levels (section 2.4.2). It is therefore a logical extension to propose that the C-3 oxygen of tropine (**35**) is also derived from acetate (**33**). This hypothesis has previously been tested within our research group by feeding sodium [1-^{13}C, $^{18}O_2$]acetate to transformed root cultures of *D. stramonium*.[9] However, whilst ^{13}C-NMR analysis of the resultant hyoscyamine showed good incorporation of the carbon-13 label (8%) into C-3, there was no associated shift of the carbon resonance to indicate that oxygen-18 was incorporated. Additionally GC-MS analysis of the isolated hyoscyamine did not detect any enrichment of the M+3 ion. Therefore, there was no experimental support for oxygen-18 retention at this site.

It was concluded that the oxygen-18 label was 'washed out' below the detectable level of GC-MS analysis (~0.4%). Although the synthetic sample fed in the previous study[9] contained 99% atom ^{13}C, the level of oxygen-18 was only 48%. Therefore, it was of interest to repeat the experiment with a sample of sodium acetate with a greater enrichment of oxygen-18, the higher level of isotope possibly facilitating the retention of a low level of isotopic label. Accordingly a sample of sodium [$^{18}O_2$]acetate (**33g**) was required for a feeding experiment in transformed root cultures of *D. stramonium*.

2.6.2 Synthesis of sodium [$^{18}O_2$]acetate

The sodium salt of [$^{18}O_2$]acetate (**33g**) was prepared by an acid-base reaction between commercially available [$^{18}O_2$]acetic acid (Aldrich) and aqueous sodium hydroxide (Figure 2.30). The reaction solvent was removed by freeze-drying to give sodium [$^{18}O_2$]acetate (**33g**) in quantitative yield. It was necessary to determine the level of oxygen isotope in the sample; however, sodium acetate (**33**) is unsuitable for mass

analysis due to its high melting point. Therefore, the *p*-phenylphenacyl-derivative (**103a**) was prepared by reaction with 2-bromo-4′-phenacetophenone[27] (Figure 2.30) and subjected to GC-MS analysis. Accordingly, sodium [$^{18}O_2$]acetate (**33g**) results in a derivative (**103a**) with *m/z* 258. The [$^{18}O_2$]acetate (**33g**) was found to be highly enriched with oxygen-18 (6% ^{18}O and 91% $^{18}O_2$) consistent with the level of oxygen-18 label (95%) in the commercial [$^{18}O_2$]acetic acid starting material. This sample has a greater enrichment of oxygen-18 than that administered in the previous study.[9]



* = ^{18}O

Figure 2.30: Synthesis of sodium [$^{18}O_2$]acetate (**33g**) and the preparation of the phenylphenacyl derivative (**103a**) for GC-MS analysis. Reagents: i, NaOH, H_2O, 5min; ii, 2-bromo-4′-phenacetophenone, 18-crown-6, MeCN/PhMe, 80°C.

2.6.3 Feeding [$^{18}O_2$]acetate

Sodium [$^{18}O_2$]acetate (**33g**) was administered to three transformed root cultures of *D. stramonium* at a final concentration of 8mmol dm^{-3} in the medium. Administering carbon-13 labelled acetates at this concentration has been shown to produce good incorporation of label into hyoscyamine (**7**) (section 2.4); therefore it was chosen as the feeding concentration for this feeding experiment. As the administered [$^{18}O_2$]acetate (**33g**) contains no carbon-13 label to facilitate indirect detection of oxygen isotope incorporation by NMR analysis, mass spectrometry must be utilised to directly detect the incorporation of oxygen-18 into the resultant hyoscyamine. Accordingly, the isolated crude alkaloid fraction was derivatised with MSTFA (**90**) in chloroform and analysed by GC-MS. The level of incorporation into hyoscyamine was calculated by comparison with a natural abundance standard (Table 2.2).

	M	M+1	M+2	M+3
Fed sodium [$^{18}O_2$]acetate	99.10	0.72	0.16	0.02
	(0.66)	(0.55)	(0.16)	(0.03)

Table 2.2: Levels of isotope in hyoscyamine (**7**) isolated from root cultures fed sodium [$^{18}O_2$]acetate (**33g**). The standard deviation of the ten sample replicates is given in parentheses.

The GC-MS analysis of the alkaloids does not show any significant enrichment of the M+2 ion, indicating that there is no detectable incorporation of oxygen-18 into the tropane moiety from [$^{18}O_2$]acetate (**33g**). This result is consistent with the previously reported feeding experiment[9] with sodium [1-^{13}C, $^{18}O_2$]acetate (section 2.6.1). Therefore, acetate (**33**) is not the biosynthetic precursor for the C-3 oxygen of hyoscyamine (**7**). In the previous study[9] it was proposed that a biosynthetic intermediate(s) between acetyl CoA (**5**) and the C_3 unit of tropine (**35**) could be susceptible to the loss of oxygen-18 by complete exchange with the medium. However, investigations of polyketide biosynthesis have revealed the incorporation of oxygen-18 from acetate (**33**) into structural motifs derived from the condensation of acetyl CoA (**5**) with malonate units.[27] It has been proposed[12] that the C_3 fragment of tropine (**35**) is biosynthesised in a similar manner (section 2.5.1), however, there must be a fundamental difference in the biosynthetic pathways of these acetate-derived molecules. Therefore, it became of interest to investigate the level of oxygen exchange in putative precursors of the acetate-derived fragment of the tropane ring system.

2.6.4 Isotope exchange experiments

2.6.4.1 Experimental considerations

As the biosynthetic precursor to the C_3 fragment of the tropane ring has not been experimentally proven a model compound was required to conduct an exchange experiment. Indirect experimental evidence implies that 1,3-acetonedicarboxylate (**96**) may be the precursor of the C_3 fragment (section 2.5.1). Additionally, the precursor to the C_3 fragment is almost certainly a β–keto or β,δ–diketo ester, a common structural

motif in acetate-derived metabolites. Therefore, dimethyl 1,3-acetonedicarboxylate (**99**) was chosen as the substrate for the model exchange experiment

To simulate *in vivo* conditions it was decided to incubate dimethyl 1,3-acetonedicarboxylate (**99**) in a solution enriched with oxygen-18 water. Therefore, this experiment investigates the 'incorporation' rather than 'washout' of isotope label. An identical incubation instead using $^{2}H_2O$ would serve as a positive control, as **99** readily exchanges its protons in aqueous solution. Therefore a method to facilitate the detection of oxygen-18 incorporation by mass analysis was required. Accordingly a GC-MS method for the analysis of dimethyl 1,3-acetonedicarboxylate (**99**) was developed.

As water is an unsuitable solvent for GC analysis due to its large expansion volume upon vaporisation it was substituted for a 10:1 THF : water mixture. This solvent system allowed direct injection of a sample from the exchange experiment into the GC-MS without extraction of **99**. Therefore it became possible to monitor the temporal level of isotope exchange by repeat injections using the auto sampler facility, with repeat injections at a fixed time interval. For that reason the GC method was adjusted to 20 minutes in length, allowing three analyses per hour.

2.6.4.2 Results and discussion

The positive control experiment was conducted by analysing a sample of **99** in a THF : $^{2}H_2O$ mixture (500:50 µl). Extracting the ion currents for *m/z* 174-178 allowed the abundance of the molecular ion to M+4 to be determined. Analysis of the positive control revealed a high level of M+3 (*m/z* 177) and M+4 (*m/z* 178) (40% and 32% respectively, uncorrected) after just 20 minutes, demonstrating that the experimental conditions were able to facilitate and detect isotope exchange. GC-MS analysis of the experiment using oxygen-18 water revealed that even over a period of 13 hours there was no increase in the level of M+2 ion above the natural abundance level. This result suggests that facile exchange of oxygen-18 label does not account for the failure to detect the incorporation of oxygen label into hyoscyamine (7) from administered [$^{18}O_2$]acetate (**33g**).

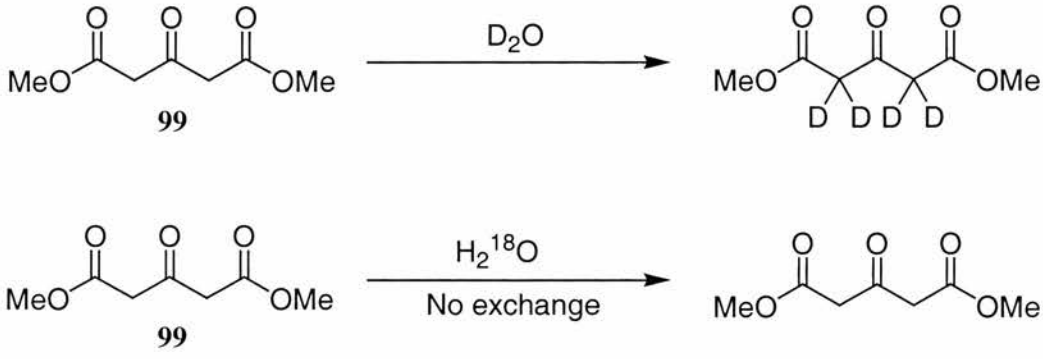


Figure 2.31: The exchange of isotope from labelled water into dimethyl 1,3-acetonedicarboxylate (**99**).

2.6.5 Discussion

As there is no experimental evidence to support the loss of oxygen-18 label *via* exchange in dimethyl 1,3-acetonedicarboxylate (**99**) other mechanisms to explain the failure of oxygen-18 isotope incorporation from acetate (**33**) into the C-3 oxygen of tropine (**35**) must be considered. Firstly it is possible that the C-3 oxygen of tropine (**35**) is derived from a molecular source other than acetate (**33**). Molecular oxygen or water could also be the source of the tropane C-3 oxygen. However, this is considered unlikely given the known precursors to tropinone (**39**). Additionally, the experimental procedures to test either of these hypotheses are not amenable to biosynthetic investigation using root cultures due to the extended growth period and large culture volumes required.

The complete loss of oxygen-18 label could also be the result of the formation of a Schiff base from the condensation of a lysine residue on a protein with an aldehyde or ketone of a biosynthetic intermediate to the C_3 fragment of tropine (**35**). A Schiff base intermediate could be formed during the biosynthesis of the known tropane precursor 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69**), or alternatively in one of transformations from **69** to tropinone (**39**) (Figure 2.32).

There is some precedent for the formation of stabilised anions at the alpha carbon to a Schiff base, e.g. aldolase[28] and transaldolase.[28] Similarly, the formation of an enolate anion has been proposed to occur during the formation of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69**) and its subsequent cyclisation to tropinone (**39**) (Figure 2.32).

However, this mechanistic hypothesis cannot be tested in plant tissue models, instead an active *in vitro* enzyme preparation, or pure protein sample is required.

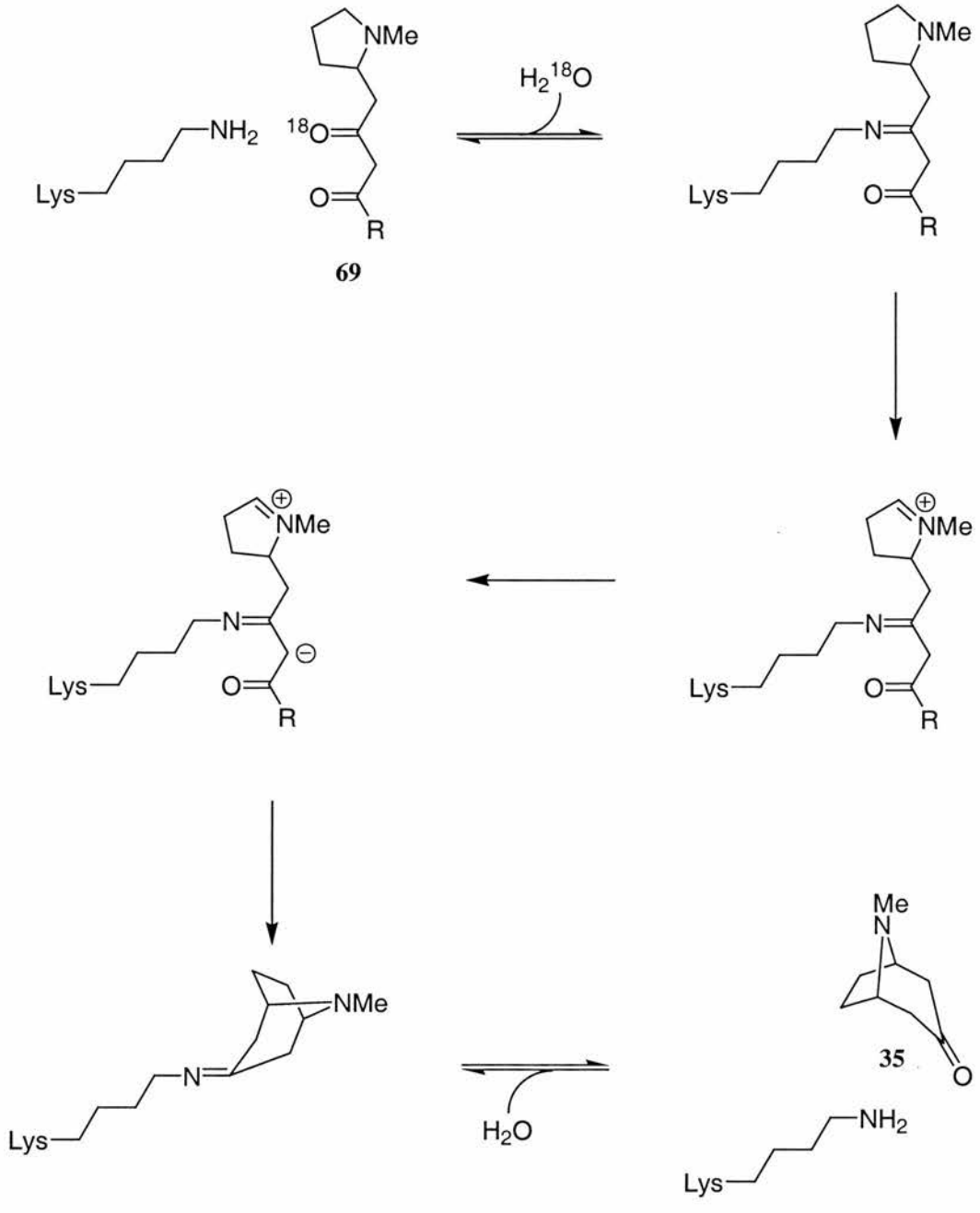


Figure 2.32: A proposed mechanism for the loss of oxygen-18 label from the acetate-derived fragment due to the formation of a Schiff base during the cyclisation of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69**) to tropinone (**35**).

2.7 Incorporation of glutamic acid in to the tropane ring

2.7.1 Previous work

Glutamic acid (**95**) is a known biosynthetic precursor to ornithine (**26**) (Figure 2.33) and therefore to the tropane ring. This has been demonstrated experimentally by a feeding experiment with [2-^{14}C, ^{15}N]glutamic acid in *D. metel*,[26] the resultant hyoscyamine incorporating isotopic label.

Through known metabolic pathways (Figure 2.10) [2-^{13}C, $^{2}H_3$]acetate (**33d**) incorporates deuterium and carbon-13 label into C-4 of glutamic acid (**95**), which subsequently enriches C-6 and C-7 of hyoscyamine (Figure 2.11).[9] However, as C-1 and C-5 of hyoscyamine (**7**) are derived from C-1 of acetate (**33**) (section 2.4.2) a more advanced precursor must be fed to successfully incorporate a deuterium label into the bridgehead positions.

2.7.2 Feeding (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid

From the accepted biosynthetic pathway to tropine (**35**) (Figure 2.10) it was anticipated that feeding commercially available (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid (**95a**) could result in the incorporation of three deuterium atoms into the tropane ring, two into C-6/C-7 and one into C-1/C-5. The incorporation of deuterium label into C-1/C-5 of hyoscyamine (**7**) from glutamic acid (**95**) has not previously been reported.

Previous investigation within the research group had shown that administering amino acids to plant tissue cultures at high concentration (5 mmol dm^{-3}) can cause tissue necrosis or a reduction in culture growth.[29] Therefore, before administering (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid (**95a**) it was necessary to determine a suitable feeding concentration using unlabelled glutamic acid (**95**). Accordingly, glutamic acid (**95**) was administered to six root cultures of *D. stramonium* at final concentrations of 2, 4 and 6 mmoldm^{-3} in the medium (two at each concentration). Feeding glutamic acid (**95**) at 2 and 4mmol dm^{-3} was found to result in a slight reduction in root growth, whilst feeding **95** at 6 mmol dm^{-3} resulted in severe growth retardation.

Therefore, (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid (**95a**) was administered to nine transformed *D. stramonium* root cultures at a final concentration of 4 mmol dm^{-3} in the media. As expected there was a reduction in the growth of root tissue. The isolated hyoscyamine was purified by preparative t.l.c. and analysed by GC-MS (performed by Dr J.T.G. Hamilton, School of Agriculture and Food Science, The Queen's University of Belfast). The level of incorporation into the resultant hyoscyamine was determined by comparison with a natural abundance hyoscyamine (**7**) standard (Table 2.3).

	M+1	M+2	M+3	M+4
Hyoscyamine	1.61 (0.192)	4.67 (0.087)	0.41 (0.018)	0.05 (0.0039)

Table 2.3: Mass enrichments into hyoscyamine from roots administered (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid (**95a**). The standard deviation of the percentage enrichments is given in parentheses.

The efficient enrichment of the M+2 ion (4.67%) in the resultant hyoscyamine suggests that two deuterium atoms are incorporated into hyoscyamine from [2, 4, 4]glutamic acid (**95a**). This is consistent with a previous feeding experiment[9] conducted with [2-^{13}C, $^{2}H_3$]acetate (**33d**), which enriches C-4 of glutamic acid with two deuterium atoms, as per the administered [2, 4, 4-$^{2}H_3$]glutamic acid (**95a**).

There is also a significant enrichment of the M+1 ion (1.61%), corresponding to the incorporation of a single deuterium atom into hyoscyamine from [2, 4, 4-$^{2}H_3$]glutamic acid (**95a**). Feeding experiments[9] with [$^{2}H_3$] and [2-^{13}C, $^{2}H_3$]acetates (**33d**) similarly observed the incorporation of a single deuterium into C-6/C-7 of hyoscyamine. This loss of deuterium can be rationalised by considering the biosynthetic steps from glutamic acid (**95**) to ornithine (**26**) (Figure 2.33).[30] One of the intermediates *N*-acetylglutamylsemialdehyde (**104**) could undergo reversible enolisation which would result in the loss of deuterium originating from C-4 of glutamic acid (**95**) (C-2 of acetate (**33**)) by facile exchange.

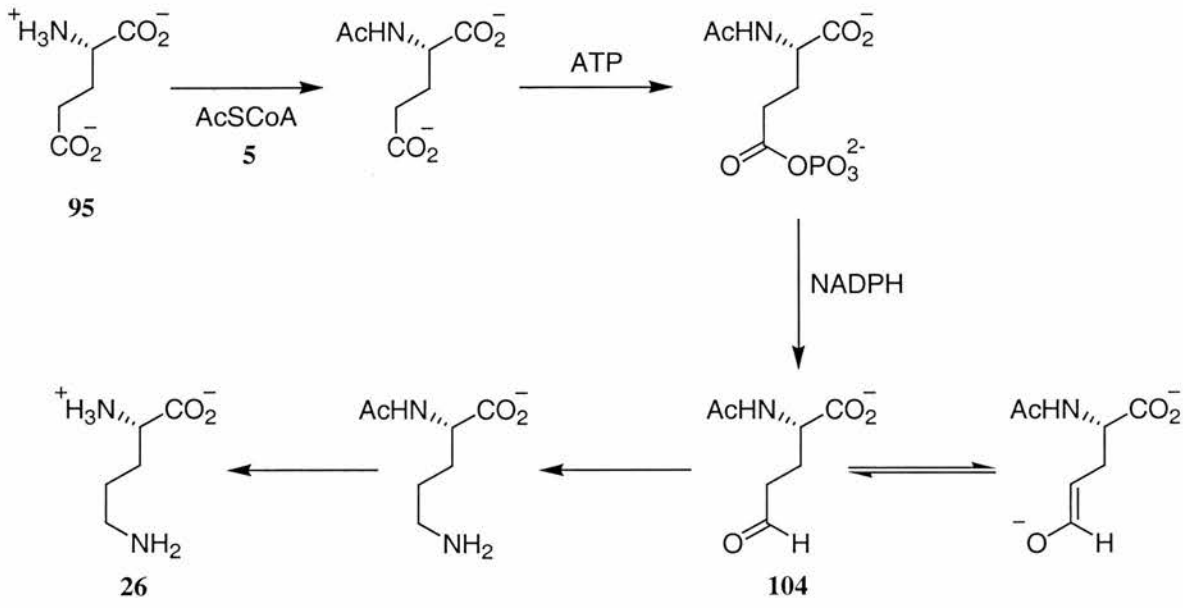


Figure 2.33: The biosynthesis of ornithine (**26**) from glutamic acid (**95**). The reversible enolisation of *N*-acetylglutamylsemialdehyde (**104**) is shown.[30]

However, the enrichment level of the M+3 ion, representing the incorporation of all three deuterium labels into hyoscyamine was very low (0.41%). This suggests that the C-2 deuterium from **95a** is almost completely lost during the biosynthetic steps from glutamic acid (**95**) to tropinone (**39**). As the percentage enrichment of the M+3 ion was low it was decided to repeat the feeding experiment to determine if the incorporation was reliable and not artefactual.

Accordingly the feeding experiment was repeated by administering (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid (**95a**) to eight transformed *D. stramonium* root cultures at a final concentration of 4 mmol dm^{-3} in the media. The alkaloids were isolated and the crude alkaloid fraction was subjected to GC-MS analysis (Dr J.T.G. Hamilton). The mass enrichments into both littorine (**80**) and hyoscyamine (7) were calculated by comparison with natural abundance standards (Table 2.4). As littorine (**80**) is the biosynthetic precursor to hyoscyamine (**7**) (section 1.6.3) both alkaloids should have the same ion enrichment pattern.

	M+1	M+2	M+3	M+4
Littorine	0.4 (0.0)	1.9 (0.1)	0.0 (0.1)	
Hyoscyamine	0.4 (0.1)	2.1 (0.0)	0.0 (0.0)	0.0 (0.0)

Table 2.4 : Mass enrichments into littorine and hyoscyamine from roots administered (*RS*)-[2, 4, 4-$^{2}H_3$]glutamic acid (**90a**). The standard deviation of the percentage enrichments is given in parentheses. Note the amount of isolated littorine was too low to assign an M+4 enrichment value.

The repeat experiment failed to detect any significant enrichment of the M+3 ion into the alkaloids, suggesting that a single deuterium atom is completely lost during the biosynthetic transformations from glutamic acid (**95**) to the tropane ring. The enrichments levels in the two alkaloids are very similar, consistent with littorine (**80**) being the biosynthetic precursor to hyoscyamine (**7**). The isotopic label has not been as efficiently incorporated in the repeat experiment, with percentage enrichments approximately half of that in the first experiment.

The mechanism of deuterium loss from C-2 of glutamic acid (**95**) is not previously described. The biosynthetic steps from glutamic acid (**95**) to ornithine (**26**) should retain the C-2 proton (Figure 2.33). Therefore the loss of deuterium must occur post ornithine (**26**) biosynthesis.

Some deuterium at C-2 could be lost in the biosynthetic steps from ornithine (**26**) to *N*-methylpyrrolinium (**58**) (Figure 2.34). The decarboxylation of ornithine (**26**) to give putrescine (**56**) has been shown to occur with a retention of configuration.[31] Subsequently tracing the fate of the C-2 deuterium of glutamic acid (**95a**) suggests that some molecules of *N*-methylpyrrolinium should retain three deuterium atoms (**58b**) and some two (**58c**) (Figure 2.34). This being based on studies of the oxidation of *N*-methylputrescine (**57**) in *E. coca*,[32] which demonstrated that the *4-pro-S* hydrogen is selectively removed from **57** during the formation of *N*-methylpyrrolinium (**58**). However, the intermediacy of the symmetrical intermediate putrescine (**56**) results in the deuterium from C-2 of glutamic acid (**95a**) being evenly distributed at C-4 and C-1 of *N*-methylputrescine (**57**). Therefore, not all of the deuterium can be lost by oxidation of C-4 of *N*-methylputrescine (**57**).

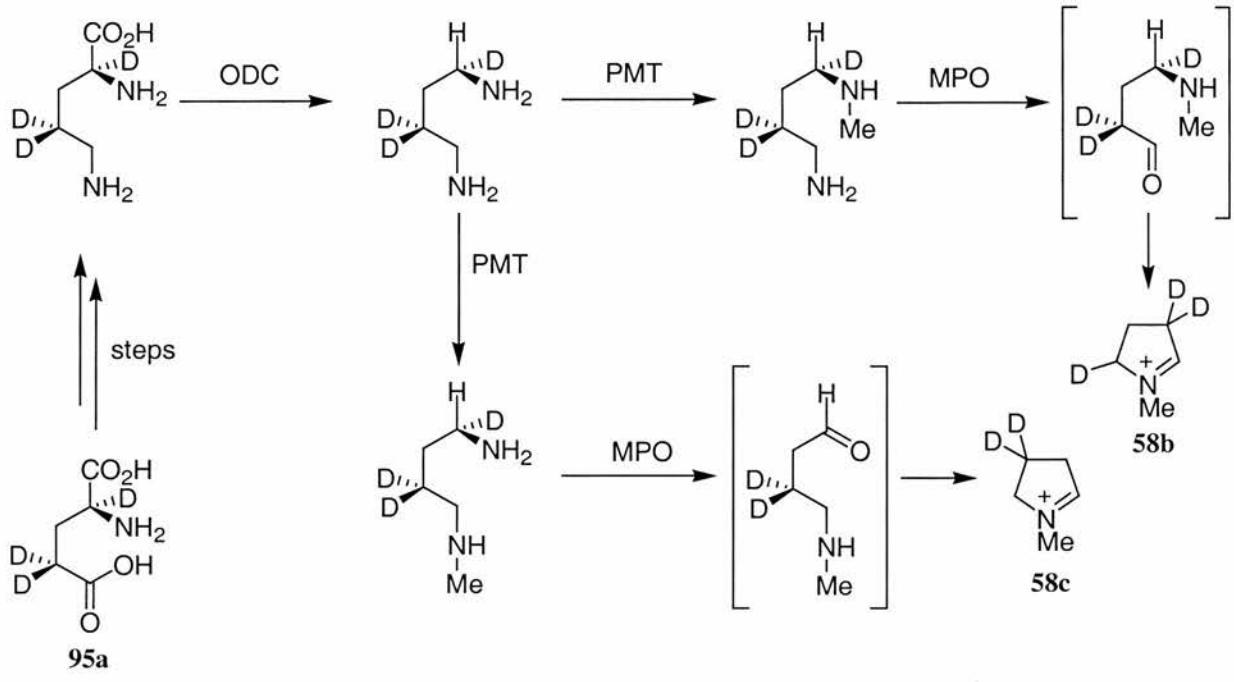


Figure 2.34: The biosynthetic steps from [2, 4, 4-$^2H_3$]glutamic acid (**95a**) to *N*-methylpyrrolinium.

Subsequent biosynthetic steps from *N*-methylputrescine (**58**) to tropinone (**39**) could account for the complete loss of deuterium derived from C-2 of [2, 4, 4-$^2H_3$]glutamic acid (**95a**). A possible mechanism is outlined in Figure 2.35.

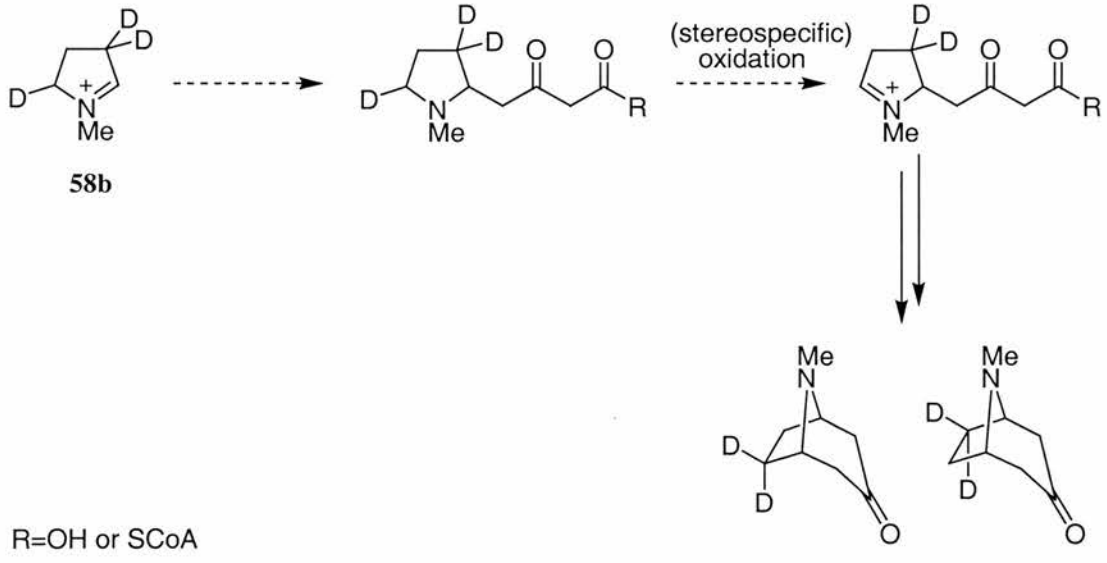


Figure 2.35: Proposed mechanism for the loss of deuterium during the biosynthesis of tropinone.

Additionally, tautomerisation of *N*-methylpyrrolinium (**58b**) could account for the loss of deuterium label (Figure 2.36). However, Leete has demonstrated that **58** does not tautomerise *in vivo* (see section 2.8.1).

Figure 2.36: Loss of deuterium label from *N*-methylpyrrolinium (**58b**) due to tautomerisation.

2.7.3 Conclusions

Deuterium from C-4 of glutamic acid (**95a**) is incorporated into the tropane ring. Deuterium from C-2 of glutamic acid (**95a**) is not incorporated in to the tropane alkaloids. The mechanism of this loss remains to be determined.

2.8 Incorporation of *N*-methylpyrrolinium into the tropanes

2.8.1 Previous work

The role of *N*-methylpyrrolinium (**58**) as a precursor of the tropanes was directly demonstrated by feeding [^{15}N]*N*-methyl[2-^{13}C, ^{14}C]pyrrolinium chloride (**58a**, X=Cl) to whole plants of *Erythroxylum coca*[33] (Figure 2.37). The resultant cocaine (**36a**) was found to be radioactive, indicating that **58** is a precursor to the tropane ring. A further feeding experiment was conducted with [1-^{13}C, ^{14}C, ^{15}N]4-(methylamino)butanal diethyl acetal (**105a**), which is transformed to labelled *N*-methylpyrrolinium (**58a**) *in vivo* (Figure 2.37). Isolation and NMR analysis of the resultant cocaine (**36a**) demonstrated that the carbon label was incorporated uniquely at C-5 of cocaine (**36a**) (Figure 2.37).

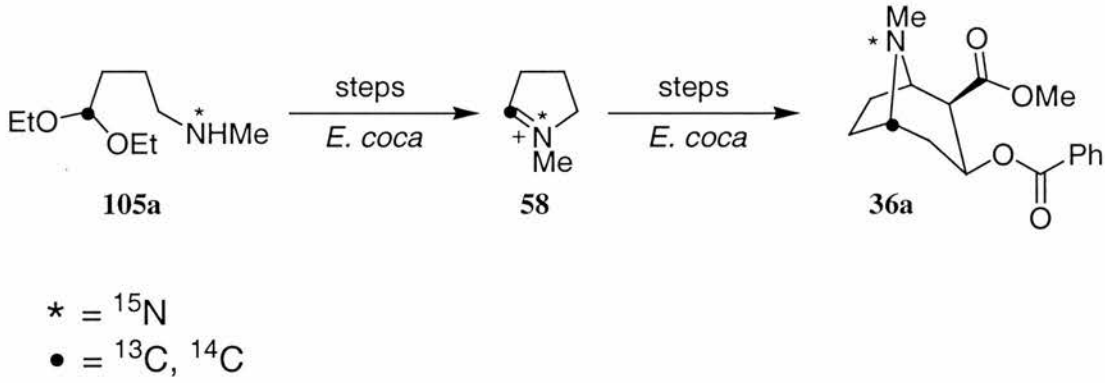
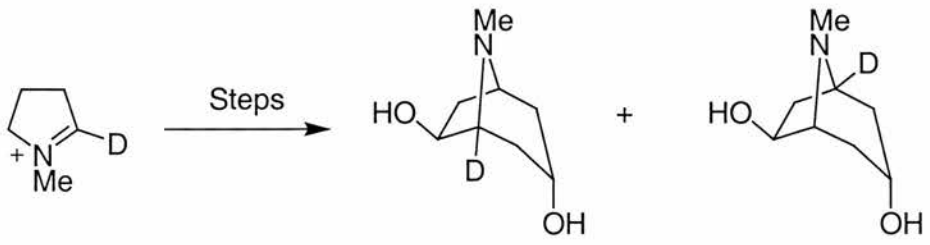


Figure 2.37: Incorporation of [1-^{13}C, ^{14}C, ^{15}N]4-(methylamino)butanal diethyl acetal (**105a**) into cocaine (**36a**) *via* [^{15}N]*N*-methyl[2-^{13}C, ^{14}C]pyrrolinium (**58a**).[33]

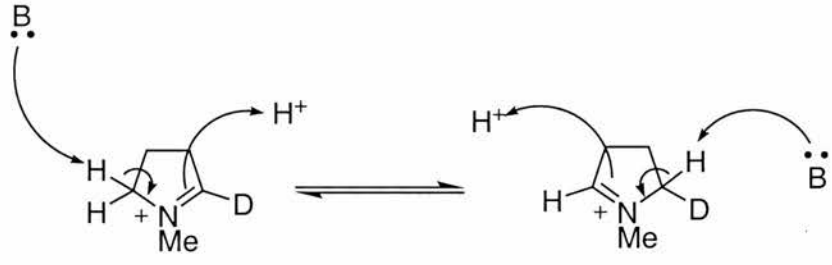
More recently as part of a series of experiments[23] *D. stramonium* was challenged with a sample of *N*-methyl[2-^{2}H]pyrrolinium (**58d**). Isolation of 6β-hydroxytropine (**106a**), and subsequent ^{2}H-NMR analysis indicated the incorporation of deuterium label into *both* of the bridgehead positions (Figure 2.38). The authors proposed that this result was consistent with earlier studies,[15,34] the results from which have been interpreted[12] to arise from the incorporation of both enantiomers of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**69**) into the tropane ring (see section 1.5.1.2).



58d **106a**

Figure 2.38: Incorporation of *N*-methyl[2-^{2}H]pyrrolinium (**58d**) into 6β-hydroxytropine (**106a**) in *D. stramonium*.

This unusual and intriguing stereochemical issue warranted further investigation. It has been proposed[20] that *N*-methyl[2-^{2}H]pyrrolinium (**58d**) tautomerises *in vivo* (Figure 2.39), resulting in both [2-^{2}H] (**58d**) and *N*-methyl[5-^{2}H]pyrrolinium (**58e**), which could then incorporate deuterium label into both of the bridgehead positions of the tropane ring (C-1 and C-5).



58d **58e**

Figure 2.39: Proposed *in vivo* tautomerisation of *N*-methyl[2-^{2}H]pyrrolinium (**58b**). Continued tautomerisation would lead to complete 'washout' of label resulting in the formation of unlabelled *N*-methylpyrrolinium (**58**).

However, if *N*-methyl[2-^{2}H]pyrrolinium (**58d**) does readily tautomerise this would result in the complete 'washout' of label yielding unlabelled *N*-methylpyrrolinium (**58**). This is at variance with the reported feeding experiment,[23] which indicated an equimolar incorporation into both bridgehead positions within the detection limits of ^{2}H-NMR, a facile tautomerisation being unlikely to result in an even distribution of label. The incorporation of label in this experiment[23] was low (0.3%), therefore accurate determination of the level of labelling at the bridgeheads is problematic.

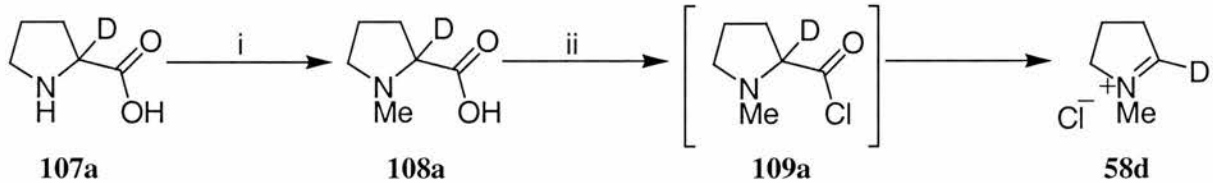
Two issues for further investigation arose from the reported[23] incorporation of label from *N*-methyl[2-^{2}H]pyrrolinium (**58d**) into the tropanes. Firstly, how readily does *N*-methylpyrrolinium (**58**) tautomerise in aqueous solution? Secondly, can the incorporation be confirmed using more sensitive GC-MS methodology?

2.8.2 Determining the level of tautomerisation of *N*-methylpyrrolinium

It was proposed that tautomerisation of *N*-methylpyrrolinium (**58**) in deuterated solvent would result in the 'incorporation' of deuterium into C-2 and C-5 of **58** by facile exchange. This incorporation could be observed by ^{1}H-NMR analysis, reduction in a peak's integral demonstrating exchange. Therefore, a sample of *N*-methylpyrrolinium (**58**) was required to perform this ^{1}H-NMR investigation.

2.8.2.1 Synthesis of *N*-methylpyrrolinium chloride

N-Methyl[2-^{2}H]pyrrolinium chloride (**58d**) has been previously prepared in a two step sequence from (*RS*)-[2-^{2}H]proline (**107a**) (Figure 2.40).[23] The first step of this synthesis is an Eschweiler-Clarke[35] *N*-methylation reaction to give (*RS*)-*N*-methyl[2-^{2}H]proline (**108a**). Subsequent conversion of the amino acid (**108a**) into its amino acid chloride (**109a**), followed by thermal decarbonylation gave the pyrrolinium salt (**58d**).[36] This synthetic strategy is equally suitable for the preparation of *N*-methyl pyrrolinium chloride (**58**) by use of (*RS*)-proline as the starting material.

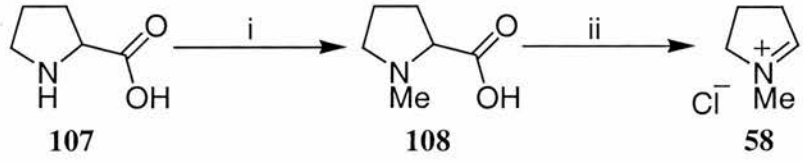


107a **108a** **109a** **58d**

Figure 2.40: Synthesis of *N*-methyl[2-^{2}H]pyrrolinium chloride (**58d**).[23] Reagents and conditions: i, HCOOH, H_2CO, H_2O, 100°C, 16hrs; ii, $POCl_3$, 100°C, 5min.

Accordingly, (*RS*)-*N*-methylproline (**108**) was prepared by treatment of (*RS*)-proline (**107**) with formaldehyde and formic acid in water at reflux.[35] Acidification of the aqueous, followed by removal of solvent gave the product **108** as its hydrochloride salt. The amino acid (**108**) was then dissolved in neat $POCl_3$ at 100°C to give the unstable α-amino acid chloride (**109**), which subsequently decarbonylated to give *N*-

methylpyrrolinium chloride (**58**) (Figure 2.41)[36]. The reaction progress was monitored by ^{1}H-NMR analysis. This was achieved by transferring an aliquot of the reaction mixture directly into an NMR tube containing a D_2O filled capillary. The reaction was found to be complete within 10-15 minutes, consistent with the short reaction times given in the literature.[36]



107 **108** **58**

Figure 2.41: Synthesis of *N*-methyl pyrrolinium chloride (**58**) from (*RS*)-proline. Reagents and conditions: i, HCOOH, H_2CO, H_2O, 100°C, 16hrs; ii, $POCl_3$, 100°C, 15min.[35,36]

Purification of the product iminium salt (**58**) proved problematic. The general method for the preparation of iminium salts[36] describes the synthesis of *N*-β-phenylethylpyrrolinium, which is purified by precipitation from water. However, *N*-methylpyrrolinium (**58**) is soluble in water, precluding the published purification protocol. Addition of diethyl ether to the reaction mixture produced a thick insoluble brown oil, presumed to be the product, whilst solubilising the $POCl_3$. The diethyl ether was decanted from the oil, and the washing procedure repeated to remove any residual phosphorous oxychloride.

2.8.2.2 NMR analysis of *N*-methylpyrrolinium chloride

A sample of *N*-methylpyrrolinium (**58**) was dissolved in D_2O and subjected to ^{1}H-NMR analysis. Deuterium oxide was chosen as the analytical solvent as it best models the aqueous *in vivo* environment of a feeding experiment. It was envisaged that any exchange between the C-2 and C-5 protons and the NMR solvent (Figure 2.42) would result in a detectable reduction of the signal integration for the C-2 and C-5 protons relative to the other non-exchangeable protons in the ^{1}H-NMR spectrum.

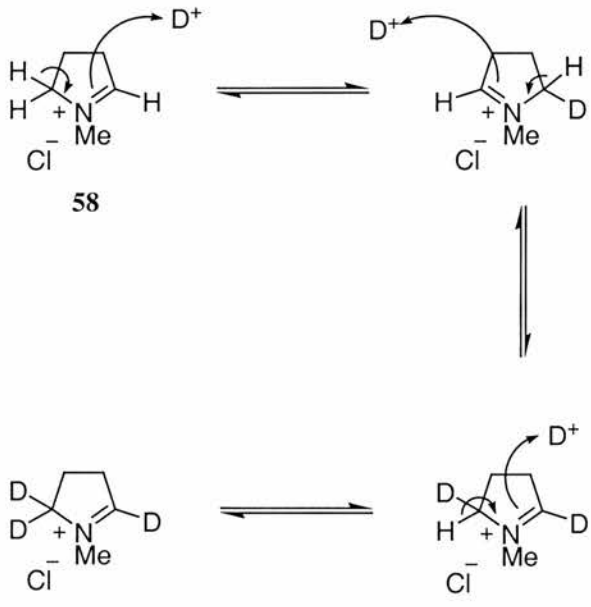


Figure 2.42: The proposed exchange of deuterium into *N*-methylpyrrolinium chloride (**58**).

However, no detectable reduction in the C-2 or C-5 proton resonance integrations was observed in the resultant ^{1}H-NMR spectrum. This indicates that *N*-methylpyrrolinium chloride (**58**) does not readily tautomerise in aqueous conditions. Re-analysis of the sample after ten days showed no reduction in the C-2 and C-5 proton signal integrals. Therefore, to conclude *N*-methylpyrrolinium (**58**) is not susceptible to tautomerisation over the duration of a feeding experiment. Therefore, tautomerisation cannot account for the apparent incorporation of deuterium equally into both C-2 and C-5 of 6-hydroxytropine (**106b**) from *N*-methyl[2-^{2}H]pyrrolinium (**58d**).

2.8.2.3 Discussion

This conclusion is consistent with a previous *in vivo* investigation of tautomerisation of *N*-methylpyrrolinium chloride (**58**).[37] In this experiment *N*-methyl[2-^{14}C]pyrrolinium chloride (**58f**) was administered to *Nicotiana tabacum* plants, the nicotine (**30a**) isolated from this experiment was found to contain radio-label exclusively at C-2′ upon chemical degradation (Figure 2.43).

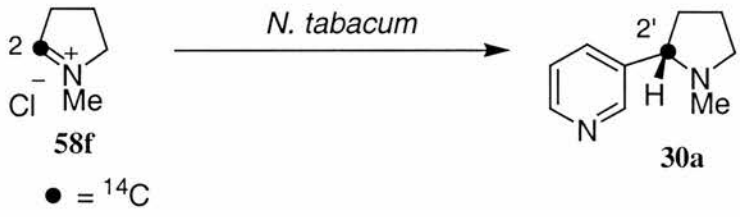


Figure 2.43: Asymmetric incorporation of [*N*-methyl2-^{14}C]pyrrolinium chloride (**58f**) into nicotine (**30a**) in *N. tabacum* plants.

2.8.3 Feeding labelled *N*-methylpyrrolinium

In order to confirm the reported[23] incorporation of *N*-methyl[2-^{2}H]pyrrolinium (**58d**) a synthetic sample of **58d** was required.

2.8.3.1 Synthesis of *N*-methyl[2-^{2}H]pyrrolinium chloride

As stated above *N*-methyl[2-^{2}H]pyrrolinium chloride (**58d**) has previously been prepared in two steps from [2-^{2}H]proline (**107a**) (Figure 2.40). In order to repeat this synthesis a sample of (*RS*)-[2-^{2}H]proline (**107a**) was required as a starting material.

There are a number of reported procedures for the preparation of proline with deuterium label at the α-carbon. Seebach has previously prepared (*S*)-[2-^{2}H]proline (**107a**) in two steps (Figure 2.44) from (*S*)-proline (**107**) in good yield.[38] Additionally, a chemo-enzymatic method for the preparation of (L)-[2-^{2}H]proline from (D)-proline has recently been reported.[39] However, as the product pyrrolinium salt (**58d**) contains no chiral centre the optical purity of the [2-^{2}H]proline (**107a**) starting material is unimportant.

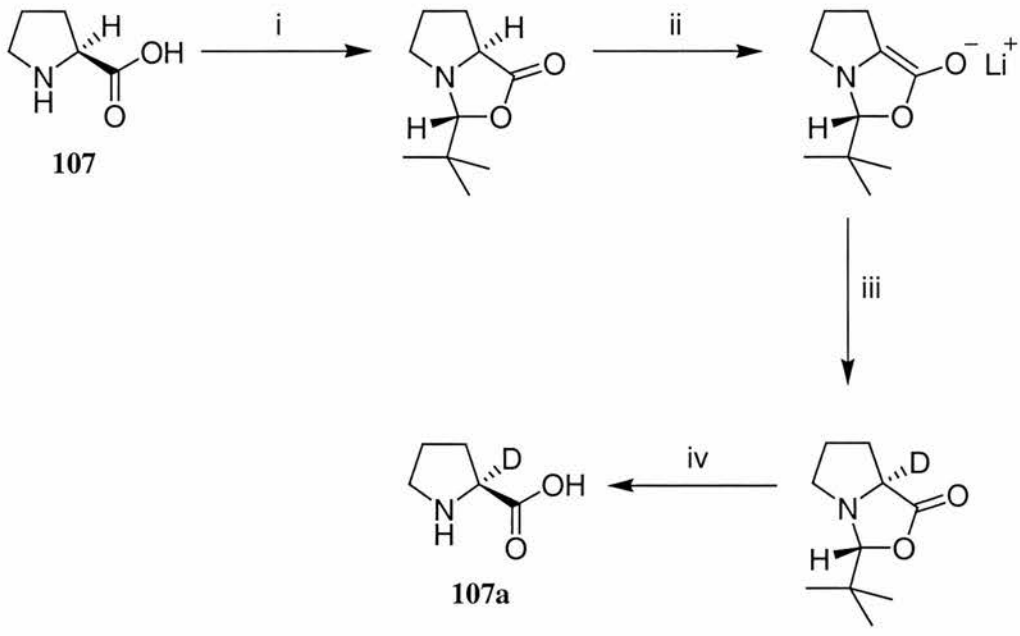


Figure 2.44: Seebach's preparation of (*S*)-[2-^{2}H]proline (**107a**).[38] Reagents and conditions: i, Pivaldehyde, TFA, pentane; ii, LDA, THF; iii, D_2O; iv, DCl.

Preferable to both of these procedures was the preparation of (*RS*)-[2-^{2}H]proline (**107a**) in a single step from (*RS*)-proline (**107**) (Figure 2.45).[40] This synthesis employs a modification of the Yamada racemisation[41] of proline (**107**) (Figure 2.46), the synthesis being performed with a deuterated acid.

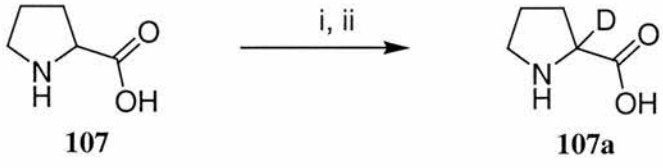


Figure 2.45: Synthesis of (*RS*)-[2-^{2}H]proline (**107a**). Reagents and conditions: i, salicylaldehyde, AcOD, 100°C, 4hrs; ii, D_2O.

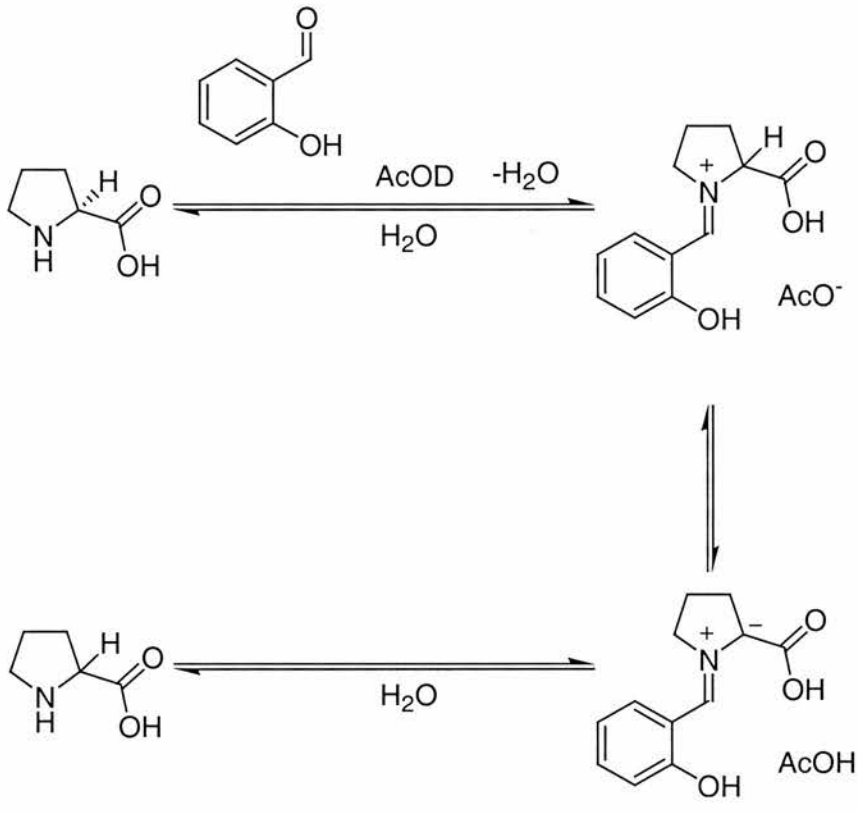


Figure 2.46: The proposed mechanism of the Yamada racemisation of (*L*)-proline to (*RS*)-proline.[41]

Accordingly, (*RS*)-proline (**107**) was treated with salicylaldehyde in [*acid*-^{2}H]acetic acid, which after workup in deuterium oxide and recrystallisation gave (*RS*)-[2-^{2}H]proline (**107**) in good yield (Figure 2.45).

This material was subsequently reacted with formaldehyde and formic acid to furnish (*RS*)-*N*-methyl [2-^{2}H]proline (**108a**) in good yield after recrystallisation. The synthesis of *N*-methyl[2-^{2}H]pyrrolinium chloride (**58d**) was achieved by heating the product (**58d**) in neat $POCl_3$ (Figure 2.40). The reaction was monitored by direct ^{1}H-NMR spectroscopy of the reaction mixture using a D_2O capillary to facilitate locking and shimming of the sample.

2.8.3.2 Feeding *N*-methyl[2-^{2}H]pyrrolinium chloride

N-Methyl[2-^{2}H]pyrrolinium chloride (**58d**) was administered to three transformed root cultures of *D. stramonium* at a final concentration of 1 mmol dm^{-3} in the medium. After the growth period of the roots the alkaloids were isolated, derivatised and analysed by

GC-MS (Table 2.5). The enrichment of an M+1 ion into both littorine (2.36%) and hyoscyamine (3.97%) demonstrates the incorporation of the deuterium from *N*-methyl [2-^{2}H]pyrrolinium chloride (**58d**), consistent with the previous feeding experiment with **58d**.[23]

	Lit			Hyo		
	M	M+1	M+2	M	M+1	M+2
Fed *N*-Me[2-^{2}H]Pyrrolinium	97.61	2.36	0.03	95.90	3.97	0.13
	(0.12)	(0.07)	(0.09)	(0.14)	(0.14)	(0.07)

Table 2.5: Percentage abundance of ions of littorine and hyoscyamine isolated from root cultures fed *N*-methyl[2-^{2}H]pyrrolinium (**58d**). The standard deviation values of the ten separate replicate analyses are given in parentheses.

Additionally, the abundances of the littorine (**80**) and hyoscyamine (**7**) in the crude alkaloid sample were approximately equal, as determined by integration of the GC chromatogram (Figure 2.47). It is possible that the biosynthesis of tropine (**35**) is increased by the feeding of an advanced intermediate and that this results is a greater accumulation of tropane alkaloids in the root tissue. The production of secondary metabolites is a tightly regulated process. Therefore, if hyoscyamine (**7**) accumulates to high concentrations in the root tissue then the expression level(s) of the enzyme(s) responsible for the conversion of littorine (**80**) to hyoscyamine (**7**) may be reduced by a negative feedback loop, which would in turn lead to the accumulation of littorine (**80**).

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Operator    : SP
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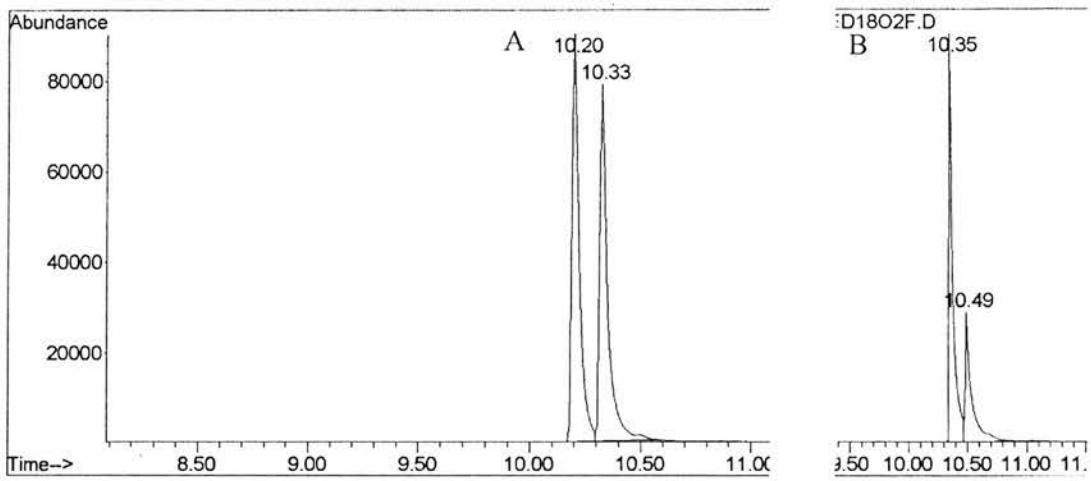

Figure 2.47: (A) GC-MS chromatogram of the crude alkaloid extract after feeding *N*-methyl[2-^{2}H]pyrrolinium chloride (**58d**) to transformed root cultures of *D. stramonium*. (B) For comparison a chromatogram of the crude alkaloid extract isolated after feeding sodium [1-$^{18}O_2$]acetate (**33g**) showing the normal hyoscyamine (**7**) (the first peak eluted) to littorine (**80**) ratio.

2.8.3.3 Discussion

GC-MS analysis cannot differentiate between incorporation into C-1 and C-5 of the tropane alkaloids. Unlike 6-hydroxytropine (**106**) the ^{1}H-NMR resonances for the protons of C-1 and C-5 are indistinguishable in hyoscyamine (**7**) and littorine (**80**). Consequently ^{2}H-NMR cannot be used to determine regiospecific incorporation in these positions. A recently developed chiral ^{2}H-NMR technique has been employed to investigate stereochemical issues in biosynthetic pathways.[42] This technique could be applied to a sample of hyoscyamine (**7**) isolated from a feeding experiment with *N*-methyl[2-^{2}H]pyrrolinium (**58d**), possibly resolving the bridgehead deuteriums. If this was achieved it would enable the regiospecific incorporation of deuterium from **58d** into hyoscyamine (**7**) to be investigated. Further studies are currently continuing within the St Andrews group with the aim of resolving this issue.

2.9 Chapter summary

Acetate has been confirmed as the precursor of the C-2/C-3/C-4 fragment of tropine (**35**) by a series of feeding experiments with carbon-13 labelled sodium acetates. The incorporation patterns into tropine (**35**) from labelled acetate (**33**) suggest that 1,3-acetonedicarboxylate (**96**) may be the precursor to this fragment. However, this remains to be proven experimentally. The origin of the C-3 oxygen of tropine (**35**) has been investigated by a feeding experiment with sodium [$^{18}O_2$]acetate (**33g**). The oxygen-18 isotope was not retained in the resultant hyoscyamine (**7**), therefore the biosynthetic origin of the C-3 oxygen is still to be defined.

The incorporation of [2, 4, 4-$^2H_3$]glutamic acid (**95a**) into the tropane ring has been investigated. The resultant hyoscyamine was found to incorporate two of the deuterium atoms. It is proposed that the third deuterium atom is lost due to oxidation processes during the biosynthesis of tropinone (**39**). The previously reported incorporation of deuterium from *N*-methyl[2-^{2}H]pyrrolinium (**58d**) into the tropane ring has been confirmed. Additionally, the tautomerisation of *N*-methylpyrrolinium (**58**) has been investigated and found not to occur, confirming previous studies.

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Chapter 3
Biosynthesis of Tropic Acid

3 BIOSYNTHESIS OF TROPIC ACID

This chapter describes experiments designed to investigate the biosynthesis of the tropic acid (**24**) ester moiety of hyoscyamine (**7**). The operation of a vicinal interchange process during the conversion of littorine (**80**) to hyoscyamine (**7**) has been investigated by synthesising and feeding deuterium labelled phenyllactic acids (**78f-h**) to transformed root cultures of *Datura stramonium*. In addition deuterium labelled hyoscyamines (**7o** and **7p**) were synthesised and incubated with root cultures in order to determine the level of reversible oxidation of hyoscyamine (**7**)

3.1 Investigating the role of a vicinal interchange process

3.1.1 A co-enzyme B_{12} type mechanism?

As previously discussed (section 1.6.4) the isomerisation of littorine (**80**) to hyoscyamine (**7**) finds some precedence in co-enzyme B_{12} dependent carbon skeleton rearrangements (Figure 3.1). It is well known that co-enzyme B_{12} mediated isomerisations are accompanied by a vicinal interchange process, where a hydrogen atom and the migrating functional group apparently swap places on adjacent carbon atoms (Figure 1.36). This arises because the co-factor adenosyl radical abstracts a hydrogen atom to generate a substrate radical (Figure 1.36). An isomerisation of the substrate radical to product radical then takes place on the surface of the enzyme and finally the product radical is quenched by abstracting back a hydrogen to regenerate the adenosyl radical.

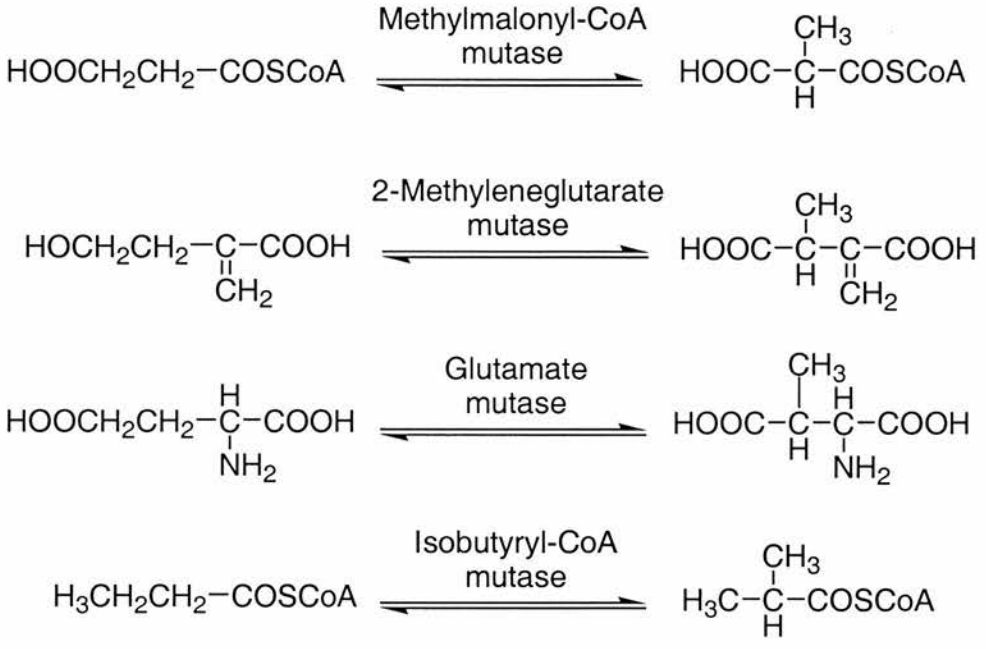
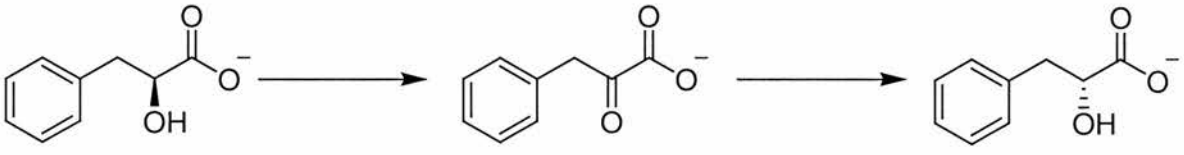


Figure 3.1: Carbon skeleton rearrangement reactions catalysed by co-enzyme B_{12} dependant enzymes.[1] All of the rearrangements operate with a vicinal interchange.

3.1.2 A role for S-adenosyl methionine

Recently a report has been published implicating SAM (**60**) as the co-factor mediating the isomerisation of littorine (**80**) to hyoscyamine (**7**).[2] In this study it was found that the addition of littorine (**80**) and SAM (**60**) to cell free extracts of *D. stramonium* root cultures resulted in a 10-20 fold increase in the levels of hyoscyamine (**7**) in the GC assay. The authors proposed that similar to co-enzyme-B_{12}, an adenosyl radical, this time derived from SAM (**60**) initiates the rearrangement. Implicit in this proposal would be the operation of a vicinal interchange process during the isomerisation reaction. Although such a mechanism is consistent with the observations of Leete,[3] it is inconsistent with more recent biosynthetic investigations,[4] which failed to detect the operation of a vicinal interchange process during the isomerisation of littorine (**80**) to hyoscyamine (**7**) (section 1.6.4). It has been demonstrated that SAM (**60**) is able to initiate enzymatic isomerisations (section 3.1.3) and has been referred to as the 'poor man's B_{12}'.[5] Importantly, whereas co-enzyme B_{12} is absent in plants,[3] SAM (**60**) is present as a methyl donating cofactor. Additionally, SAM (**60**) has been demonstrated to act as the radical generating co-factor for the plant enzyme biotin synthase (BioB),[6] a PLP-dependent enzyme that inserts a sulfur atom into dethiobiotin in the final transformation of biotin biosynthesis.[7]

However, the reported cell free assay[2] has proven to be extremely erratic[8] and has not been repeatable in our laboratory[9] (see chapter 4). Problematically, the experiment used the unnatural (*S*)-isomer of littorine and this would clearly require an *in vitro* isomerisation or racemisation with appropriate enzymes and co-factors in the cell free extract to generate some (*R*)-littorine (**80**) prior to isomerisation to hyoscyamine (**7**). Although (*R*)- and (*S*)- phenyllactic acid (**78**) are interconvertable *via* phenylpyruvic acid (**79**),[10] no enzyme system capable of isomerising (*S*)-littorine to (*R*)-littorine (**80**) has been reported previously. Nor has (*S*)-littorine been implicated as an intermediate during the isomerisation of littorine (**80**) to hyoscyamine (**7**).

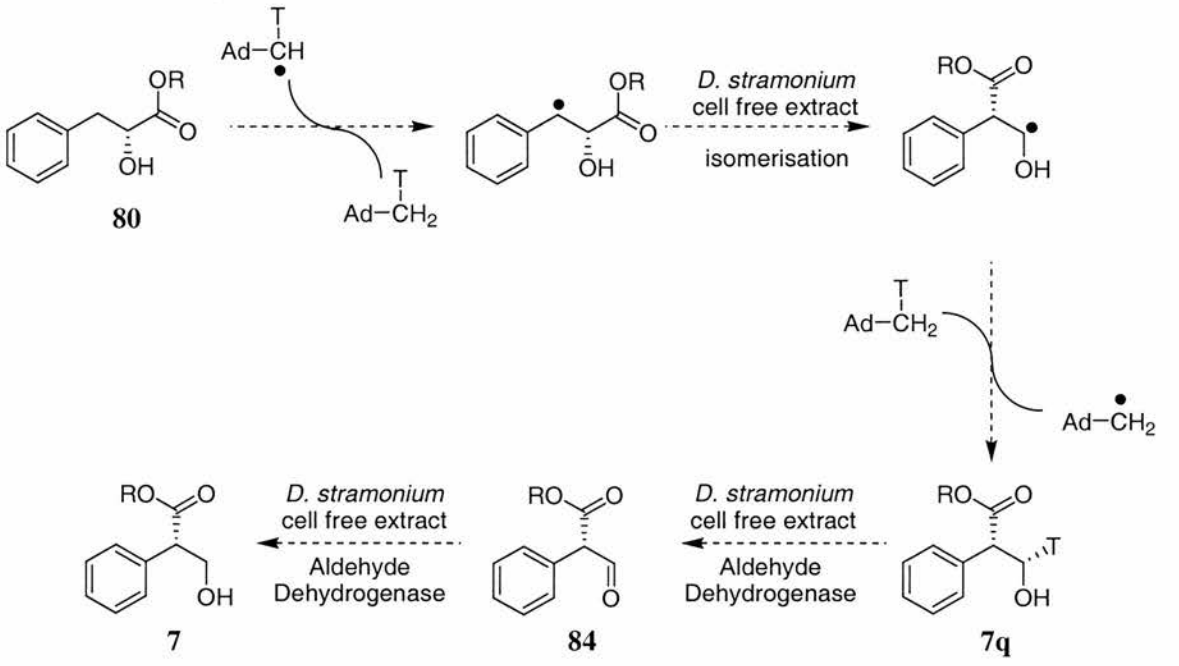


79 **78**

Figure 3.2: The isomerisation of (*S*)- to (*R*)- phenyllactic acid (**78**) *via* phenylpyruvic acid (**79**).

Additionally the experiment did not use isotopically labelled (*S*)-littorine and thus there was no direct evidence that the observed increase in hyoscyamine (**7**) was contributed from the administered (*S*)-littorine. It has been reported that hyoscyamine (**7**) can be 'carried through' several protein purification steps due to non-specific binding to proteins.[11] Therefore it is possible that the increase in hyoscyamine (**7**) observed by the GC assay is the result of endogenous hyoscyamine (**7**) present in the cell free extract.

Also when the experiment was conducted using [2,8,5´-$^{3}H_3$]SAM there was no incorporation of tritium into hyoscyamine (**7**) as might be expected from the 5´ site, but some loss of radioactivity into the bulk water. The authors proposed that the failure to detect tritium in the resultant hyoscyamine (**7**) may be due to the activity of an aldehyde dehydrogenase enzyme acting reversibly on product hyoscyamine (**7**) to generate the corresponding aldehyde (**84**) with 'washout' of tritium from the 3´-*pro-S* site (Figure 3.3), rather than the absence of a vicinal interchange process a prerequisite of a SAM-mediated isomerisations (section 3.1.3).



R = tropine

Figure 3.3: The proposed[2] generation of [3′-^{3}H]hyoscyamine (**7q**) and aldehyde dehydrogenase mediated 'washout' of tritium from the 3′-*pro-S* site of hyoscyamine (**7q**), resulting in unlabelled hyoscyamine (**7**).

Previous studies from our laboratory[12] have shed some light on the extent of washout of the C-3' oxygen in going from littorine (**80**) to hyoscyamine (**7**). The action of an oxido-reductase enzyme could result in the loss of the 3'-*pro-S* hydrogen, and at the aldehyde level (**84**) the 3′ oxygen may then be susceptible to some exchange with bulk water (Figure 3.4). To explore the level of oxygen washout a feeding experiment with (*RS*)-[2-^{2}H,^{18}O]phenyllactic acid (**78e**) to *D. stramonium* root cultures was conducted.[12] This study showed a significant but only partial loss (25-29%) of oxygen-18 label during the isomerisation of littorine (**80**) to hyoscyamine (**7**). Between 70-75% oxygen-18 was retained during the isomerisation (Figure 3.4). This level of isotope exchange at C-3' of hyoscyamine (**7**) in a whole cell experiment over 11-17 days is at variance with the complete loss of tritium from the SAM (**60**) study in a cell free extract over a few hours. Therefore, the proposed 'washout' of tritium due to the action of an aldehyde dehydrogenase enzyme accounting for the lack of a detectable vicinal interchange process is called into question. Additionally, the loss of oxygen-18 label observed in the feeding experiment is not necessarily the result of post synthetic reversible oxidation of hyoscyamine (**7**).[13]

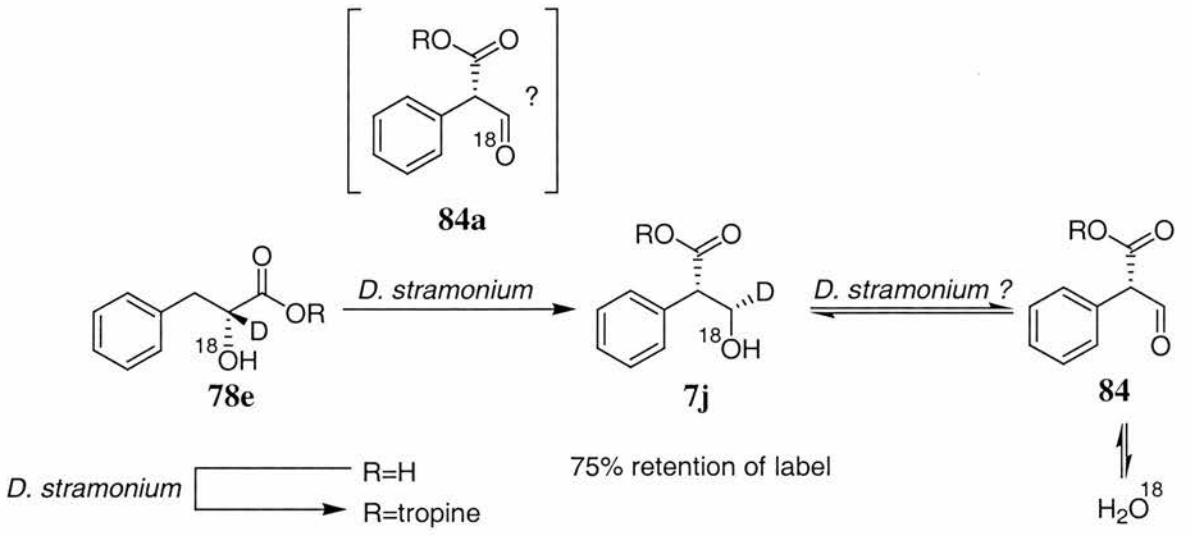


Figure 3.4: Feeding (*RS*)-[2-^{2}H, $^{18}O_2$]phenyllactic acid (**78e**) to *D. stramonium* resulted in a 70-75% retention of the label in oxygen at C-3´ of hyoscyamine (**7j**). The loss of label could be due to washout at the aldehyde level from either post-biosynthetic oxidation (**84**) or an intermediate in the isomerisation (**84a**).

3.1.3 *S*-Adenosyl-L-methionine dependent rearrangments.

Classically SAM (**60**) (Figure 3.5) is utilised as a methylating agent by methyltransferase enzymes.[1,14] In addition SAM (**60**) has also been implicated in a number of radical mediated enzyme reactions.[15,16] A recent genomic analysis[17] has revealed 645 putative SAM-dependent enzymes, forming the radical SAM superfamily. Radical SAM proteins catalyse a number of reaction types, including, sulfur insertion, ring formation, oxidation and isomerisation reactions. Proteins of the radical SAM superfamily are implicated in several biosynthetic pathways.[17]

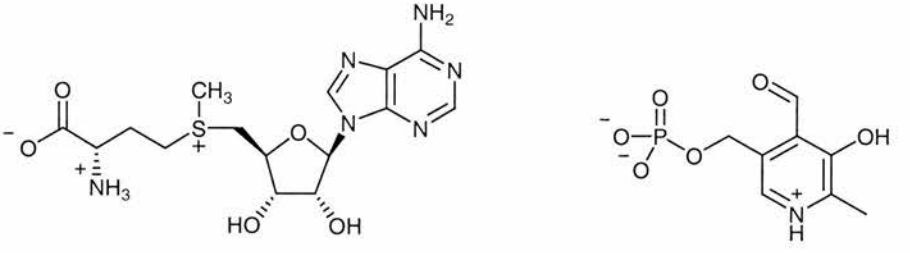


Figure 3.5: The structure of the cofactors *S*-adenosyl-L-methionine (SAM, **60**) and pyridoxal-5´-phosphate (PLP, **61**).

Lysine 2,3-aminomutase (LAM) is an extensively studied member of the radical SAM superfamily, which catalyses the isomerisation of lysine (**110**) to β-lysine (**111**) (Figure 3.6). LAM has a co-factor requirement for SAM (**60**) and pyridoxal-5′-phosphate (PLP, **61**) (Figure 3.6). The isomerisation mechanism of LAM is analogous to a co-enzyme B_{12} mediated process (Figure 3.6).

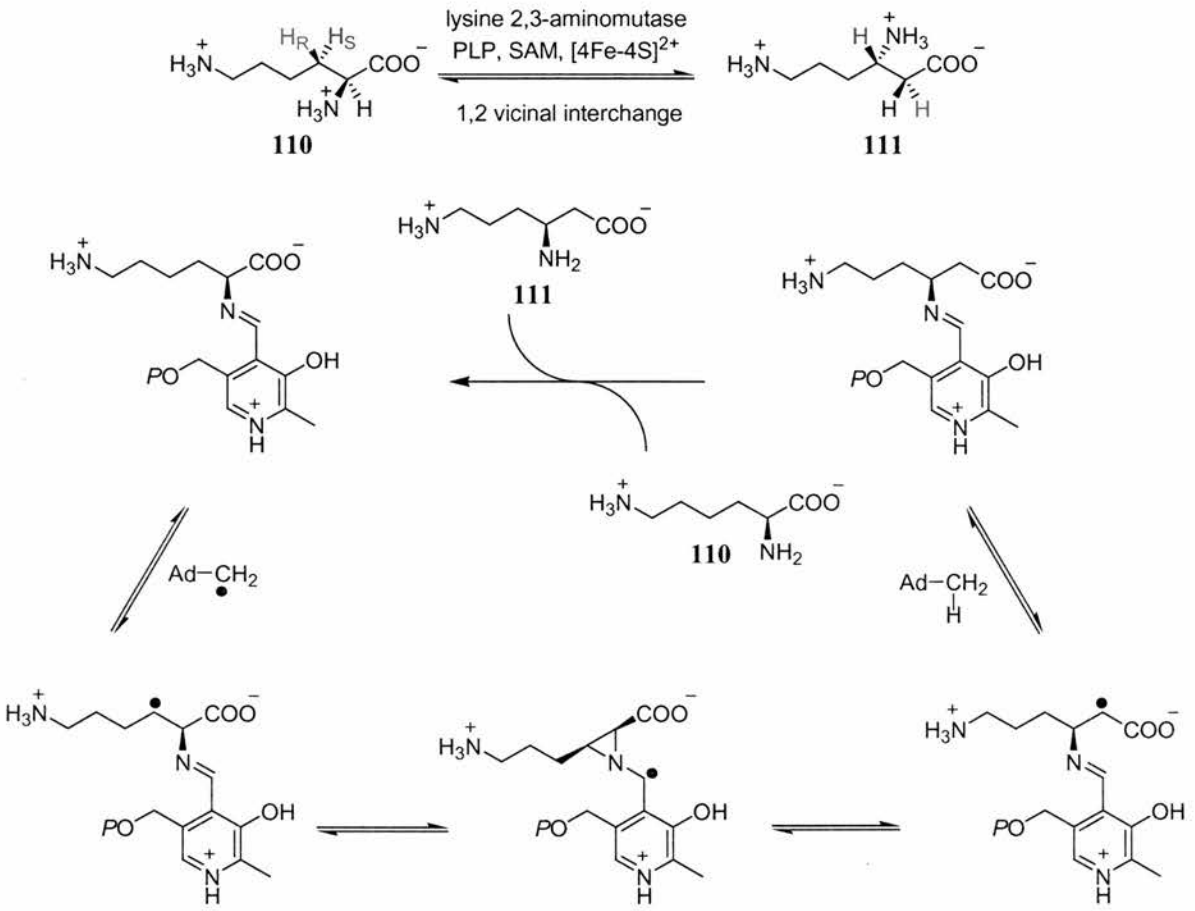


Figure 3.6: The mechanism of lysine (**110**) isomerisation to β-lysine (**111**) catalysed by lysine 2,3-aminomutase.[15] The initial condensation of PLP with lysine (**110**) has been omitted.

3.2 Feeding phenylactic acids

In light of the report[2] implicating SAM (**60**) as the co-factor in the isomerisation of littorine (**80**) to hyoscyamine (**7**) it became important to reinvestigate the mechanism of the rearrangement. Specifically it was necessary to determine if a vicinal interchange process was operating. In order to investigate whether the isomerisation of littorine (**80**) to hyoscyamine (**7**) operates with a vicinal interchange process it was necessary to examine the fate of deuterium label at C-3´ of littorine (**80**). Labelled phenyllactic acids have been shown to undergo enzymatic esterification *in vivo* to produce the corresponding labelled littorines,[10] the labelled littorines are then transformed to hyoscyamines. Therefore, phenyllactic acids containing deuterium label at C-3 represent suitable substrates for feeding experiments to investigate the isomerisation mechanism.

It was envisaged that if the isomerisation does occur *via* a vicinal interchange process then the deuterium atom abstracted from C-3´ of littorine (**80**) (C-3 of phenyllactic acid (**78**)) would be relocated to C-3´ of hyoscyamine (**7**) (Figure 3.7). Such retention of isotope label would be detectable by mass spectrometry. By utilising GC-MS (see section 2.3.3.2.2) simultaneous analysis of both littorine (**80**) and hyoscyamine (**7**) is possible enabling a direct comparison of their levels of isotope incorporation. Additionally GC-MS analysis of the isotope incorporations into hyoscyamine (**7**) offered an extremely sensitive method of analysis with a lower (<0.5%) detectable isotope incorporation than previous NMR methods. Therefore, phenyllactic acids (**78**) enriched with deuterium at C-3 became synthetic targets.

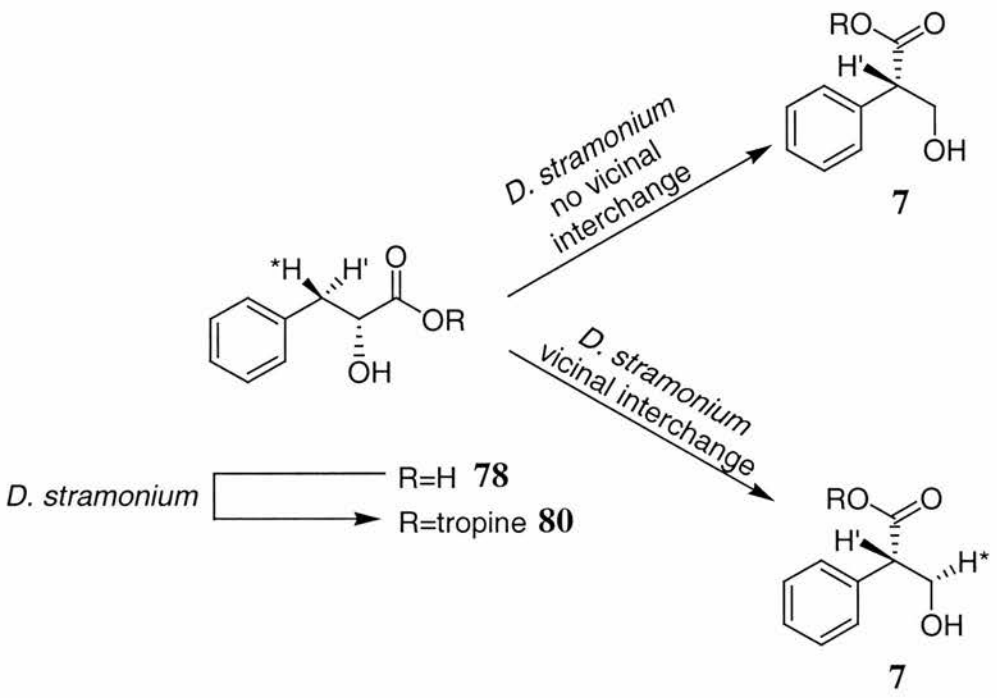
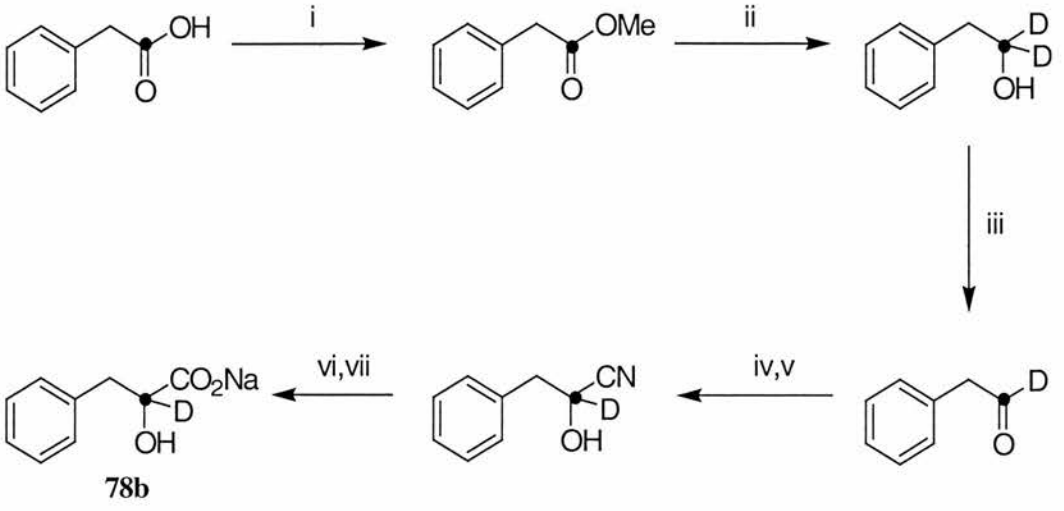


Figure 3.7: Mechanism dependent retention of hydrogen label from phenyllactic acid (**78**) into hyoscyamine (**7**) *via* littorine (**80**).

3.2.1 Synthesis of deuterium labelled phenyllactic acids

Accordingly, samples of (*RS*)-[2-^{2}H], [3,3-$^2H_2$] and [2,3,3-$^2H_3$]phenyllactic acids (**78f-h**) were required as substrates for the feeding experiments. Administering these substrates allows investigation of the fate of the C-2′ and C-3′ protons of littorine (**80**). Only labelled (*R*)-phenyllactic acid (**78**) from the administered racemic mixture will be incorporated into hyoscyamine (**7**). The enzyme(s) responsible for the biosynthesis of littorine (**80**) from phenyllactic acid (**78**) 'resolving' the racemic mixture.

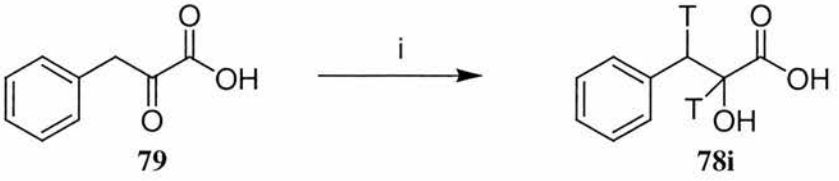
Phenyllactic acid (**78**) has previously been prepared carrying deuterium label at C-2 using a five-[12] or seven-step sequence[18] (Figure 3.8). In both of these synthetic schemes the deuterium label was introduced *via* the reduction of an ester using lithium aluminium deuteride. Although this synthesis was amenable to the preparation of the target molecule, such a lengthy procedure was utilised in previous studies to allow the incorporation of an additional isotopic label, which was not necessary in the current study. Therefore an alternative methodology was required for the synthesis of (*RS*)-[2-^{2}H]phenyllactic acid (**78f**).



78b

Figure 3.8: Preparation of sodium (*RS*)-[2-^{13}C, 2-^{2}H]phenyllactate (**78b**).[18] Sodium (*RS*)-[2-^{13}C, 3-$^{2}H_2$]phenyllactate has been prepared by an analogous method.[24] Reagents and conditions: i, CH_2N_2; ii, $LiAlD_4$; iii, PCC; iv, $NaHSO_3$; v, NaCN; vi, HCl; vii, NaOH.

Phenyllactic acid (**78**) has been previously prepared by direct chemical reduction of phenylpyruvic acid (**79**).[19,20] Such methodology has been used to prepare a sample of (*RS*)-[2, 3-$^{3}H_2$]phenyllactic acid (**78i**) by treatment of phenylpyruvic acid (**79**) with zinc amalgam in tritium oxide (Figure 3.9). The position of the tritium label in the synthetic sample was determined by chemical degradation of the product acid.[21] Whilst the use of zinc amalgam is no longer common this one-step synthesis to phenyllactic acid (**78**) is amenable to the desired introduction of deuterium label at C-2 by use of a commercially available deuterium labelled reducing agent. Sodium borohydride selectively reduces ketones in the presence of acids and is commercially available deuterium enriched. Therefore, sodium borodeuteride was chosen to perform the reduction of phenylpyruvic acid (**79**).



79 **78i**

Figure 3.9: Synthesis of (*RS*)-[2, 3-$^{3}H_2$]phenyllactic acid (**78i**) from phenylpyruvic acid (**79**). Reagents: Zinc amalgam, $^{3}H_2O$.[21]

Accordingly, a suspension of phenylpyruvic acid (**79**) in water was treated with sodium borohydride to give (*RS*)-phenyllactic acid (**78**) in moderate yield. The synthesis was repeated instead using sodium borodeuteride to furnish (*RS*)-[2-^{2}H]phenyllactic acid (**78f**) (Figure 3.10). The incorporation of deuterium label at C-2 was evident both from the decreased signal area for the C-2 proton and the reduction in multiplicity for the C-3 proton signals in the ^{1}H-NMR spectrum.

Phenyllactic acid (**78**) containing two deuterium atoms at C-3 has been previously prepared[24] by a modification of the synthetic route to sodium (*RS*)-[2-^{13}C, 2-^{2}H]phenyllactate (Figure 3.8), but again a shorter synthetic route was more desirable. To introduce deuterium label into C-3 of phenyllactic acid (**78**) using the same chemistry as the preparation of (*RS*)-[2-^{2}H]phenyllactic acid (**78f**) required the synthesis of a sample of [3, 3-$^{2}H_2$]phenylpyruvic acid (**79a**). Direct reduction of phenylpyruvic acid (**79**) in tritium oxide results in tritium enrichment of C-2[21] (Figure 3.9). Presumably reversible enolisation of the C-2 carbonyl is responsible for the introduction of tritium label at C-3. Subsequent reduction of the C-2 carbonyl group prevents the hydrogen label being 'washed out' in the product phenyllactic acid (**78i**). In a similar manner it was envisaged that deuterium could be introduced by facile exchange of the acidic hydrogens at C-3 by treatment with deuterium oxide. Accordingly phenylpyruvic acid (**79**) was stirred in D_2O and the exchange of deuterium into C-3 of phenylpyruvate (**79**) directly observed by ^{1}H-NMR analysis of the reaction mixture. The resonance corresponding to the C-3 hydrogens of phenylpyruvate (**79**) was found to have disappeared after 48 hours indicating complete exchange (within the detection limits of ^{1}H-NMR). In the previous study the duration of the zinc amalgam reduction must have only been sufficient to allow 50% of the C-3 protons to exchange.

The suspension of [3, 3-$^{2}H_2$]phenylpyruvic acid (**79a**) was then reacted directly without isolation with sodium borohydride to yield (*RS*)-[3, 3-$^{2}H_2$]phenyllactic acid (**78g**) (Figure 3.10). Reduction to the alcohol reduces the acidity of the deuterium atoms at C-3 and prevents further exchange, which would have resulted in the loss of deuterium. Similarly treatment of [3, 3-$^{2}H_2$]phenylpyruvic acid (**79a**) with sodium borodeuteride yielded (*RS*)-[2,3,3-$^{2}H_3$]phenyllactic acid (**78h**) (Figure 3.10).

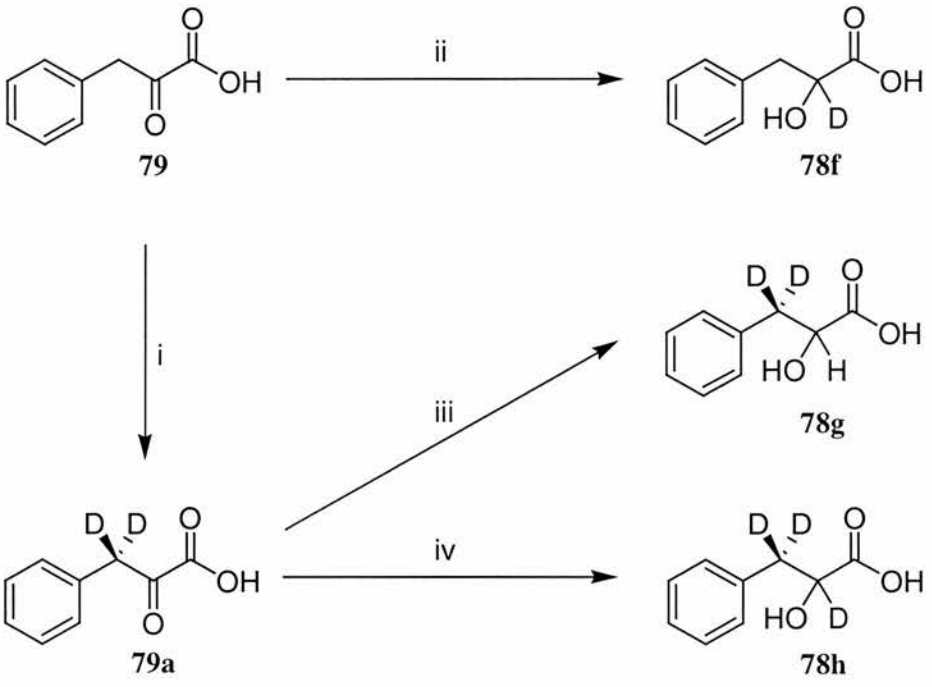


Figure 3.10: Synthetic route to labelled phenyllactic acids (**78f-h**). Reagents and conditions: i, D_2O, 48 hours; ii, $NaBD_4$, H_2O, 3.5 hours, 0-25°C, 59%; iii, $NaBH_4$, H_2O, 3.5 hours, 0-25°C, 64%; iv, $NaBD_4$, D_2O, 3.5 hours, 0-25°C, 54%.

To accurately quantify the level of deuterium enrichment of the synthetic phenyllactic acids (**78f-h**) samples of their corresponding phenyllactate esters were prepared by acid catalysed esterification in an anhydrous alcohol (methanol or ethanol) as the reaction solvent. The resultant esters were then subjected to GC-MS analysis (Table 3.1).

	M	M+1	M+2	M+3
[2-^{2}H]-PhLactate (**78f**)	15%	82%	3%	<1%
[3,3-$^2H_2$]-PhLactate (**78g**)	9%	9%	81%	1%
[2,3,3-$^2H_3$]-PhLactate (**78h**)	4%	4%	17%	75%

Table 3.1: Levels of deuterium enrichment in the synthetic phenyllactic acids (**78f-h**).

3.2.2 Feeding deuterium labelled phenyllactic acids to *D. stramonium*

Each of the deuterium labelled phenyllactic acids (**78f-h**) was administered to four transformed root cultures of *D. stramonium* at a final concentration of 0.25 mmol dm^{-3} in the medium. Addition of (*R*)-phenyllactic acid (**78**) at this concentration is known to

stimulate the production of littorine (**80**) and hyoscyamine (**7**).[22] The mass ion enrichments of isolated littorine (**80**) and hyoscyamine (**7**) were then determined by GC-MS (CI, CH_4) analysis of the derivatised crude alkaloid extract and comparison with unlabelled alkaloids (Table 3.2).

	Lit				Hyo			
	M	M+1	M+2	M+3	M	M+1	M+2	M+3
[2-^{2}H]PhLact.(**78f**)	96.07	3.67	0.20	0.07	96.15	3.82	0.02	0.02
	(0.80)	(0.80)	(0.30)	(0.10)	(0.16)	(0.16)	(0.08)	(0.04)
[3,3-$^2H_2$]PhLact.(**78g**)	87.70	3.89	8.28	0.14	95.75	4.02	0.11	0.12
	(0.50)	(0.50)	(0.46)	(0.25)	(0.46)	(0.38)	(0.15)	(0.11)
[2,3,3-$^2H_3$]PhLct(**78h**)	80.05	5.91	7.31	6.73	92.23	4.18	3.49	0.11
	(2.94)	(2.40)	(1.59)	(0.59)	(0.16)	(0.25)	(0.18)	(0.13)

Table 3.2: Percentage incorporations of deuterium labelled phenyllactic acids (**78f-h**) into littorine (**80**) and hyoscyamine (**7**).

The feeding experiment with (*RS*)-[2-^{2}H]phenyllactic acid (**78f**) resulted in an M+1 enrichment (3.6–3.9%) of both littorine (**80c**) and hyoscyamine (**7r**), consistent with the earlier observation that the C2′ hydrogen of littorine (**80**) is retained as the 3′-*pro-S* hydrogen of hyoscyamine (**7**) after isomerisation.[23] The feeding experiment with (*RS*)-[3, 3-$^2H_2$]phenyllactic acid (**78g**) resulted in a M+2 enrichment (8%) into littorine (**80d**), but significantly there was no detectable M+2 enrichment in the co-isolated hyoscyamine (**7s**). Instead, only an M+1 enrichment (4%) was observed in hyoscyamine (**7s**) indicating that one of the C3′ deuterium atoms is lost during the isomerisation of littorine (**80d**) to hyoscyamine (**7s**). Similarly the feeding experiment with (*RS*)-[2, 3, 3-$^2H_3$]phenyllactic acid (**78h**) resulted in an M+3 enrichment into littorine (**80e**) (6.7%), but only an M+2 enrichment in the resultant hyoscyamine (**7t**), again indicating the loss of one deuterium atom during the isomerisation. The results are summarised in Figure 3.12. These results reinforce the earlier study[24], which indicated that one C-3′ hydrogen (3′-*pro-S*) of littorine (**80**) is retained as the C-2′ hydrogen of hyoscyamine (**7**) and one (the 3′-*pro-R*) is lost during isomerisation. The complete loss of one deuterium atom from C3′ of littorines (**80d** and **e**) during the isomerisation to hyoscyamine (**7s** and **7t**) is inconsistent with a vicinal interchange process, which by definition, requires relocation of the isotope onto the product and

would result in an identical isotope distribution in littorine (**80**) and the corresponding hyoscyamine (**7**).

The feeding experiment with (*RS*)-[3, 3-$^2H_2$]phenyllactic acid (**78g**) also resulted in a significant M+1 enrichment (3.9%) into littorine (**80d**). This loss of isotope can be explained by oxidation of (*RS*)-[3, 3-$^2H_2$]phenyllactic acid (**78g**) to [3, 3-$^2H_2$] phenylpyruvic acid (**79a**), the deuterium at C-3 now being vulnerable to exchange with the aqueous. Subsequent enzymatic reduction of the resultant (*RS*)-[3-^2H] phenylpyruvic acid (**79b**) produces (2*R*, 3*RS*)-[3-^2H]phenyllactic acid (**78j**) (Figure 3.11). Deuterium from the (2*R*, 3*R*)-enantiomer can be incorporated into hyoscyamine (**7**) *via* littorine (**80**). Similarly (*RS*)-[2, 3, 3-$^2H_3$]phenyllactic acid (**78h**) results in M+1 and M+2 incorporations into littorine (**80**).

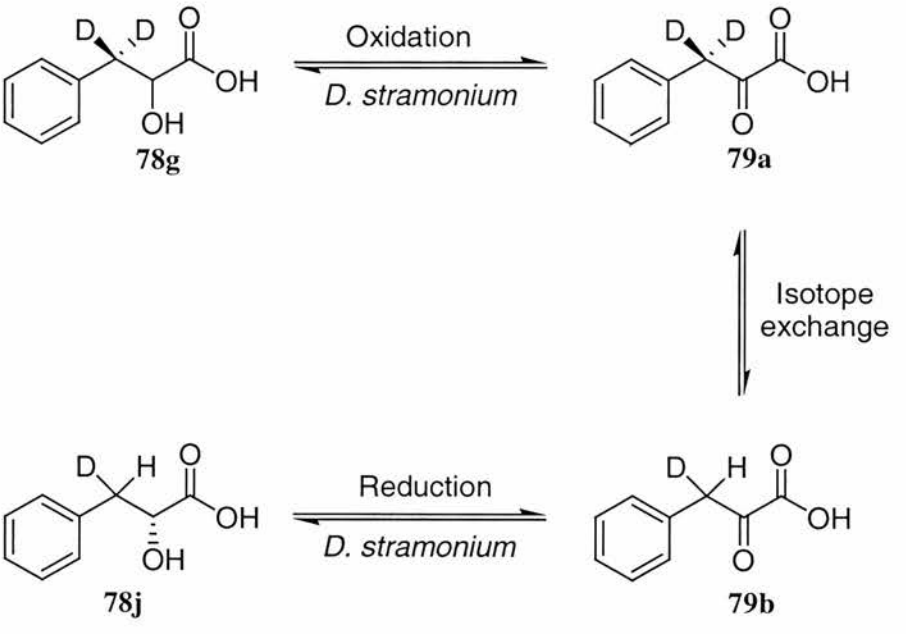


Figure 3.11: Putative *in vivo* transformation of (*RS*)-[3, 3-$^2H_2$]phenyllactic acid (**78g**) to (2*R*, 3*RS*)-[3-^2H]phenyllactic acid (**78j**) *via* (*RS*)-[3-^2H]phenylpyruvic acid (**79b**). Note only (2*R*, 3*R*)-[3-^2H]phenyllactic acid could be incorporated into hyoscyamine (**7**).

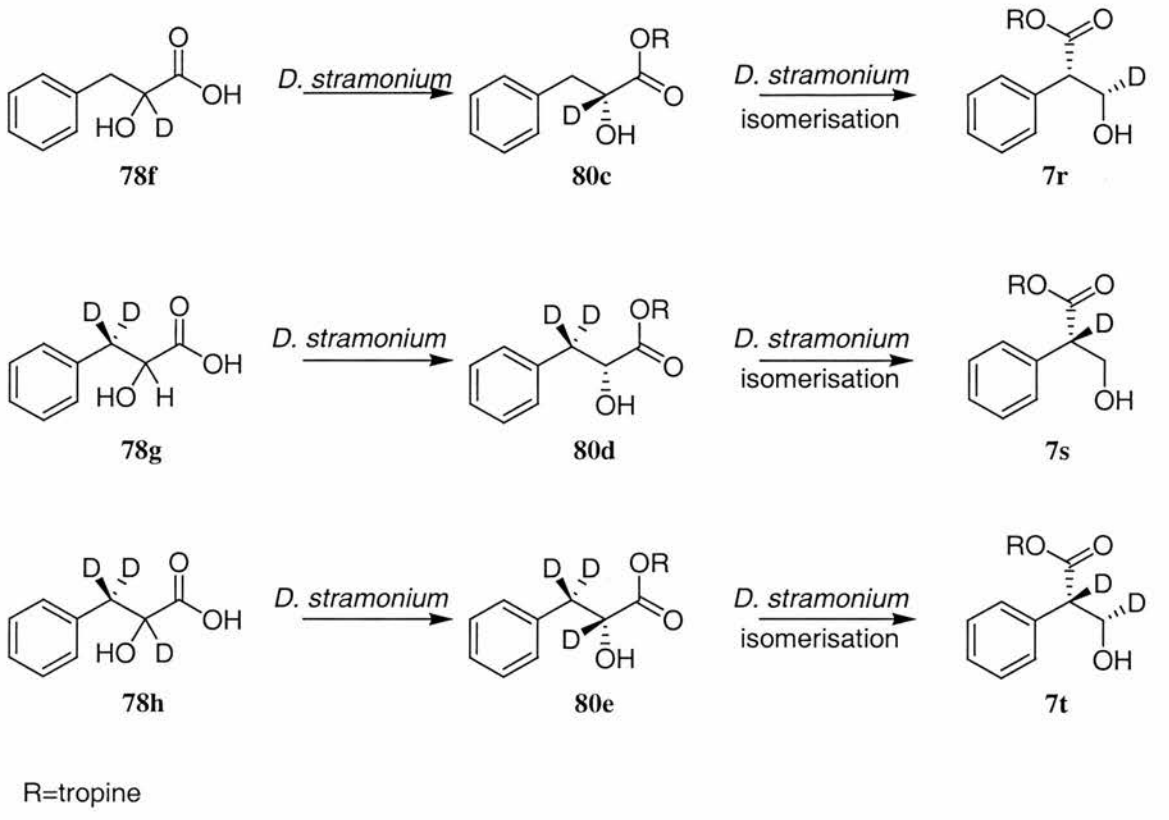


Figure 3.12: Summary of feeding isotopically labelled phenyllactic acids (**78f-h**) to root cultures of *D. stramonium*.

3.2.3 Conclusions

The results provide no support for the operation of a vicinal interchange process during the isomerisation of littorine (**80**) to hyoscyamine (**7**). However, a vicinal interchange mechanism cannot be discounted.

The proposal[2] that post biosynthetic reversible oxidation of hyoscyamine (**7**) results in 'washout' of tritium delivered to C-3´ in cell free experiments can equally account for the failure to detect a vicinal interchange in the whole cell experiments. Therefore, to be absolutely certain that a vicinal interchange process is not operating additional experiments are required to investigate this post-biosynthetic event (section 3.3).

3.3 Incubation experiments with hyoscyamines

In light of the report[2] implicating SAM (**60**) as the co-factor in the isomerisation of littorine (**80**) to hyoscyamine (**7**) it became important to design a probe to explore the level of post-biosynthetic *in vivo* oxidation at C-3´ of hyoscyamine (**7**). The proposed oxidation of hyoscyamine (**7**) to the resultant aldehyde (**84**) has a direct bearing on the observation of the vicinal interchange process by isotope labelling experiments (section 3.2). In particular it was important to determine if the isotope that is delivered to position C-3´ of hyoscyamine (**7**) (providing a vicinal interchange process is operating) is rapidly washed out *in vivo*. Accordingly a procedure to synthesise hyoscyamine (**7**) carrying deuterium label at C-3´ was required to conduct the necessary metabolic experiment. It was envisaged that the level of reversible oxidation could be determined by mass spectrometric analysis of hyoscyamine (**7**) incubated in root cultures of *D. stramonium* and subsequently extracted.

3.3.1 Synthesis of hyoscyamine

There was no published procedure to hyoscyamine (**7**) carrying hydrogen isotopic label at C-3´; therefore it became necessary to develop a new synthesis amenable to the introduction of label at C-3´. Our initial synthetic hypothesis involved esterification of a synthetically prepared labelled tropic acid (**24**) with tropine (**35**). Accordingly, the first objective was to investigate synthetic routes to hyoscyamine (**7**) *via* tropic acid (**24**).

A previous synthetic route to hyoscyamine[25] has involved the acid catalysed esterification of (*RS*)-tropic acid (**24**) and tropine (**35**). This classical formation of tropane esters had already been successfully employed in the research programme for the synthesis of littorine (**80**) (section 2.3.3.2.1). Accordingly tropic acid (**24**) and tropine (**35**) were treated with dry HCl gas. However, it proved impossible to solubilise the crude reaction product in dilute acid. This insoluble gum was attributed to the formation of polytropate produced from the competing inter-molecular esterification of tropic acid (**24**) due to its reactive primary alcohol. Therefore, direct esterification was abandoned as a viable synthetic route to hyoscyamine (**7**).

To circumvent the competing inter-molecular esterification problem, hyoscyamine (**7**) has recently been prepared from tropic acid (**24**) using a four-step protection, activation, coupling and deprotection sequence (Figure 3.13).[26] The literature synthesis involves acetylation of the primary alcohol to give *O*-acetyl tropic acid (**112**), which is in turn activated as its acid chloride (**113**) and esterified with tropine (**35**). The acetyl protecting group is then hydrolysed by treatment with aqueous acid to yield hyoscyamine (**7**). However, no reaction yields were reported, so it became necessary to repeat the procedure to determine its suitability for performing a labelled synthesis.

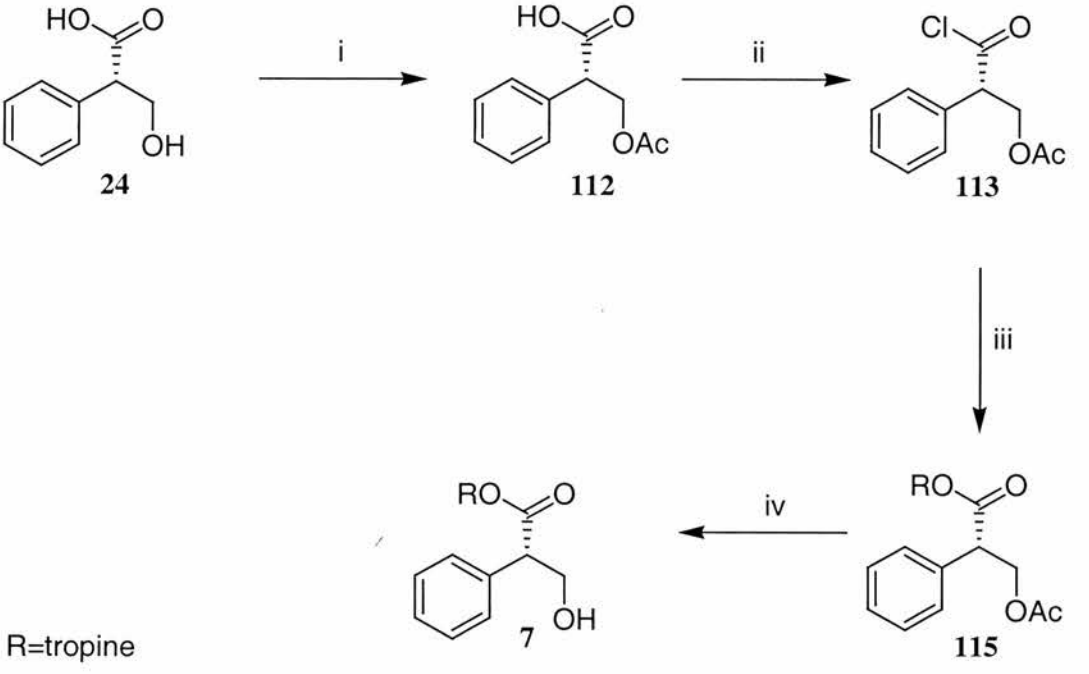
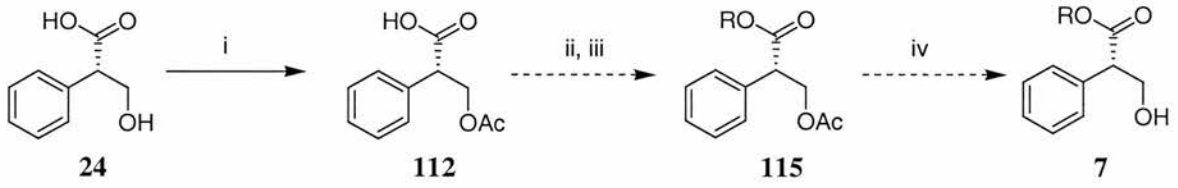


Figure 3.13: Literature synthesis of hyoscyamine (**7**) from (*S*)-tropic acid (**24**) using a four-step reaction sequence. Conditions and reagents: i, AcCl, 2 hours, reflux; ii, $SOCl_2$, 2.5 hours, 60°C; iii, tropine.HCl, $CHCl_3$, 80°C, 2 hours; iv, HCl, H_2O, 25°C, 24 hours.[26]

Accordingly, the procedure was modified and used to prepare a sample of hyoscyamine (**7**) (Figure 3.14). Treatment of tropic acid (**24**) with acetyl chloride followed by an aqueous workup generated the ester of the primary alcohol in acceptable yield. The carboxylic acid (**112**) was then activated as the acid chloride (**113**) followed by treatment with tropine (**35**). However, there was no observable formation of ester. The low esterification yield was believed to be due to the difficulty of forming the tetrahedral intermediate (**114**) adjacent to the crowded tertiary carbon. Attempts to form the ester by DCC coupling between the protected acid (**112**) and tropine (**35**)

proved unsuccessful. The inability to form the ester is consistent with a previous study, which failed to synthesise the ester of **112** with scopine (**71**) under analogous conditions.[27]



24 **112** **115** **7**

Figure 3.14: Proposed modification to the literature synthesis of hyoscyamine (**7**). Reagents and conditions: i, AcCl, DCM, RT, 16 hours; ii, $SOCl_2$, DCM, RT, 16 hours; iii, Tropine (**35**) DCM, 16 hours. iv, 4M HCl, RT, 16 hours.

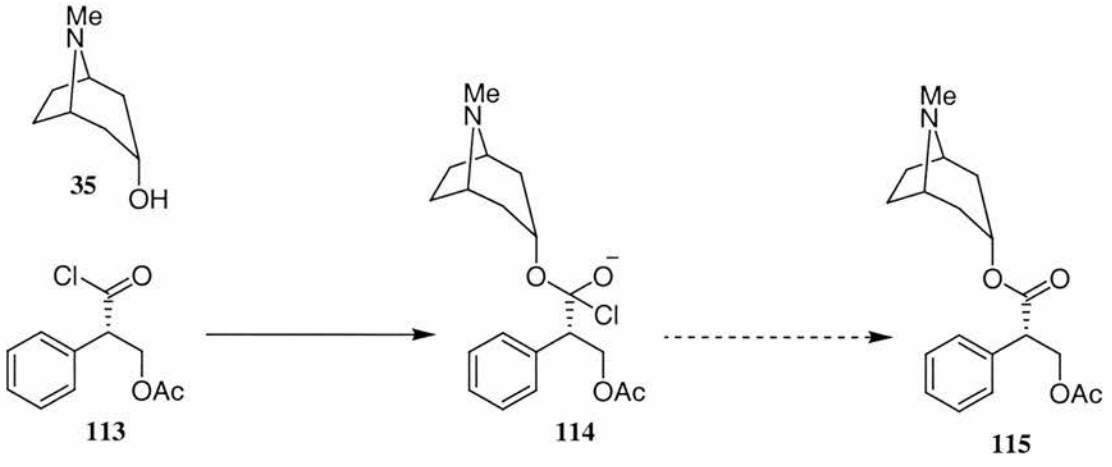


Figure 3.15: Esterification reaction of acetyl protected tropic acid chloride (**113**) with tropine (**35**) to give acetyl protected hyoscyamine (**115**). The tetrahedral intermediate (**114**) is shown.

Due to the poor yields obtained with this route to hyoscyamine (**7**) it became necessary to develop an alternative synthetic strategy to hyoscyamine (**7**). It was envisaged that the problematic primary alcohol functionality could be introduced in the final step of the synthesis, post esterification, following the disconnections shown in Figure 3.16. This approach proved appealing as it eliminated the problematic inter-molecular esterification of tropic acid (**24**) without the use of protecting groups, and the associated deprotection step, therefore reducing the number of synthetic steps from three to two. The required substrate for the addition of formaldehyde should be accessible *via*

esterification of tropine (**35**) and phenylacetic acid (**116**). This synthetic method was also attractive due to the commercial availability of [$^2H_2$]paraformaldehyde, and thus accordingly hyoscyamine can be prepared with label at position C-3´. Additionally the isotopic label is introduced in the final step of the synthesis, reducing the amount of expensive labelled reagent required.

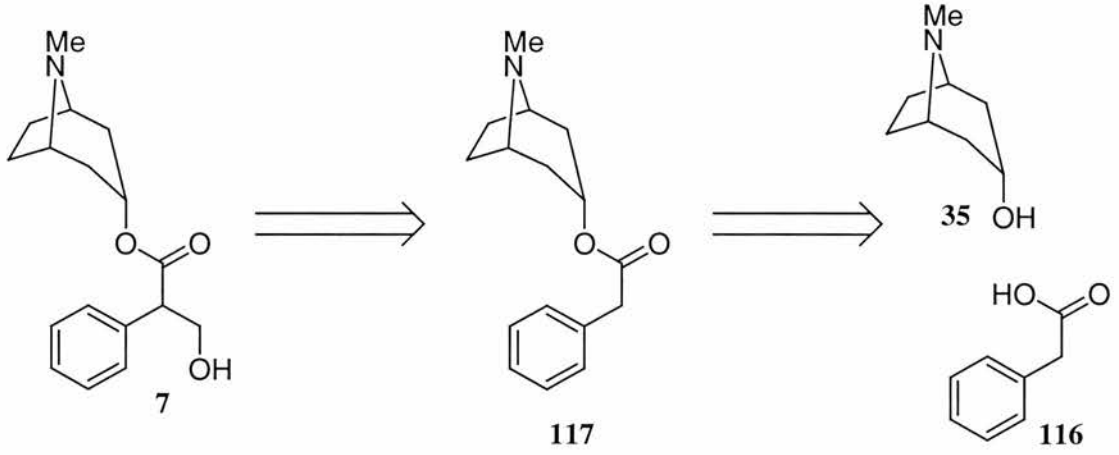


Figure 3.16: Retrosynthetic analysis for a novel synthesis of (*RS*)-hyoscyamine (**7**).

Phenylacetyltropine (**117**) was prepared following the classical preparation of tropine (**35**) esters by acid catalysed esterification of tropine (**35**) and phenyacetic acid (**116**) using dry HCl gas (Figure 3.17).[28] The product ester was dissolved in dilute sulfuric acid, treated with base and extracted into an organic solvent. The ester was recovered by this method in good yield without the need for further purification.

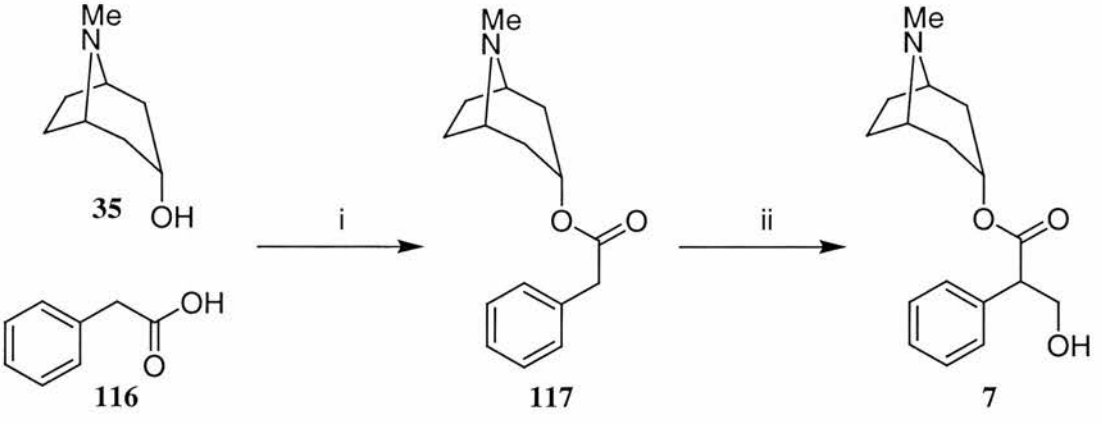


Figure 3.17: Synthesis of (*RS*)-hyoscyamine (**7**). Reagents and conditions: i, HCl gas, 145°C, 3.5 hours; ii, LDA, THF, H_2CO, –78°C.

The introduction of a hydroxymethylene group in the proposed manner had previously been achieved as part of the total synthesis of graveolide[29] by the reaction of

formaldehyde gas with an enolate anion (Figure 3.19). Accordingly, the resultant phenylacetyltropine (**117**) was treated with LDA in anhydrous THF at –78°C. This generates the enolate anion, which was subsequently quenched with formaldehyde gas (five equivalents). The gaseous formaldehyde was formed by the depolymerisation of paraformaldehyde at 150°C[30] and bubbled into the reaction using a stream of dry nitrogen. Aqueous workup and extraction into organic solvent gave a mixture of (*RS*)-hyoscyamine (**7**) and unreacted phenylacetyltropine (**117**). The resultant mixture was separated by column chromatography or preparative t.l.c. to give (*RS*)-hyoscyamine (**7**) with identical spectroscopic properties to a commercial sample (Aldrich) of the natural product (Figure 3.18).

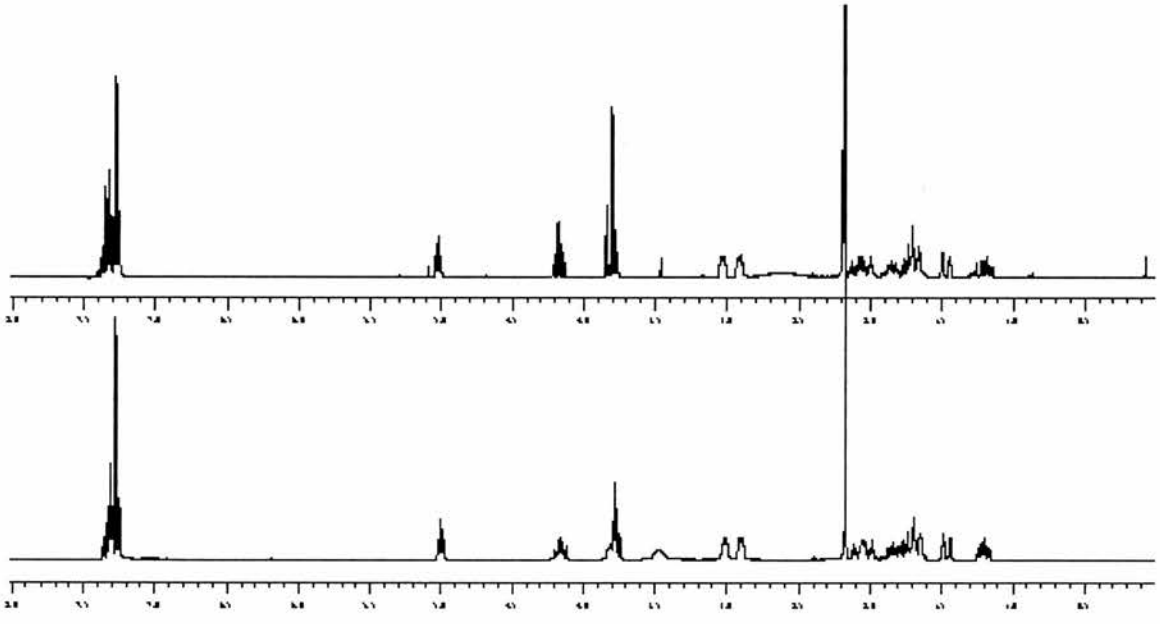


Figure 3.18: Comparison of the ^{1}H-NMR spectra of (top) synthetic (*RS*)-hyoscyamine (**7**) and (bottom) commercial hyoscyamine (**7**).

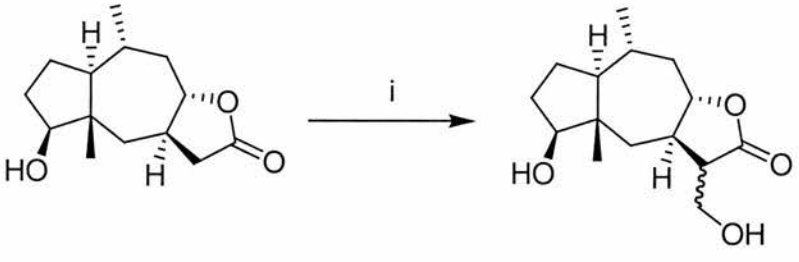


Figure 3.19: Introduction of a hydroxymethylene group as part of the synthesis of graveolide.[29] Reagents and conditions: i, LDA, $(H_2CO)_n$, -78°C.

The use of formaldehyde to introduce a hydroxymethylene group into a tropate ester has been previously achieved by use of Grignard chemistry (Figure 3.20).[31] However, this approach would have involved a greater number of synthetic steps.

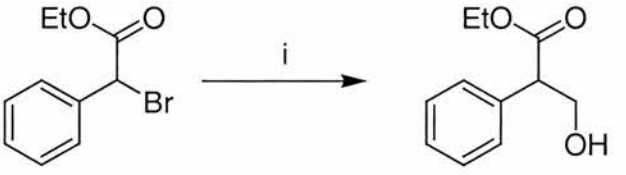


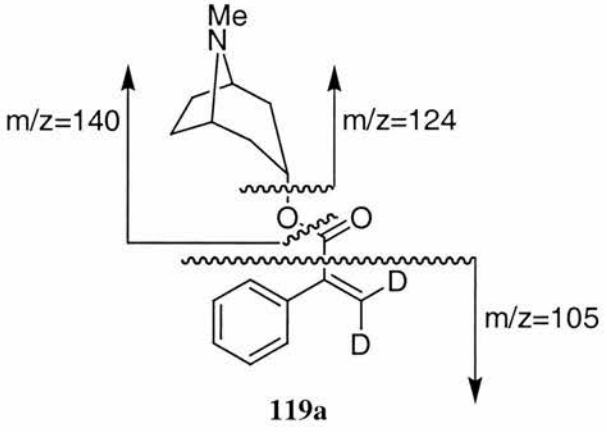
Figure 3.20: Literature synthesis of ethyl tropate (**118**). Reagents and conditions: i, magnesium, formaldehyde.[31]

3.3.2 Synthesis of (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine

An identical synthetic methodology was utilised to introduce the double deuterium label at position C-3′ of hyoscyamine (**7**). Commercially available [$^2H_2$]paraformaldehyde (Aldrich chemical company) was depolymerised to form [$^2H_2$]formaldehyde gas, seven equivalents of formaldehyde were used in an attempt increase the percentage conversion of the reaction. GC-MS analysis of the crude reaction mixture (Figure 3.23) showed no remaining phenylacetyltropine (**117**) (rt=8.5 minutes), therefore all of the starting material had reacted. However, in addition to forming (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**) (rt=10.6 minutes) two additional products were formed. Samples of (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**) were purified from the by-products by preparative t.l.c. for further analysis. Mass analysis demonstrated that the deuterium is retained in the synthesis, the resultant (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**) having a molecular weight of 363 after derivitisation. NMR analysis (^1H and ^{13}C) of the purified (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**) confirmed that the deuterium atoms are incorporated into C-3′ as expected.

The mass spectrum of the first compound (rt=9.4 minutes) revealed characteristic tropane masses of 124 and 140, and gave a mass ion peak of 273. This mass ion and a fragmentation ion of 105 suggest that the product formed is [3′, 3′-$^2H_2$]apoatropine (**119a**) (Figure 3.21). The formation of [3′, 3′-$^2H_2$]apoatropine (**119a**) could be due to LDA induced elimination. Trimethylsilyl derivitisation of hyoscyamine (**7**) should

reduce the thermal formation of apoatropine (**119**) during analysis[22], therefore the elimination product must be formed during the synthetic procedure.



119a

Figure 3.21: Proposed fragmentation of compound 1 (rt=9.4 minutes) suggesting its structure to be [3´, 3´-$^2H_2$]apoatropine (**119a**).

The mass spectrum of the second additional compound (rt=11.8 minutes) also revealed characteristic tropane masses of 124 and 140, and gave a mass ion peak of 467. It is proposed that this compound is the diol product (**120**) formed from two additions of [$^2H_2$]formaldehyde with phenylacetyltropine (**117**), or rather a second addition of [$^2H_2$]formaldehyde with (*RS*)-[3´, 3´-$^2H_2$]hyoscyamine (**7o**) (Figure 3.22). This is supported by the presence of a fragment ion of mass 362 corresponding to the loss of a single derivatised hydroxymethylene group (Figure 3.22).

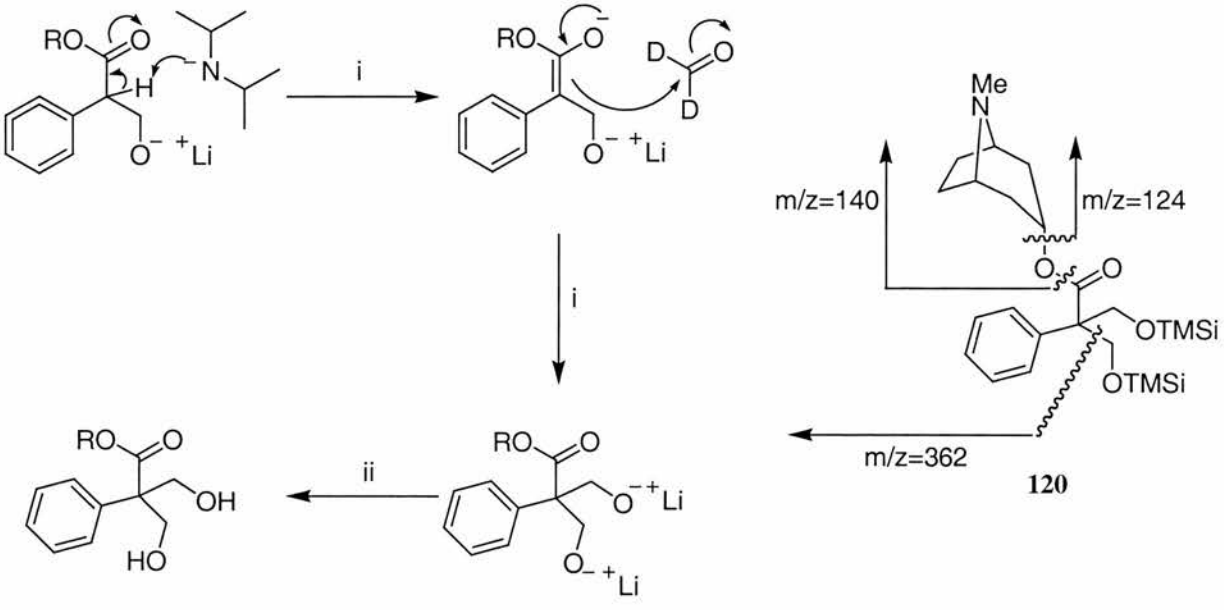


Figure 3.22: The proposed formation of the hyoscyamine diol derivative (**120a**) from addition of [$^2H_2$]formaldehyde with (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**). The fragmentation pattern of the derivatised diol (**120a**) is shown. Reagents and conditions: i, LDA, THF, D_2CO (g), -78°C; ii, H_2O, 0°C. For clarity the deuterium atoms are not shown.

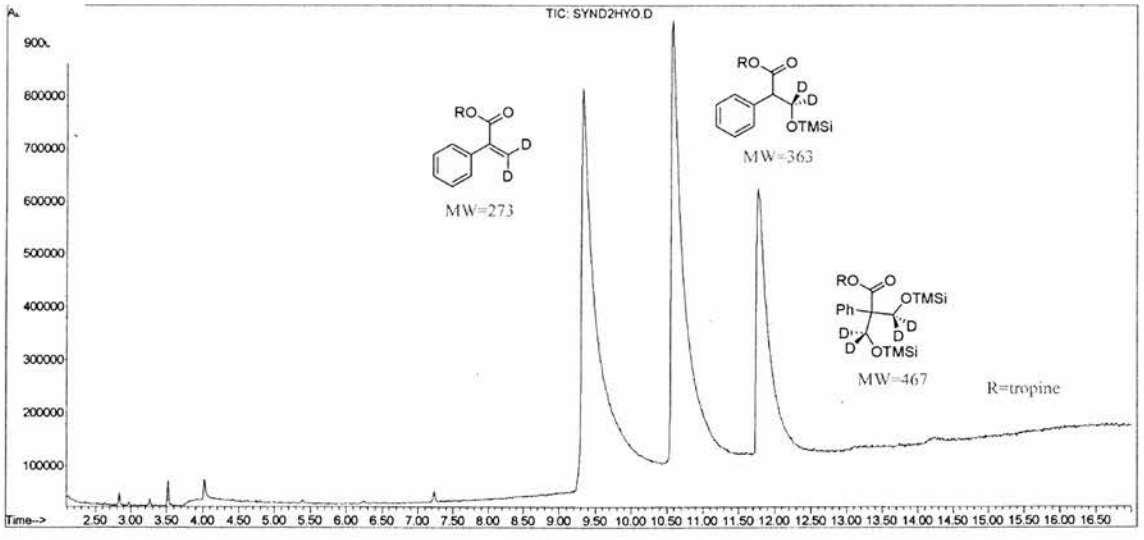


Figure 3.23: GC-MS chromatogram showing the products of the reaction of [$^2H_2$] paraformaldehyde with phenylacetyltropine (**117**). Reagents and conditions; i) LDA, THF, -78°C, 30min. ii) ^2H_2CO (g), THF, -20°C. iii) H_2O, 25°C. iv) MSTFA, 80°C, 50min.

3.3.3 Incubation experiment with (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine

A sample of (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**) was prepared by preparative t.l.c.. This was administered to twelve transformed root cultures of *D. stramonium* at a final concentration of 0.25 mmol dm^{-3} in the medium. Three flasks of roots were then incubated with the labelled hyoscyamine (**7o**) for each of 4, 8 and 12 days. The crude alkaloid extract was derivatised and subjected to GC-MS analysis. The level of isotope in the isolated hyoscyamine was determined by comparison with mass analysis of unlabelled commercial hyoscyamine (Table 3.3). It was envisaged that the incremental incubation periods would allow detection of any deuterium loss in a time dependent manner.

		M	M+1	M+2	$\frac{M+1}{M+2} \times 100$
(*RS*)-[3′, 3′-$^2H_2$]hyo (**7o**)					
	Day 4	76.40	0.59	22.70	2.60
		(0.45)	(0.12)	(0.32)	
	Day 8	82.17	0.46	17.27	2.66
		(0.18)	(0.12)	(0.16)	
	Day 12	89.11	0.70	10.06	6.96
		(0.42)	(0.21)	(0.28)	

Table 3.3: Level of isotope in isolated hyoscyamine from root cultures incubated with (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**).

An increase in the ratio of M+1: M+2 is used in this experiment as an indication of the loss of deuterium at C-3′ with time. This ratio (see Table 3.3) does increase over the course of the experiment (2.60-6.96%) indicating a low level of deuterium loss. However, deuterium loss at this low level is insufficient to account for the complete loss of label observed during the rearrangement of littorine (**80**) to hyoscyamine (**7**) (section 3.2.2). Additionally, the high standard deviation for the level of M+1 ion from hyoscyamine extracted on day 12 has a direct effect upon the reliability of the M+1: M+2 ratio, making this value less reliable. Due to the level of natural abundance of M+1 it is difficult to accurately assess any enrichment.

However, if the incubated hyoscyamine contained an additional isotopic label then changes in the M+2:M+3 ratio would represent the level of reversible oxidation. As the natural abundances of M+2 and M+3 are significantly lower than that of M+1 analysing their ratio is a more accurate representation of the level of reversible oxidation. Therefore, hyoscyamine enriched with an additional isotopic label became a target molecule to act as a metabolic probe.

3.3.4 Synthesis of (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine

The synthetic methodology used to prepare (*RS*)-[3′, 3′-$^{2}H_2$]hyoscyamine (**7o**) is also amenable to the preparation of hyoscyamine containing additional isotopic label(s) in either the tropane or tropic acid moiety. Although there are established literature procedures to isotopically labelled tropines[28,32] their synthesis would increase the number of synthetic steps. Therefore it was decided to synthesise a sample of hyoscyamine containing an extra isotopic label in the tropic acid moiety, by use of a commercially available labelled phenylacetic acid (**116**). Accordingly, having established that deuterium label is successfully introduced by this synthetic methodology a sample of (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine (**7p**) was prepared in an identical fashion, but with five equivalents of [$^{2}H_2$]formaldehyde gas and [1-^{13}C]phenyl acetotropine (**117a**) (Figure 3.24). Returning to the use of five equivalents of formaldehyde gas resulted in incomplete conversion to (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$] hyoscyamine (**7p**), but no diol product was detectable by GC-MS analysis of the crude reaction mixture. Samples of (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine (**7p**) were purified by preparative tlc for use in incubation experiments (section 3.3.5). Mass analysis demonstrated a high level of M+3 ion (93%) in the synthetic sample.

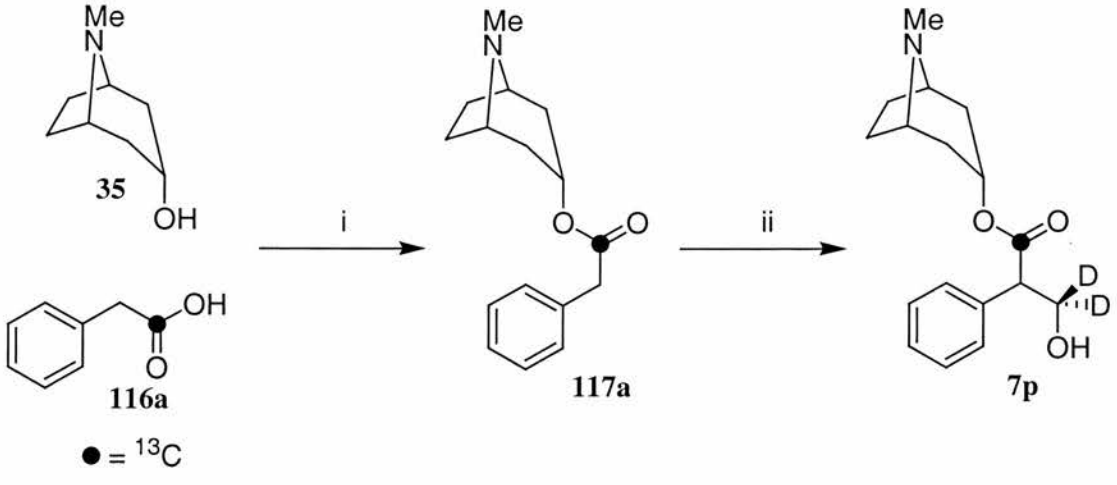


Figure 3.24: Synthesis of (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine (**7p**). Conditions and reagents; i, HCl (g) 145°C. 3 hours; ii, 1) LDA/THF –78°C. 30 minutes. 2) [$^{2}H_2$] formaldehyde (g). 3) H_2O.

3.3.5 Incubation experiment with (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine

(*RS*)-[1-^{13}C, 3′, 3′-$^{2}H_2$]Hyoscyamine (**7p**) was administered to fifteen root cultures of *D. stramonium* at a final concentration of 0.25 mmol dm^{-3}. The root cultures were then incubated for a further 6, 9, 12, 15 or 18 days (three each) before the alkaloids were extracted from each flask separately. Again the incremental incubation periods would allow detection of any deuterium loss in a time dependent manner.

This sample contained three isotopically enriched atoms. Accordingly the ratio (M+2/M+3 x 100) was used as a measure of the loss of a single deuterium due to reversible oxidation over time (Table 3.4). Although there is some variation in (M+2/M+3 x 100) the ratio remains approximately stable over this extended time period. In fact there is a tendency for the ratio to decrease a little through the experiment which is *opposite* to that expected for any loss of isotope at C-3' due to a reversible oxidative metabolism of hyoscyamine (**7**). The percentage differences in M+3 abundances observed are the result of the *de novo* production of hyoscyamine (**7**). Therefore, the proposal that a reversible *in vivo* oxidation at C-3′ of hyoscyamine (**7**) prevents the detection of a vicinal interchange is not supported by the data. Accordingly the mechanism and co-factor(s) for the isomerisation of littorine (**80**) to hyoscyamine (**7**) remain to be determined conclusively.

		M	M+1	M+2	M+3	$\frac{M+2}{M+3} \times 100$
[1′-^{13}C,3′,3′-$^{2}H_2$]hyo (**7p**)						
	Day 6	55.8 (±11.5)	0.18 (±0.03)	0.62 (±0.12)	41.7 (±10.6)	1.49
	Day 9	56.1 (±6.14)	0.27 (±0.07)	0.54 (±0.27)	41.7 (±5.7)	1.30
	Day 12	61.0 (±5.4)	0.24 (±0.15)	0.54 (±0.10)	37.1 (±5.1)	1.46
	Day 15	64.1 (±7.6)	0.16 (±0.04)	0.30 (±0.16)	34.4 (±7.2)	0.87
	Day 18	56.4 (±6.5)	0.16 (±0.07)	0.42 (±0.16)	41.7 (±6.0)	1.00

Table 3.4: Levels of hyoscyamine isotopomers after incubation of (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine (**7p**) in root cultures of *D. stramonium* for different time periods.

Each data point represents an average of three separate flasks; the greatest variance is given in parentheses.

3.4 Conclusions

In conclusion the results presented indicate that the rearrangement of littorine (**80**) to hyoscyamine (**7**) does not occur with a vicinal interchange process and are inconsistent with a mechanism involving SAM (**60**).

3.5 Chapter summary

The conversion of littorine (**80**) to hyoscyamine (**7**) has been investigated by feeding deuterium labelled (*RS*)-[2-^{2}H], [3, 3-$^{2}H_2$] and [2, 3, 3-$^{2}H_3$]phenyllactic acids (**78f-h**) to transformed root cultures of *Datura stramonium*. Isolation and GC-MS analyses of the isotope incorporation into the resultant hyoscyamine (**7r-t**) does not support the involvement of a vicinal interchange process operating during the isomerisation of littorine (**80**) to hyoscyamine (**7**).

Additionally a two-step synthesis to hyoscyamine (**7**) facilitating the incorporation of isotope label into the tropate moiety has been developed and used to prepare a sample of (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine (**7p**). A metabolism study using the (*RS*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]hyoscyamine (**7p**) has established that the alkaloid is metabolically stable at C-3′ with no evidence for a reversible *in vivo* oxidation process to the corresponding aldehyde (**84**). The data do not support a SAM/co-enzyme-B_{12} vicinal interchange mediated process for the isomerisation of littorine (**80**) to hyoscyamine (**7**).

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Chapter 4
Cell Free
Studies

4 CELL FREE EXPERIMENTS

This chapter describes experiments designed to investigate the enzymology and mechanism of the biosynthesis of the tropane alkaloids, particularly the isomerisation of littorine (**80**) to hyoscyamine (**7**). A number of cell free extract preparations of *D. stramonium* root cultures have been prepared and assayed for their ability to biosynthesise littorine (**80**) and hyoscyamine (**7**) from administered precursors. The biosynthesis of the tropane alkaloids is discussed in light of these experiments.

4.1 Enzymology of tropane alkaloid biosynthesis

Although isotopic feeding experiments have identified a number of the biosynthetic intermediates to the tropane alkaloids hyoscyamine (**7**) and scopolamine (**37**), the majority of the associated biosynthetic enzyme activities remain to be isolated (Figure 4.1). The enzyme activities that have been isolated and/or cloned are those constituting the early or late steps in the biosynthetic pathway, with the exception of the tropinone (**39**) reducing enzymes TRI and TRII (section 1.5.1.3). Additionally, no cell free preparations catalysing the biosynthesis of the phenyllactic acid (**78**) moiety of littorine (**80**) have been reported, even though the enzymatic steps (transamination and reduction) are not in themselves unusual or complex.

The final and most interesting step in the biosynthesis of the tropic acid (**24**) moiety is the isomerisation of the phenyllactoyl moiety of littorine (**80**). This rearrangement has been the subject of extensive investigation (sections 1.6.3 and 1.6.4).[1] However, the mechanistic detail and co-factors involved in the rearrangement have not been determined. It has been demonstrated that the isomerisation is catalysed by a cell free preparation of *D. stramonium* root cultures and the co-factor SAM (**60**). However, this report has not been independently corroborated, or any further investigations reported.

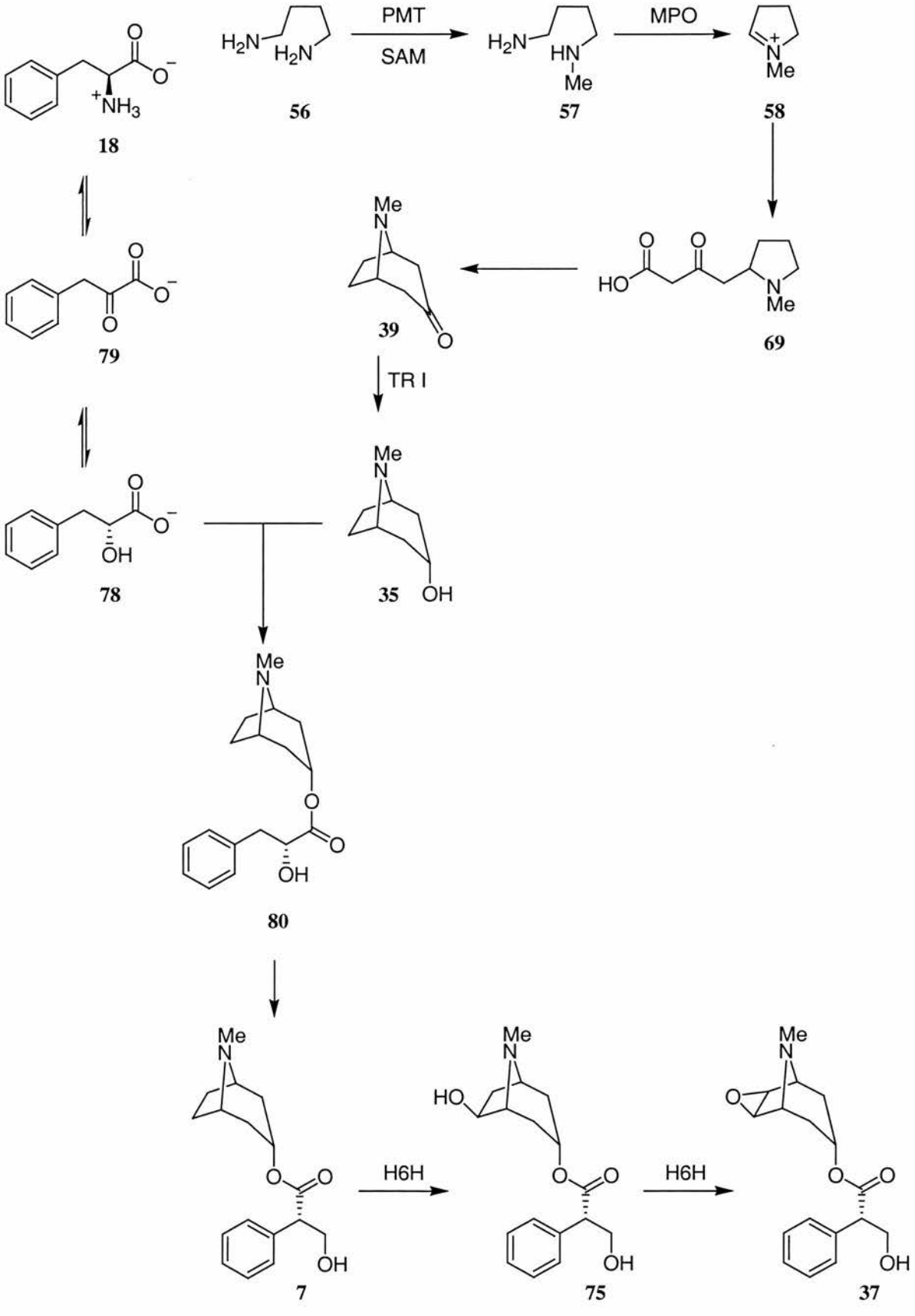


Figure 4.1: Committed biosynthetic steps to the tropane alkaloids littorine (**80**), hyoscyamine (**7**), 6β-hydroxyhyoscyamine (**75**) and scopolamine (**37**). The known enzymes are given.

4.2 The putative role of *S*-adenosyl methionine

One of the initial aims of the research programme was to verify the reported[2] SAM-dependent cell free transformation of littorine (**80**) to hyoscyamine (**7**). It was envisaged that if the reported activity could be repeated in our laboratory, the cell free preparation could be challenged with labelled littorines, in order to verify that any hyoscyamine (**7**) observed in the GC-MS assay was derived from the administered littorine. Additionally, an active cell free preparation would allow the mechanism of the littorine mutase to be investigated without the need to conduct lengthy feeding experiments.

4.2.1 Experimental considerations

The reported cell free assay[2] administered (*S*)-littorine, the unnatural isomer to the cell free preparation. It was decided to repeat the reported procedure[2] replacing (*S*)- with (*R*)-littorine (**80**) as the administered substrate; this eliminates the requirement for an *in situ* generation of (*R*)-littorine (**80**). In every other respect the protocol was performed as described in the published cell free assay.[2]

4.2.2 Preparation of protein extract[2]

Root material was harvested from culture by filtration, homogenised by pestle and mortar and suspended in cold phosphate buffer (100 mM) containing DTT (3 mM), sucrose (250 mM) and disodium EDTA (5 mM). The suspension was stirred for 30 minutes, and cell debris removed by filtration. The protein extract was clarified by low speed centrifugation to give the crude cell free preparation. The protein extract was passed through a desalting column (Amersham Biosciences), and the extract concentrated using an ultra-filtration apparatus (Amicon) fitted with a 10 kDa exclusion membrane under a positive pressure of nitrogen. The resultant concentrated extract (~2 mg ml^{-1} protein) was used for the littorine mutase assay (section 4.2.3).

4.2.3 Littorine mutase assay

Littorine mutase activity was determined by a GC-MS based assay. The GC conditions are identical to those used for the analysis of crude alkaloid mixtures, which had

consistently separated the isomeric alkaloids during the research programme (section 2.3.3.2). Assays were performed by the addition of SAM (**60**) (200 μM) and littorine (**80**) (2 mM) to an aliquot of the crude protein extract, and subsequent incubation in a water bath. The reaction was terminated by rapid cooling and addition of ammonia. The alkaloids could then be extracted directly into an organic solvent or the aqueous mixture loaded onto a hydromatrix column and eluted with a chloroform / methanol solution. The extracts were derivatised with MSTFA (**90**) prior to GC-MS analysis.

4.2.4 Results and discussion

Addition of SAM (**60**) to the assay mixture did *not* catalyse the conversion of littorine (**80**) to hyoscyamine (**7**) as determined by GC analysis. This is inconsistent with the reported cell free study,[2] where it was observed that addition of SAM (**60**) resulted in an increase in the hyoscyamine (**7**) to littorine (**80**) ratio (61:39 versus 3.5: 96.5 in assays performed without the addition of SAM (**60**)). Repetition of the assay (5 attempts) consistently failed to detect SAM-dependent activity.

This is in agreement with previous investigations in our laboratory[3] and suggests that SAM (**60**) is not involved as a co-factor during the rearrangement of littorine (**80**) to hyoscyamine (**7**). This conclusion reinforces the results of isotopic feeding experiments conducted during the research programme (see chapter 3), which failed to detect the operation of a vicinal interchange process during tropic acid (**24**) biosynthesis, a requirement of SAM-dependent isomerisations.

It has been communicated[4] to us that this assay is extremely erratic, failing to consistently catalyse the formation of hyoscyamine (**7**) from littorine (**80**). This erratic activity may be due the retention of some 'protein-associated' hyoscyamine (**7**) in the cell free preparation, which is subsequently 'released' into solution during the assay or extraction procedures.

In conclusion the putative role of SAM (**60**) as a co-factor during the isomerisation of littorine (**80**) to hyoscyamiune (**7**) is unconfirmed. Additionally, in light of isotopic feeding experiments conducted during the research programme the role of SAM (**60**) is considered doubtful. Therefore, the co-factor requirements and the mechanism by which littorine mutase operates requires further investigation.

4.3 Cell free oxidation of hyoscyamine

In an attempt to confirm the SAM-dependent nature of the littorine mutase, Ollagnier *et al* administered [2, 8, 5′-$^{3}H_3$]SAM to a cell free preparation.[2] However, there was no detectable incorporation of tritium into hyoscyamine (**7**) as would be expected if a SAM-dependent rearrangement was operating. It was proposed that the loss of radioactivity to the bulk water was the result of the reversible activity of an aldehyde dehydrogenase on the product hyoscyamine (**7**). This enzymatic oxidation could result in the loss of tritium from hyoscyamine (**7**). Therefore, it became of interest to investigate the enzymatic oxidation of hyoscyamine (**7**) in *D. stramonium* cell free extracts.

4.3.1 Previous studies

Previous work conducted in our laboratory had demonstrated that hyoscyamine (**7**) is not a substrate for a range of commercially obtained alcohol dehydrogenase enzymes (equine liver, *Saccaromyces cerevisiae* and *Thermoanaerobium brockii*).[5] This suggests that non-specific oxidation by endogenous dehydrogenase enzymes is unlikely. Therefore, the action of a hyoscyamine-specific or broad substrate tolerant dehydrogenase is required.

4.3.2 Hyoscyamine dehydrogenase assay

A cell free preparation from *D. stramonium* root cultures was prepared (section 4.4) and assayed for NAD- and NADP- dependent hyoscyamine (**7**) oxidation. The assay was performed by addition of hyoscyamine (**7**), and NAD or NADP to the cell free extract in buffer.

4.3.3 Results and discussion

Observation of the reaction over a ten-minute period showed no change in the absorbance at 340 nm. Therefore, there was no evidence for any dehydrogenase activity in the extract capable of oxidising hyoscyamine (**7**). However, it is possible that the activity level of a relevant dehydrogenase is so low in the assay that is was undetectable. However, with this caveat in mind such an activity seems unlikely to account for the

complete washout of isotope after a vicinal interchange process, as suggested in the published cell free experiment.[2]

4.4 Preparation of cell free extracts

4.4.1 Initial investigations

One of the initial aims of the research programme was to develop a procedure for the preparation of a cell free extract capable of catalysing the conversion of littorine (**80**) to hyoscyamine (**7**). The first cell free protocol utilised was based on a published preparation used to investigate the activity of hyoscyamine 6β-hydroxylase.[6]

4.4.1.1 Preparation of protein extract

Washed root tissue was homogenised by freezing in liquid nitrogen and grinding in a pestle and mortar with PVPP and extraction buffer (100 mM phosphate buffer, 200 mM sucrose and 3 mM DTT). After standing for 30 minutes the mixture was filtered and clarified by centrifugation. This crude extract was assayed for littorine mutase activity.

4.4.1.2 Assay procedure

Littorine (**80**) (2 mM) was added directly to the cell free extract, which was incubated at 33°C. Aliquots were removed at t=0, 30, 60, 90 and 120 minutes. The aliquots were cooled on ice and treated with ammonium hydroxide followed by immediate extraction into chloroform, which was removed *in vacuo*. The level of conversion was determined by dissolving the alkaloid fraction in chloroform, treatment with MSTFA (**90**) and subsequent GC analysis. The level of hyoscyamine (**7**) was compared with that in controls (no added littorine (**80**))

4.4.1.3 Results and discussions

Assay of the basic protein extract revealed no repeatable littorine mutase activity. Therefore the endogenous levels of co-factor and protein must be insufficient to facilitate significant biosynthesis of hyoascyamine (**7**).

Analysis of one cell free experiment showed an apparent accumulation of hyoscyamine (**7**) over time (Figure 4.2). However, this activity proved to be unrepeatable, casting doubt over its authenticity. The increased levels of hyoscyamine (**7**) in the cell free extract could be due to release of hyoscyamine (**7**) into solution during the course of the assay incubation.

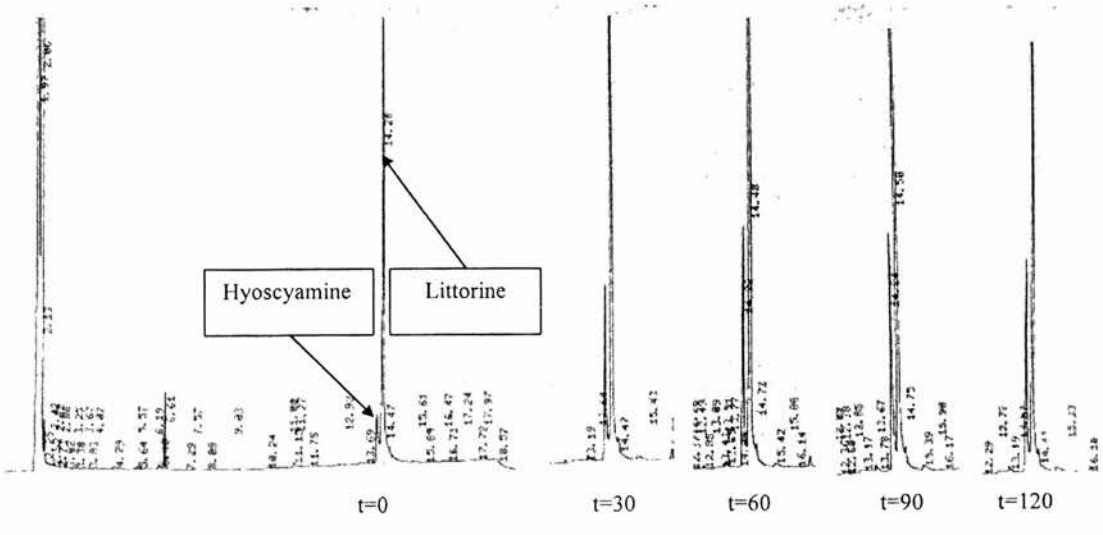


Figure 4.2: Gas chromatograms showing the apparent production of hyoscyamine (**7**).

4.4.2 Improved investigations

As the basic cell free preparation did not display any littorine mutase activity it was decided to modify the procedure with the aim of detecting cell free activity.

4.4.2.1 Experimental considerations

There are a number of plant tissue-specific problems that must be considered when developing a protocol for the preparation of an active cell free extract: the acidic cell sap, phenolases, tannins and the plant cell wall.[7]

4.4.2.1.1 Protein stability

Plant tissues contain phenolic compounds including polymeric tannins, which form both non-covalent and covalent bonds to proteins.[8] These bonds can lead to protein aggregation and precipitation. Poly(vinylpolypyrrolidone) (PVPP) complexes with

phenolics to remove them from plant homogenates preventing modification of the extracted proteins.[8,9] Therefore, insoluble PVPP was added to crude cell homogenates and then removed by centrifugation during clarification of the crude extract.

Cell disruption also exposes proteins to degradation from proteases. Accordingly the extraction buffer was supplemented with a 'cocktail' of protease inhibitors. Serine and cysteines proteases are the most abundant in plant tissues. Therefore, PMSF, chymostatin and E-64 were added to the extraction buffer.

4.4.2.1.2 Protein concentration

The ability to achieve a high protein concentration is vital for the detection of enzyme activity. Therefore, the cell free extracts were concentrated by use of an ultrafiltration unit (Amicon) fitted with a 10 kDa exclusion membrane or by precipitation with ammonium sulfate (30-70% cut), followed by dialysis or passage through a desalting column. Both of these methods produced extracts with protein concentrations of ~1.5-3 mg ml^{-1}, as determined by the Bradford assay.

4.4.2.1.3 Co-factor requirements

The mechanism and cofactor requirements of the littorine mutase enzyme have not been experimentally determined. Additionally, as the mechanism of the isomerisation is unknown, the absolute co-factor requirements cannot be inferred. It has been proposed that the littorine mutase is a cytochrome P-450 enzyme.[1] A review[10] of isolation and assay conditions for cytochrome P-450 enzymes suggests the addition of FAD, FMN, and NADPH to assay mixtures. Therefore these cofactors were added to the assay mixtures.

4.4.2.2 Preparation of cell free extract

Root tissue was disrupted by blending in a mixture of chilled extraction buffer (see experimental) and PVPP. The crude macerate was clarified and concentrated before performing the littorine mutase assay. A portion of the supernatant was ultra-centrifuged to pellet microsomes; a cellular fraction rich in P-450 enzymes.[10] The microsome pellet was resuspended in buffer containing 0.1% (v/v) Triton X-100 to solubilise membrane proteins.

4.4.2.3 Littorine mutase assay

The assay was initiated by the addition of littorine (**80**). After incubation overnight the assay mixture was basified and extracted into DCM, prior to treatment with MSTFA (**90**) and GC-MS analysis.

4.4.2.4 Results and discussion

No accumulation of hyoscyamine (**7**) was detected in the GC-MS assay. The failure to detect any littorine mutase activity could be due to a number of factors outlined below (section 4.4.3).

4.4.3 Discussion

During the research programme there has been a consistent failure to assay littorine mutase activity. A number of factors may contribute to this lack of detectable enzyme activity.

4.4.3.1 Competing reactions

It is possible that hyoscyamine (**7**) is produced under the assay conditions, but that it is subsequently degraded by the action of a second more active enzyme, such as an esterase. If this is the case an initial 'purification' step may be required to separate these two activities within the crude cell free extract.

4.4.3.2 Enzyme expression level

It may also be argued that the constitutive level of littorine mutase expression is too low to allow detection of hyoscyamine (**7**) production. It may be possible to alleviate this problem by addition of elicitors capable of inducing a greater level of enzyme expression.

4.4.3.3 Cofactor requirements

The most likely explanation for the lack of enzyme activity however, is the non-fulfilment of co-factor requirements for the conversion of littorine (**80**) to hyoscyamine (**7**) thus preventing enzyme activity.

4.4.3.4 Substrate for the rearrangment

Although littorine (**80**) is a proven precursor to hyoscyamine (**7**),[1] it is not necessarily the immediate precursor. Therefore, two or more enzyme activities may be required to produce hyoscyamine (**7**) from littorine (**80**). If this is the case, some level of substrate channelling or the formation of an enzyme complex may also be required to convert littorine (**80**) into hyoscyamine (**7**). Although such assemblies can exist *in vivo*, cellular disruption resulting in decompartmentalisation of the cell, could result in loss of these assemblies *in vitro*. Identification of an isomerisation intermediate may prove necessary to successfully assay for the production of hyoscyamine (**7**).

4.5 Hydrazine 'trapping' of putative aldehyde intermediate

It has been proposed[1] that an aldehyde (**84**) may be the initial product of the rearrangement of littorine (**80**) and that a second enzyme transformation yields hyoscyamine (**7**) (Figure 4.3) (see section 1.6.4). In order to explore the putative role of an aldehyde intermediate (**84**) (Figure 4.3) during the isomerisation of littorine (**80**) to hyoscyamine (**7**) an aldehyde 'trapping' experiment was designed.

4.5.1 Hydrazine 'trapping'

It was envisaged that if the aldehyde (**84**) were an intermediate product it would react with hydrazine (**121**) giving a product hydrazone (**122**), which could subsequently undergo an intramolecular cyclisation reaction to give the pyrazone (**123**) (Figure 4.3). This proposed 5-*exo-trig* cyclisation is a favoured transformation (Baldwin's rules).

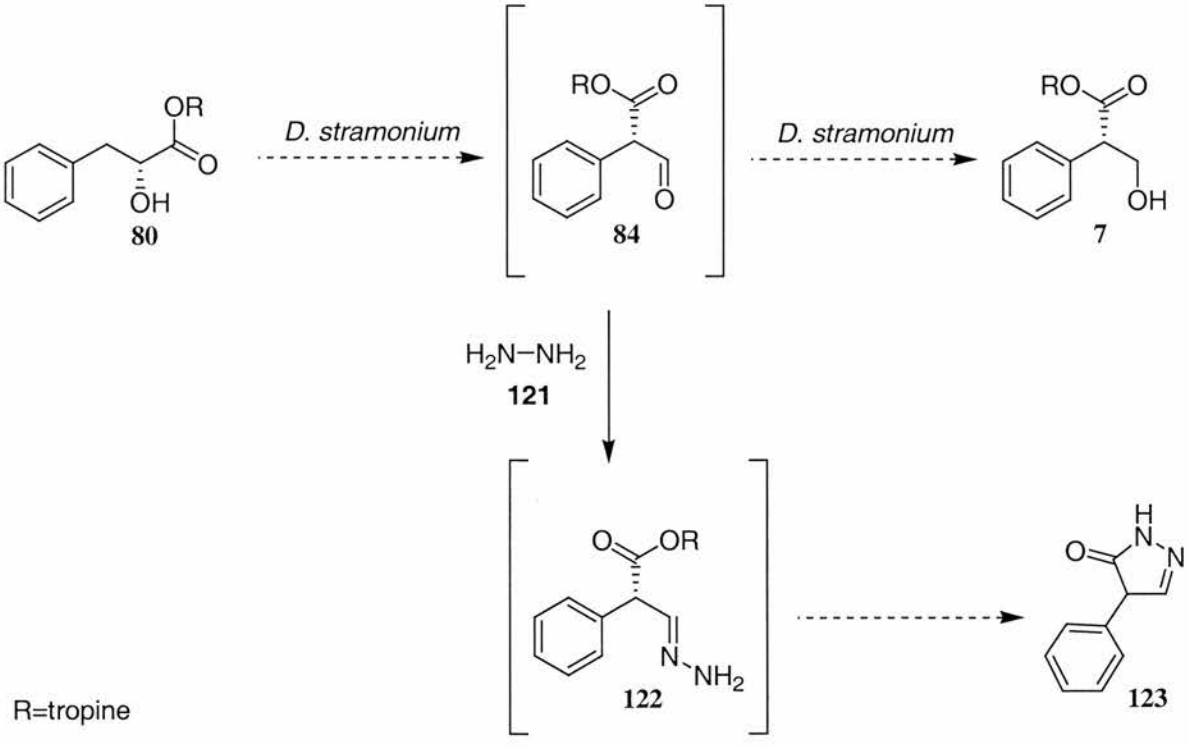


Figure 4.3: The proposed reaction of hydrazine (**121**) with the putative aldehyde intermediate (**84**) to give hydrazone (**122**). The proposed formation of the pyrazone (**123**) is also shown.

There is literature precedent for the intramolecular cyclisation of hydrazones. The reaction of methyl acetoacetate (**101**) with hydrazine (**121**) has been shown[11] to produce 5-methyl pyrazone (**124**), formed by the intramolecular cyclisation of the hydrazone product (**122**) (Figure 4.4). The putative aldehyde intermediate (**84**) is a β-keto ester like methyl acetoacetate (**101**) and so it was proposed that it would similarly undergo an intramolecular cyclisation. The product pyrazone (**123**) is reported to be insoluble in the reaction solvent forming a white crystalline precipitate.

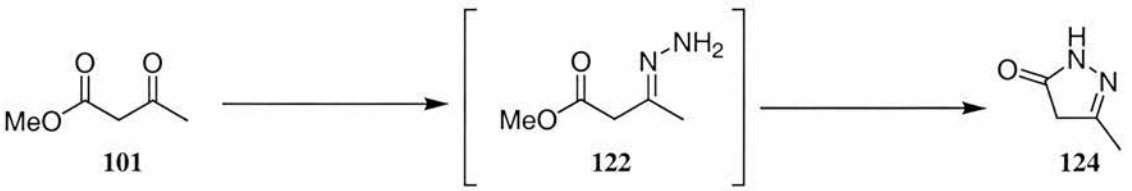
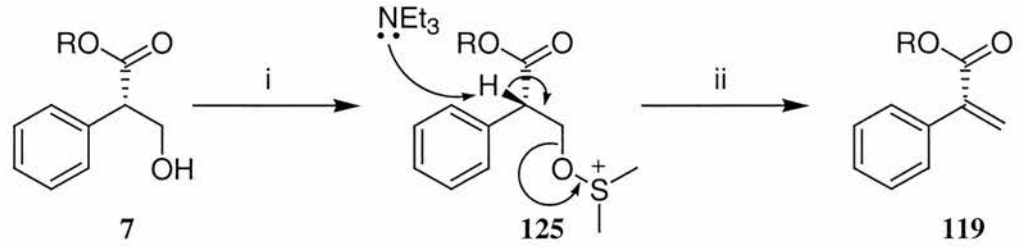


Figure 4.4: The reaction of methyl acetoacetate (**101**) with hydrazine (**121**) to produce 5-methyl pyrazone (**124**) *via* the hydrazone (**122**).

4.5.2 Synthesis of the pyrazone

In order to explore pyrazone (**123**) derivatisation, it was therefore a requirement to have a sample of the putative aldehyde intermediate (**84**) in order to investigate the proposed *in situ* derivatisation by treatment of **84** with hydrazine (**121**). It was hypothesised that **84** could be prepared in a single step from hyoscyamine (**7**) by oxidation of the primary alcohol to give the corresponding aldehyde (**84**).

However, treatment of hyoscyamine (**7**) under Swern oxidation conditions proved inefficient. ^{1}H-NMR spectroscopy of the crude reaction mixture revealed a small aldehyde peak consistent with the formation of the desired product. However, significant olefinic proton resonances were attributed to the formation of apohyoscyamine (**119**). This was confirmed by GC-MS analysis of the crude reaction mixture demonstrating that apohyoscyamine (**119**) was the most abundant product (*m/z* 271). Formation of a sulfonium salt (**125**) under the reaction conditions presumably converts the primary alcohol into a good leaving group, promoting elimination of the activated alcohol (Figure 4.5). In addition treatment of hyoscyamine (**7**) with Dess-Martin periodinane also failed to oxidise the primary alcohol in an efficient manner (t.l.c and crude ^{1}H-NMR analysis).



7 125 119

R=tropine

Figure 4.5: Proposed mechanism of apohyoscyamine (**119**) formation from hyoscyamine (**7**) under Swern oxidation conditions. Reagents and conditions: i, DMSO, oxalyl chloride; ii, NEt_3.

Given the failure to oxidise hyoscyamine (**7**) it was decided to synthesise a more accessible model aldehyde compound. Accordingly, ethyl 3-oxo-2-phenylpropionate (**126**) was chosen as **126** only differs from the putative aldehyde intermediate (**84**) in its ester functionality and has been previously prepared in a single step from commercially available materials (Figure 4.6).[12]

Accordingly, ethyl phenylacetate (**127**) was added to a suspension of sodium hydride in anhydrous toluene to form the corresponding sodium enolate, which was quenched by addition of ethyl formate, followed by aqueous workup. The resultant oil was dissolved in ethanol and treated directly with hydrazine (**121**) (Figure 4.6). A white crystalline precipitate formed, which was collected by filtration then washed with cold ethanol. ^{1}H-NMR spectroscopy of the product revealed that all of the protons were aromatic in character. This can be rationalised by enolisation of the product pyrazone (**123**) to give a pyrazole (**128**) (Figure 4.6).

The product was subsequently re-crystallised from an acetone / hexane mixture giving small white crystals. Despite considerable effort these crystals did not diffract sufficiently to determine the structure by X-ray analysis.

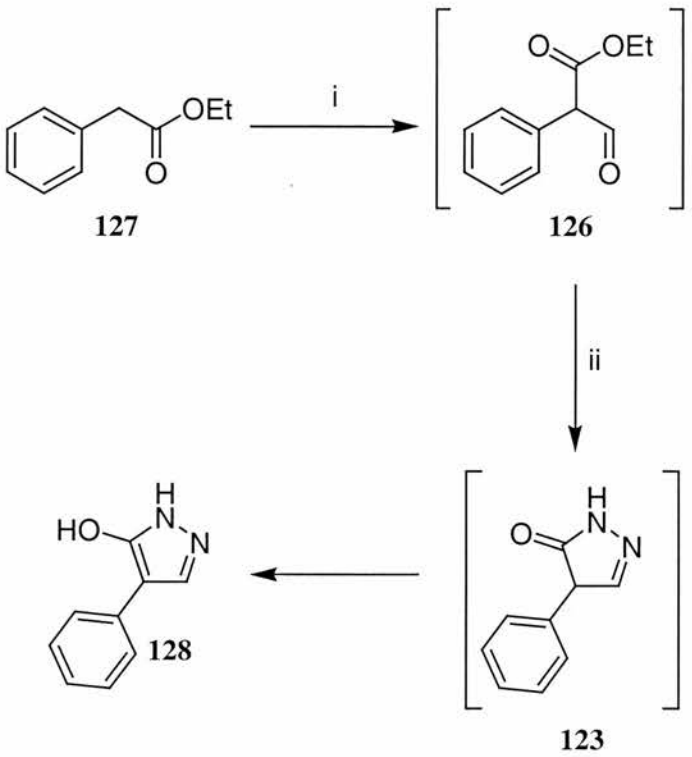


Figure 4.6: Synthesis of pyrazole (**128**). Reagents and conditions: i, NaH, HCOOEt, PhMe, 0-25°C, 24 hrs; ii, N_2H_4, EtOH, 25°C, 30 min.

The enolisation to form the pyrazole (**128**) is presumably favoured due to aromaticity. Such an enolisation is analogous to the imine to enamine conversion in the synthesis of pyrazoles from 1,3-diketones (Figure 4.7).

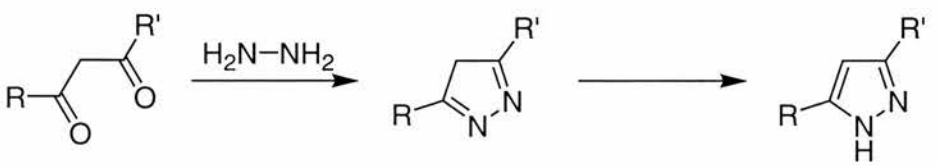


Figure 4.7: The synthesis of pyrazoles from the reaction of a 1,3-diketone and hydrazine (**121**).[13]

4.5.3 Pyrazole assay

It was an absolute requirement of the aldehyde 'trapping' experiment to have a sensitive method for detecting the pyrazole (**128**) in the complex mixtures arising from cell free experiments. Previous investigations in the research programme had used GC-MS as a method of chromatographic separation and analysis. Accordingly a GC-MS method for

the analysis of the pyrazole (**128**) was developed. The high melting point (222-224°C) of the pyrazole (**128**) made it unsuitable for direct GC analysis. Other work in this research programme and within our laboratory group had used trimethylsilyl derivatives of high boiling point compounds to facilitate their GC analysis. Accordingly, 3-hydroxy-4-phenyl pyrazole (**128**) was treated with neat MSTFA (**90**) to produce its TMS derivative (**129**) (Figure 4.8).

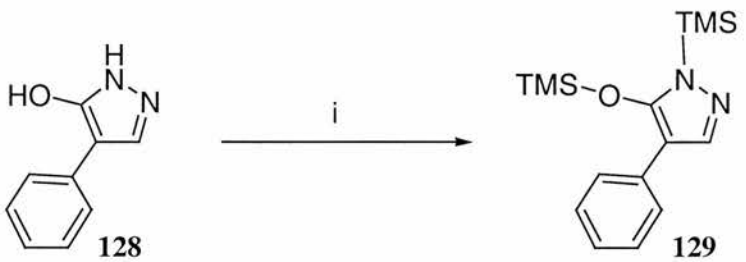


Figure 4.8: Derivatisation of 3-hydroxy-4-phenyl pyrazole (**128**) for GC-MS analysis. Reagents and conditions: i, MSTFA (**90**), 80°C, 50 minutes.

The TMS derivatised pyrazole (**129**) was subsequently subjected to GC-MS analysis and found to give a single sharp peak with *m/z* 304, corresponding to the molecular ion of doubly TMS derivatised pyrazol (**129**).

4.5.4 Experimental considerations for cell free pyrazol formation

Within the research programme all previous cell free investigations had failed to yield discernable littorine mutase activity. For this particular enzyme therefore, a 'standard' cell free preparation was not a suitable experimental model. Instead a crude 'tissue macerate' was used as an experimental system.

The recovery of the pyrazole (**128**) from the tissue macerate was an experimental requisite. Studies with the synthetic pyrazole (**128**) had shown that it was soluble in acetone. However, the use of acetone precludes direct extraction of the pyrazole (**128**) from the aqueous tissue macerate. Therefore, the root macerate was lyophilised prior to acetone extraction.

4.5.5 Preparation of protein extract

Roots were harvested from culture by filtration, suspended in cold phosphate buffer (100 mM) and homogenised by brief pulses in a Waring blender. The resultant macerate containing portions of undamaged root tissue was used directly to perform the hydrazine trapping experiment.

4.5.6 Hydrazine trapping protocol

A series of experiments (Table 4.1) were conducted to assay for the production of the putative aldehyde intermediate (**84**). All of the experiments were incubated at 30°C for 20 hours prior to freeze-drying and extraction into acetone. The organic solvent was filtered and removed under reduced pressure. The resultant crude extract was treated with MSTFA (**90**) and subjected to GC-MS analysis.

	A	B	C	D	E	F
Root macerate	20 mL	20 mL	20 mL	-	-	-
Boiled root macerate	-	-	-	20 mL	20 mL	20 mL
Littorine (**80**)	-	-	50 mM	-	-	50 mM
Hydrazine (**121**)	-	1 mL	1 mL	-	1 mL	1 mL

Table 4.1: Experimental conditions for investigations into the production of the putative aldehyde intermediate (**84**).

4.5.7 Results and discussion

The total ion chromatograms of the assay extracts were investigated by analysis of the ion current *m/z* 304. As expected in the negative controls (experiments A and D) that contained no hydrazine (**121**), there was no detectable ion current for *m/z* 304. However, the remaining experiments (B, C, E and F) all had an ion current of *m/z* 304 at the correct retention time. Closer examination of the mass spectra revealed that the most abundant ion current was *m/z* 305 not *m/z* 304. Therefore, the *m/z* 304 could arise from partial fragmentation of another compound with an identical retention time in the GC. The oven heating profile of the GC method was altered, decreasing the rate of

heating, with the aim of separating the compounds with previously identical retention times. However, the two ion currents *m/z* 304 and *m/z* 305 proved inseparable.

A control experiment was conducted, incubating commercial hyoscyamine (**7**) (Sigma) in buffer with hydrazine (**121**). The control experiment was worked up and analysed in a similar manner to the experiments described above. Again mass analysis revealed overlapping ion currents *m/z* 304 and *m/z* 305. Extraction of ion currents *m/z* 304 and *m/z* 305 revealed that although the abundance peaks elution profiles overlap, the point of greatest abundance is separated by approximately six seconds (Figure 4.9), suggesting two distinct compounds; a compound that could be the pyrazole (**128**) and a second unidentified compound.

```
Operator    : SP
Acquired    : 26 Jul 2001  22:23      using AcqMethod SPEI008
Instrument :   Instrumen
Sample Name: hyo + N2H4
Misc Info   : in MSTFA
Vial Number: 15
```

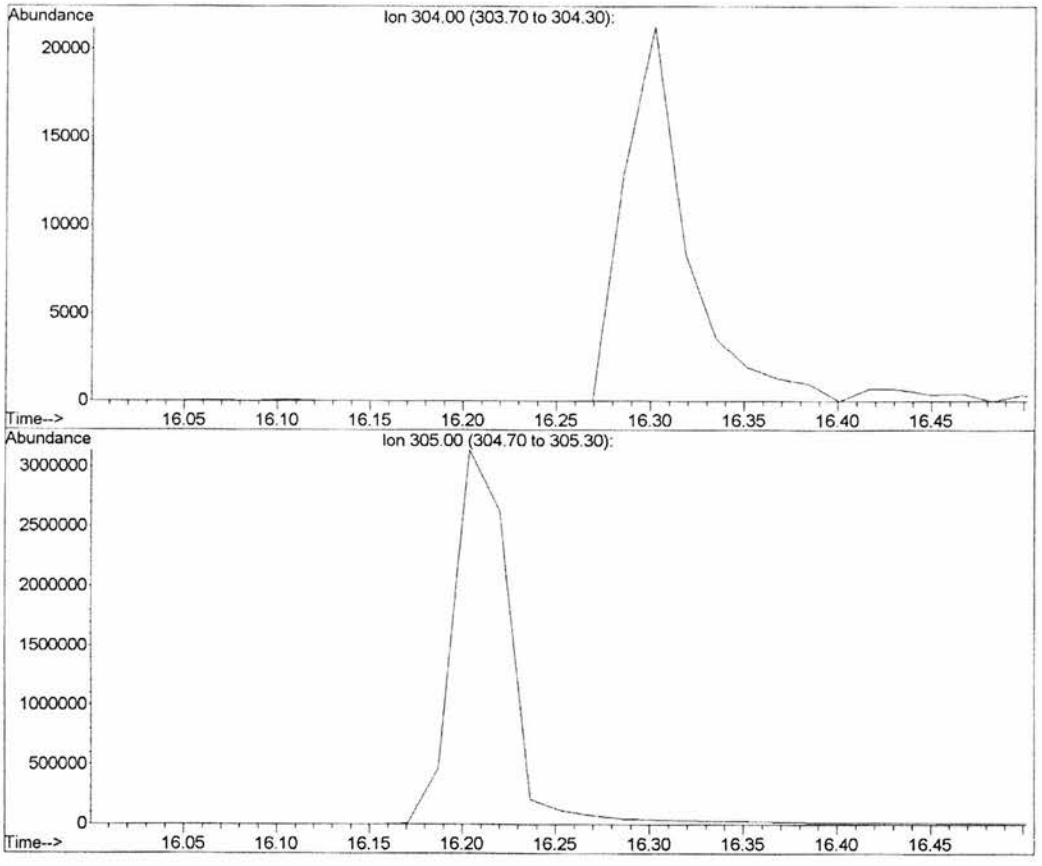


Figure 4.9: Extracted ion chromatograms for *m/z* 304 and *m/z* 305 from the GC-MS analysis of hyoscyamine (**7**) incubated with hydrazine (**121**).

Clearly the pyrazole (**128**) could arise from the reaction of hydrazine (**121**) with a small amount of aldehyde (**84**) present in the commercial sample. Therefore, a further control experiment was conducted replacing the commercial hyoscyamine (**7**) with a synthetic sample of (*RS*)-hyoscyamine prepared as described in section 3.3.1. Additionally, the buffer was also degassed and the 'derivatisation' performed under an atmosphere of nitrogen. GC-MS analysis of the isolated material produced an identical result as the incubation of commercial hyoscyamine (**7**). From the results of this experiment it can be concluded that either the reaction of hyoscyamine (**7**) with hydrazine (**121**) produces pyrazole (**128**) by an unknown process. Or, less likely another explanation of this result is that a compound with a fragmentation ion of *m/z* 304 and an identical retention time to the pyrazole (**128**) is produced in the reaction. The 'positive' results produced by the negative controls prevent any further conclusions being drawn from this series of experiments.

4.5.8 Conclusions

From this work it is not possible to prove or disprove the proposed role of an aldehyde intermediate (**84**) during the isomerisation of littorine (**80**) to hyoscyamine (**7**). Therefore, the mechanism of the littorine mutase remains to be determined.

4.6 The biosynthesis of littorine

4.6.1 Biosynthesis of tropane esters

There are a number of known esters of tropine (**35**) and pseudotropine (**40**) described in the literature (section 1.4.4). Previous investigations[14] into the biosynthesis of tropane esters have demonstrated that a number of esters are biosynthesised from tropine (**35**) or pseudotropine (**40**) and the co-enzyme A thioesters of the esterifying acids. In addition separate feeding experiments with ammonium [1-^{14}C, ^{13}C]benzoate and its *N*-acetylcysteamine thioester in *E. coca* demonstrated that the label was more efficiently incorporated into cocaine (**36**) from the latter.[15] This suggests that a thioester is the natural substrate for the esterification reaction with methyl ecgonine.

Additionally, there is a precedent for the role of co-enzyme A (**130**) thioesters in biological acylation reactions, e.g. acetyl-CoA (**5**) is the substrate for several acetyl transfer enzymes.[16] Clearly littorine (**80**) could be biosynthesised from (*R*)-phenyllactoyl-CoA (**131**) and tropine (**35**) (Figure 4.10). (*R*)-Phenyllactoyl-CoA (**131**) has previously been implicated as a biosynthetic precursor to cinnamoyl-CoA in *Clostridium sporogenes*.[17]

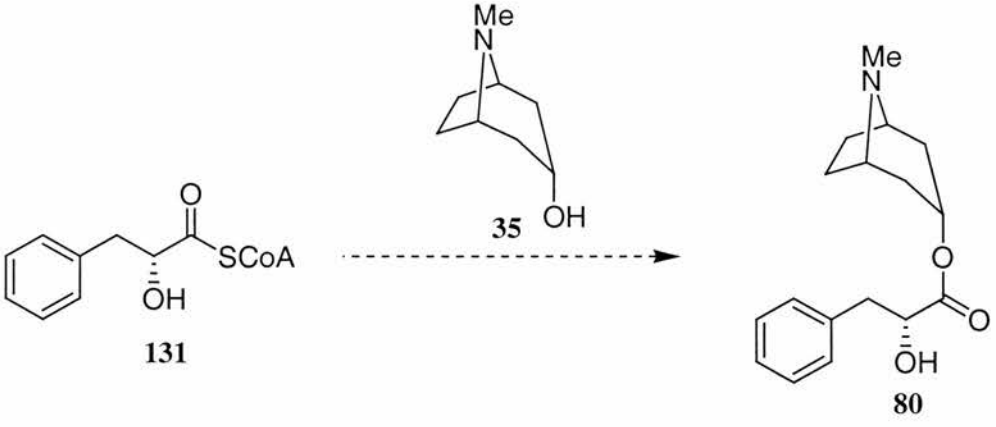


Figure 4.10: The proposed biosynthesis of littorine (**80**) from (*R*)-phenyllactoyl-CoA (**131**) and tropine (**35**).

4.6.2 Preparation of protein extract

Tigloyl-CoA: pseudotropine acyl transferase has been purified 330-fold from transformed root cultures of *D. stramonium*.[18] This published procedure was adjusted accordingly and used in preparation of a cell free extract to assay for littorine (**80**) biosynthesis.

Accordingly, root tissue was recovered by filtration, frozen in liquid nitrogen ground using a pestle and mortar. The resultant powder was mixed with PVPP and suspended in extraction buffer at 4°C for 30 minutes (see experimental for buffer constituents). Cell debris was removed from the extract by filtration followed by centrifugation. The protein extract was concentrated using a 10 kDa exclusion membrane. This concentrated extract was then assayed for its ability to catalyse littorine (**80**) formation (section 4.6.3).

4.6.3 Phenyllactoyl-CoA: tropine acyl transferase assay

The assay conditions used were a modification of the published assay[18] for tigloyl-CoA: pseudotropine acyl transferase. The formation of littorine (**80**) was determined by GC-MS of the TMS derivatised assay extract. The extraction of alkaloid from the assay mixture, derivatisation of the extract and GC-MS analysis, were performed as previously described (see section 4.2.3).

Published assays for acyl transferase enzymes in *D. stramonium* cell free preparations have been performed using coenzyme A esters as administered substrates.[14,18] However, phenyllactoyl-CoA (**131**) is not commercially available. Acetyl-CoA (**5**) is biosynthesised from acetate (**33**), ATP and coenzyme A (**130**) (Figure 4.11).[19] The biosynthesis of phenyllactoyl-CoA (**131**) may occur in an analogous fashion. Therefore, (*R*)-phenyllactic acid (**78**) (4 mM), ATP (5 mM), magnesium ions (2 mM) and coenzyme-A (**130**) (0.5 mM) were administered to the assay mixture. Administering this co-factor mixture may facilitate the *in situ* biosynthesis of (*R*)-phenyllactoyl-CoA (**131**) from phenyllactic acid (**78**). Addition of tropine (**35**) (4 mM) completed the substrate requirements for the assay.

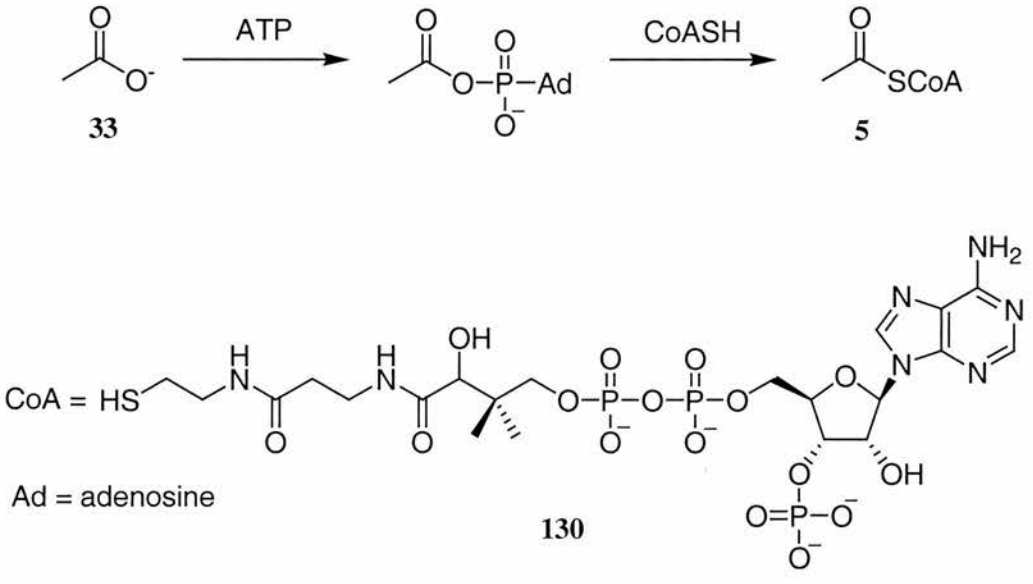


Figure 4.11: The biosynthesis of acetyl-CoA (**5**), catalysed by acetate-CoA ligase.[19] The structure of coenzyme A (**130**) is also shown.

4.6.3.1 Results and discussion

No formation of littorine (**80**) was observed by the GC-MS assay. It was proposed that the *in situ* formation of phenyllactoyl-CoA (**131**) may be insufficient to allow a detectable level of littorine (**80**) biosynthesis to occur. Therefore, it was decided to perform the assay by addition of phenyllactoyl-CoA (**131**) directly.

Accordingly the preparation of phenylactoyl-CoA (**131**) became a requirement of the research programme.

4.6.4 Synthesis of phenyllactoyl-CoA

There are a number of procedures documented in the literature for the chemical synthesis of coenzyme A esters from carboxylic acids.[20,21,22] Generally, the carboxylic acid is activated as an ester,[20,21] prior to treatment with coenzyme A (**130**) and base (Figure 4.12). The methodology used to prepare [1-^{14}C]tropoyl-CoA[20] was chosen to prepare a sample of (*R*)-phenyllactoyl-CoA (**131**) (Figure 4.13).

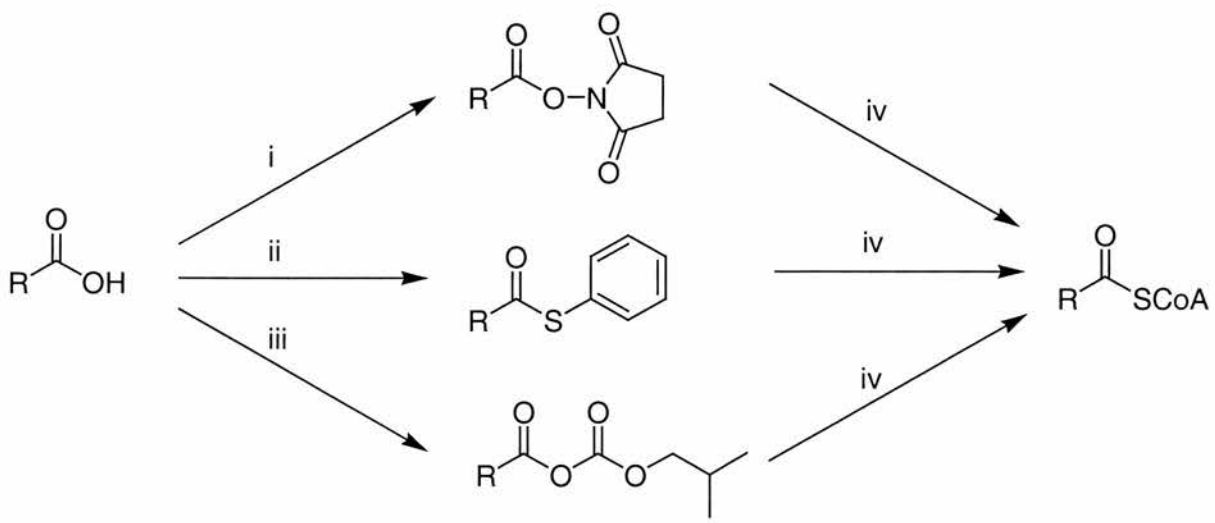


Figure 4.12: Methods for the synthesis of CoA esters.[20,21,22] Reagents: i, NHS, DCC; ii, conversion to acid chloride, thiophenol; iii, triethylamine, isobutyl chloroformate; iv CoASH (**130**), base.

Accordingly, (*R*)-phenyllactic acid (**78**) was activated as its *N*-hydroxysuccinimide ester (**132**) by treatment with DCC and *N*-hydroxysuccinimide. An aliquot of this ester (**132**) was treated with coenzyme A (**130**) and $NaHCO_3$ in a degassed water / acetone mixture to yield (*R*)-phenyllactoyl-CoA (**131**) (Figure 4.13). The product was identified by MALDI-TOF mass spectrometry and administered to the assay mixture without further purification.

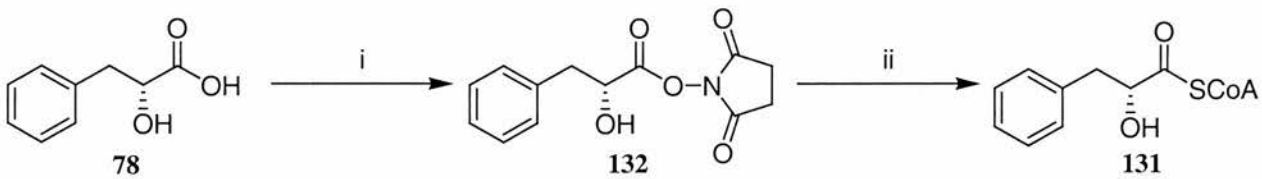


Figure 4.13: Chemical synthesis of (*R*)-phenyllactoyl-CoA (**131**) from (*R*)-phenyllactic acid (**78**). Reagents and conditions: i, DCC, NHS, THF, 25°C, 20 hours; ii, CoASH, $NaHCO_3$, H_2O / acetone.

4.6.5 Repeating phenyllactoyl-CoA: tropine acyl transferase assay

The assay was performed exactly as described above with the exception that phenyllactoyl-CoA (**131**) was used as the administered substrate.

4.6.5.1 Results and Discussions

Again no detectable biosynthesis of littorine (**80**) was observed in the GC-MS assay.

The failure to detect phenyllactoyl-CoA: tropine acyl transferase activity may be the result of hydrolysis of the coenzyme A ester (131) *in vitro*, producing insufficient substrate for the detection of enzyme activity. Alternatively, the constitutive expression level of the enzyme in the root tissue may be so low as to render enzyme activity undetectable, despite concentration of the cell free extract. In addition, the presence of a competing esterase activity may result in the hydrolysis of product littorine (**80**) preventing its accumulation to a detectable level.

4.7 Chapter summary

Attempts have been made to assay for the presence of reaction intermediates and enzyme activities implicated in the biosynthetic pathway of hyoscyamine (**7**). However, all attempted assays proved unsuccessful. Therefore, the enzymology of hyoscyamine (**7**) formation and its underlying mechanism remains to be determined.

4.8 References for chapter 4

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Chapter 5
Experimental

5 EXPERIMENTAL

5.1 General

NMR spectra were recorded on a Varian Mercury 200 MHz (^{1}H at 199.99 MHz, ^{13}C at 50.29 MHz), Varian Unity Plus 300 MHz (^{1}H at 299.91 MHz, ^{13}C at 75.41 MHz), Brucker Avance 300 MHz (^{1}H at 300.06 MHz, ^{13}C at 74.45 MHz), Varian VXR-400(S) (^{13}C at 100.57 MHz) and Varian Inova 500 MHz (^{13}C at 125.67 MHz) spectrometers. Chemical shifts are reported in parts per million quoted relative to residual $CHCl_3$ (δ_H 7.27), TMS (δ_H 0.00) or $CDCl_3$ (δ_C 77.00) in $CDCl_3$, residual H_2O (δ_H 2.60) in D_2O and CD_3OD (δ_C 49.15) in CD_3OD. Coupling constants are given in Hertz (Hz). All ^{13}C-NMR spectra are proton decoupled, with multiplicities resulting from ^{2}H-^{13}C or ^{13}C-^{13}C coupling.

Infra red analyses were recorded with using a Perkin Elmer 2000 FT-IR, absorbance values are given in cm^{-1}. Melting points were recorded on a GallenKamp GRIFFIN MPA350.BM2.5 melting point apparatus and are uncorrected.

GC–MS analyses of synthetic samples were conducted using an Agilent 5890 plus gas chromatograph equipped with a 5973N mass selective detector and 7683 series injector. Chromatographic separations were performed using an Agilent HP 19091S-433 (5% phenyl methyl siloxane, 30 m×250 μm with a film thickness of 0.25 μm). The carrier gas was helium, with a flow rate of 63.5 mL min^{-1} with a split ratio of 50:1. The injection volume was 1 μL and the injection port temperature 250°C. Mass enrichments were calculated by comparison with unlabelled synthetic standards.

Analytical and preparative thin layer chromatography separations were performed on Whatman K6F silica gel 60 Å, 0.25 mm layer thickness glass backed plates with fluorescent indicator. Compounds were visualised using a UV lamp or chemical stains. Column chromatography was carried out over silica gel, Merck, Kieselgel 60, 230-400 mesh). Petrol refers to the 40-60°C boiling fraction of petroleum ether and was redistilled before use.

Where anhydrous conditions were required reaction glassware was flame or oven dried (180°C) and allowed to cool under vacuum and the reactions conducted under an atmosphere of dry nitrogen or argon.

Reaction solvents were dried and distilled prior to use: dichloromethane (calcium hydride), THF (sodium, benzophenone), toluene (sodium), methanol (magnesium, iodine) and ethanol (magnesium, iodine).

Manipulations with *D. stramonium* root culture were carried out in a Gallenkemp flowhood, the internal surfaces of which were washed with 70% (v/v) ethanol.

Centrifugation was performed using a Beckman J2-21 M/E centrifuge fitted with a JA-20 rotor. Ultra-centrifugation was conducted using a Beckman L-60 ultracentrifuge fitted with a type 42.1 rotor.

Aqueous solutions and biological material was reduced *in vacuo* using a FTS systems flexi-dry freeze dryer.

Protein column chromatography was performed using an ÄKTA Prime FPLC system (Amersham Pharmacia Biotech).

Chemicals were obtained from commercial suppliers and isotopically enriched chemicals were obtained from Promochem Ltd except where otherwise indicated.

5.2 Culturing procedures

5.2.1 Plant material

Transformed root cultures of D. stramonium D15/5 were provided by Dr. Richard J. Robins, CNRS, Université de Nantes.

5.2.2 Growth and maintenance of *D. stramonium*[1]

Transformed root cultures of *D. stramonium* D15/5 were grown and maintained in Gamborg's B50 basal medium (Gamborg's B5 basal medium with minimal organics powder (3.2 gL^{-1}) (Sigma) and sucrose (30 gL^{-1}), adjusted to pH 5.8 with 0.1 M NaOH). Cultures were grown in Erlenmeyer flasks (250 mL) containing aliquots of sterile media (50 mL), at 27°C, under constant illumination, with lateral agitation (100 rpm). Media was sterilised in aliquots (50 mL) by autoclaving. The culture media was stored for a minimum of seven days prior to use to ensure it was free of bacterial or fungal contamination.

Subcultures were inoculated with root material (0.5-0.6 g) from cultures between 2 and 4 weeks old. Young, pale roots with several off shoots were selected preferentially for the inoculum. All manipulations were carried out under aseptic conditions.

5.3 Isolation and analysis of hyoscyamine

5.3.1 Isolation and purification of hyoscyamine (7)[2,3]

The root mass was removed from the media by filtration, and washed with distilled water to remove residual media. The roots were then dried between tissue paper, quickly frozen by submersion in liquid nitrogen and freeze-dried for 48 hours. The dried roots were ground with sand in a pestle and mortar to produce a fine powder, which was stirred in 50 mM sulfuric acid (20 mL g^{-1} dry mass) for 20 minutes, the suspension was then basified by the addition of 35% ammonia. The root macerate was then immediately filtered through glass wool onto 50 mL Hydromatrix (Varian) columns (10 mL solution per column) and the extract allowed to soak into the solid phase. The glass wool and root material was then removed and the column eluted with chloroform : methanol (20:1, 105 mL). The solvent was evaporated under reduced pressure to yield an orange-brown gum (typically 4 mg per root culture).

Hyoscyamine (**7**) was further purified by preparative t.l.c. (19:1 chloroform : diethylamine). The alkaloids were visualised by u.v. by use of Dragendorff's spray reagent (Sigma). Hyoscyamine (**7**) was identified by comparison with a commercial sample (Sigma). The silica containing hyoscyamine (**7**) was removed from the glass plate and extracted with dichloromethane (4×25 mL). The combined organics were evaporated under reduced pressure to yield hyoscyamine (**7**) as an orange gum (typically 0.5-1 mg per root culture).

^{1}H-NMR (200 MHz) ($CDCl_3$): 7.36-7.28 (5H, m, aromatics), 5.04 (1H, t, $^3J_{HH}$ = 5.1, C3-H), 4.23-4.11 (1H, m, C3´-Hα), 3.88-3.74 (2H, m, C3´-Hβ, C2´-H), 3.13-3.02 (1H, m, C1/C5-H), 2.97-2.89 (1H, m, C1/C5-H), 2.21 (3H, s, NCH_3), 2.2-0.8 (8H, m, C2-H_2, C4-H_2, C6-H_2, C7-H_2).

5.3.2 GC-MS analysis of alkaloid samples

Prior to GC analysis the alkaloid extract was dissolved in chloroform (500 μL) and treated with MSTFA (200 μL) at 80°C for 50 min to yield the trimethylsilyl ether derivatives of littorine (**91**) and hyoscyamine (**92**).

GC-MS analysis of the alkaloid extract was performed as above except chromatographic separations were conducted using a SGE fused-silica wall-coated open tubular capillary column (25 m×0.22 mm) with Cydex-B (0.25 μm film thickness) as the bonded phase. The GC-MS oven-heating programme was 150°C to 260°C at a rate of 10°C min^{-1}, then to 275°C at a rate of 3°C min^{-1}, the temperature was then kept stationary at 275°C for 1 minute. The analyses were performed with a flow rate of 64.8 mL min^{-1}, in splitless mode.

The MSD was operated in SIM mode to observe ion currents *m/z* 361 (molecular ion, M), *m/z* 362 (M+1) etc.

5.3.3 HPLC separation of alkaloids

HPLC analyses were performed using a Varian 9012 solvent delivery system equipped with a Varian 9050 variable wavelength UV-VIS detector. Chromatographic separations were performed using a Hypersil 5 μm C-18 column (250×4.6 mm, Macherey-Nagel).

The flow rate was 1mL min^{-1}, the run time 20 minutes, the solvent mix was of 80:20 water : acetonitrile (+0.01% TFA).

5.4 Feeding protocols

5.4.1 General

Putative substrates were fed as a filter (0.22 μm) sterilised solution in methanol or water on day 6 or 7 after sub culture. The alkaloids were extracted from the root cultures on day 17 or 18 after sub culture as described in section 5.3.1 above.

5.4.2 Feeding sodium [1-^{13}C]acetate (33e)

Sodium [1-^{13}C]acetate (299 mg) was fed as a solution in water (18 mL) to nine root cultures of *D. stramonium* at a concentration of 8 mmol dm^{-3} in the media. Hyoscyamine (4 mg) was isolated and studied by ^{13}C-NMR.

5.4.3 Feeding sodium [2-^{13}C]acetate (33f)

Sodium [2-^{13}C]acetate (299 mg) was fed as a solution in water (18 mL) to nine root cultures of *D. stramonium* at a concentration of 8 mmol dm^{-3} in the media. Hyoscyamine (5 mg) was isolated and subjected to ^{13}C-NMR spectroscopy.

5.4.4 Feeding sodium [1,2-$^{13}C_2$]acetate (33a)

A mixture of sodium [1,2-$^{13}C_2$]acetate (76 mg) and unlabelled sodium acetate (**33**) (221 mg) was fed as a solution in water (18 mL) to nine root cultures of *D. stramonium* at a final concentration of 2 mmol dm^{-3} and 6 mmol dm^{-3} in the media. Hyoscyamine (4 mg) was isolated and studied by ^{13}C-NMR spectroscopy.

5.4.5 Feeding sodium [$^{18}O_2$]acetate (33g)

Sodium [$^{18}O_2$]acetate (103 mg) was fed as a solution in water (6 mL) to three root cultures of *D. stramonium* at a concentration of 8 mmol dm^{-3} in the media. The crude alkaloid extract (23 mg) was derivatised and subjected to GC-MS analysis.

5.4.6 Feeding (*RS*)-[2,4,4-$^2H_3$]glutamic acid (95a)

(*RS*)-[2,4,4-$^2H_3$]Glutamic acid (270 mg) was fed as a solution in water (9 mL) to nine root cultures of *D. stramonium* at a concentration of 4 mmol dm^{-3} in the media. Hyoscyamine (5 mg) was purified as described above (section 5.3.1) and subjected to GC-MS analysis. The feeding experiment was repeated feeding to eight flasks at 4 mmol dm^{-3} and the resultant crude alkaloid extract (52 mg) was analysed directly by GC-MS. Dr J.T.G. Hamilton, School of Agriculture and Food Science, The Queen's University of Belfast performed the GC-MS analyses.

5.4.7 Feeding *N*-methyl[2-^{2}H]pyrrolinium chloride (58d)

N-Methyl[2-^{2}H]pyrrolinium chloride (**58d**) was administered as an ethanol/water mixture (3 mL) to three transformed root cultures of *D. stramonium* at a final concentration of 1 mmol dm^{-3} in the medium. After the growth period of the roots the crude alkaloid extract (26 mg) derivatised and analysed by GC-MS

5.4.8 Feeding (*RS*)-[2-^{2}H]phenyllactic acid (78f)

(*RS*)-[2-^{2}H]phenyllactic acid (**78f**) (8.4 mg) was fed as a solution in water/methanol (4 mL) to four root cultures of *D. stramonium* at a concentration of 0.25 mmol dm^{-3} in the media. The crude alkaloid extract (15 mg) was derivatised and subjected to GC-MS analysis.

5.4.9 Feeding (*RS*)-[3,3-$^2H_2$]phenyllactic acid (78g)

(*RS*)-[3,3-$^2H_2$]phenyllactic acid (**78g**) (8.4 mg) was fed as a solution in water/methanol (4 mL) to four root cultures of *D. stramonium* at a concentration of 0.25 mmol dm^{-3} in the media. The crude alkaloid extract (16 mg) was derivatised and subjected to GC-MS analysis.

5.4.10 Feeding (*RS*)-[2,3,3-$^2H_3$]phenyllactic acid (78h)

(*RS*)-[2,3,3-$^2H_3$]phenyllactic acid (**78h**) (6.3 mg) was fed as a solution in water/methanol (3 mL) to three root cultures of *D. stramonium* at a concentration of

0.25 mmol dm^{-3} in the media. The crude alkaloid extract (13 mg) was derivatised and subjected to GC-MS analysis.

5.4.11 Incubating (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (7o)

Twelve subcultured flasks of roots were fed a solution of (*RS*)-[3′, 3′-$^2H_2$]hyoscyamine (**7o**) (54 mg) in methanol/ water (6 mL). Root cultures were then incubated for a further 4, 8, or 12 days (four each) before alkaloid extraction. The alkaloid fractions were derivatised and subjected to GC-MS analysis.

5.4.12 Incubating (*RS*)-[1′-^{13}C, 3′, 3′-$^2H_2$]hyoscyamine (7p)

Fifteen subcultured flasks of roots were fed a solution of (*RS*)-[1′-^{13}C, 3′, 3′-$^2H_2$] hyoscyamine (**7p**) (68 mg) in methanol/ water (7.5 mL). The root cultures were then incubated for a further 6, 9, 12, 15 or 18 days (three each) before alkaloid extraction. All root cultures were freeze-dried separately prior to individual alkaloid extraction, derivatisation and GC-MS analysis.

5.5 Preparation of cell free extracts

5.5.1 General

All procedures were conducted at 4°C using pre-chilled laboratory apparatus. Roots grown in culture for 14-17 days were selected preferentially for the preparation of cell free extracts. Roots were harvested from culture by filtration, washed with distilled water and blotted dry prior to further homogenisation.

5.5.2 Determination of protein concentration

Protein concentration was determined by the dye-binding method of Bradford.[4] The assay solution was prepared by dissolving Coomassie Brilliant Blue G-250 (600 mg L^{-1}) in 2% perchloric acid, followed by filtering to remove undissolved dye. The assay solution was stored at 4°C in a brown glass bottle and allowed to warm to room temperature prior to use. The assay was performed by mixing dye solution (750 μL) with a known dilution of the protein extract (750 μL). After incubation (5 minutes) the absorbance values (λ 595 nm) were measured using a spectrophotometer. The protein concentration was then calculated by comparison with a standard curve plotted from measurements with known concentrations with BSA.

5.5.3 Preparation 1 SAM-dependent activity[5]

The root tissue was selected and isolated as described above (5.5.1). The root tissue (~10 g) was homogenised with acid washed sand and buffer A (20 mL) in a pestle and mortar for 30 minutes. The crude slurry was then filtered through 3 layers of muslin, and remaining cell debris removed from the filtrate by centrifugation (30,000×g, 4°C, 30 minutes). The protein extract (17 mL) was then passed down a Hi-Trap™ desalting column (Amersham Biosciences), equilibrated with 10% (v/v) buffer A and the proteins eluted using the same buffer. The protein containing fractions were pooled and concentrated to 3 mL using an ultrafiltration device (Amicon) fitted with a 10 kDa exclusion membrane under a positive pressure of N_2. This extract was assayed for littorine mutase activity as described below with the addition of SAM (200 μM).

Buffer A: 100 mM K_2HPO_4 (pH 8), 3 mM DTT, 5 mM EDTA Na_2, 250 mM sucrose.

5.5.4 Littorine mutase assay

To perform the cell free assay the protein preparations (0.5-2 mL) were heated to 33°C in a water bath with the appropriate additions and littorine (2 mM). After the incubation time 0, 30, 60, 90, 120 minutes or overnight, the assay mixture was rapidly cooled, basified by the addition of ammonia (35%) and extracted into chloroform or DCM (10 mL). The organic solvent was then removed under reduced pressure and the crude gum redissolved in chloroform (500 μL) and MSTFA (200 μL). After heating at 80°C for 1 hour the product was analysed by GC-MS.

The GC-MS assay was carried out as described for the analysis of the alkaloid fraction isolated from *D. stramonium* root cultures (section 5.3.2).

GC analyses were conducted using a Hewlett Packard 5890A gas chromatograph equipped with a flame ionisation detector coupled to a Spectra Physics SP4290 integrator. The injector temperature was 250°C and the detector 275°C. Chromatographic separations were performed using a chiral SGE column using the heating profile described for GC-MS analysis above (section 5.3.2). The carrier gas was nitrogen and the injection volume 0.5 μL.

5.5.5 Preparation 2 oxidation of hyoscyamine

The root tissue was selected and isolated as described above (5.5.1). The cell masses from six root cultures (~30 g) were harvested by filtration, washed with distilled water and blotted dry. The roots were then frozen in liquid nitrogen and ground in a pre-chilled pestle and mortar with acid washed sand, PVPP (10 g), and buffer B (60 mL) and allowed to stand for 30 minutes. The resultant slurry was filtered through muslin and clarified by centrifugation (10,000×g, 4°C, 30 minutes).

Buffer B: 100 mM K_2HPO_4 (pH 7.5), 20% glycerol, 5 mM DTT, 0.05% cysteine, 10 mM β-mercaptoethanol, 5 μM FAD, 5 μM FMN, 50 mM sodium ascorbate.

5.5.6 Dehydrogenase assay

In a cuvette (1.5 mL) was added 0.2 M Tris-HCl (1.3 mL, pH 7.3), NAD or NADP (50 μL of 6.6 mM) and hyoscyamine (**7**) (50 μL of 30 mM). This mixture was incubated for 5 minutes before the addition of 100 μL of cell free extract. The absorbance at 340 nm was recorded over 10 minutes. No change was observed.

5.5.7 Preparation 3^6 initial studies

The root tissue was selected and isolated as described above (section 5.5.1). The root tissue (~50 g) from 6 culture flasks was wrapped in aluminium foil and frozen with liquid N_2, then homogenised in a pestle and mortar with acid washed sand. The frozen cell homogenate was then suspended in buffer C (100 mL) and mixed with PVPP (10 g). The suspension was then left for 30 minutes with occasional stirring. The crude homogenate was then passed through three layers of muslin and clarified by centrifugation (11,000×g, 4°C, 30 minutes). This cell free homogenate was used as the crude enzyme preparation and assayed by the addition of littorine (2 mM).

Buffer C: 100 mM K_2HPO_4 (pH 7.8), 3 mM DTT, 200 mM sucrose.

5.5.8 Preparation 4^7 improved studies

The root tissue was selected and isolated as described above (5.5.1). The cell masses from four root cultures (~25 g) were harvested by filtration, washed with distilled water and blotted dry. The roots were then frozen in liquid nitrogen and ground in a pre-chilled pestle and mortar with acid washed sand, PVPP (8 g), and buffer D (50 mL) and allowed to stand for 30 minutes. The resultant slurry was filtered through muslin and clarified by centrifugation (10,000×g, 4°C, 30 minutes). The protein extract was concentrated using an ultrafiltration device (Amicon) fitted with a 10 kDa exclusion membrane under a positive pressure of N_2. This concentrated extract was assayed for littorine production in the presence of several co-factors:NADPH (0.5 mM), NADP (0.5 mM),

Buffer D: 100 mM K_2HPO_4 (pH 7.5), 20% glycerol, 5 mM DTT, 0.05% cysteine, 10 mM β-mercaptoethanol, 5 μM FAD, 5 μM FMN, 50 mM sodium ascorbate, 1 mM PMSF.

5.5.9 Microsome preparation

Protein extract from section 5.5.8 was centrifuged (200,000×g, 4°C, 70 minutes). The pellet was resuspended in buffer D plus 0.1% (v/v) triton X-100.

5.5.10 Preparation of root macerate for 'hydrazine trapping' experiment

Five root cultures (fresh weight ~55 g) were blended in 110 mL of phosphate buffer (100 mM, pH 7.5) giving 120 mL of crude extract.

5.5.11 Pyrazole formation assay

The crude extract was assayed with the additives described in table 4.1. After incubation overnight the extract was rapidly frozen in liquid nitrogen and lyophilised. The resultant solid residue was ground with a spatula and suspended in acetone (50 mL) with stirring. The acetone was removed under reduced pressure and the resultant gum treated with MSTFA (200 μL) at 80°C for 1 hour prior to GC-MS analysis.

5.5.12 Cell free preparation 4^8 phenyllactoyl-CoA: tropine acyl transferase

Root tissue was isolated from culture as described above (section 5.5.1). The root tissue (~25 g) from 5 culture flasks was wrapped in an aluminium foil parcel and frozen with liquid N_2. The frozen roots were homogenised in a pestle and mortar with acid washed sand and insoluble PVPP (3 g). The powdered roots were suspended in 55 mL of Buffer E for 30 minutes. The crude homogenate was then passed through three layers of muslin and clarified by centrifugation (15,000×g, 4°C, 30 minutes). The cell free extract was concentrated by ammonium sulfate fractionation. Accordingly the 30% cut was made by addition of 9.7 g ammonium sulfate, with stirring for 20 minutes. The solution was then centrifuged (17,700×g, 4°C, 30 minutes). The supernatant was further saturated to 80% by addition of 19.3 g of ammonium sulfate, and the protein

precipitated by centrifugation (17,700×g, 4°C, 30 minutes). The pellet was resuspended in buffer F and desalted by passage through a Hi-Trap™ column eluted with buffer F and assayed for its ability to catalyse the formation of littorine (section 5.5.13).

Buffer E: 200 mM K_2HPO_4 (pH 7.0), 3 mM DTT, 20 mM disodium EDTA, 125 mM sucrose.

Buffer F: 50 mM K_2HPO_4 (pH 7.0), 3 mM DTT, 5 mM disodium EDTA, 125 mM sucrose.

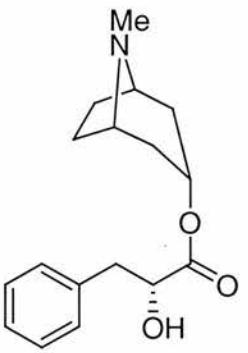
5.5.13 Phenyllactoyl: tropine acyl transferase assay[8]

The assay buffer (total volume 500 μL) contained cell free extract (250 μL), tropine (4 mM), and phenyllactic acid (4 mM), ATP (5 mM), CoASH (0.5 mM), $MgCl_2$ (2 mM) or the crude phenyllactoyl-CoA mixture (1.4 mg). The assay mixture was incubated at 30°C overnight and analysed by GC-MS using the usual alkaloid analysis method. Assays in 'neat' cell free extract were also performed.

Assay buffer: 300 mM glycine buffer (pH 9).

5.6 Synthetic procedures

5.6.1 Littorine ((*R*)-phenyllactoyltropine) (80) [9]



80

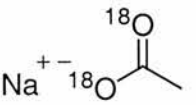
(*R*)-Phenyllactic acid (**78**) (990 mg, 6.0 mmol) was added to tropine (**35**) (600 mg 4.3 mmol) and heated with stirring to 145°C under a dry nitrogen atmosphere. A stream of dry hydrochloric acid gas was then passed intermittently over the mixture with stirring for 3.5 hours. After cooling the mixture was dissolved in 50 mM sulfuric acid (20 mL) with gentle heating, basified with 35% ammonia and extracted into chloroform (4×50 mL). The organics were combined, dried over $MgSO_4$, filtered, and removed under reduced pressure to yield a thick orange oil. The mixture was purified by column chromatography over silica ($CHCl_3$: EtOH : 35% NH_3, 7:7:0.2)[10] to give an orange gum (980 mg, 80%). The TMS derivative (**91**) was synthesised by treatment with MSTFA for GC-MS analysis.

^{1}H-NMR (200 MHz) ($CDCl_3$): δ 7.30-7.22 (5H, m, aromatics), 5.05 (1H, t, $^3J_{HH}$=5.2, C3-H), 4.40 (1H, dd, $^3J_{HH}$=5.7, C2´-H), 3.30-2.23 (2H, m, C1-H, C5-H), 3.08 (2H, ddd, $^3J_{HH}$=5.7, $^3J_{HH}$=6.9, $^2J_{HH}$=31, C3´-H_2), 2.29 (3H, s, NCH_3), 2.1-1.25 (8H, m, C2-H_2, C4-H_2, C6-H_2, C7-H_2).

^{13}C-NMR (50 MHz) ($CDCl_3$): δ 173.3 (C1´), 136.4 (Ca), 129.4 (Cb), 128.6 (Cc), 126.8 (Cd), 71.5 (C2´), 68.9 (C3), 59.7 (C1, C5), 40.6 (NCH_3), 40.1 (C3´), 36.3 (C2/C4), 36.2 (C2/C4), 25.4 (C6/C7), 25.3 (C6/C7).

GC-MS (EI, 70eV): 361 (M^+, 7%), 193 (4%), 140 (5%), 124 (100%), 94 (10%), 82 (11%), 73 (13%).

5.6.2 Sodium [1,1-$^{18}O_2$]acetate (33g)[11]



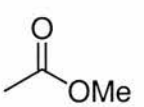
33g

To a solution of [$^{18}O_2$]acetic acid (100 mg, 1.56 mmol) in water (1 mL) was added a solution of sodium hydroxide (62.5 mg, 1.56 mmol) in water (125 μL), at 0°C. After 5 minutes stirring the solution was frozen and the solvent removed by freeze-drying to give a white solid (33 g) (134 mg, 100%). A sample (2 mg) was treated with 18-crown-6 (1 mg) and 2-bromo-4′-phenacetophenone (10 mg) in toluene : acetonitrile (2 mL) for 20 hours at 80°C to yield the *p*-phenylphenacyl-derivative,[12] which was subjected to GC-MS analysis.

GC-MS (EI): 258 (M^+, 4%), 181 (100%), 152 (45%).

This ratio has been corrected for natural abundance and demonstrates an isotope content of 6% ^{18}O and 91% $^{18}O_2$ in the sample.

5.6.3 Methyl acetate (100)



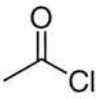
100

A suspension of sodium acetate (**33**) (500 mg, 6.1 mmol) in dimethyl sulfate (1.73 mL, 18.3 mmol) was heated to 160°C under nitrogen. Distillation of the reaction mixture afforded methyl acetate (**100**) (410 mg, 91%) as a colourless liquid.

^{1}H-NMR (200 MHz) ($CDCl_3$): δ 3.43 (3H, s, $OC\underline{H}_3$), 1.86 (3H, s, $C\underline{H}_3$).

^{13}C-NMR (125 MHz) ($CDCl_3$): δ 170.9 (C-1), 51.0 ($O\underline{C}H_3$), 20.0 (C-2).

5.6.4 Acetyl chloride (102)[11]

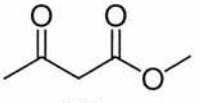


102

A suspension of sodium acetate (**33**) (500 mg, 6.1 mmol) in benzoyl chloride (2.97 mL, 25.6 mmol) was heated to 160°C under nitrogen. Distillation of the reaction mixture afforded acetyl chloride (**102**) (292 mg, 61%) as a colourless liquid.

^{1}H-NMR (200 MHz) ($CDCl_3$): δ 2.64 (3H, s, $C\underline{H}_3$).

5.6.5 Methyl acetoacetate (101)[11]



101

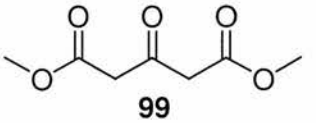
Method A: Methyl actetate (**100**) (500 mg, 6.76 mmol) was added to a suspension of sodium hydride (60% in oil, 300 mg, 7.5 mmol) in THF (25 mL). The reaction was refluxed for 20 hours. After cooling water (10 mL) was added followed by dilution by addition of diethyl ether (10 mL). The aqueous layer was separated and the pH adjusted to 5-6 by addition of 1M HCl. The aqueous was extracted with ether (4x20 mL), the organic layers combined, dried over magnesium sulfate, filtered and the solvent removed under reduced pressure to give a pale yellow oil (146 mg, 38%).

^{1}H-NMR (200 MHz) ($CDCl_3$): 3.70 (3H, s, $OC\underline{H}_3$), 3.46 (2H, s, $C\underline{H}_2$) 2.26 (3H, s, $C\underline{H}_3$).
^{13}C-NMR (125 MHz) ($CDCl_3$): 200.5 (C-3), 167.5 (C-1), 52.3 ($O\underline{C}H_3$), 49.8 (C-2), 30.1 (C-4)

Method B: n-BuLi (1.6 M in hexanes, 4.6 mL, 7.36 mmol) was added to a solution of hexamethyldisilazane (1.2 g, 7.45 mmol) in THF (20 mL) at 0°C and allowed to stir for 15 minutes. The reaction was then cooled to –45°C followed by the addition of methyl acetate (**100**) (250 mg, 3.38 mmol). After a further 30 minutes stirring acetyl chloride

(**102**) (265 mg, 3.38 mmol) in THF (3 mL) was added slowly. The reaction was stirred for 90 minutes and the temperature allowed to rise to 0°C. The work up procedure was as for method A above. The product was recovered as a yellow oil (131 mg, 33%).

5.6.6 Dimethyl 1,3-acetonedicarboxylate (99)[11]

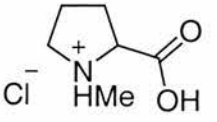


99

n-BuLi (1.6 M in hexanes, 4.2 mL, 6.72 mmol) was added to a solution of diisopropylamine (675 mg, 6.68 mmol) in THF (20 mL) at 0°C and allowed to stir for 15 minutes. The temperature was then reduced to –45°C. Methyl acetoacetate (**101**) (250 mg, 2.16 mmol) in THF was then added slowly over 10 minutes and the mixture allowed to stir for a further 30 minutes. To this was added dimethyl carbonate (215 mg, 2.39 mmol) in THF (2 mL) over 15 minutes. The resultant mixture was stirred for 90 minutes and the temperature allowed to rise to 0°C. Water (10 mL) was added, followed by dilution with diethyl ether (10 mL). The aqueous layer was separated and the pH adjusted to 5-6 by the addition of 1 M HCl (aq). The organic was extracted with diethyl ether (4x20 mL), the organics combined, dried over magnesium sulfate, filtered and the solvent removed under reduced pressure to give a dark orange oil (Yield (by NMR) 16%, 60 mg).

^{1}H-NMR (200 MHz) ($CDCl_3$): 3.73 (6H, s, $OC\underline{H}_3$), 3.61 (4H, s, $C\underline{H}_2$).
^{13}C-NMR (100 MHz) ($CDCl_3$): 195.2 (C3), 167.1 (C1), 52.4 (C2), 48.6 (OCH_3).

5.6.7 (*RS*)-*N*-Methylproline hydrochloride (108)[13]



108

To (*RS*)-proline (**107**) (230 mg, 2 mmol) in water (15 mL) at 0°C was added 90% formic acid (112 µL, 2.2 mmol) and 37% formaldehyde (243 µL, 3 mmol) and the

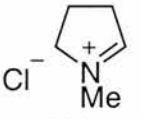
reaction refluxed for 15 hours. After cooling to room temperature 1 mL of 1N HCl was added and the water removed under reduced pressure to give a yellow solid, which was further dried with gentle heating under vacuum and re-crystallised from methanol/ether. Yield 256 mg, 78%

^{1}H-NMR (300 MHz) (D_2O): δ 4.03 (1H, dd, $^3J_{HH}$=7.8, $^3J_{HH}$=9.6, C2-H), 3.77-3.67 (1H, m, C5-H), 3.24-3.13 (1H, m, C5-H), 2.94 (3H, s, NCH_3), 2.60-2.48 (1H, m, C3-H), 2.23-1.93 (3H, m, C4-H_2, C3-H).

^{13}C-NMR (75 MHz) (D_2O): δ 171.7 (C-1), 68.8 (C-2), 56.9 (C-5), 41.1 (NCH_3), 28.5 (C-3), 22.6 (C-4).

ESI (+ve): 152 $[M+Na]^+$

5.6.8 *N*-Methylpyrrolinium chloride (58)



58

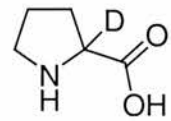
N-Methylproline hydrochloride (**108**) (165 mg, 1 mmol) was heated in $POCl_3$ (1mL) for 10 minutes followed by addition of the reaction mixture to diethyl ether. The diethyl ether was decanted off the resultant thick oil, which was re-washed with diethyl ether to give a crude dark oil (132 mg, crude).

^{1}H-NMR (300 MHz) (D_2O): δ 8.58 (1H, br s, C2-H), 4.12 (2H, t, $^3J_{HH}$=7.8, C5-H), 3.60 (3H, s, NCH_3), 3.21-3.10 (2H, br m, C3-H_2), 2.32 (2H, p, $^3J_{HH}$=7.8,)

^{13}C-NMR (75 MHz) (CD_3OD): 183.7 (C2), 62.2 (C-5), 54.3 (NCH_3), 37.5 (C3), 21.3 (C4).

ESI (+ve): 84 $[M]^+$

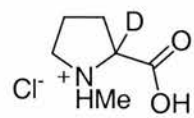
5.6.9 (*RS*)-[2-^{2}H]Proline (107a)[14]



(D/L) Proline (**107**) (500 mg, 4.35 mmol) was dissolved in acetic acid-^{2}H (9.4 mL) to which was added salicylaldehyde (40 μL, 0.4 mmol). The reaction was heated for 4 hrs at 100°C, and the acetic acid removed under reduced pressure to leave a dark oil, which was stirred in D_2O (5 mL) for 15 minutes. The solution was then stirred with activated carbon and filtered. Removal of water under reduced pressure yielded a yellow solid, which was re-crystallised from methanol/ether. Yield 370 mg, 88%.

^{1}H-NMR (300 MHz) (D_2O): 3.44-3.26 (2H, m, C5-$\underline{H}_2$), 2.36-2.25 (1H, m, C3-$\underline{H}$), 2.08-1.93 (3H, m, C3-$\underline{H}$ & C4-$\underline{H}_2$).
^{13}C-NMR (75 MHz) (D_2O): 175.1 (C1), 61.85 (t, $^1J_{CD}$=23, C2), 46.8 (C5), 29.5 (C3), 24.4 (C4).
ESI (+ve): 233 $[2M+H]^+$, 255 $[2M+Na]^+$

5.6.10 *N*-Methyl[2-^{2}H]proline hydrochloride (108a)

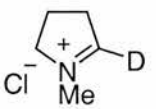


108a

To (*RS*)-[^{2}H]proline (**107a**) (240 mg, 2.07 mmol) in water (15 mL) at 0°C was added 90% formic acid (115 μL, 2.2 mmol) and 37% formaldehyde (245 μL, 3 mmol) and the reaction refluxed for 15 hours. After cooling to room temperature 1N HCl (1 mL) was added and the water removed under reduced pressure to give a yellow solid, which is further dried with gentle heating under vacuum. The crude product was recrystalised from methanol/ether. Yield 211 mg, 61%.

^{1}H-NMR (300 MHz) (D_2O): 3.76-3.69 (1H, m, C5-$\underline{H}$), 3.21-3.11 (1H, m, C5-H), 2.93 (3H, s, NC$\underline{H}_3$), 2.53-2.46 (1H, m, C3-$\underline{H}$), 2.20-1.93 (3H, m, C4-$\underline{H}_2$, C3-$\underline{H}$).
^{13}C-NMR (75 MHz) (CD_3OD): δ 173.3 (C-1), 70.7 (m, C-2), 57.4 (C-5), 41.7 (N$\underline{C}H_3$), 29.3 (C-3), 23.6 (C-4).

5.6.11 *N*-Methyl[2-^{2}H]pyrrolinium chloride (58d)

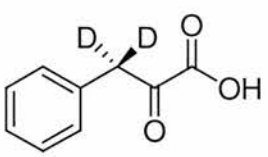


58d

(*RS*)-*N*-Methyl[2-^{2}H]proline hydrochloride (**108a**) (100mg, 0.6 mmol) was heated to 105°C in $POCl_3$ (1 mL) for 20 minutes followed by addition of the solution to to diethyl ether (20 mL) to give a crude oil, which was further washed with diethyl ether (3x20 mL) to give *N*-methyl[2-^{2}H]pyrrolinium chloride (**108a**) as a dark brown oil (71mg, crude).

^{1}H-NMR (300 MHz) (CD_3OD): 3.99 (2H, t, $^3J_{HH}$=8.4, C5-H), 3.51 (3H, s, NMe), 3.07-2.96 (2H, m, C2-H), 2.17 (2H, p, $^3J_{HH}$=8.4, C4-H).
ESI (+ve): 85 $[M]^+$

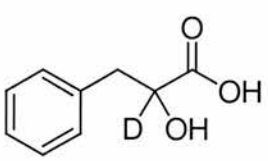
5.6.12 [3, 3-$^2H_2$]Phenylpyruvic acid (79a)



79a

An emulsion of phenylpyruvic acid (**79**) (259 mg, 1.6 mmol) in deuterium oxide (30 mL) was stirred at room temperature for 48 hours. The level of deuterium exchange was monitored directly by ^{1}H-NMR spectroscopyof the filtered reaction mixture. The product was treated directly as described below (sections 5.6.14 and 5.6.15).

5.6.13 (*RS*)-[2-^{2}H]Phenyllactic acid (78f)



To a solution of sodium borodeuteride (118 mg, 2.8 mmol) in water (10 mL) at 0°C was added dropwise an emulsion of phenylpyruvic acid (**79**) (259 mg, 1.6 mmol) in water (30 mL). The mixture was then stirred at room temperature for 2.5 hours. 2N HCl (aq) (10 mL) was then added to the solution with stirring for 10 minutes followed by extraction into CH_2Cl_2 (4×40 mL). The CH_2Cl_2 extractions were combined, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield 157 mg (59%) of a white solid. The ethyl ester was formed for GC-MS analysis by stirring in dry EtOH in the presence of *p*-toluenesulfonic acid (catalytic) for 24 hours.

^{1}H-NMR (300 MHz) ($CDCl_3$): δ 7.35-7.24 (5H, m, aromatics), 3.10 (2H, dd, $^2J_{HH}$=14.1, C-$\underline{H}_2$).

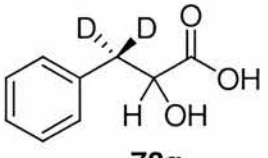
^{13}C-NMR (75 MHz) (CD_3OD): δ 41.6 (C-3), 72.5 (C-2, t, $^1J_{CD}$=23), 127.6, 129.3, 130.6, 139.0 (aromatics), 177.2 (C-1).

ν^{KBr} cm^{-1}; 3446 (OH), 2927 (CO$\underline{OH}$), 1726 ($\underline{CO}$OH).

mp 95-97°C (lit.[15] 97-98°C)

GC-MS (EI, 70eV), *m/z* (rel. int.): 195 $[M]^+$ (0.5), 177 $[M-H_2O]^+$ (65), 149 $[M-EtOH]^+$ (13), 132 $[M-H_2O-Et]^+$ (24), 122 $[M-73]^+$ (40), 104 $[M-PhCH_2]^+$ (33), 91 $[PhCH_2]^+$ (100), 77 $[Ph]^+$ (9), 65 $[M-130]^+$ (10).

5.6.14 (*RS*)-[3, 3-$^2H_2$]Phenyllactic acid (78g)



78g

To a solution of sodium borohydride (106 mg, 2.8 mmol) in water (10 mL) at 0°C was added dropwise an emulsion of [3, 3-$^2H_2$]phenylpyruvic acid (**79a**) prepared as above (section 5.6.12) in D_2O (30 mL). The mixture was then stirred at room temperature for 2.5 hours. 2N HCl (aq) (10 mL) was then added to the solution with stirring for 10 minutes followed by extraction into CH_2Cl_2 (4×40 mL). The CH_2Cl_2 extractions were combined, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield 172 mg (64%) of a white solid. The methyl ester was prepared in anhydrous MeOH (as 5.6.13) for GC-MS analysis.

^{1}H-NMR (300 MHz) ($CDCl_3$): δ 7.34-7.23 (5H, m, aromatics), 4.51 (1H, s, C<u>H</u>OH).

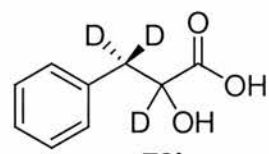
^{13}C-NMR (75 MHz) (CD_3OD): δ 41.4 (m, C-3), 72.9 (C-2), 127.6, 129.3, 130.7, 140.0 (aromatics), 177.2 (C-1).

ν^{KBr} cm^{-1}; 3448 (OH), 2962 (CO<u>OH</u>), 1733 (<u>CO</u>OH).

mp 96-97°C (lit.[15] 97-98°C).

GC-MS (EI, 70eV), *m/z* (rel. int.): 182 $[M]^+$ (1.7), 163 $[M\text{-}HDO]^+$ (45), 132 $[M\text{-}HDO\text{-}MeO]^+$ (8), 123 $[M\text{-}COOMe]^+$ (15), 105 $[M\text{-}Ph]^+$ (13), 93 $[PhCD_2]^+$ (100), 78 $[M\text{-}104]^+$ (5), 66 $[M\text{-}116]^+$ (5).

5.6.15 (*RS*)-[2, 3, 3-$^2H_3$]Phenyllactic acid (78h)



78h

To a solution of sodium borodeuteride (118 mg, 2.8 mmol) in D_2O (10 mL) at 0°C was added dropwise an emulsion of [3, 3-$^2H_2$]phenylpyruvic acid (**79a**) prepared as above (section 5.6.12) in D_2O (30 mL). The mixture was then stirred at room temperature for 2.5 hours. 2N HCl (aq) (10 mL) was then added to the solution with stirring for 10 minutes followed by extraction into CH_2Cl_2 (4×40 mL). The CH_2Cl_2 extractions were combined, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield 147 mg (54%) of a white solid. The methyl ester was prepared (see section 5.6.14) for GC-MS analysis.

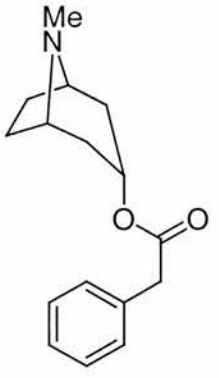
^{1}H-NMR (300 MHz) ($CDCl_3$): δ 7.34-7.24 (5H, m, aromatics).

^{13}C-NMR (75 MHz) (CD_3OD): δ 41.1 (m, C-3), 72.3 (m, C-2), 127.5, 129.2, 130.5, 138.7 (aromatics), 177.1 (C-1).

ν^{KBr} cm^{-1}; 3451 (OH), 2930 (CO<u>OH</u>), 1733 (<u>CO</u>OH).

GC-MS (EI, 70eV), *m/z* (rel. int.): 183 [M] (1.5), 164 [M-HDO] (45), 133 [M-HDO-MeO] (8), 124 [M-COOMe] (15), 106 [M-Ph] (11), 93 $[PhCD_2]$ (100), 78 [M-105] (4), 66 [M-106] (5).

5.6.16 Phenylacetyl tropine (117)



117

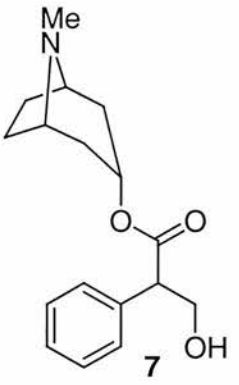
A solid phase mixture of phenylacetic acid (**116**) (408 mg, 3.0 mmol) and tropine (**35**) (424 mg, 3.0 mmol) was heated to 145°C under nitrogen. A stream of dry hydrochloric acid gas was then passed over the mixture for 3 hours with stirring. After cooling the reaction mixture was dissolved in 50 mM sulfuric acid (20 mL), basified with 35% ammonia solution and the product was extracted into chloroform (4×30 mL). The combined organics were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield the product as a clear oil (620 mg, 80%).

^{1}H-NMR (300 MHz) ($CDCl_3$): 7.32-7.18 (5H, m, aromatics), 4.96-4.90 (1H, m, C3-H), 3.55 (2H, s, C2´-H_2), 3.00-2.92 (2H, m, C1-H, C5-H), 2.18 (3H, s, NCH_3), 2.03 (2H, dt, $^2J_{HH}$=15.0, $^3J_{HH}$=4.3, C2-Hα, C4-Hα), 1.90-1.76 (2H, m, C6- Hα, C7-Hα), 1.63-1.52 (4H, m C2-Hβ, C4-Hβ, C6-Hβ, C7-Hβ).

^{13}C-NMR (75 MHz) ($CDCl_3$): 170.4 (C1'), 133.9, 129.1, 128.4, 126.84 (aromatics), 67.8 (C3), 59.5 (C1, C5), 42.1 (C2'), 40.2 (NCH_3), 36.3 (C2, C4), 25.3 (C6, C7).

GCMS: (EI, 70eV), *m/z* (rel. int.): 259 (M^+, 29%), 140 (9.5%), 124 (100%), 94 (19%), 91 (18%), 82 (21%), 67 (9.5%).

5.6.17 (*RS*)-Hyoscyamine (7)



7

n-BuLi (1.6 M in hexanes; 1.9 mL, 3.0 mmol) was added to diisopropylamine (3 mmol, 420 μL) in THF (10 mL) at 0°C and the reaction was stirred for 30 minutes. The reaction was then cooled to -78°C and phenylacetyl tropine (**117**) (518 mg, 2 mmol) in THF (5 mL) was added slowly over 5 minutes and the reaction stirred for a further 30 minutes. The reaction mixture was warmed to -20°C and formaldehyde gas, generated by heating paraformaldehyde (300 mg, 10 mmol) to 150°C, was bubbled through the mixture with a stream of dry nitrogen, until all of the paraformaldehyde had sublimed. After warming to room temperature the reaction was quenched by the addition of water (10 mL). The aqueous layer was separated and extracted into diethylether (4×30 mL). The organic extracts were combined and evaporated under reduced pressure to yield a pale orange gum (480 mg), which was purified over silica gel ($CHCl_3$:MeOH:NEt_3, 75:25:1) to yield (*RS*)-hyoscyamine (**7**) (121 mg, 21%) and recovered phenylacetyl tropine (164 mg). For GC-MS analysis the TMS derivative was prepared using MSTFA.[16]

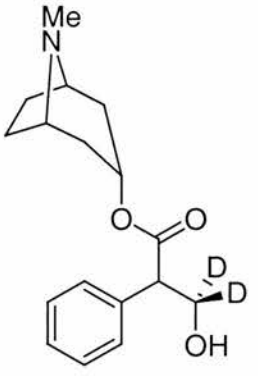
^{1}H-NMR (300 MHz) ($CDCl_3$): 7.39-7.22 (5H, m, aromatics), 4.99 (1H, t, $^3J_{HH}$ = 5.2, C3-H), 4.18-4.10 (1H, m, C3´-Hα), 3.80-3.72 (2H, m, C3´-Hβ, C2´-H), 3.10-3.05 (1H, m, C1/C5-H), 3.00-2.95 (1H, m, C1/C5-H), 2.18 (3H, s, NCH_3), 2.13-2.03 (2H, m, C2-Hα, C4-Hα), 1.89-1.63 (3H, m, C6-Hα, C7-Hα, C6/C7-Hβ), 1.67-1.50 (1H, m, C2/C4-Hβ), 1.60-1.45 (1H, m, C2/C4-Hβ), 1.24-1.15 (1H, m, C6/C7-Hβ).

^{13}C-NMR (75 MHz) ($CDCl_3$): 172.3 (C1´), 135.6, 128.8, 128.1, 127.7 (aromatics), 68.1 (C3), 64.1 (C3´), 59.6 (C1/C5), 59.5 (C1/C5), 54.4 (C2´), 40.2 (NCH_3), 36.3 (C2/C4), 36.1 (C2/C4), 25.4 (C6/C7), 24.8 (C6/C7).

NB where two carbon numbers are given for diastereomeric carbon atoms e.g. C6/C7 the assignment is uncertain.

GCMS (EI, 70eV): 361 (M^+, 17%), 140 (7%), 124 (100%), 104 (7%), 94 (11%), 82 (13%), 73 (10%), 67 (7%).

5.6.18 (*RS*)-[3′, 3′-2H_2]Hyoscyamine (7o)



n-BuLi (1.6 M in hexanes; 1.9 mL, 3.0 mmol) was added to diisopropylamine (3 mmol, 420 μL) in THF (10 mL) at 0°C and the reaction was stirred for 30 minutes. The reaction was then cooled to -78°C and phenylacetyl tropine (**117**) (518 mg, 2 mmol) in THF (5 mL) was added slowly over 5 minutes and the reaction stirred for a further 30 minutes. The reaction mixture was warmed to -20°C and [$^2H_2$]formaldehyde gas, generated by heating [$^2H_2$]paraformaldehyde (538 mg, 16.8 mmol) to 150°C, was bubbled through the mixture with a stream of dry nitrogen, until all of the [$^2H_2$] paraformaldehyde had sublimed. After warming to room temperature the reaction was quenched by the addition of water (10 mL). The aqueous layer was separated and extracted into diethylether (4×30 mL). The organic extracts were combined and evaporated under reduced pressure to yield a pale orange gum (520 mg), which was estimated to be 38% (*RS*)-[3′, 3′-2H_2]hyoscyamine (**7o**) by GC-MS analysis. Aliquots were purified by preparative t.l.c. ($CHCl_3$:MeOH:NEt_3, 75:25:0.5), and the silica extracted with chloroform/methanol (10 mg of product was recovered from 30 mg of the oil). For GCMS analysis the TMS derivative was prepared using MSTFA.[16]

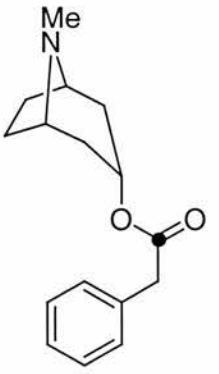
^{1}H-NMR (300 MHz) ($CDCl_3$): 7.38-7.24 (5H, m, aromatics), 5.03 (1H, t, $^3J_{HH}$=5.4, C3-H), 3.78 (1H, s, C2'-H), 3.06-3.00 (1H, m, C1/C5-H), 2.94-2.88 (1H, m, C1/C5-H), 2.20 (3H, s, NCH_3), 2.14-2.00 (2H, m, C2-Hα, C4-Hα), 1.88-1.65 (3H, m, C6-Hα, C-7Hα, C6/C7-Hβ), 1.70-1.50 (1H, m, C2/C4-Hβ), 1.65-1.44 (1H, m, C2/C4-Hβ), 1.22-1.13 (1H, m, C6/C7-Hβ).

^{13}C-NMR (75 MHz) ($CDCl_3$): 172.0 (C1'), 135.7, 128.7, 128.0, 127.6 (aromatics), 67.4 (C3), 59.8 (C1/C5), 59.7 (C1/C5), 54.5 (C2'), 39.8 (NCH_3), 35.6 (C2/C4), 35.8

(C2/C4), 25.1 (C6/C7), 24.6 (C6/C7). NB The signal for C3' was below the detectable threshold due to $^{1}J_{CD}$ coupling.

GC-MS (EI): 363 (M^{+}, 9%), 140 (7%), 124 (100%), 106 (8%), 94 (13%), 82 (15%), 73 (13%), 67 (8%).

5.6.19 [1´-^{13}C]Phenylacetyl tropine (117a)



117a

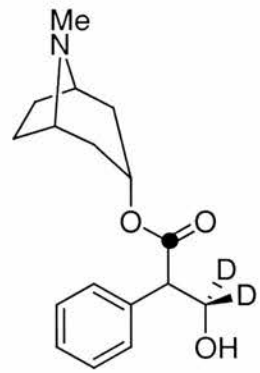
A solid phase mixture of [1-^{13}C]phenylacetic acid (**116a**) (411 mg, 3.0 mmol) and tropine (**35**) (424 mg 3.0 mmol) was heated to 145°C under dry nitrogen. A stream of dry hydrochloric acid gas was then passed over the mixture intermittently with stirring for 3 hours. After cooling the mixture was dissolved in 50 mM sulfuric acid (30 mL), basified with 35% ammonia and extracted into chloroform (4×30 mL). The combined organic layers were dried over $MgSO_4$, filtered and removed under reduced pressure to yield a clear oil (620 mg, 79%).

^{1}H-NMR (300 MHz) ($CDCl_3$): 7.32-7.20 (5H, m, aromatics), 4.98-4.93 (1H, m, C3-H), 3.56 (2H, d, $^{2}J_{CH}$=7.8, C2'-H_2), 3.01-2.94 (2H, m, C1-H, C5-H), 2.19 (3H, s, NCH_3), 2.05 (2H, dt, $^{2}J_{HH}$=15.0, $^{3}J_{HH}$=4.3, C2-Hα, C4-Hα), 1.89-1.81 (2H, m, C6-Hα, C7-Hα), 1.65-1.55 (4H, m, C2-Hβ, C4-Hβ, C6-Hβ, C7-Hβ).

^{13}C-NMR (75 MHz) ($CDCl_3$): 170.4 (C1´), 133.9, 129.1, 128.3, 126.8 (aromatics), 67.8 (C3), 59.5 (C1, C5), 42.1 (C2´, d, $^{1}J_{CC}$=57.6), 40.2 (NCH_3), 36.28 (C2, C4), 25.3 (C6, C7).

GCMS (EI, 70eV): 260 (M^+, 16%), 140 (8%), 124 (100%), 94 (22%), 91 (24%), 82 (26%), 67 (11%).

5.6.20 (*RS*)-[1′-^{13}C, 3′, 3′-$^2H_2$]Hyoscyamine (7p)



7p

n-BuLi (1.6 M in hexanes; 1.9 mL, 3.0 mmol) was added to diisopropylamine (3 mmol, 420 μL) in THF (10 mL) at 0°C and the reaction was stirred for 30 minutes. The reaction was then cooled to -78°C and [1′-^{13}C]phenylacetyl tropine (**117a**) (620 mg, 2.38 mmol) in THF (5 mL) was added slowly over 5 minutes and the reaction stirred for a further 30 minutes. The reaction mixture was warmed to -20°C and [$^2H_2$]formaldehyde gas, generated by heating [$^2H_2$]paraformaldehyde (382 mg, 11.9 mmol) to 150°C, was bubbled through the mixture with a stream of dry nitrogen, until all of the [$^2H_2$]paraformaldehyde had sublimed. After warming to room temperature the reaction was quenched by the addition of water (10 mL). The aqueous layer was separated and extracted into diethylether (4×30 mL). The organic extracts were combined and evaporated under reduced pressure to yield a pale orange gum (506 mg), which was estimated to be 56% (*RS*)-[1′-^{13}C, 3′, 3′-$^2H_2$]hyoscyamine (**7p**) by GC-MS analysis. An aliquot was purified by preparative t.l.c. ($CHCl_3$: MeOH : NEt_3, 75 : 25 : 0.5), and the silica extracted with chloroform/methanol (11mg of product was recovered from 30mg of the oil). For GCMS analysis the TMS derivative was prepared using MSTFA.[16]

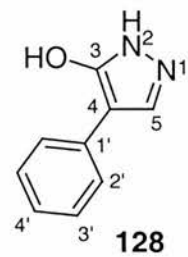
^{1}H-NMR (300 MHz) ($CDCl_3$): 7.36-7.23 (5H, m, aromatics), 5.03-4.96 (1H, m, C3-H), 3.76 (1H, d, $^2J_{CH}$=7.4, C2'-H), 3.08-3.02 (1H, m, C1/C5-H), 2.97-2.91 (1H, m, C1/C5-H), 2.18 (3H, s, NCH_3), 2.17-2.00 (2H, m, C2-Hα, C4-Hα), 1.85-1.60 (3H, m, C6-Hα,

C7-Hα, C6/C7-Hβ), 1.70-1.50 (1H, m, C2/C4-Hβ), 1.67-1.46 (1H, m, C2/C4-Hβ), 1.25-1.12 (1H, m, C6/C7-Hβ).

^{13}C-NMR (75 MHz) ($CDCl_3$): 172.2 (C1'), 135.3, 128.8, 128.1, 127.7 (aromatics), 68.1 (C3), 59.6, 59.5 (C1/C5), 54.2 (d, $^1J_{CC}$=56, C2'), 40.3 (NCH_3), 36.4, 36.1 (C2/C4), 25.4, 24.9 (C6/C7). NB The signal for C3' was below the detectable threshold due to $^1J_{CD}$ coupling.

GC-MS (EI): 364 (M^+, 10%), 140 (6%), 124 (100%), 106 (8%), 94 (12%), 82 (14%), 67 (8%).

5.6.21 3-Hydroxy-4-phenyl pyrazole (128) [17,18]



128

A solution of ethyl phenylacetate (**127**) (657 mg, 4 mmol) in anhydrous toluene (10 mL) was added slowly to a stirred suspension of sodium hydride (60% in mineral oil, 240 mg, 6 mmol) in toluene (20 mL) at 0°C. The mixture was allowed to warm to room temperature and stirred for 1 hour before the drop wise addition of ethyl formate (326 mg, 4.4 mmol). The solution was stirred for 16 hours, followed by the addition of water (10 mL). The aqueous layer was acidified, and extracted with diethyl ether (3×20 mL). The combined organics were dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to give a yellow oil. The oil was dissolved in ethanol (10 mL), cooled to 0°C, followed by the addition of hydrazine hydrate (5 mL). The reaction was stirred for 1 hour, the precipitate recovered by filtration and washed with cold ethanol to give a white, crystalline solid (55 mg, 9%)

^{1}H-NMR (300 MHz) (DMSO-d6): 7.85 (1H, s, C5-H), 7.67 (2H, dd, $^3J_{HH}$=4.5, $^5J_{HH}$=0.9, C2´-H), 7.30 (2H, t, $^3J_{HH}$=4.5, C3´-H), 7.08 (1H, dt, $^3J_{HH}$=4.5, $^5J_{HH}$=0.9, C4´-H).

^{13}C-NMR (75 MHz) (DMSO-d6): 158.7 (C3), 133.6 (C1´), 128.7 (C3´), 127.7 (C5), 125.3 (C2´), 125.0 (C4´), 104.4 (C4).

ν^{KBr} cm^{-1}: 3253, 2972, 1608, 1575, 1519, 1442, 1389, 1299, 1274, 1225, 1164, 1080, 1057, 1013, 915, 845, 761, 737, 699, 674, 568, 507.

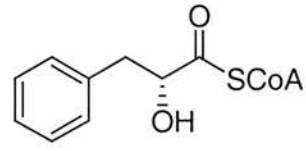
MS (EI), *m/z* (rel. int.): 160 [M] (100%), 103 [M-57] (27%), 89 [M-71] (9%), 77 [C_6H_5] (15%), 63 [M-97] (7%), 51 [M-109] (8%).

HRMS (EI), $C_9H_8N_2O$ calculated (M^+) 160.0636: found 160.0631 (3.2ppm).

GC-MS (EI, 70eV), *m/z* (rel. int.): 304 [M] (100%), 289 [M-CH_3] (75%), 261 (11%), 230 (7%), 147 (12%), 73 (67%).

m.p. 222-224°C

5.6.22 (*R*)-Phenyllactoyl coenzymeA (131)[19,20]



131

A solution of (*R*)-phenyllactic acid (**78**) (498 mg, 3 mmol), *N*-hydroxysuccinimide (345 mg, 3 mmol) and 1,3-dicyclohexylcarbodiimide (681 mg, 3.3 mmol) in anhydrous THF (30 mL) was stirred at room temperature. After 24 hours the 1,3-dicyclohexylurea was removed by filtration and the filtrate extracted with 1M $NaHCO_3$ (aq) (30 mL). The organic was dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to yield a thick cloudy oil. To a degassed solution of co-enzyme A (7.7 mg, 10 μmmol) and $NaHCO_3$ (8.4 mg, 100 μmol) in water (2 mL) was added an aliquot (13.2 mg) of the crude phenyllactoyl *N*-hydroxysuccinimide ester in acetone (3 mL). The solution was then stirred at 4°C for 24 hours. The acetone was removed with a stream of nitrogen and the aqueous extracted with ethylacetate (1x10 mL) prior to removal by freeze-drying to give a white solid (15 mg).

MALDI-TOF: 914 (M-H)

5.6.23 References for Chapter 5

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