Biosynthetic studies on tropic acid and piliformic acid

Chesters, Nicola C.J.E.

BIOSYNTHETIC STUDIES ON TROPIC ACID AND PILIFORMIC ACID

Nicola C. J. E. Chesters, B.Sc. (Hons)

Ph.D. Thesis

University of Durham

1995

COPYRIGHT

DECLARATION

The work contained in this thesis was carried out in the Department of Chemistry at the University of Durham between October 1992 and September 1995. All the work was carried out by the author, unless otherwise indicated. It has not been previously submitted for a degree at this or any other university.

ABSTRACT

Biosynthetic Studies on Tropic Acid and Piliformic Acid

This thesis is divided into two parts and covers biosynthetic studies on two secondary metabolites, tropic acid in Part I and piliformic acid, in Part II.

(S)-Tropic acid is the acid moiety of the alkaloids hyoscyamine and scopolamine, which are produced by a number of plants of the *Solanacae* family. An intriguing rearrangement of the L-phenylalanine side chain gives rise to the isopropanoid (S)-tropic acid skeleton. The detailed nature of the rearrangement has however remained elusive despite continued interest over the years. In chapter two the identification of intermediates between L-phenylalanine and (S)-tropic acid is discussed, which has placed (R)-D-phenyllactic acid as an immediate precursor. The stereochemical features of the rearrangement are described in chapter 3 and finally in chapter 4 a mechanism for the rearrangement is proposed. This is based on information obtained from the incorporation of various isotopically labelled precursors to tropic acid into two of the minor alkaloids, 3α-2'-hydroxyacetoxytropane and 3α-phenylacetoxytropane. This work was carried out in collaboration with Dr Richard Robins at the AFRC Institute of Food Research in Norwich.

Piliformic acid is elaborated by the slow growing fungus *Poronia piliformis*. The incorporation of a number of isotopically labelled substrates into piliformic acid has revealed a mixed biosynthetic origin, comprising C_8 and C_3 fragments. These have been shown to be of acetogenic and citric acid cycle origins respectively. The C_8 fragment has been further demonstrated to be a degradation product of a longer chain fatty acid. The mode of coupling of the two fragments has been investigated and suggests the intermediacy of a novel α-carboxyoctanoate. A pathway for the assembly of piliformic acid, involving a 1,3-hydrogen shift, is proposed, consistent with the above findings. These results are the subject of chapter 6.

To Mum and Dad

...And scientists, who ought to know,

Tell us that it must be so.

Oh, let us never, never doubt,

What nobody is sure about.

Hilair Belloc

The Microbe

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, David O'Hagan. Without his continuous help, encouragement, seemingly endless supply of ideas and enthusiasm the project would never have been possible. My grateful thanks also go to Richard Robins, initially at the AFRC Institute of Food Research and now at the University of Nantes, for supplying the transformed root cultures of *D. stramonium*, feeding [2-^{3}H]-phenyllactate and sodium [2-^{13}C, ^{2}H]-phenyllactate, GC-MS analysis of the alkaloid extracts, teaching me the subculturing and extraction techniques and for very many lively discussions on the biosynthesis of tropic acid. I thank Professor Heinz Floss and two members of his group, at the University of Washington, Seattle: Allen Kastelle, for the analysis of the chiral acetic acid and Kevin Walker for the syntheses of (2R, 3S)-[2-^{13}C, 3-^{2}H, *ring*-^{2}H$_5$]-phenyllactic acid and (2R, 3R)-[2-^{13}C, ^{2}H, 3-^{2}H]-phenyllactic acid. I also thank Dr Raymond Edwards, at the University of Bradford, for supplying the cultures of *P. piliformis* and *X. mali*. Funding from the EPSRC is gratefully acknowledged.

Many thank must go to the technical staff, particularly to Alan Kenwright, Ian McKeag, Julia and Barry Say for service NMR analysis and help with the interpretation of some very interesting spectra, Jimmy and Joe at the stores for, among other things, yards and yards of aluminium foil, Gordon and Ray for some amazing glassware creations and Hazel, Jean, Maurine, Brenda for keeping lab 8C clean (quite a challenge!) and a plentiful supply of caffein. A big 'thank you' also to all the members of the O'Hagan group, Caragh, Sarah, Russell, Raju, Manju, Jon, Jeff, Dave, Ruhul, Colin, Adam, Mustafa and Naveed.

Finally, last, but certainly not least, a very big thank you to Mum and Dad for all their support, financial and otherwise, and, of course a special thank you to Nick.

ABBREVIATIONS

Ac	acetyl
ACP	acyl carrier protein
ADC	arginine decarboxylase
AFRC	Agricultural and Food Research Council
ATP	adenosine triphosphate
Bn	benzyl
Bu	butyl
Bros	*p*-bromobenzenesulphonyl
CCL	*Candida cylindracea* lipase
CHIRAPHOS	2,3-bis(diphenylphosphino)butane
CI	chemical ionisation
CoA	coenzyme-A
d	doublet
DCC	dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMSO	dimethylsulphoxide
ee	enantiomeric excess
EI	electron impact
Et	ethyl
FAB	fast atom bombardment
FAS	fatty acid synthase
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
hfc	3-(heptafluoropropylhydroxymethylene)-(+)-camphorato
INADEQUATE	incredible natural abundance double quantum experiment
LDA	lithium diisopropylamide
L-LDH	L-lactate dehydrogenase
m	multiplet

Me	methyl
Mes	methanesulphonyl
m.p.	melting point
MPO	N-methylputrescine oxidase
MS	mass spectroscopy
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
ODC	ornithine decarboxylase
q	quartet
p	pentet
PCC	pyridinium chlorochromate
Ph	phenyl
PKS	polyketide synthase
PMT	putrescine methyltransferase
s	singlet
t	triplet
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TMSBr	trimethylsilyl bromide
Tos	toluenesulphonyl

CONTENTS

FORWARD

Radio- and Stable Isotope Labelling as a Tool for Biosynthetic Studies

Classically radioisotope labelled molecules have been used to follow the fate of substrates administered to biological systems, primarily ^{14}C, ^{3}H and ^{32}P. The attraction of this technique lies primarily in its sensitivity. The tracer is detected by scintillation counting and a very low level of activity can be recorded. In addition the half-life of ^{14}C is 5600 years and that for ^{3}H is 12.26 years, therefore losses due to decay can be essentially neglected. However, a significant disadvantage of radiolabelling is that scintillation detection is not site-specific. To follow the fate of an individual carbon atom from the precursor into the molecule of interest, each carbon must be identified uniquely by chemical degradation and the degradation products rigorously purified. In essence, each carbon of interest must be individually carved out. There are also problems associated with handling radioactive material, such as contamination of personnel and cross contamination between high and low level sources.

With the advent of high field NMR analysis the use of stable isotopes became widely used in biosynthetic studies[1,2] with ^{13}C NMR being most commonly used as an analytical tool. It has a low natural abundance ($\approx$1.1%) which means that even low levels of incorporation can be readily detected. Comparison between a labelled and unlabelled ^{13}C-NMR spectrum quickly shows which sites have become enriched by comparing peak heights. The level of incorporation can also be determined from the peak integrals. The interpretation of spectra is facilitated by proton decoupling and the wide chemical shift range of carbon.

Deuterium labelling, combined with ^{2}H NMR analysis offers an even more sensitive probe, since the natural abundance of ^{2}H is only 0.016%. The short relaxation time of deuterium avoids saturation problems and the relative levels of deuterium incorporation can therefore be accurately assessed by integration. The ^{2}H chemical

shifts correspond very closely to the ^{1}H chemical shifts and therefore a fully assigned ^{1}H NMR spectrum enables the ^{2}H resonances to be assigned, although the range of resonances (in Hz) is only about 15% of that for ^{1}H. Deuterium is a quadrupole nucleus (spin =1) and consequently the principal disadvantages of ^{2}H NMR analysis are the low sensitivity to detection (low magnetogyric ratio) and broadening of the signals.

Tritium can also be assayed directly by ^{3}H NMR and since tritium has the highest magnetogyric ratio of any known element, and a nuclear spin of $^{1}/_{2}$, the lines in the spectra are sharp and relatively small quantities can be detected. Again, the chemical shifts correspond to proton resonances. The very low natural abundance of tritium (10^{-15}%) also facilitates detection of tritium enrichment, although this method is not as sensitive as scintillation counting. The assessment of tritium by ^{3}H NMR is site specific. However the principal disadvantage of ^{3}H NMR is that the level of tritium required risks contamination between high and low level sources.

The integrity of chemical bonds in the administered precursors and final metabolite can be probed with double labelling techniques. Since the abundance of ^{13}C is low the probability of two ^{13}Cs lying adjacent to one another in the same molecule is very small (1.1% x 1.1%) and coupling between two ^{13}Cs is not normally observed. If however a precursor, with adjacent ^{13}Cs such as 1,2-[$^{13}C_2$]-acetate, is introduced any residual coupling in the recovered metabolite indicates that the (acetate) C-C bond has remained intact during the biosynthetic process.

The fate of deuterium can also be followed with ^{13}C NMR. Deuterium has a spin of 1 and will couple to the ^{13}C that it is attached to. Thus when a ^{2}H is tagged to a ^{13}C atom it can be readily observed in the ^{13}C NMR spectrum. Furthermore a carbon attached to a deuterium will be shifted upfield by 0.25-0.3ppm per deuterium. This is known as the α-effect (^{13}C-D). The number of deuteriums may then be directly observed by the magnitude of the shift related to that at natural abundance. Where the spectrum is complicated by the coupling of a carbon atom with one two or three

deuterium atoms attached in different molecules, the signal can be collapsed to show a series of singlets by simultaneously decoupling deuterium and hydrogen (*i.e.* $^{13}CH_3$, $^{13}CH_2D$, $^{13}CHD_2$, $^{13}CD_3$) by use of a NMR machine with a triple resonance probe.

Carbon-13 adjacent to a C-D group is also shifted to lower frequency. This is the β-effect (^{13}C-C-D). It is a smaller effect resulting in a shift of only 0.05-0.08ppm per deuterium atom but it is still observable and a useful tool. When an enriched carbon is placed next to a site with several deuterium atoms attached, the shift is additive, and hence the presence and number of deuterium atoms can be deduced directly without loss of signal to noise, as there is no $^2J_{^{13}C-^2H}$ coupling. The fate of ^{18}O can similarly be followed by ^{13}C NMR, through the induced chemical shift in the ^{13}C spectrum.[3]

Another technique, increasingly used for isotope detection, is mass spectral analysis, often combined with GC or HPLC purification. The principal attractions of this technique are that much smaller samples can be analysed compared to NMR techniques. The number of isotopes in a particular molecule can also be assessed. The disadvantage however, as with radioisotope labelling, is that the detection of incorporation is not site specific and chemical degradation may prove necessary to locate the isotope.

Techniques using isotopes other than carbon-13 are particularly useful as they give information beyond the assembly of the carbon skeleton of the molecule, revealing details of the origin of the peripheral hydrogens or heteroatoms. For example, the incorporation of ^{18}O from $^{18}O_2$ into isoflavonone (chapter 4) indicated a radical rather than an ionic process for the rearrangement of flavonone to isoflavonone.[4] Deployment of these techniques with suitably designed substrates allows the site specific enrichment of carbon atoms and the integrity of C-C and C-D bonds to be evaluated directly in a biosynthetic study, without recourse to the more tedious, traditional radiolabelling approach.

References

1. J. C. Vederas, *Nat. Prod. Rep.*, 1987, **4**, 277
2. M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, 539
3. J. C. Vederas, *J. Am. Chem. Soc.*, 1980, **102**, 374
4. T. Hakamatsuka, M. F. Hashim, Y. Ebizuka, U. Sankawa, *Tetrahedron*, 1991, **47**, 5969

PART I

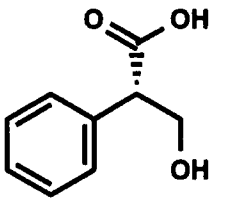
THE BIOSYNTHESIS OF TROPIC ACID

CHAPTER 1

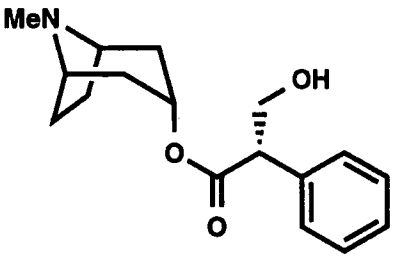
Introduction and Background

1.1 Introduction

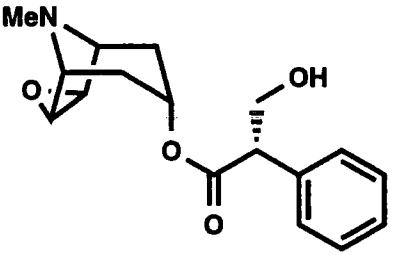
(S)-Tropic acid (**1**) is the acid moiety of the tropane alkaloids, hyoscyamine (**2**) and scopolamine (**3**) (fig. 1), secondary metabolites which are produced by plants of the *Solanacae* family. The hallucinogenic and hypnotic properties of these alkaloids, together with their high toxicity has led to many of the tropane alkaloid producing plants becoming infamous through their associated folklore.[1,2] These plants include mandrake (*Mandragora officinarum*), henbane (*Hyoscyamus niger*), deadly nightshade (*Atropa belladona*) and jimson weed (*Datura stramonium*).



(S)-tropic acid (**1**)



hyoscyamine (**2**)



scopolamine (**3**)

Fig. 1

The use of extracts of these plants as poisons, in magico-religious activities and as medicines has been recorded since biblical times. Mandrake has been long associated with fertility and the priestesses of the Delphic Oracle would inhale the smoke from burning henbane. As mandrake plants became prized for their medicinal and devinatory

uses a wealth of legends grew regarding the collection of the plants. The mandrake, when uprooted was believed to utter a shriek so terrible that any who heard it would die. Consequently a number of collection rituals arose, commonly using dogs to pull up the plants. The use of tropane alkaloid producing plants in witchcraft did not become widespread until the Middle Ages when a means of avoiding the risks associated with oral administration of the plant extracts was discovered. Extracts of various combinations of mandrake, henbane and deadly nightshade were combined with fats to make 'witches salves' or 'flying ointments' which were spread onto the skin, thus the transdermal absorption of the readily fat soluble alkaloids was facilitated. The hallucinogenic effects of hyoscyamine intoxication undoubtedly accounts for the experiences of flying and lycanthropy reported by witches and werewolves. Deadly nightshade later found a cosmetic use. Renaissance ladies would use the juice of the berries in their eyes, to dilate the pupils, which was considered to be attractive. The name *belladona* derives from the Italian, meaning beautiful woman. Atropine (racemic hyoscyamine), the principal alkaloid found in *Atropa belladona,* was used for the same purpose in ophthalmology until recently when it was replaced by safer synthetic analogues.

Both hyoscyamine and scopolamine are acetylcholine antagonists, selectively blocking the muscarinic class of acetylcholine receptors. The physiological effects of hyoscyamine or scopolamine intoxication are an increased heart rate, vasodilatation, (which causes the skin to become flushed), smooth muscle relaxation, pupil dilation, and drying up of the glandular secretions of the nose, throat and bronchial passages. In overdose death is ultimately a result of respiratory failure. Hyoscyamine and scopolamine, in controlled doses, have also found much use in modern medicine. The hypnotic effect of scopolamine is used in premedication prior to surgery and in sleeping pills, while the antispasmodic, antiemetic properties of hyoscyamine have made it useful in the treatment of gastroenteritis, motion sickness and ulcers. Hyoscyamine is also an antidote to nerve gas.[3] Nerve gas contains fluorophosphoric acids which inhibit choline esterase, the enzyme responsible for the breakdown of acetylcholine in

synapses. Extracts from tropane alkaloid producing plants are still used in some parts of the world to treat asthma, and synthetic analogues, which relax the bronchial tubes without the associated effects on the heart, are used in modern anti-asthma drugs.

1.2 The Use of Transgenic Root Cultures in Biosynthetic Studies

The biosynthesis of hyoscyamine (**2**) has been shown to occur exclusively in the roots of the plants, although the oxidative elaboration of the tropane skeleton to generate scopolamine occurs predominantly after translocation to other parts of the plant.[4] It has further been observed that root cultures of plants have the ability to synthesise alkaloids characteristic of the plants from which they are derived. In one experiment where reciprocal grafts between tobacco plants and tomato were performed it was found that nicotine only accumulated in the plants with the tobacco roots.[5]

Transgenic root cultures, or 'hairy roots' offer advantages over the use of whole plants for biosynthetic studies, since they grow rapidly and can be harvested after only two weeks growth, whereas whole plants take several months. Transformed root cultures are also amenable to manipulation in the same way as callus or suspension cultures without the associated problems of genetic stability or maintenance of secondary metabolism. They are cultured in sterile conditions and will grow and produce secondary metabolites in the absence of phytohormones.[4,6] There is potential for the use of transformed root cultures in the generation of commercially important natural products as well as for biosynthetic studies. Transgenic root cultures are produced by a natural genetic engineering process that occurs when a plant is infected by the plant pathogenic bacterium *Agrobacterium rhizogenes*, responsible for hairy root disease in plants. To effect the transformation an aseptic tissue culture is stabbed with a hypodermic needle containing the bacteria. A plasmid from the bacteria enters the plant cell and encodes the proliferation of fast growing 'hairy roots' at the site of inoculation. After antibiotic treatment to kill the remaining bacteria, the hairy roots can be grown and subcultured indefinitely.[7]

1.3 The Biosynthesis of the Tropane Skeleton

The carbon atoms of the tropane skeleton are derived from ornithine (**4**) and acetate (**5**) as shown in fig. 2.

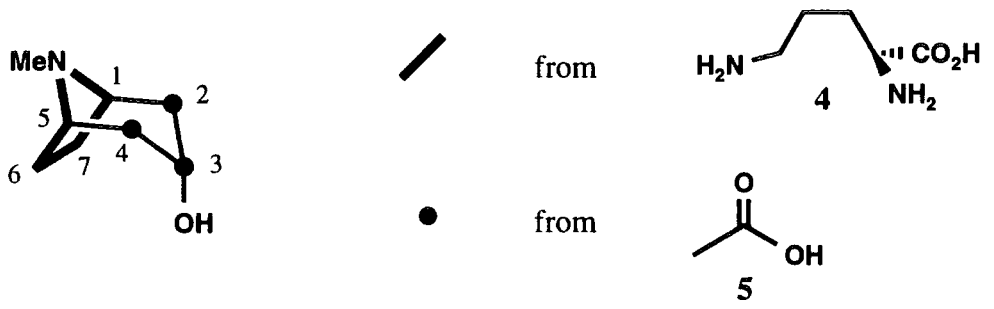


Fig. 2

The incorporation of ornithine into the pyrrolidine portion of the tropane skeleton was first established by Leete in 1954[8] and was later shown to be unsymmetrical, [2-^{14}C]-labelling only the C-1 bridgehead carbon[9,10] (fig.3).

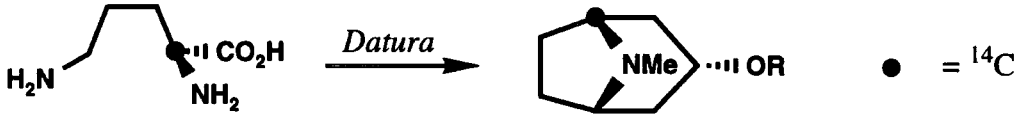
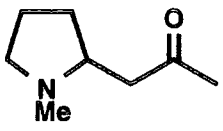
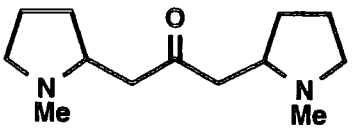


Fig. 3

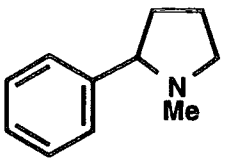
This pyrrolidine ring is however common to a number of alkaloids including hygrine (**6**), cuscohygrine (**7**), nicotine (**8**) and the tropane alkaloid cocaine (**9**) (fig. 4). Many of these alkaloids are produced by a number of different species and while ornithine is incorporated unsymmetrically into some, as is the case with hyoscyamine produced by *D. stramonium*, in others symmetrical incorporation is observed and label from [2-^{14}C]-ornithine is distributed equally between the C-1 and C-5 carbons.[11-13] The most obvious interpretation of the unsymmetric incorporation of ornithine into the pyrrolidine moiety of hyoscyamine is the absence of a symmetrical intermediate in the biosynthetic pathway. δ-N-Methylornithine (**10**)[14-16] was proposed as a biosynthetic intermediate, thus retaining the identity of the two C-2 and C-5 carbons of ornithine through N-methylputrescine (**11**), an established intermediate.[17]



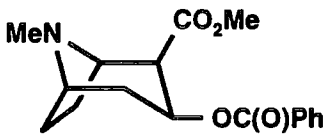
hygrine (**6**)



cuscohygrine (**7**)



nicotine (**8**)



cocaine (**9**)

Fig. 4

N-Methylputrescine was then assumed to undergo transammination to 4-N-methylaminobutanal (**12**) and cyclise to form an N-methylpyrrolinium salt (**13**) which is then further elaborated to tropinone (**14**) (fig. 5).

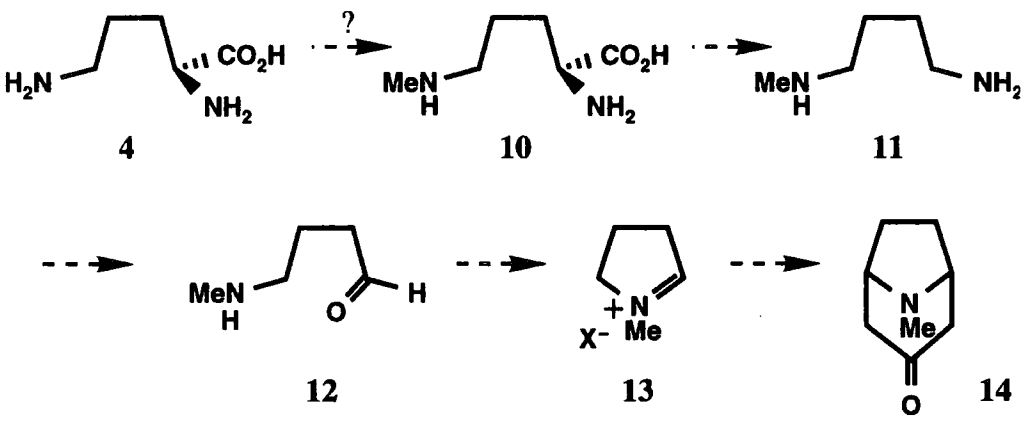


Fig. 5

The conversion of labelled ornithine (**4**) to 4-N-methylaminobutanal (**12**) has been demonstrated *in vivo*.[18] It was originally proposed that the double bond of the N-methylpyrrolinium salt might isomerise and so explain the symmetrical labelling patterns observed in some species. Leete has however failed to observe this phenomenon both *in vitro* and *in vivo*, in *Nicotiana tabacum* to generate nicotine labelled only on the C-2' carbon of the pyrrolidine ring[19] (fig. 6).

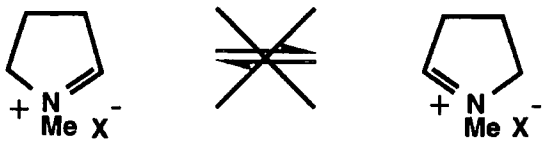


Fig. 6

Putrescine (**15**) itself has also been successfully incorporated into the tropane alkaloids[20,21] although this was initially interpreted as an aberrant pathway since it is a symmetrical molecule.[13] However, since the symmetrical incorporation of ornithine by some species cannot be explained through the isomerisation of the N-methylpyrrolinium salt and the incorporation of putrescine has been demonstrated, it was necessary to invoke putrescine as an intermediate. The separate identity of the C-2 and C-5 carbons of ornithine could be explained by suggesting that *free* putrescine was not an intermediate, rather an enzyme bound form resulting from the decarboxylation of ornithine which is then methylated to N-methylputrescine.[13] This has been verified as the pathway to the N-methylpyrrolinium salt *in vivo* (fig. 7) and the enzymes involved, ornithine decarboxylase (ODC), putrescine methyltransferase (PMT) and N-methylputrecine oxidase (MPO) are well described .[22]

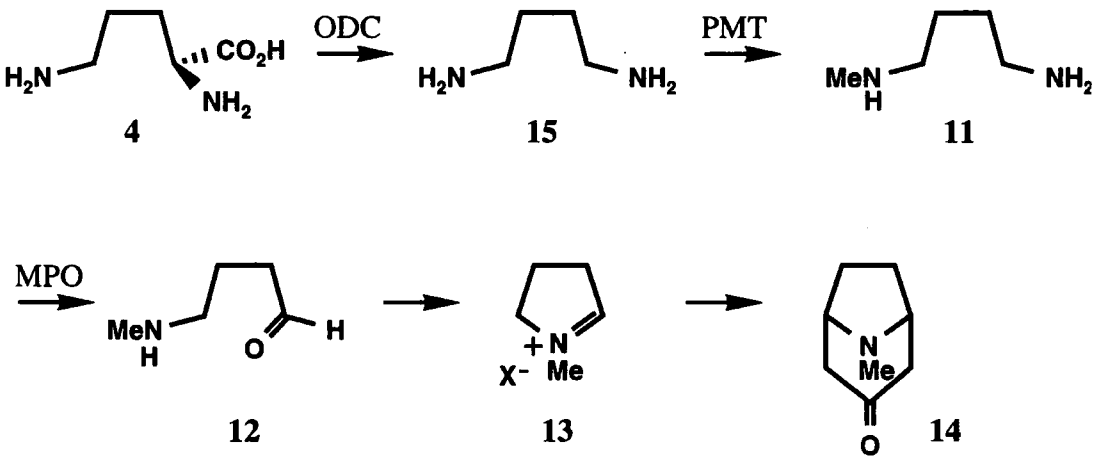


Fig. 7

It is now known that in some species the predominant precursor to putrescine is actually arginine (**16**), rather than ornithine. The metabolic relationship between these amino acids is shown in fig. 8. The incorporation of agmantine (**17**), an intermediate between

arginine and putrescine, has been demonstrated and through the selective inhibition of either ornithine or arginine decarboxylase activity, the major pathway has been revealed.[23]

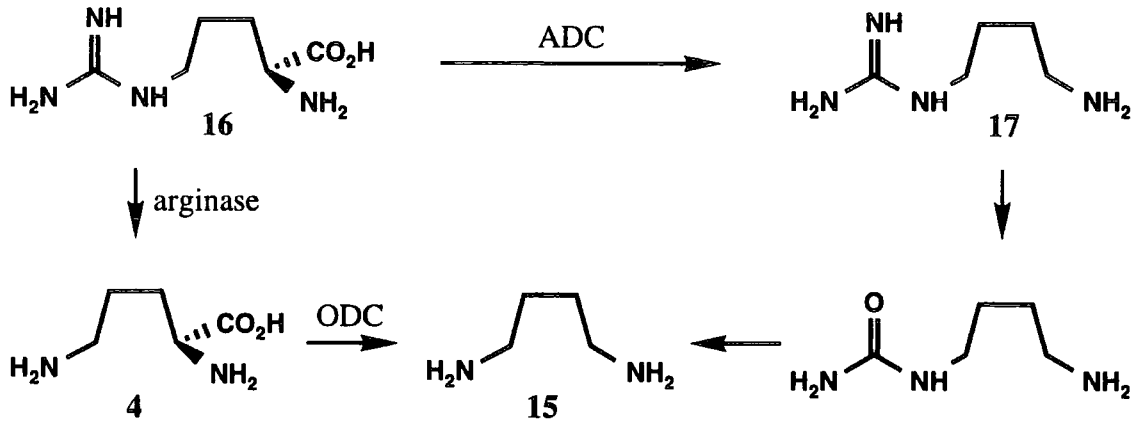


Fig. 8

The remaining carbons of the tropane skeleton have been shown to derive from acetate. Acetoacetate (**18**) is also efficiently incorporated and had gained general acceptance as a *bona fide* intermediate.[24] This would involve condensation between the N-methylpyrrolinium salt and acetoacetate, possibly activated as its coenzyme-A ester to generate α-carboxyhygrine (**19**). Decarboxylation to generate hygrine (**6**), an established intermediate in hyoscyamine biosynthesis[24,25] would be followed by oxidation of the pyrrolidine ring, and finally the cyclisation of hygrine would generate tropinone (fig. 9).

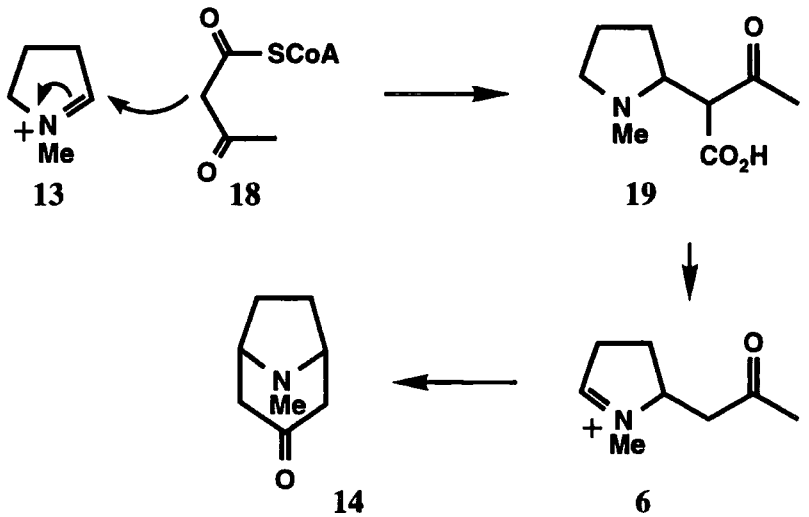


Fig. 9

[1-^{14}C]-Acetate has been shown to label C-9 of cocaine, consistent with this hypothesis (fig. 10). However, the mode of incorporation of acetate into tropinone has remained a contentious issue and this generally accepted pathway, utilising acetoacetate, has been called into question, and has recently been re-evaluated.

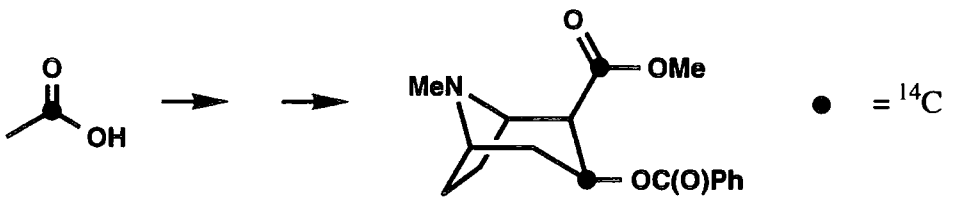


Fig. 10

The reaction between the N-methylpyrrolinium salt and acetoacetate is a facile process and occurs spontaneously *in vitro*[26] and there has been speculation that the incorporation of acetoacetate *in vivo* simply represents an alternative, non-enzymatic pathway.[13,22] It became clear that an alternative biosynthetic pathway to the tropane skeleton was operating when Leete and Kim[27] observed the incorporation of [1-^{13}C, ^{14}C, ^{15}N]-(methylamino)butanal diethylacetal (**20**) into cocaine and were surprised to observe that C-5, rather than C-1 became enriched (fig. 11). If acetoacetate is a true intermediate this would require the C-4 of acetoacetate (or its coenzyme-A ester) to react with the N-methylpyrrolinium salt, a pathway which is less reasonable than condensation with C-2.

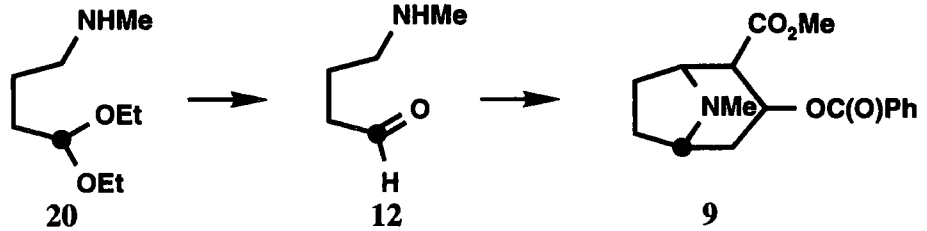


Fig. 11

This led to a new proposal where the acetate derived carbons were incorporated *via* malonyl-CoA (**21**), as shown in (fig. 12).[24] Hydrolysis and decarboxylation of **22** would then be a necessary prerequisite for hyoscyamine biosynthesis .

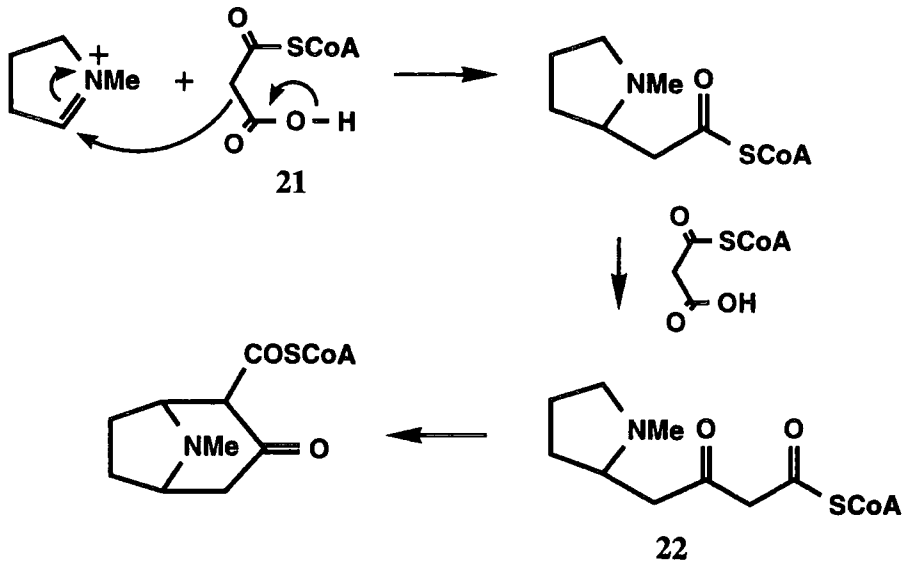


Fig. 12

The intermediacy of acetoacetate was finally discredited by Hemscheidt and Spenser[28] who demonstrated that [1,2,3,4-$^{13}C_4$]-acetoacetate was not incorporated intact into hyoscyamine, but instead gave the same labelling pattern as [1,2-$^{13}C_2$]-acetate in hyoscyamine from *D. stramonium* (fig. 13), indicating that the acetoacetate had been degraded to acetate prior to incorporation.

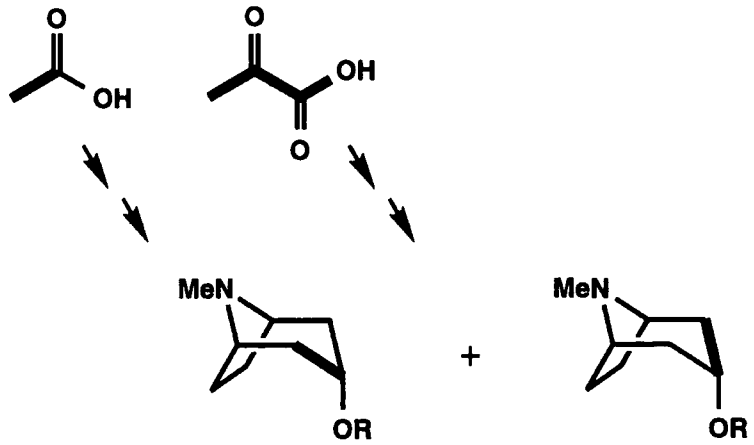


Fig. 13

It was also observed[28] that the two bridgehead carbons were equally labelled in these experiments, and that N-methyl-[2-^{2}H]-pyrrolinium chloride (**13a**) placed deuterium equally at the two bridgehead positions (fig. 14). This suggested that perhaps either achiral N-methylpyrrolidine or racemic hygrine may be involved in tropinone biosynthesis.

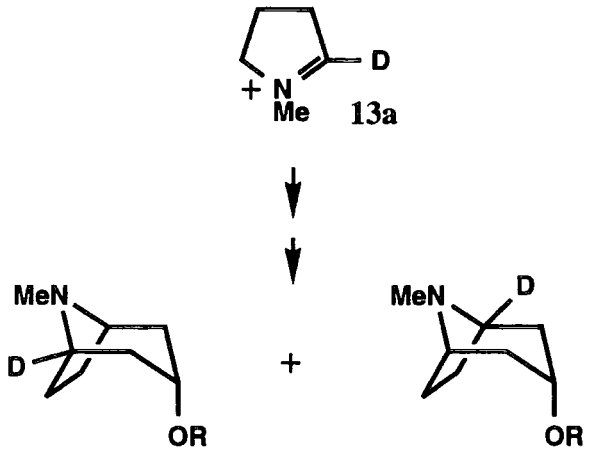


Fig. 14

A similar result was obtained through feeding [2'3'-$^{13}C_2$]-hygrine (**6a**) and ethyl [2',3'$^{13}C_2$]-N-methyl-2-pyrrolidinyl-3-oxobutyrate (**22a**) to *D. innoxia* (fig. 15).[29]

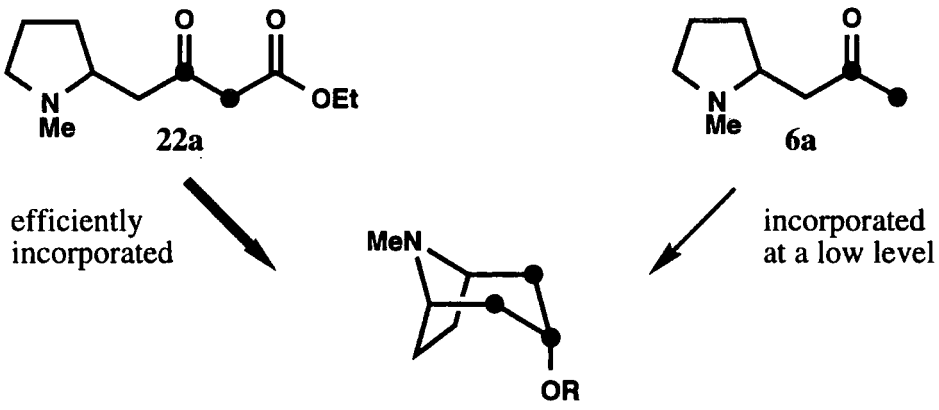


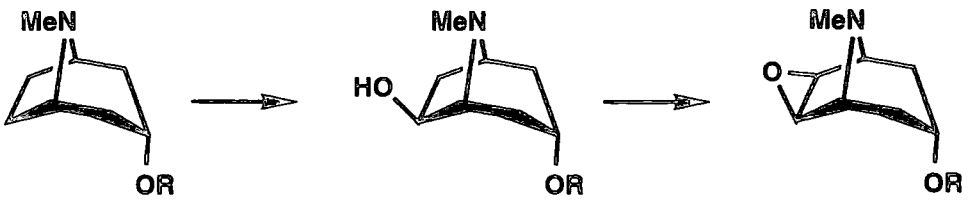
Fig. 15

The ketone functionality of tropinone is finally reduced to generate tropine prior to esterification. Two tropinone reductases have been identified, and purified from several sources.[22] They are specific for either the reduction of tropinone to tropine (**23**) or its 3-β-epimer, pseudotropine (**24**) (fig. 16).

tropine (**23**) pseudotropine (**24**)

Fig. 16

The conversion of the tropine moiety to the 6,7-epoxide moiety of scopolamine (3) occurs after hyoscyamine formation (fig. 17).[30] The first stage of this conversion is C-6 hydroxylation to give 6-β-hydroxyhyoscyamine (25), an isolable intermediate.



2 25 3

Fig. 17

The enzyme responsible for the conversion of hyoscyamine to scopolamine has been shown to require iron (II), oxygen, and oxoglutarate.[31] It has so far proved impossible to separate the hydroxylation and epoxidation activities and it cannot be discounted that a single protein may be responsible for both activities.[32,33]

1.4 The Biosynthesis of Tropic Acid: An Historical Perspective

The biosynthesis of tropic acid (1) has attracted interest over many years[34,35] and was first investigated by Leete in the 1960s.[36] It was initially proposed that tropic acid may be derived from prephenic acid (**26**),[37] which is an established intermediate in the

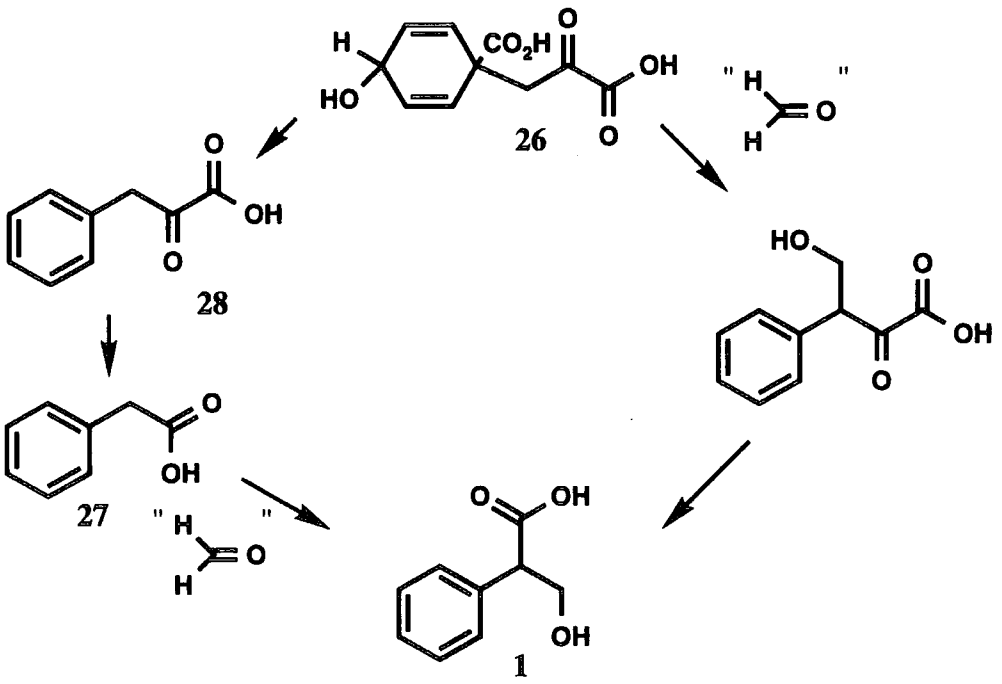


Fig. 18

biosynthesis of many aromatic compounds. Two plausible routes were suggested, differing only in the timing of the introduction of the hydroxymethyl group (fig. 18). It was envisaged that either formaldehyde or a biosynthetic equivalent would attack prephenic acid itself, or that prephenic acid would first be degraded to phenylacetic acid (**27**) *via* phenylpyruvic acid (**28**).

Leete administered DL-[3-^{13}C]-phenylalanine to *D. stramonium* plants to investigate the intermediacy of phenylpyruvic acid. The incorporation of phenylalanine would clearly implicate phenylpyruvate as an intermediate, since the two can interconvert *via* transammination (fig. 19).

29 **28**

Fig. 19

In the event DL-[3-^{13}C]-phenylalanine was efficiently incorporated, labelling the benzylic carbon (fig. 20), a result later confirmed by Underhill and Youngken.[38] However, attempts to probe the postulated C-1 origin of the hydroxymethyl group, by feeding [^{14}C]-formate, were unsuccessful, although this negative result was inconclusive as formate is a poor C-1 source in plants.

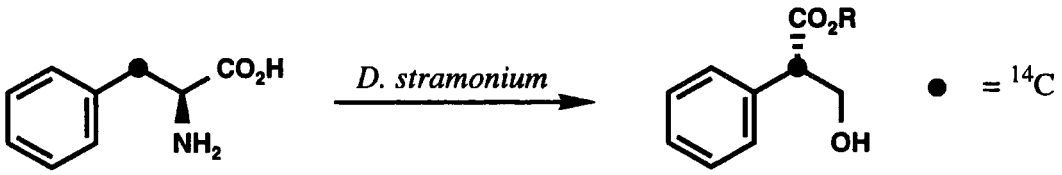


Fig. 20

In a second experiment Leete and Louden[39] administered DL-[2-^{14}C]-phenylalanine, expecting to see incorporation into the carboxyl carbon of the tropate moiety, however, it was the hydroxymethyl group which was uniquely enriched. This then led to the

suggestion that C-1 of phenylpyruvic acid might be lost (fig. 21) during the biosynthesis.

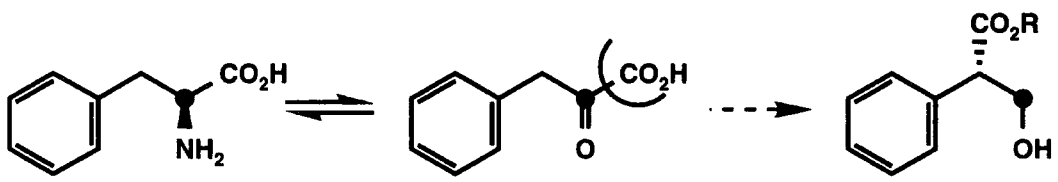


Fig. 21

L-[1-^{14}C]-Phenylalanine was later shown to specifically and efficiently label the carboxyl group of tropic acid (fig. 22), which demonstrated that all the carbons of tropic acid were derived from L-phenylalanine.[40, 41]

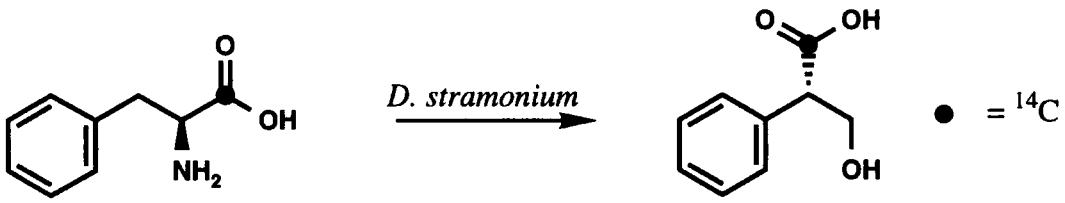
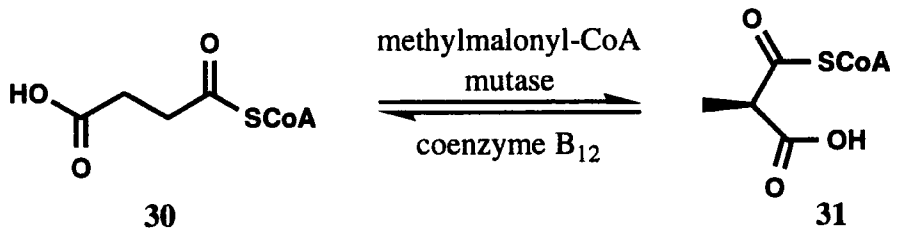


Fig. 22

The discovery of the coenzyme B_{12} mediated rearrangement of succinyl-CoA (**30**) to methylmalonyl-CoA (**31**) (fig. 23)[42] at the time prompted Leete to speculate[41] that the rearrangement of the linear propanoid L-phenylalanine side chain to the isopropanoid tropate side chain might have a related mechanism.



30 **31**

Fig. 23

Leete *et al*[43] later carried out a definitive experiment administering DL-[1,3-$^{13}C_2$]-phenylalanine to *D. innoxia* plants and observed that the ^{13}C enriched sites were

coupled in the ^{13}C NMR spectrum of the isolated hyoscyamine. This study, made possible by the advent of high field NMR, demonstrated unequivocally that tropic acid was formed as the result of an *intramolecular* rearrangement of L-phenylalanine (fig. 24).

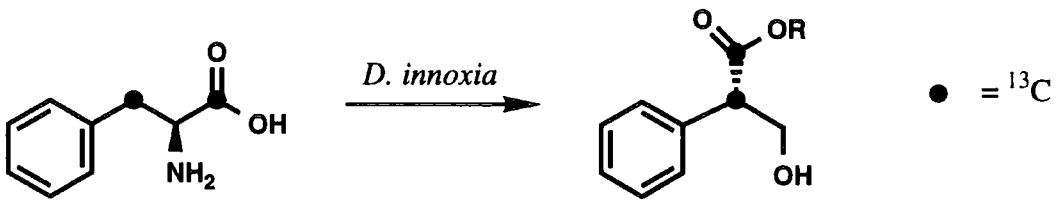


Fig. 24

1.5 Identification of Intermediates Between L-Phenylalanine and Hyoscyamine

Since the realisation that all the carbons of L-phenylalanine are retained in the tropate skeleton, interest in the pathway has been maintained although intermediates between L-phenylalanine and hyoscyamine have only recently emerged. The identification of (R)-D-phenyllactic acid (**32b**) as a direct precursor is the subject of Chapter 2.[44,45] Further, Robins *et al*[46] have recently shown that littorine is the most immediate precursor to hyoscyamine to date. The rearrangement thus occurs at the tropine ester level (fig. 25).

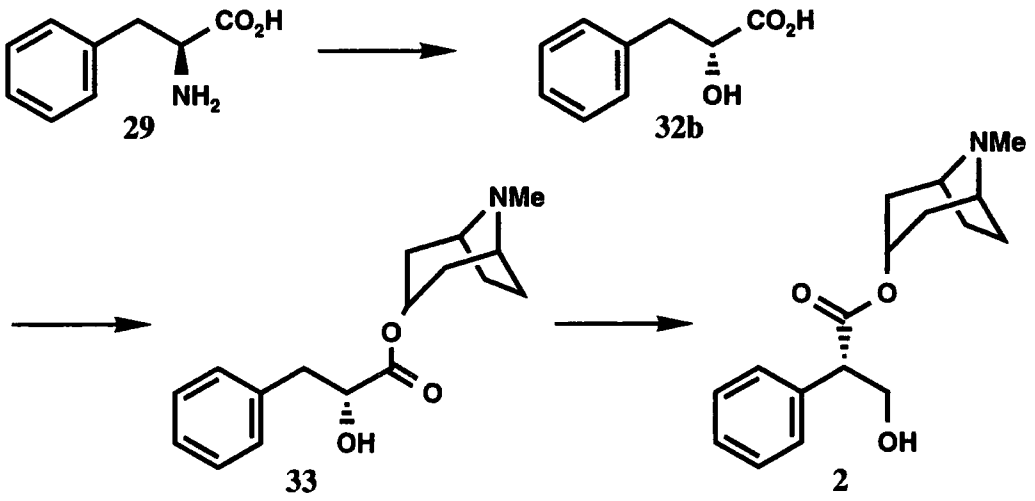


Fig. 25

1.6 The Stereochemistry of Tropic Acid Biosynthesis

The fate of the hydrogens at C-3 of phenylalanine was first investigated independently by Haslam *et al*[47] and Leete[48,49] who came to contradictory conclusions. Leete's interpretation was that a vicinal interchange process was operating, *i.e.* that a hydrogen from C-3 of L-phenylalanine migrated in the reverse direction to the carboxyl moiety. He further concluded it was the 3-*pro*-S hydrogen of L-phenylalanine which underwent the backmigration, resulting in retention of configuration at C-2 of tropic acid (fig. 26). This view has gained general acceptance.

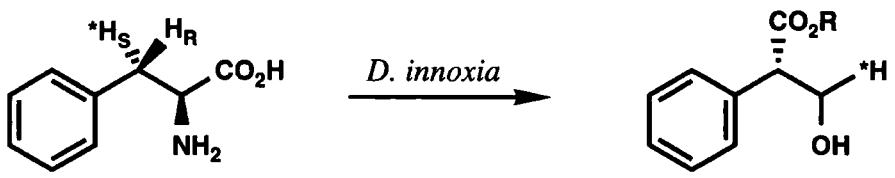


Fig. 26

The stereochemical course at the other migration terminus had not previously been investigated. It was not known whether the C-2 hydrogen of (R)-D-phenyllactic acid occupied the 3'-*pro*-R of 3'-*pro*-S site of hyoscyamine after the rearrangement. Through the use of chiral methyl methodology we have demonstrated that this hydrogen occupies the 3'-*pro*-S site, and that the new hydrogen is introduced to the 3'-*pro*-R site, and therefore the configuration at this centre is inverted (fig. 27) during the biosynthesis.[50]

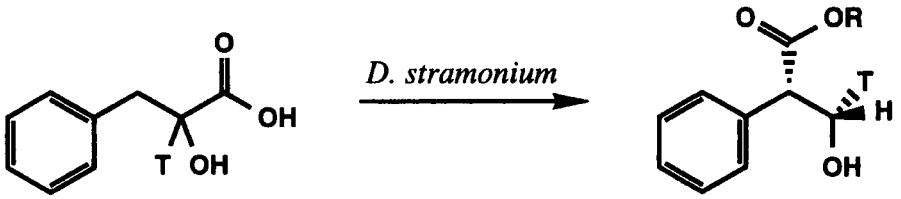


Fig. 27

This result indicated the operation of a retention - inversion process, which was difficult to reconcile with the accepted view of a vicinal interchange process, as the hydrogen would have to be removed from and delivered to opposite faces of the molecule. This

prompted us to re-evaluate the stereochemical course of the rearrangement at C-2' of hyoscyamine.[51] From our results we could find no evidence for a vicinal interchange process, but observed the complete loss of the 3'-*pro*-R hydrogen of (R)-D-phenyllactic acid and the retention of the 3'-*pro*-S hydrogen at C-2' of hyoscyamine (fig. 28). These conclusions, which contradict Leete's results, are discussed in Chapter 3.

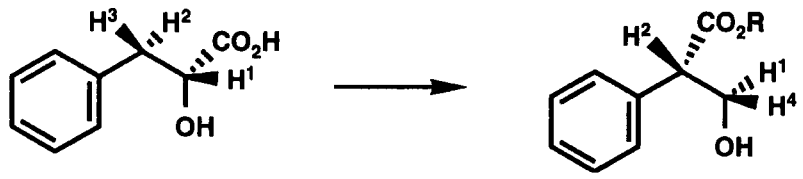
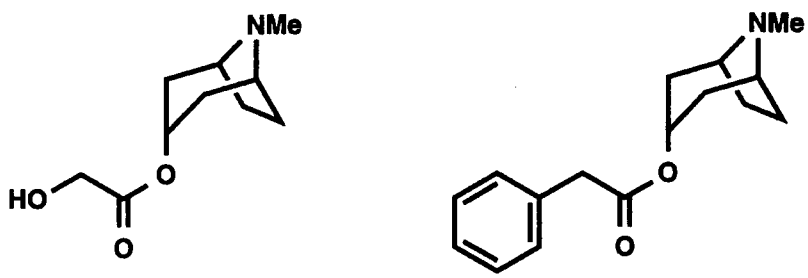


Fig. 28

Finally, the incorporation of a number of phenyllactic acids into two of the minor tropane alkaloids, 2'-hydroxyacetoxytropane (**34**) and phenylacetoxytropane (**35**) (fig. 29) was investigated in collaboration with Dr Richard Robins, then at the AFRC Institute of Food Research in Norwich. We present evidence, in Chapter 4, that these two alkaloids are shunt metabolites of the rearrangement of littorine to hyoscyamine and propose a putative mechanism for the rearrangement, involving an iron-oxo initiated radical process.[52]



2'-hydroxyacetoxytropane (**34**) phenylacetoxytropane (**35**)

Fig. 29

1.7 References

1. J. Mann, *Murder, Magic and Medicine*, Oxford Unversity Press, Oxford, New York, Tokyo, 1992, p23-29 and p76-84
2. W. H. Lewis and M. P. F. Lewis, *Medicinal Botany,* John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, 1977
3. H. E. Flores, *Chem. Ind,* 1992, 374
4. K. Saito, M. Yamazaki, A. Kawaguchi and I Murakoshi, *Tetrahedron*, 1991, **47**, 5955
5. R. F. Dawson, *Science*, 1941, **94**, 396
6. J. Payne, J. D. Hamill R. J. Robins and M. C. J. Rhodes, *Planta Med.,* 1987, **53**, 474
7. J. D. Hamill, A. J. Parr, M. C. J. Rhodes, R. J. Robins, and N. J. Walton, *Biotechnology*, 1987, **5**, 800
8. E. Leete, L. Marion and I. D. Spencer, *Can. J. Chem.*, 1954, **32**, 1116
9. E. Leete, *J. Am. Chem. Soc.,* 1962, **84**, 55
10. E. Leete, *Tetrahedron Lett.*, 1964, **24**, 1619
11. T. Hashimoto, Y. Yamada and E. Leete, *J. Am. Chem. Soc.,* 1989, **111**, 1141
12. E. Leete, *J. Am. Chem. Soc.*, 1982, **104**, 1043
13. E. Leete, *Planta Med.*, 1990, **56**, 339
14. A. Ahmad and E. Leete, *Phytochemistry*, 1970, **9**, 2324
15. F. E. Baralle and E. G. Gross, *J. Chem. Soc., Chem. Commum.*, 1969, 721
16. E. Leete, *Planta Med.*, 1979, **36**, 97
17. H. W. Leibisch, W. Maier and H. R. Schütte, *Tetrahedron Lett.*, 1966, **34**, 4079
18. S. Mizusaki, T. Kisaki and E. Tamaki, *Plant Physiol,*. 1968, **4**, 393
19. E. Leete, *J. Am. Chem. Soc.*, 1967, **87**, 7081
20. J. Kaczkowski and L. Marion, *Can. J. Chem.*, 1963, **26**, 1725
21. E. Leete and M. C. Louden, *Chem. Ind.,* 1963, **26**, 1725
22. R. J. Robins and N. J. Walton, *The Alkaloids*, 1993, **44**, 115, and references cited therein
23. N. J. Walton, R. J. Robins and A. C. J. Peerless, *Planta*, 1990, **182**, 136

24. H. W. Leibisch, K. Peisker, A. S. Radwan and H. R. Shütte, *Z. Pflanzenphysiol.*, 1972, **67**, 1

25. D. G. O'Donovan and M. F. Keogh, *J. Chem. Soc., C,* 1969, 223

26. T. Endo, N. Hamaguchi, T. Hashimoto and Y. Yamada, *FEBS Lett.*,1988, **234**, 86

27. E. Leete and S. H. Kim, *J. Am. Chem. Soc.,* 1988, **110**, 2976

28. T. Hemscheidt and I. D. Spencer *J. Am. Chem. Soc.*,1992, **114**, 5472

29. T. W. Abraham and E. Leete, *J. Am. Chem. Soc.*, 1995, **117**, 8100

30. G. Fodor, A. Romeike, G. Janzo and I. Koczar, *Tetrahedron Lett.*,1959, **7**, 19

31. T. Hashimoto and Y. Yamada, *Plant Physiol.*, 1986, **81**, 619

32. T. Hashimoto and Y. Yamada, *Eur. J. Biochem.*, 1987, **164**, 277

33. T. Hashimoto, J. Kohno and Y. Yamada, *Phytochemistry*, 1989, **28**, 1077

34. R. Robinson, *Proceedings of the University of Durham Philosophical Society*, 1927 - 1933, **8**, 14

35. E. M. Trautner, *Aust. Chem. Inst. J. and Proc.,* 1947, **14**, 411

36. E. Leete, *J. Am. Chem. Soc.,* 1960, **82**, 612

37. E. Wenkert, *Experienta*, 1959, **15**, 165

38. E. W. Underhill and H. W. Youngken, *J. Pharm. Sci.*, 1962, **51**, 121

39. E. Leete and M. L. Louden, *Chem. Ind.*, 1961, 1405

40. M. L. Louden and E. Leete, *J. Am. Chem. Soc.*, 1962, **84**, 1510

41. M. L. Louden and E. Leete *J. Am. Chem. Soc.,* 1962, **84**, 4507

42. R. Stjernholm and H. G. Wood, *Proc. Natl. Acad. Sci.,* 1961, **47**, 303

43. E. Leete, N. Kowanko and R. A. Newmark, *J. Am. Chem. Soc.*, 1975, **97**, 6826

44. N. C. J. E. Chesters, D. O'Hagan, R. J. Robins, *J. Chem. Soc. Perkin Trans, I,* 1994, 1159

45. N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, *J. Chem. Soc., Chem. Commun.,* 1995, 127

46. R. J. Robins, P. Bachmann and J. G. Woolley, *J. Chem. Soc., Perkin Trans.* I, 1994, 615

47. R. V. Platt, C. T. Opie and E. Haslam, *Phytochemistry*, 1984, **23**, 2211

48. E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271

49. E. Leete, *Can. J. Chem.*, 1987, **65**, 226

50. N. C. J. E. Chesters, D. O'Hagan, R. J. Robins, A. Kastelle and H. G. Floss, *J. Chem.* Soc., *Chem. Commun.*, 1995, 129

51. N. C. J. E. Chesters, K. Walker, D. O'Hagan and R. J. Robins, *J. Am. Chem. Soc.*, in press

52. R. J. Robins, N. C. J. E. Chesters, D. O'Hagan, A. J. Parr, N. J. Walton and J. G. Woolley, *J. Chem. Soc., Perkin Trans. I*, 1995, 481

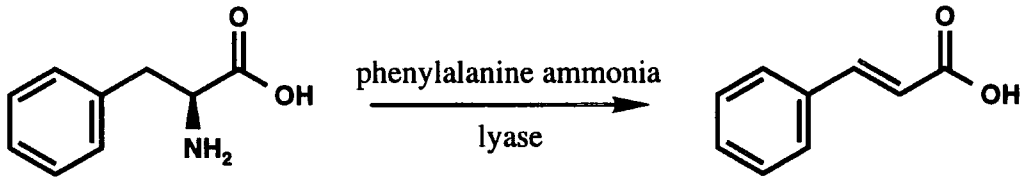
CHAPTER 2

Intermediates Between L-Phenylalanine and Hyoscyamine

2.1 The Intermediacy of Phenyllactic Acid

It has been established since 1962 that all the carbon atoms of the phenylalanine side chain are utilised in tropic acid biosynthesis.[1] Further, an intramolecular carbon skeletal rearrangement is involved[2] between L-phenylalanine and hyoscyamine. However, the true intermediates have only recently emerged from an array of candidate precursors.

Cinnamic acid (**36**) is a key intermediate in the biosynthesis of a wide variety of non-nitrogenous aromatic plant secondary metabolites. These include cinnamoyl esters, alcohols, flavanones, xanthones and benzoic ester derivatives.[3] Cinnamic acid is derived directly from L-phenylalanine by the action of phenylalanine ammonia lyase (fig. 30).



29 **36**

Fig. 30

Its widespread occurrence in the biosynthesis of plant phenylpropanoids (C_6-C_3 compounds) made it an attractive intermediate in tropic acid biosynthesis.[4] However, Woolley[5] and Leete[6,7] have independently failed to observe the incorporation of radiolabelled cinnamic acids into the tropane alkaloids. Experiments have also been carried out with (R)- and (S)-3-hydroxy-3-phenylpropionic acids (**37**)[6] and epoxycinnamic acid (**38**)[7], both plausible oxygenated derivatives of cinnamic acid. Some support for this hypothesis was provided by the evidence that the epoxide of cinnamic acid, as its phenyl thiolester (**39**) could be induced to rearrange *in vitro* with

the addition of boron trifluoride etherate,[8] through carboxyl migration,[9] to furnish the tropate skeleton as α-formylphenylthiolacetate (**40**). The tropate oxidation level could then be achieved by a selective reduction with sodium cyanoborohydride[9] (fig. 31). However, no *in vivo* incorporations of these acids have been observed.

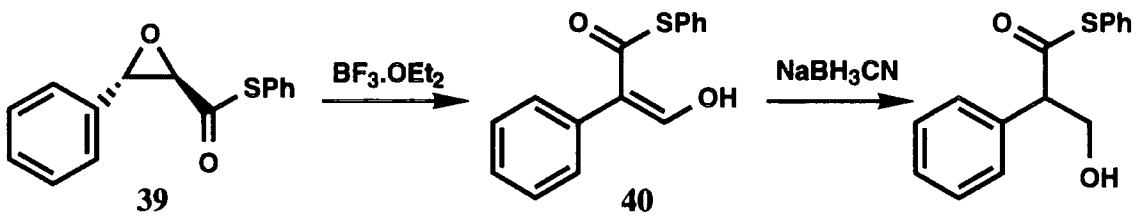


Fig. 31

There is one isolated report of the incorporation of cinnamic acid into tropate esters.[10] However, this has subsequently been disregarded as the probable result of radiochemical contamination of the alkaloids with a cinnamic acid derivative.[6] More recently the intermediacy of 2-phenyl-3-aminopropionic acid (**41**) has been investigated,[11] the putative product of a rearranging enzyme acting directly on phenylalanine. However, in the event this compound was not incorporated into hyoscyamine in *D. innoxia* plants. These results are summarised in fig. 32.

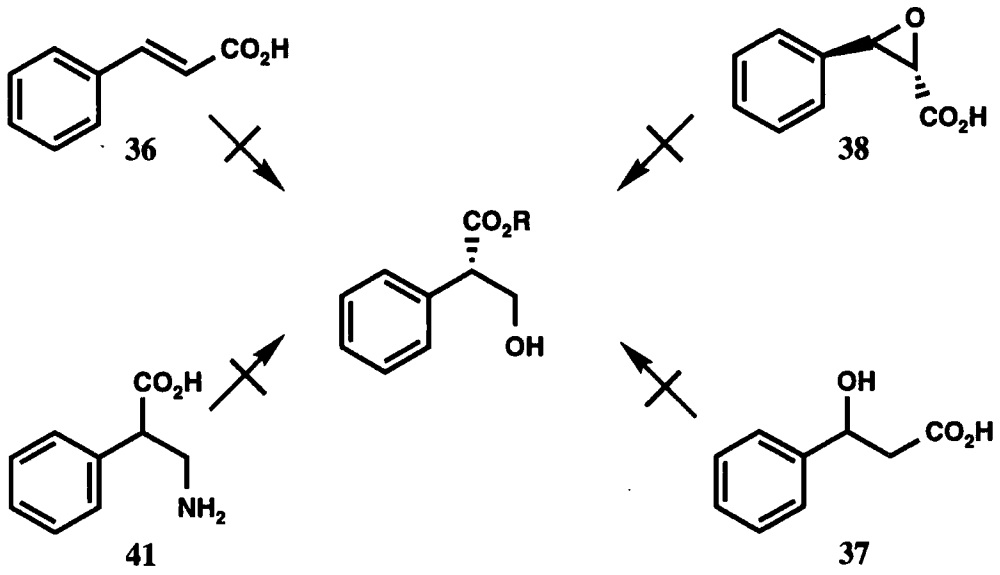


Fig. 32

Attention then turned to two other phenylpropanoid carboxylic acids, phenylpyruvic acid (**28**) and phenyllactic acid (**32**). These were clearly potential precursors since the two can interconvert with L-phenylalanine (**29**), through transammination and reduction, processes which are facile and well documented. [12-15] (fig. 33).

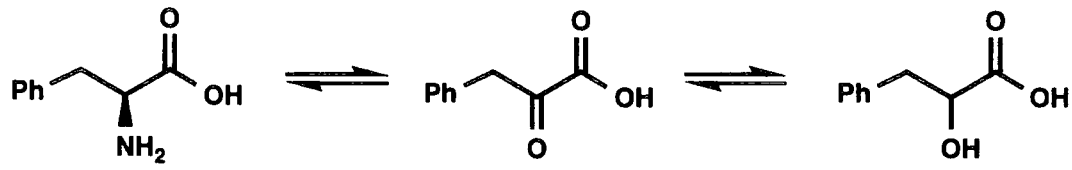


Fig. 33

All of these acids are incorporated into the tropane alkaloids at high and approximately equal levels.[5,7,16-18] Consequently feeding experiments with these precursors, carrying isotopic labels on the carbon skeleton, had not delineated the closer precursor. Phenyllactic acid is an attractive precursor as the functional group interconversion of the -NH_2 to -OH is then set up to furnish the hydroxymethyl moiety of tropic acid.

A mounting body of circumstantial evidence has appeared in the literature over the past 25 years in support of this view. In 1969 Evans and V. A. Woolley[19] observed the incorporations of ^{14}C labelled phenylalanines into littorine (**33**), the ester of

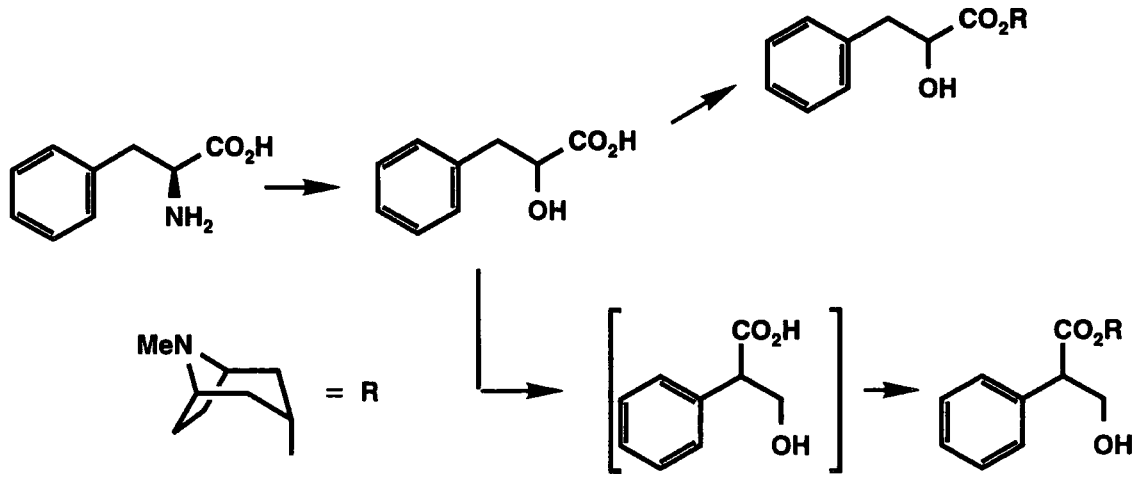


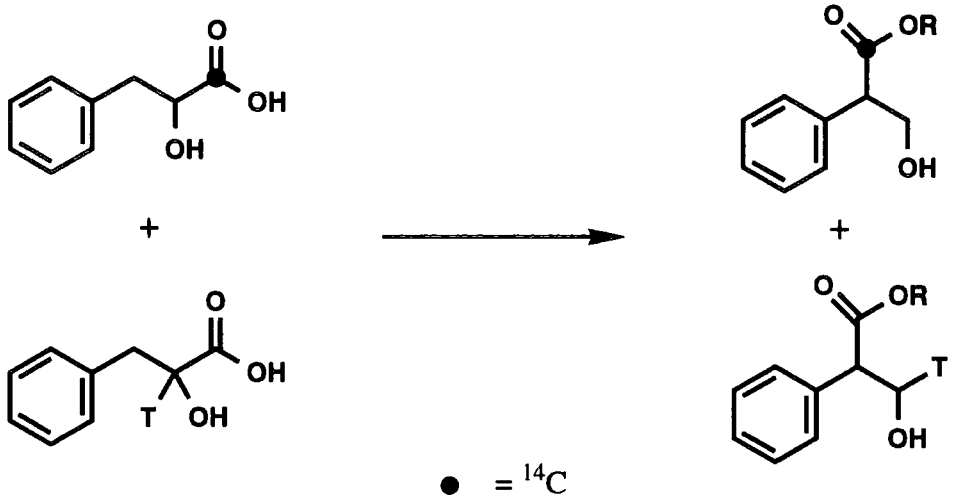
Fig. 34

phenyllactic acid (**32**) and tropine (**23**), hyoscyamine (**2**) and scopolamine (**3**). Their observation that the level of incorporation of radioactivity decreased in the order littorine > hyoscyamine > scopolamine was consistent with phenylalanine being a more direct precursor to phenyllactate than tropate (fig. 34).

More recently Kitamura *et al.*[20] have directly observed the interconversion of phenylalanine (**29**) and phenyllactic acid (**32**) in tropane alkaloid producing root cultures of *Duboisia lecharditti.* This was shown by using an isotope trapping technique. The technique involved the simultaneous feeding of [1-^{14}C]-phenylalanine and unlabelled phenyllactic acid. After incubation, the phenyllactic acid isolated from the root cultures was rigorously purified and found to be radioactive, *i.e.* the radioactive phenylalanine had been "trapped" as phenyllactate. Incorporations of radioactivity into the alkaloids were reduced in comparison to previous experiments feeding only the [1-^{14}C]-phenylalanine without phenyllactic acid. Trapping techniques have precedence in the determination of biosynthetic intermediates, however, the possibility that the trapped phenyllactate was not used for the production of tropane alkaloids, but for some other metabolite, could not be excluded. Attempts were also made to trap both phenylpyruvic acid (**28**) and tropic acid (**1**) without success. Phenylpyruvic acid (**28**) is a necessary intermediate between phenylalanine (**29**) and phenyllactic acid (**32**) and the failure to trap this acid has been attributed to the inability of the cultures to directly utilise exogenously added phenylpyruvic acid in alkaloid biosynthesis.

Competitive feeding experiments with [1-^{14}C]-phenyllactic acid and [2-^{14}C]-phenylpyruvic acid together, with unlabelled precursors, placed phenyllactic acid closer than phenylpyruvic acid to the tropate moiety.[16] Subsequently Ansarin and Woolley[21] inferred the obligate intermediacy of phenyllactic acid from the observation that tritium from (RS)-[1-^{14}C, 2-^{3}H]-phenyllactic acid is incorporated into the tropoyl moiety of hyoscyamine (fig. 35). Over a series of experiments the ^{3}H:^{14}C ratio of the recovered tropic acid remained similar to the administered phenyllactic acid. This result, although surprising, given that racemic phenyllactic acids were administered and despite the very

low incorporation (0.01%), provided the strongest evidence to that date that phenyllactic acid was an obligatory intermediate in the biosynthesis of tropic acid.



● = ^{14}C

Fig. 35

In light of the mounting evidence for the intermediacy of phenyllactic acid, a definitive experiment was conducted to lay the issue to rest.

2.2 Experimental Work to Determine the Role of Phenyllactic Acid

As discussed previously, phenylalanine (**29**), phenylpyruvic acid (**28**) and phenyllactic acid (**32**) readily interconvert and all are incorporated efficiently into the tropoyl moiety of hyoscyamine (**2**) and scopolamine (**3**). Labelling solely on the carbon skeleton of these molecules is therefore insufficient to fully assess their position in the tropic acid biosynthetic pathway. Here a dual ^{13}C-^{2}H isotopic labelling strategy was employed. If deuterium retention from the chiral centre of phenyllactic acid (**32**) could be demonstrated then this would place it as a more direct precursor to tropate than phenylpyruvic acid (**28**) or phenylalanine (**29**), since oxidation of the phenyllactate to phenylpyruvate would result in irretrievable loss of deuterium (fig. 36). The deuterium was tagged to a ^{13}C reporter atom to render its presence identifiable in the ^{13}C NMR spectrum. This technique can also be used to assess the extent of deuterium washout. To this end [2-^{13}C,^{2}H]-phenyllactic acid (**32a**) was synthesised and administered to transformed root cultures of *Datura stramonium*.[22]

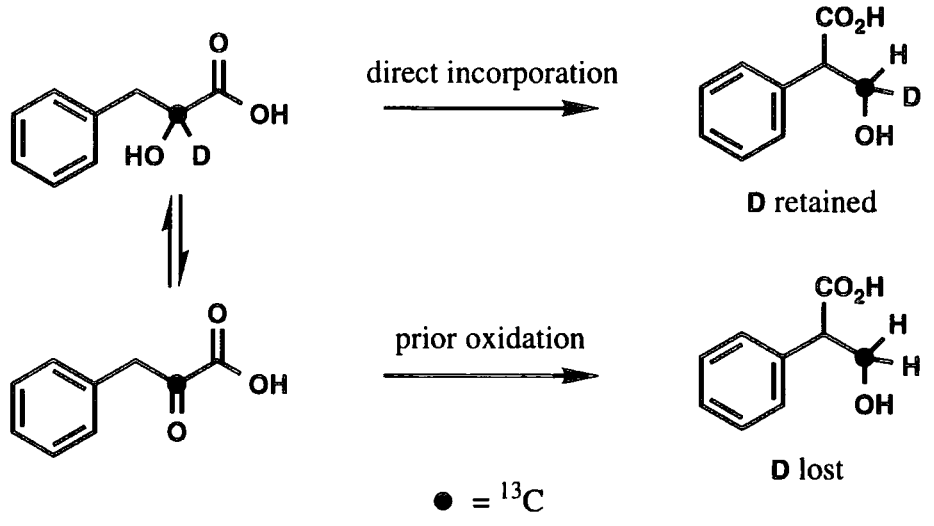
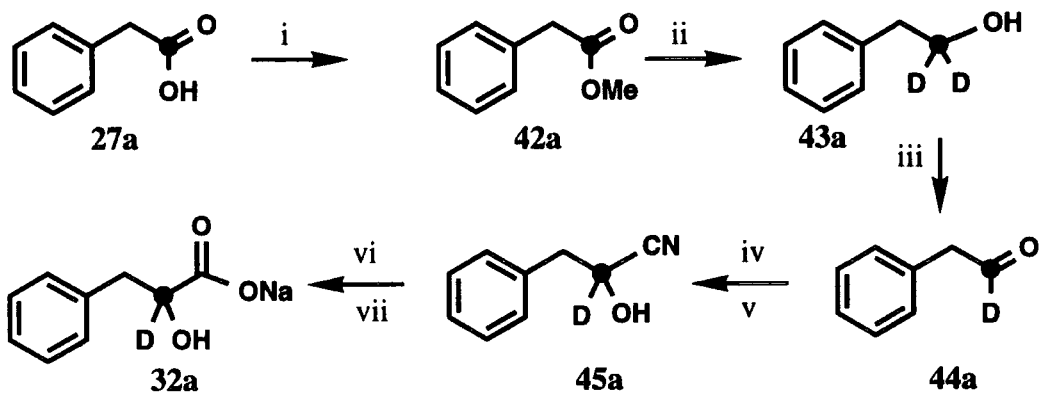


Fig. 36

2.3 The Synthesis of [2-^{13}C, ^{2}H]-Phenyllactate (32a)

[2-^{13}C,^{2}H]-Phenyllactic acid was synthesised according to the route outlined in scheme 1, starting from the commercially available [1-^{13}C]-phenylacetic acid (**27a**). The carboxylic acid was converted to its methyl ester (**42a**) and then the deuterium was introduced by reduction with lithium aluminium deuteride. Oxidation to phenylacetaldehyde (**44a**) with PCC followed by formation of the cyanohydrin (**45a**), and then hydrolysis, generated the required phenyllactic acid. This route proved versatile in the syntheses of a number of other labelled phenyllactic acids and has the potential to be further modified to place a ^{13}C label at either C-1 or C-3 of phenyllactic acid by the use of sodium [^{13}C]-cyanide or [2-^{13}C]-phenylacetic acid respectively.



(i) CH_2N_2, (ii) $LiAlD_4$ (iii) PCC, 3Å sieves, (iv) $Na_2S_2O_5$,
(v) NaCN (vi) 50% HCl (vii) 0.1M NaOH

Scheme 1

2.4 [1-^{13}C, $^{2}H_2$]-2-Phenylethanol (43a)

[1-^{13}C]-Phenylacetic acid (**27a**) was converted to its methyl ester (**42a**) in quantitative yield by treatment with an excess of diazomethane in ether. This modification proved necessary in order to effect a smooth reduction in the next step. Reduction with lithium aluminium deuteride then generated [1-^{13}C, $^{2}H_2$]-2-phenylethanol (**43a**) in 86% yield.

2.5 [1-^{13}C, ^{2}H]-Phenylacetaldehyde (44a)

The oxidation of 2-phenylethanol to phenylacetaldehyde was a key step in the synthesis of phenyllactic acid. This route was selected over an alternative strategy which involved introducing the deuterium by the direct reduction of either methyl phenylacetate or benzyl cyanide to phenylacetaldehyde, with an appropriately labelled reducing agent. Also a route to [1-^{13}C]-benzyl cyanide has already been established by Cox.[23] A preparation of $Li(OEt)_3H$ from lithium aluminium hydride and ethanol has been published by Brown and Shoaf[24] which would be amenable to the preparation of the deuterated analogue. This reagent proved versatile for the reduction of a variety of aliphatic and aromatic nitriles to generate aldehydes in good yield, however in the case of benzyl cyanide the enhanced acidity of the methylene protons simply led to the evolution of hydrogen rather than reduction. It was anticipated that this would be a general problem with this methodology and thus the route was abandoned.

Attention then focused on the optimisation of the oxidation reaction. The Swern oxidation (DMSO and $(COCl)_2$) is a classic and general route to aldehydes and ketones from alcohols. In an early paper discussing this reaction, Omura and Swern report only a 23% yield for the oxidation of 2-phenylethanol to phenylacetaldehyde by this method,[25] the remaining material being principally unreacted starting material. Both benzyl alcohol and 3-phenylpropanol were oxidised efficiently under these conditions and no explanation for this anomalous result was offered.

Another potential approach involved the use of ruthenium dioxide and potassium periodate.[26] This reaction is carried out in a biphasic solution of water : acetonitrile : tetrachloromethane (3:1:1). Initially water soluble ruthenium dioxide is oxidised by the periodate to organic soluble ruthenium tetroxide. The ruthenium tetroxide is then reduced back to ruthenium dioxide with the concomitant oxidation of the alcohol. However, after 24 hours the oxidation of 2-phenylethanol to phenylacetaldehyde was largely incomplete (33%).

Another classic reagent for the oxidation of alcohols to aldehydes is pyridinium chlorochromate (PCC).[27] While this is a versatile and mild oxidising agent reactions involving PCC characteristically produce sticky brown tars which hinder product isolation. In the event, such reactions were incomplete and low yielding. A modification of the PCC oxidation involves supporting the reagent on either molecular sieves[28] or alumina.[29] This has been shown to accelerate the reaction and also to facilitate the work up. Accordingly, 2-phenylethanol was oxidised efficiently using PCC supported on 3Å molecular sieves, and the crude product could be isolated simply by filtering the reaction mixture away from a brown powder through a silica pad. The product after evaporation, was slightly green, presumably due to contamination with a chromium (III) species, and was purified by distillation. Yields for this reaction were between 70 and 90%. Phenylacetaldehyde is susceptible to polymerisation, which is apparent in the product as a slight cloudiness or as a fine white precipitate. For this reason characterisation of labelled compounds was limited to ^{1}H NMR to avoid loss of material after redistillation immediately prior to use.

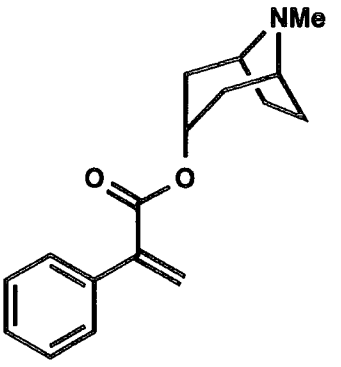
2.6 (RS)-[2-^{13}C,^{2}H]-Phenyllactic Acid (32a)

The cyanohydrin (**45a**) derived from [1-^{13}C, ^{2}H]-phenylacetaldehyde (**44a**) was prepared according to an established procedure.[30] Initially the bisulfite adduct was formed and this was reacted with sodium cyanide. The cyanohydrin was hydrolysed directly without prior purification, using 50% hydrochloric acid, to generate (RS)-[2-^{13}C, ^{2}H]-phenyllactic acid (**32a**) in typically around 40% yield over the two steps. The

acid was then purified by recrystallisation from chloroform. The first time that (RS)-[2-^{13}C,^{2}H]-phenyllactic acid (**32a**) was prepared it became apparent that the material was contaminated with approximately 3.6% [1-^{13}C]-phenylacetic acid (**43a**). This was detected from the unusually prominent carbonyl peak in the ^{13}C NMR spectrum, which had not been visible in unlabelled runs. A small doublet at 3.41ppm was apparent in the ^{1}H NMR and re-examination of the ^{1}H NMR spectra from some of the unlabelled runs revealed a minor singlet at 3.41ppm, consistent with this interpretation. This material was however used directly since phenylacetic acid is not esterified with tropine in *D. stramonium* to any measurable extent, if at all. Some evidence for an enzyme capable of esterifying phenylacetic acid and tropine to generate phenylacetyl tropine has been detected at very low levels on some Solanaceous species[31] but this is not the primary route to phenylacetyl tropine. This assessment was validated by the result of the experiment, which is further discussed in chapter 4. The contamination in the prepared material was presumed to arise as a result of incomplete cyanohydrin formation. Such contamination was avoided in subsequent runs by the repeated recrystallisation of phenyllactic acid.

2.7 Incorporation Studies with (RS)-Phenyllactic Acid

Sodium DL-[2-^{13}C,^{2}H]-phenyllactate was administered to 5 day old transformed root cultures of *D. stramonium* and after 17 days growth the alkaloids were extracted. Initially the crude alkaloid extract was analysed by GC-MS and the mass enhancements of the alkaloids hyoscyamine (**2**), littorine (**33**), and apoatropine (**46**) (the dehydrated derivative of hyoscyamine, (fig. 37)) were assessed. These are given in Table 1. The figures are adjusted to take into account the natural abundance of heavy isotopes, and therefore represent actual incorporations. These results clearly indicate the incorporation of both ^{13}C and ^{2}H labels into hyoscyamine, apoatropine and littorine to give clear M+2 mass enhancements, with smaller M+1 mass enhancements.



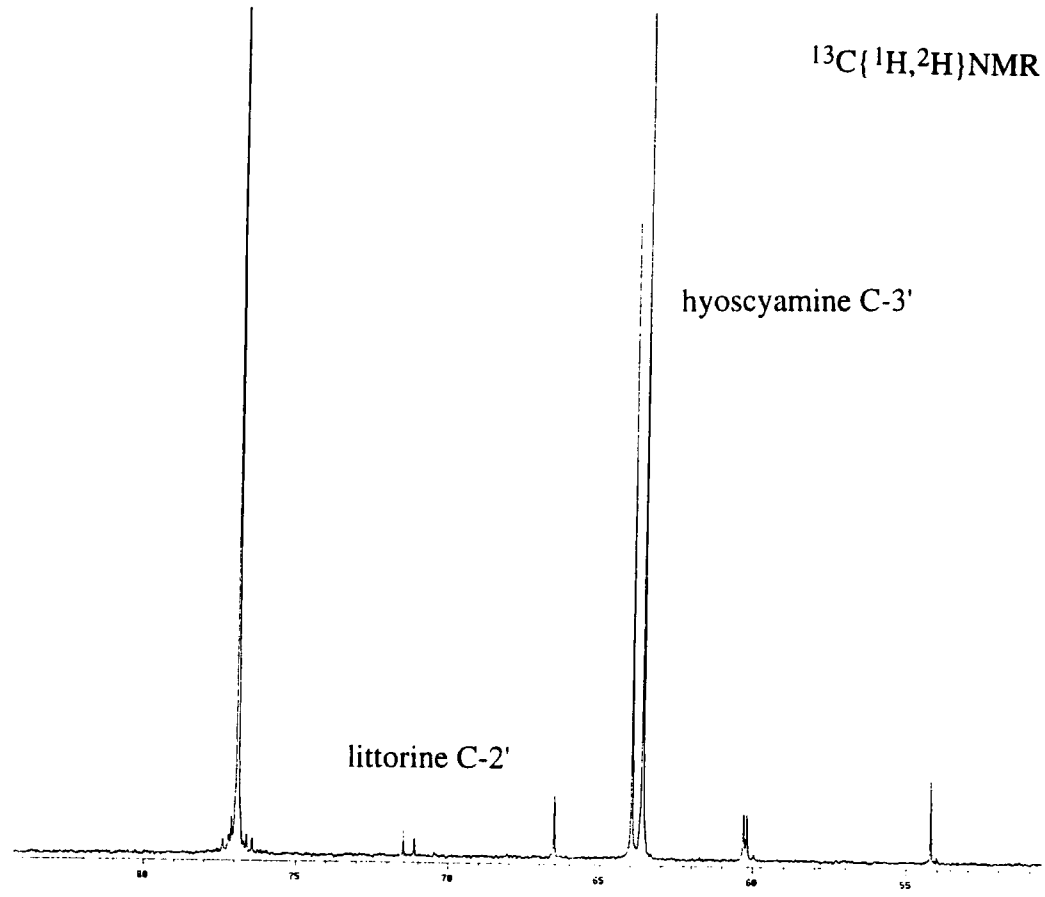
apoatropine (**46**)

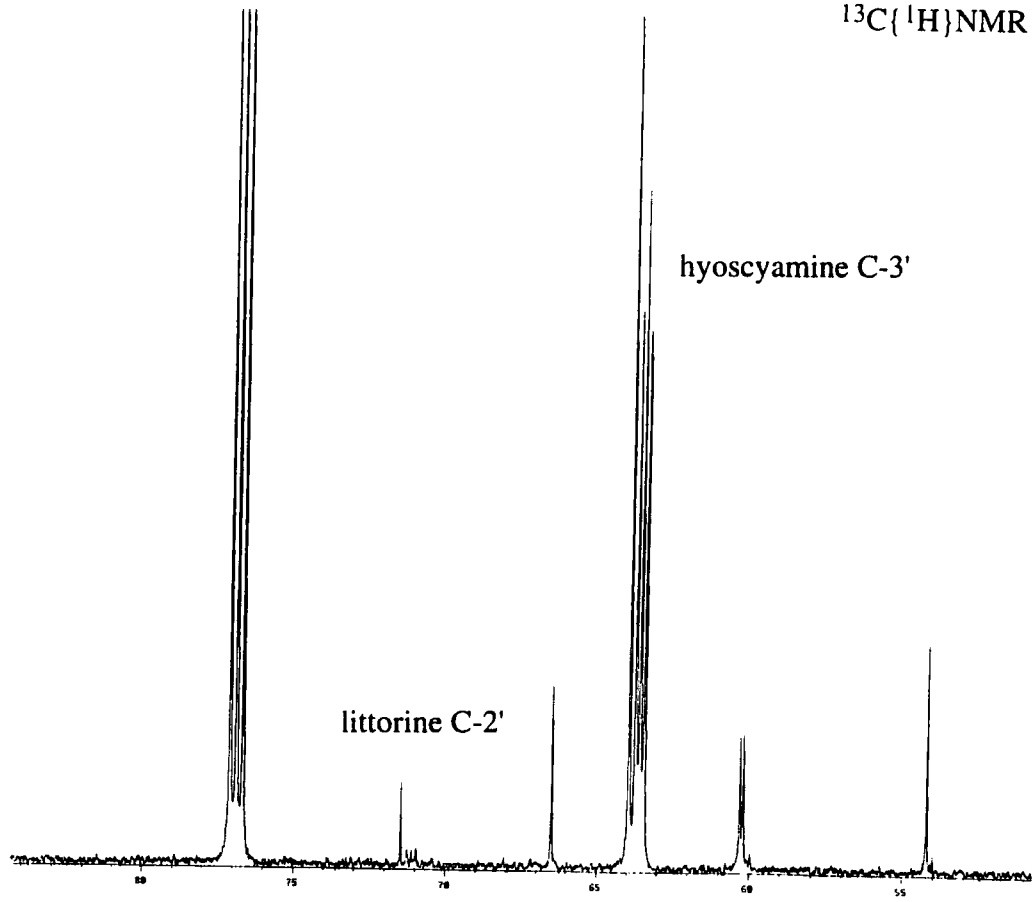
Fig. 37

	Littorine	Hyoscyamine	Apoatropine
M^+	76.92%	77.36%	81.22%
M^++1	5.05%	5.69%	5.83%
M^++2	18.03%	16.95%	12.95%

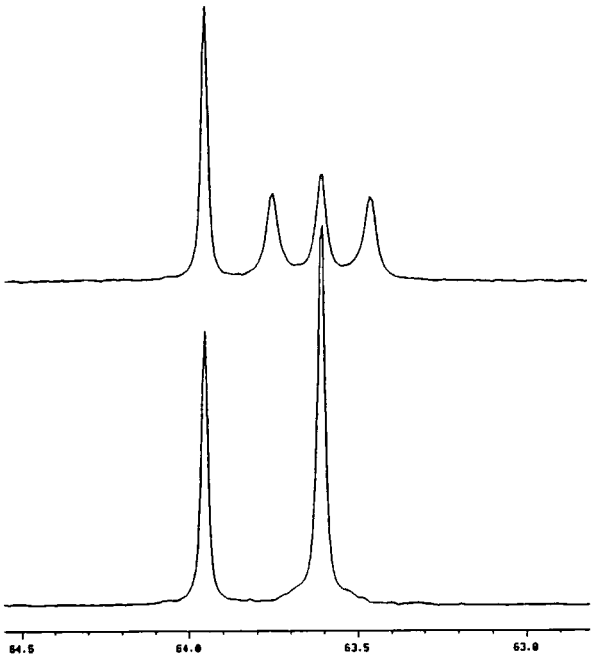
Table 1

Preparative TLC gave hyoscyamine containing a small amount (4.6%) of littorine. Hyoscyamine and littorine have similar chromatographic properties, however, the ^{13}C NMR resonances for the C-1', C-2' and C-3' carbons are distinct, so the small amount of littorine did not interfere with the NMR analysis of the hyoscyamine. The fate of the deuterium label was determined by $^{13}C\{^1H\}$ and $^{13}C\{^1H, ^2H\}$NMR (fig. 38 and fig. 39) analysis. The incorporation of ^{13}C from [2-^{13}C, ^2H]-phenyllactate into C-3' of the tropate ester was clearly visible in the $^{13}C\{^1H\}$NMR spectrum from the enhancement of the peak at 63.9ppm. The adjacent coupled peak 0.35ppm upfield (α-shift) shows the intact incorporation of the ^{13}C-^2H bond from [2-^{13}C, ^2H]-phenyllactic acid. This triplet collapsed to a singlet in the $^{13}C\{^1H,^2H\}$NMR spectrum as expected. A similar pattern is observed for the peaks at 71.5ppm (singlet) and 71.1ppm (triplet) which are attributed to both single and double labelled components with C-2' of littorine.





Hyoscyamine isolated after feeding [2-^{13}C, ^{2}H]-phenyllactic acid
Fig. 38



Hyoscyamine isolated after feeding [2-^{13}C, ^{2}H]-phenyllactic acid
expansion of C-3' region.
Fig. 39

2.8 Discussion

It is clear from these results that the (C-2)-H bond of phenyllactic acid (**32**) can remain intact during the rearrangement process to become the (C-3')-H bond of hyoscyamine. This experiment demonstrates unambiguously that phenyllactic acid (**32**) is a more direct precursor to the tropate ester of hyoscyamine than phenylpyruvic acid (**28**) or L-phenylalanine (**29**), confirming the evidence previously obtained by inference[16,20,21] (fig. 40). It is also apparent from the incorporations into littorine that phenyllactic acid is esterified directly with tropine to form this alkaloid.

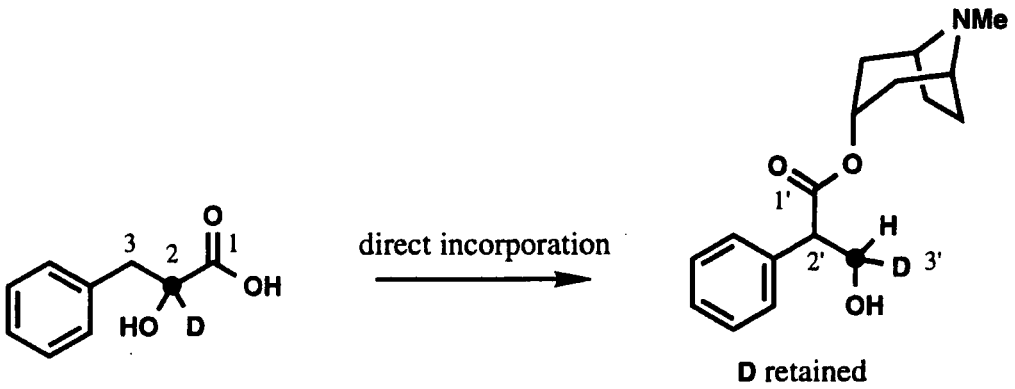


Fig. 40

It is also interesting to note that while the predominantly intact incorporation of the ^{13}C-^{2}H bond (16.95%, hyoscyamine and 18.0% littorine, by GC-MS) was observed there was significant deuterium washout (5.69%, hyoscyamine and 5.05% littorine by GC-MS). This is also clearly evidenced from the ^{13}C enriched singlets at 63.9ppm and 71.5ppm in the $^{13}C\{^{1}H\}$ NMR spectrum corresponding to C-3' of hyoscyamine and C-2' of littorine respectively. The M+1 enhancements in the CG-MS analysis of hyoscyamine (**2**), littorine (**33**) and apoatropine (**46**), are approximately one third of the M+2 enhancements. The most reasonable explanation for this is an interconversion between phenyllactic acid (**32**) and phenylpyruvic acid (**28**) (fig. 41) as expected, especially as only one of the enantiomers present can be a substrate for direct rearrangement. Alternatively a phenyllactate racemase may be operating, interconverting the enantiomeric forms of phenyllactic acid prior to esterification with tropine (**23**).

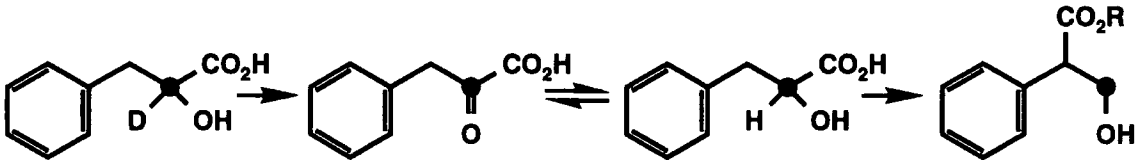


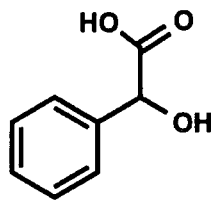
Fig. 41

With this result, the previous contrasting report that negligible loss of ^{3}H relative to ^{14}C occurred[21] is surprising. However, the operation of a kinetic isotope effect and the different biological system used (plants *v.s.* root cultures) may invalidate too critical a comparison between the two experiments.

2.9 The Role of Free Tropic Acid

It was considered for some time that the esterification of tropic acid (**1**) or its co-enzyme-A ester[33] with tropine (**23**) may be a key step in the pathway from L-phenylalanine (**29**) to hyoscyamine (**2**). Evidence has however been accumulating which indicates that *free* tropic acid itself is not an intermediate in hyoscyamine biosynthesis. The first piece of evidence came from the observation of Robins *et al.* [34, 35] that exogenously added tropic acid decreased rather than increased the tropane

alkaloid production in *Datura* root cultures. This result was mirrored with mandelic acid (**47**) (fig. 42), a tropic acid analogue, which was not esterified.



mandelic acid (**47**)

Fig. 42

However, no such decrease in alkaloid production was observed when phenyllactic acid (**32**), a known precursor, was administered. Hyoscyamine (**2**), when fed to the cultures, was found to accumulate to levels 30-fold in excess of natural concentrations and Robins *et al.* therefore concluded that it was neither the ability to accumulate hyoscyamine, nor the availability of the esterifying acid which limited hyoscyamine production, but rather that the tropic acid could not be directly utilised. More evidence to support this view can be drawn from the observation of Simola *et al.* that the addition of tropine (**23**) and tropic acid (**1**) to suspension cultures of *Atropa belladonna* failed to stimulate alkaloid production.[36]

In contrast there have been some reports that tropic acid *increased* the tropane alkaloid production,[37-39] although this has only been observed in low yielding cultures.[18] Further, one early report describes the esterification of exogenously added [^{14}C]-tropic acid and tropine to form hyoscyamine in *Nicotiana tabacum,* however, these plants do not normally produce tropane alkaloids and this is the only report of this esterification taking place in a non-tropane alkaloid producing species.[40] These conflicting reports prompted two groups to carry out some definitive studies to establish or otherwise the intermediacy of free tropic acid.

In 1992 Kitamura *et al.*[41] compared the efficiency of incorporation of radiolabelled L-phenylalanine (**29**) and tropic acid (**1**) into the hyoscyamine (**2**) from

Duboisia leichardtii. While L-phenylalanine was incorporated into the acid moiety of the alkaloid at the high level expected from Leete's observations[42] the tropic acid was incorporated at a very low level (less than 1%). Their subsequent failure to trap tropic acid in a similar manner to the trapping of phenyllactic acid[20] combined to provide the strongest evidence to date that tropic acid is not an intermediate. The authors could not however preclude the possibility that tropic acid was metabolised to non-alkaloidal products before reaching the site of alkaloid biosynthesis. The conversion of tropic acid to glucose esters has been observed in *Datura innoxia* and to its glycoside in *Eucalyptus periniana,*[43] equally, such processes could explain the failed incorporation.

It has been extensively demonstrated that free phenyllactic acid (**32**) is incorporated into hyoscyamine (**2**)[16,17] and that it is a direct precursor to the tropate moiety.[21,22] The relationship between phenyllactic acid (**32**) and tropic acid (**1**) has recently been evaluated by Robins *et al.*[18,44] [1,3-$^{13}C_2$]-Phenyllactic acid was administered to transformed root cultures of *D. stramonium* or *Brugmansia candida* x *aurea* and the expected high level of incorporation was observed. However, when the labelled phenyllactate was fed in competition with unlabelled tropic acid no reduction in the phenyllactate incorporations into hyoscyamine (**2**) or apoatropine (**46**) were observed, as would be expected if the exogenously added tropic acid was competing with tropic acid derived from phenyllactic acid for esterification with tropine. Incorporations of the labelled phenyllactic acid into littorine (**33**) were enhanced, which suggests the excess tropic acid had stimulated the metabolism of phenyllactate to littorine. From these results it is clear that tropic acid (**1**) cannot be placed as a more immediate precursor to hyoscyamine (**2**) than phenyllactic acid (**32**).

These experiments combine to demonstrate that free tropic acid in not an intermediate in the biosynthesis of hyoscyamine. None of these results however negate the possibility that an activated from of tropic acid, such as its co-enzyme A ester, is involved. This is discussed in the following section.

2.10 Littorine: The Substrate for the Rearranging Enzyme

The direct rearrangement of littorine (**33**) was first investigated by Leete,[7] in whole plants of *D. innoxia*, who observed that the ^{3}H : ^{14}C ratio in hyoscyamine after feeding [3β-^{3}H, 1'-^{14}C]-littorine was substantially different to the ^{3}H : ^{14}C ratio in the administered substrate (fig. 43). This result was consistent with the hydrolysis of the [3β-^{3}H, 1'-^{14}C]-littorine to [3β-^{3}H]-tropine and [1-^{14}C]-phenyllactic acid and separate use of the acid and base in the formation of hyoscyamine after dilution from the non-radioactive pools, *i.e.* the phenyllactyl moiety of littorine did not undergo rearrangement to tropate while esterified to tropine. It is known that tropine esters will hydrolyse *in vivo*[45, 46] and Leete did not ignore the possibility that the facile hydrolysis of littorine was also reversible. Hydrolysis followed by dilution of the label and re-esterification would also effect a change in the ^{3}H : ^{14}C ratio, masking any small amount of littorine rearranging directly. This was however dismissed as improbable since the ^{3}H ; ^{14}C ratio in the recovered littorine was essentially the same as in the administered littorine. If such hydrolysis and re-esterification was occurring then the littorine ^{3}H : ^{14}C ratio would also be expected to change.

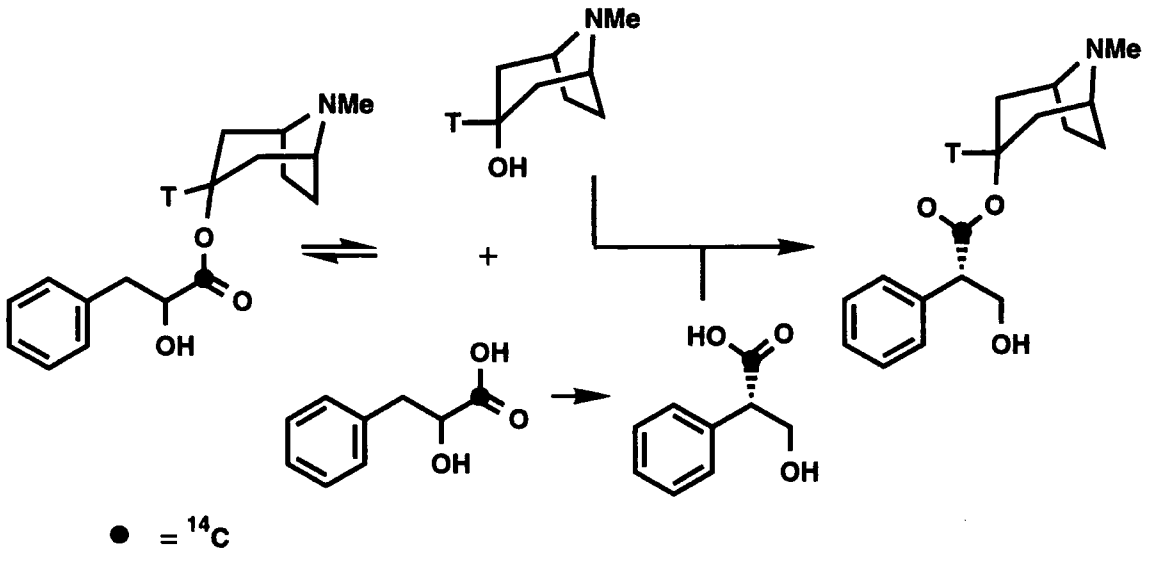


Fig. 43

Subsequent studies in root cultures of *D. stramonium* have however placed littorine as the substrate for the rearranging enzyme. The intermediacy of littorine was

first implied by Sauerwein *et al.*[47] They observed that the level of incorporation of [1-^{13}C, 2-$^{2}H_3$]-acetate into the acetate derived carbons of the tropane skeleton decreased in the order tropinone > tropine > littorine > hyoscyamine > 6-hydroxyhyoscyamine and scopolamine. This was seen as an indication as to their position in the biosynthetic pathway, the earlier intermediates being the more highly enriched. The intermediacy of littorine was directly and recently re-examined by Robins *et al.*[48] Quintupally labelled littorine, prepared from [*methyl*-$^{2}H_3$]-tropine and [1,3-$^{13}C_2$]-phenyllactic acid (fig. 44). and was administered to transformed root cultures of *D. stramonium*.

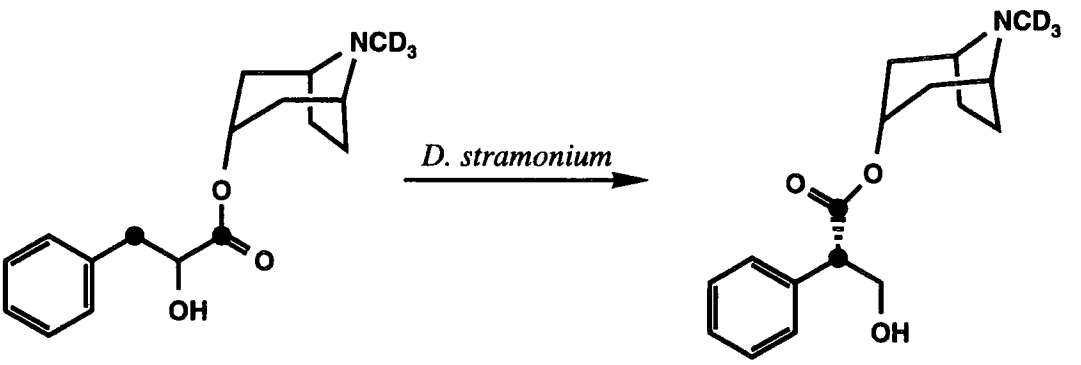


Fig. 44

The alkaloids were examined by GC-MS analysis and the results from feeding the quintupally labelled littorine, without dilution, are summarised in Table 2.

	Littorine	**Hyoscyamine**	**Tropine**	**Methyl Phenyllactate**
M +5	8.7%	4.4%	___	___
M +3	3.9%	4.7%	12.2%	___
M+2	10.1%	3.1%	___	18.0%

Table 2

The M+5 mass enhancement in hyoscyamine of 4.4% was considered to be too high to arise from hydrolysis of the littorine and separate use of the labelled tropine and

phenyllactic acid after dilution from the unlabelled pools. From examination of the M+3 and M+2 incorporations into hyoscyamine it can be seen that statistical recombination of labelled tropine and tropic acid from independently rearranged phenyllactic acid could only account for an M+5 enrichment 3.1% x 4.7% ≈ 0.2%. Further, the intact incorporation was increased rather than diminished by the exogenous addition of unlabelled phenyllactic acid or tropine. These results are therefore best explained by the direct rearrangement of littorine, *i.e.* littorine is indeed the substrate for the rearranging enzyme.

It was also apparent from this experiment that the expected hydrolysis of littorine was still occurring. M+2 and M+3 mass enhancements in hyoscyamine of 3.1% and 4.4% respectively were observed arising form either the separate use of the [1,3-$^{13}C_2$]-phenyllactate or the [*methyl*-$^{2}H_3$]-tropine. The tropine and methyl phenyllactate pools were enriched to 12.2% and 18.0% respectively. Similarly the littorine recovered had an M+5 mass enhancement of 8.7% together with M+2 and M+3 mass enhancements of 10.1% and 3.9% respectively. Clearly littorine is hydrolysed and reformed from labelled phenyllactate and tropine. The greater M+2 mass enhancement indicates a greater re-use of the labelled phenyllactic acid than the tropine.

The hydrolysis of the esters and recombination of the two labels would adequately explain the change in ^{3}H : ^{14}C ratio that Leete observed in hyoscyamine in the corresponding radioactive experiment.[7] It does not however fully explain the consistent ^{3}H : ^{14}C ratio observed in the recovered littorine, although it is interesting to note that the M+5 mass enhancement in littorine in Robins' experiment is approximately twice the magnitude of the M+5 enhancement in the hyoscyamine. Another interesting feature which cannot be fully explained is that the M+2 mass enhancement in the littorine is approximately three times greater than the M+2 mass enhancement in the hyoscyamine. From the argument that hyoscyamine arises solely from the rearrangement of littorine it follows that M+2 and M+3 hyoscyamine should only arise from M+2 and M+3 littorine. If this is the case it would be expected that the levels of

M+2 and M+3 enrichment in littorine and hyoscyamine would be similar. A statistical level of M+5 enrichment into hyoscyamine resulting from the separate use of the phenyllactic acid and tropine has been discussed. However, the possibility that the pools of phenyllactic acid / tropic acid and tropine, in a form primed for esterification, may become saturated with labelled material, must be considered. This would make the probability of label recombination events more likely than the statistical 0.2%. While the proportion of M+5 littorine and hyoscyamine is higher in the presence of added phenyllactate or tropine the figures are still small, even bearing in mind that only the natural R-(-)-littorine would be processed.

In light of these still unexplained features it would be of interest to confirm this result. Two further experiments would be helpful. Firstly, it may be useful to re-evaluate this recombination by another analytical method, NMR. If littorine were synthesised from [1-^{13}C]-phenyllactic acid and [^{18}O]-tropine (fig. 44) intact incorporation could be seen directly by NMR as the ^{18}O will induce an α-shift in the enriched ^{13}C signal. The use of a NMR analysis should still arrive at essentially the same conclusion. Here again however, higher than simply statistical levels of re-combination would not be detected.

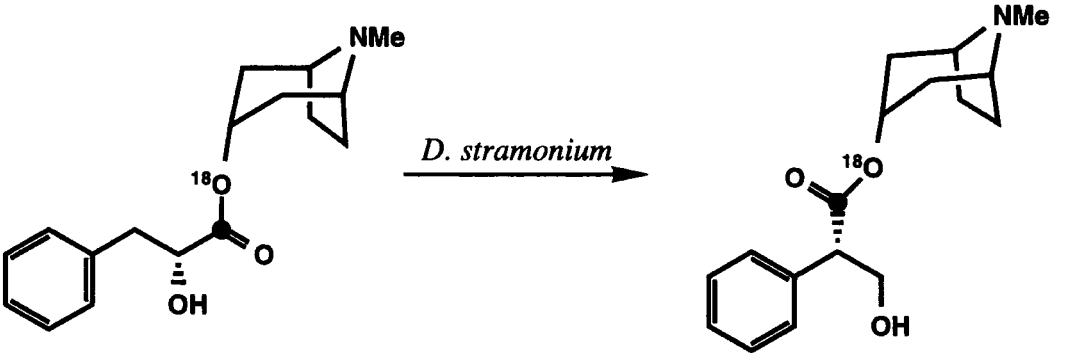


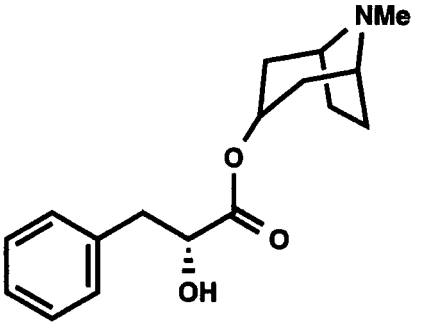
Fig. 44

The extent of recombination of labels after hydrolysis could be assessed from a crossover experiment. If the quintupally labelled littorine that Robins *et al.*[48] fed was introduced as an admixture with littorine synthesised from [2-^{13}C]-phenyllactic acid then M+5 mass enhancements into hyoscyamine would indicate intact incorporation

and M+2 and M+3 mass enhancements, would necessarily arise after hydrolysis as described above. M+1 mass enhancements could indicate either direct incorporation or incorporation after hydrolysis and re-esterification. M+4 incorporation would however indicate the use of phenyllactic acid from the M+1 littorine after hydrolysis together with tropine from the M+5 littorine and so the extent of re-combination of labelled material *in vivo* could be assessed. As studies move away from whole cells to cell free extracts it is important to be confident of the substrate for the rearranging enzyme. While the Robins result provides strong and convincing evidence that littorine is the substrate the conversion of littorine to hyoscyamine in a cell free extract will probably be required for definitive proof.

2.11 (R)-D- or (S)-L-Phenyllactic Acid?

It became relevant to establish whether (R)-D or (S)-L-phenyllactic acid (**32a** or **32b**) was the true intermediate in the biosynthesis of hyoscyamine. Littorine (**33**) (fig. 46), the ester of (R)-phenyllactic acid (**32b**) and tropine (**23**), has emerged as the most likely substrate for the rearranging enzyme.



littorine (**33**)

Fig. 46

Littorine (**33**) was first isolated as the major alkaloid from a Western Australian plant *Anthocercis littorea*[49] and has subsequently been shown to be widespread in *Datura* together with hyoscyamine.[50] This provided circumstantial evidence that the (R)-enantiomer of phenyllactic acid is further processed to (S)-tropic acid. The incorporations of both L-(S)- and D-(R)-[1,3-$^{13}C_2$]-phenyllactic acids into hyoscyamine

have been observed at approximately equal levels.[51] This can only be explained by their rapid interconversion *in vitro via* phenylpyruvate (fig. 47). However, more recent studies have indicated that (R)-D-phenyllactic acid is incorporated at slightly higher levels than (S)-L-phenyllactic acid.[55] In order to identify which enantiomer of phenyllactic acid is the true substrate for rearrangement, the separate enantiomers were fed to *D. stramonium* root cultures. The dual labelled methodology, employing a ^{13}C-^{2}H combination at C-2 was deployed once again (section 2.2).

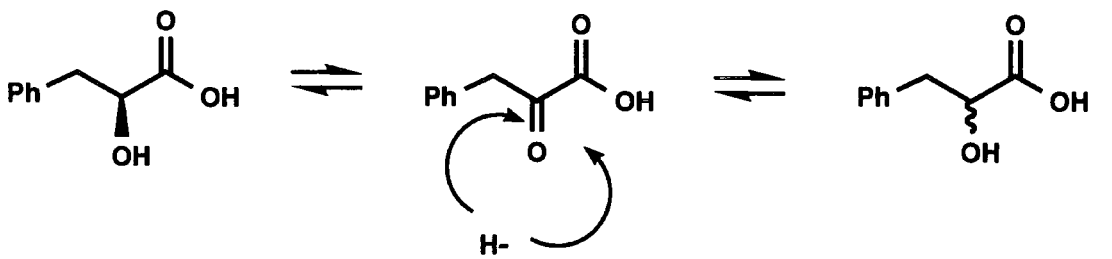


Fig. 47

2.12 Incorporation Studies with (R)-D- and (S)-L-Phenyllactic Acid

Since the two potential substrates, (R)-D- and (S)-L-phenyllactic acids can interconvert *in vivo,* ^{13}C labelling alone has proved insufficient to determine the closer intermediate to tropic acid. However, if the retention of deuterium from the chiral centre of either (R)-D- or (S)-L-phenyllactic acid could be demonstrated, then this would confirm the role of a particular enantiomer. Following the argument presented in section 2.2, if one enantiomer was incorporated directly this would result in the intact incorporation of the ^{13}C-^{2}H bond. Conversely, if prior epimerisation was a requirement the deuterium will be lost during the oxidation to phenylpyruvic acid (**28**) and only ^{13}C incorporation would be observed (fig. 48) in the resultant hyoscyamine (**2**). To this end DL-[2-^{13}C,^{2}H]-phenyllactic acid (**32a**) was synthesised, using the route previously described in section 2.3 and the material was then resolved into its component enantiomers (**32d** (R) and **32e** (S)) as dicussed in the next section. The (R)-D- and (S)-L-phenyllactic acids were then fed in separate experiments to transformed root cultures of *D. stramonium*[53] and the isotope incorporation into hyoscyamine determined, both by NMR and GC-MS analysis.

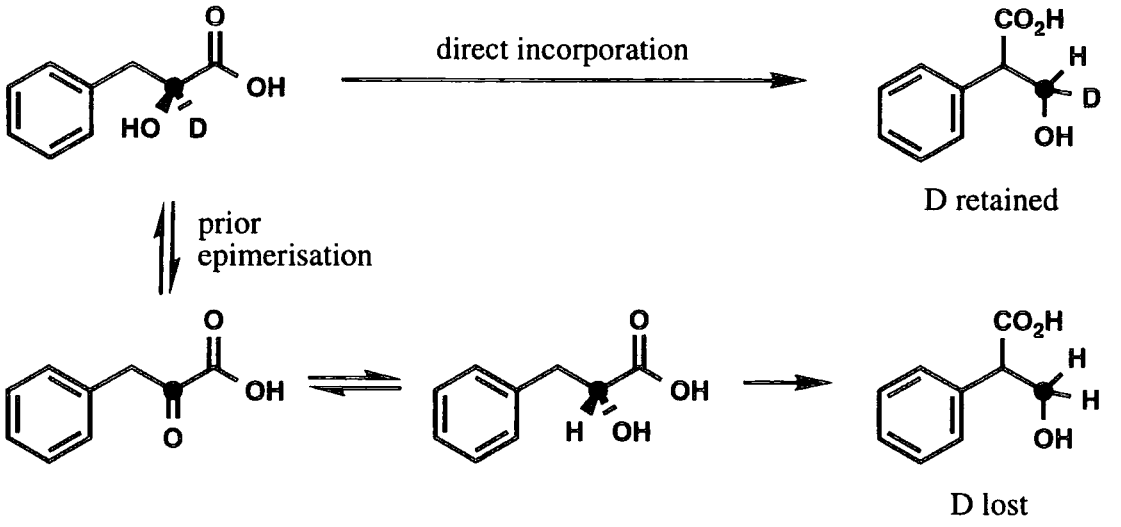


Fig. 48

2.13 The Resolution of Phenyllactic Acid

Initially two enzymatic methods for the resolution of phenyllactic acid were considered, both using *Candida cylindracea (rugosa)* lipase (CCL). In the first case the selective formation of the butyl ester of phenyllactate (**48**) has been described, with an enantiomeric excess of 74% (fig. 49).[54] The enantiomeric excess is rather low for our purposes, but recrystallisation of the free acid may have improved it sufficiently.

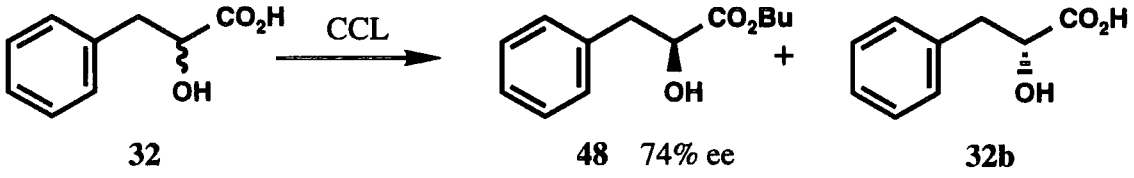


Fig. 49

The second method described the resolution of methyl O-acetylmandelate (**49**) by the selective hydrolysis of the acetyl ester.[55] It was envisaged that this method could have been extended to methyl O-acetyl phenyllactate (**50**) (fig. 50).

A disadvantage common to both these methods is that the phenyllactic acid would need to be derivatised and later recovered, modifications that would clearly compromise the yield.

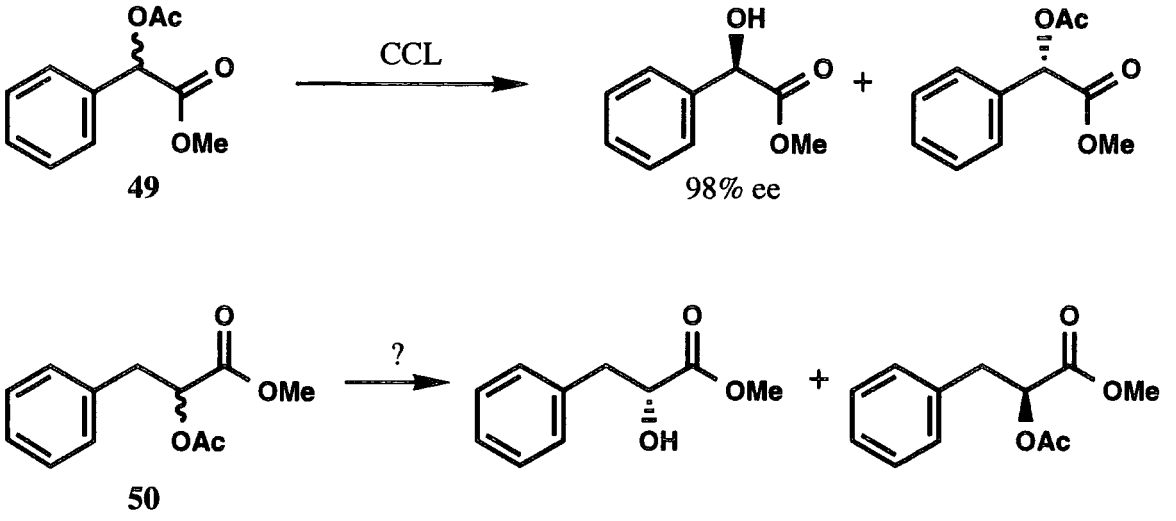
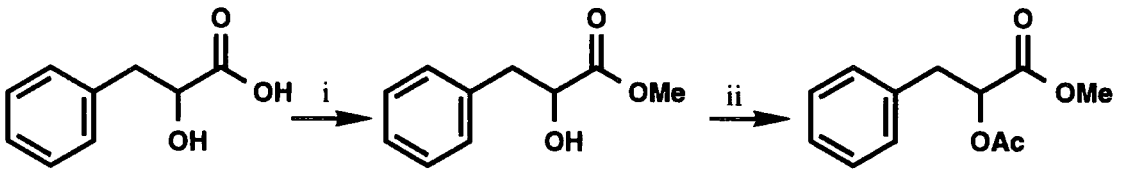


Fig. 50

Alternative methodology used the recrystallisation of the diastereomeric salts of (RS)-phenyllactic acid and a suitable chiral base. Methods using morphine[56] and strychnine[57] are already described but it was judged prudent to avoid the use of either controlled or very toxic substances. The method developed by Saigo *et al.* was eventually used,[58] which involved the recrystallisation of diastereomeric salts of phenyllactic acid (**32**) and (R)- or (S)-2-phenylglycinol (**53**). After recrystallisation to constant optical rotation the free phenyllactic acid was liberated simply by decomposing the salt in dilute mineral acid. Both enantiomers of 2-phenylglycinol are commercially available and so each enantiomer of phenyllactic acid was readily accessed. This methodology was initially tested on unlabelled material and the enantiomeric purity of the resolved material was judged to be very high by both optical rotation and melting point. However, since the quantity of the material was small and the rotations were low the enantiomeric excess was also assessed by ^{1}H NMR studies on the methyl O-acetylphenyllactate derivative (**50**). This demonstrated that the resultant material had an enantiomeric excess greater that 95% ee. When the [2-^{13}C,^{2}H]-phenyllactic acid sample was resolved the optical resolutions recorded were slightly lower than that from the unlabelled material from both the (R)- and (S)-phenyllactates ($[\alpha]_D^{20}$ = +17° and -16° versus $[\alpha]_D^{20}$ = +19° and -19° respectively) (see experimental). We attribute this anomaly to the presence of ^{2}H at the chiral centre.

2.14 NMR Determination of the Optical Purity of the Resolved Phenyllactic Acids

The enantiomeric excesses of the resolved phenyllactic acids were determined by ^1H NMR analysis of the methyl O-acetylphenyllactate derivatives with the chiral shift reagent *tris*-[2-heptafluoropropylhydroxymethylene]-(+)-camphoratol europium III derivative ($Eu(hfc)_3$). Methyl O-acetylphenyllactates from racemic material, an authentic sample of (S)-phenyllactic acid and the resolved phenyllactic acids (**32b** (R) and **32c** (S)) were prepared using the route shown below (scheme 2).



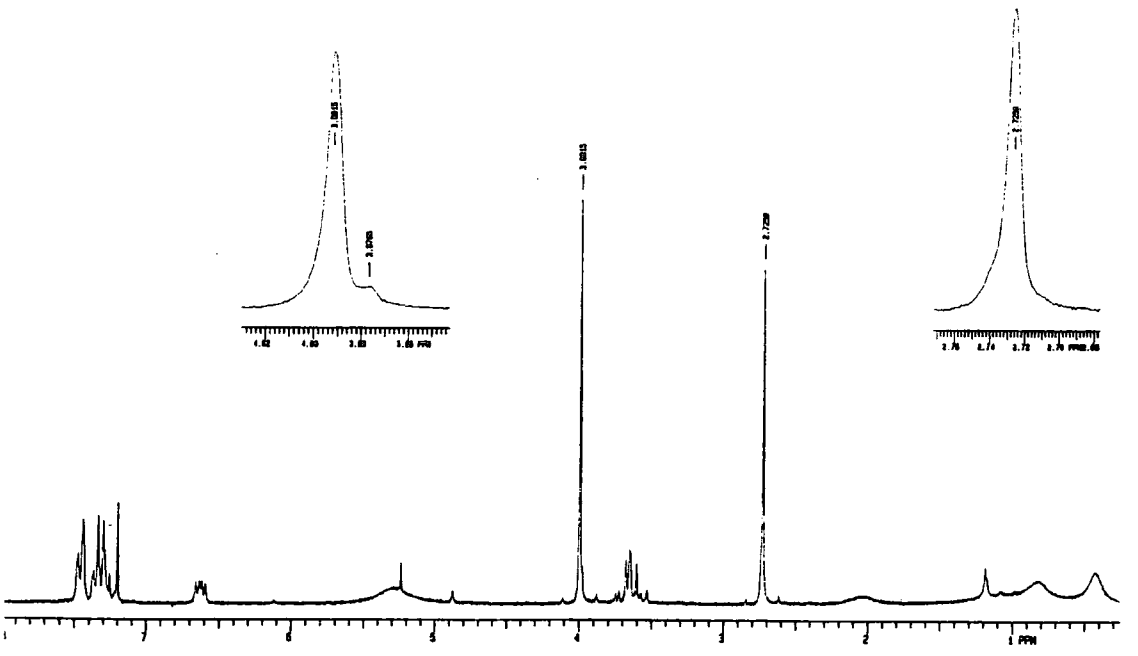
32 **52** **50**

(i) CH_2N_2, (ii) AcOH, DCC, DMAP

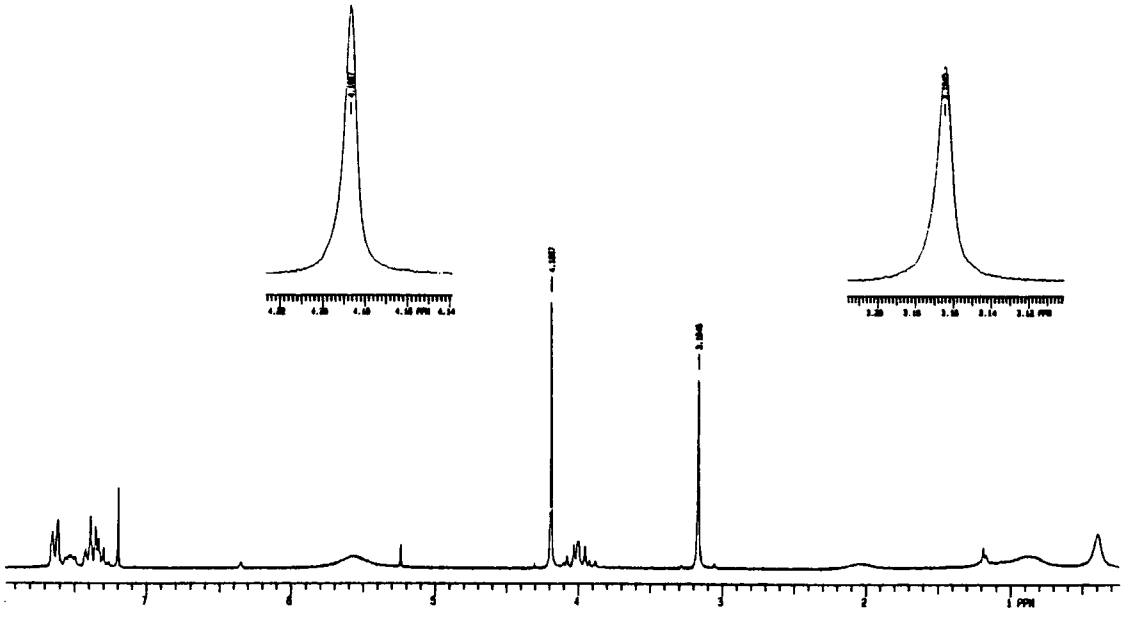
Scheme 2

Initially the phenyllactic acid was converted to its methyl ester (**52**) with diazomethane. This was then coupled with acetic acid using dicyclohexylcarbodiimide (DCC) and a catalytic quantity of 4-*N,N*-dimethylaminopyridine (DMAP) to give methyl O-acetylphenyllactate (**50**) in 84% yield over the two steps.

In the ^1H NMR analysis the authentic (S)- methyl O-acetylphenyllactate no splitting of the methyl signals of the ester or acetate could be seen. Racemic methyl O-acetylphenyllactate was then added in small portions until a concentration of 10% racemate (*i.e.* 5% of the other enantiomer) had been reached. At this concentration small additional peaks were visible to higher frequency of the methyl peak and to lower frequency of the acetate methyl peak. The derivatives of the resolved phenyllactic acids were analysed in the same manner and again, no additional signals appeared until 10% racemic material had been added. These spectra are shown in fig. 51 and fig. 52. From this analysis the enantiomeric excess of the resolved material was demonstrated to be at least 95%.

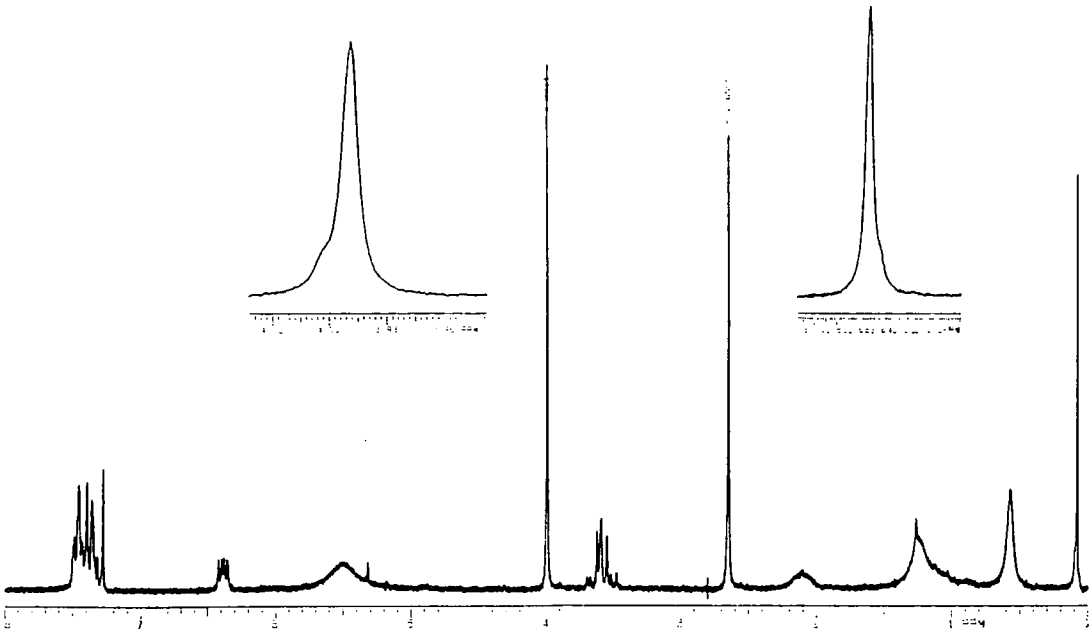


(R)- methyl O-acetylphenyllactate + $Eu(hfc)_3$
10% racemate added

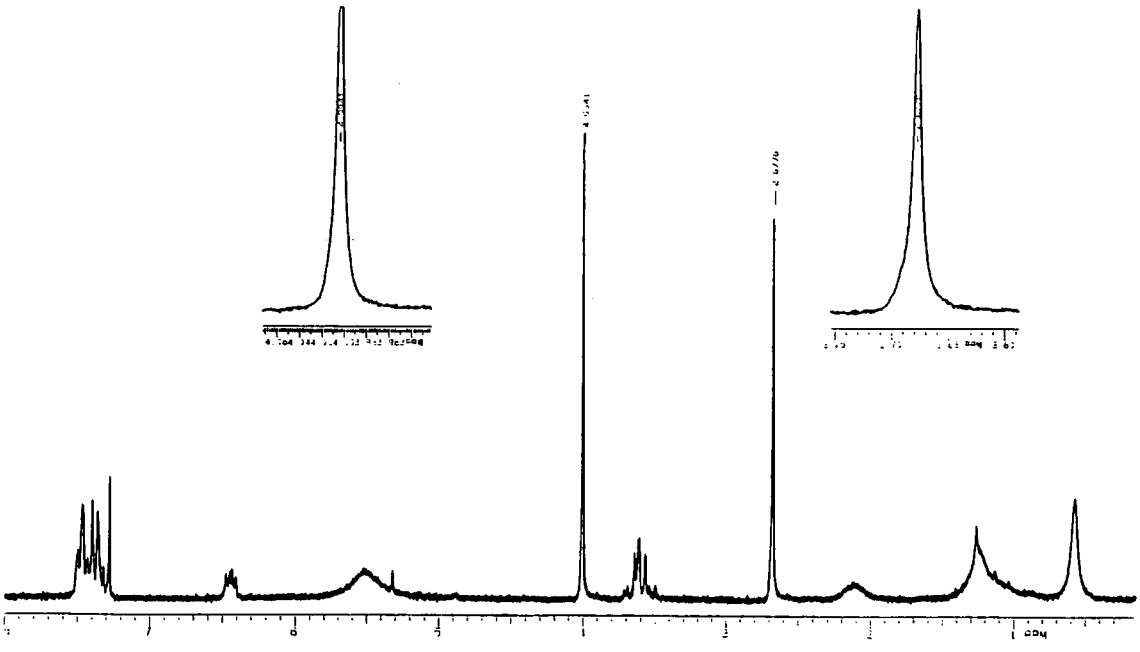


(R)-methyl O-acetylphenyllactate + $Eu(hfc)_3$

Fig. 51



(S)- methyl O-acetylphenyllactate + $Eu(hfc)_3$
10% racemate added



(S)- methyl O-acetylphenyllactate + $Eu(hfc)_3$

Fig. 52

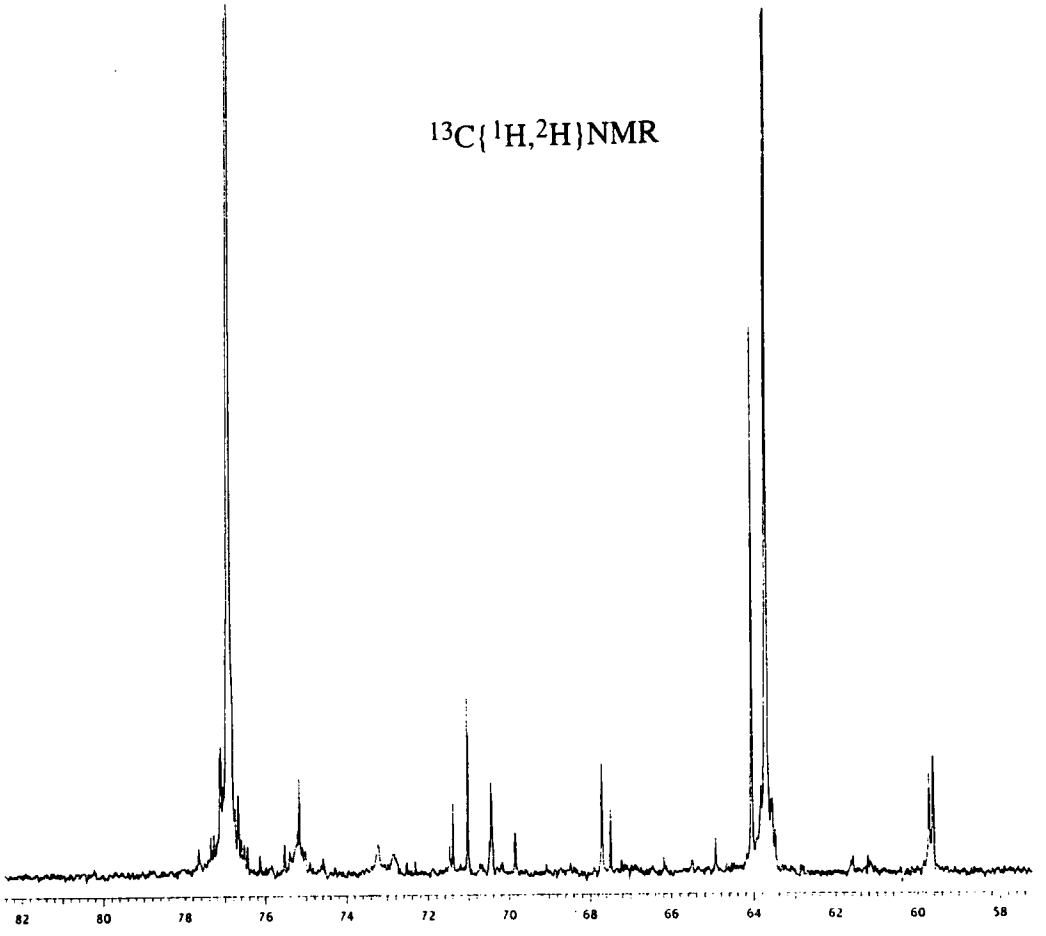
2.15 (R)-D-Phenyllactic Acid and (S)-L-Phenyllactic Acid Incorporations and Conclusions

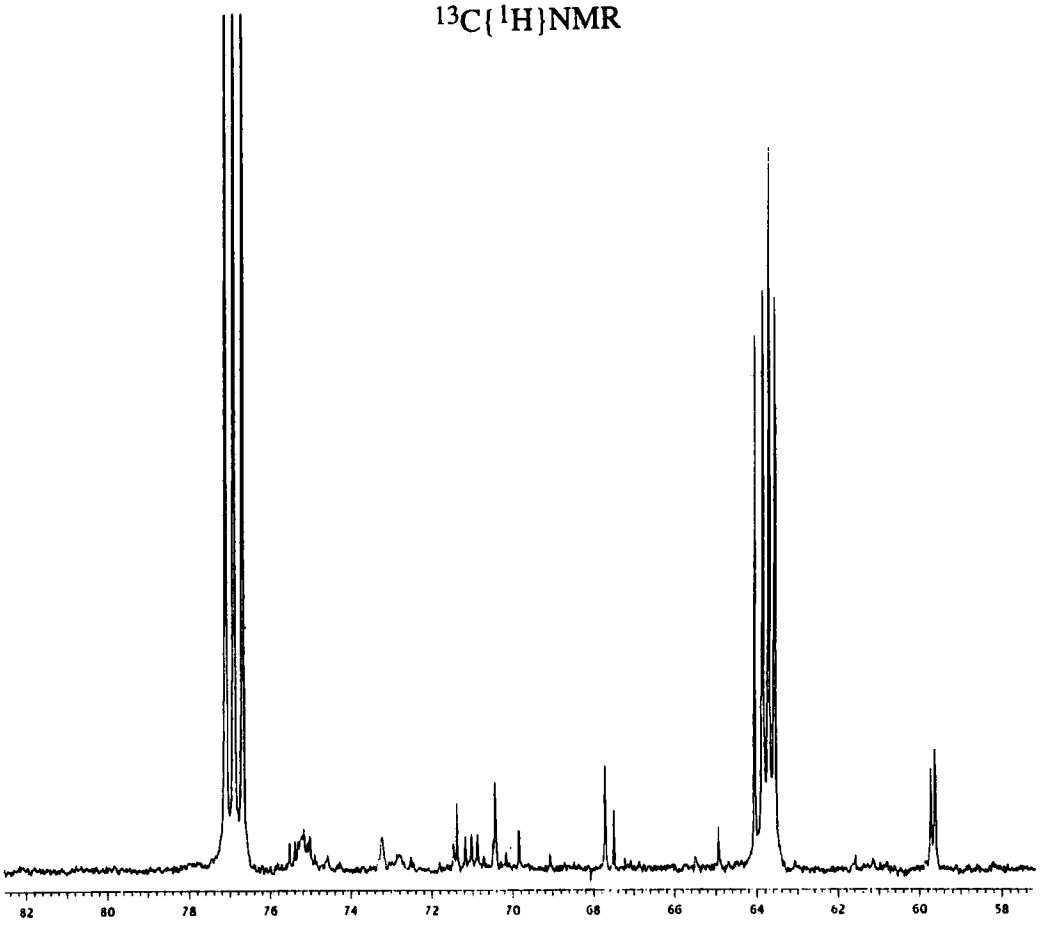
The resolved (R)-D- and (S)-L-[2-^{13}C, ^{2}H]-phenyllactic acids (**32d** and **32e**) were administered to 4 day old transformed root cultures of *D. stramonium*. The (R)-D-phenyllactate supplemented cultures showed signs of bacterial contamination after 6 days growth, which was controlled by the addition of the antibiotic ampicillin. Although growth was slightly affected the alkaloids were still produced at a normal level. The hyoscyamine and littorine alkaloid components were isolated and analysed by ^{13}C{^{1}H} and ^{13}C{^{1}H,^{2}H} NMR, and relevant sections of the spectra are shown in fig. 53 and fig. 54. M+2 and M+1 mass enhancements were determined by GM-MS (Table 4).

	Littorine		**Hyoscyamine**		**Apoatropine**	
	R	**S**	**R**	**S**	**R**	**S**
M+	69.7%	86.5%	77.6%	92.5%	79.2%	92.9%
M^{+}+1	1.5%	10.3%	0.8%	5.1%	1.4%	7.1%
M^{+}+2	28.9%	3.3%	21.7%	2.4%	19.5%	0.1%

Incorporations of (R)-D- and (S)-L-phenyllactic acids
into littorine, hyoscyamine and apoatropine
Table 4

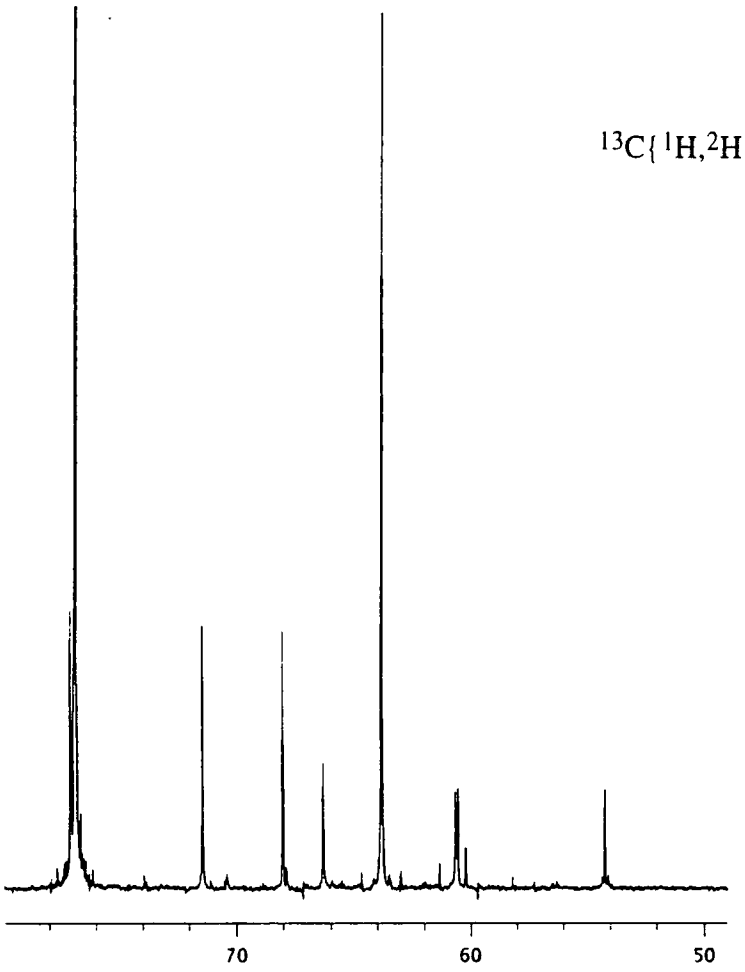
^{13}C NMR analysis of the hyoscyamine isolated for the (R)-D-phenyllactic acid (**32d**) experiment shows a highly enriched singlet at 64.05ppm, corresponding to C-3' of hyoscyamine, with an associated triplet shifted to lower frequency. This triplet collapses to a singlet when deuterium decoupling is applied, as can be seen in the ^{13}C{^{1}H,^{2}H} NMR spectrum. The ratio of the two C-3' signals corresponding to ^{13}C and ^{13}C-^{2}H enrichment in the deuterium decoupled spectrum is about 1:3. This clearly shows that

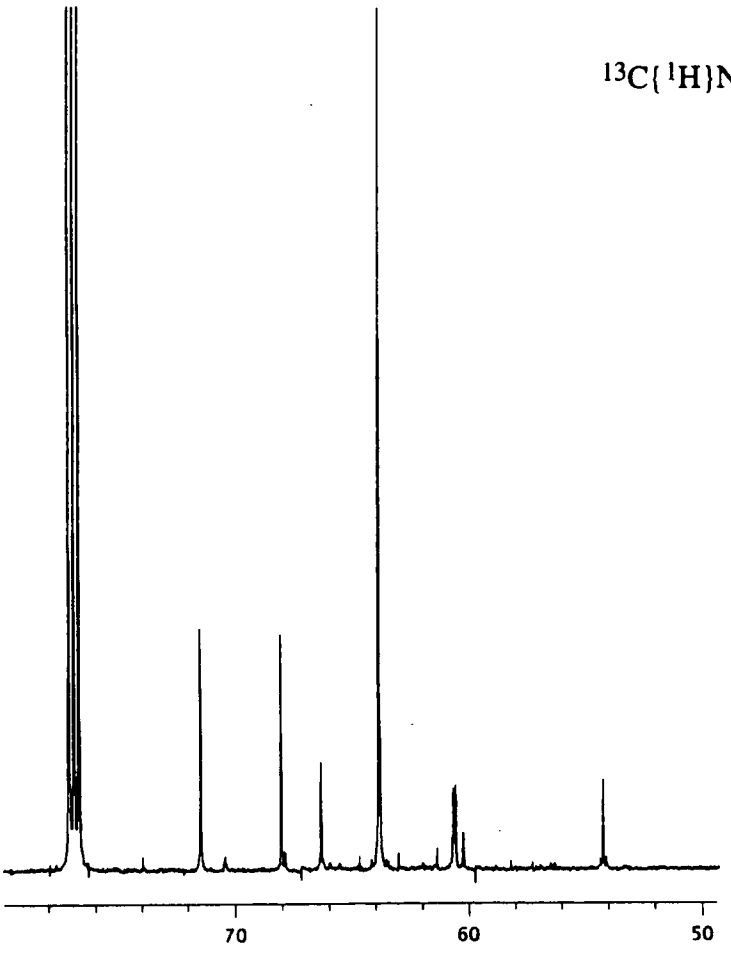




Hyoscyamine isolated after feeding (R)-D-[2-^{13}C, ^{2}H]-phenyllactic acid.

Fig. 53





Hyoscyamine isolated after feeding (S)-L-[2-^{13}C, ^{2}H]-phenyllactic acid.
Fig. 54

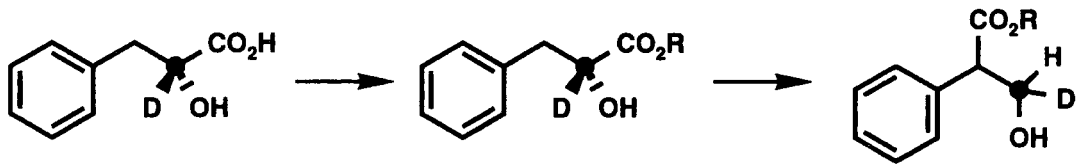
the ^{13}C-^{2}H bond has been incorporated largely intact. Conversely, for the (S)-L-phenyllactic acid (**32e**) there is no α-shifted component associated with the signal at 64.05ppm and this indicates very clearly that all of the deuterium from C-2 of (S)-L-[2-^{13}C, ^{2}H]-phenyllactic acid has been washed out.

An interesting feature of these spectra is the signal at 71ppm corresponding to C-2' of littorine. The (R)-D-phenyllactic acid fed material shows a similar pattern to the C-3' signal of hyoscyamine in the $^{13}C\{^{1}H\}$ NMR spectrum: an enriched singlet with an associated α-shifted triplet which collapses to a singlet in the $^{13}C\ \{^{1}H,^{2}H\}$ NMR spectrum. This again demonstrates the predominantly intact incorporation of (R)-D-phenyllactic acid with a little deuterium washout. More significant however is the resonance from C-2' of littorine after feeding (S)-L-phenyllactic acid. Here there is only an enriched singlet at 71ppm in the $^{13}C\{^{1}H\}$ NMR spectrum with no α-shifted component. This demonstrates that the (S)-phenyllactic acid is not able to esterify with tropine to form the unnatural epimer of littorine.

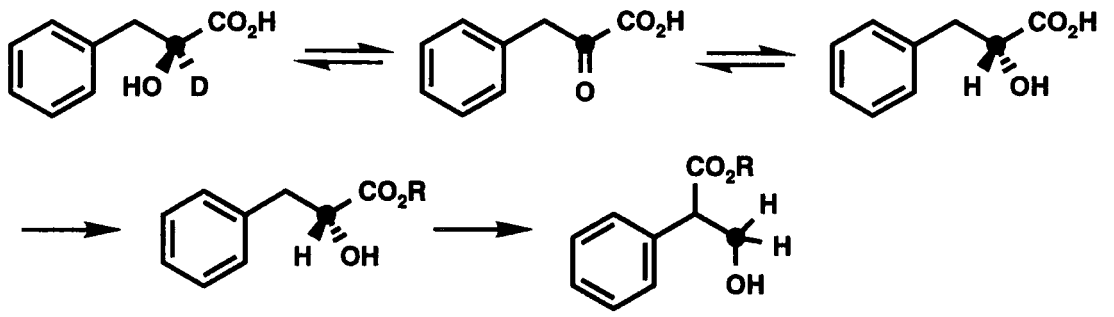
These experiments demonstrate unambiguously that (R)-D-phenyllactic acid (**32b**) is processed more directly than (S)-L-phenyllactic acid (**32c**) in the biosynthesis of hyoscyamine (**2**) and littorine (**33**) (fig. 55). This is consistent with only (R)-D-littorine being formed and that (R)-D-littorine is the direct precursor to hyoscyamine in *D. stramonium*. The result also implicates the operation of a (R)-D-phenyllactate dehydrogenase enzyme at a pivotal point between L-phenylalanine metabolism and alkaloid biosynthesis.

While vertebrates and higher plants contain only L-lactate dehydrogenases, invertebrates, lower fungi and prokaryotic organisms may contain either L- or D-lactate dehydrogenases or sometimes both.[59-61] The racemisation of lactate in some bacteria has been shown to occur as a consequence of the activities of both L- and D-lactate dehydrogenases operating in the same organism,[62] and in other examples the operation

of a lactate racemase has been shown.[63] The ability of *D. stramonium* to process L-phenyllactic acid as well as the required D-phenyllactic acid suggests that either both D- and L-phenyllactate dehydrogenases are present in this plant, or perhaps that a phenyllactate racemase is present which interconverts the two enantiomers.



R-Phenyllactic acid is directly incorporated into littorine and rearranged.



S-Phenyllactic acid is epimerised before incorporation.

Fig. 55

2.16 References

1. M. L. Louden and E. Leete *J. Am. Chem. Soc.*, 1962, **84**, 1510
2. E. Leete, N. Kowanko, R. A. Newmark, *J. Am. Chem. Soc.*, 1975, **97**, 6826
3. G. G. Gross in *The Biochemistry of Plants*, **7** Ed P.K. Sumpf and E. E. Conn Academic Press, New York, London, Toronto, Sydney, San Francisco, 1981 p. 301
4. I. D. Spencer in *Comprehensive Biochemistry*, **20** Ed M Florkin and E. H. Stortz, 1968, p. 294
5. W. C. Evans and J. G. Woolley, *Phytochemistry*, 1976, **15**, 287
6. E. Leete, *Phytochemistry*, 1983, **22**, 933
7. E. Leete and E. P.Kirven, *Phytochemistry*, 1974, **13**, 1501
8 J. Wemple, *J. Am. Chem. Soc.*, 1970, **92**, 6694
9 J. Domagala and J. Wemple *Tetrahedron Lett.*, 1973, **14**, 1179
10. B. V. Prabbu, C. A. Gibson and L. C. Schramm, *Lloydia*, 1976, **39**, 79
11. R. J. Cox and D. O'Hagan, *J. Chem. Soc., Perkin Trans. I*, 1991, 2537
12. A. Jindra, A. Kovacs, Z. Pittnerova and M. Psenak, *Phytochemistry*, 1966, **5**, 1303
13. G. A. Ravisharikov and A. R. Menhta, *Biochem. Inter.*, 1991, **23**, 679
14. A. C. Neish, *An. Rev. Plant. Physiol.*, 1960, **11**, 55
15. P. Chandra, G. Read and L. C. Vining, *Can. J. Biochem.*, 1966, **44**, 403
16. M. Ansarin and J. G. Woolley, *J. Nat. Prod.*, 1993, **56**, 1211
17. M. Ansarin and J. G. Woolley, *Phytochemistry*, 1994, **35**, 935
18. R. J. Robins, J. G. Woolley, M. Ansarin, J. Eagles and B. J. Goodfellow, *Planta*, 1994, **194**, 86
19. W. C. Evans and V. A. Woolley, *Phytochemistry*, 1969, **8**, 2183
20. Y. Kitamura, S. Nishimi, H. Muira and T. Kinoshita, *Phytochemistry*, 1993, **34**, 425
21. M. Ansarin and J. G. Woolley, *Phytochemistry*, 1993, **32**, 1183
22. N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, *J. Chem. Soc., Perkin Trans. I*, 1994, 1159

23. R. J. Cox, *PhD Thesis*, Durham, 1992.

24. H. C. Brown and C. J. Shoaf, *J. Am. Chem. Soc.*, 1964, **86**, 1079

25. K. Omura and D. Swern, *Tetrahedron*, 1978, **34**, 1651

26. P. H. J. Carlsen, T. Kasuki and K. B. Sharpless, *J. Org. Chem.*, 1981, **46**, 3936

27. G. Piancatelli, A. Scettri and M. D'Auria, *Synthesis*, 1982, 245

28. J. Herscovici and K. Antonakis, *J. Chem. Soc., Chem. Commun.*, 1980, 561

29. Y-L. Cheng, W-L. Liu and S. Chen, *Synthesis*, 1980, 223

30. E. Pierson, M. Giella and M. Tishler *J. Am. Chem. Soc.*, 1948, **70**, 1450

31. R. J. Robins, P. Bachmann, A. C. J. Peerless and S. Rabot, *Plant Cell Tissue Cult.*, in press

32. E. Leete, *Planta Med.*, 1990, **56**, 339

33. E. Leete, *Planta Med.*, 1979, **36**, 97

34. R. J. Robins, A. J. Parr, J. Payne, N. J. Walton and M. C. J. Rhodes *Planta*, 1990, **182**, 414

35. R. J. Robins, A. J. Parr, E. G. Bent and M. C. J. Rhodes, *Planta*, 1991, **183**, 185

36. L. K. Simola, R. Parviainen, A. Martinsen, H. Huhtikangas, R. Jokela and M. Lounasmaa, *Phytochemistry*, 1990, **29**, 3517

37. M. Konoshima, M. Tabata, N. Hiraoka and H. Miyake, *Shoyakugaku Zasshi*, 1967, **21**, 108

38. N. Hiraoka, M. Tabata, and M. Konoshima, *Phytochemistry*, 1973, **12**, 795

39. T. Hashimoto and Y. Yamada, *Agric. Biol. Chem.*, 1987, **51**, 2769

40. S. J. Stohs, *J. Pharm. Sci.* 1969, **58**, 703

41. Y. Kitamura, A. Taura, Y. Kajiya and H. Muira, *J. Plant Physiol.* 1992, **140**, 141

42. E. Leete, *J. Am. Chem. Soc.*, 1960, **82**, 612

43. M. Ushiyama and T. Furuya, *Phytochemistry*, 1989, **28**, 2333

44. R. J. Robins, J. Eagles, I. Colquhoun, J. G. Woolley and M. Ansarin, 1992, *Abtracts, Phytochemistry and Agriculture* p.66, Ed. T. van Beek, Phytochemical Society / Royal Netherlands Chemical Society Symposium, Wageningen, April 1992

45. D. Neumann and K. H. Tschöpe, *Flora (Jena)*, 1966, **156**, 521

46. R. Achari, W. C. Evans and F. Newcombe, *Naturwissenschaften*, 1966, **56**, 88

47. M. Sauerwein, K. Shimomura and M. Wink, *Phytochemistry*, 1993, **32**, 905

48. R. J. Robins, P. Bachmann and J. G. Woolley, *J. Chem Soc., Perkin Trans. I*, 1994, 615.

49. J. R. Cannon, K. R. Joshi, G. V. Meehan and J. R. Williams, *Aust. J. Chem.*, 1969, **22**, 221

50 W. C. Evans, A. Ghani and V. A. Woolley *Phytochemistry*, 1972, **11**, 2527

51. R. J. Robins and N. J. Walton, *The Alkaloids*, 1993, **44**, 115

52. M. Ansarin and J. G. Woolley, *J. Chem. Soc., Perkin Trans. I*, 1994, 487

53. N. C. J. E. Chesters, D. O'Hagan and R. J. Robins *J. Chem. Soc., Chem. Commun.*, 1995, 127

54. S. Parida and J. S. Dordick, *J. Am. Chem. Soc.*, 1991, **113**, 2253

55. H. S. Bevinakatti, A. A. Banerji and R. V. Newadkar, *J. Org. Chem.*, 1989, **54**, 2453

56. A. McKenzie and H. Wren, *J. Chem. Soc.*, 1910, **97**, 1355

57. D. Wright, S. A. Brown and A. C. Neish, *Can. J. Biochem. Physiol.*, 1958, **36**, 1037

58. K. Saigo, H. Miura, K. Ishizaki and H. Nohira, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 1188

59. J. J. Holbrook, A. Liljas, S. J. Steindel and M. G. Rossmann *The Enzymes*, **11**, part A, Ed. P. D. Boyer, Academic Press, New York, San Francisco, London, 3rd Edition, 1975

60. H. Taguaki and T. Ohta, *J. Biol. Chem.*, 1991, **266**, 12588

61. G. L. Long and N. O. Kaplan, *Science*, 1968, **162**, 685

62. D. Dennis and N. O. Kaplan, *J. Biol. Chem.*, 1960, **235**, 810

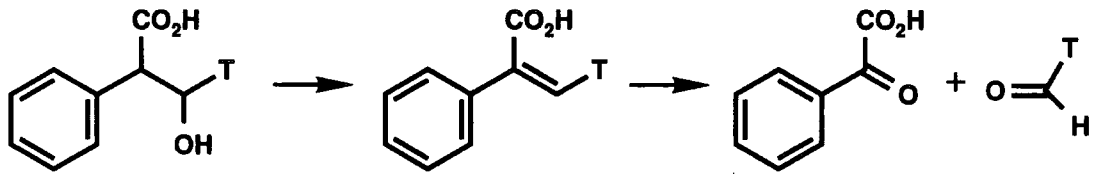
63. E. I. Garvi, *Microbiol. Rev.*, 1980, **44**, 106

CHAPTER 3

Stereochemical Features of the Tropic Acid Biosynthesis

3.1 The Fate of the Hydrogens at C-3 of L-Phenylalanine

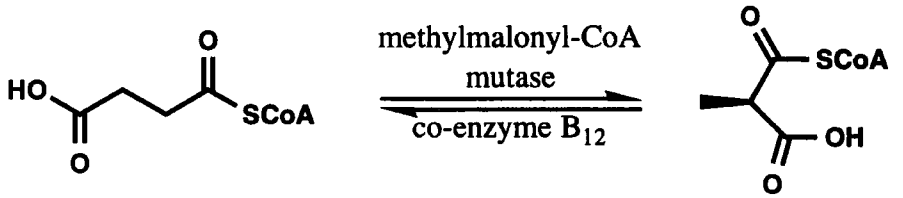
The fate of the hydrogens at C-3 of L-phenylalanine was investigated by Leete in 1984,[1] in a study which has gained general acceptance. A mixture of all four possible stereoisomers of [1-^{14}C, 3-^{3}H]-phenylalanine was administered to *D. stramonium* and *D. innoxia* plants. The location of the tritium at C-3' of the tropate esters, hyoscyamine and scopolamine, was determined after hydrolysis, by dehydration to atropic acid (**53**) and degradation of the tropic acid to benzoyl formic acid (**54**) (analysed as an oxime) and formaldehyde (**55**) (analysed as dimedone) (fig. 56).



53 **54** **55**

Fig. 56

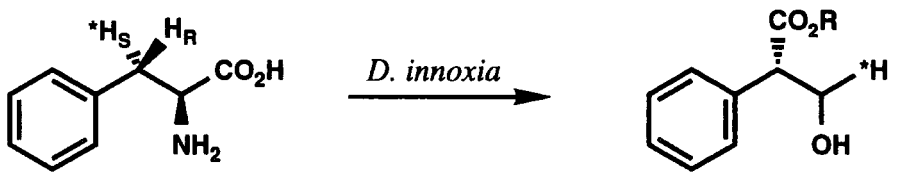
The presence of tritium in the formaldehyde derivative was consistent with its location at C-3 of tropic acid. From this result Leete concluded that a hydrogen from C-3 of phenylalanine was migrating in the reverse direction to the carboxyl group during the rearrangement, *i.e.* a *mutase* enzyme was mediating a *vicinal interchange* process. Leete highlighted the potential analogy with the (2R)-methylmalonyl-CoA mutase mediated interconversion of (2R)-methylmalonyl-CoA and succinyl-CoA (fig. 57).



30 **31**

Fig.57

In a subsequent study[2] Leete administered stereospecifically labelled (2S,3R)-[1-^{14}C, 3-^{3}H]-phenylalanine and (2S, 3S)-[1-^{14}C, 3-^{3}H]-phenylalanine to the plants. From analysis of the radioactive tropic acid obtained, after barium hydroxide hydrolysis of the alkaloids, he concluded that it was the 3-*pro*-S hydrogen of L-phenylalanine which had migrated to appear at C-3' of (S)-tropic acid (fig 58), *i.e.* the configuration at C-2' of the tropate ester was retained during the rearrangement.



Leete's conclusions[1,2]

Fig. 58

These experiments are discussed in the context of more recent results later in this chapter.

3.2 The Cryptic Stereochemistry at C-3' of Tropate

At this point there was one remaining stereochemical aspect of tropic acid biosynthesis that had not been previously addressed. The hydrogen delivered to C-3' of the tropate, moiety after rearrangement must occupy either the 3'-*pro*-R or 3'-*pro*-S site at C-3 of tropate (fig. 59). Also the new bond must be formed with either retention or inversion of configuration.

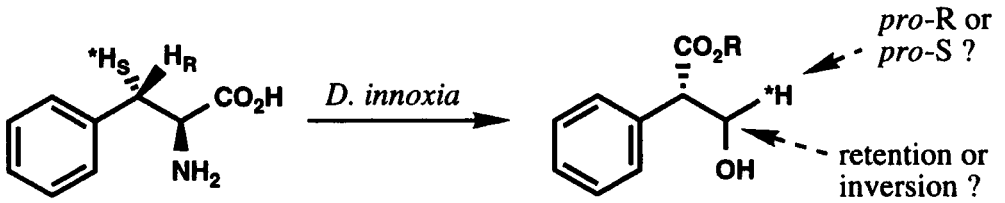


Fig. 59

Enzyme reactions which involve the transfer of hydrogen to generate a *pro*-chiral centre do not result in the creation of a new chiral centre but are none the less stereospecific. The stereochemical course of such reactions cannot be determined simply by

examination of the substrate or product and as such the stereochemistry of such processes is said to be cryptic.[3] The stereochemical course of the delivery of hydrogen to C-3' of hyoscyamine was probed using chiral methyl methodology. Such a strategy involves the generation of a methyl group made chiral by virtue of isotopic substitution with all three isotopes of hydrogen. Thus, delivery of tritium to the C-3' prochiral centre of hyoscyamine in a biosynthetic experiment followed by chemical modification introducing deuterium allowed generation of a chiral methyl moiety. This chiral methyl group was then carved out as chiral acetic acid for analysis (fig. 60).[4]

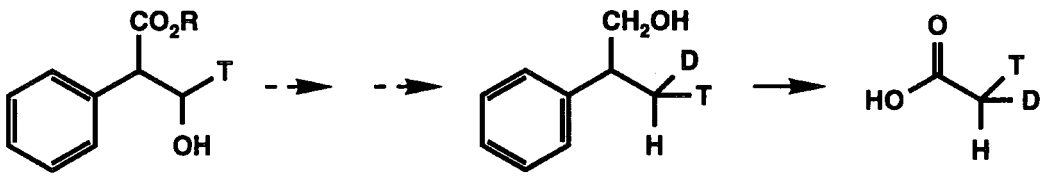
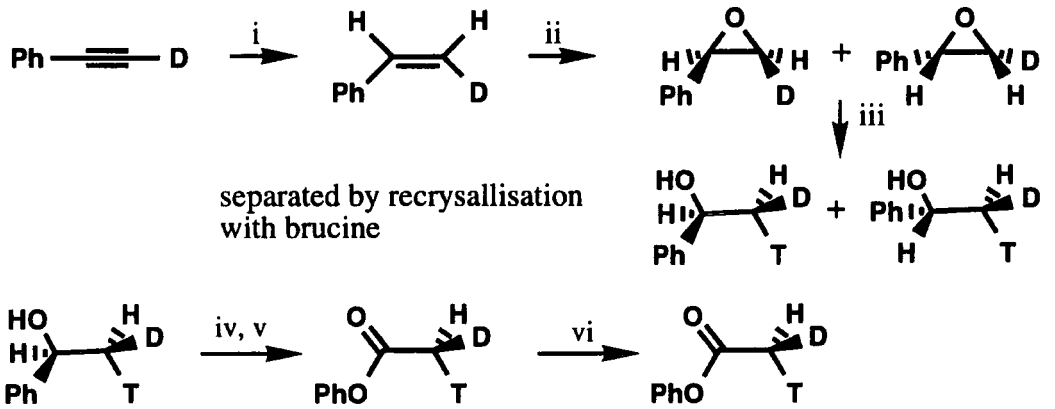


Fig. 60

3.3 The History of the Chiral Methyl Group

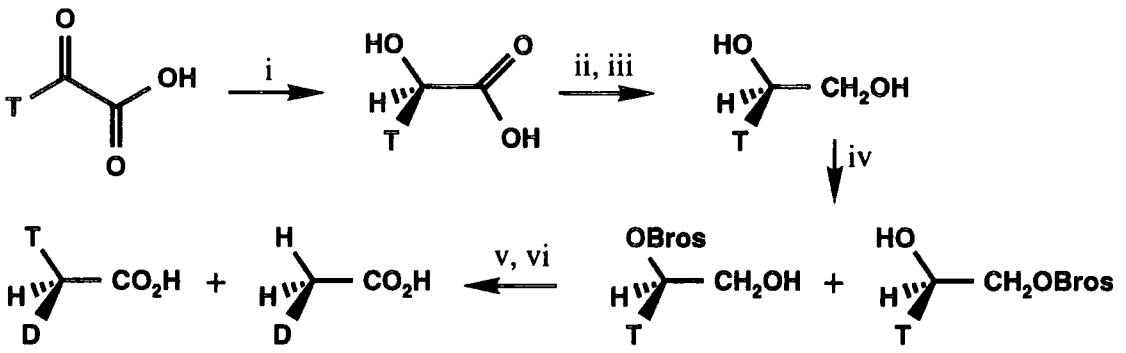
The chiral methyl group was first synthesised, as chiral acetate, in 1969 independently by Cornforth *et al.*[5,6] and Arigoni *et al.*[7] Two different approaches were used. In the Cornforth *et al.* synthesis a chiral methyl group was synthesised racemically, but diastereoselectively, bearing a chiral auxiliary, R*. The two enantiomers were then separated by recrystallisation of the diastereomeric salts with respect to R* with the chiral base brucine. Subsequent derivatisation led to the chiral acetate (scheme 3).



(i) Diimide, (ii) $PhC(O)O_2H$, (iii)$LiBT_4$, (iv) $H_2Cr_2O_7$, (v) $CF_3C(O)O_2H$, (vi) NaOH

Scheme 3

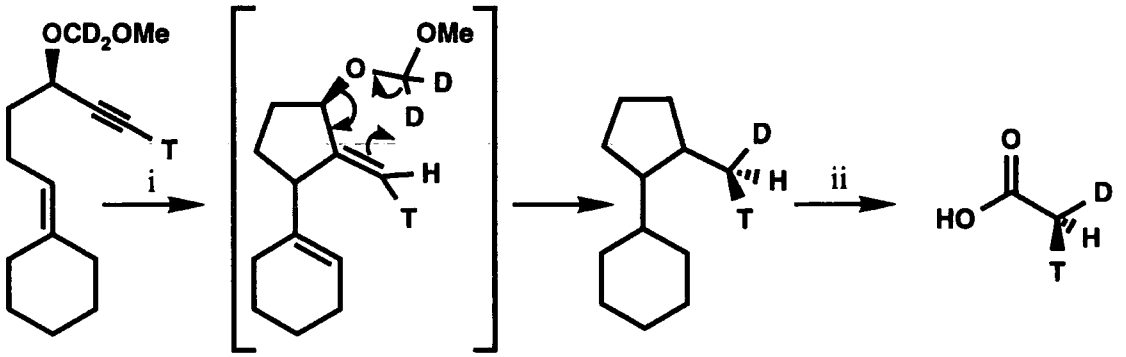
Alternatively, Arigoni *et al.* relied on the stereospecific introduction of the three isotopes of hydrogen to the carbon centre. The key step in this route is the formation of either (S)- or (R)-glycolic acid by reduction of glyoxylate with either L-lactate dehydrogenase or glyoxylate reductase from spinach leaves respectively (scheme 4).



(i) L-LDH, (ii) MeOH, (iii) BrosCl, (iv) $LiAlH_4$, (vi) O_2, Pt

Scheme 4

Perhaps the most elegant synthesis was that devised by Townsend, Scholl and Arigoni,[8] where the three isotopes of hydrogen were introduced stereospecifically in a series of electrocyclic reactions (scheme 5).



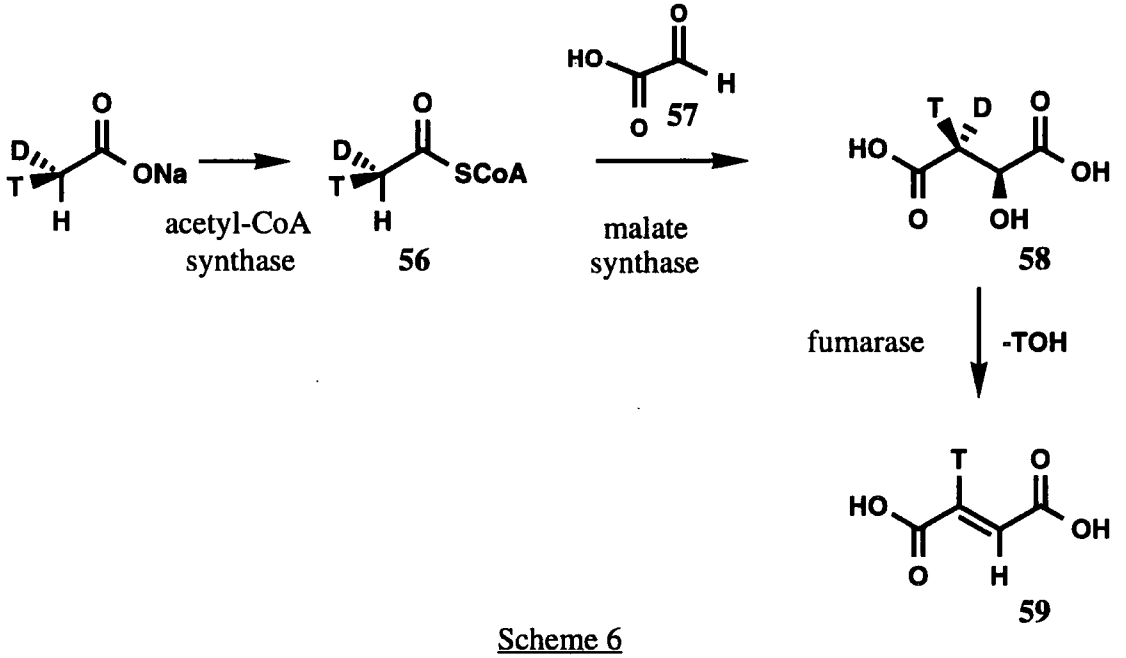
(i) Δ, (ii) $H_2Cr_2O_7$, H_2SO_4

Scheme 5

Since its development chiral methyl methodology has been used to probe many cryptic stereochemical problems in chemistry and biochemistry. A number of other syntheses have appeared and the subject has been extensively reviewed.[9-12]

3.4 Analysis of the Chiral Methyl Group

Tritiated compounds cannot be used in an isotopically pure form due to the practical difficulties associated with handling radioactive compounds. Chiral methyl preparations therefore invariably contain a predominant -CH_2D component and consequently chiroptical methods of analysis cannot be used. Both the Cornforth[5,6] and Arigoni[7] groups developed and enzymatic method of analysis, based on radioactive assay, which hinged on the operation of a kinetic isotope effect resulting in the preferential fission of the C-H over the C-D over the C-T bonds (scheme 6) in the condensation of acetyl-CoA (**56**) and glyoxylic acid (**57**). First the acetic acid is activated to its coenzyme-A thioester and is then condensed with glyoxylic acid (**57**), to form malate (**58**) mediated by malate synthase (k_H/k_D =3.8[13]). Subsequent dehydration, mediated by fumarase, results in the stereospecific loss of water. The tritium remaining in the fumarate (**59**) is then determined by scintillation counting and the percentage of tritium remaining in the fumarate is known as the *F value*. As the initial kinetically controlled malate synthase reaction with glyoxylic acid (**57**) results in the preferential, but not exclusive breaking of the C-H bond the results are interpreted thus: 79% retention of radioactivity indicates the exclusive presence of the (R)- enantiomer and 21% the exclusive presence of the (S)- enantiomer.



Scheme 6

Tritium NMR has also been used to determine the optical purity of chiral acetate.[9] The malate produced, by condensation of acetyl-CoA and glyoxylic acid, possess a chiral methylene group with the tritium occupying one site, geminal to deuterium, or the other, geminal to hydrogen. Whether a tritium is next to a deuterium or hydrogen is readily assessed from the ^{1}H-^{3}H coupling or a ^{2}H induced ^{3}H shift. Thus from the assignment of the *pro*-R and *pro*-S resonances the optical purity can be determined. Direct NMR analysis of diastereomers containing the chiral methyl group is usually confounded due to the free rotation of the methyl group. However, Anet and Kopelevich[14] have demonstrated that it is possible to determine the enantiomeric purity of a chiral methyl group directly, by creating a diastereomer in which there is a strong preference for one conformational isomer (rotamer) (fig. 61).

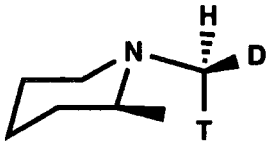
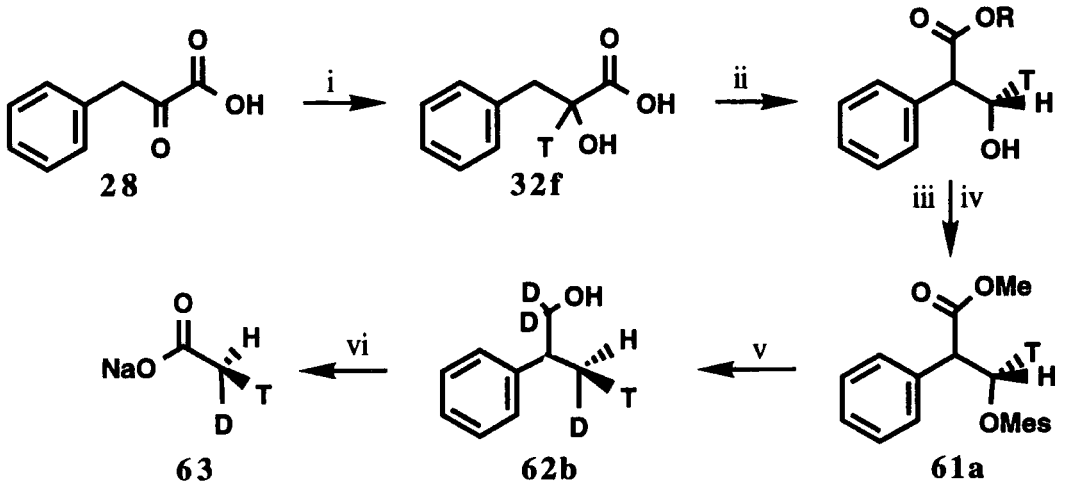


Fig. 61

3.5 Generating a Chiral Methyl Group at C-3 of Tropic Acid

The tritium at C-3 of tropic acid was introduced by incubating transformed root cultures of *D. stramonium* with [2-^{3}H]-phenyllactic acid (**32f**) (918μCimmol^{-1}). The retention of

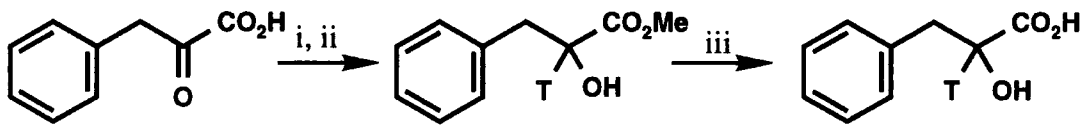


(i) $NaBT_4$, (ii) *D. stramonium*, (iii) saturated $Ba(OH)_{2(aq)}$ (iv) CH_2N_2
(v) MesCl, DMAP, Py, (vi) $LiAlD_4$, (vii) KIO_4, $KMnO_4$

Scheme 7

the C-2 hydrogen of phenyllactic acid in the hydroxymethylene group of tropic acid had previously been established from the stable isotope studies using (RS)-[2-^{13}C, ^{2}H]-phenyllactic acid (**32a**) (section 2.7). The isolated hyoscyamine, containing tritium, was then hydrolysed and derivatised in a stereocontrolled manner as outlined in scheme 7, to stereospecifically generate chiral acetic acid for analysis.

3.6 [2-^{3}H]-Phenyllactic Acid (32f)



28 **52b** **32f**

(i) $NaBT_4$, (ii) CH_2N_2, (iii)TMSBr

Scheme 8

[2-^{3}H]-Phenyllactic acid (**32f**) (specific acitivity 918μCimmol^{-1}) was generated by the reduction of phenylpyruvic acid (**28**) with sodium boro-[^{3}H]-hydride in methanol. The crude phenyllactic acid was converted directly to its methyl ester (**52b**) with diazomethane, to enable purification by flash chromatography. The ester was then hydrolysed with TMS bromide using an established procedure[15] to regenerate the free acid. This proved effective as it is a mild process and the excess reagent is volatile. Purification of the product was achieved simply by converting the acid to its sodium salt with dilute sodium hydroxide solution and then washing with chloroform. This generated sodium [2-^{3}H]-phenyllactate (**32f**) in 95% yield and a 1% radiochemical yield (scheme 8).

3.7 Feeding Experiment and the Isolation of [3-^{3}H]-Tropic Acid

[2-^{3}H]-Phenyllactic acid (**32f**) was administered (0.2μCiml^{-1}) to 4 day old transformed root cultures of *D. stramonium*. The hyoscyamine (204μCimmol^{-1}) isolated was diluted 10-fold with cold carrier. The material was hydrolysed by refluxing in saturated barium

hydroxide solution for 30 minutes and was then acidified to generate tropic acid. The free acid was then converted to its methyl ester (**60a**), using diazomethane, in 67% yield over the two steps.

3.8 Methyl [3-^{3}H]-3-Methanesulphonyltropate (61a)

A key step in the derivatisation of tropic acid was the conversion of the hydroxymethyl group into a functionality amenable to dispacement with lithium aluminium deuteride. An obvious first choice was the *p*-toluenesulphonyl derivative (**64**) since it is an excellent leaving group and generates a crystalline product. A crystalline product at some stage in a radioactive synthesis is attractive as recrystallisation ensures a highly pure product for the determination of specific activity. A high conversion was also an important criterion since the quantities of material were small. Unfortunately, this derivative could not be prepared in good yield due to a subsequent elimination reaction (fig. 62); the presence of **65** was evidenced in the ^{1}H NMR spectrum: 5.89ppm (^{1}H, d, J 1.25Hz) and 6.36ppm (^{1}H, d, J 1,25Hz). The use of pyridine as a mild base left the reaction largely incomplete (23% yield) and the use of the stronger base, triethylamine, resulted in further elimination (19% yield).

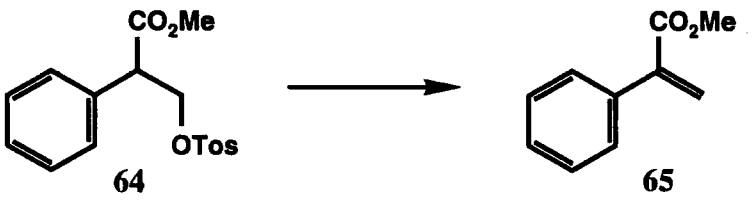


Fig. 62

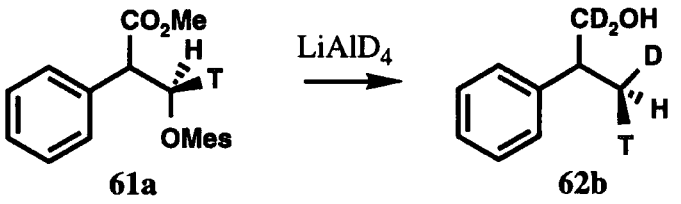
The methanesulphonate derivative (**61**) was then prepared. The more reactive methanesulphonylchloride reacted efficiently, at lower temperature in the presence of pyridine and DMAP and achieved a good conversion (74% yield) without any sign of competing elimination. The product was also crystalline, which was an added advantage.

3.9 [1-$^2H_2$, 3-^2H,^3H]-2-Phenylpropanol (62b)

Deuterium was introduced, to fully furnish the chiral methyl group by reduction with lithium aluminium deuteride. This generated [1-$^2H_2$, 3-^2H,^3H]-2-phenylpropanol (**62b**) in 96% yield. It is important to note that the displacement of the methanesulponyl group by deuteride is in this case an S_N2 reaction which proceeds with inversion of configuration. This clearly has to be taken into account in the final analysis.

3.10 A Mechanistic Investigation

The deuterium isotope was introduced into the chiral methyl group by the displacement of the methanesulphonyl group with lithium aluminium deuteride. This reaction was anticipated to occur stereospecifically, through an S_N2 mechanism, resulting in an inversion of stereochemistry at this centre (fig. 63).



61a **62b**

Fig. 63

It was however important to establish that the elimination of the methanesulphonate was not occurring prior to reduction, since this would result in racemisation (fig. 64), particularly as it had already been demonstrated that the *p*-toluenesulphonate derivative (**64**) was prone to elimination under basic conditions.

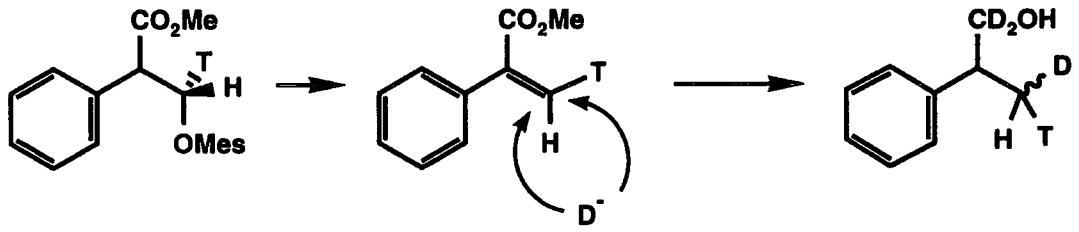
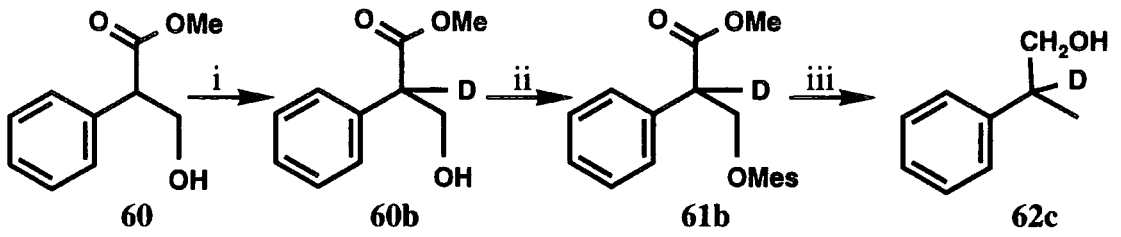


Fig. 64

To investigate this potential problem, methyl tropate (**60b**) was generated enriched with deuterium at the chiral centre. To achieve this the relatively labile hydrogen at C-2 of methyl tropate was exchanged by stirring methyl tropate in a solution of sodium methoxide in MeOD. The methanesulphonate (**61b**) was thus prepared and then reduced to the alcohol (**62c**) using the conditions described in sections 3.8 and 3.9, which had been optimised previously with unlabelled material (scheme 9).



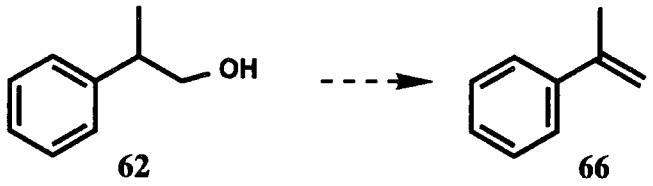
Scheme 9

It was evident from the ^1H NMR spectrum of the isolated 2-phenylpropanol (**62c**), that the deuterium had been fully retained through the reduction step. The methyl and methylene protons appeared as broad singlets at 1.18ppm and 3.60ppm respectively and there was no resonance at 2.79ppm for the methine proton. It was therefore concluded that the reaction proceeded by the expected S_N2 process, and without any evidence for an elimination / reduction process. This placed the methodology on a firm footing for the preparation of the chiral methyl group in a stereospecific manner.

3.11 Chiral Acetic Acid (63)

The Kuhn Roth oxidation[15,16] was developed in the 1930s as an analytical technique for the quantitative determination of methyl groups in a molecule. The substrate is heated with a mixture of chromic and sulphuric acids and then the acetic acid is collected by steam distillation. The methyl group of acetic acid is derived from the pendant methyl groups of the original molecule. The reaction has also been used extensively in the synthesis of chiral acetic acid.[8-10, 18-20] This methodology however proved unsuccessful for 2-phenylpropanol (**62**) in our hands. No acetate was detected after steam-distillation. It is possible that the oxidation did not proceed beyond the formation of 2-phenylpropionic acid.

Another method commonly used for the oxidative degradation of compounds to generate acetic acid, utilises potassium periodate and a catalytic amount of potassium permanganate.[21,22] This methodology is typically used for unsaturated molecules and attracted our attention as it was known to be effective in the oxidative generation of chiral acetic acid from cumene (**66**).[23] It is noteworthy that cumene (**66**) would be obtained by the dehydration of 2-phenylpropanol (**62**) in our system (fig. 65).



62 **66**

Fig. 65

The periodate/permanganate oxidation of cumene was thus re-evaluated and in our hands was found to be efficient. However, in the event, the dehydration of 2-phenylpropanol to cumene was unnecessary since the periodate/permanganate oxidation of 2-phenylpropanol itself was found to generate acetic acid in adequate yield. Acetate generated from unlabelled reactions was characterised as the *p*-phenylphenacyl acetate (**67**) derivative. The only contaminant in the acetate samples was some formate (acetate : formate 2:1), as expected, however, formate does not effect the analysis of chiral acetic acid when co-administered to the coupled enzyme assay. The relative amounts of sodium acetate to sodium formate in the chiral acetate sample was determined by ^{1}H NMR analysis of the mixture. This analysis also distinguished two populations of acetate *i.e.* those carrying a single deuterium and unlabelled acetate as evidenced from the singlet at 1.90ppm and the ^{2}H-shifted triplet at 1.89ppm in the ^{1}H NMR spectrum (fig. 66). In retrospect, the unlabelled acetate was deduced to arise from a source other than 2-phenylpropanol. Re-analysis of the ^{1}H NMR spectrum of the 2-phenylpropanol recorded prior to oxidation revealed a small quantity of diethyl ether, the solvent used in the lithium aluminium deuteride reduction. The presence of unlabelled acetate is attributed to the oxidation of this residual diethyl ether to (unlabelled) acetate.

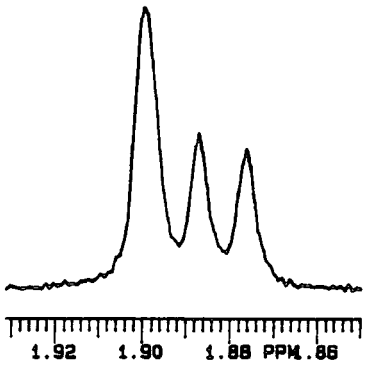
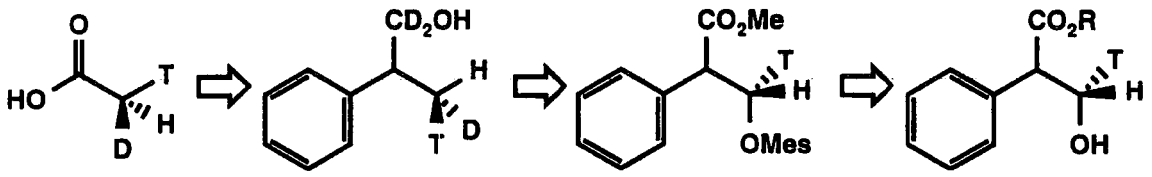


Fig. 66

3.12 Analysis of the Chiral Acetate and Discussion

The optical purity of the acetate sample was determined using the coupled malate synthase / fumarase assay described in section 3.4. This was kindly carried out by H. G. Floss and A. Kastelle in Washington, Seattle. The analysis indicated that the acetate had the (R)- configuration with an enantiomeric excess of 96% (F value 77.9). From this the stereochemical location of tritium in the tropate moiety can be deduced as outlined in scheme 10, taking into account one stereochemical inversion during the S_N2 displacement of the methanesulphonyl group.



Scheme 10

The tritium therefore occupied the 3'-*pro*-S site of hyoscyamine, and thus the hydrogen that is delivered to this carbon, following rearrangement, appears at the 3'-*pro*-R site. It has already been demonstrated in this thesis that it is (R)-D- and not (S)-L-phenyllactic acid which is utilised, therefore it is further deduced that the C-3'-H bond of hyoscyamine replaces the C-1'-C-2' bond of littorine with *inversion* of configuration (fig. 67).

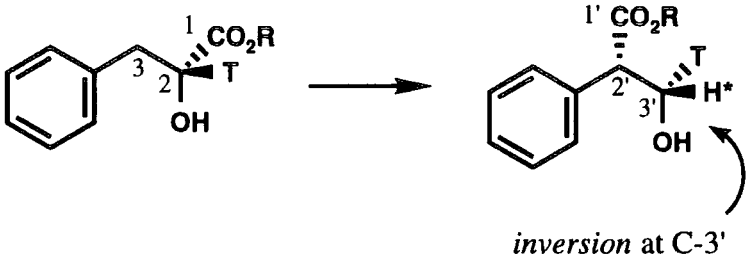


Fig. 67

3.13 Is There a Vicinal Interchange?

Leete[1] had concluded that a vicinal interchange process was operating during tropic acid biosynthesis. That is, a hydrogen from C-3 of L-phenylalanine (and therefore littorine) migrates in the reverse direction to the carboxyl group, to appear at C-3' of hyoscyamine. He also concluded that it was the 3-*pro*-S hydrogen of L-phenylalanine that underwent the 1,2-migration which forced the conclusion that a retention of configuration took place at this migration terminus[2] (fig. 68).

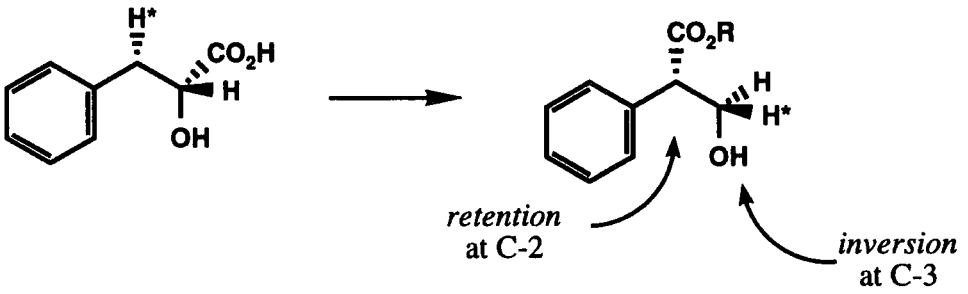


Fig. 68

The rearrangement of littorine to hyoscyamine has obvious, but perhaps superficial similarities to the co-enzyme B_{12} mediated rearrangements[24,25] of methylmalonyl-CoA mutase,[26] isobutyryl-CoA mutase,[27,28] glutamate mutase[29] and α-methyleneglutamate mutase (fig. 69). All of these reactions are vicinal interchange processes involving the 1,2-migration of a carboxyl moiety with the concurrent 1,2-backmigration of a hydrogen atom. This similarity to the rearrangement of the L-phenylalanine (D-phenyllactate) skeleton to tropic acid has been highlighted for some time.[30]

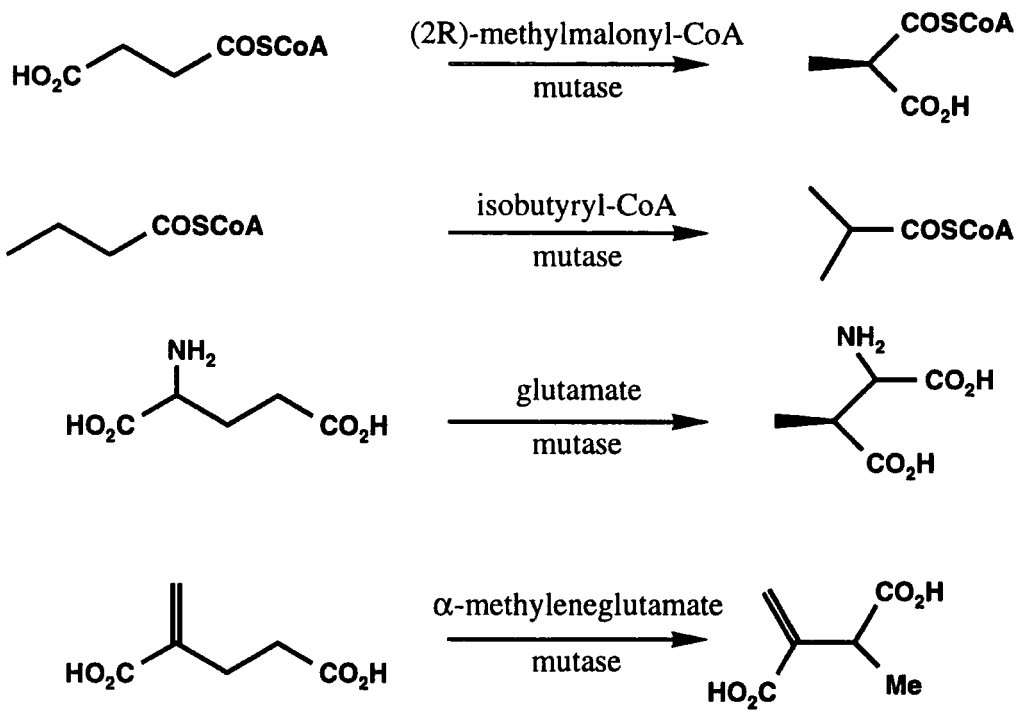


Fig. 69

In the cases of methylmalonyl-CoA mutase and isobutyryl-CoA mutase the migrating hydrogen replaces the COSCoA group with retention of configuration. We have demonstrated that the hydrogen delivered to C-3 of tropic acid after rearrangement is delivered with inversion of configuration and so the steric course in tropic acid biosynthesis, in this respect, is opposite. However, in the case of glutamate mutase the COSCoA group is replaced with inversion of configuration. The occurrence of vitamin B_{12} in plants is however not well documented. Leete has failed to detect any of this vitamin in *Datura* plants,[2] and there is only a single report that vitamin B_{12} occurs in comfrey (*Symphytum officinale*).[31] The involvement of the co-factor in the biosynthesis of hyoscyamine must be considered unlikely. Also the extension of the analogy such that the rearrangement occurs at the co-enzyme-A ester level has been countered by Robins *et al*[32] who placed littorine, and not phenyllactyl-CoA as the substrate for the rearranging enzyme.

A surprising feature to emerge from the combined stereochemical studies of the rearrangement is that to effect an inversion at C-3 of tropic acid and invoke a vicinal

interchange process, then the 3-*pro*-S hydrogen of littorine must be abstracted from one side of the molecule and subsequently returned to the opposite side (fig. 70).

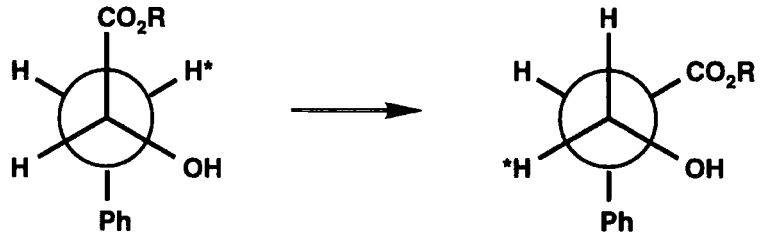
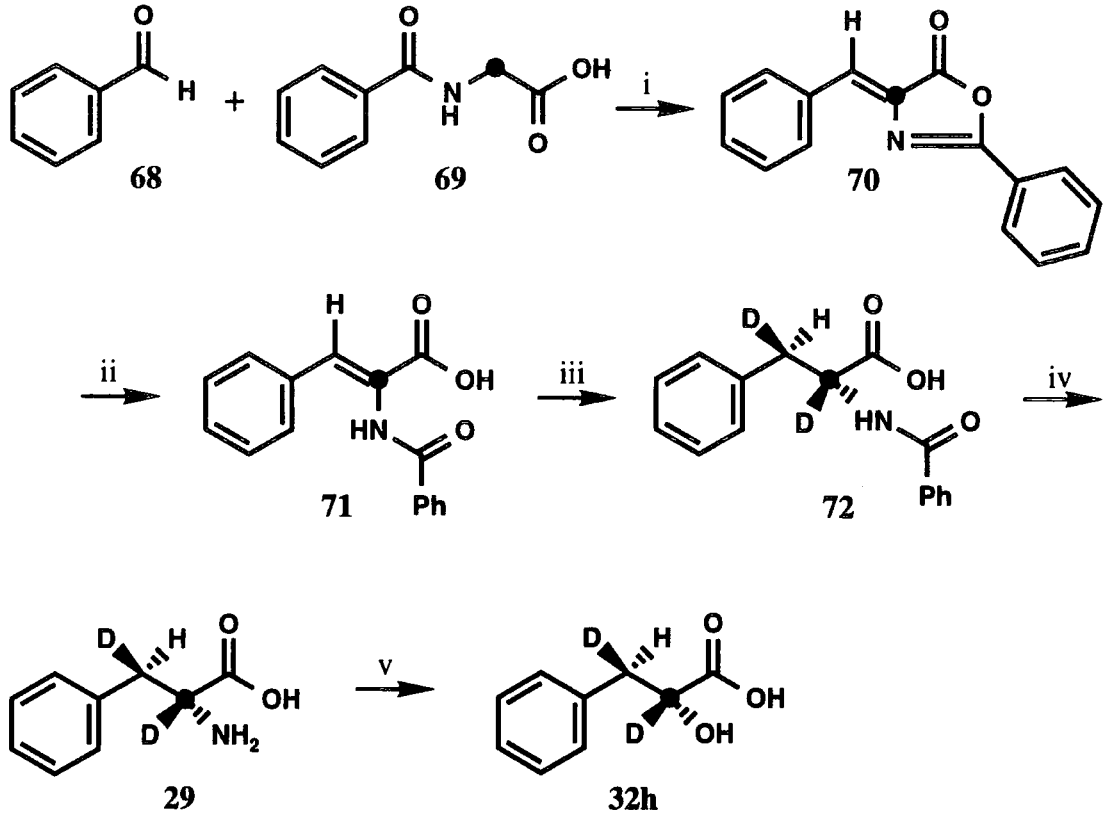


Fig. 70

Intuitively, this would seem to be an unlikely scenario as it would require a substantial change in the conformation of the substrate or active site of the rearranging enzyme to allow the abstracted hydrogen to be delivered to the opposite face. This prompted us to re-evaluate the stereochemical course at the other migration terminus.[33]

3.14 Investigation of the Fate of the C-3 Hydrogens of Phenyllactic Acid

A series of experiments was carried out to determine the fate of the C-3 hydrogens of phenyllactic acid. A number of phenyllactic acids labelled with ^{2}H at C-3 were administered to *D. stramonium* transformed root cultures. In each experiment a ^{13}C reporter atom was placed at C-2 of phenyllactic acid and deuterium at C-3 such that any migration of the deuterium to C-3 of tropic acid could be detected directly by ^{13}C NMR. Initially (2R,3S)-[2-^{13}C, 3-^{2}H, *ring*-D_5]-phenyllactic acid (**32g**) and (2R, 3R)-[2-^{13}C,^{2}H, 3-^{2}H]-phenyllactic acid (**32h**) were administered to 4 day old transformed root cultures of *D. stramonium.* These two phenyllactic acids were kindly supplied by Professor H. G. Floss, and Kevin Walker, at the University of Washington, Seattle. The synthetic route to (2R, 3R)-[2-^{13}C,^{2}H, 3-^{2}H]-phenyllactic acid (**32h**) is outlined in scheme 11 and (2R,3S)-[2-^{13}C, 3-^{2}H, *ring*-D_5]-phenyllactic acid (**32g**) was synthesised by an analogous procedure, starting from [$^{2}H_6$]-benzaldehyde and reducing with hydrogen at step (iii) rather than using deuterium gas.



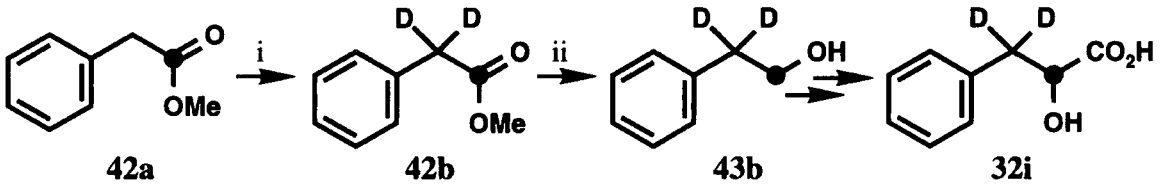
(i) Ac_2O 80°C, (ii) 1 equiv NaOH, (iii) D_2, Rh(S,S)CHIRAPHOS,
(iv) 6N HCl, reflux, (v) $NaNO_{2(aq)}$

Scheme 11

Compound **69** was synthesised from glycine and benzoyl chloride using an established procedure.[34] The synthesis developed by Wrightman *et al*[35] was then used to prepare the stereospecifically labelled D-phenylalanines. In the original procedure the hydrogenation (step iii) was carried out simply using palladised charcoal as a catalyst. The reaction occurred, as expected, with *syn* stereospecificity to generate the racemic (2RS, 3SR)-[3-^{2}H]-phenylalanine. This was then converted to the corresponding N-chloroacetylphenylalanine which was enantioselectively hydrolysed using hog renal acylase to generate (2S, 3R)-[3-^{2}H]-phenylalanine and (2R, 3S)-[3-^{2}H]-N-chloroacetylphenylalanine. The latter product could be hydrolysed non-enzymatically. This methodology generates the phenylalanines with approximately 93% correct stereochemistry at C-3. Kevin Walker however used a modification of this procedure developed by Fryzuk and Bosnich[36] whereby the hydrogenation (or deuteration) is carried out stereospecifically in the presence of Rh(SS)CHIRAPHOS.

D-phenylalanine obtained from this reaction was then deprotected and diazotised with retention of configuration[37] to generate the required phenyllactic acid. The stereochemical integrity of the synthetic route is already demonstrated and as expected the (2R, 3S)-[2-^{13}C, 3-^{2}H, *ring*-$^{2}H_5$]-phenyllactic acid (**32g**) and (2R, 3R)-[2-^{13}C, 3-^{2}H]-phenyllactic acid (**32h**) were judged to be >98% ee by GC analysis of the Mosher's ester derivative of the methyl ester of the phenyllactates.

(RS)-[2-^{13}C, 3-$^{2}H_2$]-Phenyllactic acid (**32i**) was synthesised using a modified version of the procedure used to prepare (RS)-[2-^{13}C,^{2}H]-phenyllactic acid (**32a**), as described in section 2.3. Deuterium was exchanged into methyl [1-^{13}C]-phenylacetate (**42a**), by deprotonation with LDA and quenching the resulting anion with MeOD. Reduction with lithium aluminium hydride to generate [1-^{13}C, 2-$^{2}H_2$]-phenylethanol (**43b**) which was converted to [2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid (**32i**), as previously described (scheme 12).



42a **42b** **43b** **32i**

(i) LDA, -78°C, MeOD, (ii) $LiAlH_4$

Scheme 12

Two more isotopically labelled phenyllactic acids were synthesised. The aim of this last experiment was to investigate the possibility of any crossover of deuterium between substrate and product molecules (fig. 71). [1-^{13}C]-Phenyllactic (**32j**) acid was synthesised from [1-^{13}C]-methyl phenylacetate (**42a**), again, using the methodology described in section 2.3. [3-$^{2}H_2$]-Phenyllactic acid (**32k**) was prepared by initially exchanging deuterium into phenylpyruvic acid (**28**) followed by reduction with sodium borohydride. These compounds were then admixed at a 1:1 ratio for the feeding experiment.

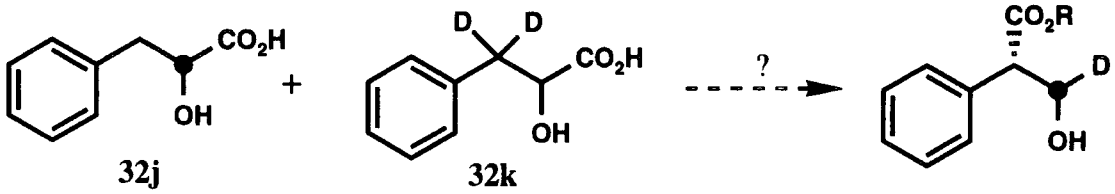
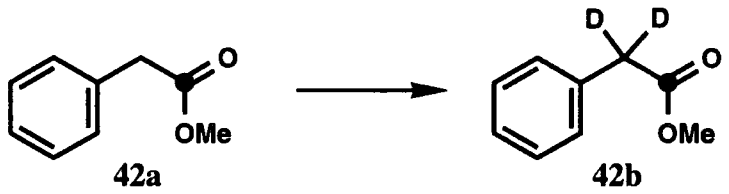


Fig. 71

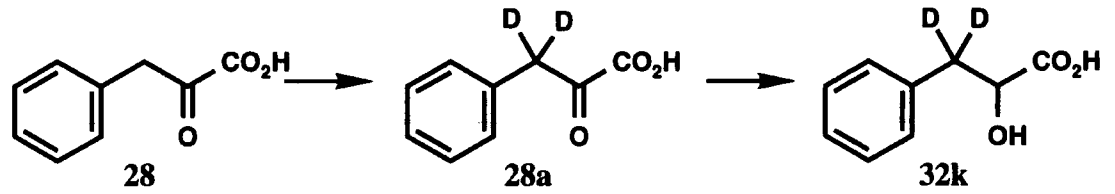
3.15 Methyl [1-^{13}C, 2-$^{2}H_2$]-Phenylacetate (44b)



Scheme 13

Methyl [1-^{13}C]-phenylacetate was deprotonated with LDA (-78°C) and the deep blue dianion was then quenched with MeOD to generate methyl [1-^{13}C,2-$^{2}H_2$]-phenylacetate in 78% yield (scheme 13) containing approximately 20% [1-^{13}C,3-^{2}H]-phenylacetate.

3.16 [3-$^{2}H_2$]-Phenyllactic Acid (32k)



(i) K_2CO_3, D_2O, (ii) $NaBH_4$, (iii) CH_2N_2, (iv) 1M KOH

Scheme 14

[3-$^{2}H_2$]-Phenyllactic acid (**32k**) could have been prepared in an analogous manner to the [2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid (**32i**) by exchanging deuterium into methyl phenylacetate. However, since no ^{13}C label was required the alternative method of

exchanging deuterium into phenylpyruvic acid followed by reduction with sodium borohydride was selected as a higher deuterium incorporation could be achieved (scheme 14). Phenylpyruvic acid (**28**) was dissolved in a solution of potassium carbonate in D_2O (pH11) and left at 4°C for 12 hours. After extraction into ether the phenylpyruvic acid was reduced and directly methylated according to the procedure described in section 3.5.1 to generate methyl [3-$^2H_2$]-phenyllactate (**52c**). Hydrolysis then gave the required [3-$^2H_2$]-phenyllactic acid, (**32k**) in 31% yield over the three steps. No monodeuterated material was apparent by analysis of the ^1H NMR spectrum.

3.17 Results From Feeding (2R,3S)-[2-^{13}C, 3-^{2}H, *ring*-$^2H_5$]-Phenyllactic Acid (32g)

The incorporation of the ^{13}C label into the C-3' of hyoscyamine was evident by the highly enriched singlet at 64.33ppm in the ^{13}C NMR spectrum. There was no evidence of deuterium coupling. The major component of the enriched peak was clearly β-shifted, as indicated from the natural abundance peak at 64.30ppm. A similar pattern was also visible at 71.69ppm, the signal corresponding to C-2' of littorine. In this case the highly enriched singlet also had a β-shift induced component, caused by the presence of deuterium at C-3' (fig. 72). In contradiction to Leete's results[1,2] the 3-*pro*-S deuterium of the administered phenyllactic acid *had not* migrated to C-3' of the tropate moiety of hyoscyamine. The β-shift associated with the C-3' enriched peak is however consistent with the retention of deuterium at C-2' of the tropate ester (fig. 73).

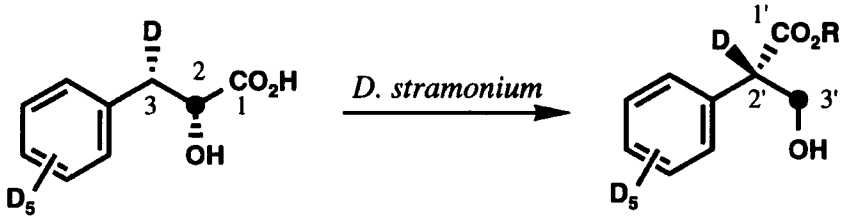


Fig. 73

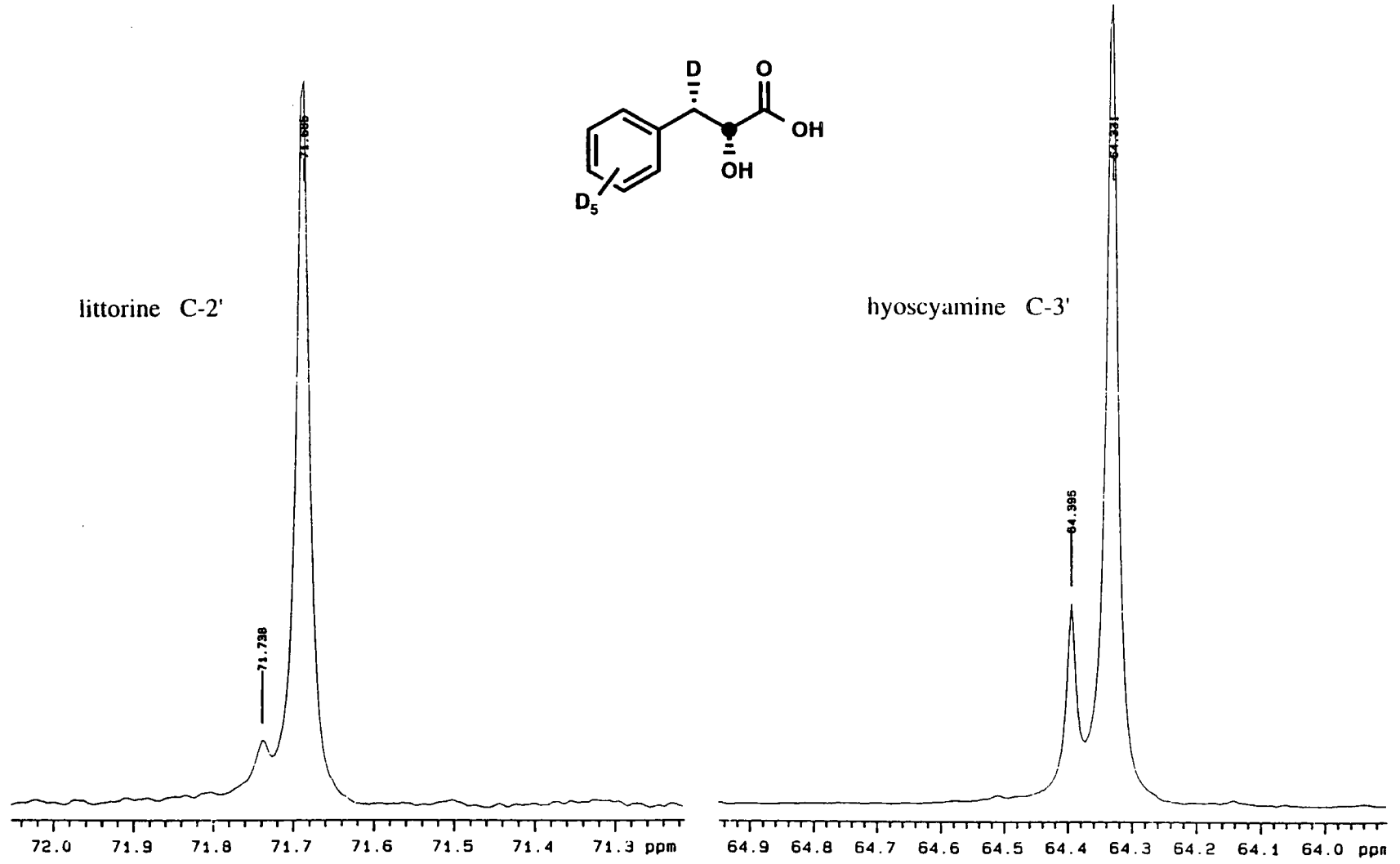

littorine C-2'
71.738
71.805
72.0 71.9 71.8 71.7 71.6 71.5 71.4 71.3 ppm
D
O
OH
OH
D5
hyoscyamine C-3'
64.395
64.381
64.9 64.8 64.7 64.6 64.5 64.4 64.3 64.2 64.1 64.0 ppm

Fig. 72

3.18 Results From Feeding (2R, 3R) [2-^{13}C,^{2}H, 3-^{2}H]-Phenyllactic Acid (32h)

The carbon-13 in this phenyllactic acid was already directly bonded to a deuterium atom, which we know from feeding (R)-[2-^{13}C,^{2}H]-phenyllactic acid is retained at C-3 of tropic acid. The backmigration of the 3-*pro*-R deuterium would therefore have been obvious by the presence of two deuterium atoms attached to C-3 of tropic acid. This would produce a charactaristic quintet with a large associated α-shift in the ^{13}C NMR spectrum, at 64ppm.

The ^{13}C NMR spectrum of hyoscyamine (fig. 74) showed a large enriched singlet at 63.90ppm with an associated α-shifted triplet at 63.55ppm. This indicates the intact incorporation of the C-2-^{2}H bond of the administered phenyllactic acid with partial deuterium washout. This complements the (R)-[2-^{13}C, ^{2}H]-phenyllactic acid (**32d**) experiment (section 2.15). There was no doubly deuterated component, and the peaks were not β-shifted. This is consistent with the *complete loss* of the 3-*pro*-R deuterium of (2R, 3R)-[2-^{13}C,^{2}H, 3-^{2}H]-phenyllactic acid (fig. 75).

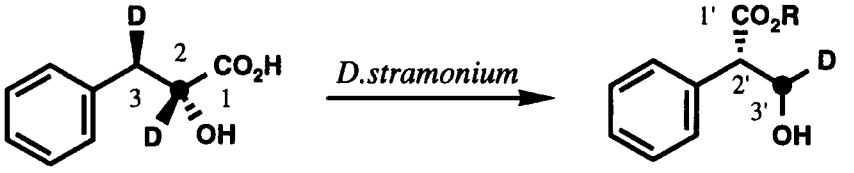
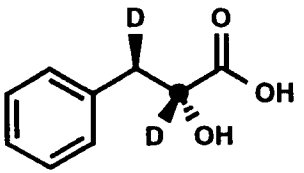


Fig.75

By comparison the C-2' signal of littorine (71ppm) signal showed both (α+β)- and β-shifted components which indicated the predominantly intact incorporation of the (C-2)-^{2}H bond of the phenyllactic acid, with some deuterium washout and the retention of the 2-*pro*-R deuterium. This result is consistent with the direct esterification of (2R, 3R)-[2-^{13}C,^{2}H, 3-^{2}H]-phenyllactic acid to form littorine, as previously demonstrated through by feeding (R)- and (S)-[2-^{13}C, 2-^{2}H]-phenyllactic acids (section 2.15).



littorine C-2'

hyoscyamine C-3'

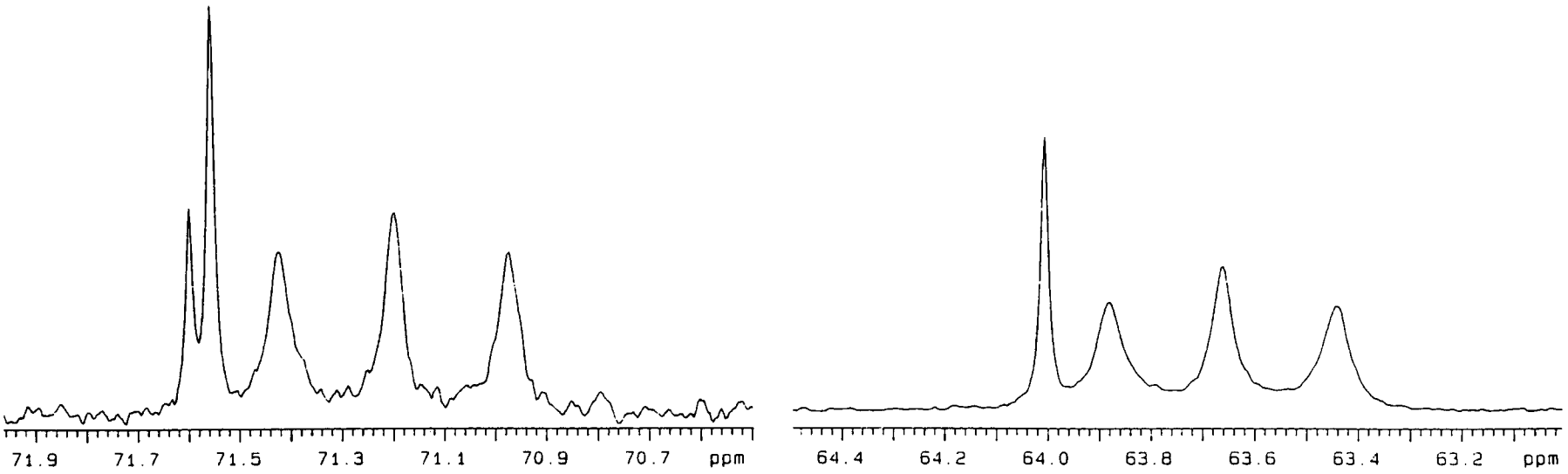


Fig. 74

3.19 Results Form Feeding (RS)-[2-^{13}C,3-$^{2}H_2$]-Phenyllactic Acid

(RS)-[2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid (**32i**) was fed to reinforce the above results, and in particular to establish unambiguously that there is no backmigration. It was also necessary to demonstrate that the shift of the C-3' enriched peak observed in the ^{13}C NMR spectrum of the (2R, 3S)-[2-^{13}C, 3-^{2}H, *ring*-$^{2}H_5$]-phenyllactic acid (**32g**) experiment hyoscyamine was indeed a β-shift and not, the less likely scenario, that it is a shift induced by the perdeuterated phenyl ring. Here again in the resultant ^{13}C NMR (fig. 76) spectrum of hyoscyamine, the major component of the enriched peak at 64.0ppm is β-shifted with a minor peak occurring at 64.06ppm. The peak is not coupled, therefore no deuterium is directly bonded to the C-3' atom. This is again consistent with the retention of one deuterium at the chiral centre, C-2', of the tropate ester, the other being abstracted and not returned to C-3' (fig. 77). Some loss of ^{2}H is apparent from C-3 of the [2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid, since the non-β-shifted peak is larger than that observed in the (2R, 3S)-[2-^{13}C, 3-^{2}H, *ring*-$^{2}H_5$]-phenyllactic acid experiment. This was expected to occur to some extent since the [2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid contained approximately 20% [2-^{13}C, 3-^{2}H]-phenyllactic acid, however, the loss of deuterium may also be a consequence of the exchange of deuterium during epimerisation of the (S)-[2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid present in the racemic substrate.

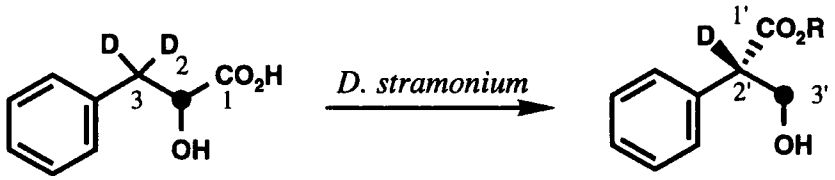
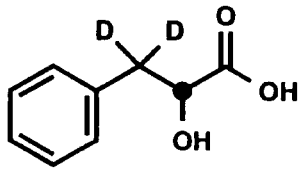


Fig. 77

The littorine enriched C-2 signal showed the expected pattern. The major component is the doubly β-shifted signal at 71.44pm with a singly β-shifted component at 71.49ppm and a non-β-shifted, natural abundance peak, at 71.54ppm. This is the singly deuterated component again arising due to the [2-^{13}C, 3-^{2}H]-phenyllactic acid in the administered substrate.



littorine C-2'

hyoscyamine C-3'

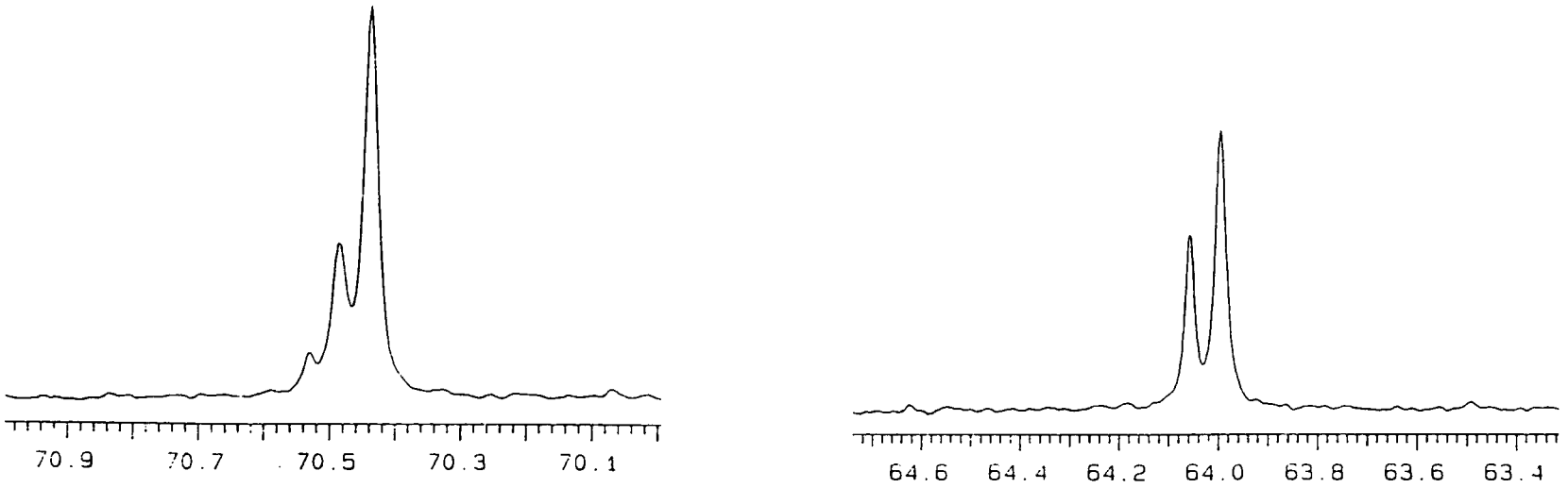


Fig. 76

3.20 Results From Feeding (RS)-[2-^{13}C]-Phenyllactic Acid (32j) and (RS)-[3-$^{2}H_3$]-Phenyllactic Acid (32k) as an Admixture

The ^{13}C NMR spectrum of the isolated hyoscyamine showed only an enriched singlet at 64.10ppm (fig. 78). There was no evidence of any crossover of deuterium between the labelled substrates (fig. 79). The ^{13}C NMR spectrum of littorine showed only a highly enriched singlet at 71.53ppm as expected.

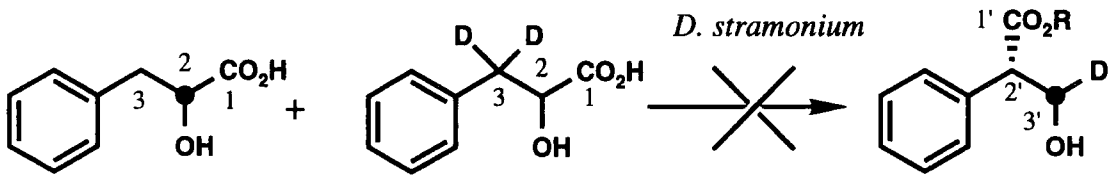


Fig. 79

3.21 Conclusions

The results from these experiments are summarised in fig. 80.

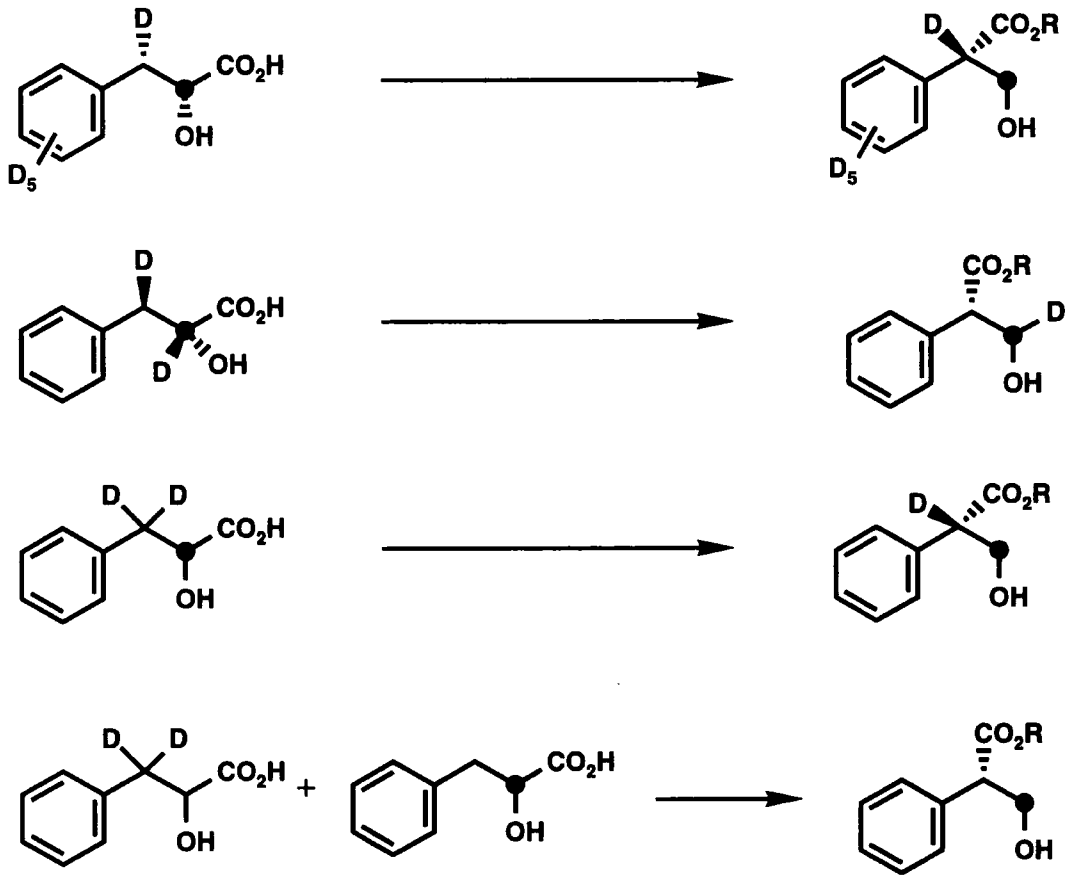
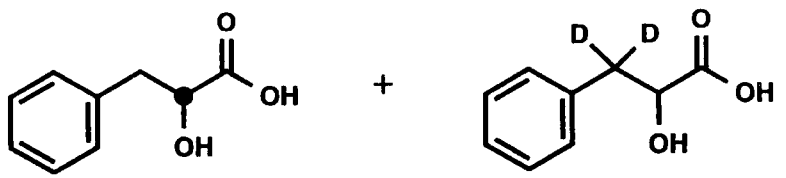
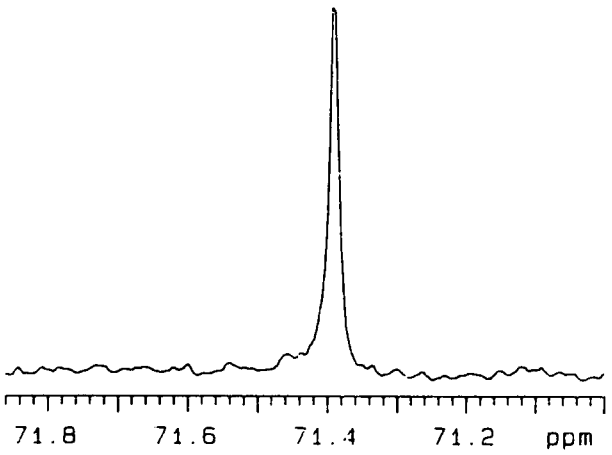


Fig. 80



littorine C-2'



hyoscyamine C-3'

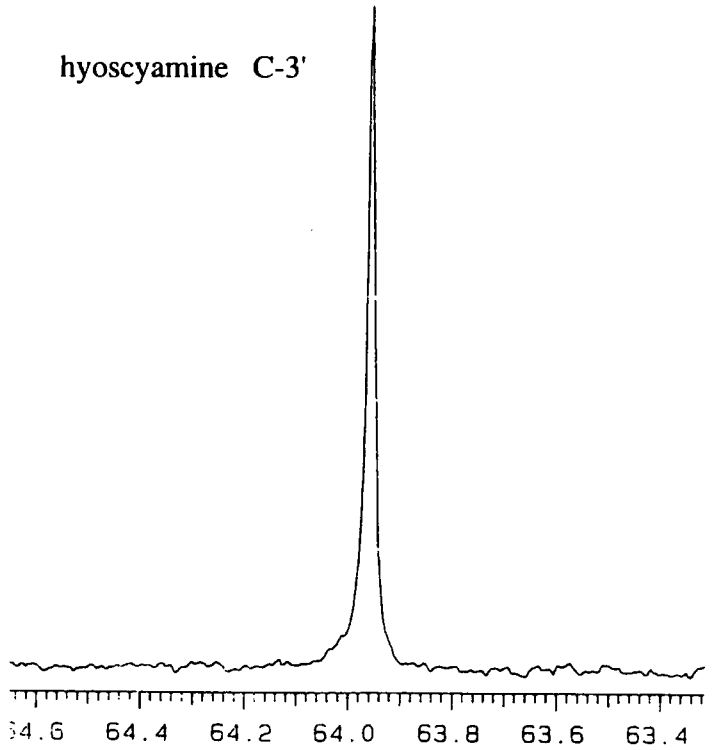


Fig. 78

These results are unambiguous and demonstrate the loss of the 3-*pro*-R hydrogen of phenyllactic acid (and therefore littorine) during the rearrangement. From these experiments there is no evidence that this hydrogen is returned and accordingly a vicinal interchange discounted. The 3'-*pro*-S hydrogen is retained at C-2', the chiral centre, of the tropate moiety. The implications of these results are firstly that the rearrangement does not involve a vicinal interchange, as was generally accepted, and secondly that there is *inversion* of configuration at both migration termini (fig. 81).

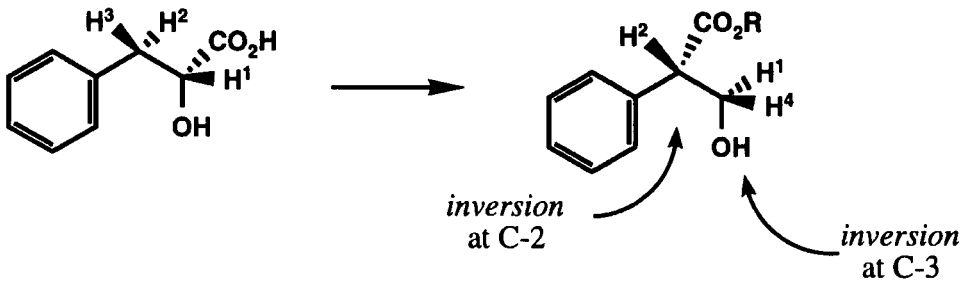
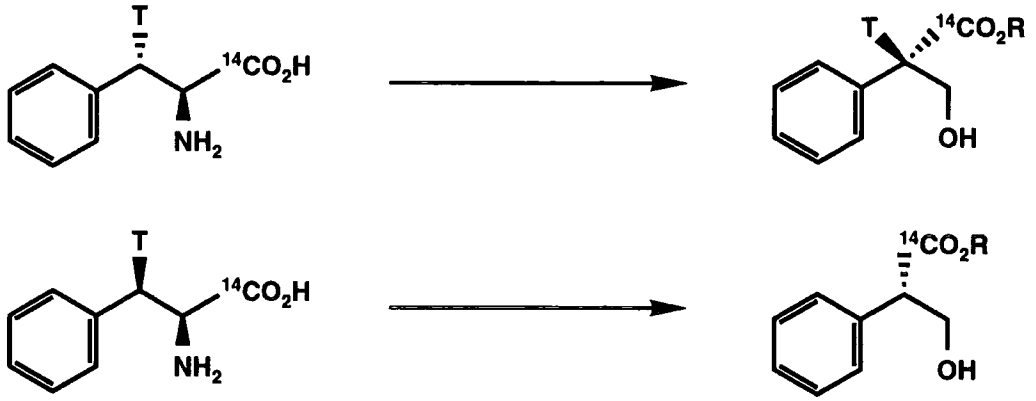


Fig. 81

3.22 Discussion

Clearly these results are inconsistent with Leete's conclusions of a mutase enzyme mediating a vicinal interchange process. Earlier work by Haslam *et al.*[38] however reports findings entirely consistent with our own. Both (2S, 3R)- and (2S, 3S)-[1-^{14}C, 3-^{3}H]-phenylalanines were synthesised using Wrightman's procedure[35] which gives the desired phenylalanines with 90% enantiomeric excess of the desired stereochemistry at C-3 (this also being the synthesis that Leete used). The phenylalanines were administered to whole *D. stramonium* plants and the isolated hyoscyamine and scopolamine were hydrolysed to tropic acid and dehydrated to atropic acid to locate the tritium. The results from these experiments were considered to be consistent with the loss of the 3-*pro*-R hydrogen of phenylalanine, which was not returned, and with the retention of the 3-*pro*-S hydrogen at the chiral centre (fig. 82).



Haslam's conclusions

Fig. 82

Leete's criticism of the work hinged on the argument that both hyoscyamine and tropic acid are readily racemised under basic conditions. If this were the case then the loss of the 3-*pro*-R tritium could be explained by wash out at the chiral centre of tropic acid during the hydrolysis in 10% NaOH, and not by abstraction during the rearrangement process. He considered the retention of the 3-*pro*-S tritium a consequence of its migration to C-3 of tropic acid, rather than its persistence from C-2 of tropic acid (fig. 83).

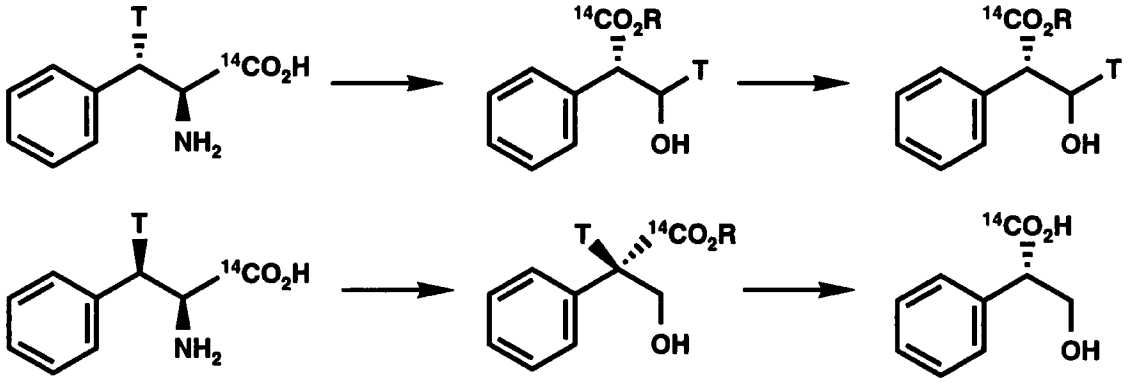


Fig. 83

The key issue here is the readiness of the tropic acid to racemise under basic conditions, a matter we felt warranted investigation.

3.23 A Study on the Rate of Deuterium Exchange From the Chiral Centre of Tropic Acid

Methyl [2-^{2}H]-tropate was synthesised as described in section 3.6. This served as a model system to study the rate of racemisation of hyoscyamine/tropic acid during the hydrolysis of the alkaloid in either $Ba(OH)_2$ or $Na(OH)_2$ (fig. 84).

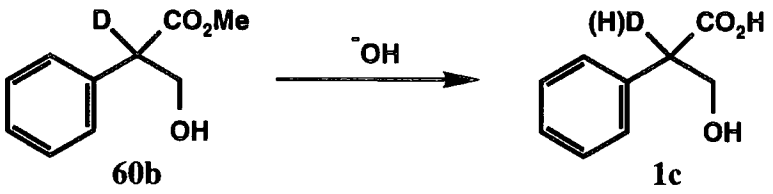
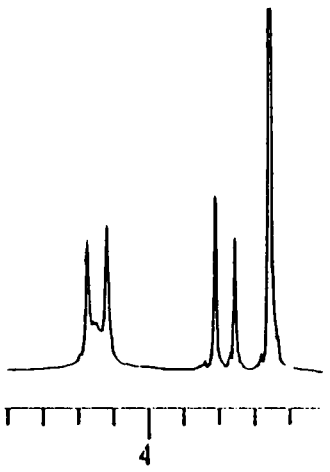


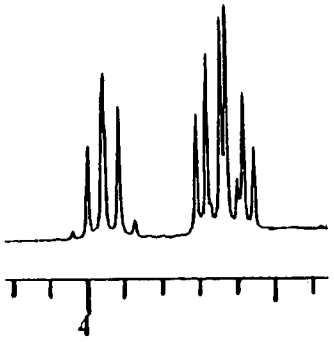
Fig. 84

Methyl [2-^{2}H]-tropate (**60b**) was initially subjected to hydrolysis in saturated barium hydroxide solution for 30 minutes, the conditions used by Leete to hydrolyse hyoscyamine and scopolamine. It was apparent from the ^{1}H NMR spectrum of the resulting tropic acid that the deuterium had remained largely intact at the chiral centre of tropic acid, with only a small level of washout. This is evidenced from the coupling of the hydroxymethyl protons and the integral of one of the hydroxymethyl and methine resonances, which are coincident.

[2-^{2}H]-Tropic acid (**1c**) was then refluxed in 10% sodium hydroxide removing aliquots of the reaction mixture at different times to assess the rate of exchange. The aliquots were immediately acidified to prevent further exchange and the isolated tropic acid was analysed by ^{1}H NMR. From these spectra it can be seen that a substantial quantity of deuterium is still present after 5 minutes and 10 minutes, washout is however substantial after 20 minutes (fig. 85) and essentially complete after 30 minutes. These results indicate that the exchange may not be as fast as suggested by Leete.[2] The kinetic isotope effect operating in the exchange of tritium from the chiral centre of tropic acid would be expected to be significantly larger than that for deuterium (k_H/k_D = 1-7 k_H/k_T = 1-16[39]). It is quite conceivable that there was residual tritium



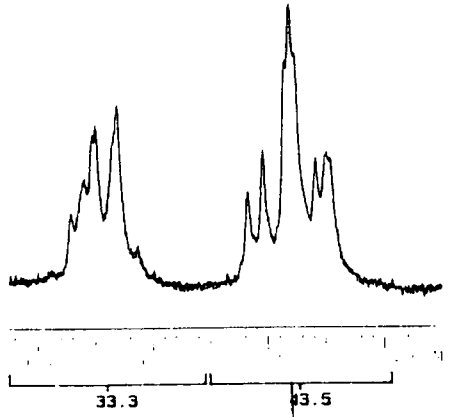
methyl (2-^{2}H)-tropate



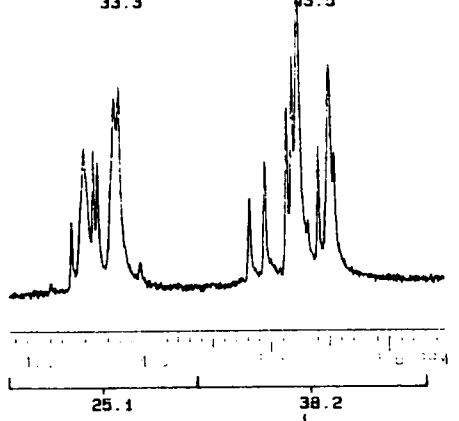
tropic acid

NaOH hydrolysis

20min



10min



5min

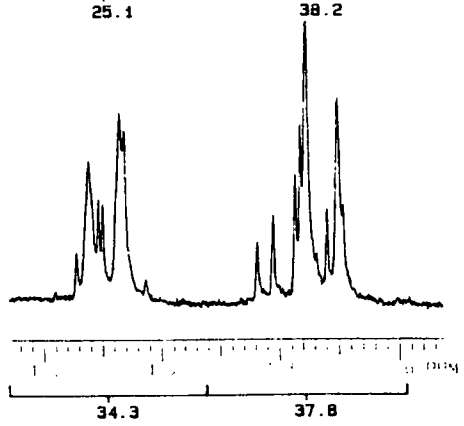


Fig. 85

present at the chiral centre of tropic acid after $Ba(OH)_2$ hydrolysis and probably after 10% NaOH hydrolysis carried out by Haslam *et al.* and the interpretation of their result is in our view correct.

The inconsistency between our results and Leete's[1,2] are however not so easily explained. From his degradation studies, in particular the conversion of C-3 of tropic acid into formaldehyde dimedone, there can be no question that Leete did indeed observe tritium at C-3 of tropic acid, albeit at a low level ($\approx 0.1\%$). However, there are some important differences between the two studies which probably account for the different results. Firstly, in the earlier experiments L-phenylalanine was administered rather than the more immediate precursor phenyllactic acid. It is now known that the incorporation of L-phenylalanine must proceed *via* phenylpyruvic acid. The stereospecific loss of tritium from phenylpyruvic acid into the water in the cells cannot be discounted (fig. 86).

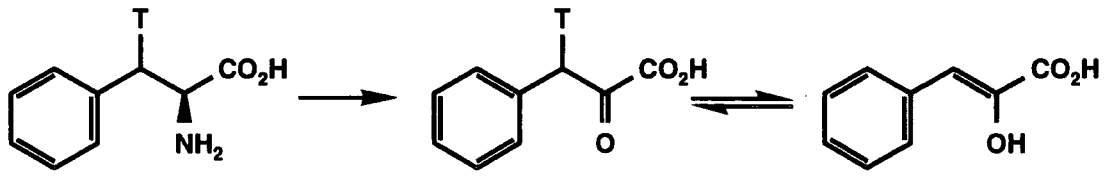


Fig. 86

The incorporation of tritium into NAD(P)H could allow for its re-introduction into C-3 of phenyllactic acid *via* a (2R)-phenyllactate dehydrogenase (fig. 87). Additional delivery of tritium to C-3' of hyoscyamine may also occur after rearrangement in a manner which is discussed in section 4.5.

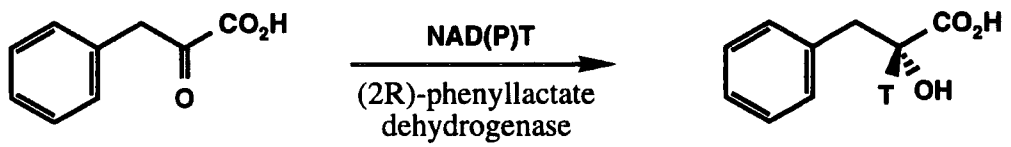


Fig. 87

It is interesting to compare here the results from feeding (2R, 3S)-[2-^{13}C, 3-^{2}H, *ring*-$^{2}H_5$]-phenyllactic acid (**32g**) and (RS)-[2-^{13}C, 3-$^{2}H_2$]-phenyllactic acid (**32i**). The non-β-shifted component is much larger in the latter case, indicating some loss of deuterium relative to ^{13}C. This is unlikely to be an artefact due to chemical manipulation as identical alkaloid isolation procedures were used. The loss of ^{2}H in the latter case could be explained as loss from the (S)-enantiomer, which must epimerise either *via* oxidation to phenylpyruvic acid and reduction with a (2R)-phenyllactate dehydrogenase or a racemase as discussed in section 2.15. The second significant factor is the use of whole plants rather than submerged transformed root cultures. If tritium were lost into the water in the cell, its incorporation into NAD(P)H and re-introduction *via* a dehydrogenase would be more likely in the whole plant situation since the volume of water is limited to the small volume of the cell and its immediate environs. Conversely, with the submerged root cultures exchange of tritiated water with water from the medium would be expected and so dilute the labelled water available in the cells. Another relevant feature of whole plant experiments is the much lower levels of L-phenylalanine incorporation observed. This is in the region of 0.02%, compared to the 15-40% incorporation of phenyllactic acid in the transformed root culture studies.

A further feature is the stereospecifically labelled L-phenylalanines used in these experiments were only approximately 90% enantiomerically pure at C-3, compared to the (2R)-phenyllactic acids which were 98% enantiomerically pure at C-3. It is possible that the results were perturbed significantly by contaminating isotope from the small amount of the other enantiomer present, particularly in the light of the low level of incorporations observed.

Whatever the origin of the misinterpretation of Leete's study, this re-evaluation using stable isotopes offers an unambiguous assessment of the stereochemical course in hyoscyamine biosynthesis.

3.24 References

1. E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271
2. E. Leete, *Can. J. Chem.*, 1987, **65**, 226
3. K. R. Hansen and I. A. Ross, *Acc. Chem. Res.*, 1975, **8**, 1
4. N. C. J. E. Chesters, D. O'Hagan, R. J. Robins, A. Kastelle and H. G. Floss, *J. Chem Soc., Chem Commun.*, 1995, 129
5. J. W. Cornforth, J. W. Redwood, H. Eggerer, W. Buckel and C. Gutschow, *Nature*, 1969, **221**, 1212
6. J. W. Cornforth and J. W. Redwood, *Eur. J. Biochem.*, 1970, **14**, 1
7. J. Lüthy, J. Rétey and D. Arigoni, *Nature*, 1969, **221**, 1213
8. C. A. Townsend, T. Scholl and D. Arigoni *J. Chem. Soc., Chem. Commun.*, 1975, 921
9. H. G. Floss and M-D. Tsai, *Adv. Enzymol.*, 1979, **50**, 243
10. H. G. Floss in *Methods in Enzymology,* **87**, part C, Ed. D. L. Purich, Academic Press, New York, London, Paris, San Deigo, São Paulo, Sydney, Tokyo, Toronto, 1982, p. 126
11. H. G. Floss, M-D Tsai and J. W. Redwood in *Topics in Stereochemistry* , **15**, Ed. E. L. Eliel, S. H. Wilen and N. L. Allinger, John Wiley and Sons, New York, Chichester, Brisbane, Toronto and Singapore, 1984, p. 253
12. H. G. Floss and S. Lee, *Acc. Chem. Res.,* 1993, **26**, 116
13. H. Lenz and H. Eggerer, *Eur. J. Biochem.*, 1976, **65**, 237
14. F. A. L. Anet and M. Kopelevich, *J. Am. Chem. Soc.*, 1989, **111**, 3429
15. T. Marita, Y. Okamoto and H. Sakurai, *Bull. Chem. Soc. Jpn.*, 1978, **51**, 2169
16. R. Kuhn and H. Roth, *Chem. Ber.,* 1933, **66**, 1274
17. M. Hudlicky *Oxidations in Organic Chemistry* Ed. A. M. R. Rouhi, American Chemical Society, Washington D. C., 1990, p. 224
18. R. W. Woodward, L. Mascaro Jr., R. Hörhammer, S. Eisenstein and H. G. Floss. *J. Am. Chem. Soc.*, 1980, **102**, 6314

19. J. J. Lee, P. M. Dewick, C. P. Garst-Allman, F. Spregifico, C. Kowal, C-J. Chang, A. G. McInnes J. A. Walter and P. J. Kever and H. G. Floss, *J. Am. Chem Soc.*, 1987, **109**, 5426

20. K. Kobayashi, P. K. Jadhav, T. M. Zydowsky and H. G. Floss, *J. Org. Chem.*, 1983, **48**, 3510

21. H. G. Floss in *Asymmetric Reactions and Processes in Chemistry*, Ed. E. L. Eliel and S. Otsuka, American Chemical Society, Washington D.C. 1982, p. 229

22. J. T. Kealy, S. Lee, H. G. Floss and D. V. Santi, *Nucleic Acids Res.*, 1991, **19**, 6465

23. H. G. Floss, personal communication, 1993

24. R. H. Ables, *Acc. Chem. Res.*, 1976, **9**, 114

25. H. A. Barker, *Annu. Rev. Biochem.*, 1972, **41**, 55.

26. M. Sprecher, M. J. Clark and D. B. Sprinson, *J. Biol. Chem.*, 1966, **241**, 872

27. K. A. Reynolds, D. O'Hagan, D. Gani and J. A. Robinson, *J. Chem. Soc., Perkin Trans. I,* 1988, 3195

28. G. Brendelberger, J. Rétey, D. M. Ashworth, K. Reynolds, F. Willenbrock and J. A. Robinson, *Angew. Chem. Int. Ed. Engl.*, 1988, **27**, 1089

29. M. Sprecher, M. J. Clark and D. B. Sprinson, *J. Biol. Chem.*, 1966, **241**, 864

30. M. L. Louden and E. Leete, *J. Am. Chem. Soc.,* 1962, **84**, 1510

31. D. R. Briggs, K. F. Ryan and H. L. Bell, *J. Plant Foods,* 1983, **5**, 143

32. R. J. Robins, P. Bachmann and J. G. WoolleyJ, *Chem. Soc., Perkin Trans. I*, 1994, 615

33. N. C. J. E. Chesters, K. Walker, D. O'Hagan and H. G. Floss, *J. Am Chem. Soc.,* in press

34. A. W. Ingersoll and S. H. Babcock, *Org. Synth. Coll. Voll. II*, 1943, 328

35. R. H. Wrightman, J. Staunton, K. R. Hanson and A. R. Battersby, *J. Chem. Soc., Perkin Trans. I*, 1972, 2355

36. M. D. Fryzuk and B. Brosnich, *J. Am. Chem. Soc.*, 1977, **99**, 6262

38. B. Wünsch and M. Zott, *Liebig's Ann. Chem.*, 1992, 39

38. R. V. Platt, C. T. Opie and E. Haslam, *Phytochemistry*, 1984, **23**, 2211

39. A Fersht, *Enzyme Structure and Mechanism*, 2nd Edition, W. H. Freeman and Co. New York, 1985, p. 93

CHAPTER 4

Mechanistic Insights

4.1 Introduction

The intermediates between L-phenylalanine (**29**) and hyoscyamine (**2**) are now identified and all the stereochemical features of the rearrangement of the linear propanoid side chain, to generate the branched tropate moiety, are now evaluated. L-Phenylalanine is initially transamminated and then reduced to form (R)-D-phenyllactic acid[1] (**32b**). Esterification with tropine (**23**), to generate littorine (**33**) has been shown to precede the rearrangement.[2] Littorine is then converted to hyoscyamine, with the loss of the 3'-*pro*-R hydrogen, and with inversion of configuration at both migration termini.[3] These results are summarised in fig. 88.

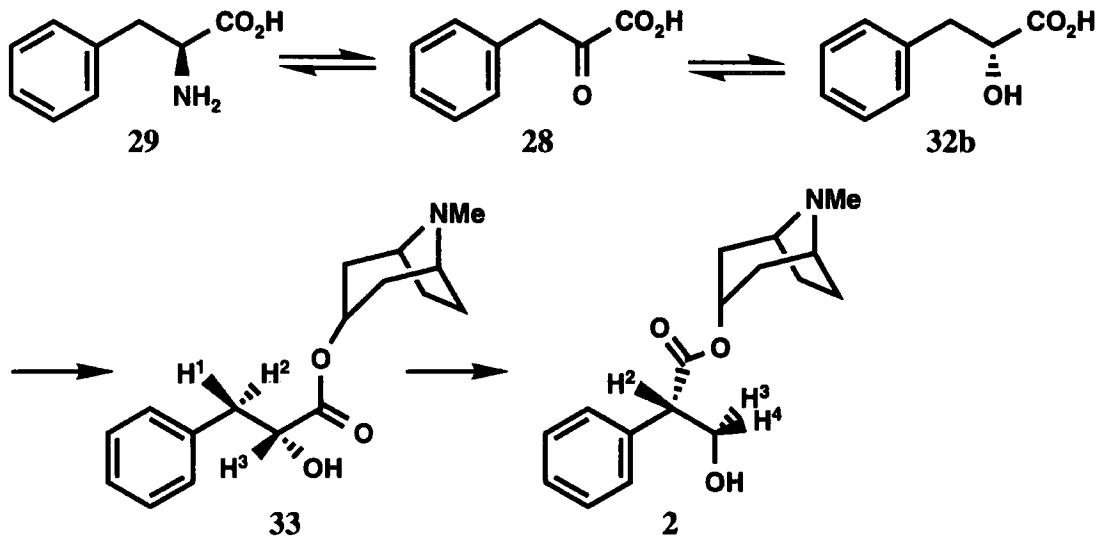
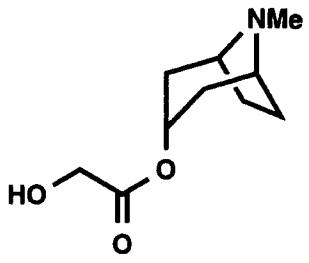
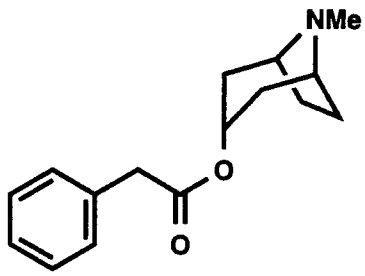


Fig. 88

4.2 3α-2'-Hydroxyacetoxytropane and 3α-Phenylacetoxytropane

3α-2'-Hydroxyacetoxytropane (**34**) and 3α-phenylacetoxytropane (**35**) (fig. 89) occur as minor alkaloids in *Datura* species[4] and these two alkaloids would appear to be shunt metabolites from the rearrangement of littorine (**33**) to hyoscyamine (**2**).





2'-hydroxyacetoxytropane (**34**) phenylacetoxytropane (**35**)

Fig. 89

3α-2'-Hydroxyacetoxytropane (**34**) and 3α-phenylacetoxytropane (**35**) are produced only in small quantities compared to hyoscyamine, approximately 0.01% and 0.02% of the total alkaloid content of the roots of *Brugmansia candida* x *aurea*, compared to hyoscyamine which accounts for approximately 30%.[5] These minor alkaloids are therefore not amenable to NMR analysis. In collaboration with Dr Richard Robins (then) at the AFRC Insitute of Food Research in Norwich, the incorporations of a number of isotopically labelled substrates into 2'-hydroxyacetoxytropane (**34**) and phenylacetoxytropane (**35**) was assessed by GC-MS analysis. Alkaloid extracts from roots, which had been supplemented with [1,3-$^{13}C_2$]-phenyllactic acid and [1',3'-$^{13}C_2$ N-*methyl*-$^{2}H_3$]-littorine, and my own alkaloid extracts from feeding (RS)-, (R)- and (S)-[2-^{13}C,^{2}H]-phenyllactic acid, were examined.[6]

(RS)-[1,3-$^{13}C_2$]-Phenyllactic acid was administered to transformed root cultures of *D. stramonium* and *B. candida* x *aurea* hybrid. Efficient incorporation into phenylacetoxytropane, 2'-hydroxyacetoxytropane and hyoscyamine was observed, at approximately equal levels (≈40%). This supports the view that these different compounds became enriched by closely related processes. These results are summarised in Table 5. Additionally, no reduction in the level of these incorporations was observed when exogenous unlabelled phenyllactic or tropic acids were added, consistent with previous results.[6]

		Hydroxyacetoxy--tropane	Phenylacetoxy--tropane	Hyoscyamine
Datura stamonium	M^++1	43.3%	16.4%	6.1%
	M^++2	/	37.2%	37.7%
Brugmansia candida x *aurea*	M^++1	41.7%	9.5%	7.5%
	M^++2	/	40.9%	41.3%

Incorporations of [1,3-$^{13}C_2$]-phenyllactic acid into 2'-hydroxyacetoxytropane, phenylactetoxytropane and hyoscyamine (determined R. J. Robins).

Table 5

The M+1 mass enhancements in 2'-hydroxyacetoxytropane (**34**) when fed with (RS)-DL-[1,3-$^{13}C_2$]-phenyllactic acid is consistent with the loss of the ((C-3) + phenyl) of the phenyllactic acid. Correspondingly, the M+2 incorporations into phenylacetoxytropane (**35**) clearly indicate that both the ^{13}C labels have been retained and therefore C-2 of phenyllactic acid had been lost (fig. 90) and not C-1, as might be anticipated as a result of an oxidative degradation of phenyllactic acid *in vivo*. M+1 incorporations into both hyoscyamine (**2**) and phenylacetoxytropane (**35**) are much lower than the M+2 incorporations, although they are still significantly above natural abundance. These enrichments can however be attributed to the presence of approximately 9% of (RS)-DL-[1-^{13}C]-phenyllactic acid and (RS)-DL-[3-^{13}C]-phenyllactic acid in the (RS)-DL-[1,3-$^{13}C_2$]-phenyllactic acid administered.[8]

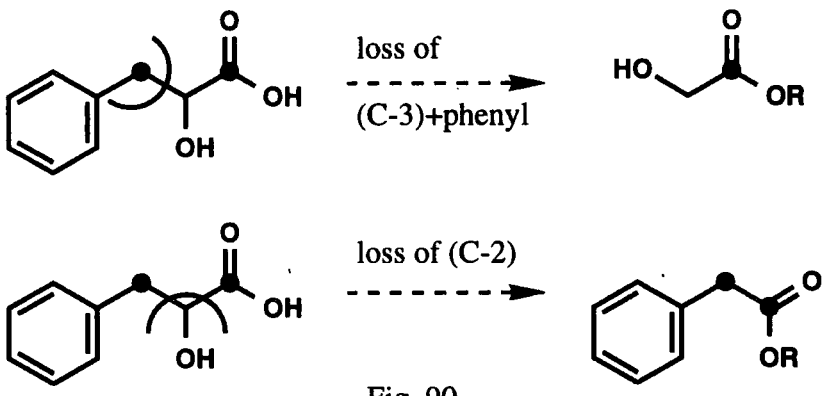


Fig. 90

Isotope from (RS)-DL-[2-^{13}C, ^{2}H]-phenyllactic acid did not enrich phenylacetoxytropane (**35**), a result which is again consistent with the loss of C-2 of phenyllactate (fig. 91).

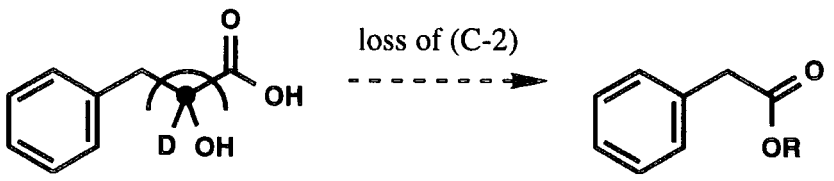


Fig. 91

This result was confirmed by examining the simultaneous incorporations into phenylacetoxytropane (**35**) and 2'-hydroxyacetoxytropane (**34**) from (R)-D- and (S)-L-[2-^{13}C, ^{2}H]-phenyllactic acids.[1] These results are presented in Table 6.

		Hydroxyacetoxy -tropane	Phenylacetoxy -tropane	Hyoscyamine
(R)	$M^{+}+1$	1.4%	-1.5%	0.8%
	$M^{+}+2$	18.4%	-0.4%	21.7%
(S)	$M^{+}+1$	9.8%	-0.6%	5.1%
	$M^{+}+2$	1.0%	-0.4%	2.4%

Incorporations of (R)- and (S)-[2-^{13}C, ^{2}H]-phenyllactic acids into 2'-hydroxyacetoxytropane, phenylacetoxytropane and hyoscyamine. Negative numbers represent an error of approximately 2% in determination.

Table 6

Again no incorporations into phenylacetoxytropane (**35**) were observed from either enantiomer of [2-^{13}C, ^{2}H]-phenyllactic acid, consistent with the loss of C-2. The possibility that this alkaloid was formed *via* oxidation of the phenyllactic acid (**32**) to phenylpyruvic acid (**28**), with subsequent decarboxylation to generate free phenylacetic

acid (**27**), and can be discounted from the results as in this event a strong M+1 mass enhancement would result but is not observed (fig. 92).

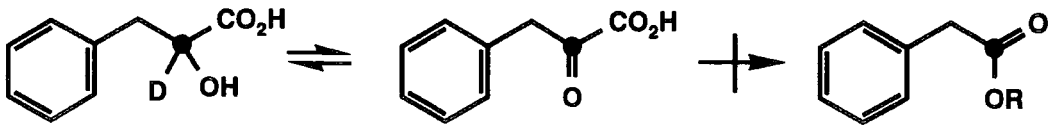


Fig. 92

The 18.4% M+2 mass enhancement evaluated for 2'-hydroxyacetoxytropane (**34**) after feeding (R)-D-[2-^{13}C, ^{2}H]-phenyllactic acid, demonstrates that one of the C-H bonds of the hydroxymethyl group is retained intact from the C-H bond of C-2 at (R)-D-phenyllactic acid. However, the corresponding 9.8% M+1 mass enhancement, apparent after feeding (S)-L-[2-^{13}C, ^{2}H]-phenyllactic acid demonstrates that incorporation from this enantiomer occurs only after epimerisation to (R)-D-phenyllactic acid, as observed for both littorine and hyoscyamine.

In order to reinforce the hypothesis that phenylacetoxytropane (**35**) and 2'-hydroxyacetoxytropane (**34**) are products closely related to the rearrangement process, the incorporations of quintupally labelled (RS)-[1',3'-$^{13}C_2$ *methyl*-$^{2}H_3$]-littorine (**33**) were examined by Richard Robins (Table 7). The M+5 incorporation of this quintupally labelled littorine into hyoscyamine was deemed too high to be the result of the separate use of the tropine and phenyllactic acids after hydrolysis of the administered ester. Similarly, phenylacetoxytropane was found to have a significant M+5 mass enhancement ($\approx$ 3.5%). Although lower than the M+5 mass enhancement evaluated for hyoscyamine this was still too high to be the result of the hydrolysis of the quintupally labelled littorine and recombination of the [1,3-$^{13}C_2$]-phenyllactic acid and [*methyl*-$^{2}H_3$]-tropine. Further, the M+2 and M+3 mass enhancements were smaller then the M+5 mass enhancement which again indicates that the hydrolysis of the littorine was not the predominant route to incorporation. [1',3'-$^{13}C_2$, *methyl*-$^{2}H_3$]-Littorine was efficiently incorporated into 2'-hydroxyacetoxytropane to give predominantly a M+4 mass enhancement, as expected from the loss of ((C-3')+phenyl) (fig. 93).

	Hydroxyacetoxy-tropane		Phenylacetoxytropane			Hyoscyamine		
Additional compound fed	**M^++3**	**M^++4**	**M^++2**	**M^++3**	**M^++5**	**M^++2**	**M^++3**	**M^++5**
None	5.1%	13.0%	nd	nd	nd	3.3%	4.8%	4.5%
Tropine (0.25mM)	4.9%	18.8%	2.2%	1.9%	4.0%	4.3%	3.8%	6.5%
(RS)-Phenyllactate (0.25M)	4.1%	16.0%	1.8%	1.6%	3.3%	3.5%	3.5%	6.1%

Incorporations of [1',3'-$^{13}C_2$, *methyl*-$^2H_3$]-littorine into 2'-hydroxyacetoxytropane, phenylacetoxytropane and hyoscyamine, nd = insufficient area under GC-MS peak to determine incorporation (determined by R. J. Robins).

Table 7

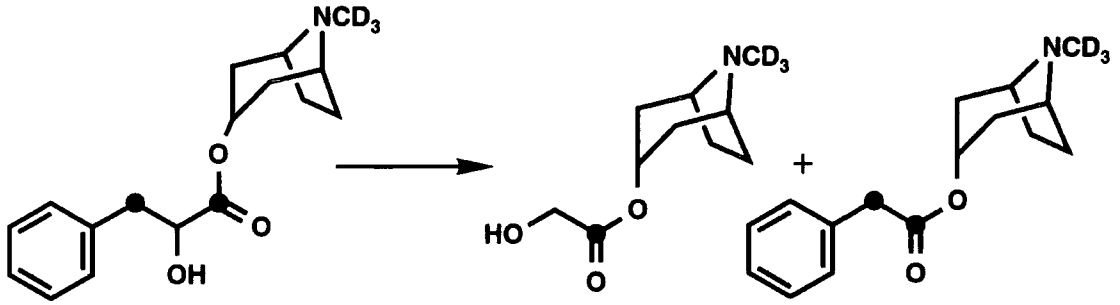


Fig. 93

Following the argument outlined in section 2.10 it is concluded that phenylacetoxytropane (**35**) and 2'-hydroxyacetoxytropane (**34**) are necessarily shunt metabolites of hyoscyamine biosynthesis.

A comparison of the littorine (**33**) to hyoscyamine (**2**) rearrangement with the rearrangements mediated by the coenzyme-B_{12} mutases (of which the rearrangement of methylmalony-CoA mutase is an illustrative example (fig. 94)), has been discussed (section 3.13).

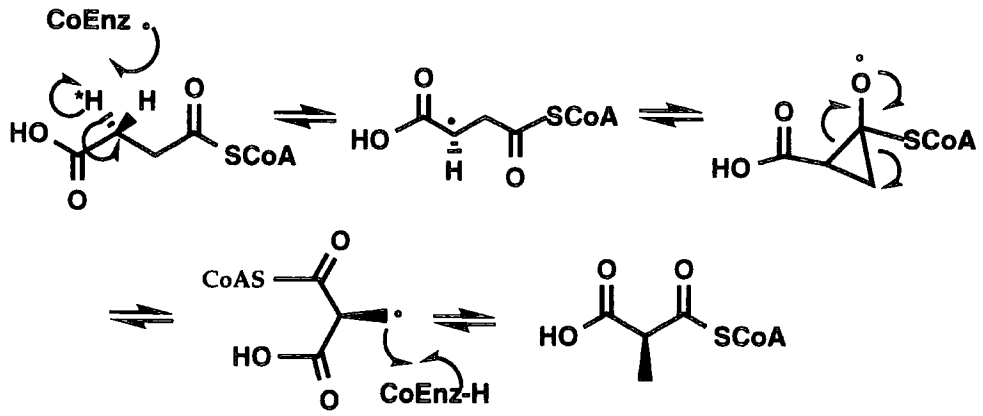


Fig. 94

The rearrangement is most probably initiated by abstraction of the 3'-*pro*-R hydrogen of littorine. Such a process could be either radical or ionic in nature. However, a radical process is attractive since radical processes have precedent in biochemistry whereas anionic and cationic processes are rare (fig. 95).[9]

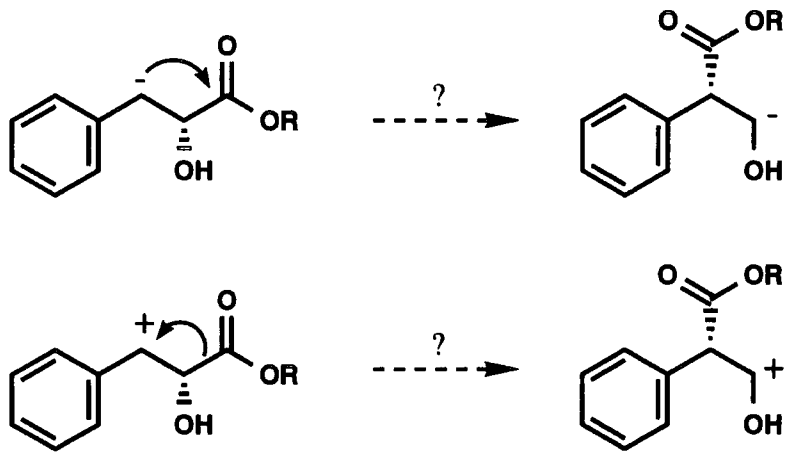


Fig. 95

A benzylic radical should find stability *via* a transient cyclopropyl intermediate (**74**) which can open across the (C-1')-(C-2') bond to furnish the tropate skeleton. This would be the normal, predominant process resulting in the interconversion of hyoscyamine and littorine (fig. 96). However, hydroxylation of radical (**73**) could initiate a pathway to 2'-hydroxyacetoxytropane (**34**) and hydroxylation of radical (**75**) could initiate a pathway to phenylacetoxytropane (**35**). This is discussed in section 4.5.

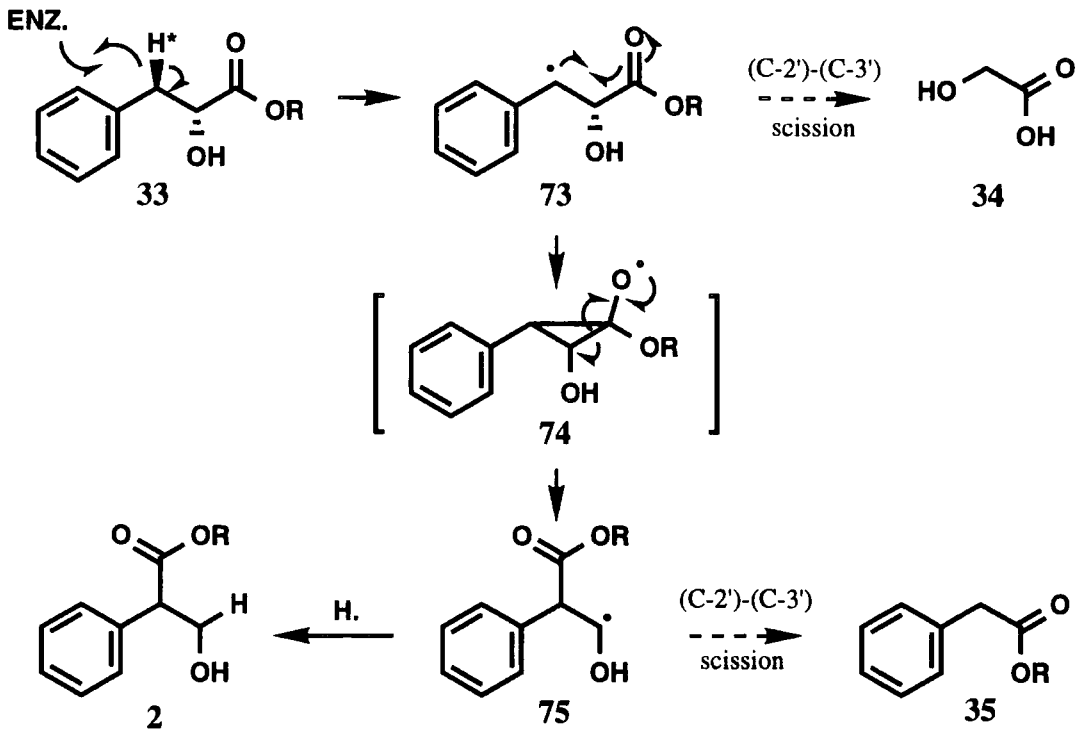


Fig. 96

4.3 Iron-oxo Enzymes

The evidence that vitamin B_{12} is not found in plants,[10] and the recent conclusion that there is no vicinal interchange process operating, limits the extention of the analogy to methylmalonyl-CoA mutase or a related coenzyme B_{12} process. However, a more reasonable suggestion is radical initiation by an iron-oxo enzyme, a process which has some precedent in the rearrangement of flavanone to isoflavone in plants.[11-13]

Iron-oxo enzymes[14] mediate oxidative reactions. These are predominantly hydroxylation, however, some alcohol to aldehyde oxidations, desaturations and C-C bond cleavage reactions are also found. Enzymes of this type include the cytochrome P-450 enzymes which contain a protoporphyrin ring as a prosthetic group and a cystein residue which forms as an axial ligand to the haem iron (fig. 97); others are non-haem and contain iron ligated to histidine residues.

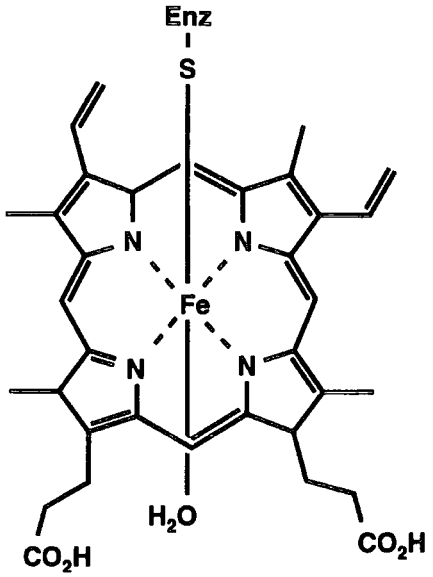


Fig. 97

These enzymes require both NADPH and O_2 and mediate the stepwise cleavage of molecular oxygen. Initially a superoxide is formed which abstracts hydrogen to form a hydroperoxide. The remaining O-O bond is then cleaved heterolytically to generate a $Fe^{IV}O\cdot$ radical, which is responsible for the abstraction of hydrogen from the C-H bond of the substrate. The carbon radical generated is then quenched by a hydroxyl radical from the iron species. This association process is known as *oxygen rebound* [14] (fig. 98).

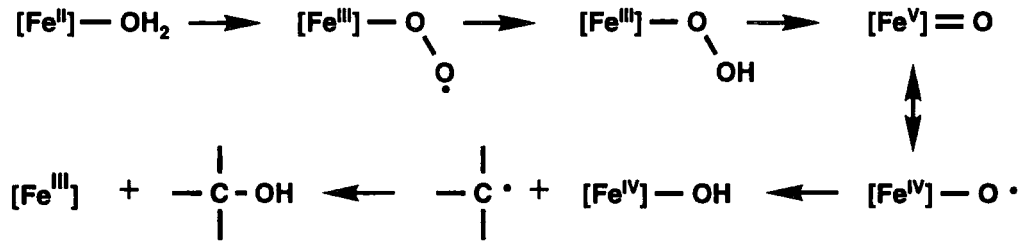


Fig. 98

4.4 The Flavonoids

The flavonoids[15] are a group of L-phenylalanine derived secondary metabolites which occur almost universally in higher plants. They are normally present as glycosides and have a variety of functions including flower pigments and antimicrobial activity. The flavonoids are biosynthesised, like many phenylpropanoids, from cinnamate, which is in turn derived from L-phenylalanine (**29**) by the action of phenylalanine ammonia

lyase. Cinnamic acid (**36**) is then hydroxylated to form coumaric acid, which is activated to its coenzyme-A ester (**76**). Coumaryl-CoA then condenses with three units of malonyl-CoA (**21**), in a manner analogous to polyketide biosynthesis. The condensation of malonate units and subsequent cyclisation are mediated by chalone synthase, to generate chalcone (**77**), an isolable intermediate. Rapid cyclisation then furnishes the flavanone skeleton (**78**), which is then further modified to the flavonoids characteristic of the individual plant (fig. 99).

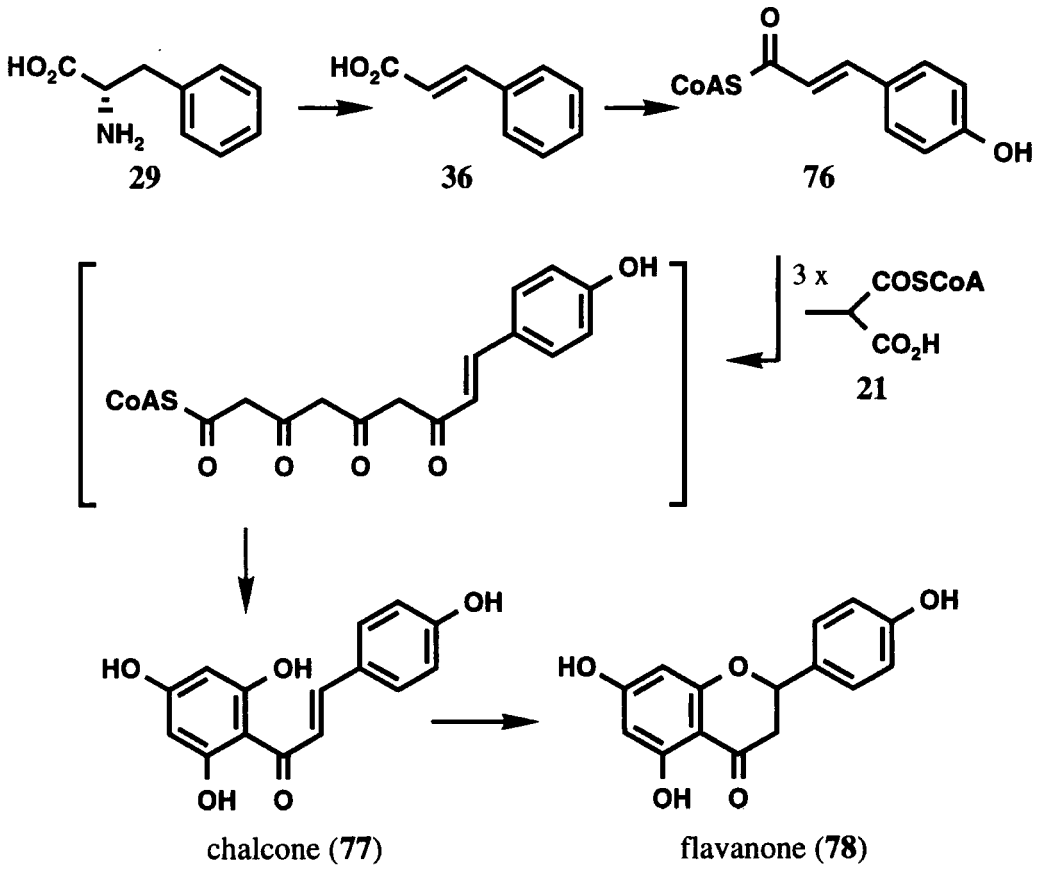
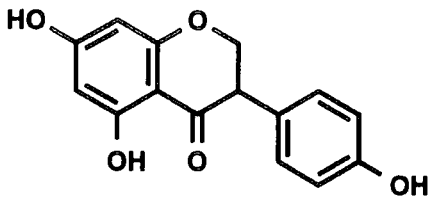


Fig. 99

The isoflavonoids (**79**)[15] are a relatively small group of secondary metabolites found predominantly in the *Leguminosae*. They are elaborated from the flavonoids by a 1,2-aryl migration from C-2 to C-3 of the pyrone ring[17,18] (fig. 100). This has been shown to be a two step process, involving an oxidative aryl migration and subsequent dehydration (fig. 101). The operation of a P-450 enzyme in the first reaction was implicated by the observation that the process required both NADPH and O_2, was inhibited by CO and P-450 inhibitors and was partially reversed by light.[12,13]



isoflavonoid aglycone (**79**)

Fig. 100

The C-2 hydroxyl was shown to be derived from molecular oxygen, not water, which is consistent with a radical mechanism. The operation of a P-450 enzyme was eventually demonstrated unequivocally by Hakamatsuka *et al.*[11] and a possible mechanism was suggested and is reproduced in fig. 102.

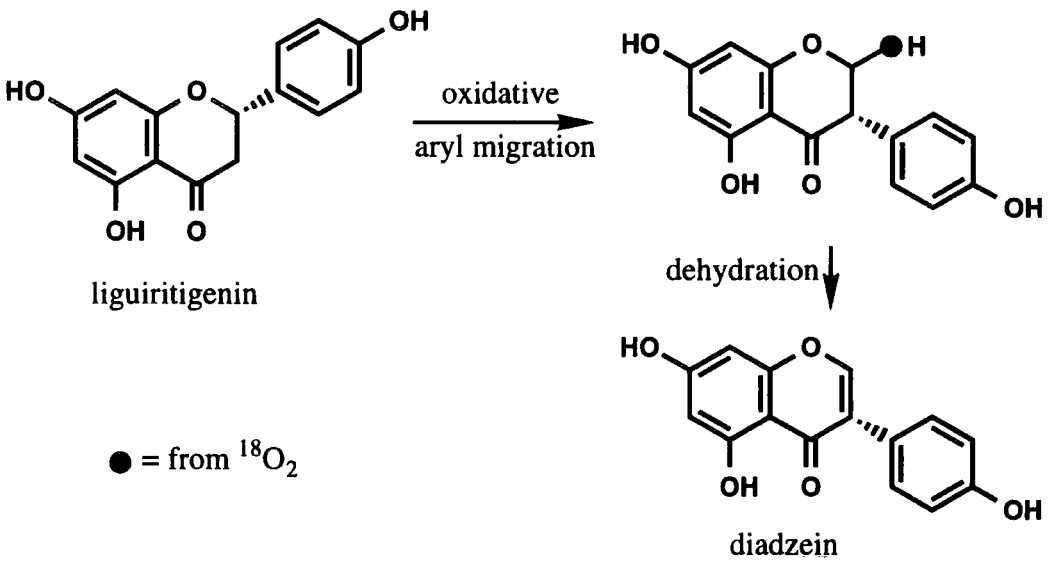


Fig. 101

The precise mechanism of the flavanone - isoflavanone rearrangement is still the subject of some debate and some modifications regarding the detail of the mechanism have subsequently been suggested.[19] Hakamatsuka *et al.*[11] have further speculated the involvement of iron-oxo enzymes in a number of other natural product biosyntheses which can all be rationalised by invoking a mechanism of hydroxylation associated with radical rearrangement.

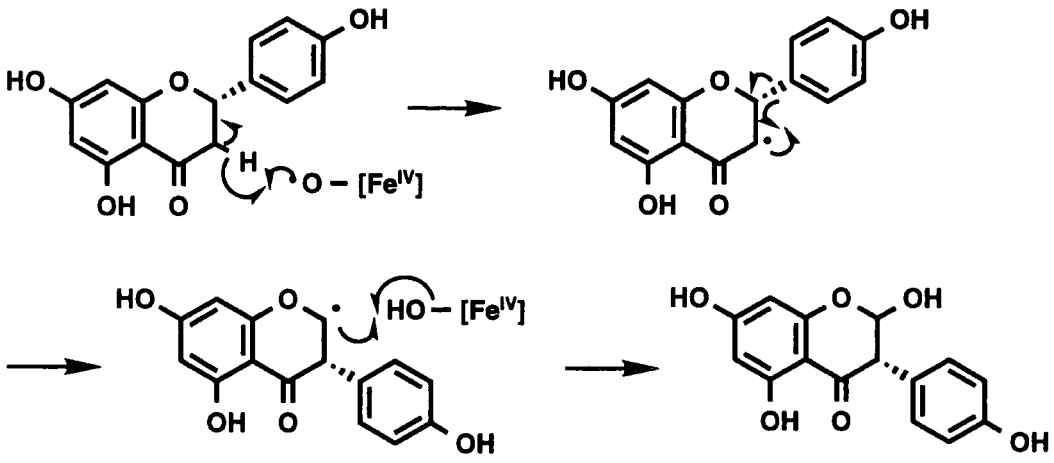


Fig. 102

4.5 A Modified View of the Mechanism of the Littorine Rearrangement

A mechanism for the littorine rearrangement (fig. 103) utilising an iron-oxo enzyme clearly requires modification of the original proposal, since the radical generated by the iron-oxo species is subsequently quenched by a hydroxyl radical in an oxygen rebound process, rather than a hydrogen radical. This could happen before or after the rearrangement. The radical would then rearrange *via* the postulated cyclopropyl transition state to generate a hyoscyamine radical (**75**). This could then be quenched by a hydroxyl radical and generate the aldehyde hydrate moiety (**80**). The action of a dehydrogenase on this aldehyde hydrate would then generate hyoscyamine (**2**). However, if the radical (**73**) was aberrantly quenched with HO· instead of rearranging, the dihydroxy species (**82**) would be susceptible to oxidative cleavage across the (C-2')-(C-3') bond. The scission of this bond would then result in the loss of ((C-3') + phenyl) to generate 2'-hydroxyacetoxytropane (**34**). The aldehyde hydrate (**80**) or α-formylphenylacetoxytropane (**81**) formed after quenching the product radical (**75**) would similarly be susceptible to the loss of (C-3') and generate phenylacetoxytropane in a manner consistent with the labelling patterns observed (section 4.2). This pathway is attractive for a number of reasons. Firstly, it rationalises the low level formation of phenylacetoxytropane (**35**) and 2'-hydroxyacetoxytropane (**34**). Also, the introduction of one of the hydroxymethylene hydrogens from a reductase enzyme may account for Leete's[10,20] observation that tritium from C-3 of L-phenylalanine was re-introduced to this site. As discussed in section 3.22, tritium lost into the water in the cells could

become incorporated into NAD(P)H by a circuitous route and result in its delivery to this site in the final reductive step. The 'same face' carboxyl migration is attractive, as according to Akhtar and Wright's[14] postulate, the stereochemical course of iron-oxo enzymes is not a consequence of the mechanism, but of the lack of mobility of the substrate within the Michaelis complex. This is believed to be an evolutionary feature arising to prevent the highly reactive intermediate radicals participating in random reactions and thereby denaturing the active site.

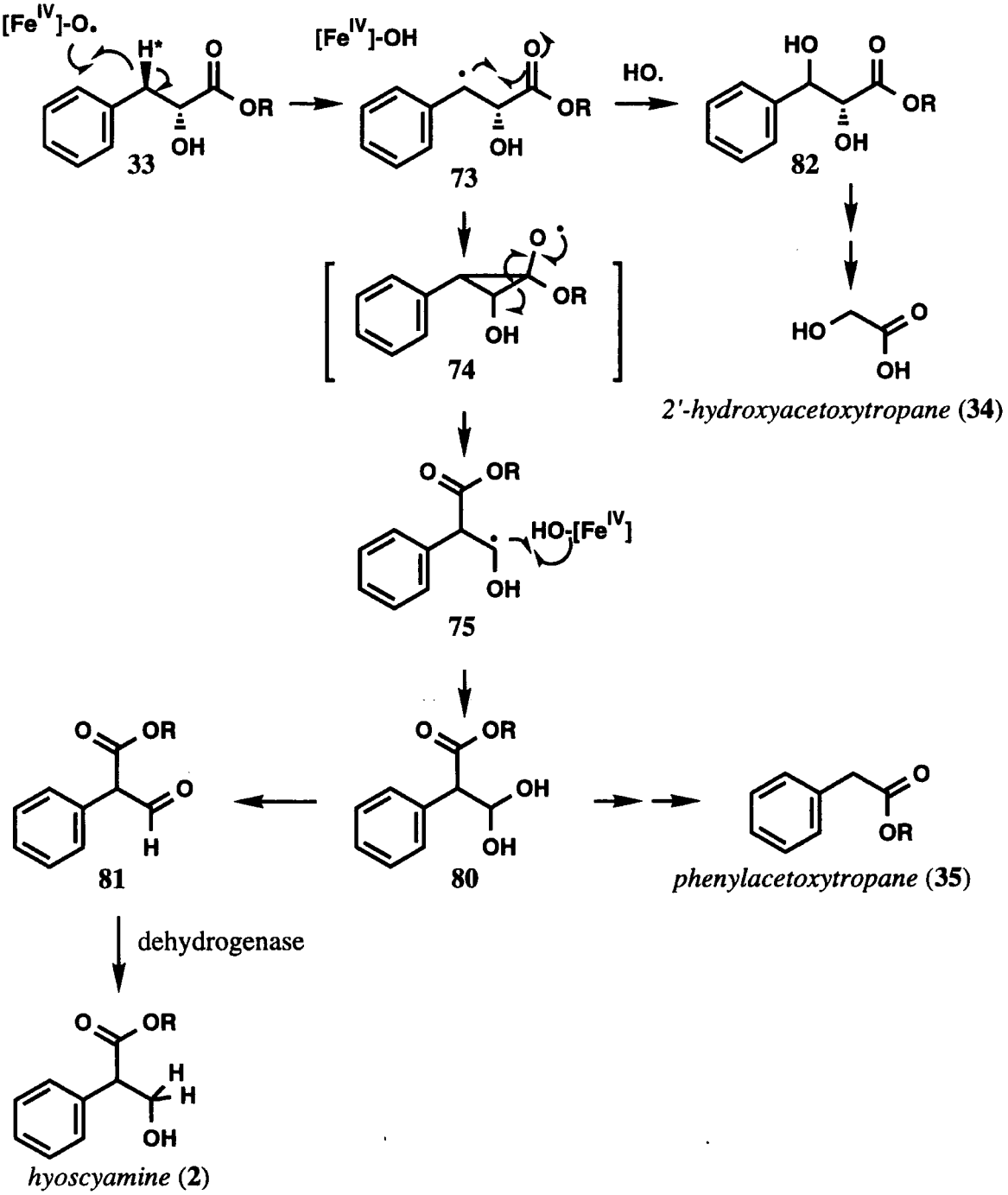


Fig. 103

A modification of this mechanism can be considered, arising for the recent view of Golding *et al.*[21] concerning the mechanism of coenzyme-B_{12} mediated mutase reactions. Here a fragmentation-recombination process is envisaged, rather than the previously accepted view of a cyclopropyl transition state (**74**). The extension of this hypothesis to the littorine / hyoscyamine rearrangement is outlined in fig. 104.

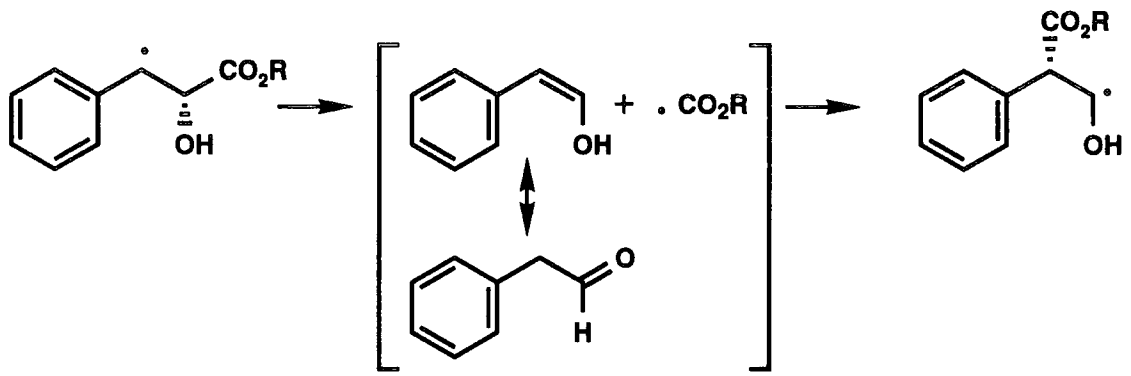


Fig. 104

If such a process were operating then inhibition by both phenylacetaldehyde and formyl tropine would be anticipated, in the same way as glycine and acrylate have been shown to inhibit glutamate mutase (fig. 105).[21]

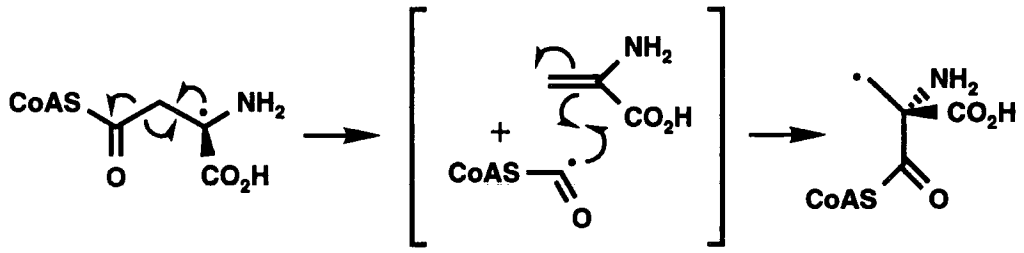


Fig. 105

The intermediacy of α-formylphenylacetoxytropane (**81**) has been previously suggested, when the utilisation of a C_6-C_2 fragment derived from phenylalanine was still under discussion.[22-24] α-Formyphenylacetic acid has been synthesied by Gross *et al.*[25] but proved to be very unstable in aqueous media, making feeding studies impossible with this intermediate.

Further experimental work is now required to test the iron-oxo hypothesis. Initially it would be interesting to determine the ^{18}O incorporation from [2-^{13}C,^{18}O,^{2}H]-phenyllactic acid. The deuterium would be necessary to distinguish between losses due to interconversion with phenylalanine and losses after rearrangement. It is anticipated that half of the ^{18}O relative to ^{2}H would be lost from the aldehyde hydrate (fig. 106), determined from the heavy isotope shifts associated with the C-3' ^{13}C NMR signal.

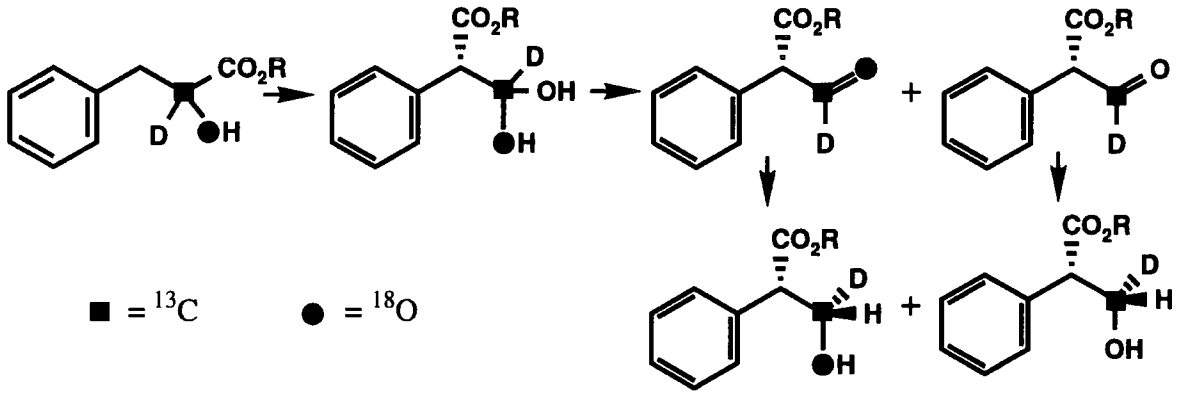


Fig. 106

The operation of a iron-oxo enzyme could also be probed using a cell free system able to convert littorine to hyoscyamine. The dependence or otherwise of the process on NADPH and O_2 could be determined, and the incorporation of ^{18}O from $^{18}O_2$ into the hydroxymethylene group investigated (fig. 107).

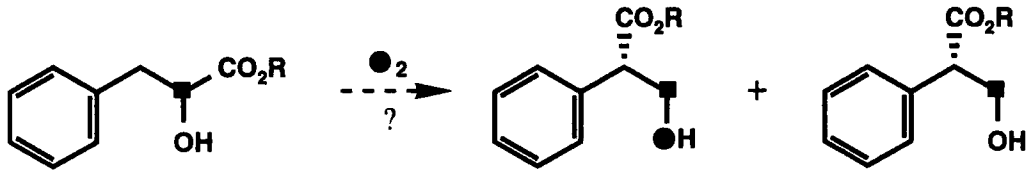


Fig. 107

4.6 References

1. N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, *J. Chem Soc., Chem. Commun.*, 1995, 127
2. R. J. Robins, P. Bachmann and J. G. Woolley *J. Chem. Soc., Perkin Trans. I,* 1994, 615
3. N. C. J. E. Chesters, D. O'Hagan, R. J. Robins, A. Kastelle and H. G. Floss, *J. Chem. Soc., Chem. Commun.*, 1995, 129
4. A. J. Parr, J. Payne. J. Eagles, B. T. Chapman, R. J. Robins and M. C. J. Rhodes, *Phytochemistry*, 1990, **29**, 2545
5. R. J. Robins, A. J. Parr, J. Payne, N. J. Walton and M. C. J. Rhodes, *Planta,* 1990, **182**, 414
6. R. J. Robins, N. C. J. E. Chesters, D. O'Hagan, A. J. Parr, N. J. Walton and J. G. Woolley, *J. Chem. Soc., Perkin Trans.*, 1994, 481
7. R. J. Robins, J. G. Woolley, M. Ansarin, J. Eagles, B. J. Goodfellow, *Planta,* 1994, **194**, 86
8. M. Ansarin and J. G. Woolley, *Phytochemistry*, 1994, **35**, 935
9. R. G. Finke in *Molecular Mechanisms in Bioorganic Processes*, Ed. C. Bleasdale and B. T. Golding, Royal Society of Chemistry, 1990, p.425
10. E. Leete, *Can. J. Chem.*, 1987, **65**, 226
11. T. Hakamatsuka, M. F. Hashim, Y. Ebizuka and U. Sankawa, *Tetrahedron,* 1991, **47**, 4969
12. G. Kochs and H. Grisebach, *Eur. J. Biochem.*, 1986, **155**, 311
13. M. F. Hashim, T. Hakamatsuka, Y. Ebizuka and U. Sankawa, *FEBS Lett.*, 1990, **271**, 219
14. M. Akhtar and J. N. Wright, *Nat. Prod. Rep.,* 1991, **8** 527
15. K. Hahlbrook in *Comprehensive Biochemistry*, **7**, Ed. P. K. Stumpf and E. E. Conn, Academic Press, New York, London, Toronto, Sydney, San Francisco, 1981, 425
16. P. M. Dewick in *The Flavonoids,* Ed. J. B. Harborne, Chapman and Hall, London, Glasgow, New York, Tokyo, Melbourne, Madras, 1994, 117

17. L. Crombie and M. B. Thomas, *J. Chem. Soc., C.*, 1967, 1796

18. L. Crombie, P. M. Dewick and D. A. Whiting, *J. Chem. Soc., Perkin Trans. I*, 1973, 1285

19. L. Crombie and D. A. Whiting, *Tetrahedron Lett.*,1992, **33**, 3663

20. E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271

21. B. Beatrix, W. Buckel, C. J. Edwards, B. T. Golding, F. K. Kroll, O. Zelder, *Abstracts, Biological Challenges for Organic Chemistry*, Royal Society of Chemistry, St Andrews, July 1995

22. M. L. Louden and E. Leete, *J. Am. Chem. Soc.*, 1962, **64**, 4507

23. E. W. Underhill and H. W. Youngken, *J. Pharm. Sci.*, 1962, **51**, 121

24. C. A. Gibson and H. W. Youngken, *J. Pharm. Sci.*, 1967, **56**, 854

25. G. G. Gross, K. J. Koelen and A. Müller, *Z. Naturforsch*, 1981, **36**, 611

PART II

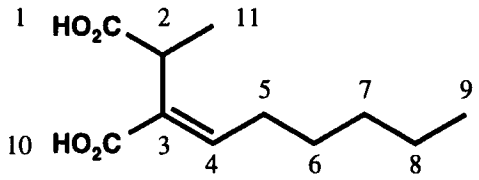
THE BIOSYNTHESIS OF PILIFORMIC ACID

CHAPTER 5

Introduction and Background

5.1 Introduction

Piliformic acid (**82**), a secondary metabolite of the slow growing dung fungus *Poronia piliformis,* was first isolated by Edwards in 1985[1] (fig. 108).

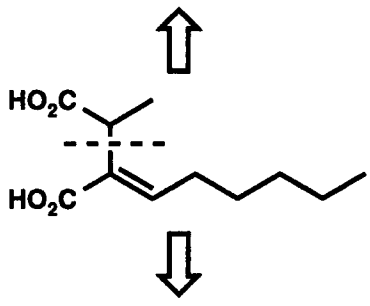


piliformic acid (**82**)

Fig. 108

Poronia are morphologically related to the genus *Xylaria*, fungi which grow on dead and decaying wood. Four *Xylaria* have been identified[1] which produce piliformic acid and *Poronia piliformis* was selected for this study as the highest levels of the metabolite are produced from this organism. No previous biosynthetic work has been carried out on piliformic acid. However, on inspection there is no obvious contiguous chain, and the molecule can be viewed as being constructed from a C_3 and C_8 chain. A mixed biosynthetic origin can therefore be hypothesised comprising a fatty acid or polyketide fragment and a C_3 fragment of different origin (fig. 109).

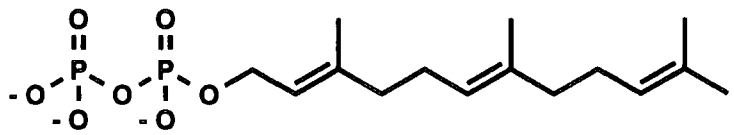
C_3 fragment



polyketide / fatty acid fragment

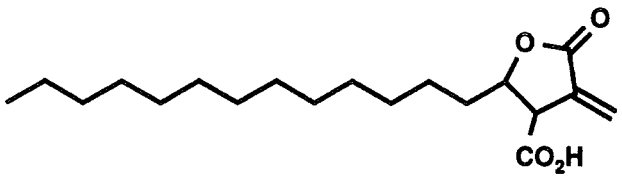
Fig. 109

Piliformic acid has a structural relationship to a number of other metabolites from lichens and fungi. In the case of the lichens these secondary metabolites are assumed to be products of the mycobiont.[2] A number of examples are illustrated,[1-8] all of which can be considered to derive from a polyacetate chain and either a C_3 or C_4 fragment (fig. 110 and fig. 111). Some of these compounds have been shown to have useful pharmacological properties, in particular, protolichesterinic (**83**) (*Cetraria islandica*) acid has antifungal, antibacterial, antitumour and growth regulating effects.[9] Chaetomellic acid-A (**98**) (*Chaetomella acutiseta*) has potential as an anticancer agent[8,10] and has been shown to act through the inhibition of the first stage in the post translational processing of the *ras*-oncogene, farnesylation, mediated by farnesyl-protein transferase.[11] A mutated form of this gene is involved with 25% of human tumours.[12] Molecular modelling[8] has shown that chaetomellic acid mimics farnesyl pyrophosphate (**100**) (fig. 112), which similarly comprises a hydrophilic head and hydrophobic tail.

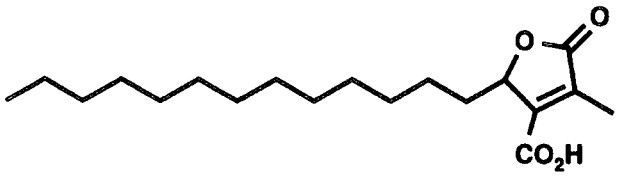


farnesyl pyrophosphate (**100**)

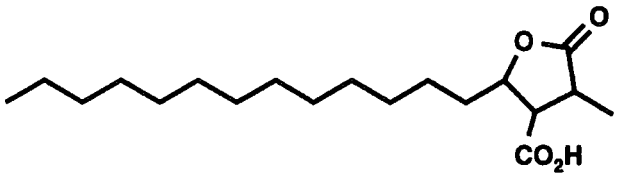
Fig. 112



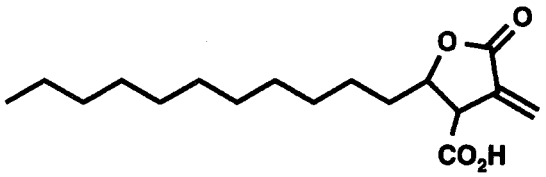
(+)-protolichesterinic acid (**83**)
Cetraria islandica



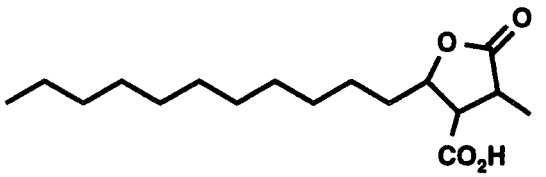
lichesterinic acid (**84**)
Cetraria islandica



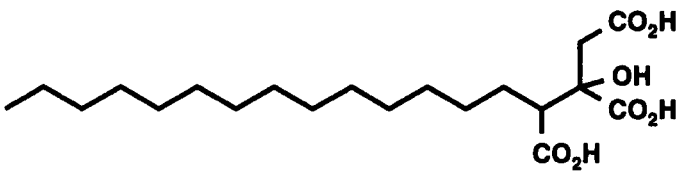
nephromopsic acid (**85**)
Nephromopsis stracheyi



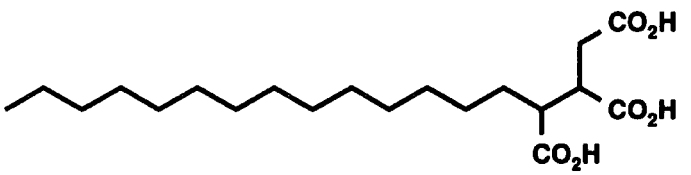
nephrosterinic acid (**86**)
Cetraria endocrocea



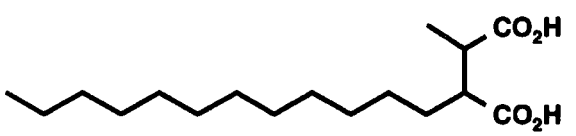
nephrosteranic acid (**87**)
Cetraria endocrocea



caperatic acid (**88**)
Parmelia caperata



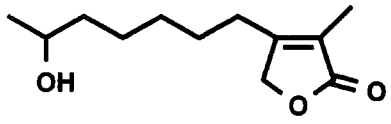
rangiformic acid (**89**)
Cladonia rangiformis



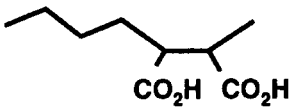
roccellic acid (**90**)
Roccella trictoria

Fig. 110

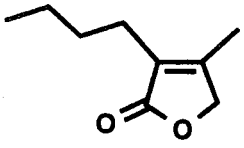
Fungal Metabolites



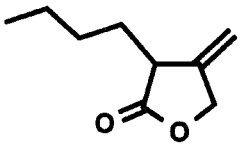
seiridin (**91**)
Seiridium cardinale



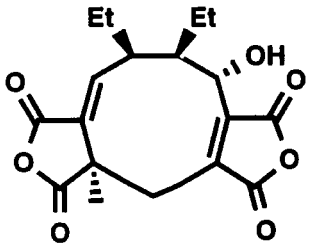
2-methyl-3-carboxyhexanoic acid (**92**)
Hypoxylon illitium



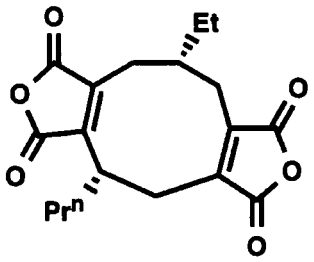
3-butyl-4-methylfuran-2(5H)-one (**93**)
Hypoxylon serpens



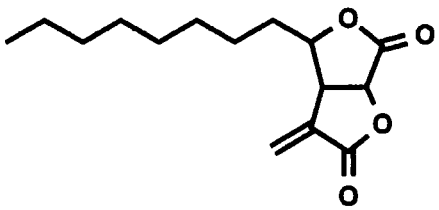
3-butyl-4-methylenefuran-2(5H)-one (**94**)
Hypoxylon serpens



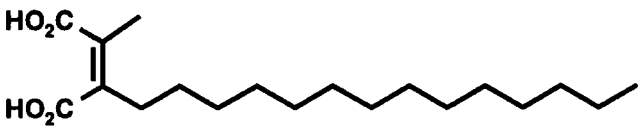
glauconic acid (**95**)
Penicillium purpurogenum



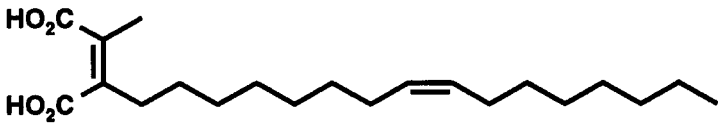
byssochlamic acid (**96**)
Penicillium purpurogenum



avenaciolide (**97**)
Aspergillus avenaceus



chaetomellic acid A (**98**)
Chaetomella acutiseta



chaetomellic acid B (**99**)
Chaetomella acutiseta

Fig. 111

5.2 The Structure of Piliformic Acid

From comparison of the proton resonances with various crotonic acids[13] Edwards[1] assigned the double bond stereochemistry as E. The structure and double bond geometry were confirmed by synthesis. Consistent with this assignment, in my own studies, an NOE effect was seen between the C-2 methine proton and the methylene protons on C-6 (fig. 113).

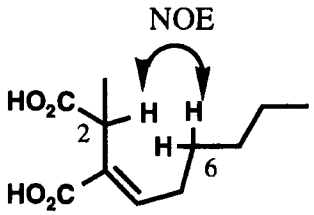
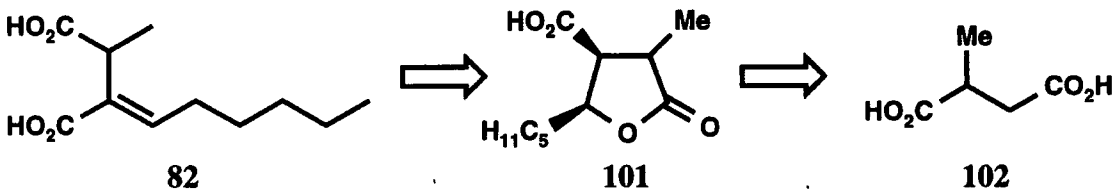


Fig. 113

Piliformic acid has an asymmetric carbon at C-2 and it is interesting to observe that while *Poronia piliformis, Xylaria longipes, and Xylaria polymorpha* all produce piliformic acid with a strong negative rotation: $[\alpha]_D^{23}$ = -89° (c=1, MeOH), the metabolite isolated from *Xylaria mali* and *Xylaria hypoxylon* is racemic and *Hypoxylon deustum* generates piliformic acid with a positive rotation, smaller in magnitude: $[\alpha]_D^{23}$ = +31°(c=1, MeOH). The absolute stereochemistry of piliformic acid has yet to be determined from any of the optically active sources. Edwards attempted to degrade piliformic acid using the reverse of the scheme he used to synthesise it. The proposed strategy involved converting it first to the lactone (**101**) and then carrying out a reverse aldol reaction to generate a methylsuccinic acid (**102**), which could be compared to authentic (R)- and (S)- samples (fig. 114). In the event the acid could not, however, be converted to the lactone.[1]



82 **101** **102**

Fig. 114

5.3 The Biosynthesis of Piliformic Acid

As discussed above, a mixed biosynthetic origin for piliformic acid can be inferred, comprising C_3 and C_8 fragments. Isotopic labelling studies have been used to determine much of the detailed origin of piliformic acid in *Poronia piliformis*. The long chain fragment was suggestive of a fatty acid or polyketide origin, both involving the head to tail coupling of acetate units. An initial series of experiments, in which cultures of *P. piliformis* were supplemented with labelled acetates, confirmed this hypothesis. It was subsequently shown that the precursor to the C_8 fragment of piliformic acid is in fact an intact octanoate chain (**103**) (fig. 115).

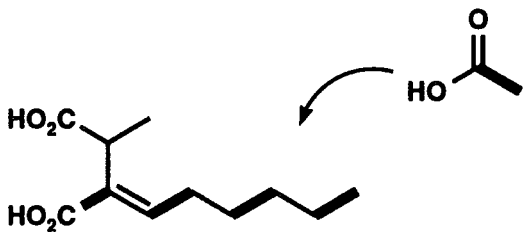


Fig. 115

There were two possible origins for the C_8 fragment, either a fully saturated unbranched chain originating from the β-oxidation of a long chain fatty acid or from a fragment which is generated from acetate condensation mediated by a dedicated polyketide synthase. To distinguish between these two possibilities the stereochemical course of the enoyl reductase enzyme was determined. It has recently become apparent[14-17] that the stereochemical courses of the fatty acid synthase enoyl reductase and the polyketide synthase enoyl reductase are opposite in fungi and that each type follows the same stereochemistry within this class of organism. The outcome of this experiment was that the enoyl reductase enzyme involved in the biosynthesis of piliformic acid followed the stereochemical course expected for a fatty acid synthase and it was therefore concluded that the octanoate fragment of piliformic acid is the degradation product of a long chain fatty acid, rather than the product of a dedicated polyketide synthase (fig. 116).

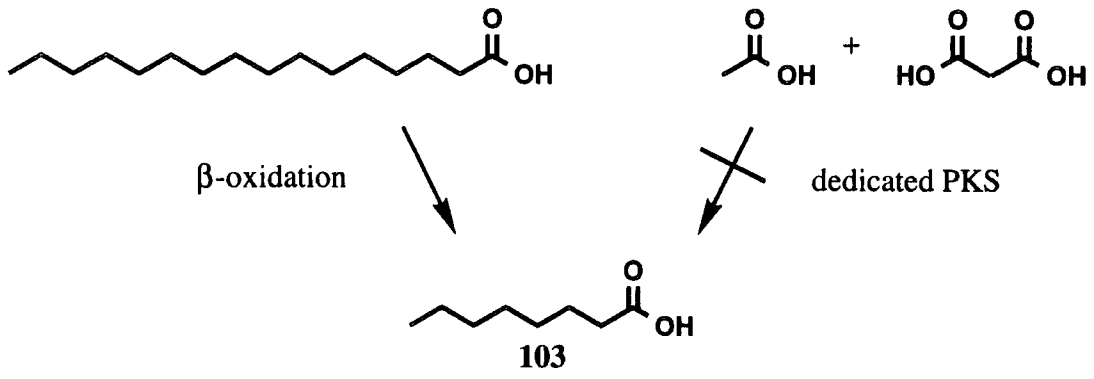


Fig. 116

It was envisaged that the C_3 and C_8 units of piliformic acid become joined through the attack of an activated form of octanoate (**103**) onto a suitable funcionalised C_3 or C_4 fragment (fig. 117).

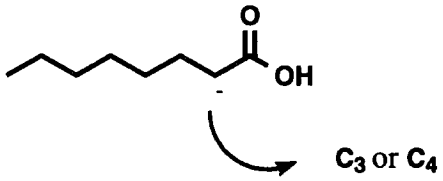


Fig. 117

As such, the precursor to the C_3 fragment has two likely origins. It would be chemically reasonable to suggest pyruvate (**104**), which could be generated from the glycolytic pathway or from the transammination of L-alanine (**105**). This would be quite an unusual precursor. A second possibility is that this fragment is not originally a C_3 fragment but a C_4 fragment. Oxaloacetate (**106**), a dicarboxylic acid generated in the citric acid cycle, also provides a suitable α-ketone functionality and could decarboxylate to generate the methyl group at C-11 of piliformic acid. Both molecules fulfil the electronic requirements for piliformic acid assembly. The delineation of the closer intermediate is however hampered by the fact that these two molecules can interconvert *in vivo* (fig. 118).

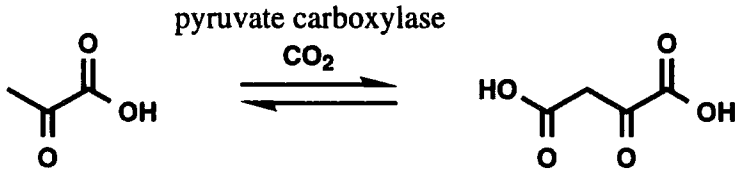


Fig. 118

The formation of oxaloacetate (**106**) through the citric acid cycle involves the back to back coupling of acetate units (fig. 119). Incorporation of acetate in this manner, into piliformic acid, was clearly observed in our initial acetate experiments.

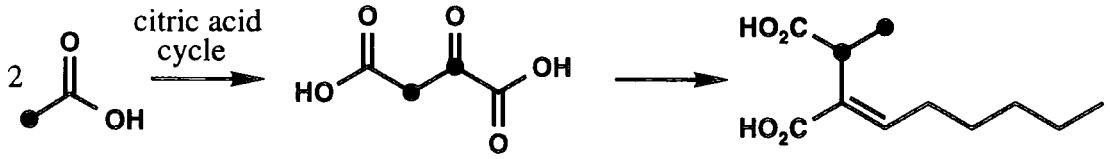


Fig. 119

Pyruvate (**104**) proved to be a difficult substrate to synthesise carrying appropriate isotopic labels. To investigate the possible C_3 origin of the C_3 fragment of piliformic acid, L-alanine (**105**) was administered to *P. piliformis*. This provides an equivalent result since pyruvate (**104**) and L-alanine (**105**) interconvert *in vivo* through transammination (fig. 120).

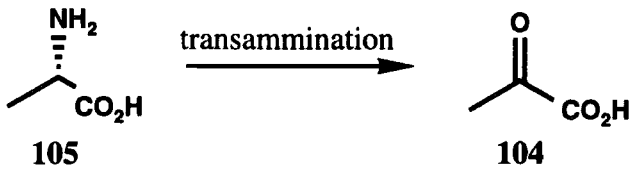
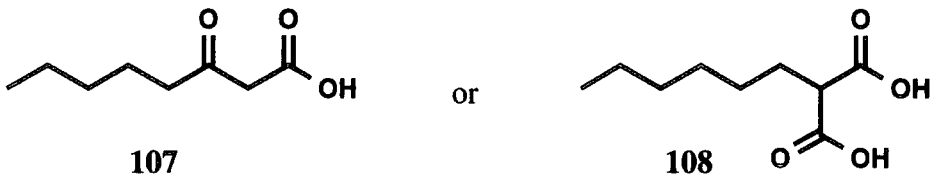


Fig. 120

The low incorporations of isotopically labelled alanines indicated that alanine was not an efficient precursor to piliformic acid and it was therefore considered unlikely that pyruvate was an immediate or obligate precursor to piliformic acid. Succinate (converted to oxaloacetate in the citric acid cycle) was however incorporated efficiently into piliformic acid. This observation, and that of the acetate incorporations, provides convincing evidence that oxaloacetate (**106**) rather than pyruvate (**104**) condenses with the octanoate unit during the biosynthesis of piliformic acid.

As discussed above, it is envisaged that an octanoate fragment and the oxaloacetate (**106**) react by the nucleophillic attack of the C_8 unit onto the oxaloacetate α-ketone functionality. This then requires the activation of C-2 of octanoate towards

deprotonation. Again two reasonable possibilities exist. Firstly the intermediate could simply be a β-keto-octanoate (**107**), derived directly as an intermediate in the β-oxidation of fatty acids, or alternatively α-carboxylation could generate a suitably activated octanoate unit (**108**) (fig. 121). The latter option, employing a putative octanoate carboxylase, is consistent with the incorporation of acetate, propionate and butyrate into polyketides more generally, although an octanoate carboxylase has never previously been identified.



107 **108**

Fig. 121

These two possibilities were investigated by determining the retention, or otherwise, of deuterium from the β-carbon of the octanoate fragment. Clearly β-oxidation would result in the loss of deuterium from this site, whereas α-carboxylation would not (fig. 122).

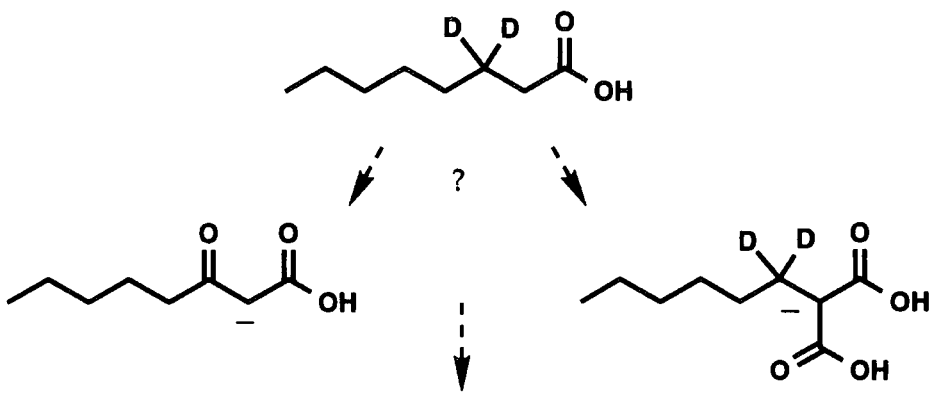


Fig. 122

In the event this study demonstrated that deuterium was retained, and thus it is proposed that the activation of the octanoate chain occurs through an α-carboxy octanoate. It also became apparent during this study that a 1,3-hydrogen shift from C-4 to C-2 occurs during piliformic acid biosynthesis (fig. 123), a result which has shed some light on possible mechanisms of piliformic acid biosynthesis.

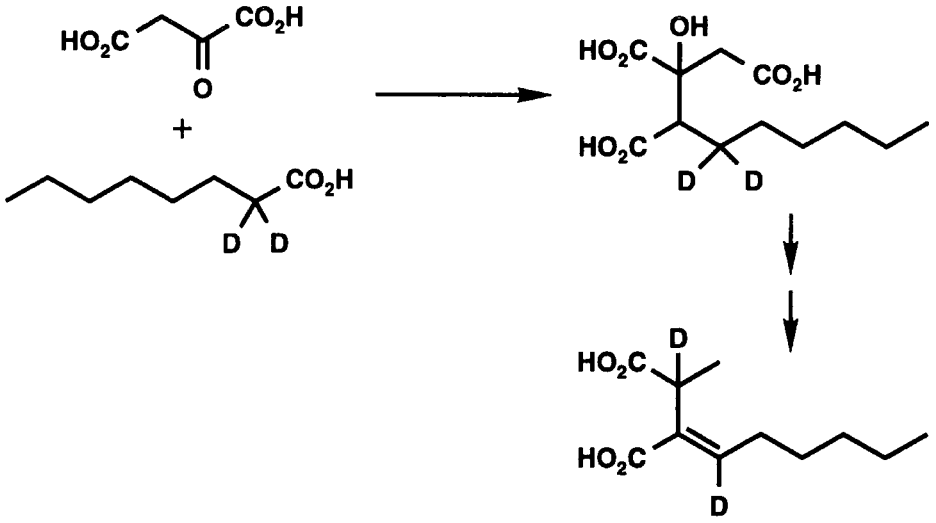
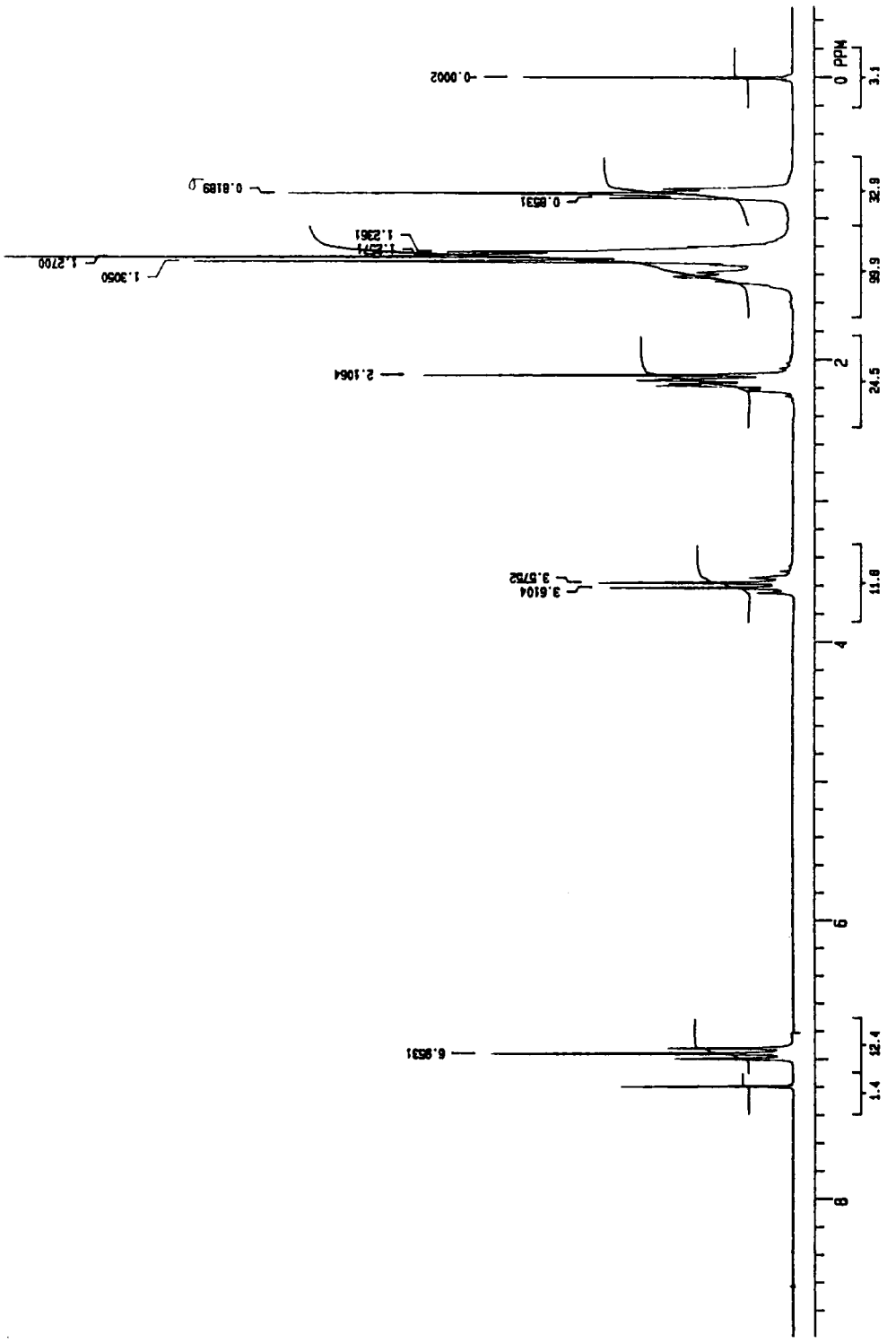


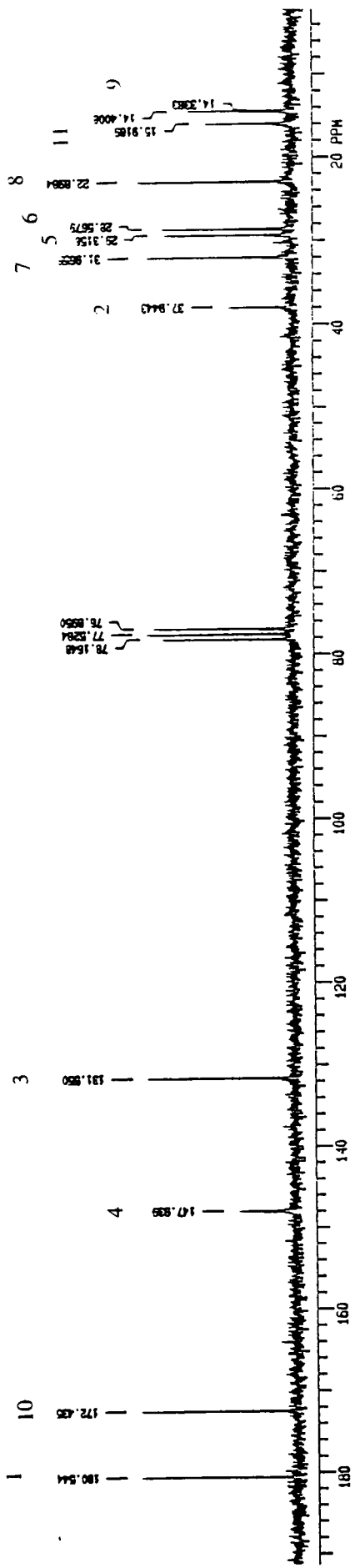
Fig. 123

These results are discussed in detail in the following chapter and a pathway for the biosynthsis of piliformic acid is proposed which is consistent with the labelling patterns observed. The biosynthesis of piliformic acid is discussed in the context of biosynthetic studies that have been carried out by other workers on the related metabolites introduced in section 5.1. The ^{1}H and ^{13}C NMR spectra of piliformic acid are shown in fig. 124 and fig. 125.



^{1}H NMR spectrum of piliformic acid

Fig. 124



13C NMR spectrum of piliformic acid

Fig. 125

5.4 References

1. J. R. Anderson, R. L. Edwards and A. J. S. Whalley, *J. Chem. Soc., Perkin Trans. I*, 1985, 1481
2. Y. Ashahina and S. Sibata, *Chemistry of the Lichen Substances*, Japanese Society for the Promotion of Science, Ueno, Tokyo, 1954
3. H. Thomas and J. Vogelsang, *Ann.*, 1945, **357**, 1907
4. R. L. Edwards and A. J. S. Whalley, *J. Chem. Soc., Perkin Trans. I*, 1979, 803
5. E. Evidente, G. Randazzo and A. Balio, *J. Nat. Prod.*, 1986, **49**, 593
6. J. L. Bloomer C. E. Moppet and J. K Sutherland, *J. Chem. Soc., Chem. Commun.*, 1965, 619
7. M. Tanabe, T. Hamasaki and Y. Suzuki, *J. Chem. Soc., Chem. Commun.*, 1973, 212
8. S. B. Singh, D. L. Zink, J. M. Liesch, M. A. Goetz, R. G. Jenkins, M. Nallin-Omstead, K. C. Silverman, G. F. Bills, R. T. Mosely, J. B. Gibbs, G. Albers-Schonberg and R. B. Lingham, *Tetrahedron*, 1993, **49**, 5917
9. M. M. Murta, M. B. A. de Azevedo and A. E. Greene, *J. Org. Chem.*, 1993, **58**, 7537.
10. S. B. Singh, *Tetrahedron Lett.*, 1993, **34**, 6524
11. J. B. Gibbs, *Cell*, 1991, **65**, 1
12. M. Barbacid, *Ann. Rev. Biochem.*, 1987, **56**, 779
13. M. D. Nair and R. Adams. J. *Am. Chem. Soc.*, 1960, **82**, 3786
14. C. R. Hutchinson, L. Schu-Wen, A. G. McInnes and J. R. Walter, *Tetrahedron*, 1983, **39**, 3507
15. P. B. Rheese, B. J. Rawlings, S. E. Ramer and J. C. Vederas, *J. Am. Chem. Soc.*, 1988, **110**, 316
16. C. A. Townsend, S. W. Brobst, S. E. Ramer and J. C. Vederas, *J. Am. Chem. Soc.*, 1988, **110**, 318
17. B. J. Rawlings, P. B. Rheese, S. E. Ramer and J. C. Vederas, *J. Am. Chem. Soc.*, 1989, **111**, 3382

CHAPTER 6

Isotopic Labelling Studies on Piliformic Acid

6.1 Fatty Acid Biosynthesis[1]

Fatty acids are primary metabolites built up from the head to tail condensation of acetate units. This gives rise to even chain lengths, although odd chain lengths may arise from the incorporation of non acetate starter units. The chain length, which is determined by the steric constraints of the fatty acid synthase (FAS), usually terminates at C_{16} or C_{18}.

The initial process in fatty acid biosynthesis is the activation of acetyl-CoA (**56**) to malonyl-CoA (**21**) by acetyl-CoA carboxylase, an enzyme which requires biotin as a co-factor (fig. 126).

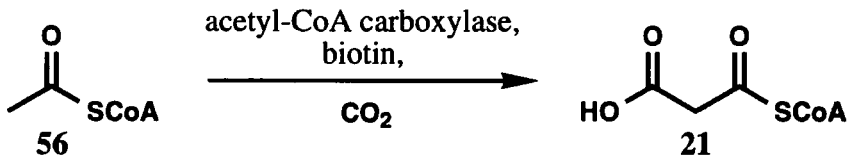


Fig. 126

This activated form of acetate is then transesterified onto the acyl carrier protein and undergoes a decarboxylative condensation with a molecule of acetyl-CoA (**56**) to generate β-ketobutyryl-ACP (**109**). This is then fully reduced by the sequential operation of a β-keto-ACP reductase, a β-hydroxy-ACP dehydratase and finally a *trans*-enoyl-ACP reductase. These reactions are illustrated in fig. 127. The butyryl-ACP (**112**) is then transesterified and re-enters the cycle to condense with another molecule of malonyl-ACP and initiate chain elongation. These four enzymes and associated co-factors, which are constituents of the fatty acid synthase, mediate the synthesis of a fully saturated fatty acid. Any functionality in the final product, such as unsaturation, is introduced by post assembly modification (with the exception of some bacteria).

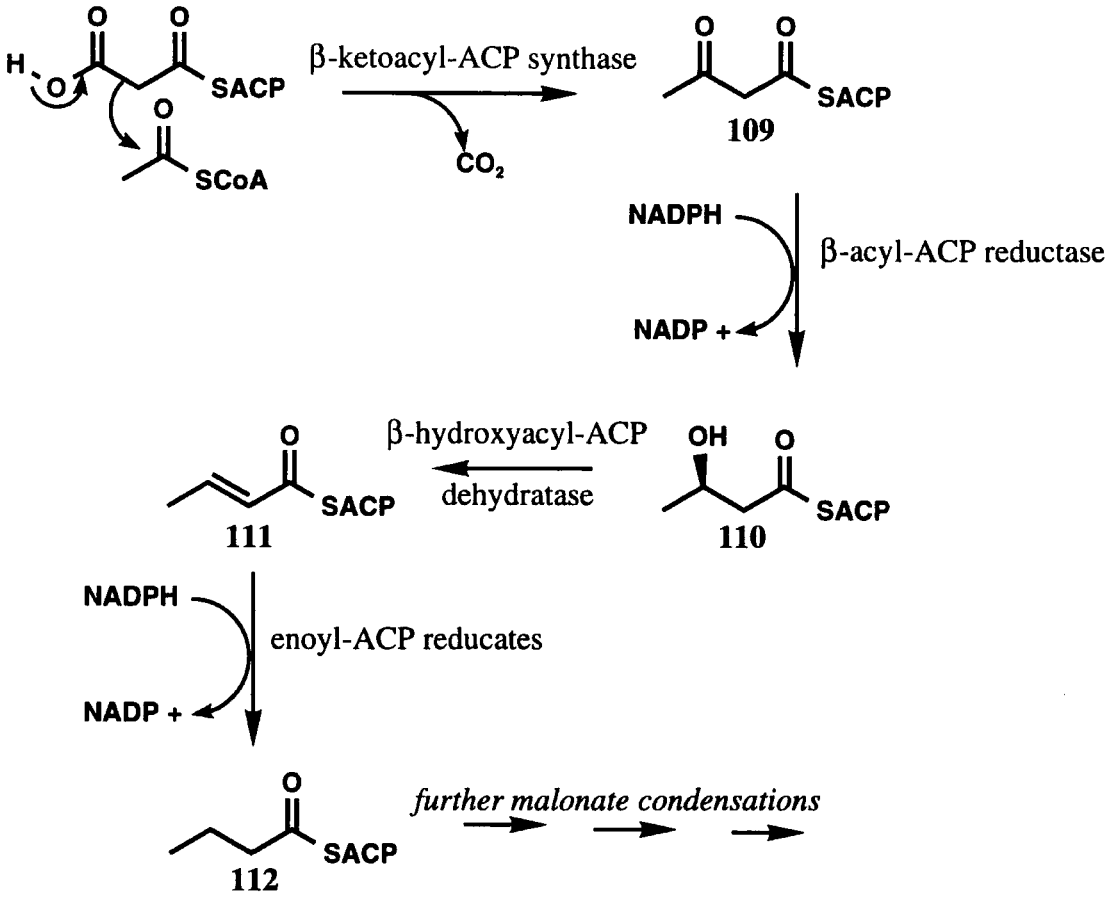


Fig. 127

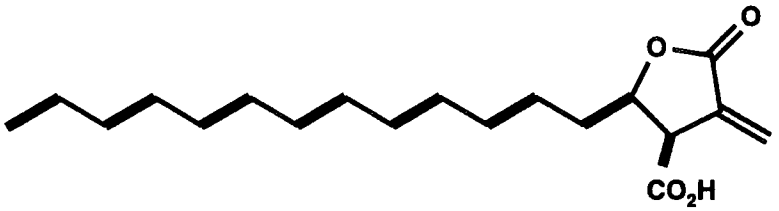
The nature of the fatty acid synthase (FAS) depends on the organism and fatty acid synthases have been classified into two basic types. A Type I FAS consists of one multifunctional protein. These are found in higher organisms such as mammals, birds and insects. Type II FASs are found in bacteria and plants and consist of up to seven discrete enzymes and a separate acyl carrier protein. Fungi fall somewhere in between these two extremes having two almost identical multifunctional proteins, α and β, which function as an $\alpha_6\beta_6$ oligomer. In fatty acid assembly it would appear that there is a level of commitment for the first few condensation reactions. Chain lengths below C_{14} are rare, as are chain lengths above C_{18}.

Polyketides are secondary metabolites, constructed in a similar manner from the head to tail condensation of acetate units. The enzymes that mediate the synthesis of these molecules almost certainly evolved as a result of genetic mutations of the genes responsible for fatty acid synthases. The principle difference between the two systems

is that any functionality residual in polyketide chains is introduced as the chain is assembled. This is known as a "processive assembly" process. The fatty acid cycle is selectively modified so that functionality is not removed during synthesis. A carbonyl is introduced by not performing the reductions at all, an alcohol by reducing once only and an alkene is generated by missing out the final reduction.

6.2 The Origin of the Long Chain Fragments of Related Metabolites

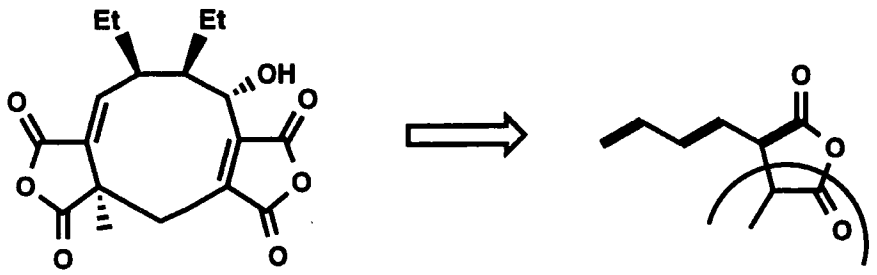
The acetogenic origin of the long chain fragments of a number of metabolites, apparently related to piliformic acid, has been demonstrated through feeding isotopicallly enriched acetates. Studies by Bloomer[2] on the lichen metabolite, protolichesterinic (**83**) acid serve as an illustrative example (fig. 128).



from acetate

Fig. 128

A similar experiment was carried out on glauconic acid (**95**),[3] a metabolite elaborated by *Penicillium purpurogenum.* This metabolite is constructed from two C_9 units with identical carbon skeletons each constructed from an acetate derived C_6 unit and a C_3 unit of different origin (fig. 129)



C_3 fragment

from acetate

Fig. 129

A further example is provided by avenaciolide (**97**),[4] produced by *Aspergillus avenaceus*. This metabolite was labelled in separate experiments by [1-^{13}C]-acetate and [2-^{13}C]-acetate revealing a C_{12} polyacetate fragment, and again a C_3 fragment (fig. 130).

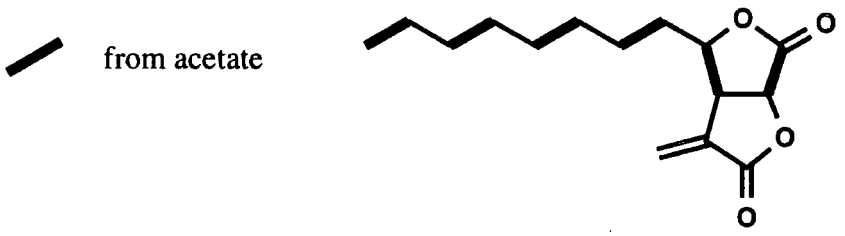


Fig. 130

A feature of the long chain fragments, common to all these metabolites, is the lack of any functionality (oxygenation, unsaturation, methylation etc.). This is also a feature of fatty acid biosynthesis and is therefore suggestive of a fatty acid origin of these polyacetate chains. Further to this evidence, there is a strong implication for a fatty acid origin of these fragments in some lichen metabolites[5] simply on structural grounds, *i.e.* the chain lengths of the polyacetate fragments correspond exactly to the chain lengths of common fatty acids. The polyacetate chains of protolichesterinic acid (**83**), lichesterinic acid (**84**), nephromopsic acid (**85**), caperatic acid (**88**) and rangiformic acid (**89**) have C_{16} chains from which a palmitic acid origin is implied. Similarly nephrosterinic acid (**86**), nephrosteranic acid (**87**) and roccellic acids (**90**) have C_{14} chains suggesting a myristic acid origin. However, there are many examples, including piliformic acid (**82**), where the chain length does not correspond to a common fatty acid. These metabolites include glauconic acid (**95**) and avenaciolide (**97**), which are discussed above, and the metabolites from various species of Xylaraceous fungi: 2-methyl-3-carboxyhexanoic acid (**92**),[6] 3-butyl-4-methylenefuran-2(5H)-one (**93**) and 3-butyl-4-methylfuran-2(5H)-one (**94**).[7] Fatty acids with chain lengths shorter than C_{14} are rare so the origin of the polyacetate fragments in these metabolites is not clear. As outlined in chapter 5, there are two possibilities, either these fragments are generated from acetate and malonate by a dedicated polyketide synthase or alternatively they arise by degradation (β-oxidation) of a longer chain fatty acid.

6.3 The Origin of the C_8 Fragment of Piliformic Acid

The expected polyacetate origin of the long chain fragments of a number of related metabolites has been demonstrated and it was therefore necessary to verify the acetogenic origin of the C_8 fragment of piliformic acid. A number of isotopically labelled acetates were administered to *P. piliformis* and high incorporations were observed, in the expected head to tail manner, characteristic of fatty acids and polyketides. The scene was then set to probe in detail the origin of the C_8 fragment. In the first instance we were able to demonstrate the intact incorporation of octanoate by feeding [1-^{13}C]-octanoic acid. These results are described in detail in the following paragraphs.

The acetate origin of the side chain carbons of piliformic acid was demonstrated by feeding [1,2-$^{13}C_2$]-acetate at 15mmolar to cultures of *P. piliformis*. This was incorporated into the C_8 fragment at 5.7%. The residual ^{13}C-^{13}C couplings in the ^{13}C NMR spectrum (fig. 131) of the isolated metabolite confirmed the expected connectivity (fig. 132). A lower level of incorporation (0.48%) was also seen in the C_3 fragment and this is discussed later. The high concentration of labelled acetate in the medium led to the occasional incorporation of two units of [1,2-$^{13}C_2$]-acetate incorporated adjacent to each other, in the same molecule. This is evidenced from the minor couplings in the ^{13}C NMR of the isolated piliformic acid. The magnitudes of the $^1J_{^{13}C^{13}C}$ couplings were determined by an INADEQUATE experiment (Table 8).

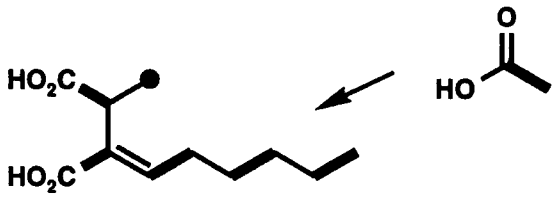
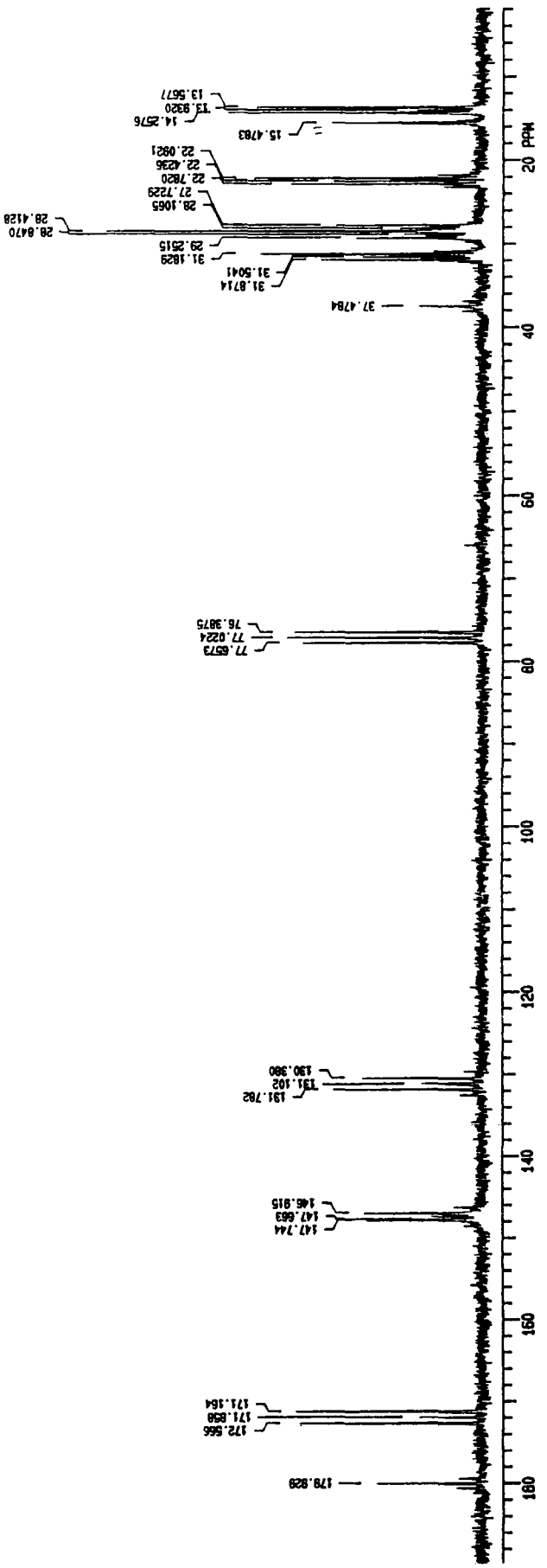


Fig.132

Bond	$^1J_{^{13}C^{13}C}$
(C-8)-(C-9)	35Hz
(C-6)-(C-7)	35Hz
(C-4)-(C-5)	42Hz
(C-10)-(C-3)	71Hz
(C-1)-(C-2)	54Hz

Table 8



^{13}C NMR spectrum of piliformic acid after feeding [1,2-$^{13}C_2$]-acetate

Fig. 131

The origin of the hydrogens of the C_8 chain was investigated by supplementing *P. piliformis* with [1-^{13}C, 2-$^{2}H_3$]-acetate (15mmolar). This material was incorporated at 4.6%, a similar level to the [1,3-$^{13}C_2$]-acetate (fig. 133).

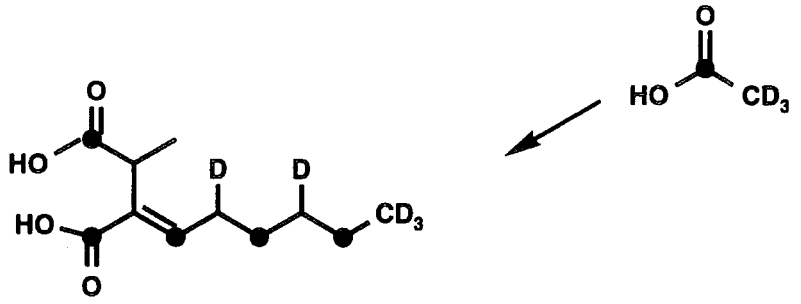


Fig. 133

The presence of deuterium was detected by the characteristic β-shift associated with the ^{13}C reporter atom in the ^{13}C NMR spectrum (fig. 134 and fig. 135). The signal corresponding to C-8 is predominantly β-shifted by three deuteriums attached to C-9, with smaller components to higher frequency corresponding to two, one and no deuterium atoms at this site. A fifth minor peak is also apparent, to lower frequency of the others. This is interpreted as a result of four β-shifts on the ^{13}C atom as a consequence of two molecules of labelled acetate being incorporated adjacent to each other in the same molecule (fig. 136). This phenomenon has already been demonstrated in the [1,3-$^{13}C_2$]-acetate result. This additional signal cannot be interpreted as a $^{2}J_{^{13}C^{13}C}$ coupling as the magnitude of the chemical shift difference between this peak and the other associated peaks is too large, the $^{2}J_{^{13}C^{13}C}$ couplings from alkanes being typically around 3Hz[8].

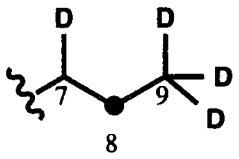
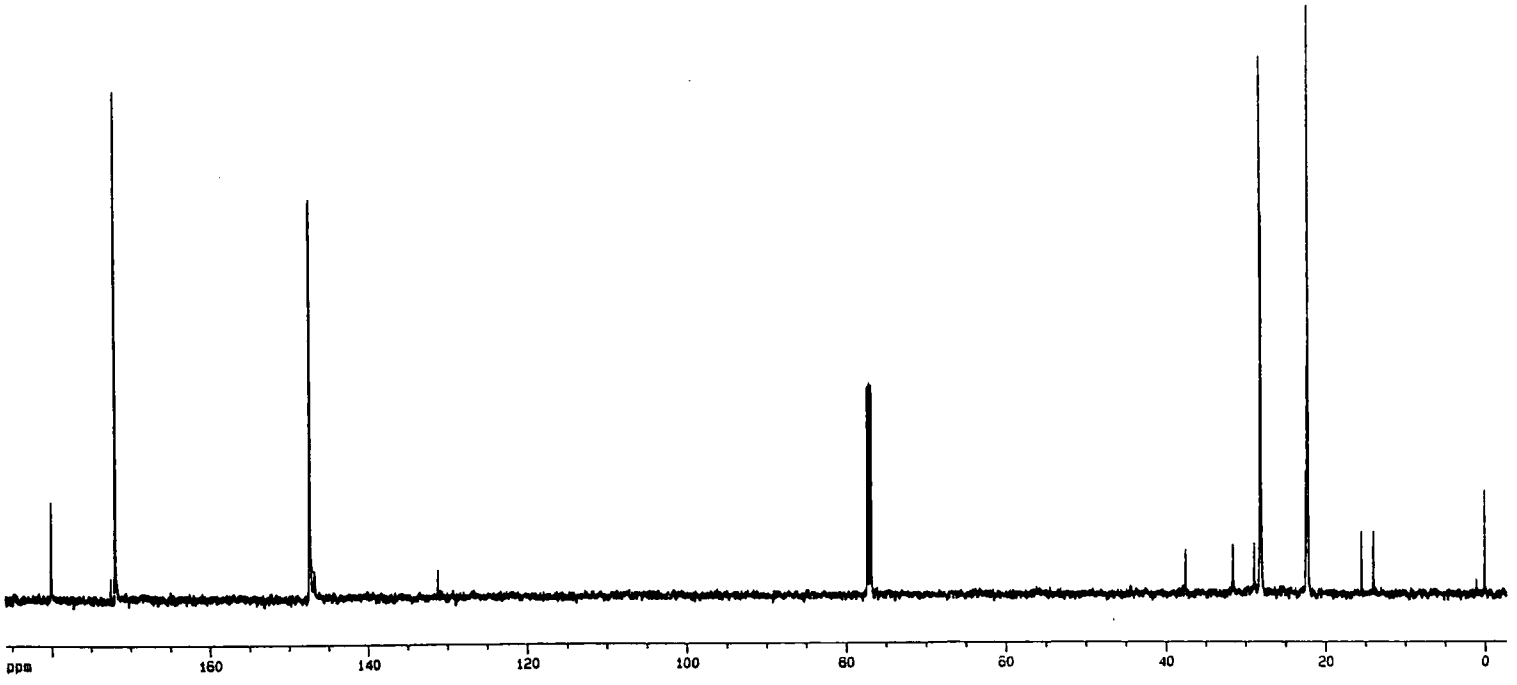


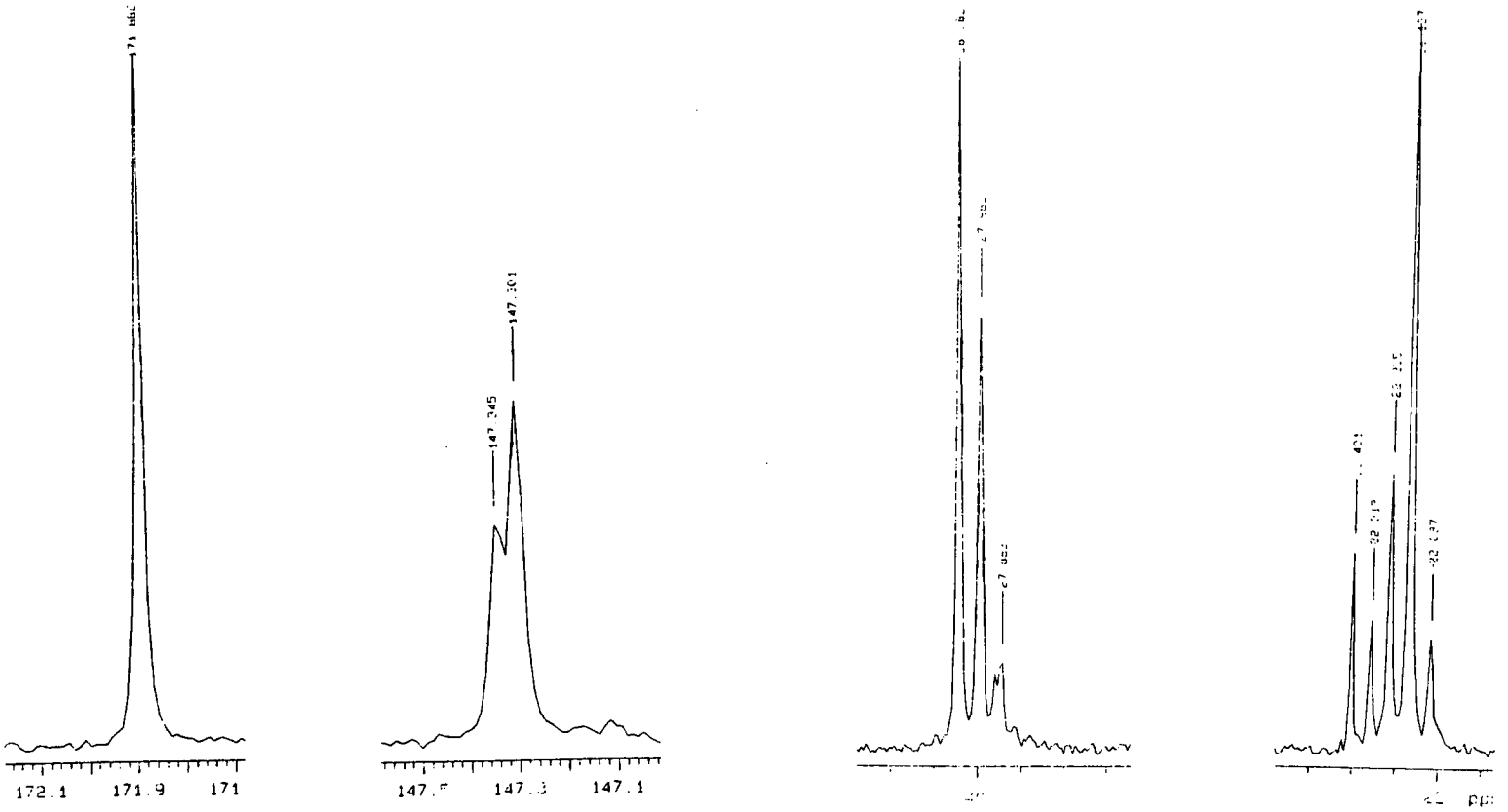
Fig. 136

The incorporation of deuterium was also detected directly by ^{2}H NMR (fig. 137). There is some evidence for a "starter effect," *i.e.* there is a particularly prominent enrichment in the terminal acetate methyl group of the polyacetate chain due to the more facile direct incorporation of labelled acetate from the acetate pool, rather than the more



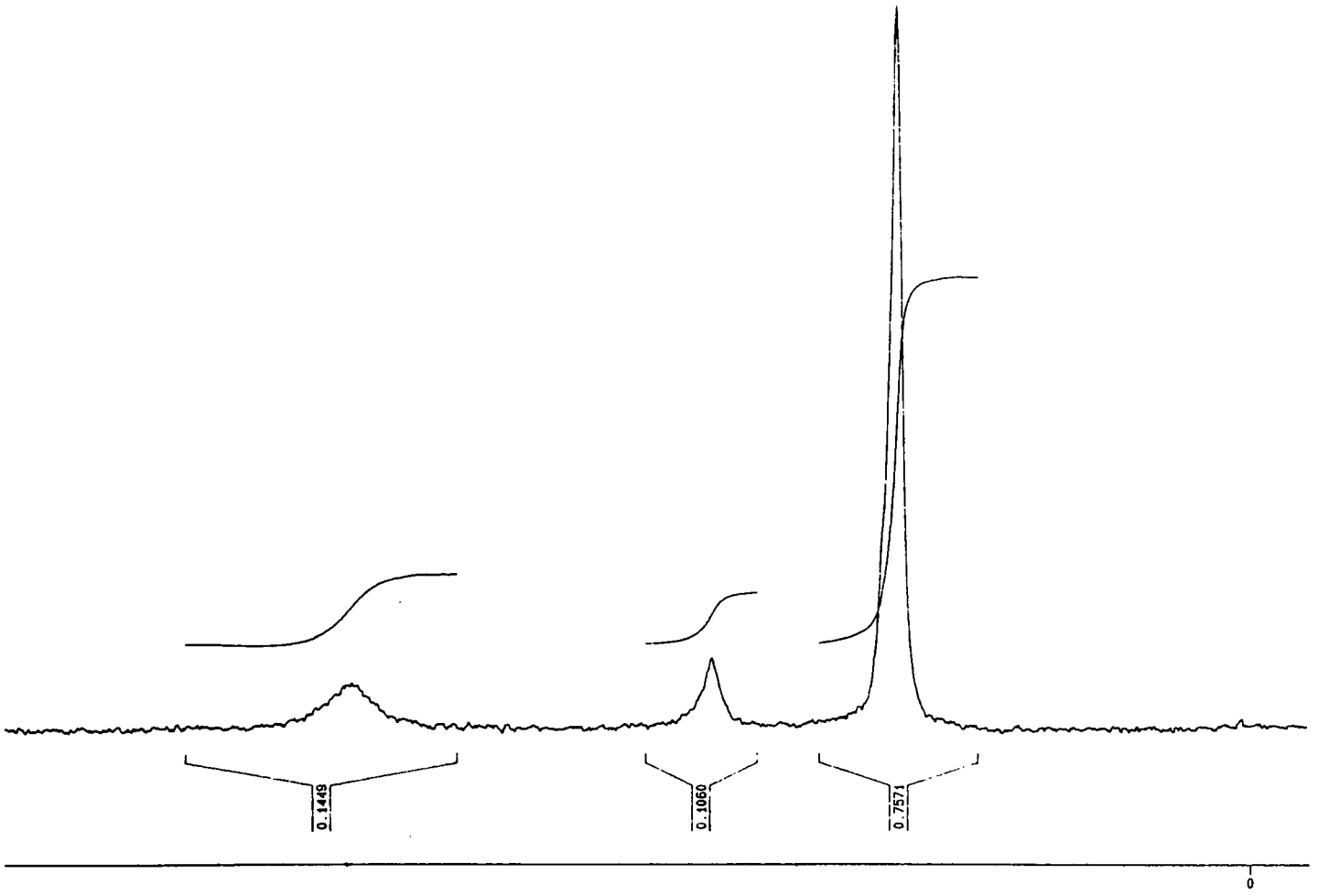
^{13}C NMR spectrum of piliformic acid after feeding [1 ^{13}C, 2-$^{2}H_3$]-acetate

Fig. 134



^{13}C NMR spectrum of piliformic acid after feeding [1-^{13}C, 2-$^{2}H_3$]-acetate
expansion of ^{13}C enriched peaks

Fig. 135



^{2}H NMR spectrum of piliformic acid after feeding [1-^{13}C, 2-$^{2}H_3$]-acetate

Fig. 137

indirect incorporation from the malonate pool. This is a common phenomenon as there is less exchange of deuterium with the medium from acetate than from the malonate.

In a complementary experiment [2-^{13}C, $^{2}H_3$]-acetate was fed (4.8mmolar) to cultures of *P. piliformis*. Again the expected ^{13}C enrichments into the C_8 fragment were observed (2.4%) (fig. 138) in the ^{13}C {^{1}H} and ^{13}C{^{1}H, ^{2}H} NMR spectra (fig. 139) of the isolated piliformic acid and the deuterium could be clearly seen through the α-shifts associated with the enriched peaks. A low level of deuterium enrichment was also observed into the C-11 methyl group, the implications of this result are discussed in section 2.12.

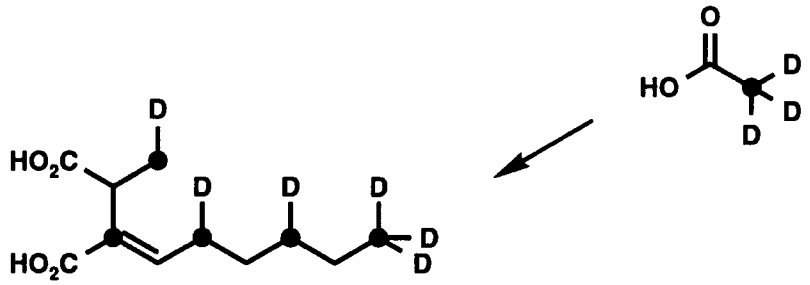


Fig. 138

The intermediacy of an intact octanoate chain was next investigated by administering sodium [1-^{13}C]-octanoate to cultures of *P. piliformis*. The ^{13}C NMR (fig. 140) of the isolated piliformic acid (fig. 141) showed a very large enhancement (5.5%) at C-10 and smaller enhancements at the carbons expected to be labelled by [1-^{13}C]-acetate in the side chain (2%).

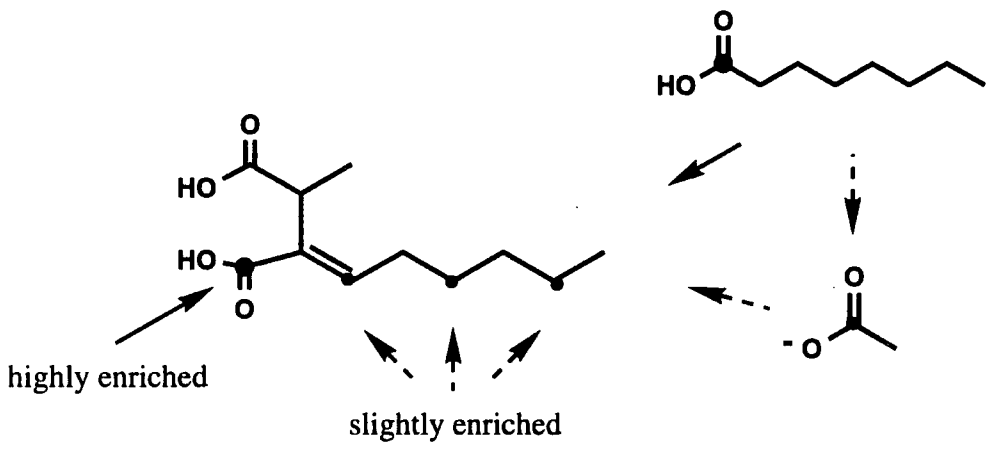
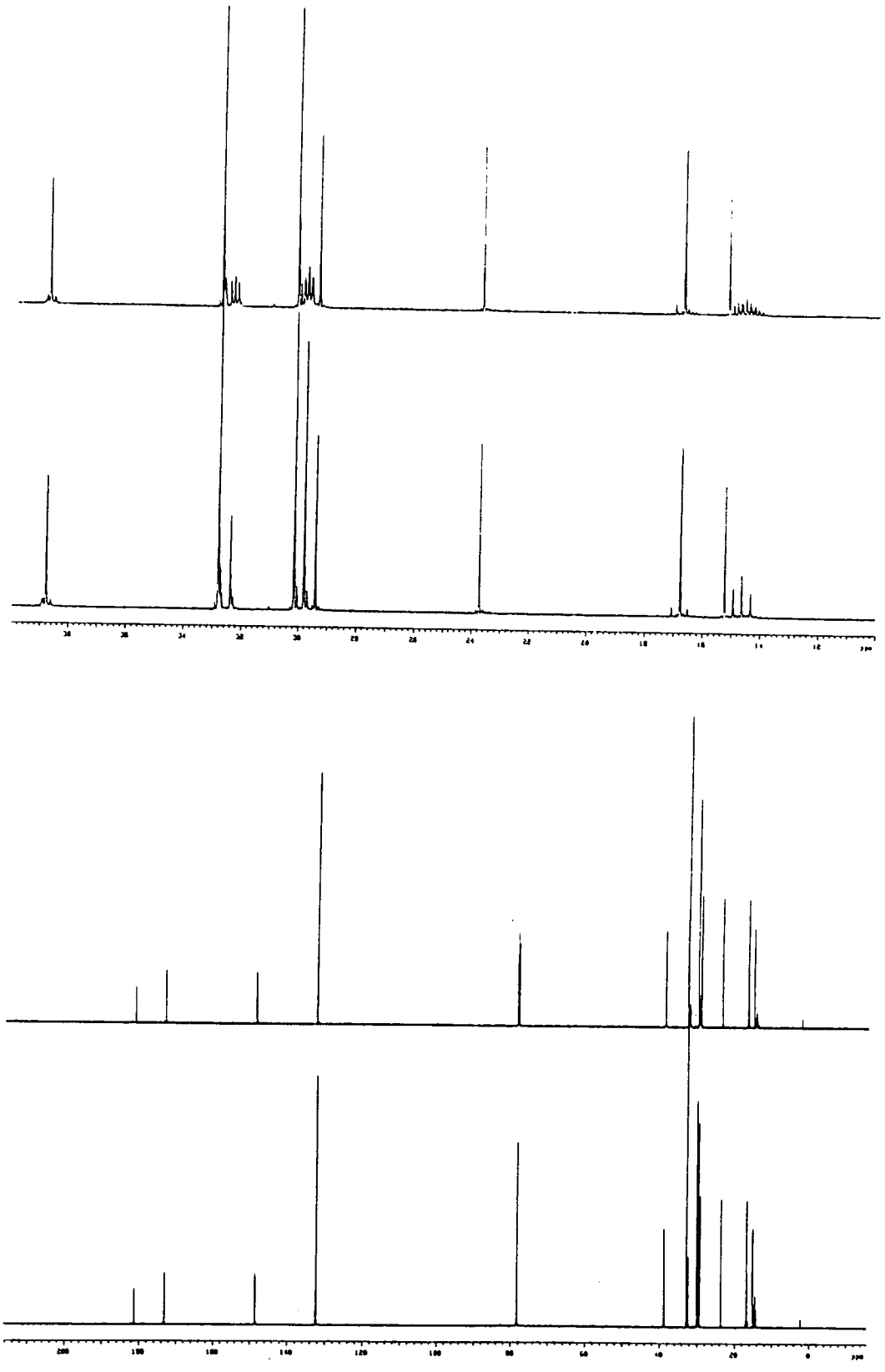
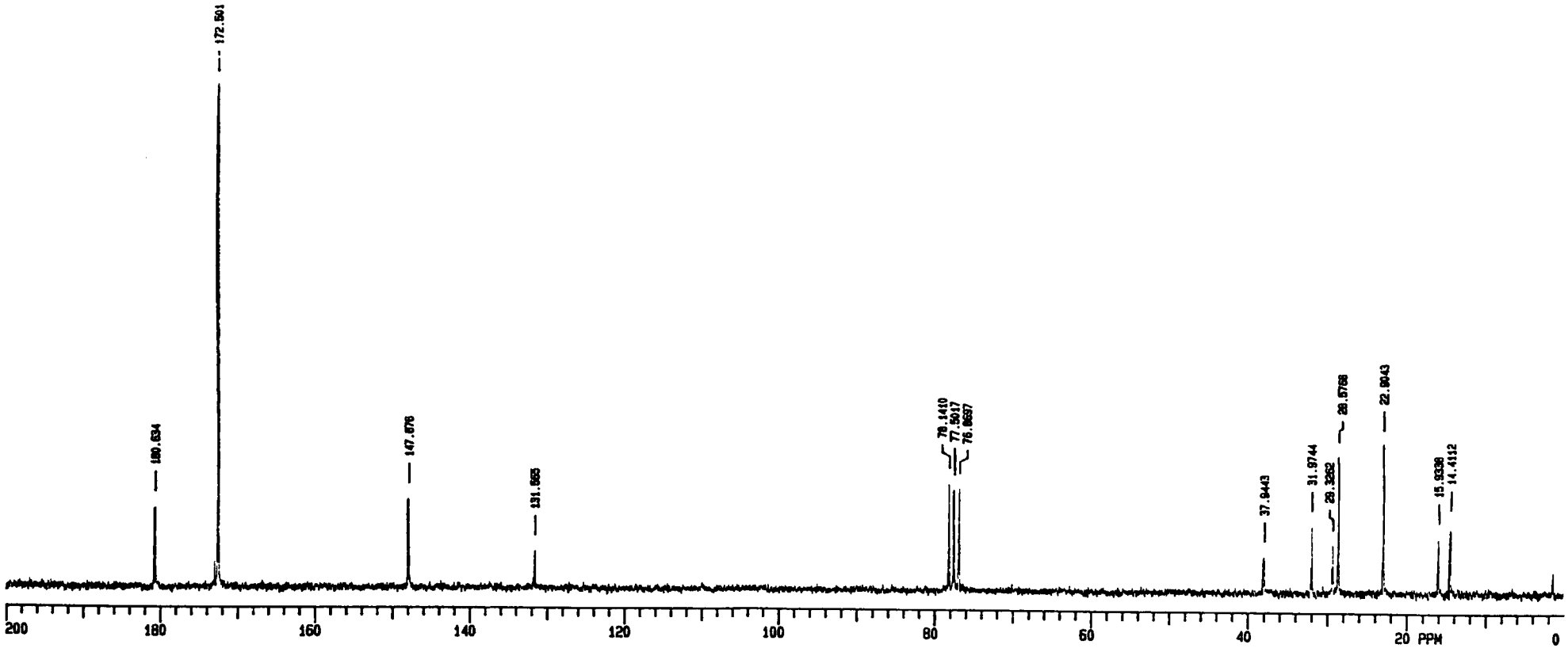


Fig. 141



$^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ NMR spectra of piliformic acid after feeding [2-^{13}C, $^{2}H_3$]-acetate

Fig. 139



^{13}C NMR spectrum of piliformic acid after feeding [1-^{13}C]-octanoate

Fig. 140

This shows that the greater portion of the substrate had been incorporated *intact* although some β-oxidation had occurred. This experiment clearly demonstrates that octanoate is an intermediate in piliformic acid biosynthesis.

6.4 Fatty Acid or Polyketide?

The experiments described above demonstrate that the side chain of piliformic acid is derived from the head to tail assembly of acetate units and further that octanoate is a biosynthetic intermediate. It then remained to delineate more specifically the origin of the C_8 fragment. It could arise either as a degradation product of a long chain fatty acid, or as the product of a dedicated C_8 polyketide synthase. The incorporation of carboxylic acids into polyketide secondary metabolites is rare, since there is a tendency for micro-organisms to degrade longer chain fatty acid, or polyketide substrates, to acetate prior to incorporation.[9,10] However, the incorporation of longer chain carboxylic acids is not without precedent. Averufin (**113**),[11] a precursor to aflatoxin

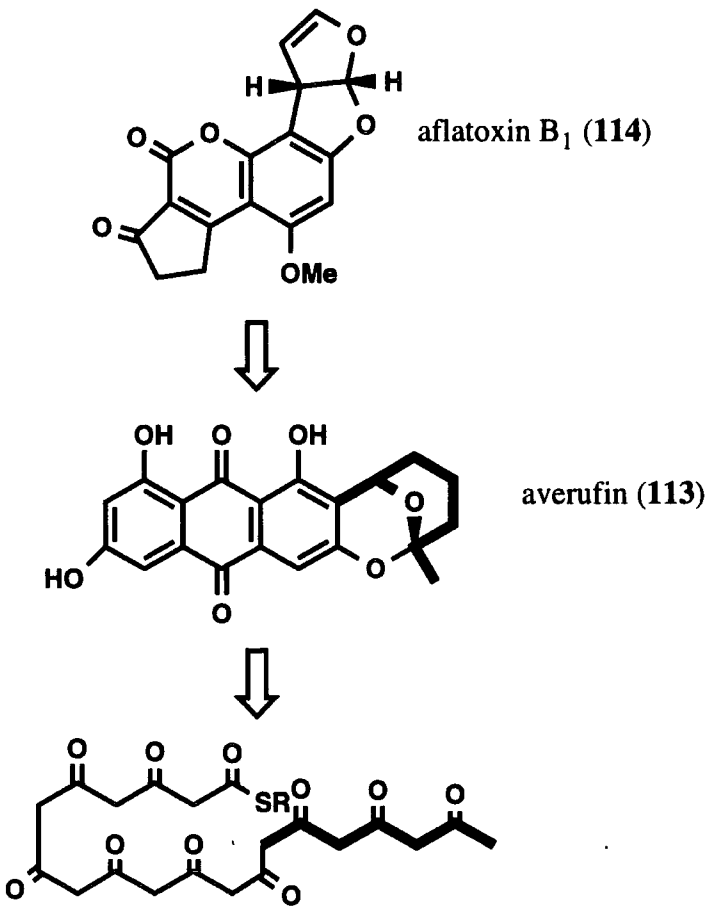
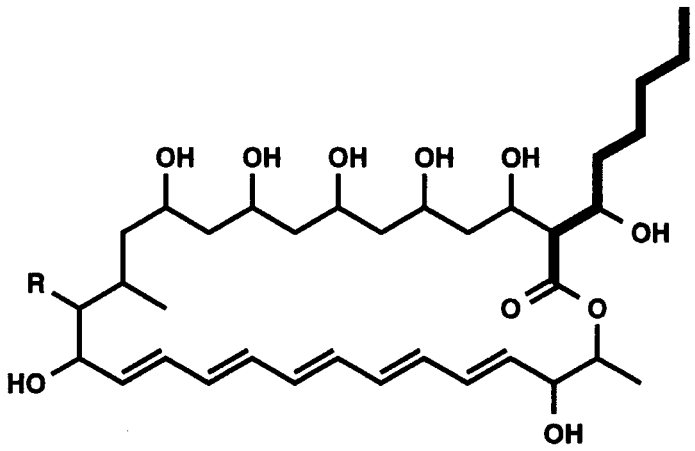


Fig. 142

(**114**) elaborated by a mutant strain of the fungus *Aspergillus parasiticus,* has been shown to incorporate an intact hexanoate starter unit (fig. 142). This hexanoate has been shown to be fatty acid in origin.[12]

A more unusual example is the bacterial polyketide fungichromin (**115**), and the related metabolite filipin (**116**),[13] produced by *Streptomyces cellulosae*. Here an intact octanoate unit is condensed at the end of the polyketide chain (fig. 143). It has subsequently been demonstrated that the octanoate chain is derived from oleate.[14]



R = OH fungichromin (**115**)
R = H filipin (**116**)

Fig. 143

Initially, a straight forward experiment was carried out to investigate the possible fatty acid origin of the octanoate fragment. 16-Fluoropalmitate (**117**) was introduced to *P. piliformis* cultures. It was anticipated that β-oxidation to a C_8 chain would lead to incorporation of a terminal fluorine atom in the polyacetate chain (fig. 144). This would be readily detected by ^{19}F NMR analysis. However, no such incorporation was observed in the resultant piliformic acid. There are several possible reasons for this failure. The substrate was not readily dissolved in the medium and/or could not be absorbed easily by the mycelium. The growth of the mycelium was certainly inhibited and a slight soapy film could be seen on the surface of the malt extract broth. Alternatively the 16-fluoropalmitate (**117**) may have been readily degraded to fluoroacetetate which would be toxic to the fungus. There was however no

evidence either for the incorporation of fluorine *via* fluoroacetate. Finally of course palmitate or another saturated fatty acid may not be a precursor. The study of fungichromin (**115**),[14] discussed above, identified oleic acid as a precursor, and an origin from this unsaturated fatty acid may exist for piliformic acid also.

16-fluoropalmitate

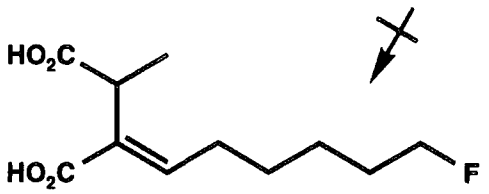


Fig. 144

The fatty acid or polyketide origin of the polyacetate C_8 fragment of piliformic acid was next investigated by probing the cryptic stereochemistry of the enoyl reductase enzyme.

6.5 Enoyl Reductase Enzymes

The stereochemistry of fatty acid biosynthesis was investigated by Sedgewick and Cornforth during the late 1970s.[15,16] The fate of the hydrogen atoms from chiral acetate, when administered to various fatty acid synthases, was determined and it was revealed that carboxylation of acetyl-CoA (**56**) occurs with retention of configuration, that the decarboxylative condensation occurs with inversion of configuration and that reduction of the β-ketoacyl-ACP (**109**) occurs to the *si*-face, followed by a *syn*-elimination. The stereochemistry of these reactions would appear to be conserved in all organisms. However, the stereochemical course of the final reduction, mediated by the enoyl reductase, varies from organism to organism. These results are illustrated below (fig. 145) and all four possible stereochemical combinations are known, in different classes of organism.

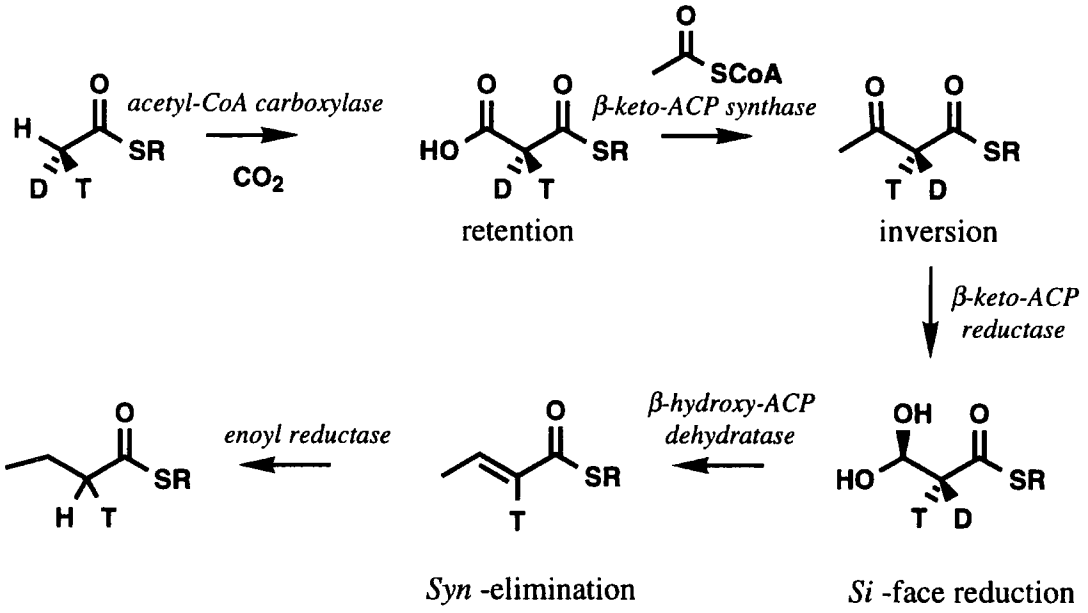


Fig. 145

There are several possibilities for the enoyl reductase stereochemistry. Hydride may be presented to either the *si-* or *re-* face of C-3, and either the *si-* of *re-* face of C-2 can be protonated (fig. 146).

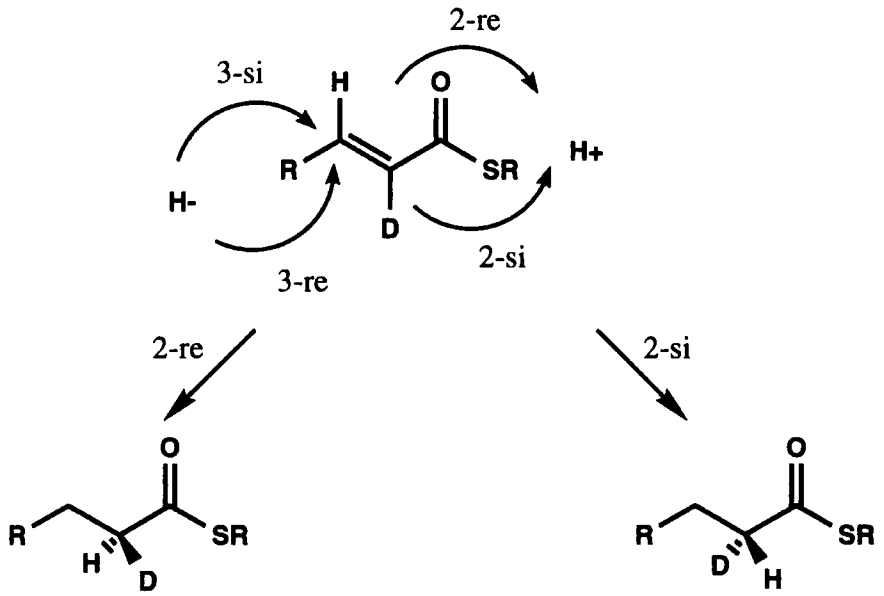
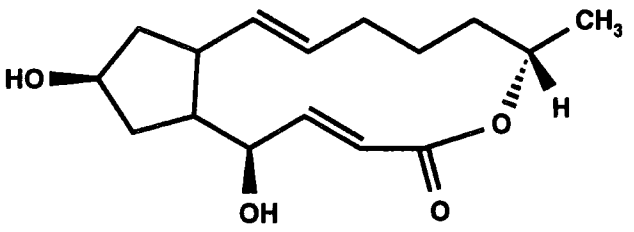


Fig. 146

Four fungal secondary metabolites have been studied where the stereochemistry of the polyketide synthase enoyl reductase has been compared with that of the fatty acid synthase enoyl reductase in the same organism. In each case [2-$^2H_3$]-acetate was administered to the systems and oleic acid and the polyketide metabolite were both

isolated. The stereochemical location of the acetate hydrogens was then determined either directly by NMR or indirectly after degradation to an α-deuterated aliphatic acid. This was achieved by generating a diastereomer for ^{2}H NMR analysis by coupling to an enantiomer of methyl mandelate prior to analysis.

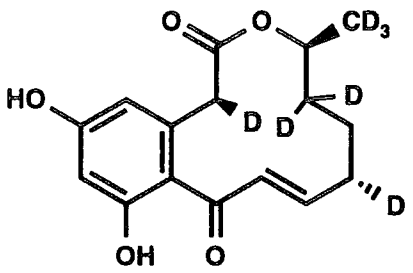
The first metabolite to be studied in this manner was brefeldin-A (**118**), a macrolide elaborated by *Penicillium brefeldianum*[17, 18](fig. 147).



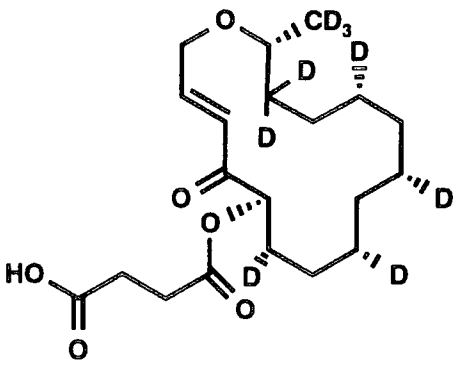
Brefeldin-A (**118**)

Fig. 147

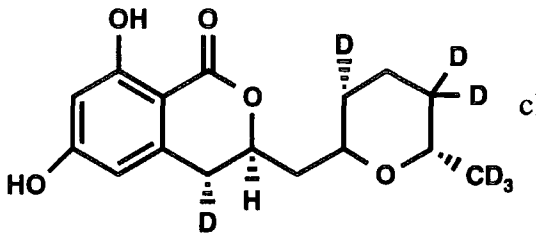
This molecule is an octaketide with a macrocyclic ring rendering it structurally similar to the prostaglandins, although unlike the prostaglandins, it is not a modified fatty acid. Further examples are provided by Vederas on the fungal polyketides dehydrocurvularin (**119**), from *Alternaria cineraria*, antibiotic A26771B (**120**)[19] from *Penicillium turbatum* and cladosporin (**121**)[20, 21] from *Cladosporium cladosporiodes* (fig. 148). In all of these cases the deuterium retained from [2-$^{2}H_3$]-acetate appeared at the *pro*-S site in the polyketide, indicating protonation to the *re*-face. In the fatty acid, oleic acid (**122**), the opposite stereochemistry is apparent, indicating protonation to the *si*-face. Although the sample size is small it would appear to be a general phenomenon in fungi that the stereochemical course of the protonations, mediated by the enoyl reductase enzymes, are *opposite* in the fatty acid and polyketide synthases of the *same organism*. It also appears that the polyketide or fatty acid synthases follow the same stereochemical course within this class of organism. This feature was used to delineate the fatty acid or polyketide origin of the polyacetate fragment in piliformic acid, by establishing the stereochemical location of deuterium atoms derived from acetate in the side chain.



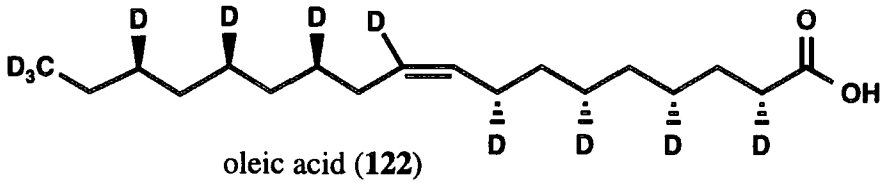
dehydrocurvularin (**119**)



antibiotic A26771B (**120**)



cladosporin (**121**)



oleic acid (**122**)

Fig. 148

A further example was a study on averufin (**113**) carried out by Townsend *et al.*[12] This polyketide was shown to incorporate hexanoate as a starter unit, as discussed above. The cryptic stereochemistry of both the hexanoate unit and oleic acid co-produced by the organism was investigated. In the event the stereochemistries were the same, the deuterium from [2-$^2H_3$]-acetate appearing at the *pro*-R site in both instances (fig. 149). There are no fully reduced methylene sites in the rest of the averufin molecule amenable to stereochemical comparison. However, from the above result and the results known for other fungal metabolites, it was concluded that the hexanoate unit is a product of fatty acid degradation.

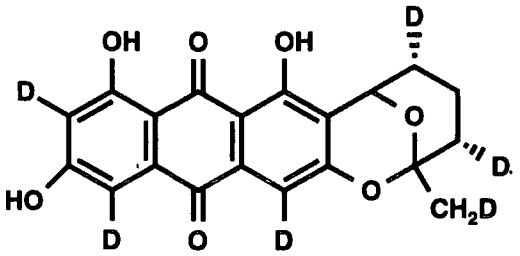


Fig. 149

6.6 Piliformic Acid Enoyl Reductase Stereochemistry

An analogous strategy was carried out to determine the origin of the polyacetate chain of piliformic acid. From experiments with both [1-^{13}C,2-$^{2}H_3$]-acetate and [2-^{13}C,2-$^{2}H_3$]-acetate the labelling pattern from [2-$^{2}H_3$]-acetate was evaluated (fig. 150).

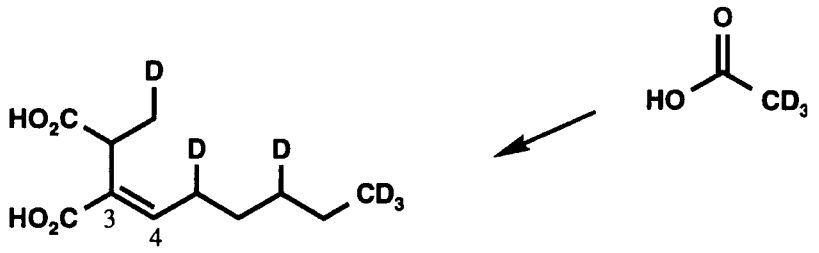


Fig. 150

The double bond between C-3 and C-4 provides a site in the molecule amenable to oxidative cleavage which was achieved using ruthenium tetroxide and potassium periodate. This relatively mild oxidation process has been applied to polyketide metabolites before by Sharpless[22] and also used to investigate the stereochemistry of the methyl branched side chain of the fungal polyketide metabolite, tenellin (**123**)[23,24] (Fig. 151).

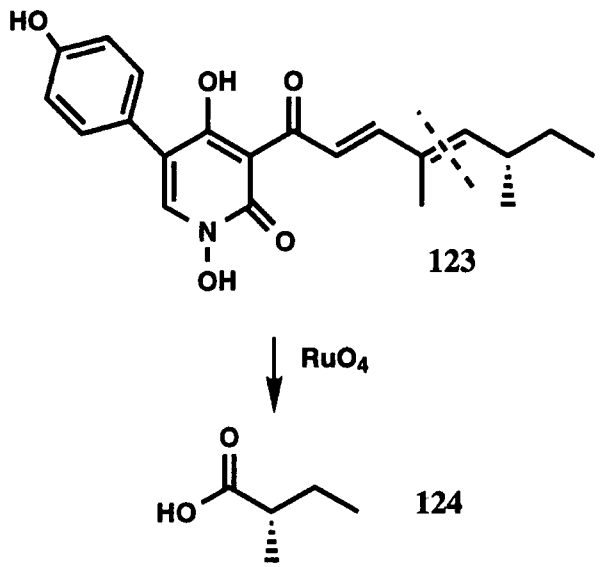
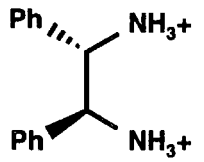


Fig. 151

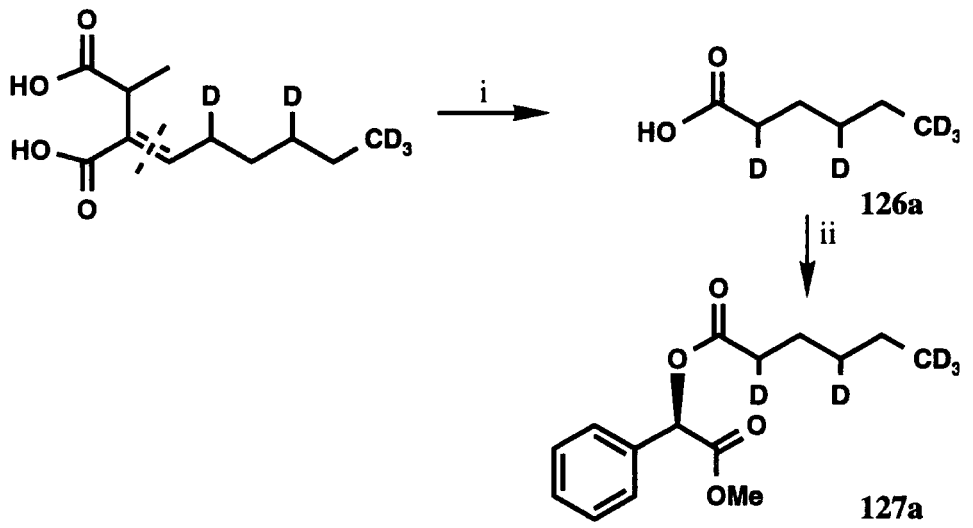
The polyketide was cleaved as shown and the stereochemistry of the resulting methylbutyric acid (**124**) determined by complexing with a chiral diamine (**125**)[26] (fig. 152) and NMR analysis the resulting complex.



125

Fig. 152

As [2-$^2H_3$]-acetate is relatively cheap, several grams was fed to cultures of *P. piliformis* to generate ≈1g of isotopically enriched piliformic acid. Oxidative cleavage of the double bond then generated isotopically enriched hexanoic acid (**126a**), which was analysed by ^2H NMR as its (2R)-methyl mandelate ester (scheme 15).



(i) RuO_4, KIO_4, (ii) (2R)-methyl mandelate, DCC, DMAP

Scheme 15

The configuration of deuterated hexanoic acid (**126a**) generated from piliformic acid could be determined by NMR because (2R)-methyl mandelate moiety renders the prochiral α-hydrogens / deuteriums non-equivalent (fig. 153).

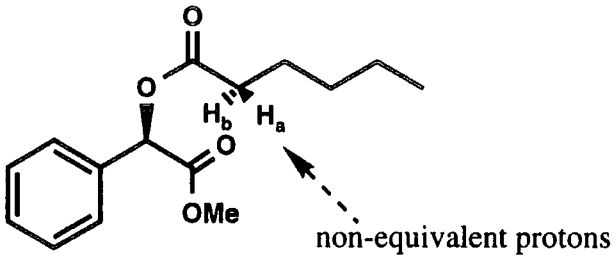


Fig. 153

The coupling patterns in the ^1H NMR spectrum of this molecule are complex but a single peak for each site is observed in the proton decoupled ^2H NMR spectrum. The relative chemical shifts for α-deuterated carboxylic acid / mandelate esters have previously been established[26,27] and comparison of the metabolite derived hexanoic acid (**126a**) with racemic (C-2) deuterated material (**127b**) (fig. 154) revealed the stereochemistry of the deuterated piliformic acid to be (R).

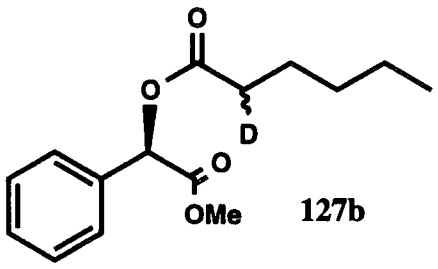


Fig. 154

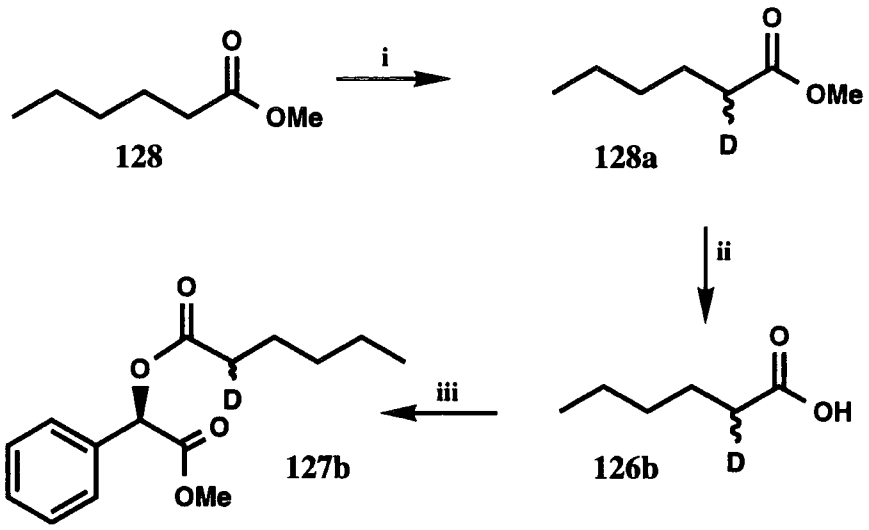
6.7 Methyl O-Hexanoyl Mandelate from Piliformic Acid (127a)

Piliformic acid, isolated after supplementing cultures of *P. piliformis* with [2-$^2H_3$]-acetate at 15mmolar was oxidatively cleaved with ruthenium tetroxide and potassium periodate in a biphasic solution of water : acetonitrile : carbon tetrachloride (3:1:1) to give hexanoic acid in 77% yield. The hexanoic acid was then esterified with (2R)-methyl mandelate, using DCC and a catalytic quantity of DMAP in 65% yield.

6.8 Methyl (RS)-[2-^{2}H]-O-Hexanoyl-(2'R)-mandelate (127b)

Methyl hexanoate was deprotonated with LDA at -78°C. The anion was then quenched with MeOD to generate racemic α-deuterated methyl [2-^{2}H]-hexanoate containing approximately 50% ^2H at C-2, as judged by ^1H NMR analysis. Base hydrolysis of the

ester, by refluxing in a 1M KOD in D_2O for 12 hours, generated (RS)-[2-^{2}H]-hexanoic acid in 74% yield. This deuterium enriched acid was then coupled to (2R)-methyl mandelate in the same manner as that for piliformic acid derived hexanoic acid (scheme16).

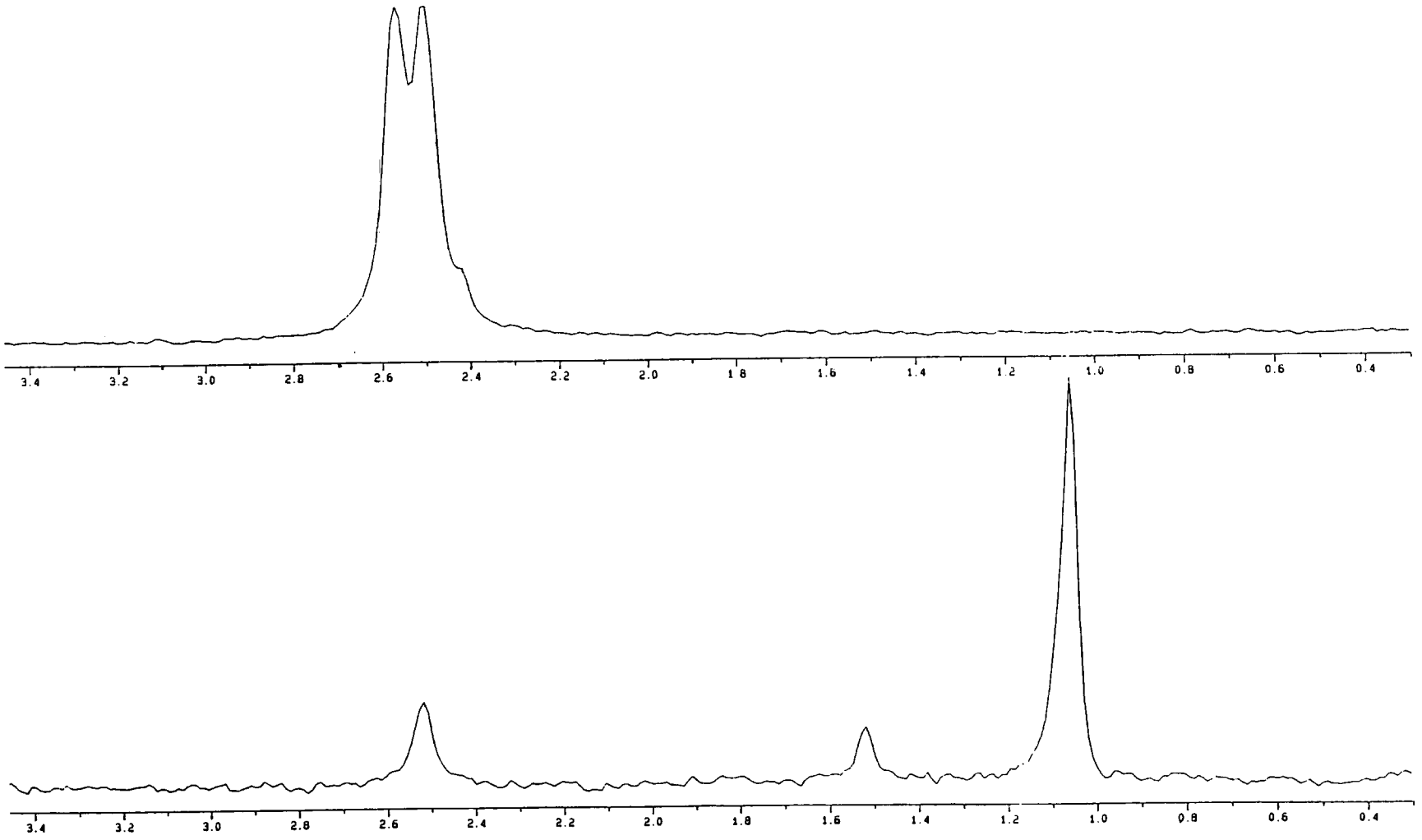


(i) LDA, THF, -78°C, 1h then MeOD, -78°C, 30min, (ii) 1M KOD/D_2O, (iii) (2R)- methyl mandelate, DCC, DMAP.

Scheme 16

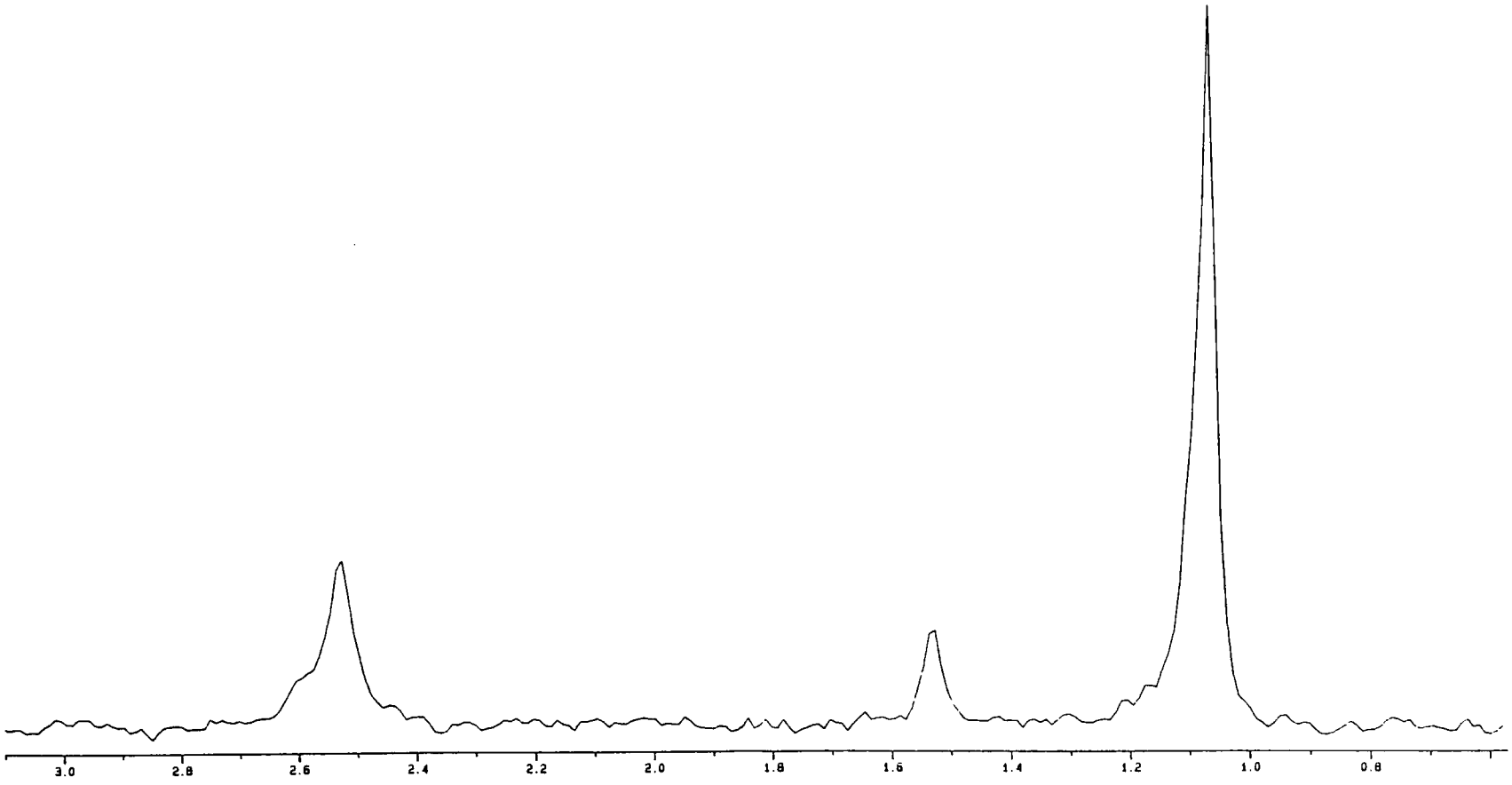
6. 9 ^{2}H NMR Analysis of the Methyl O-Hexanoylmandelate Derivatives

The ^{2}H NMR spectra of each of the deuterated mandelate derivatives (**127a** and **127b**) were recorded. The piliformic acid derived material (**127a**) was then spiked with a small quantity of racemate such that a second peak at the α-position, became apparent to higher frequency of the existing peak. This confirmed the direction of the shift. These spectra are shown below (fig. 155 and fig. 156). The spectrum of the spiked material shows a small peak appearing to higher frequency of the existing peak. From previous assignments determined by Parker[26,27] it was deduced that the deuterium in the metabolite derived hexanoate is situated exclusively in the *pro*-R site. Protonation by the enoyl reductase therefore occurs to the *si*-face. This study clearly suggests that the side chain of piliformic acid has a fatty acid, rather than a polyketide origin.



^{2}H NMR spectrum of methyl O-hexanoylmandelate, from (RS)-[2-^{2}H]-hexanoic acid (top) and piliformic acid derived hexanoic acid (bottom)

Fig. 155



^2H NMR spectrum of methyl O-hexanoylmandelate spiked mixture

Fig. 156

6.10 The Origin of the C_3 Fragment of Piliformic Acid

It is envisaged that piliformic acid is formed by the condensation of an activated octanoate unit with a suitably functionalised C_3 or C_4 fragment. Pyruvate (**104**) and oxaloacetate (**106**) are candidate intermediates in piliformic acid biosynthesis since both of these molecules provide a suitable electrophilic ketone moiety for condensation (fig. 157).

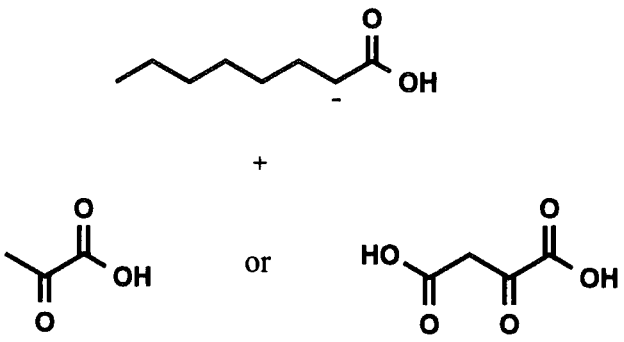


Fig. 157

While pyruvate (**104**) and oxaloacetate (**106**) can interconvert *in vivo*[28] their metabolic origin is quite different. Pyruvate (**104**) is the final product of glycolysis. Glycolysis[29a] is the sequence of reaction which converts glucose (**130**) to pyruvate (**104**) with the concomitant production of ATP. This pathway is universal to almost all organisms and takes place in the cell cytosol. The reactions of the glycolytic pathway are summarised in fig.158 to illustrate how [2-^{14}C]-glucose is catabolised to [2-^{14}C]-pyruvate.

Oxaloacetate (**106**) is a dicarboxylic acid generated in the citric acid cycle. The citric acid cycle[29b] is the final common pathway for the oxidation of fuel molecules. Acetate enters the cycle as acetyl-CoA (**56**) and is completely oxidised to carbon dioxide and water. In aerobic organisms glycolysis and the citric acid cycle are linked by the decarboxylation of pyruvate. This reaction, and the reactions of the citric acid cycle, occur within the mitochondrial matrix. The reactions of the citric acid cycle result in the back to back coupling of acetate units. This is illustrated in fig. 159 , which follows the fate of [2-^{13}C]-acetate. In addition to its role in generating energy for the cell (by generating ATP) the citric acid cycle is a source of biosynthetic intermediates.

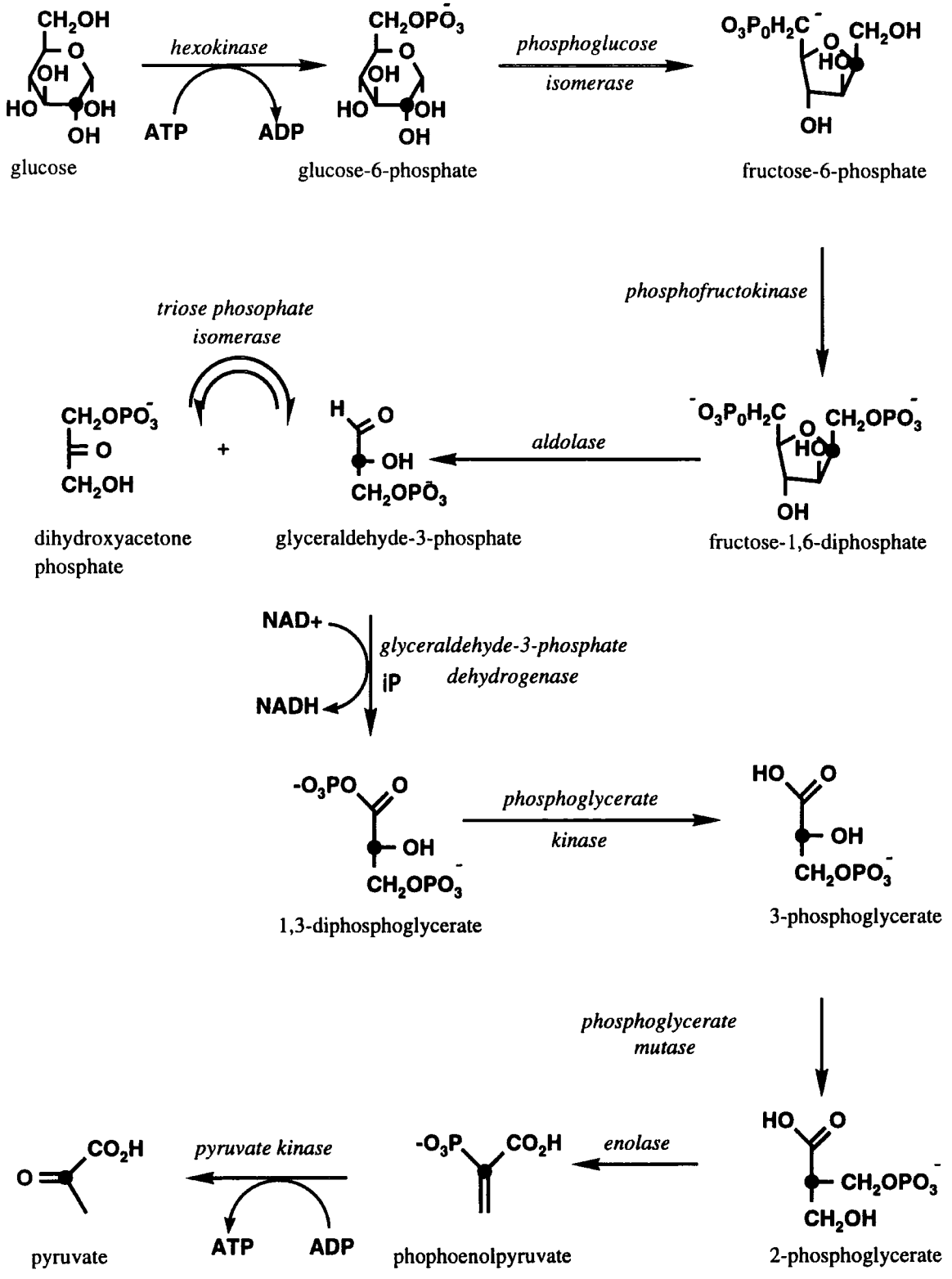

hexokinase
ATP
ADP
phosphoglucose
isomerase
glucose
glucose-6-phosphate
fructose-6-phosphate
phosphofructokinase
triose phosphate
isomerase
aldolase
dihydroxyacetone
phosphate
glyceraldehyde-3-phosphate
fructose-1,6-diphosphate
NAD+
NADH
iP
glyceraldehyde-3-phosphate
dehydrogenase
phosphoglycerate
kinase
1,3-diphosphoglycerate
3-phosphoglycerate
phosphoglycerate
mutase
pyruvate kinase
ATP
ADP
enolase
pyruvate
phophoenolpyruvate
2-phosphoglycerate

Fig. 158

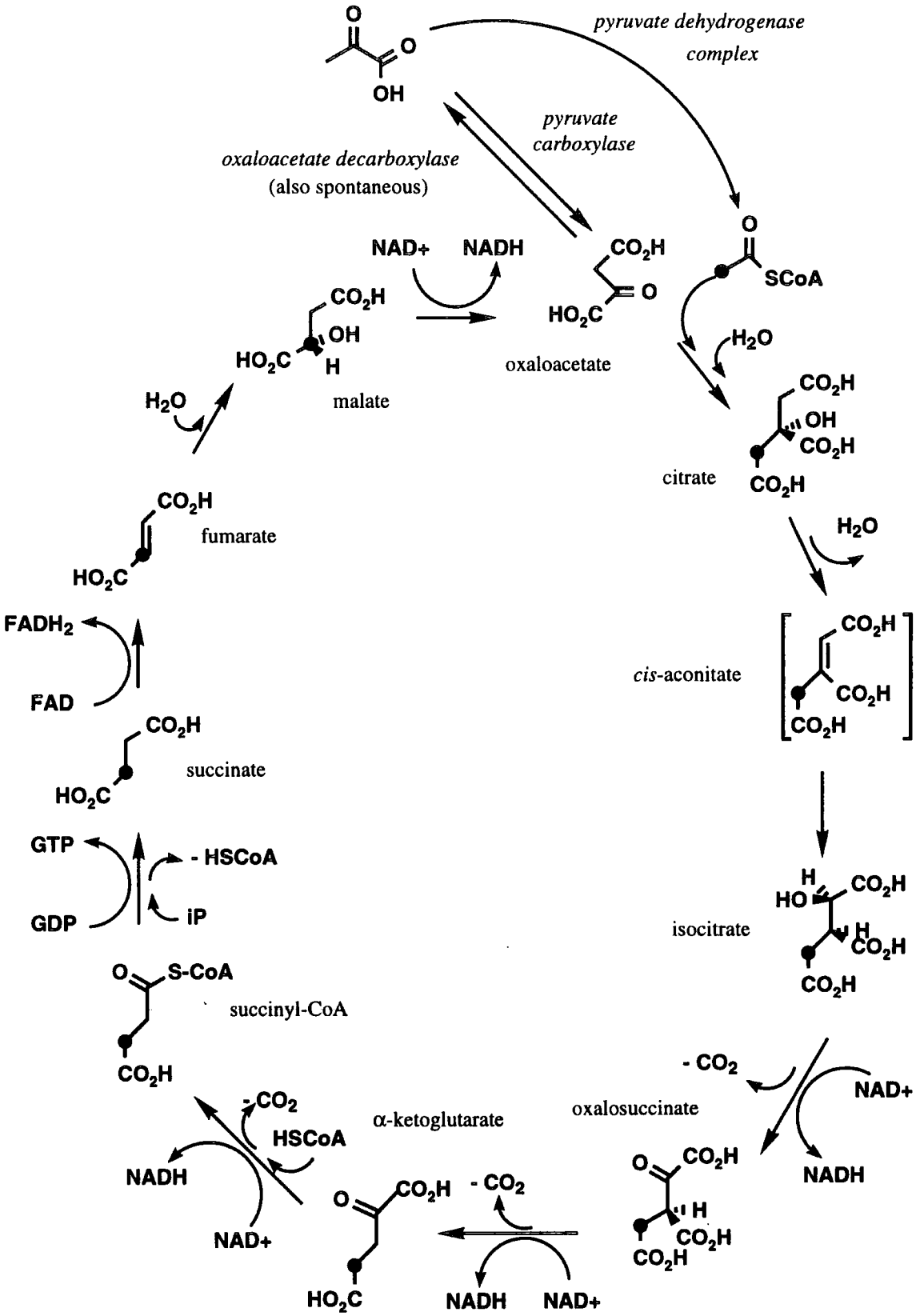

pyruvate dehydrogenase complex
pyruvate carboxylase
oxaloacetate decarboxylase (also spontaneous)
oxaloacetate
citrate
cis-aconitate
isocitrate
oxalosuccinate
α-ketoglutarate
succinyl-CoA
succinate
fumarate
malate
NAD+
NADH
H2O
- CO2
HSCoA
- HSCoA
iP
GTP
GDP
FAD
FADH2
SCoA
S-CoA

Fig. 159

6.11 The Origin of the C_3 or C_4 Fragment in Related Metabolites

[1-^{14}C]-Acetate incorporation into protolichesterinic acid (**83**)[2,30,31] revealed the expected high level of labelling in the long chain fragment and also a lower level of labelling at C-1 (fig. 160) in the C_3 fragment. This latter enrichment is consistent with the incorporation of acetate *via* a citric acid cycle dicarboxylic acid. It is interesting to note that positions C-2 and C-5 were also labelled in this experiment. This cannot however be the result of acetate metabolism through the citric acid cycle as incorporation by this pathway would result in the C-2 (methyl) carbons of acetate becoming joined, not the C-1 (carboxyl) carbons. Subsequently the citric acid cycle origin of this fragment was further implicated by the successful incorporation of [1,4-$^{14}C_2$]-succinate (**130**) into protolichesterinic acid.

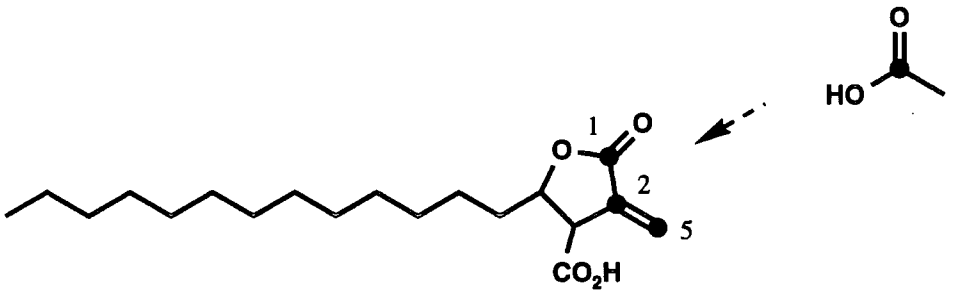


Fig. 160

Comparing protolichesterinic acid (**83**) to the apparently related lichen metabolites caperatic acid (**88**) and rangiformic acid (**89**), reveals an important structural difference. In caperatic and rangiformic acids the non-acetate derived fragment is a four carbon unit, which provides circumstantial evidence that in these lichen metabolites, oxaloacetate (**106**) is the more likely precursor.

Glauconic acid (**95**)[3] provides further evidence of a citric acid cycle intermediate. Glauconic acid (**95**) is the product of two C_9 fragments which are themselves constructed from C_6 and C_3 units as discussed in section 6.2. Here again the incorporation of both [1-^{14}C]-acetate and [2-^{14}C]-acetate was entirely consistent with the involvement of a citric acid cycle intermediate. Subsequently succinate (**130**)

incorporation was also shown. In later experiments the incorporation of [2-^{14}C]-pyruvate (**104**) and [2-^{14}C]-glucose (**130**), which would generate [2-^{14}C]-pyruvate *in vivo* through glycolysis, were also shown. [2-^{14}C]-Pyruvate is readily converted into [1-^{14}C]-acetate *in vivo*, however, the high level of incorporation observed at C-6, compared to the sites enriched by acetate, indicate that this had largely not happened. This experiment therefore demonstrated that a *symmetrical* C_4 intermediate, such as succinate (**130**) was not required, as such an intermediate would result in the scrambling of the label between C-6 and C-7. Thus of the citric acid cycle C_4 dicarboxylic acids, oxaloacetate (**106**) was the only candidate precursor, although a direct incorporation of pyruvate (**104**) is not precluded (fig. 161).

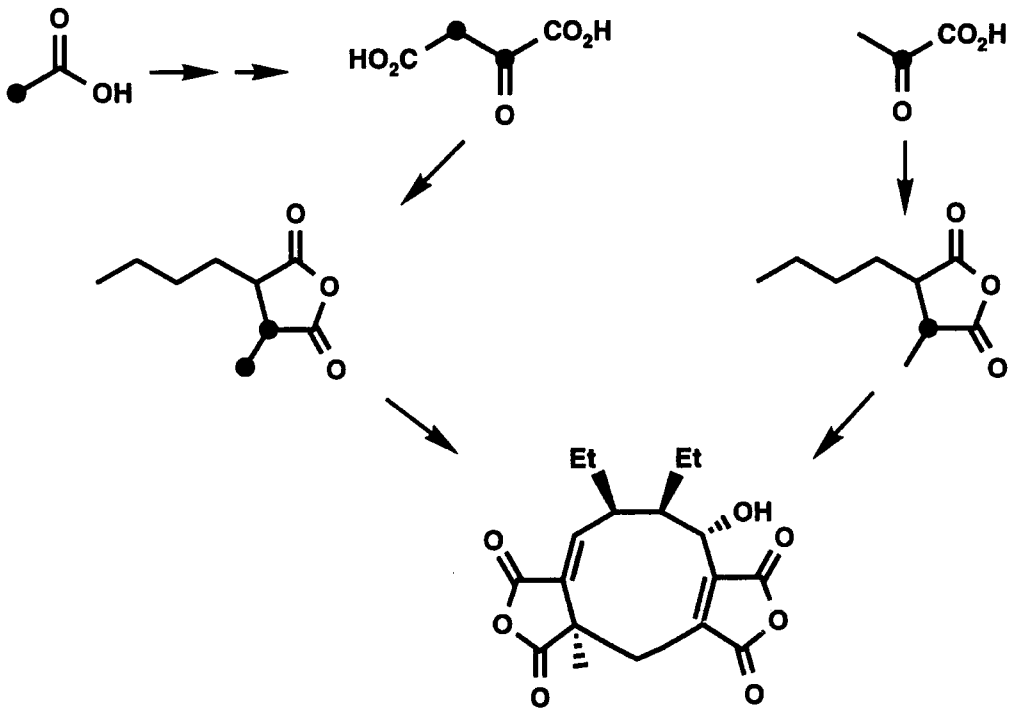


Fig. 161

The citric acid cycle origin of the C_3 fragment of avenaciolide (**97**) has also been demonstrated.[4] Here [2-^{13}C]-acetate was incorporated at a sufficiently high level that coupling between C-11 and C-12 was apparent in the ^{13}C NMR (fig. 162).

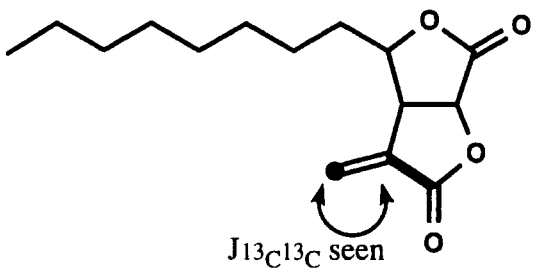


Fig. 162

However, only a cautious biosynthetic comparison between this metabolite and piliformic acid can be made as the mode of succinate incorporation is clearly quite different. This is inferred by the structural difference which suggests a condensation in the opposite direction to the β-carbon, rather than the α-carbon of the long chain moiety (fig. 163).

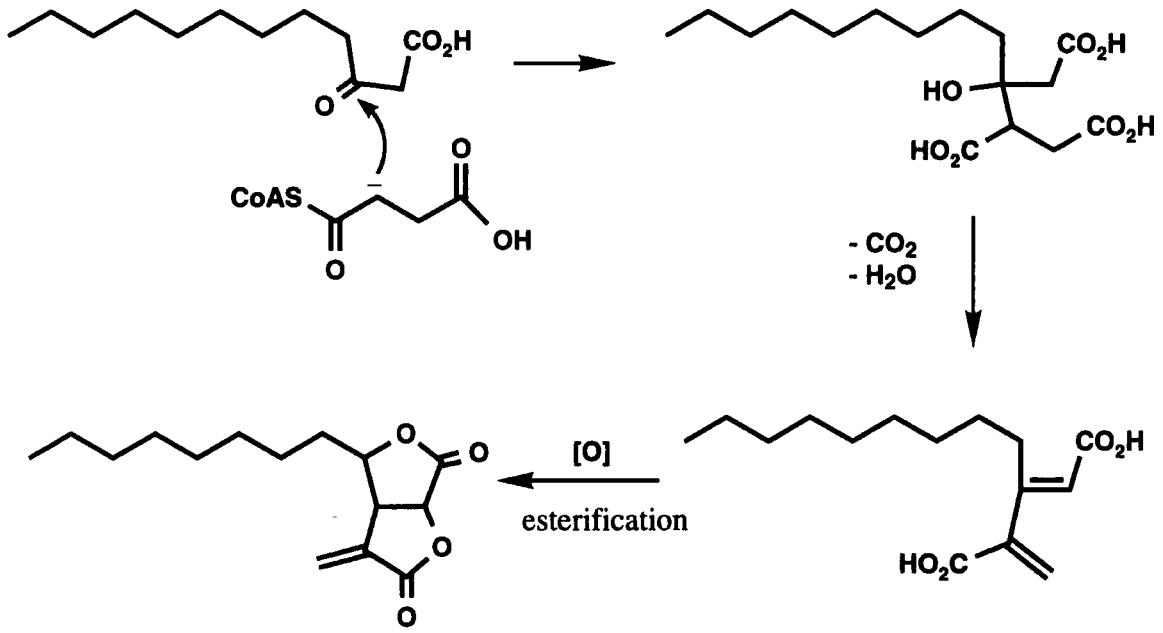


Fig. 163

Another group of secondary metabolites for which a citric acid cycle dicarboxylic acid origin has been suggested are the tetronic acids, of which carlosic acid (**131**)[32,33] is an illustrative example. Acetate was shown to label six of the carbons in a head to tail manner while [2,3-$^{14}C_2$]-succinate (**130**) efficiently labelled C-4 and C-9 (fig. 164).

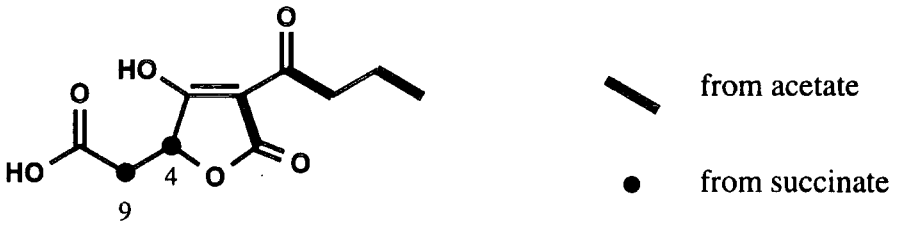


Fig. 164

Leibing and Reio[33] suggested that β-keto-hexanoate condensed with malate (citric acid cycle) to generate carlosic acid (**131**) in *Penicillium charlesii*. However, this hypothesis was later modified by Bentley *et al*[32] who suggested that malate might first condense with malonyl-CoA followed by the addition of an acetate / malonate derived C_4 fragment, to account for the co-production of smaller quanities of methyltetronic acid (**132**) by the fungus (fig. 165). It is noteworthy that labelled acetates were not incorporated in the C_4 fragment of carlosic acid (**131**).

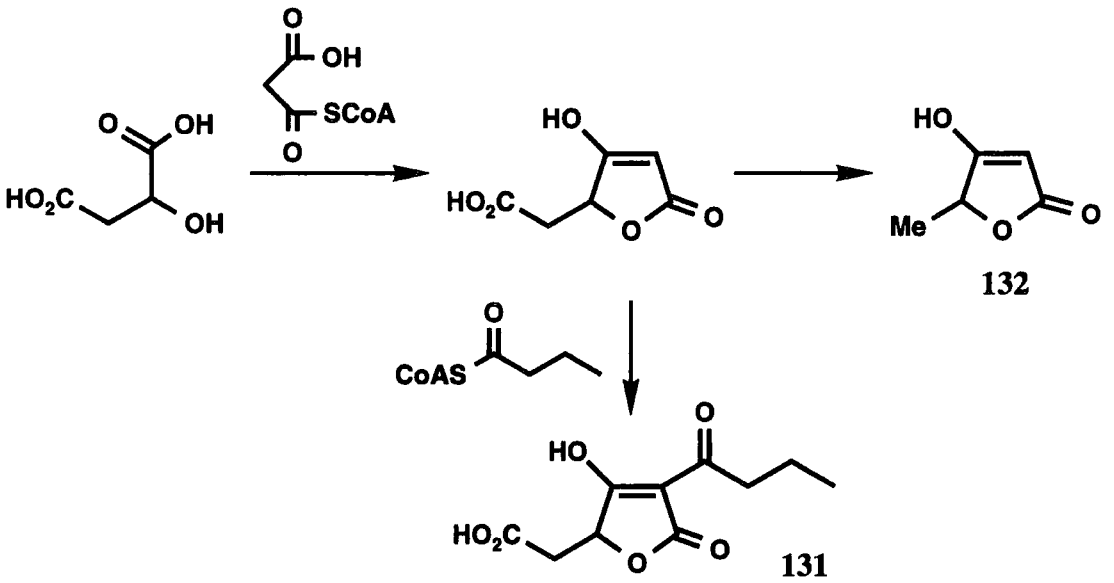
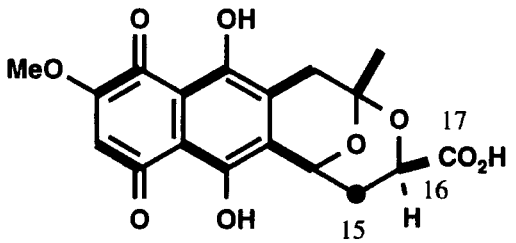


Fig. 165

A further example of the incorporation of a citric acid cycle derived fragment is found in the fungal polyketide marticin (**133**),[34] elaborated by *Fusarium martii*. From the incorporation of [1,2-$^{13}C_2$]-acetate a heptaketide with a C_3 fragment of different origin was apparent, rather than a nonaketide having lost one carbon. C-16 and C-17 were

coupled, consistent with the intact incorporation of an acetate unit, whereas C-15 and C-16 showed smaller satellites indicating back to back coupling through the citric acid cycle (fig. 166).



marticin (**133**)

Fig. 166

6.12 Isotopic Incorporations into the C_3 Fragment of Piliformic Acid

It has been demonstrated in a number of other metabolites that the C_3 fragments incorporate acetate in a manner consitent with a citric acid cycle origin. In the case of glauconic acid (**95**) a possible glycolytic origin was also indicated.[3] With this background the biosynthesis of the C_3 fragment of piliformic acid was investigated.

The first evidence for the origin of the C_3 fragment of piliformic acid came from the initial acetate feeding experiments. ^{13}C NMR studies of piliformic acid, supplemented with [1,2-$^{13}C_2$]-acetate, indicated that the isotope was principally incorporated into the side chain (section 6.3) and also at a lower level (0.48%) into the C_3 unit. C-1 and C-2 were enriched, and coupled, indicating the incorporation of an intact acetate unit, and C-11 was enriched but divorced from an adjacent isotope, indicating the incorporation of one carbon of an acetate molecule. This is illustrated in fig. 167, showing incorporations into the C_3 fragment only. This is consistent with the back to back coupling of acetate through the citric acid cycle to generate oxaloacetate (**106**), which has lost one carbon atom by decarboxylation.

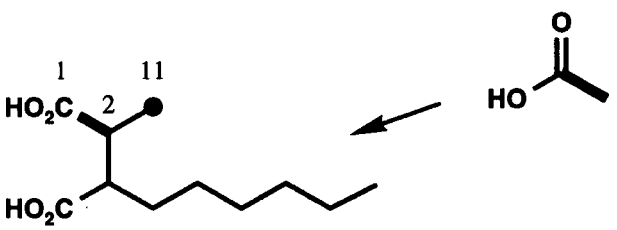


Fig. 167

When [2-^{13}C, $^{2}H_3$]-acetate was administered to *P. piliformis* C-2 and C-11 were again enriched and also a single low level deuterium enrichment was observed attached to the C-11 methyl group. The incorporations of [2-^{13}C, $^{2}H_3$]-acetate into the C_3 fragment are illustrated in fig. 168.

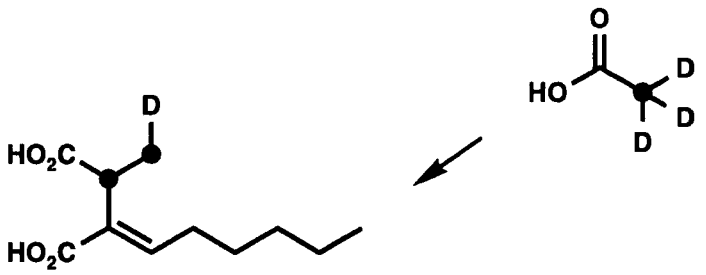


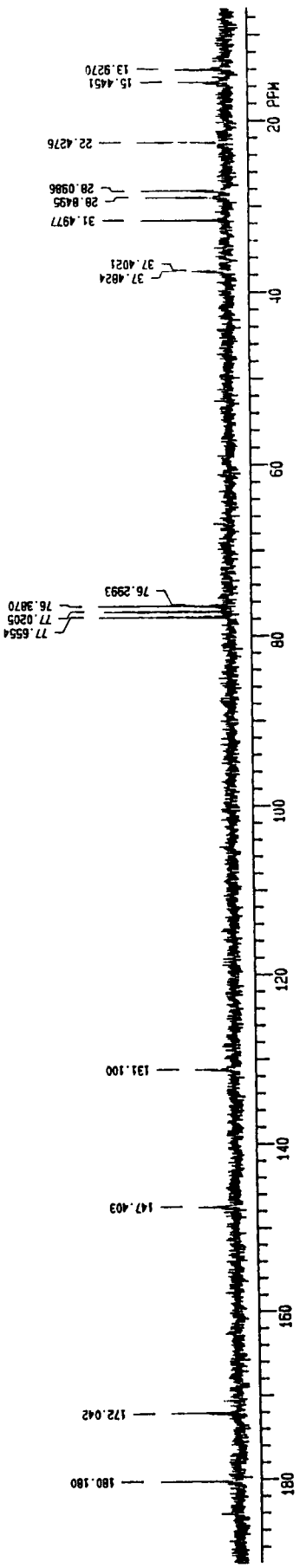
Fig. 168

These experiments do not however indicate whether decarboxylation of oxaloacetate had occurred before or after coupling with the octanoate fragment.

To investigate the origin of this C_3 fragment in greater detail, commercially available DL-[1-^{13}C]-alanine (**105a**) was administered to *P. piliformis*. The biosynthetic implications of alanine (**105**) incorporation are the same as for pyruvate (**104**) as the two can interconvert through transammination. In the event the ^{13}C enrichment of C-1 was small but apparent (0.09%) in the ^{13}C NMR spectrum (fig. 169) indicating the expected regiospecific labelling. However, the low level of incorporation suggests that alanine (and therefore pyruvate) had only been incorporated rather inefficiently and possibly by an indirect route.

6.13 Pyruvate or Oxaloacetate?

In order to establish whether pyruvate (**104**) could indeed be incorporated without prior carboxylation to oxaloacetate it was proposed to feed [3-^{13}C, $^{2}H_3$]-pyruvate, with deuterium tagged to ^{13}C to enable detection by ^{13}C NMR analysis. Carboxylation would entail loss of one deuterium and therefore the retention of all three deuterium atoms would delineate oxaloacetate or pyruvate as the closer precursor (fig. 170).



^{13}C NMR spectrum of piliformic acid after feeding [1-^{13}C]-alanine

Fig. 169

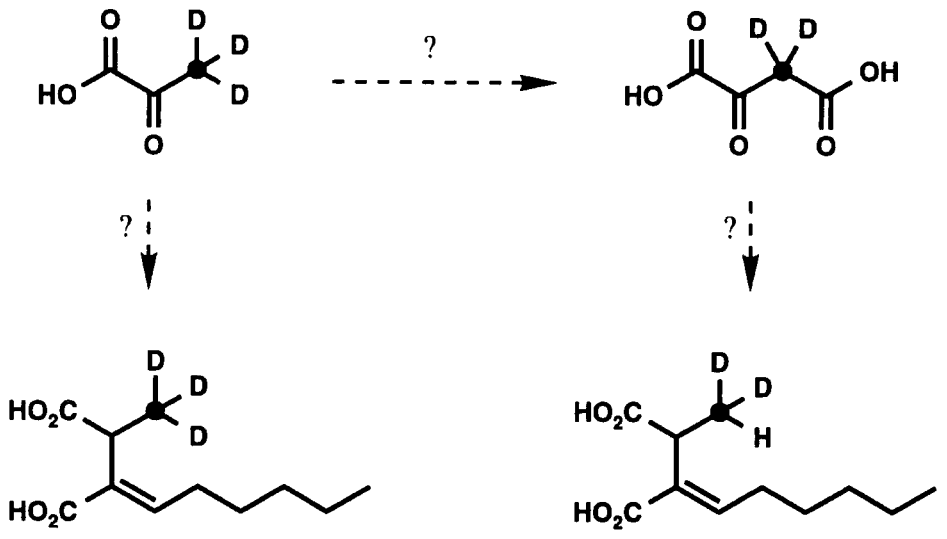
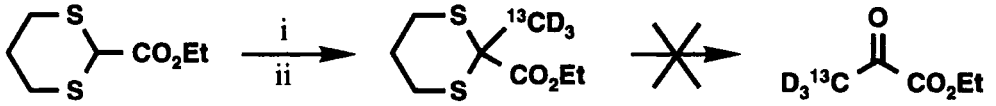


Fig.170

Initially a synthetic route to pyruvate *via* [2-^{13}C, $^{2}H_3$]-acetate was examined.[35] Unfortunately the low overall yield of this procedure meant that starting from an expensive label such as [2-^{13}C, $^{2}H_3$]-acetate would render the experiment too expensive.

Another route was also investigated which utilised commercially available ethyl 1,3-dithiane-2-carboxylate (**134**), introducing the label from [^{13}C, $^{2}H_3$]-methyl iodide (scheme 17). The methylation was accomplished in almost quantitative yield. However attempts to hydrolyse the methylated dithiane product (**135**), to generate ethyl pyruvate (**136**), resulted only in the destruction of starting material under all of the conditions tried, including N-bromosuccinimide,[36] mercuric oxide / boron trifluoride etherate[37], silver oxide[38], silver nitrate[39] and trichlorocyanuric acid[40].



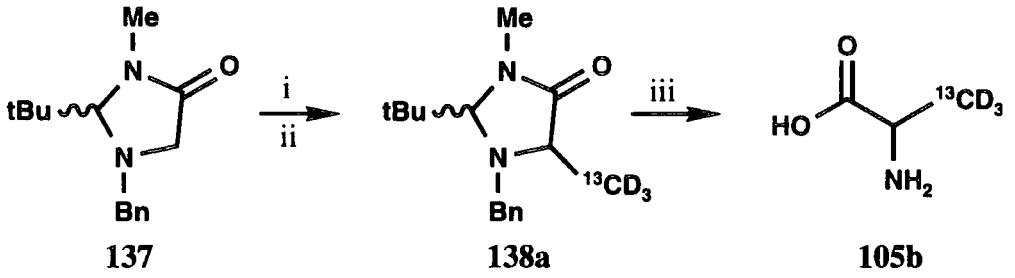
134 **135** **136**

(i) *n*-BuLi, (ii) [^{13}C, $^{2}H_3$]-MeI

Scheme 17

As this pyruvate synthesis proved impractical [3-^{13}C, $^{2}H_3$]-alanine (**105b**) was prepared instead. It was judged that the retention of three deuterium atoms from this

precursor would provide a definitive result as *in vivo* transammination would lead to [3-^{13}C, $^{2}H_3$]-pyruvate. In the first instance unlabelled DL-alanine (**105**) was synthesised from 1-benzyl-2-(*t*-butyl)-3-methylimidazolin-4-one (**137**) using the methodology developed by Seebach[41] (scheme 18).

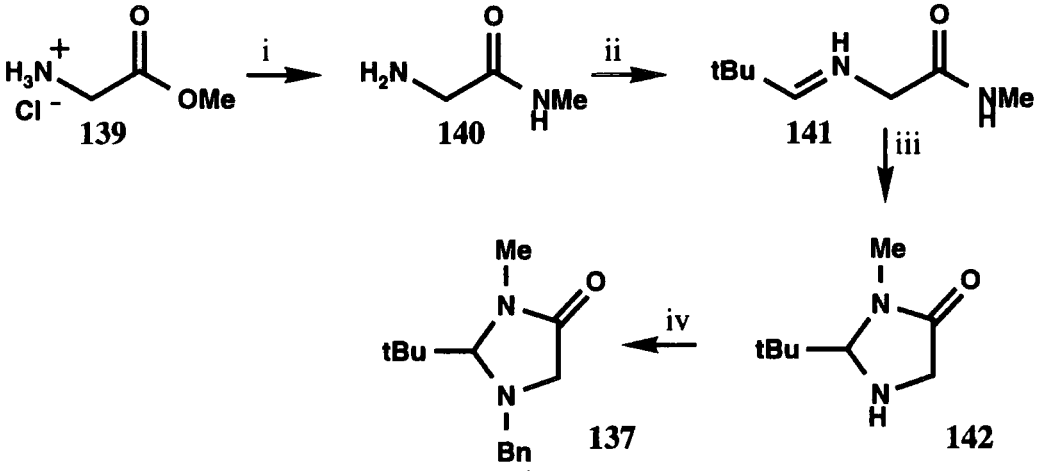


137 **138a** **105b**

(i) *n*-BuLi, (ii) [^{13}C, $^{2}H_3$]-MeI, (iii) 6N HCl 180°C, sealed tube

Scheme 18

6.14 DL-1-Benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one[43, 44]



139 **140** **141** **137** **142**

(i) 8M $MeNH_2$, (ii) t-BuCHO, (iii) saturated methanolic HCl (iv) BnCl, NaOH

Scheme 19

Glycine methyl ester hydrochloride (**139**) was initially converted to the corresponding amide (**140**) by stirring in 8M ethanolic methylamine for 15h. After this time the crude product was used to generate oxime (**141**) with pivalaldehyde. The oxime was cyclised directly by stirring in methanolic HCl for 12h to generate 2-(*t*-butyl)-3-methylimidazolidin-4-one in 49% yield, over 3 steps. 2-(*t*-Butyl)-3-methylimidazolidin-4-one (**142**) was then treated with benzyl chloride to generate 1-benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one (**137**) in 56% yield as shown in scheme 19.

6.15 DL-1-Benzyl-2-(*t*-butyl)-3,-methyl-[5-^{13}C,^{2}H$_3$-*methyl*]-imidazolidin-4-one (138a)[41]

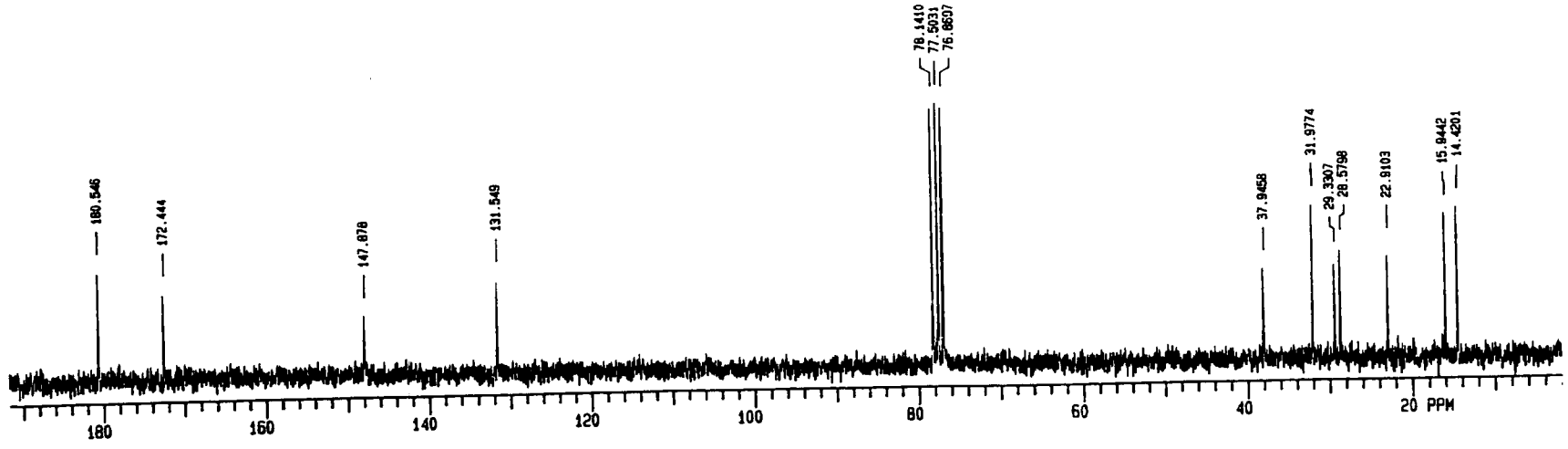
DL-1-Benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one (**137**) was deprotonated with LDA at -78°C and the anion was then quenched with methyl iodide. After aqueous work-up a mixture of the required 1-benzyl-2-(*t*-butyl)-3,5-dimethylimidazolidin-4-one (**138a**) and a little unreacted starting material (**137**) was obtained. Although chromatographically similar the product was separable by repeated flash chromatography and DL-2-(*t*-butyl)-3-methyl-[5-^{13}C,^{2}H$_3$-*methyl*]-imidazolidin-4-one (**138a**) was isolated in 56% yield.

6.16 DL-[3-^{13}C, ^{2}H$_3$]-Alanine (105b)[44]

Seebach[44] has demonstrated that alkylated imidazolidinones are difficult to hydrolyse, and that they require vigorous conditions. Consistent with this 1-benzyl-2-(*t*-butyl)-3,5-dimethylimidazolidin-4-one (**138**) did not hydrolyse even after refluxing in 6N HCl for two hours, thus a more vigorous method was required. Success was achieved by heating a suspension of the methylated imidazolidinone (**138a**) in 6N HCl at 180°C in a sealed tube for 12 hours. This produced the labelled alanine hydrochloride mixed with methylamine, hydrochloride. One concern was that the hydrolysis may generate the partially hydrolysed product, alanine N-methyl amide, but in the event this was not the case. The free amino acid (**105b**) was liberated by ion exchange chromatography, free from methylamine, and the identity of the product was confirmed by ^{1}H NMR and by TLC comparison to an authentic sample of DL-alanine.

6.17 Incorporation of [3-^{13}C, ^{2}H$_3$]-Alanine into Piliformic Acid

The piliformic acid isolated from *P. piliformis,* supplemented with [3-^{13}C, ^{2}H$_3$]-alanine, was analysed by ^{13}C{^{1}H} and ^{13}C{^{1}H,^{2}H}-NMR (fig. 171). There was no evidence for any incorporation of deuterium into piliformic acid although the C-11 resonance appeared to have a low level of enrichment (≈ 0.1%), indicating incorporation with deuterium washout. Also, low level enhancements of the C-9, C-7,



^{13}C NMR spectrum of piliformic acid after feeding [3-^{13}C, $^{2}H_3$]-alanine

Fig. 171

C-5, C-3 resonances suggested that a small amount of ^{13}C had been incorporated into these sites *via* the oxidative decarboxylation of pyruvate to generate [2-^{13}C]-acetate. From this result it can only be concluded that L-alanine is not an efficient precursor to piliformic acid and the direct incorporation of pyruvate is by implication, negated.

6.18 Incorporation of [2,3-$^2H_4$]-Succinic Acid into Piliformic Acid

The low level and indirect incorporation of alanine (**105**), coupled with the conclusive evidence that acetate can be incorporated into the C_3 fragment *via* a citric acid cycle intermediate, placed oxaloacetate (**106**) as a candidate precursor to piliformic acid. To test this hypothesis [2,3-$^2H_4$]-succinic acid (**130a**) was administered to *P. piliformis*. It was anticipated that metabolism of the deuterated succinate (**106a**), through the citric acid cycle, would furnish oxaloacetate bearing a single deuterium at C-3. Incorporation into piliformic acid, followed by decarboxylation would then label the C-11 methyl group with deuterium (fig. 172).

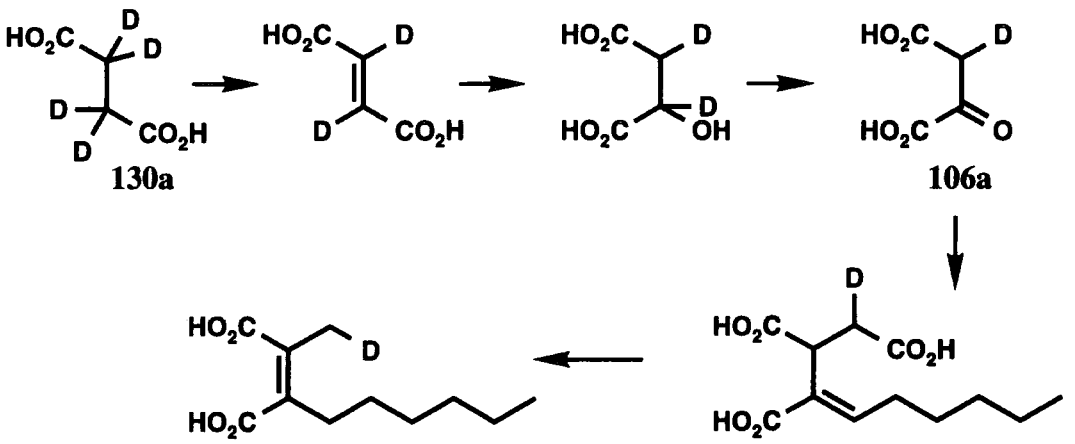
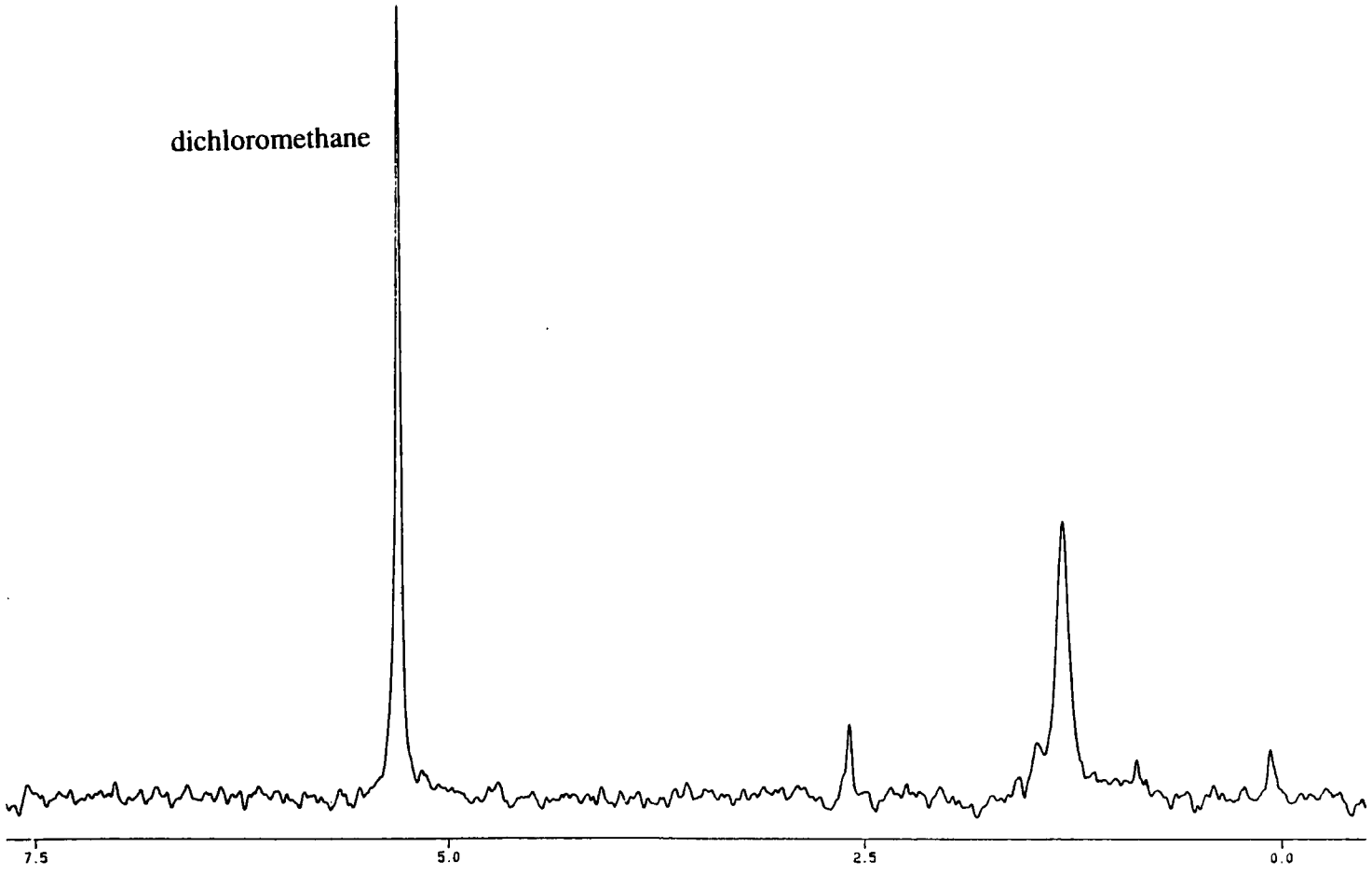


Fig. 172

The resultant piliformic acid isolated was analysed by ^2H NMR (fig. 173) and in this case revealed a prominent enhancement at 1.31ppm. Three minor peaks at 0.87ppm, 1.57ppm and 2.59ppm indicated some incorporation of deuterium into the C_8 fragment consistent with the formation of [2-^2H]-acetate *in vivo*, presumably *via* succinate (**130**) → oxaloacetate (**106**) → pyruvate (**104**) → acetate. The marked incorporation of [2,3-$^2H_4$]-succinic acid clearly places oxaloacetate (**106**) as a *bona fide* intermediate.



^{2}H NMR spectrum of piliformic acid after feeding [2,3-$^{2}H_4$]-succinic acid

Fig. 173

6.19 Mechanismistic Considerations for the Coupling of the C_8 Fragment with Oxaloacetate

As discussed previously the attachment of the octanoate (**103**) unit to oxaloacetate (**106**) requires the α-carbon of octanoate to be activated towards deprotonation. This could be satisfied with either a β-keto-octanoate (**107**) or a α-carboxyoctanoate (**108**) intermediate. With the knowledge that the polyacetate chain is a degradation product of a fatty acid, the β-keto-octanoate emerged as the more likely intermediate as this would arise by an extension of the normal β-oxidation process. α-Carboxylation on the other hand would require a dedicated octanoate carboxylase.

Delineation of these possibilities was probed by investigating deuterium retention at the β-carbon of octanoate, which becomes the olefinic proton of piliformic acid. Clearly conversion to a β-keto-octanoate (**107**), prior to incorporation, would result in the complete loss of deuterium from this site, whereas the alternative α-carboxylation, to generate the α-carboxyoctanoate (**108**), would lead to deuterium retention. Of course only one deuterium would be retained in piliformic acid due to the introduction of the double bond. These mutually exclusive processes are illustrated in Fig. 174.

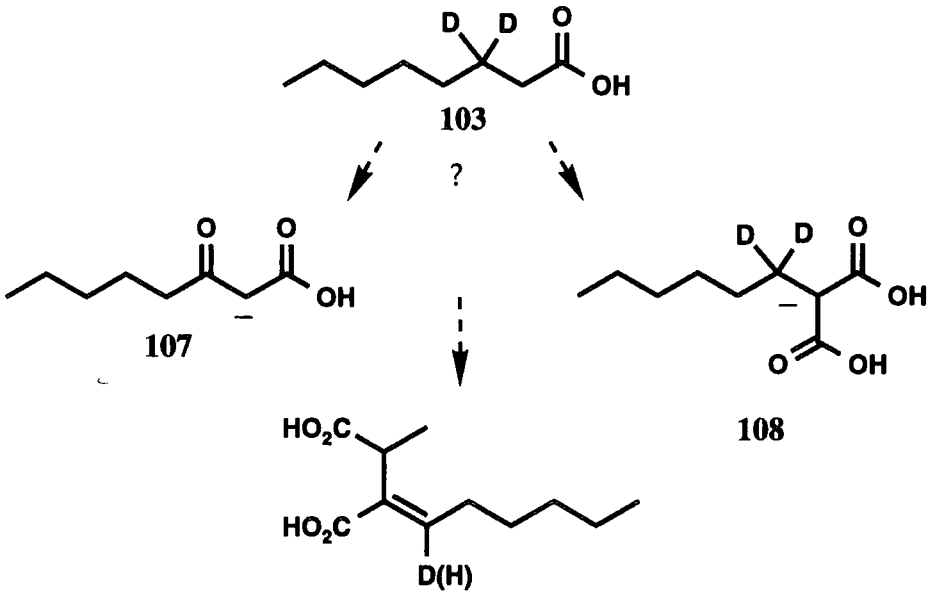
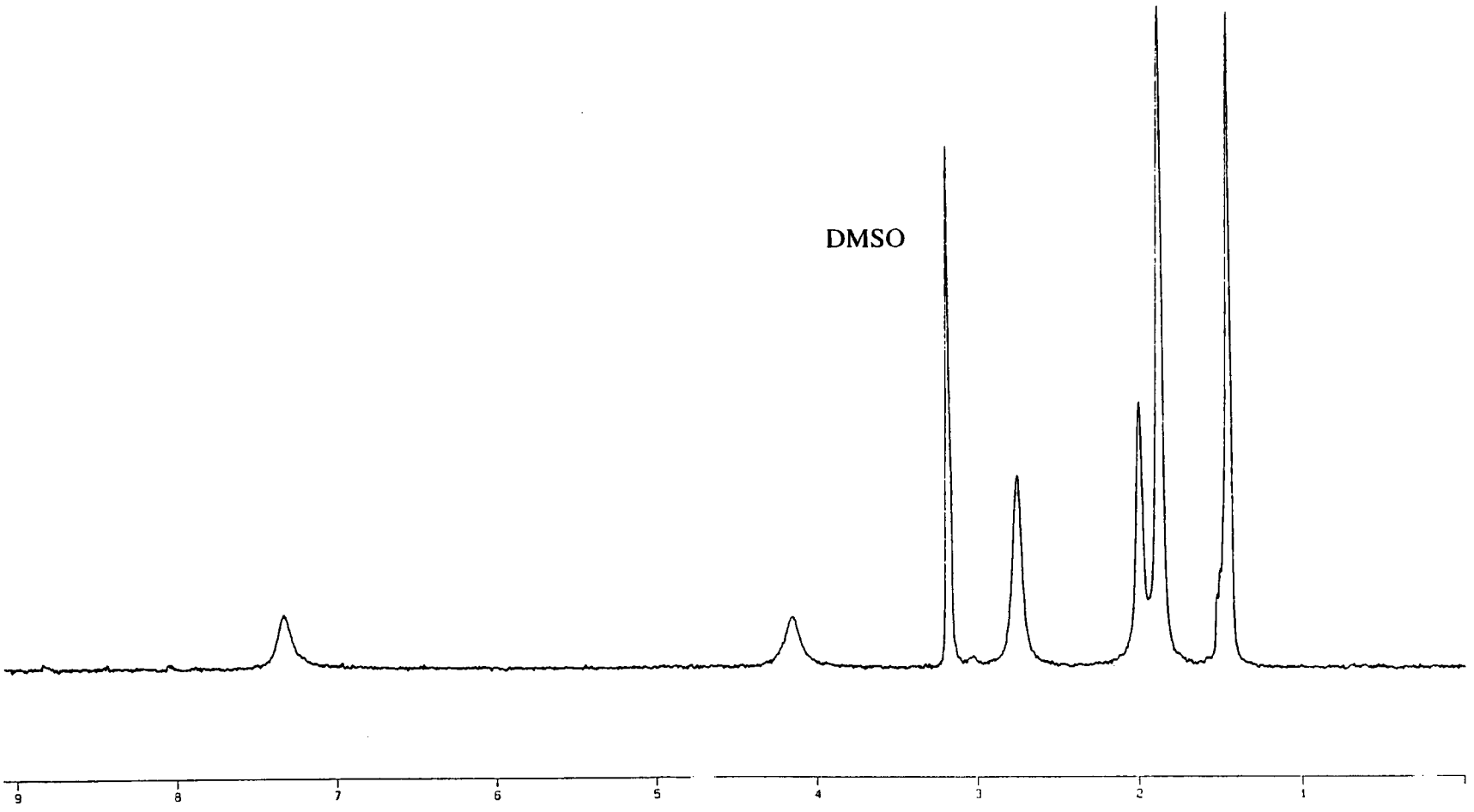


Fig. 174

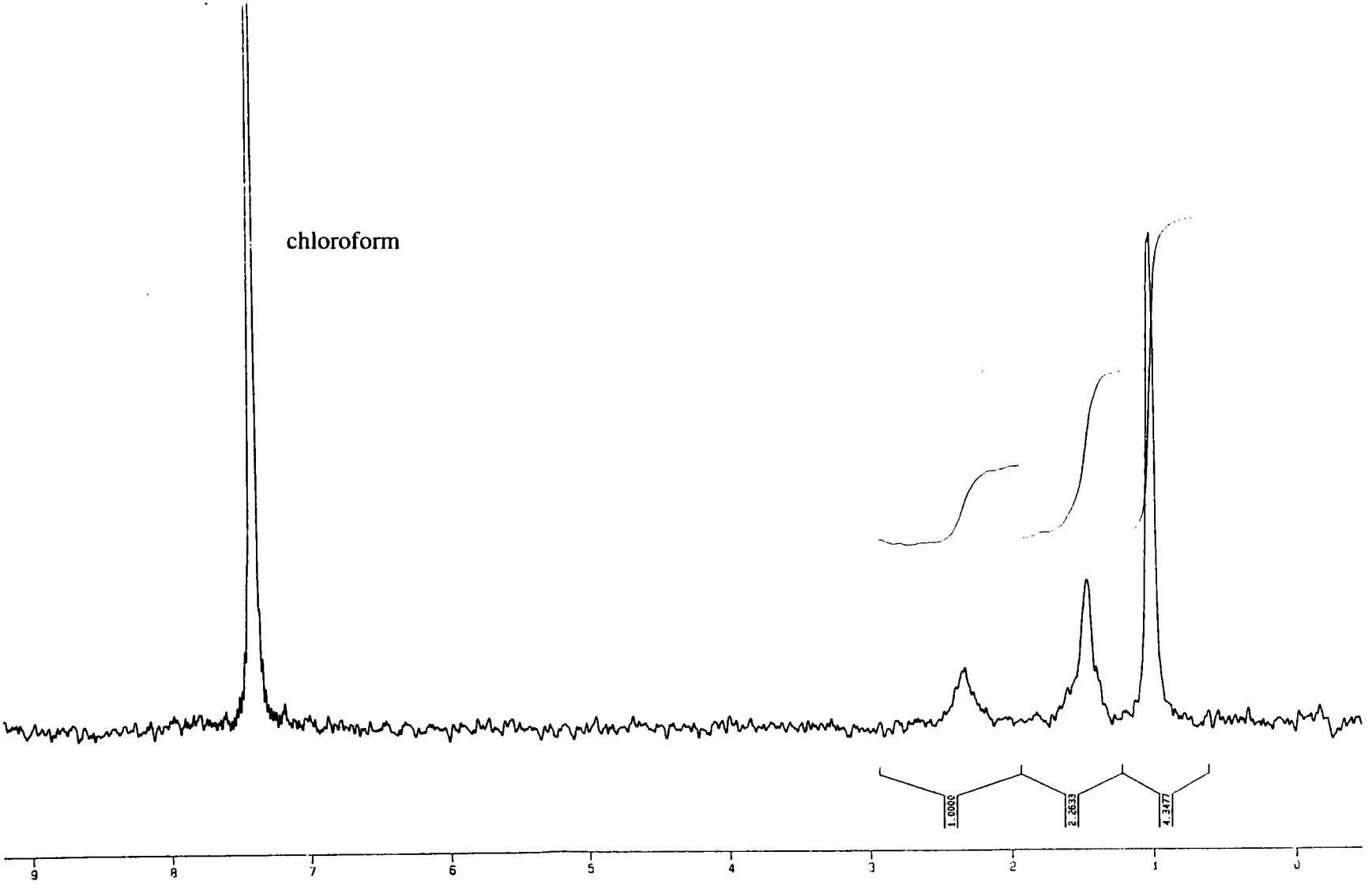
Commercially available perdeuterated octanoate was administered to cultures of *P. piliformis*. The use of perdeuterated material, instead of selectively labelling the β-carbon of octanoic acid meant that the intact incorporation of octanoate could be easily assessed by comparing enrichments at the sites of interest with other sites on the hydrocarbon chain. Perdeuterated hexanoate and butyrate were also administered to *P. piliformis*, in separate experiments to establish, or otherwise, if shorter acids could be incorporated directly as starter units. The ^{2}H NMR spectra from these three experiments are shown below (fig. 175 fig. 176 and fig. 177). Firstly, a background acetate labelling pattern can be clearly seen in the ^{2}H NMR spectra from the hexanoate and butyrate feeding experiments, with no evidence of direct incorporation. Therefore in the shorter chain precursors only β-oxidation to acetate has occurred. In the octanoate derived spectrum however all of the side chain proton sites are deuterium enriched showing again the intact incorporation of octanoate. A signal of particular interest is the enriched olefinic site at 7.0ppm. This clearly indicates deuterium retention at the β-carbon of octanoate showing that, although unprecedented, the octanoate intermediate must have been activated by α-carboxylation. Thus, this result implies that a dedicated octanoate carboxylase is operating during the biosynthesis and that hypotheses involving a β-keto-octanaoate become redundant.

Another mechanistically revealing and unexpected enrichment is seen at the methine site of the C_3 fragment (C-2 of piliformic acid). The occurrence of the deuterium atom here indicates the operation of a 1,3-hydrogen shift occurring during the biosynthesis. This deuterium integrates fully as one, thus no loss or exchange with the medium has occurred. The transfer of a deuterium atom while the piliformic acid is tightly held in the active site of an enzyme would explain this complete transfer. On the basis of these results a mechanism for the coupling of the C_3 and C_8 fragments is proposed below (fig. 178). It is envisaged that the decarboxylation is driven by the loss of the hydroxyl moiety. The migration of the double bond is then required to furnish the piliformic acid skeleton. One 1,3-hydrogen shift has already been demonstrated and is shown. It is possible that a prior 1,3-hydrogen shift is involved in the first double bond migration to generate **144** from **145**.



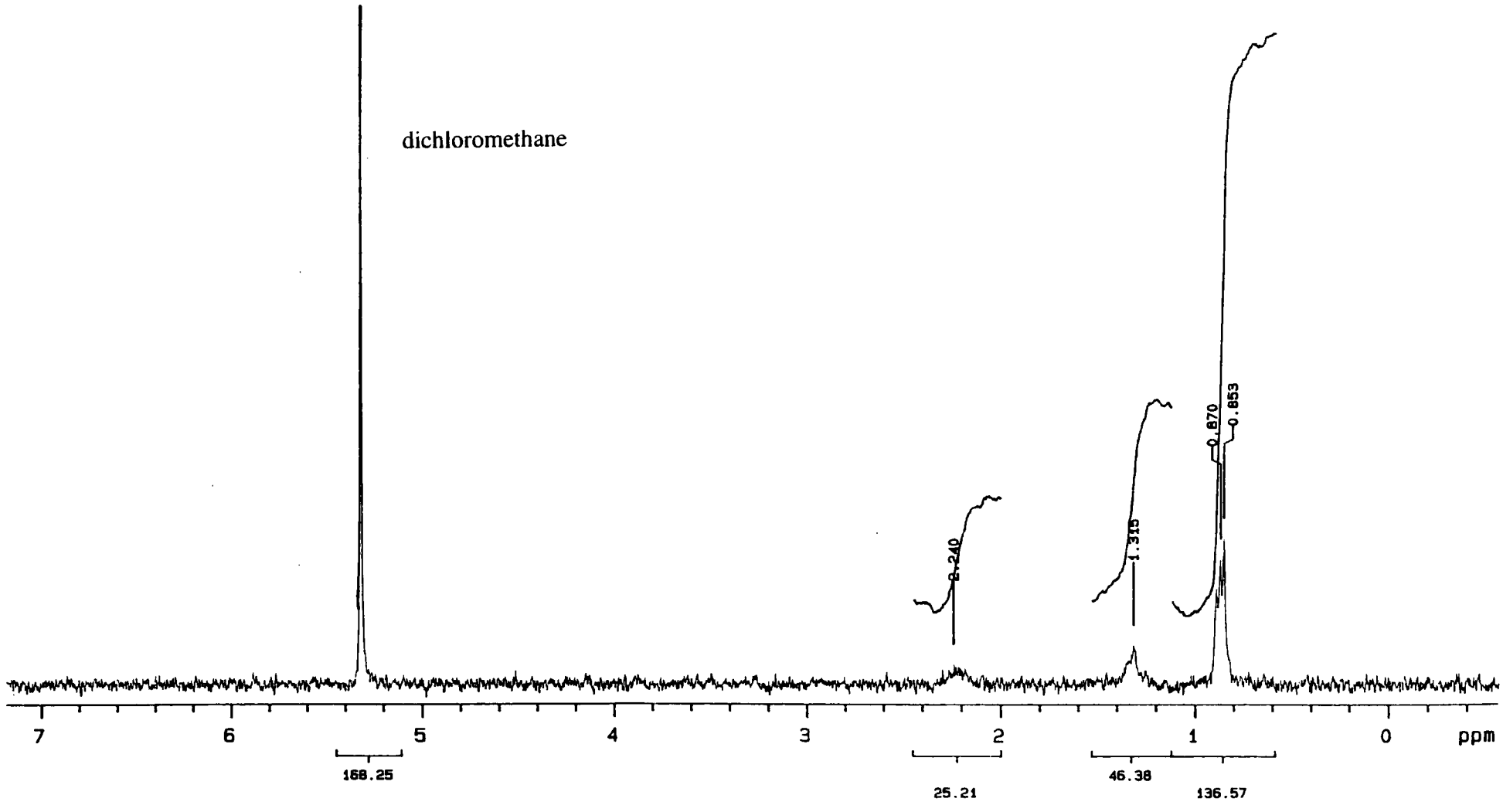
^{2}H NMR spectrum of piliformic acid after feeding perdeuterated octanoic acid

Fig. 175



^{2}H NMR spectrum of piliformic acid after feeding perdeuterated hexanoic acid

Fig. 176



^{2}H NMR spectrum of piliformic acid after feeding perdeuterated butyric acid

Fig. 177

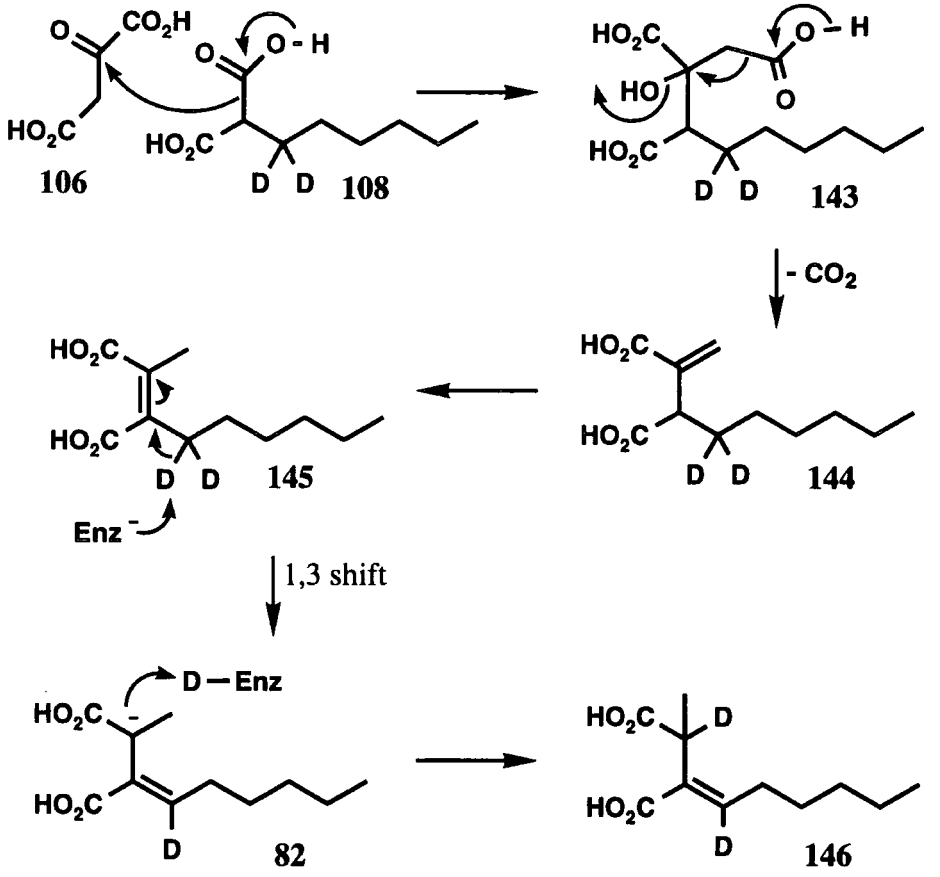


Fig. 178

The incorporations of [$^{2}H_{15}$]-octanoic acid allow the fate of all the deuteriums from this precursor to be followed except those at C-2. The C-2 of octanoic acid is incorporated to furnish C-3 of piliformic acid, which does not have any hydrogens attached (fig. 179). To be confident that the deuterium enrichment at the methine site of piliformic acid did not arise from the C-2 deuterium atoms of [$^{2}H_{15}$]-octanoic acid, [1-^{13}C, 2-^{2}H]-octanoic acid (**106b**) was administered to *P. piliformis*.

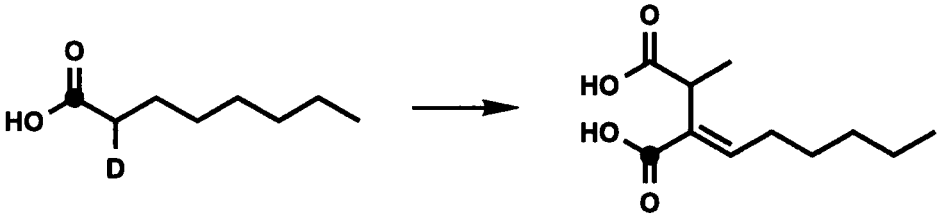
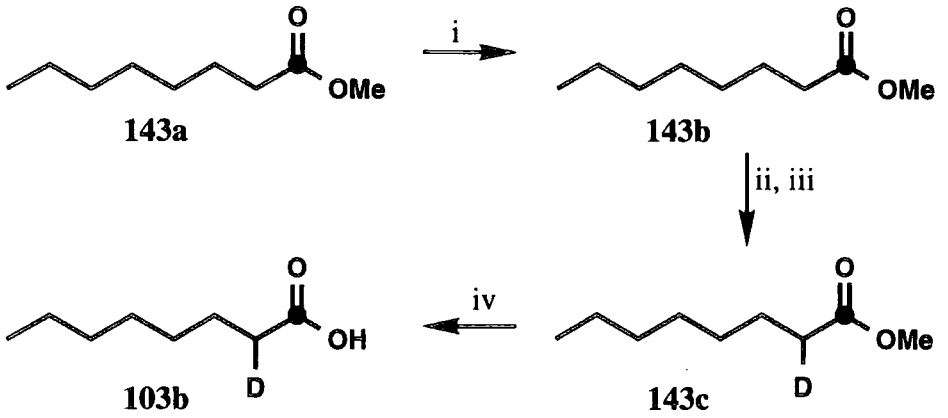


Fig. 179

This substrate was synthesised from [1-^{13}C]-octanoic acid following the procedure described for [2-^{2}H]-hexanoic acid in section 6.8 (scheme 20).

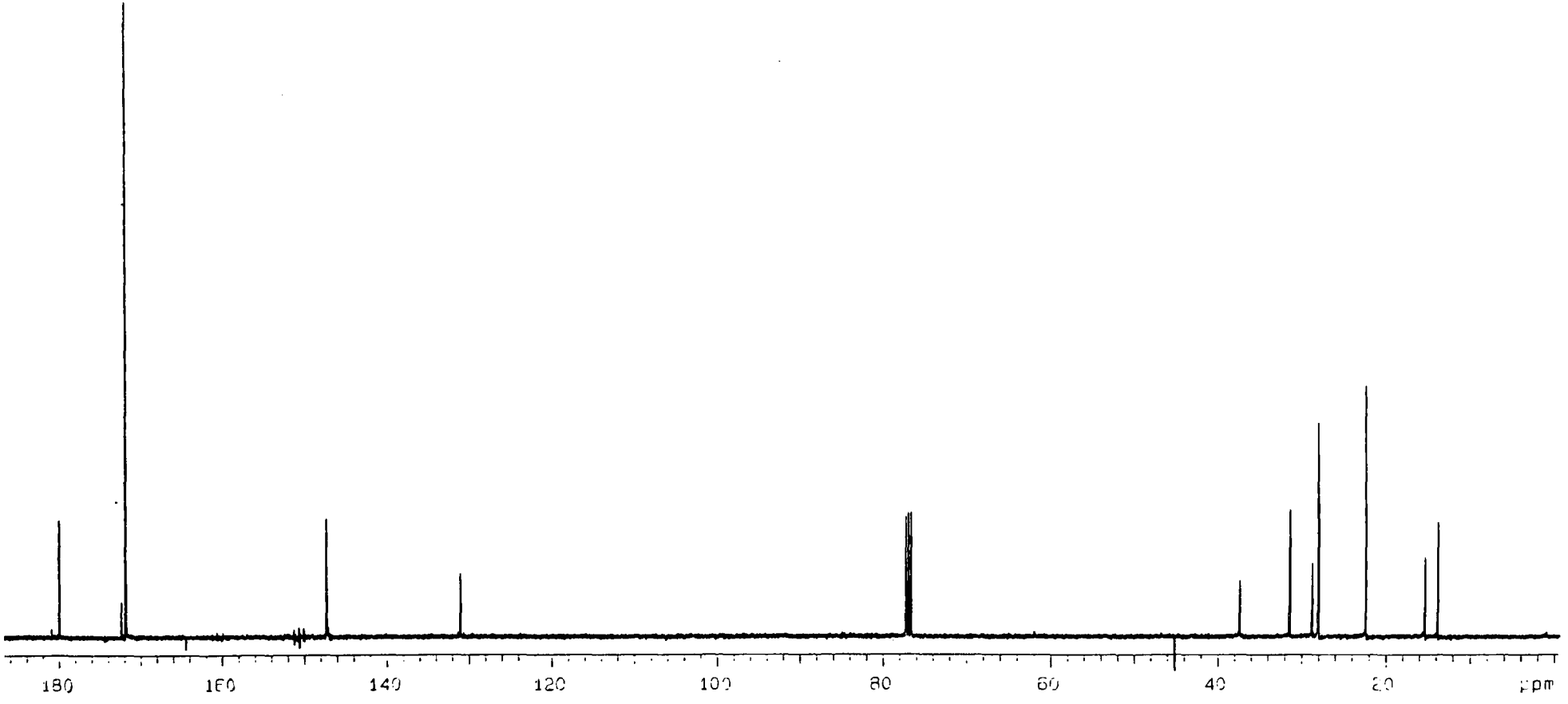


(i) CH_2N_2, (ii) LDA, -78°C, (iii) MeOD, (iv) KOD, D_2O

Scheme 20

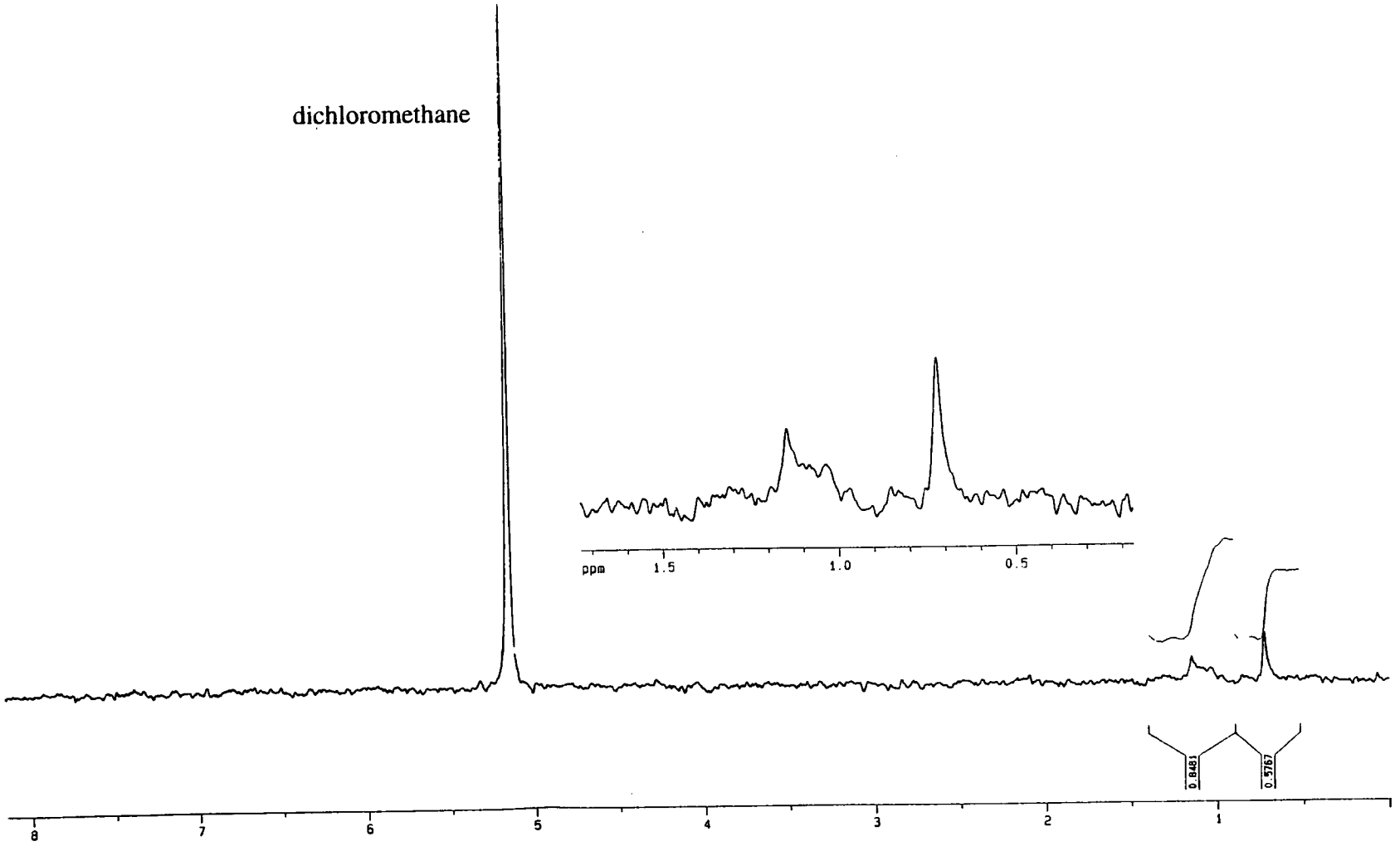
The expected high level of intact incorporation was seen by ^{13}C NMR analysis (fig. 180) (4.9%) with a lower level of incorporation *via* [1-^{13}C, 2-^{2}H]-acetate (1.4%). ^{2}H NMR analysis revealed that deuterium had only been incorporated into piliformic acid *via* [1-^{13}C, 2-^{2}H]-acetate as predicted (fig. 181). This result in no way contradicts the conclusion that the deuterium incorporation into the methine site of piliformic acid from [$^{2}H_{15}$]-octanoic acid was a consequence of a 1,3 shift from C-4 as described above.

The mechanism proposed may also explain the different optical rotations of piliformic acid obtained from different biological sources. The metabolite isolated from *P. piliformis, X. longipes* and *X. polymorpha* has a strong negative rotation which suggests that piliformic acid from these sources is the same enantiomer and the stereochemistry of hydrogen delivery to the methine site is tightly controlled. The racemic material isolated from *X. mali* and *X. hypoxylon* suggests an intermediate which is less tightly held and is free to invert. Correspondingly the material from *H. deustum* with a rotation smaller in magnitude than *P. piliformis*' metabolite suggests again a less rigidly held system but with preferential hydrogen delivery to the opposite face. It was anticipated that if hydrogen was delivered equally to either face of piliformic acid at C-2 after double bond migration that only 50% of the deuterium from C-4 would appear at the methine site in racemic piliformic acid.



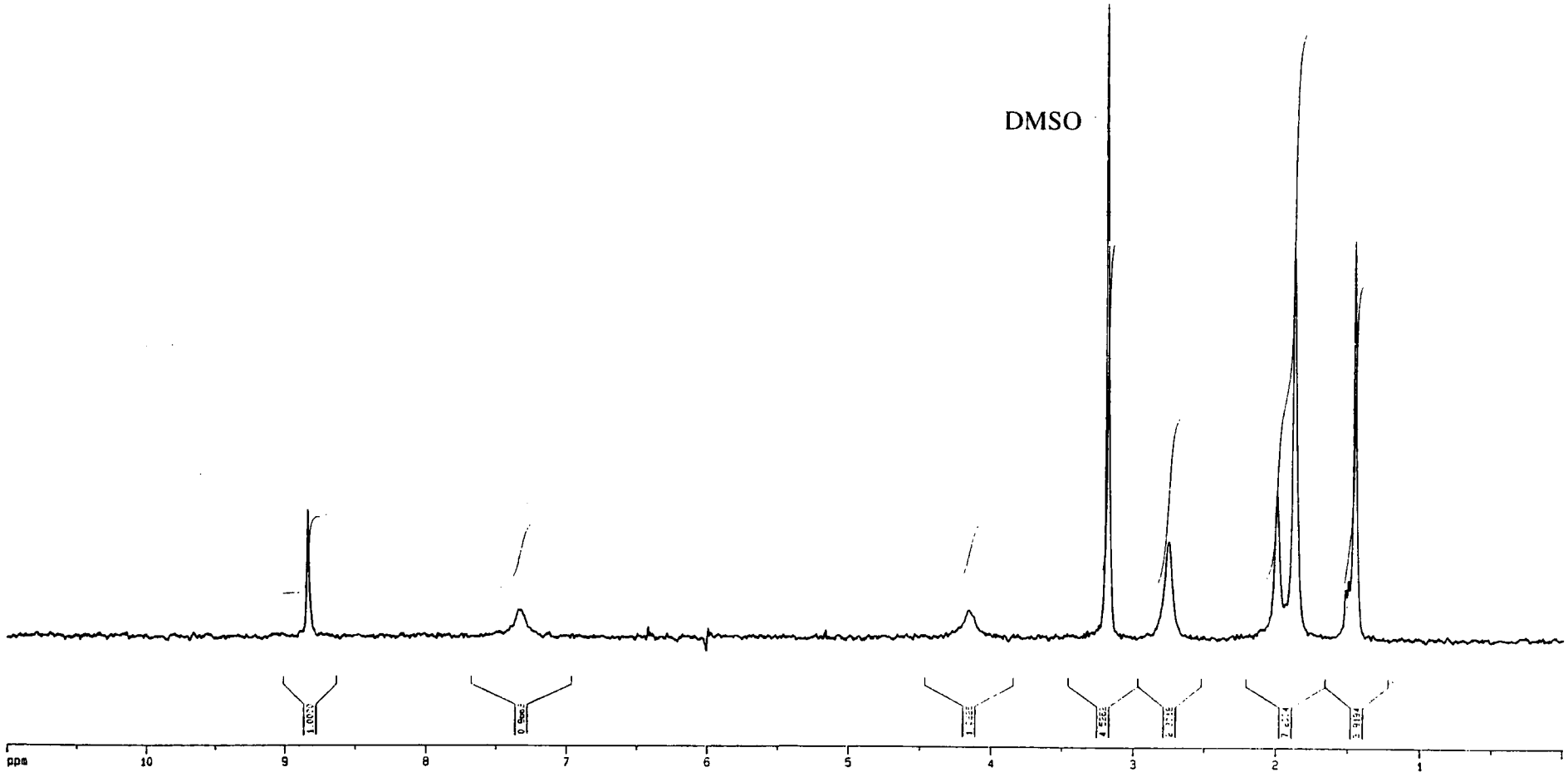
^{13}C NMR spectrum of piliformic acid after feeding [1-^{13}C, 2-^{2}H]-octanoic acid

Fig. 180



^{2}H NMR spectrum of piliformic acid after feeding [1-^{13}C, 2-^{2}H]-octanoic acid

Fig. 181



^{2}H NMR spectrum of piliformic acid isolated from *X. mali*
after feeding perdeuterated octanoic acid

Fig. 182

To investigate this possibility [$^2H_{15}$]-octanoic acid was administered to *Xylaria mali* and the racemic piliformic acid isolated for ^2H NMR analysis (fig. 182). Once again the intact incorporation of octanoate into the C_8 fragment was apparent and significantly the olefinic and methine sites were enhanced at an equal level (1:1) indicating that complete transfer had again occurred. At present this interesting observation is not satisfactorily explained.

6.20 Conclusions

Piliformic acid is demonstrated to be of mixed biosynthetic origin, comprising a C_8 polyacetate and a C_3 fragment. It has further been shown that the octanoate chain is the product of a fatty acid synthase and that the C_3 fragment is almost certainly derived from oxaloacetate. Experiments with perdeuterated octanoic acid have revealed an intriguing 1,3-hydrogen shift in two producing organisms.

Another interesting aspect of these findings is apparent when piliformic acid is compared to some structurally related fungal and lichen metabolites. The lichen metabolites with very long chains: protolichesterinic acid (**83**), lichesterinic acid (**84**), nephromopsic acid (**85**), caperatic acid (**88**), rangiformic acid (**89**), nephrosterinic acid (**86**) and nephrosteranic acid (**87**) are all oxygenated at the β-position of their polyacetate chains. The presence of oxygen at this particular site strongly suggests that the activated form of these acids, which condenses with the C_3/C_4 unit, is indeed the β-ketoacid, the ketone then being reduced to an alcohol and forming a lactone with one of the carboxyl groups as proposed by Bloomer *et al*[2] (fig. 183).

Inspection of the related fungal metabolites 2-methyl-3-carboxyhexanoic (**92**) acid, 3-butyl-4-methylfuran-2(5H)-one (**93**) and 3-butyl-4-methylenefuran-2(5H)-one (**94**) and also glauconic acid (**95**) reveals that they, and piliformic acid, are not oxygenated at the β- site. This difference, and the shorter length of the chains, highlights the possibility that the mechanisms of assembly of the lichen metabolites may differ from the shorter chain fungal metabolites.

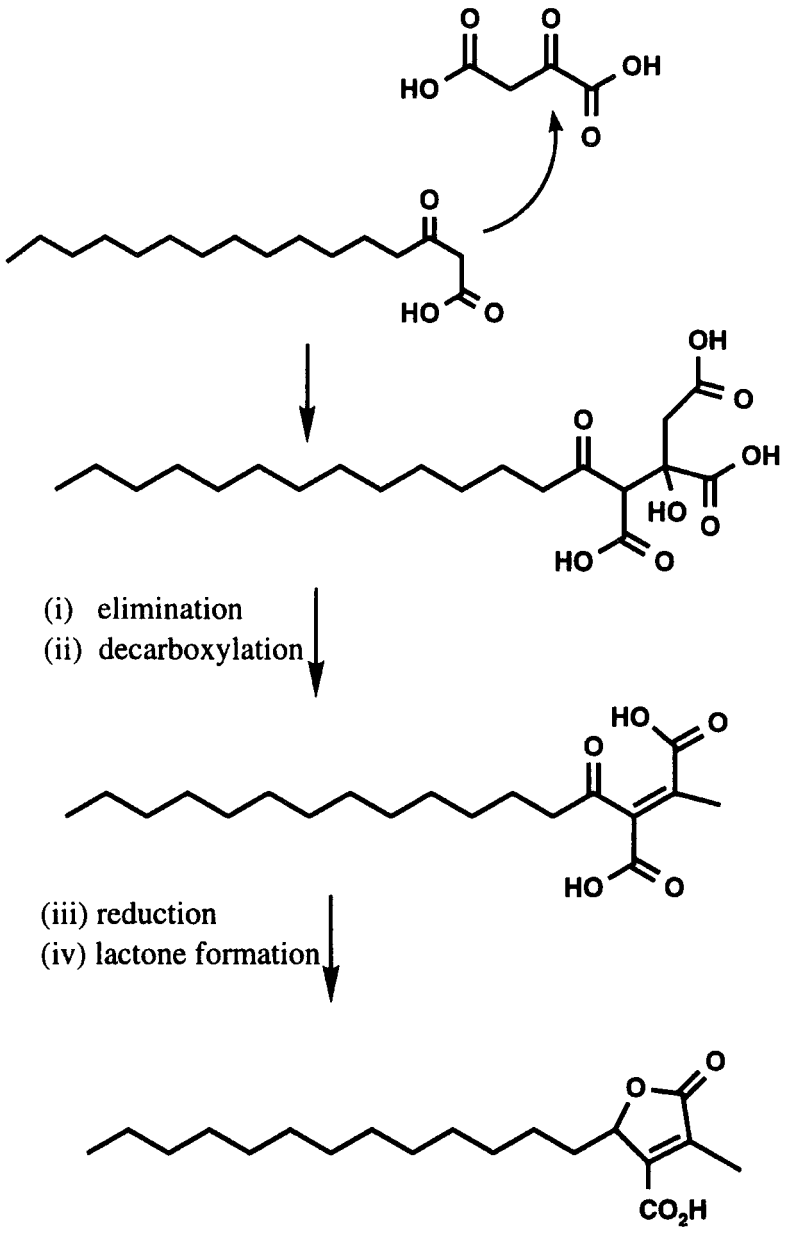

(i) elimination
(ii) decarboxylation
(iii) reduction
(iv) lactone formation

Fig. 183

6.21 References

1. D. O'Hagan *"The Polyketide Metabolites"* Ellis Horwood Ltd, Chichester, 1991
2. J. L. Bloomer, W. R. Eder and W. F. Hoffman, J. *Chem. Soc., Chem. Commun.*, 1968, 354
3. J. L. Bloomer, C. E. Moppet and J. K. Sutherland, *J. Chem. Soc., Chem. Commun.*, 1965, 618
4. M. Tanabe, T. Hamasaki and Y. Suzuki, *J. Chem. Soc., Chem. Commun.*, 1973, 212
5. Y. Ashahina, and S. Sibata, *"Chemistry of the Lichen Substances"* Japanese Society for the Promotion of Science, Ueno, Tokyo, 1954
6. J. R. Anderson, R. L. Edwards, A. J. S. Whalley *J. Chem. Soc., Perkin Trans. I,* 1985, 1481
7. R. L. Edwards and A. J. S. Whalley, J. *Chem. Soc., Perkin Trans. I,* 1979, 803
8. H. O. Kalinowski, S. Berger ans S. Braun, *Carbon-13 NMR Spectroscopy*, John Wiley and Sons, Chichester, New York, Brisbane, Toronto, Singapore, 1988, p. 577
9. J. C. Vederas, W. C. Graf, L. David and C. Tamm, *Helv. Chim. Acta.*, 1975, **58**, 1866
10. D. O'Hagan, *Nat. Prod. Rep.*, 1992, **8**, 447
11. C. A. Townsend S. B. Christensen and J. K. Trautwein, *J. Am. Chem. Soc.*, 1984, **106**, 3838
12. C. A. Townsend, S. W. Brobst, S. E. Ramer and J. C. Vederas, *J. Am. Chem. Soc.*, 1988, **110**, 318
13. P. H. Harrison, H. Noguari and J. C. Vederas J. *Am. Chem. Soc.*, 1986 **108**, 3833
14. B. J. Rawlings, P. H. Harrison and J. C. Vederas, *J. Antibiot.*, 1989, **42**, 577
15. B. Sedgewick and J. W. Cornforth, *Eur J. Biochem.*, 1977, **75**, 465
16. B. Sedgewick and J. W. Cornforth, *Eur J. Biochem.*, 1977, **75**, 481
17. C. R. Hutchinson, L. Shu-Wen, A. G. McInnes and J. A. Walter, *Tetrahedron*, 1983, **39**, 3507

18. M. Gonzalez-de-la Parra and C. R. Hutchinson, *J. Am. Chem. Soc.,* 1986, **108**, 2448

19. K. Arai, B. J. Rawlings, Y. Yoshizawa and J. C. Vederas, *J. Am. Chem. Soc.,* 1989, **111**, 3391

20. P. B. Rheese, B. J. Rawlings, S. E. Ramer and J. C. Vederas, *J. Am. Chem. Soc.,* 1988, **110**, 316

21. B. J. Rawlings, P. B. Rheese, S. E. Ramer, and J. C. Vederas, *J. Am. Chem. Soc.,* 1989, **111**, 3382

22. P. H. J. Carlsen, T. Kasuki and K. B. Sharpless, *J. Org. Chem*, 1981, **46**, 3936

23. R. J. Cox, *PhD Thesis*, Durham University, 1992

24. N. C. J. E. Chesters, *3rd Year Project*, Durham University, 1992

25. R. Fullwood and D. Parker, *Tetrahedron Asymmetry*, 1992, **3**, 25
The stereochemistry of 2-methylbutyrates determined using the chiral amine has subsequently been reassigned, the correct stereochemistry is shown.

26. D. Parker, *J. Chem. Soc., Perkin Trans. I*, 1983, 83

27. D. Parker, *Chem. Rev.,* 1991, 1441

28. H. L. Kornberg, *Angew. Chem., Int. Ed. Engl.*,1965, **4**, 558

29. D. E. Metzler, *Biochemistry*, Academic Press, New York, San Francisco, London, 1977 a: p. 539. b: p. 517

30. J. L. Bloomer and W. F. Hoffman, *Tetrahedron Lett.*, 1969, **50**, 4339

31. J. l. Bloomer, W. R. Eder and W. F. Hoffman, *J. Chem. Soc., C.*, 1970, 1848

32. R. Bentley, D. S. Bhate and J. G. Keil, *J. Biol. Chem.*, 1962, **3**, 237

33, S. Leibing and L. Reio, *Acta. Chem. Scand.*, 1958, **8**, 1575

34. J. E. Holenstein, A. Stoessel, H. Kern and J. B. Stothers, *Can. J. Chem.*, 1984, **62**, 1971

35. P. Roth, A. Hädner and C. Tamm, *Helv. Chim. Acta.*, 1990, **73**, 476

36. B. T. Gröbel and D. Seebach, *Synthesis*, 1977, 357

37. E. Vedejs and P. L. Fuchs, *J. Org. Chem.*, 1971, **36**, 366

38. D. Gravel, C. Vaziri and S. Rahal, *J. Chem. Soc., Chem. Commun.* 1972, 1323

39. C. A. Rheese, J. O. Rodin, R. G. Brownlee, W. G, Duncan and R. M. Silverstein, *Tetrahedron*, 1968, **24**, 4249

40. G. A. Olah, S. C. Narang and G. F. Salem, *Synthesis*, 1970, 659

41. D. Seebach, J. D. Aebi, R. Naef and T. Weber, *Helv. Chim. Acta.*, 1987, **70**, 237

42. R. Naef and D. Seebach, *Helv. Chim. Acta.*, 1985, **68**, 135

43. R. Fitzi and D. Seebach, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 345

44. D. Seebach, J. D. Aebi, R. Naef and T. Weber, *Helv. Chim. Acta.*, 1985, **68**, 144

CHAPTER 7

Experimental

7.1 General

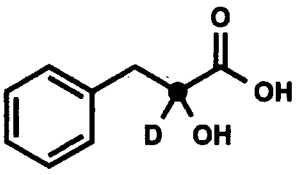
IR spectra were recorded on a Perkin-Elmer F.T. 1720X or 1600 spectrometer. Low resolution mass spectra were recorded on a VG Analytical 7070E Organic mass spectrometer. NMR spectra were recorded on Varian Gemini 200MHz (^{1}H at 199.977MHz, ^{13}C at 50.30MHz), Varian XL-200 (^{1}H at 200.057MHz) Varian VXR-400(S) (1H at 399.952 ^{13}C at 100.577MHz), Bruker AMX-500MHz (^{1}H at 500.137MHz, ^{13}C at 125.759MHz, ^{2}H at 76.775MHz), Bruker AC-250 (^{1}H at 250.133MHz, ^{13}C at 62.257MHz) and Varian VXR-600, (^{13}C at 150.869MHz, University of Edinburgh) spectrometers. Chemical shifts are quoted relative to TMS (δ=0). Radioactive analysis was carried out using a Packard 1600TR scintillation analyser in Ecoscint A. GC-MS was recorded on a VG TRIO-1S mass spectrometer (VG Masslab Ltd, Manchester) fitted with a Hewlett Packard 5890 series II gas chromatograph (Hewlett Packard Inc. Fort Collins USA) and a DB17 column (J&W Scientific, Folsom, USA) was used for separation. Flash chromatography was carried out using Fluka silica gel-60 (35-70mm) or Sorbsil -C60-H (40-60mm). Melting points were determined using a digital Gallenkamp melting point apparatus and are uncorrected. The solvents used in reactions were dried and distilled prior to use: tetrahydrofuran and diethyl ether (sodium benzophenone, under nitrogen), dichloromethane (calcium hydride, stored over molecular sieves), triethylamine (calcium hydride), diisopropylamine (calcium hydride) and pyridine (calcium hydride). Petrol refers to petroleum ether (30-60°C) and ether refers to diethyl ether. Non-aqueous reactions were carried out under an atmosphere of dry nitrogen or dry argon.

PART 1

7.2 Growth of *D. stramonium* and Isolation of Hyoscyamine

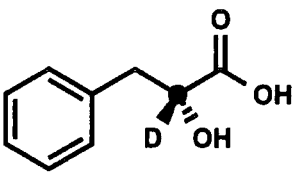
Transformed root culture of *D. stramonium* were subcultured at ≈ 0.5g per flask (50ml B50 medium) after 10-14 days growth.[1] The phenyllactates were pulse fed in sterile, neutral solution on days 5, 7 and 9 to a final concentration of 0.42 - 0.60mmoldm^{-3} in the medium. The roots were harvested after 17 days and freeze dried. The freeze dried roots were then ground with acid washed sand and extracted into 5% H_2SO_4 (10ml per 0.5g dry weight) by stirring for 15min. The aqueous extract was then made basic with 35% ammonia solution, filtered through Kieselguhr (Varian Bondelut) and eluted with chloroform : methanol (20 : 1). The eluant was evaporated under reduced pressure to give a brown oil. Purification by preparative TLC (chloroform : diethylamine (9: 1)) afforded hyoscyamine, usually contaminated with trace amounts of littorine and lipids. $\delta_C(CDCl_3)$[2,3] 24.20 (C-6 or C-7), 24.76 (C-6 or C-7), 34.85 (C-2 or C-4), 35.06 (C-2 or C-4), 42.22 (-Me), 54.24 (C-2'), 60.50 (C-1 or C-5), 60.61 (C-1 or C-5), 64.05 (C-3'), 66.57 (C-3), 127.94 (C-7'), 128.09 (C-5'), 129.03 (C-6'), 135.38 (C-4'), 171.87 (C-1')

7.2.1 Sodium (RS)-[2-^{13}C, ^{2}H]-Phenyllactate



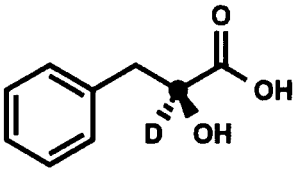
Sodium (RS)-[2-^{13}C, ^{2}H]-phenyllactate was pulse fed to transformed root cultures of *D. stramonium* (9x50ml) to a final concentration of 0.47mmoldm^{-3}. Hyoscyamine (18mg), contaminated with trace amounts of littorine, was isolated with incorporations of 16.95% (^{13}C and ^{2}H) and 5.69% (^{13}C only) as analysed by CG-MS. The littorine in the hyoscyamine sample had incorporations of 18.03% (^{13}C and ^{2}H) and 5.05% (^{13}C only). The values for incorporations into hyoscyamine determined by NMR analysis were 13% (^{13}C and ^{2}H) and 9% (^{13}C only) (fig. 38 and fig. 39).

7.2.2 Sodium (R)-D-[2-^{13}C, ^{2}H]-Phenyllactate



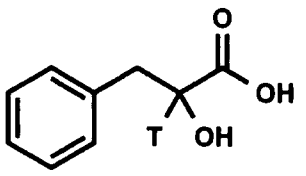
Sodium (R)-D-[2-^{13}C, ^{2}H]-phenyllactate was pulse fed to transformed root cultures of *D. stramonium* (5x50ml) to a final concentration of 0.42mmoldm^{-3}. On day six baterial contamination was apparent and was controlled by the addition of the antibiotic ampicillin (250µl, 25mgml^{-1}). Hyoscyamine (20mg), contaminated with trace amounts of littorine, was isolated with incorporations of 21.7% (^{13}C and ^{2}H) and 0.8% (^{13}C only) as analysed by CG-MS. The littorine in the hyoscyamine sample had incorporations of 29.8% (^{13}C and ^{2}H) and 1.5% (^{13}C only). The values for incorporations into hyoscyamine, determined by NMR analysis, were 25% (^{13}C and ^{2}H) and 4% (^{13}C only) (fig. 53).

7.2.3 Sodium (S)-L-[2-^{13}C, ^{2}H]-Phenyllactate



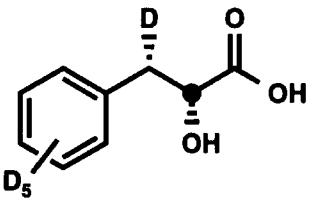
Sodium (S)-L-[2-^{13}C, ^{2}H]-phenyllactate was pulse fed to transformed root cultures of *D. stramonium* (10x50ml) to a final concentration of 0.47mmoldm^{-3}. Hyoscyamine (30mg), contaminated with trace amounts of littorine, was isolated with an incorporation of 5.1% (^{13}C only) as analysed by CG-MS. The littorine in the hyoscyamine sample had an incorporation of 10.3% (^{13}C only). The values for incorporation into hyoscyamine determined by NMR analysis were 16% (^{13}C only) (fig. 54).

7.2.4 Sodium (RS)-[2-^{3}H]-Phenyllactate



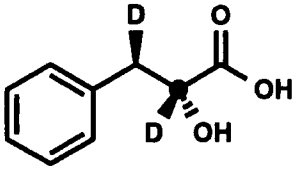
Sodium (RS)-[2-^{3}H]-phenyllactate (918μCimmol^{-1}) was pulse fed to transformed root cultures of *D. stramonium* (22x50ml) to a final concentration of 0.54mmoldm^{-3}. Hyoscyamine (50mg, 214mCiμmol^{-1}), was isolated with an incorporation of 22%, analysed scintillation counting.

7.2.5 Sodium (2R, 2S)-[2-^{13}C, 3-^{2}H, *ring*-^{2}H$_5$]-Phenyllactate



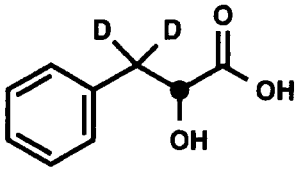
Sodium (2R, 2S)-[2-^{13}C, 3-^{2}H, *ring*-^{2}H$_5$]-phenyllactate was pulse fed to transformed root cultures of *D. stramonium* (9x50ml) to a final concentration of 0.58mmoldm^{-3}. Hyoscyamine (22mg), contaminated with trace amounts of littorine, was isolated with an incorporation of 42% (^{13}C only, β-shifted) as analysed by ^{13}C NMR (fig. 72).

7.2.6 Sodium (2R, 3R)-[2-^{13}C, ^{2}H, 3-^{2}H]-Phenyllactate



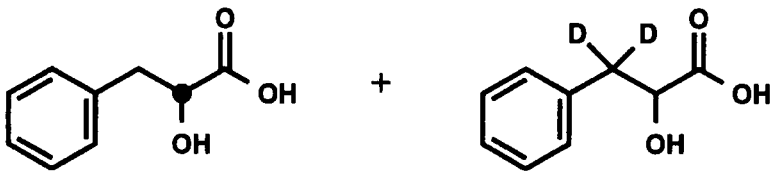
Sodium (2R, 3R)-[2-^{13}C, ^{2}H, 3-^{2}H]-phenyllactate was pulse fed to transformed root cultures of *D. stramonium* (5x50ml) to a final concentration of 0.54mmoldm^{-3}. Hyoscyamine (18mg) was isolated with incorporations of 14% (^{13}C and ^{2}H, not β-shifted) and 6% (^{13}C only, not β-shifted) as analysed by ^{13}C NMR (fig. 74).

7.2.7 Sodium (RS)-[2-^{13}C, 3-$^{2}H_2$]-Phenyllactate



Sodium (RS)-[2-^{13}C, 3-$^{2}H_2$]-phenyllactate was pulse fed to transformed root cultures of *D. stramonium* (5x50ml) to a final concentration of 0.60mmoldm^{-3}. Hyoscyamine (10mg) was isolated with incorporations of 28% (^{13}C only, β-shifted) and 17% (^{13}C only, not β-shifted) as analysed by ^{13}C NMR (fig. 76).

7.2.8 Sodium (RS)-[2-^{13}C]-Phenyllactate and Sodium (RS)-[3-$^{2}H_2$]-Phenyllactate



Sodium (RS)-[2-^{13}C]-phenyllactate and sodium (RS)-[3-$^{2}H_2$]-phenyllactate were pulse fed to transformed root cultures of *D. stramonium* (8x50ml) as an admixture, to a final concentration of 0.30mmoldm^{-3} of each phenyllactate. Hyoscyamine (14mg) was isolated with incorporations of 26% (^{13}C only) (fig. 78).

7.3 Methyl Phenylacetate (42)

A solution of phenylacetic acid (2.5g, 18.36mol) in ether (60ml) was quenched with an excess of diazomethane (25mmol) generated from Diazald (5.35g, 25mmol) and potassium hydroxide (2g, 35.71 mmol) in ether (100ml). The excess diazomethane was quenched by the addition of a few drops of glacial acetic acid such that the yellow colour disappeared. The solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was chromatographed over silica gel eluting with dichloromethane (100%) to give methyl phenylacetate as a clear colourless oil (2.70g, 98%). ν_{max}: 3031, 2952, 1738, 1257, 1160, 704. *m/z*(EI+) 150 (M^+, 21.41%), 91 (M^+-59, 100%). $\delta_H(CDCl_3)$: 3.63 (2H, s, -CH_2), 3.69 (3H, s, -OMe), 7.29 (5H, m, -Ar).

$\delta_C(CDCl_3)$: 41.68 (C-2), 52.56 (-OMe), 127.61 (C-6), 129.08 (C-4), 129.77 (C-5), 134.45 (C-3), 172.52 (C-1). Found 150.06823, $C_9H_{10}O_2$ required 150.06807.

7.4 Methyl [1-^{13}C]-Phenylacetate (42a)

[1-^{13}C]-Phenylacetic acid (2.0g, 14.58mmol) was methylated according to the procedure described in section 7.3 to give methyl [1-^{13}C]-phenylacetic acid (2.22g, 100%). ν_{max}: 3031, 2951, 1696, 1235, 1138, 704. *m/z*(EI+) 151 (M^+, 35.75%), 91 (M^+-60, 100%). $\delta_H(CDC_3)$: 3.64, (2H, d, $J1_H13_C$ 7.9Hz, -CH_2), 3.70 (3H, d, $J1_H13_C$ 3.6Hz -OMe), 7.29Hz (5H, m, -Ar). $\delta_C(CDCl_3)$: 41.19 (d $J13_C13_C$ 28.7Hz C-2), 52.06 (-OMe), 127.10 (C-6), 138.38 (C-4), 129.25 (C-5), 134.45 (C-3), 172.03 (C-1).

7.5 Methyl [2-$^2H_2$]-Phenylacetate (42c)

Methyl phenylacetate (1.0g 6.66mmol) was added dropwise over a period of 5min to a stirred solution of LDA (16.65mmol), generated from *n*-butyllithium (10.40ml, 1.6M in hexanes, 16.65mmol) and diisopropylamine (1.68g, 2.18ml, 16.65mmol), in THF (20ml) at -78°C. The temperature of the solution was kept below -60°C during the addition. When all the methyl phenylacetate was added the solution became a deep blue colour. The solution was left to stir at this temperature for 30min then MeOD (5ml, excess) was added dropwise and the blue colour then disappeared. The solution was left to warm to 18°C and was then stirred at this temperature for a further 1h. The reaction mixture was poured into a saturated solution of ammonium chloride (30ml) and then was extracted into ether (2x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure, and the residue was chromatographed over silica gel, eluting with dichloromethane (100%) to give methyl [2-$^2H_2$]-phenylacetate as a yellow oil (819mg, 81%). ν_{max}: 3028, 2952, 1740, 1253, 1214, 699. *m/z*(EI+) 152 (M^+ CD_2, 50.68%), 151 (M^+ -CHD, 10.30%), 93 (M^+ -59 -CD_2, 100%), 92 (M^+ -59 CHD, 23.65%). $\delta_H(CDCl_3)$: 3.62 (0.27H, t, -CHD), 3.70 (3H, s, -OMe), 7.30 (5H, m, -Ar). $\delta_C(CDCl_3)$: 40.82 (m, C-2), 51.92 (-OMe) 127.03 (C-6), 128.50 (C-4), 129.41 (C-5), 133.83, (C-3), 171.93 (C-1).

7.6 Methyl [1-^{13}C, 2-$^{2}H_2$]-Phenylacetate (42b)

Methyl [1-^{13}C]-phenylacetate (1.03g, 6.80mmol) was deprotonated using LDA (17mmol) and quenched with MeOD (5ml, excess) according to the procedure described in section 7.5 to give methyl [1-^{13}C, 2-$^{2}H_2$]-phenylacetate (811mg, 78%). ν_{max}: 3028, 2952, 1695, 1207, 1151, 701). *m/z*(EI+) 153 (M^+ -$^{13}CD_2$, 25.36%), 152 (M^+ -^{13}CHD, 20.0%), 93 (M^+ -59 -$^{13}CD_2$), 92 (M^+ -59, 84%). δ_H($CDCl_3$): 3.62, (0.2H, m, -CHD), 3.69 (3H, d, J_{1H13C} 3.9Hz -OMe), 7.29 (5H, m, -Ar). δ_C($CDCl_3$): 40.89 (m, C-2), 52.04 (-OMe), 127.09 (C-6), 128.56 (C-4), 129.19 (C-5), 133.99 (C-3), 172.03 (C-1).

7.7 2-Phenylethanol (43)

Methyl phenylactetate (1.85g, 12.32mmol) was added, dropwise to a stirred suspension of lithium aluminium hydride (1.87g, 49.21mmol) in ether (50ml) and the mixture was heated under reflux for 2h. After cooling to 18°C the reaction was quenched by the addition of wet ether (30ml) then the reaction mixture was poured into 5% sulphuric acid (50ml). The ether layer was retained and the aqueous layer extracted into ether (2x50ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue was chromatographed over silica gel, eluting with dichloromethane (100%) to give 2-phenylethanol (1.32g, 88%). ν_{max}: 3340br, 2941, 2375, 1496, 1453, 1045, 696. *m/z*(EI+) 122 (M^+ 18.53%), 91 (M^+ -31, 100%). δ_H ($CDCl_3$):2.78 (2H, t, J_{vic} 6.8Hz, -CH_2), 2.97 (1H, s, -OH), 3.74 (2H, t, J_{vic} 6.8Hz, -CH_2), 7.20 (5H, m, -Ar). δ_C ($CDCl_3$): 39.7 (C-2), 64.04 (C-1), 126.92 (C-6), 129.06 (C-4), 129.60 (C-5), 139.23 (C-3). Found: 122.06698, $C_8H_{10}O$ required: 122.07316.

7.8 [1-$^{2}H_2$]-2-Phenylethanol (43c)

Methyl phenylacetate(2.20g, 14.68mmol) was reduced using lithium aluminium deuteride (1.90g, 45.24 mmol) according to the procedure described in section 7.7 to give [1-$^{2}H_2$]-2-phenylethanol (1.56g, 85%). ν_{max}: 3338br, 3086, 2862, 2208, 1604, 1496, 1454. *m/z* (EI+) 124 (M^+ 39.05%), 91 (M^+ -32, 100%). δ_H ($CDCl_3$): 1.94 (1H, s,

-OH), 2.81 (2H, s, $-CH_2$), 2.71 (5H, m, -Ar). δ_C ($CDCl_3$): 38.97 (C-2), 62.85 (p, $J_{^{13}C-^2H}$ 21.7Hz C-1), 126.40 (C-6), 128.80 (C-4), 129.01 (C-5), 138.54 (C-3).

7.9 [1-^{13}C, $^2H_2$]-2-Phenylethanol (43a)

Methyl [1-^{13}C]-phenylacetate (2.22g, 14.58mmol) was reduced using lithium aluminium deuteride (2.45g, 58.32mmol) according to the procedure described in section 7.7 to give [1-^{13}C, $^2H_2$]-2-phenylethanol (1.56g, 86%). ν_{max} : 3346br, 3027, 2931, 2190, 2090, 1603, 1496, 1453. *m/z* (EI+) 125 (M^+ 38.60%), 91 (M^+ -35, 100%). δ_H ($CDCl_3$): 2.15 (1H, s, -OH), 2.81 (2H, d, $J_{^1H^{13}C}$ 5.3Hz $-CH_2$), 7.25 (5H, m, -Ar). δ_C ($CDCl_3$): 39.49 (d, $J_{^{13}C^{13}C}$ 35.8Hz C-2), 63.37 (p, $J_{^{13}C^2H}$ 21.9Hz C-1), 126.93 (C-6), 129.06 (C-4), 129.54 (C-5), 139.10 (C-3).

7.10 [1-^{13}C]-2-Phenylethanol (43d)

Methyl [1-^{13}C]-phenylacetate (998mg 6.60mmol) was reduced using lithium aluminium deuteride (1.00g, 26.4mmol) according to the procedure described in section 7.7 to give [1-^{13}C]-2-phenylethanol (832mg 99%). ν_{max}: 3346br, 2937, 2865, 1496, 1453, 1027, 698. *m/z*(EI+) 125 (M^+ 38.60%), 91 (M^+ -32, 100%). δ_H($CDCl_3$): 1.37 (1H, dt, $J_{^1H^1H}$ 6.2Hz $J_{^1H^{13}C}$ 2.9Hz, -OH), 2.88 (2H, td, J_{vic} 12.4Hz, $-J_{^1H^{13}C}$ 6.1Hz, $-CH_2$), 3.87 (2H, dtd $J_{^1H^{13}C}$ 143.3Hz, J_{vic} 12.4Hz, $J_{^1H^1H}$ 6.2Hz, $-CH_2$), 7.29 (5H, m, -Ar). δ_C($CDCl_3$): 39.19 (d, $J_{^{13}C^{13}C}$ 17.85Hz, C-2), 63.79 (C-1), 126.50 (C-6). 128.59 (C-4), 129.03 (C-5), 139.20 (C-3).

7.11 [2-$^2H_2$]-2-Phenylethanol (43e)

Methyl [2-$^2H_2$]-phenylacetate (819mg, 5.39mmol) was reduced using lithium aluminium hydride (818mg, 21.56mmol) according to the procedure described in section 7.7 to give [2-$^2H_2$]-2-phenylethanol (612mg 93%). ν_{max}: 3345br, 2925, 2877, 1496, 1448, 1059, 698. *m/z*(EI+) 124 (M^+ -CD_2, 27.94%), 123 (M^+ -CHD, 7.26%), 93 (M^+ -31 -CD_2, 100%) 92 (M^+ -31 -CHD 29.78%). δ_H($CDCl_3$): 1.17 (1H, s, -OH),

2.84 (0.2H, dt, -CHD), 3.83 (2H, s, $-CH_2$), 7.26, (5H,m,-Ar). $\delta_C(CDCl_3)$: 38.82 (m, C-2), 63.56 (C-1), 126.45 (C-6), 128.56 (C-4), 129.01 (C-5), 138.40 (C-3).

7.12 [1-^{13}C, 2-$^{2}H_2$]-Phenylethanol (43b)

Methyl [1-^{13}C, 2-$^{2}H_2$]-phenylacetate (745mg, 4.87mmol) was reduced using lithium aluminium hydride (740mg, 19.48mmol) according to the procedure described in section 7.7 to give [1-^{13}C, 2-$^{2}H_2$]-2-phenylethanol (459mg, 75%). ν_{max}: 3346br, 2917, 2860, 1495, 1448, 1028, 699. *m/z*(EI+) 125 (M^+ -$^{13}CD_2$, 13.61%), 124 (M^+ -^{13}CHD, 6.78%), 93 (M^+ -32 -$^{13}CD_2$, 100%), 92 (M+ -32 -^{13}CHD, 52.92%). $\delta_H(CDCl_3)$: 1.40 (1H, td J_{1H1H} 5.93Hz, J_{1H13C} 2.84Hz, -OH), 2.85 (0.3H, m, -CHD), 3.86 (2H, dd J_{1H13C} 143.7Hz, J_{1H1H} 5.93Hz, $-CH_2$) 7.29 (5H, m, -Ar). $\delta_C(CDCl_3)$: 38.79 (m, C-2), 63.63 (C-1), 126.46 (C-6), 128.57 (C-4), 129.00 (C-5), 138.34 (C-3).

7.13 Phenylacetaldehyde (44)[4]

2-Phenylethanol was added dropwise to a stirred suspension of pyridinium chlorochromate (PCC) (5.29g, 24.56mmol) and molecular sieves (150g, 3Å Sigma, 1/16" pellets, dried and ground) in dichloromethane (200ml) at 18°C and the reaction mixture was left to stir at this temperature for 2h. The molecular sieves were then removed by filtration and the filtrate was washed through a thick silica pad, washing liberally with dichloromethane (≈500ml). The solvent was evaporated under reduced pressure and the residue was purified by bulb to bulb distillation (0.2mmHg, furnace temperature 40°C) to give phenylacetaldehyde (859mg, 69%).ν_{max} : 3020, 1725, 1500, 1455, 750, 700. *m/z*(EI+) 120 (M^+ 20.78%), 19 (M^+-29 100%) δ_H ($CDCl_3$): 3.66 (2H, d, J_{vic} 2.3Hz $-CH_2$), 7.30 (5H, m, -Ar), 9.72 (1H, t, 2.3Hz -CHO). δ_C ($CDCl_3$): 50 54 (C-2), 127.38 (C-6), 129.98 (C-4), 129.62 (C-5), 131.88 (C-3), 199.38 (C-1).

As this product is susceptible to polymerisation to give polymeric material the products from labelled reactions was used directly after distillation without full characterisation.

7.14 Preparation of Phenylacetaldehyde using RuO_4 (44)[5]

2-Phenylethanol (2.05g, 16.38mmol) was added to a vigorously stirred biphasic solution of potassium periodate (14g, 61mmol) and ruthenium trichloride (34mg) in H_2O : CH_3CN : CCl_4 (3:1:1) and the reaction was left to stir for 24h. The reaction mixture was then transferred to a separatory funnel where ether (60ml) and water (60ml) were added. The organic layer was separated and the aqueous layer washed with two further portions of ether (2x60ml). The organic extracts were combined, dried ($MgSO_4$), filtered and evaporated under reduced pressure. The crude product was chromatographed over silica gel eluting with dichloromethane : hexane (2:1) to give phenylacetaldehyde as a clear oil, (590mg, 33%). This material had identical spectroscopic data to that described above.

7.15 [1-^{2}H]-Phenylacetaldehyde (44c)

[1-$^2H_2$]-2-Phenylethanol (763mg, 6.25mmol) was oxidised using PCC (2.69g 12.48mmol) and 3Å molecular sieves (35g) according to the procedure described in section 7.13 to give [1-^{2}H]-phenylacetaldehyde (578mg, 76%). $\delta_H(CDCl_3)$: 3.71 (2H, s, -CH_2), 7.30 (5H, m, -Ar).

7.16 [1-^{13}C, ^{2}H]-Phenylacetaldehyde (44a)

[1-^{13}C, $^2H_2$]-2-Phenylethanol (1.56g, 12.47mmol) was oxidised using PCC (5.38g 24.94mmol) and 3Å molecular sieves (150g) according to the procedure described in section 7.13 to give [1-^{2}H]-phenylacetaldehyde (578mg, 76%). $\delta_H(CDCl_3)$: 3.60 (2H, d, J_{1H13C} 7.2Hz, -CH2), 7.30 (5H, m -Ar).

7.17 [1-^{13}C]-Phenylacetaldehyde (44d)

[1-^{13}C]-2-Phenylethanol (812mg, 6.60mmol) was oxidised using PCC (2.85g, 2.85mmol) and 3Å molecular sieves (80g) according to the procedure described in section 7.13 to give [1-^{13}C]-phenylacetaldehyde (720mg, 90%). $\delta_H(CDCl_3)$: 3.69 (2H,

dd, J_{1H13C} 7.16Hz, J_{vic} 2.38Hz, -CH_2), 7.30 (5H, m, -Ar), 9.75 (1H, dt, J_{1H13C} 175.5Hz, J_{vic} 2.38Hz, -CHO).

7.18 [2-$^2H_2$]-Phenylacetaldehyde (44e)

[2-$^2H_2$]-2-Phenylethanol (545mg, 4.39mmol) was oxidised using PCC (1.89g, 8.78mmol) and 3Å molecular sieves (50g) according to the procedure described in section 7.13 to give [2-$^2H_2$]-phenylacetaldehyde (499mg, 91%). $\delta_H(CDCl_3)$: 3.67 (0.3H, m, -CHD), 7.30 (5H, m, -Ar), 9.80 (1H, s, -CHO).

7.19 [1-^{13}C, 2-$^2H_2$]-Phenylacetaldehyde (44b)

[1-^{13}C, 2-$^2H_2$]-2-Phenylethanol (447mg, 3.57mmol) was oxidised using PCC (1.54g, 7.14mmol) and 3Å molecular sieves (43g) according to the procedure described in section 7.13 to give [1-^{13}C, 2-$^2H_2$]-phenylacetaldehyde (385mg, 88%). $\delta_H(CDCl_3)$: 3.67 (0.35H, m, CHD), 7.30 (5H, m, -Ar), 9.75 (1H, d, J_{1H13C} 175.3Hz, -CHO).

7.20 Phenyllactic Acid (32)

A solution of sodium metabisulfite (3.26g, 17.16mmol) in water (10ml) was added to phenylacetaldehyde (2.05g, 2.0ml, 17.16mmol) and the mixture shaken vigorously for 10min at 18°C, after which time the bisulfite adduct formed as a white precipitate. Sodium cyanide (2.10g, 42.9mmol) was added to the mixture in several portions while swirling the flask gently. The white precipitate dissolved and the cyanohydrin was seen forming as clear, oily droplets.[6] The solution was extracted into benzene (3x30ml) and the combined organic extracts were dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give the cyanohydrin as a clear oil, which was immediately covered with 50% HCl (30ml) and the stirred solution heated under reflux for 2.5h. After cooling, the solution was extracted into ether (3x30ml) and the combined organic extracts were dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The residue was recrystallised form chloroform to give phenyllactic acid as a white solid (1.14g, 43%). m.p. 96-98°C lit. 97-98°C,[7] ν_{max}: 3348, 2956, 1732, 1240, 1191, 1091,

1062. *m/z*(CI+) 184 (M^++18, 8.92%). $\delta_H(CDCl_3)$: 2.98 (1H, dd, J_{gem} 14.0Hz, J_{vic}, 6.9Hz, -CH_2), 3.19 (1H, dd, J_{gem} 14.0Hz, J_{vic} 4.2Hz, -CH_2), 4.50, (1H, dd, J_{vic} 6.9Hz, J_{vic} 4.2Hz, -CH), 7.30 (5H, m, -Ar). $\delta_C(CDCl_3)$: 40.1 (C-3), 71.0 (C-2), 127.1 (C-7), 129.6 (C-5), 129.5 (C-6), 135.8 (C-4), 178.6 (C-1). Found: C 64.7%, H 5.9% $C_9H_{10}O_3$ requires: C 65.0%, H 6.02%.

7.21 [2-^{2}H]-Phenyllactic Acid (32l)

[1-^{2}H]-Phenylacetaldehyde (578mg, 4.77mmol) was reacted with sodium metabisulfite (906mg, 4.77mmol) and sodium cyanide (584mg, 11.93mmol) according to the procedure described in section 7.20 to give [2-^{2}H]-phenyllactic acid (198mg, 25%). m.p. 97.5-98.5°C,ν_{max}: 3350, 2966, 1731, 1287, 1190, 1109, 1059. *m/z*(CI+) 185 (M^++NH3, 1.15%), 167 (M^+, 0.9%), $\delta_H(CDCl_3)$: 2.99 (1H, d, J_{gem} 14.0Hz, -CH_2), 3.21 (1H, d, J_{gem} 14.0Hz, -CH_2), 7.30 (5H, M, -Ar). $\delta_C(CDCl_3)$: 40.07 (C-3), 70.61 (t, $J_{^{13}C^2H}$ 22.6Hz, C-2), 127.14 (C-7), 128.58 (C-5), 129 50 (C-6), 135.77 (C-4), 178.17 (C-1).

7.22 [2-^{13}C, 2-^{2}H]-Phenyllactic Acid (32a)

[1-^{13}C, ^{2}H]-Phenylacetaldehyde (1.29g, 10.40mmol) was reacted with sodium metabisulfite (1.98g, 10.40mmol) and sodium cyanide (1.27g, 26.0mmol) according to the procedure described in section 7.20 to give [2-^{13}C, ^{2}H]-phenyllactic acid (319mg, 18%). m.p. 97-99°C, ν_{max}: 3346, 2962, 1740, 1262, 1190, 1098, 1024. m/z(CI+) 186 (M^++NH_3, 2.68%), 168 (M^+ 0.31%), $\delta_H(CDCl_3)$: 2.99 (1H, dd J_{gem} 14.0Hz, $J_{^1H^{13}C}$ 4.8Hz, -CH_2), 3.20 (1H, d, J_{gem}, 14.0Hz, $J_{^1H^{13}C}$ 4.4Hz, -CH_2), 7.30 (5H, m, -Ar). $\delta_C(CDCl_3)$: 40.03 (d, $J_{^{13}C^{13}C}$ 34.6Hz, C-3), 70.62 (t, $J_{^{13}C^2H}$ 22.7Hz, C-2), 127.11 (C-7), 128.56 (C-5), 129.47 (C-6), 135.47 (d $J_{^{13}C^{13}C}$ 2.6Hz, C-4), 178.34 (d, $J_{^{13}C^{13}C}$ 57.5Hz, C-1).

7.23 [2-^{13}C]-Phenyllactic Acid (32j)

[1-^{13}C]-Phenylacetaldehyde (720mg, 5.95mmol) was reacted with sodium metabisulfite (1.13mg, 5.95mmol) and sodium cyanide (729mg, 14.88mmol) according to the procedure described in section 7.20 to give [2-^{13}C]-phenyllactic acid (512mg, 3.06mmol) 51%. m.p. 95-97°C, ν_{max}; 3349, 2956, 1728, 1240, 1188, 1084, 1057. *m/z*(CI+) 185 (M^++18, 10.37%). $\delta_H(CDCl_3)$: 2.99 (1H, ddd, J_{gem} 14.0Hz, J_{vic} 7.0Hz, J_{1H13C} 4.8Hz, -CH_2), 3.20 (1H, ddd J_{gem} 14.0Hz, J_{vic} 4.4Hz, J_{1H13C} 4.4Hz, -CH_2), 4.51 (1H ddd, J_{1H13C} 148.6Hz, J_{vic} 7.0Hz, J_{vic} 4.4Hz, -CH), 7.30 (5H, m -Ar). $\delta_C(D_2O)$: 40.04 (d J_{13C13C} 35.5Hz, C-3), 71.16 (C-2), 126.36 (C-7), 128 28 (C-5), 129.21 (C-6), 137.91 (C-7), 180.44 (d, J_{13C13C} 54.5Hz, C-1).

7.24 [3-$^2H_2$]-Phenyllactic Acid (32k) (preparation 1)

[2-$^2H_2$]-Phenylacetaldehyde (446mg, 3.65mmol) was reacted with sodium metabisulfite (694mg, 3.65mmol) and sodium cyanide (447mg, 9.13mmol) according to the procedure described in section 7.20 to give [2-$^2H_2$]-phenyllactic acid (323mg, 53%). m.p. 95-97°C, ν_{max}: 3447, 2962, 1733, 1262, 1231, 1054, 1028. *m/z*(CI+) 186 (M^++18 -CD2, 100%), 185 (M^++18 -CHD, 33.2%). $\delta_H(CDCl_3)$ 2.99 (0.2H, m, -CHD), 3.20 (0.2H, m, -CHD), 4.50 (1H, s, -CH), 7.30 (5H, m, -Ar). $\delta_C(D_2O)$: 39.38 (m, C-3), 73.07 (C-2), 126.38 (C-7), 128.29 (C-5), 129.21 (C-6), 137.85 (C-3), 180 47 (C-7).

7.25 [2-^{13}C, 3-$^2H_2$]-Phenyllactic Acid (32k)

[1-^{13}C, 2-$^2H_2$]-Phenylacetaldehyde (385mg, 3.13mmol) was reacted with sodium metabisulfite (595mg, 3.13mmol) and sodium cyanide (383mg, 7.83mmol) according to the procedure described in section 7.20 to give [2-^{13}C, 3-$^2H_2$]-phenyllactic acid (298mg, 1.76 mmol) 36%. m.p. 95-96°C, ν_{max}: 3347, 2950, 1733, 1776, 1231, 1091, 1058. m/z(CI+) 187 (M^++NH_3, 3.13%) 169 (M^+ 0.76%), $\delta_H(CDCl_3)$: 2.99 (0.2H, m, -CHD), 3.20 (0.2H, m, -CHD), 4.41 (1H, d, J_{1H13C} 148.6Hz, -CH), 7.30 (5H, m, -Ar). $\delta_C(CDCl_3)$: 40.25 (m, C-3), 71.43 (C-2). 127.63 (C-7), 129.07 (C-5), 130.02 (C-6), 136.29 (C-4), 179.07 (d, J_{13C13C} 57.7Hz, C-1).

7.26 Methyl [3-$^2H_2$]-Phenyllactate (52a)

A solution of phenylpyruvic acid (300mg, 1.83mml) in D_2O (30ml) was adjusted to pH11 with K_2CO_3 and left at 4°C for 48h. The solution was then acidified by the careful addition of D_2SO_4 (conc) and extracted into ether (3x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was dissolved in MeOD (30ml) at 0°C and a solution of sodium borohydride (600mg 12.12mmol) in D_2O (12ml) and 1M KOD (3ml) was added dropwise, maintaining the temperature below 5°C during the addition. The reaction mixture was allowed to warm to 18°C and was then left to stir for 12h. The methanol was evaporated, the residue dissolved in 10% hydrochloric acid (30ml) and the solution extracted into ether (3x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue was re-dissolved in ether (20ml) and quenched with an excess of an ethereal solution of diazomethane (10mmol). The excess diazomethane was removed by the addition of a few drops of glacial acetic acid, so that the yellow colour disappeared and the solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was chromatographed over silica gel, eluting with dichloromethane (100%) to give methyl [3-$^2H_2$] phenyllactate (104mg, 31%). ν_{max}: 3455br, 3038, 2954, 2630, 1740, 1236, 1112. *m/z*(EI+) 182 (M^+ 1.85%), 93 (M^+-89, 100%). $\delta_H(CDCl_3)$: 2.70 (1H, s, -OH), 3.69 (1H, s, -OMe), 4.37 (1H, s, -CH), 7.19 (5H, m, -Ar). $\delta_C(CDCl_3)$: 40.38 (p $J_{^{13}C^2H}$ 19.1Hz, C-3), 52.96 (-OMe), 71.69 (C-2), 127.41 (C-7), 128.92 (C-5), 129.95 (C-6), 136.76 (C-4), 175.08 (C-1).

7.27 [3-$^2H_2$]-Phenyllactic Acid (32k) (preparation 2)

Methyl [3-$^2H_2$]-phenyllactate (84.7mg, 0.465mmol) was covered with 1M KOH (6ml) and heated under reflux for 12h. The solution was then acidified with 10% HCl and extracted into ether (3x15ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give [3-$^2H_2$]-phenyllactic acid (77.3mg, 0.465mmol) 100%. m.p. 96-98°C. ν_{max}: 3343, 2348, 1729, 1261, 1232, 1108.

m/z(CI+) 186 (M^++18, 100%). $\delta_H(D_2O)$: 4.19 (1H, s, -CH), 7.30 (5H, m, -Ar). $\delta_C(D_2O)$: 39.38 (m, C-3), 73.07 (C-2), 126.38 (C-7), 128.29 (C-5), 129.21 (C-6), 137.85 (C-3), 180 47 (C-7).

7.28 Resolution of (RS)-Phenyllactic Acid to Obtain (R)-Phenyllactic Acid (32b)[8]

(RS)-Phenyllactic acid (200mg, 1.2mmol) and (S)-2-phenylglycinol (165mg 1.2mmol) (99%ee, Aldrich Chemical Co.) were dissolved in refluxing ethyl acetate : isopropanol (7:2) (10.8ml) and the solution cooled to 0°C for 4h. The white precipitate was collected and recrystallised to constant optical rotation from ethyl acetate : isopropanol (2:1). The salt was then decomposed by stirring with 10% HCl (5ml) for 5min at 18°C and then the aqueous solution was extracted into ether (3 x 10ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to generate (R)-phenylactic acid as a white solid. This material was recrystallised from chloroform (22.2mg, 22%). m.p. 121-122°C lit. 124-125°C,[9] $\nu_{max.}$ 3348, 2956, 1734, 1243, 1192, 1092, 795, 701, *m/z*(CI+): 184 (M^++NH_3, 100%), $\delta_H(CDCl_3)$: 2.92 (1H, dd, J_{gem}, 14.0Hz, J_{vic} 7.0Hz, -CH_2), 3.13 (1H, dd, J_{gem} 14.0Hz, J_{vic}, 4.2Hz, -CH_2), 4.44 (1H, dd, J_{vic} 7.0Hz, J_{vic} 4.2Hz, -CH), 7.22 (5H, m, -Ar), $\delta_C(CDCl_3)$: 40.65 (C-3), 71.49 (C-2), 127.66 (C-7), 129.09 (C-5), 130.03 (C-6), 136.32 (C-4), 178.69 (C-1), $[\alpha]_D^{21°C}$ +21.81° (c=0.055, EtOH), lit. $[\alpha]_D^{25°C}$ +19° (c = 3.1 EtOH)[9] Found C 65.12%, H 6.42%, $C_9H_{10}O_3$ requires C 65.05%, H 6.07%.

7.29 Resolution of (RS)-Phenyllactic Acid to Obtain (S)-Phenyllactic Acid (32c)

(RS)-Phenyllactic acid (150mg, 0.90mmol), was resolved using (R)-2-phenylglycinol (125mg, 0.90mmol) (Aldrich Chemical Co. 99%ee) according to the procedure described in section 7.28 to generate (S)-phenyllactic acid as a white crystalline solid (14.8mg, 20%). m.p. 122-124°C lit 123-124°C,[8] $\delta_H(CDCl_3)$:2.93 (1H, dd, J_{gem} 13.8hz, J_{vic} 6.9Hz, -CH_2,), 3.15 (1H, dd, J_{gem} 13.8Hz, J_{vic}, 4.2Hz, -CH_2), 4.45 (1H, dd, J_{vic} 6.9Hz, J_{vic}, 4.2Hz, -CH), 7.19 (5H, m-Ar), $\delta_C(CDCl_3)$: 40.65 (C-3), 71.49 (C-2),

127.66 (C-7), 129.09 (C-5), 130.03 (C-6), 136.32 (C-4), 178.69 (C-1), $[\alpha]_D^{21°C}$ -21.28 (c=0.024, EtOH) lit. $[\alpha]_D^{12°C}$ -18.7° (c = 2.9, EtOH).[7]

7.30 Resolution of (RS)-[2-^{13}C,^{2}H]-Phenyllactate to Obtain (R)-[2-^{13}C,^{2}H]-Phenyllactic Acid (32d)

(RS)-[2-^{13}C,^{2}H]-Phenyllactic acid (200mg, 1.19mmol) was resolved, using (S)-2-phenylglycinol (163mg, 1.19mmol) according to the procedure described in section 7.28, to generate (R)-[2-^{13}C,^{2}H]-phenyllactic acid (16.2mg, 16%). This material was converted to its sodium salt with 0.1M sodium hydroxide, prior to feeding. m.p. 119-121°C, $\delta_H(D_2O)$ 2.74 (1H, dd, J_{gem} 14.1Hz $J_{^{13}C^{1}H}$ 5.0Hz, $-CH_2$), 2.97 (1H, dd, J_{gem} 14.1Hz, $J_{^{13}C^{1}H}$ 3.5Hz, $-CH_2$), 7.20 (5H, m, -Ar), $\delta_C(D_2O)$: 42.55 (d, $J_{^{13}C^{13}C}$ 35Hz C-3), 75.88 (t, $J_{^{13}C^{2}H}$ 22,45Hz, C-2), 129.46 (C-7), 131.38 (C-5), 132.29 (C-6), 141.00 (D, $J_{^{13}C^{13}C}$ 2.5Hz, C-4), 183.56 (d $J_{^{13}C^{13}C}$ 55Hz, C-1) $[\alpha]_D^{21°C}$ +19.78° (c=0.015, EtOH)

7.31 Resolution of (RS)-[2-^{13}C,^{2}H]-Phenyllactic Acid to Obtain (S)-[2-^{13}C,^{2}H]-Phenyllactic Acid (32e)

(RS)-[2-^{13}C,^{2}H]-Phenyllactic acid (199mg, 1.185mmol) was resolved using (R)-2-phenylglycinol (163mg 1.185mmol), according to the procedure described in section 7.28, to generate (S)-[2-^{13}C,^{2}H]-phenyllactic acid (50mg, 50%). This material was converted to its sodium salt with 0.1M sodium hydroxide prior to feeding. m.p. 120-121°C, $\delta_H(D_2O)$: 2.73 (1H, dd, J_{gem} 14.0Hz, $J_{^{13}C^{1}H}$ 5.07 $-CH_2$), 2.97 (1H, dd, Jgem 14.0Hz $J_{^{13}C^{1}H}$ 3.7Hz -CH2), 7.19 (5H, m, -Ar), $\delta_C(D_2O)$ 43.06 (d, $J_{^{13}C^{13}C}$ 35Hz, C-3), 75.89 (t, $J_{^{13}C^{2}H}$, 22.7Hz, C-2), 129.47 (C-7), 131.39(C-5), 132.30 (C-6), 140.99 (d, $J_{^{13}C^{13}C}$ 2.5Hz, C-4), 183 57 (d, $J_{^{13}C^{13}C}$ 54.4Hz), $[\alpha]_D^{21°C}$ -16.00° (c=0.10 EtOH).

7.32 (RS)-Methyl O-Acetylphenyllactate (50)

A stirred solution of phenyllactic acid (185mg, 1.11mmol) in ether was quenched with an ethereal solution of diazomethane (excess) at 18°C, so that the bright yellow colour

remained. The excess diazomethane was removed by the addition of a few drops of glacial acetic acid and then the solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was chromatographed over silica gel eluting with dichloromethane (100%) to give (RS)-methyl phenyllactate as a white solid. (RS)-Methyl phenyllactate (200mg, 1.11mmol) and dicyclohexylcarbodiimide (229mg, 1.11mmol) were added to a stirred solution of acetic acid (67mg, 1.11mmol) and 4-dimethylaminopyridine (4mg, 0.033mmol) in dichloromethane (6ml), at -10°C, and the reaction mixture was left to stir at this temperature for 3h. The resultant precipitate was removed by filtration and the filtrate concentrated under reduced pressure. The residue was then redissolved in dichloromethane (3ml), filtered again and the filtrate concentrated under reduced pressure The residue was chromatographed over silica gel eluting with dichloromethane (100%) to give (RS)-methyl O-acetylphenyllactate as a colourless oil (209mg, 84%). ν_{max}: 3030, 2954, 1746, 1374, 1218, 1082, *m/z*(EI+) 223 (M^++1, 0.46%) $\delta_H(CDCl_3)$: 2.10 (3H, s, -Me), 3.10 (1H, dd, J_{gem}, 14.4Hz, J_{vic} 8.4Hz, $-CH_2$), 3.21 (1H, dd, J_{gem} 14.4Hz, J_{vic} 4.8Hz, $-CH_2$), 3.75 (3H, s, -OMe), 5.24 (1H, dd, J_{vic} 8.4Hz, J_{vic}, 4.8Hz, -CH), 7.30 (5H, m, -Ar), with (+)-Eu(hfc)$_3$ -Me: (R)- to lower frequency, -OMe (S)- to higher frequency, $\delta_C(CDCl_3)$: 21.09 (C-9), 37.86 (C-3), 52.81 (-OMe), 73.46 (C-2), 127.54 (C-7), 128.97 (C-5), 129.76 (C-6), 136.40 (C-4), 170.65 (C-1). Found 223.09761, $C_{12}H_{15}O_4$ (M^++1) requires 223.09703.

7.33 (R)-Methyl O-Acetylphenyllactate (50a)

(R)-Phenyllactic acid (24mg 0.145mmol) was methylated and acetylated according to the procedure described in section 7.32 to generate (R)-methyl O-acetylphenyllactate (14mg, 44%). Selected spectroscopic data: $\delta_H(CDCl_3)$: 2.01 (3H, s, -Me), 3.01 (1H, dd, J_{gem} 14.4Hz J_{vic} 8.4 Hz, $-CH_2$), 3.12 (1H, dd, Jgem 14.54Hz, J_{vic} 4.8Hz, $-CH_2$), 3.67 (3H, s, -OMe), 5.15 (1H, dd, J_{vic} 8.4Hz, J_{vic} 4.8Hz, -CH), 7.20 (5H, m, -Ar). Using Eu(hfc$_3$), no splitting was visible until 10% racemate (5% other enantiomer) had been added. This was obvious as an additional set of singlet resonances to lower frequency of the methyl ester and to higher frequency of the acetyl methyl peaks.

7.34 (S)-Methyl O-Acetylphenyllactate (50b)

(S)-Phenyllactate (14.8mg, 0.086mmol) was methylated and acetylated according to the procedure described in section 7.33 to give (S)-methyl O-acetylphenyllactate (10.4mg, 54%). Selected spectroscopic data: $\delta_H(CDCl_3)$: 2.10 (3H, s, -Me), 3.01 (1H, dd, J_{gem} 14.5Hz, J_{vic} 9.0Hz, $-CH_2$), 3.20 (1H, dd, J_{gem} 14.5Hz, J_{vic} 4.1Hz, -CH2), 3.74 (3H, s, -OMe), 5.22 (1H, dd, J_{vic} 9.0Hz, J_{vic}, 4.1Hz, -CH), 7.28 (5H, m, -Ar). Using Eu(hfc_3), no splitting was visible until 10% racemate (5% other enantiomer) had been added this was obvious as an additional set of singlet resonances to higher frequency of the methyl ester and to lower frequency of the acetyl methyl peaks.

7.35 Methyl Phenyllactate (52)

A solution of sodium borohydride (600mg, excess) in water (10ml) and sodium hydroxide (2ml, 1M aqueous solution) was added dropwise, to a stirred solution of phenylpyruvic acid (300mg, 1.82mmol) in methanol (50ml) at 0-5°C over a period of 30 min. The reaction mixture was allowed to warm to 18°C and was left to stir overnight. The reaction was then quenched by addition of 10% hydrochloric acid (20ml) at 0°C and the methanol was evaporated under reduced pressure. Water (50ml) was added and the phenyllactic acid extracted into ether (3 x 50ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was dissolved in ether (60ml) then methylated directly by quenching with an excess of ethereal diazomethane (9.35mmol), generated from Diazald (2g) and potassium hydroxide (0.8g), in ether (60ml). The excess diazomethane was removed by bubbling a stream of dry N_2 through the solution until the yellow colour had completely disappeared. The solution was then dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The residue was chromatographed over silica gel, eluting with dichloromethane (100%) to give methyl phenyllactate, (264mg, 80%) as a waxy solid. ν_{max} : 3470br 3030, 2953 1740, 1497, 1455, 1275, 1097, 817, 748. *m/z*(CI+) 198 (M^+ + NH_4, 14.0%) $\delta_H(CDCl_3)$: 2.96 (1H, dd, J_{gem} 13.8Hz, J_{vic} 6.9Hz $-CH_2$), 3.13 (1H, dd, J_{gem} 13.8Hz, J_{vic} 4.6 $-CH_2$), 3.24 (1H, s, -OH), 3.73 (3H, s, -Me), 4.44 (1H, dd, J_{vic}

6.9Hz, J_{vic} 4.6Hz, -CH), 7.27 (5H,m -Ar). δ_C ($CDCl_3$): 40.05 (C-3), 52.88 (-Me), 71.85 (C-2), 127.37 (C-7), 128.92 (C-7),129.80 (C-5), 136.93 (C-4), 175.10 (C-1). Found 180.07626, $C_{10}H_{12}O_3$ requires 180.07864.

7.36 Methyl [2-^{2}H]- Phenyllactate (52b)

Phenylpyruvic acid (300mg, 1.82mmol) was reduced using sodium boro-[$^2H_4$]-hydride (500mg, excess) according to the procedure described in section 7.35, to generate methyl [2-^{2}H]-phenyllactate (252mg, 76%). ν_{max}: 3469br, 3030, 2954, 1741, 1605, 746, 701 *m/z*(CI+) 199 (M+1+NH_3, 100%) δ_H($CDCl_3$): 2.81 (1H, s, -OH), 2.84 (1H,d, J_{gem} 11.6Hz, -CH_2), 3.00 (1H,d, J_{gem} 11.6Hz, -CH_2) 3.63 (3H, S, Me), 7.14 (5H, m, -Ar). δ_C($CDCl_3$): 40.98 (C-3), 52.86 (C-2), 71.5 (t, $J_{^{13}C\text{-}^2H}$ 23 Hz), 27.37 (C-7), 128.91 (C-5), 129.98 (C-6), 136.93 (C-4), 175.09 (C-1).

7.37 Methyl [2-^{3}H]- Phenyllactate (52c)

Phenylpyruvic acid (300mg, 1.82mmol), was reduced using sodium boro-[$^3H_4$]-hydride (100mCi) with cold carrier sodium borohydride (600mg, excess) according to the procedure described in section 7.35, to generate methyl [2-^{3}H]-phenyllactate (252mg, 77%), with a specific activity of 918μCimmol^{-1} (radiochemical yield, 0.9%). δ_H($CDCl_3$): 2.94 (1H, dd, J_{gem} 13.9 Hz, J_{vic} 6.9Hz -CH_2), 2.79 (1H, s, -OH), 3.12 (1H, dd, J_{gem} 13.9Hz, J_{vic} 4.5Hz-CH_2) 3.73 (3H, s, -Me), 4.42 (1H, dd, J_{vic} 6.9Hz, J_{vic} 4.5Hz-CH_2), 7.21 (5H, m, -Ar), δ_C($CDCl_3$):41.05 (C-3), 52.89 (-Me), 71.86 (C-2), 127.37 (C-7), 128.91(C-5), 129.99 (C-6), 136.97 (C-4), 175.10 (C-1).

7.38 Sodium Phenyllactate (32)

Bromotrimethylsilane (2.77mmol, 425mg), was added to a stirred solution of methyl phenyllactate (200mg, 1.11mmol) in dry dichloromethane at 0°C and the mixture was left to stir for 2h at 18°C. The volatiles were removed under reduced pressure and then the residue was dissolved in chloroform (20ml), washed with 10% hydrochloric acid (20ml) and the solvents removed again under reduced pressure. The residue was

resuspended in choroform (4ml), distilled water (10ml) was added and the aqueous layer was adjusted to pH7 by the addition of dilute sodium hydroxide solution. The aqueous layer was then separated and evaporated under reduced pressure to give sodium phenyllactate, (174mg 83%) as an amorphous white solid. ν_{max} 3395br, 3032, 2959, 2908, 1156, 1417, 1311, 1090, 699. *m/z*(FAB) 165 (M^+-23, 100%), $\delta_H(D_20)$: 2.75 (1H, dd, J_{gem} 13.8Hz J_{vic} 7.9Hz $-CH_2$), 2.98 (1H, dd, J_{gem} 13.8Hz, Jvic 4.2Hz, $-CH_2$), 4.13 (1H, dd, J_{vic} 7.9Hz, J_{vic} 4.2Hz, -CH), 7.21 (5H, m, -Ar). $\delta_C(D_20)$: 43.20 (C-3), 76.28 (C-2), 129.48 (C-7), 131.40 (C-5), 132.26 (C-6), 141.02 (C-4), 183.55 (C-5).

7.39 Sodium [2-^{2}H]-Phenyllactate (32l)

Methyl [2-^{2}H]-phenyllactate (0.227g 1.25mmol) was hydrolysed according to the procedure described in section 7.38 to give sodium [2-^{2}H]-phenyllactate, (82mg, 0.438mmol 35%). ν_{max}: 3395br, 3253br, 3034, 2859, 2918, 1563, 1407, 1219, 1113, 698. *m/z* (EI+) 149 (M-39, 48.47%) $\delta_H(D_20)$: 2.74 (1H, d, J_{gem} 13.9Hz, $-CH_2$), 2.97 (1H, d J_{gem} 13.9Hz, $-CH_2$), 7.21 (5H, m, -Ar). $\delta_C(D_2O)$: 43.04 (C-3), 75.87 (t, J_{13C2H} 22.3, C-2), 129.45 (C-7), 131.37 (C-6), 140.98 (C-4), 183.54 (C-1).

7.40 Sodium [2-^{3}H]-Phenyllactate (32f)

Methyl [2-^{3}H]-phenyllactate (0.252g, 1.40mmol) was hydrolysed according to the procedure described in section 7.37 to give [2-^{3}H]-phenyllactic acid, (251mg, 95%) as an amorphous white solid. $\delta_H(D_2O)$: 2.77(1H, dd J_{gem} 13.9Hz, J_{vic} 7.5Hz, $-CH_2$), 2.99 (1H, dd, J_{gem} 13.9Hz, J_{vic} 3.9Hz, $-CH_2$), 4.18 (1H, dd, J_{vic} 7.5Hz, J_{vic} 3.9Hz, -CH), 7.22 (5H, m, -Ar), $\delta_C(D_2O)$: 43.09 (C-3), 75.95 (C-2), 129.56 (C-7), 131.44 (C-5), 132.23 (C-6), 140.85 (C-4), 183.58 (C-1).

7.41 Hydrolysis of Hyoscyamine

Commercial hyoscyamine (500mg, 1,13mmol), was dissolved in saturated barium hydroxide solution (25ml) and heated under reflux for 30min. The solution was allowed

to cool and was then extracted into ether (3x30ml). The aqueous extract was acidified with conc. HCl and was also extracted into ether (3x30ml). The combined organic extracts from the second extraction were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give tropic acid, which was recrystallised from chloroform, (221mg, 77%). m.p. 117-118°C, lit: 118°C,[10] ν_{max}: 3404, 2899, 1707, 1261, 1274, 1019. *m/z*(CI+) 184 (M^++NH_3, 47.32%), δ_H(DMSO): 3.4 (1H, s, br, -OH), 3.60 (2H, m, -CH_2), 3.93 (1H, dd, J_{vic} 8.4 Hz, -CH), 7.30 (5H, m, Ar). δ_C(DMSO): 54.60 (C-3), 63.70 (C-2), 127.34 (C-7), 128.33 (C-5), 128.70 (C-6), 137.34 (C-4), 174.03 (C-1). Found 166.06285, $C_9H_{10}O_3$ requires 166.06299.

7.42 Methyl Tropate (60)

An ethereal solution of diazomethane (9.35mmol), generated from Diazald (2.0g, 9.35mmol) and potassium hydroxide (0.8g) in ether (60ml), was added to a solution of tropic acid, (500mg, 3.00mmol) in ether (60ml). The excess diazomethane was removed by the addition of a few drops of glacial acetic acid until the yellow colour completely disappeared. The solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue was chromatographed over silica gel, eluting with dichloromethane : ether (5%) to yield methyl tropate as a colourless oil, (539mg, 100%). ν_{max}: 3441br, 3031, 2953, 1736, 1168, 1045, 737, 701. *m/z* (EI+) 181 (M+1, 96.5%), 150 (M-30, 100%), 118 (M-62, 81%). δ_H ($CDCl_3$): 3.59 (1H, dd, J_{gem} 10.0Hz, J_{vic} 5.2Hz, -CH_2), 3.86 (1H, dd, J_{vic} 8.3Hz, J_{vic} 5.2 Hz, -CH), 4.14 (1H, dd, J_{gem} 10.0Hz, J_{vic} 8.3Hz, -CH_2), 7.27 (5H, M, -Ar). δ_C ($CDCl_3$): 52.73 (-Me), 54.43 (C-2), 65.08 (C-3), 128.28 (C-7), 128.66 (C-5), 129.38 (C-6), 136.07 (C-4), 174.15 (C-1).

7.43 Hydrolysis of [3-^{3}H]-Hyoscyamine: Methyl [3-^{3}H]-Tropate (60a)

Cold carrier hyoscyamine (500mg, 1,73mmol) was added to the [3-^{3}H]-hyoscyamine (47mg, 0.162mmol, 204μCimmol^{-1}) isolated from the root cultures. The mixture was then stirred in saturated barium hydroxide solution (25ml) until dissolved and heated under reflux for 1h. The solution was cooled and then extracted into ether (3x30ml).

The aqueous extract was acidified with 50% HCl and further extracted into ether (3x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give crude tropic acid. This material was used directly without further purification. An ethereal solution of diazomethane (9.35mmol), generated from Diazald (2.0g, 9.35mmol) and potassium hydroxide (0.8g), in ether (60ml) was added to a solution of the tropic acid, in ether (60ml). The reaction was quenched by the addition of a few drops of glacial acetic acid until the yellow solution became colourless. The solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure and then the residue was chromatographed over silica gel eluting with dichloromethane : ether (5%) to give methyl [3-^{3}H]-tropate as a colourless oil (0.23g, 67%). δ_H ($CDCl_3$): 3.59 (1H, dd, J_{gem} 10.0Hz, J_{vic} 5.2Hz, -CH_2), 3.86 (1H, dd, J_{vic} 8.3Hz, J_{vic} 5.2 Hz, -CH), 4.14 (1H, dd, J_{gem} 10.0Hz, J_{vic} 8.3Hz, -CH_2), 7.27 (5H, M, -Ar). δ_C ($CDCl_3$): 52.73 (-Me), 54.43 (C-2), 65.08 (C-3), 128.28 (C-7), 128.66 (C-5), 129.38 (C-6), 136.07 (C-4), 174.15 (C-1).

7.44 Methyl [2-^{2}H]-Tropate (60b)

A small piece of sodium metal (≈ 250mg) was added to a stirred solution of methyl tropate (1.0g, 6.0mmol) in MeOD (15ml) and the reaction was left to stir at 18°C for 2h. The solution was adjusted to pH4 with 5% D_2SO_4 in D_2O, the solvent evaporated under reduced pressure and the residue was dissolved in water (30ml) and extracted into ether (3 x 30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was re-suspended in ether (50ml) and was re-methylated by adding an ethereal solution of diazomethane (≈ 8mmol, excess), until the solution remained bright yellow. The reaction was quenched after 5min by the dropwise addition of glacial acetic acid until the solution just became colourless. The solvent was removed under reduced pressure and the residue chromatographed over silica gel, eluting with dichloromethane : ether (9:1), to give methyl [2-^{2}H]-tropate as a colourless oil, (876mg, 81%). ν_{max}: 3447br, 1734,1450, 1436, 700. *m/z* (EI+) 182 (M^++1 41.52%), 151 (100%), 119 (86.68%). δ_H ($CDCl_3$): 3.06 (3H, s -Me), 3.78 (1H, d, J_{gem} 11.0Hz, -CH_2), 4.14 (1H, d, Jgem 11.0Hz, -CH_2), 7.29 (5H, m, -Ar). δ_C

($CDCl_3$): 52.61 (-Me), 54.22 (t, J_{C-D} 19.7Hz, C-2), 64.80 (C-3), 128.22 (C-7), 128.66 (C-5), 129.31 (C-6), 136.18 (C-4), 174.11 (C-1).

7.45 Methyl 2-Phenyl 3-methanesulphonylpropionate (61)

Methanesulphonyl chloride (687mg, 6.00mmol) was added to a stirred solution of methyl tropate (540mg 3.00mmol) and DMAP (36mg, 0.30mmol) in pyridine (10ml) at 18°C and the mixture was stirred for 30min. The reaction was quenched by the addition of 10% HCl (30ml) and then dichloromethane (30ml) was added. The organic extract was washed sequentially with 10% HCl (30ml) and saturated copper sulphate solution (30ml), dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was chromatographed over silica gel eluting with dichloromethane (100%) and the product recrystallised from ethanol to give methyl 2-phenyl-3-methanesulphonylpropionate as a white crystalline solid, (574mg, 74%). m.p. 59-60°C, ν_{max}: 3020, 2970, 1740, 1345, 1180, 1170, 990, 815, 700, 515, 500. *m/z* (EI+) 259 (M^++1 34.94%), 162 (89.79%), 121 (100%). δ_H ($CDCl_3$): 2.97 (3H, s, $-SO_2CH_3$), 3.72 (3H, s, $-OCH_3$), 4.03 (1H, dd, J_{vic} 5.45Hz, J_{vic} 9.55Hz, -CH), 4.37 (1H, dd, J_{gem} 9.95Hz, J_{vic} 5.45Hz $-CH_2$), 4.72 (1H, dd, J_{gem} 9.95Hz, J_{vic} 9.55Hz, $-CH_2$), 7.35 (5H, m, -Ar). δ_C ($CDCl_3$): 37.73 ($-SO_2CH_3$), 51.26 (C-2), 53.05 (-0Me), 70.41 (C-3), 128.56 (C-5), 129.02 (C-7), 129.67 (C-6), 134.05 (C-4), 171.78 (C-1). Found C 51.21%, H 5.37%, N 0%, $C_{11}H_{14}O_5S$ requires C 51.15%, H 5.46%, N 0%.

7.46 Methyl 2-Phenyl [3-^{3}H]-3-methanesulphonylpropionate (61a)

Methyl [3-^{3}H]-tropate (228mg 1.27mmol) and DMAP (0.13mmol, 15mg) in pyridine (5ml) were reacted with methanesulphonyl chloride (290mg, 2.54mmol) according to the procedure described in section 7.45, to give methyl 2-phenyl-[3-^{3}H]-3-methanesulphonylpropionate as a white crystaltalline solid, (574mg, 55%) m.p. 58-59°C. δ_H(CDCl): 2.96 (3H, s, $-SO_2Me$), 3.72 (3H, s, -Me), 4.04 (1H, dd, J_{vic} 5.43Hz, J_{vic} 9.57Hz, -CH), 4.37 (1H, dd, J_{gem} 9.99Hz, J_{vic} 5.43 Hz $-CH_2$), 4.72 (1H, dd, J_{gem}

9.99Hz, J_{vic} 9.57Hz, -CH_2), 7.34 (5H, m, -Ar), δC($CDCl_3$): 37.74 (-SO_2Me), 51.24 (C-2), 53.02 (Me), 70.41 (C-3), 128.56 (C-5), 129.02 (C-7), 129.67 (C-6), 134.04 (C-4), 171.79 (C-1).

7.47 Methyl [2-^{2}H]-2-Phenyl-3-methanesulphonylpropionate (61b)

Methyl [2-^{2}H]-tropate (300mg, 1.66mmol) and DMAP (20mg 0.166mmol), in pyridine (10ml), were reacted with methanesulphonyl chloride (368mg, 3.32mmol) according to the procedure described in section 7.45 to give methyl [2-^{2}H]-2-phenyl-3-methanesulphonylpropionate, as a white crystalline solid (263mg, 61%). m.p. 59-60°C ν_{max}: 1758, 1540, 1258, 1178. *m/z* (CI+) 277 (M^+ + 18, 72.55%). δ_H ($CDCl_3$): 2.88 (3H, s, -SO_2Me), 3.63 (3H,s, -OMe), 4.29 (1H,d, J_{gem} 7.2Hz, -CH_2) 4.64 (1H, d, J_{gem} 7.2Hz, -CH_2), 7.23 (5H, m, -Ar). δC ($CDCl_3$): 37.69 (-SO_2Me), 50.88 (t, J_{C-D} 17Hz), 53.04 (-OMe), 128.55 (C-5), 128.92 (C-7), 129.67 (C-6), 134.01 (C-4), 171.78 (C-1).

7.48 Methyl 2-Phenyl-3-*p*-toluenesulphonylpropionate (64) (preparation 1)

p-Toluenesulphonyl chloride (620mg, 3.26mmol), was added to a stirred solution of methyl tropate (532mg, 2.96mmol) in pyridine (6ml) at 0°C and the reaction mixture left to stir at 20°C for 2h. Dichloromethane (100ml) was added, and the solution was washed with 10% hydrochloric acid (3x100ml). The organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue was chromatographed over silica gel eluting with ethyl acetate: hexane (1:4). Unreacted starting material was recovered and methyl 2-phenyl-3-*p*-toluenesulphonylpropionoate, which was recrystallised from ethanol, gave white, needle-shaped crystals (195mg, 23%). m.p. 93°C lit. 88°C,[11] ν_{max}: 2955, 1730, 1595, 1360, 1170, 995, 835, 545. *m/z* (EI+) 335 (M^++1, 57%), 162 (100%), 91 (92%). δ_H ($CDCl_3$): 2.2 (3H, s, Ar-CH_3), 3.65 (3H, s, -OCH_3), 3.97 (1H, dd, J_{vic} 9.2Hz, J_{vic} 5.8 Hz, -CH), 4.19 (1H, dd, Jgem 9.8Hz, J_{vic} 5.8Hz, -CH_2), 4.52 (1H, dd, J_{gem} 9.8Hz, J_{vic} 9.2Hz, -CH_2), 7.22 (7H, m, -Ar), 7.70 (2H, d, J_{vic} 7Hz, -Ar). δ_C ($CDCl_3$): 21.63 (Ar-CH_3), 50.71 (C-2), 52.42 (OMe), 70.03, (C-3), 127.93, (C-5), 127.98 (C-6), 128.27 (C-7), 128.95 (C-3' or C-2'), 129.81 (C-2' or

C-3'), 132.53 (C-4'), 133.69 (C-4), 144.81 (C-1'), 170.99 (C-1). Found C 61.35%, H 5.51%, N 0%, $C_{17}H_{18}O_5S$ requires C 61.06%, H 5.42%, N 0%.

7.49 Methyl 2-Phenyl-3-*p*-toluenesulphonylpropionate (64) (preparation 2)

Triethylamine (287mg, 2.84mmol) and *p*-toluenesulphonylchloride (454mg, 2.38mmol) were added to a stirred solution of methyl tropate (410mg, 2.27mmol) in dichloromethane (30ml), at -20°C. The reaction was left to stir at -20°C for 15h. TLC analysis indicated that both methyl 2-phenyl-3-tosylpropionate and the eliminated product, methyl 2-phenylacrylate, as well as a little residual starting material were present. TLC analysis after a further 3h showed an increase in the eliminated product w.r.t. the desired product. The solution was washed with aqueous tartaric acid solution (10%, 30ml) and brine (30ml). The organic extract was dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue was chromatographed over silica gel, eluting with dichloromethane (100%) to yield methyl 2-phenyl-3-tosylpropionoate, which was recrystallised from ethanol (145mg, 19%). This material had identical spectroscopic data to that described above.

7.50 2-Phenylpropanol (62)

2-Phenyl-3-methanesulphonylpropionate (180mg, 0.70mmol) in ether (10ml) was added dropwise to a stirred suspension of lithium aluminium hydride (106mg, 2.79mmol) in ether (25ml) and the mixture heated under reflux for 2h. The reaction was quenched by the addition of wet ether (20ml) and then poured into 5% H_2SO_4. The product was extracted into ether (2x 30ml) and the combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was chromatographed over silica gel, eluting with dichloromethane (100%) to give 2-phenylpropanol as a clear, colourless oil (150mg, 88%).ν_{max}: 3368br, 3029, 3028, 1494,1453, 1014, 762, 701. *m/z*(EI+) 136 (M+ 30.66%), 105 (M-31 100%). δ_H ($CDCl_3$): 1.28 (3H, d, J_{vic} 7.0 Hz, $-CH_3$), 1.55 (1H, s, -OH), 2.97 (1H, m, -CH), 3.7

(2H, d, J_{vic} 6.8 Hz, -CH_2), 7.25 (5H, m, -Ar). δ_C ($CDCl_3$): 18.08 (C-3), 42.93 (C-2), 69.19 (C-3), 127.17 (C-7), 127.99 (C-5), 128.66 (C-4), 129.13 (C-6). Found 136.08891, $C_9H_{12}O$ requires 136.08881.

7.51 [1-$^2H_2$, 3-^{2}H]-2-Phenylpropanol (62a)

Methyl 2-phenyl-3-methanesulphonylpropionate (180mg, 0.70mmol) was reduced with lithium aluminium deuteride (117mg, 2.79mmol) according to the procedure described in section 7.50 to give [1-$^2H_2$, 3-^{2}H]-2-phenylpropanol as a clear, colourless liquid, (80mg, 82%). ν_{max}: 3345br, 3027, 2928, 1492, 1452, 970, 749, 698, *m/z*(EI+) 139 (M+ 16.56%), δ_H($CDCl_3$):1.17 (3H, dt J_{1H1H} 7.0Hz, J_{1H2H} 1.8Hz, -CH_2D), 1.50 (1H, s, -OH), 2.83 (1H, t, J_{vic} 7.0Hz -CH), 7.17 (5H, m, -Ar), δ_C($CDCl_3$): 17.22 (t, J_{13C2H} 19.18 Hz, C-3), 42.14 (C-2), 67.88 (p, J_{13C2H} 21.7Hz, C-1), 126.61 (C-7), 127.44 (C-5), 128.59 (C-6), 143.62 (C-4).

7.52 [1-$^2H_2$, 3-^{2}H,^{3}H]-2-Phenylpropanol (62b)

Methyl 2-phenyl-3-methanesulphonyl-[3-^{3}H]-propionate (156mg, 0.6mmol) was reduced with lithium aluminium deuteride (102mg, 2.42mmol) according to the procedure described in section 7.50 to give [1-$^2H_2$, 3-^{2}H,^{3}H]-2-phenylpropanol (80mg, 96%). δ_H($CDCl_3$) 1.25 (3H, dt J_{1H1H} 7.0Hz, J_{1H2H} 1.8Hz -CHDT(H)) 1.44 (1H, s, -OH), 2.92 (1H, t, J_{vic} 7.0Hz -CH), 7.30 (5H, m, -Ar), δ_C($CDCl_3$) 17.19 (t J_{13C2H} 19.25Hz C-3), 42.10 (C-2), 60.80 (p J_{13C2H} 21.7Hz C-1), 126.56 (C-7), 127.31 (C-5) 128.54 (C-6), 143.64 (C-4).

7.53 [2-^{2}H]-2-Phenylpropanol (62c)

Methyl 2-[^{2}H]-2-phenyl-3-mesylpropionate was reduced with lithium aluminium hydride (150mg, 4.00mmol) according to the procedure described in section 7.50 to give [2-^{2}H]-2-phenylpropanol (105mg, 77%). ν_{max}: 3354br, 3024, 2928, 1493, 1446,

1090, 756, 699. *m/z*(EI+) 137 (M^+ 12.84%), 106 (100%). δ_H ($CDCl_3$): 1.18 (3H, s, $-CH_3$), 1.59 (1H, s, -OH), 3.60, (2H, s, $-CH_2$), 7.20 (5H, m, -Ar). δ_C ($CDCl_3$): 16.46 (C-3), 40.95 (t, J_{C-D} 17.43Hz, C-2), 67.55 (C-1), 125.61 (C-7), 126.44 (C-5), 127.58 (C-6), 142.64 (C-4).

7.54 Periodate/Permanganate Oxidation of 2-Phenylpropanol: Sodium Acetate (5)

2-Phenylpropanol (232mg, 1.7mmol) was added to a stirred solution of potassium periodate (30g, 0.13mol) and potassium permanganate (80mg, 0.51mmol) in distilled water (200ml). The reaction mixture was left to stir vigorously at 18°C for 4 days, then the insoluble salts were removed by filtration. The filtrate was made basic with 1M sodium hydroxide solution and the solvent evaporated under reduced pressure. The residue was re-dissolved in 5% sulphuric acid and lyophilised. The lyophilisate was then adjusted to pH8.5 with 0.01M sodium hydroxide solution. The water was evaporated under reduced pressure to give a white solid (50mg) which contained sodium acetate (43%) and sodium formate (57%) (calculated from NMR integrals). Yield of acetate 17%. $\delta_H(D_2O)$: 1.76 (3H, s) 8.31 (1.3H, s), $\delta_C(D_2O)$: 26.15, (C-2), 173.94 (C-1).

7.54.1 *p*-Phenylphenacyl Acetate (67)

2-Bromo-4'-phenylacetophenone (80mg, 0.29mmol), was dissolved in warm ethanol (2ml) and was added to a stirred solution of the sodium acetate (24mg 0.29mmol) in water (1ml) and the mixture was heated under reflux for 1h. The solution was cooled to 0°C and after the addition of a few drops of water a white precipitate formed and was collected by filtration. The precipitate was dissolved in dichloromethane (10ml), washed with water (10ml) then the solution was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was chromatographed over silica gel, eluting with dichloromethane (100%) to give 2-acetyl-4'-phenylacetophenone (17mg, 0.067mmol) 33%. m.p. 109-111°C, lit:111°C,[12] ν_{max}: 2961, 2933, 2360, 1754, 1746, 1701, 1600, 120, 1091, 760. m/z(CI+) 272 (M^++18, 0.70%), 255 (M^++1, 2.53%), formate

derivative: 141 (M+, 0.56%) $\delta_H(CDCl_3)$: 2.25 (3H, s, $-CH_3$), 5.38 (2H, $-CH_2$), 7.60 (9H, m, -Ar). $\delta_C(CDCl_3)$: 20.62 (C-1), 66.03 ($-CH_3$), 127.27 (-Ar), 127.47 (-Ar), 128.34 (-Ar), 128.99 (-Ar), 132.82 (-Ar), 139.60 (-Ar), 170 .47 (CO), 191.73 (C0).

7.55 Periodate/Permanganate of [1-$^2H_2$, 3-^2H,^3H]-2-Phenylpropanol: Sodium [2-^2H,^3H]-Acetate (5a)

2-Phenyl-[1-$^2H_2$, 3-^2H,^3H]-propanol (80mg, 0.59mmol) was treated with periodate /permanganate according to the procedure described in section 7.54 to give a white solid (62mg) which contained deuterated acetate (43%), non-deuterated acetate (29%) and formate (28%). $\delta_H(D_2O)$: 1.89 (2H, t, J_{1H-2H} 2.15Hz), 1.90 (2.1 H, s), 8.44 (0.7H, s).

7.56 Periodate/Permanganate Oxidation of Cumene

Cumene (200mg, 1.7mmol) was added to a stirred solution of potassium periodate (30g, 0.13mol) and potassium permanganate (80mg, 0.51mmol) in distilled water (200ml). The reaction mixture was left to stir vigorously at 18°C for 2h and then the insoluble salts were removed by filtration. The filtrate was made basic with 1M sodium hydroxide solution and the solvent evaporated under reduced pressure. The residue was re-dissolved in 5% sulphuric acid and lyophilised. The lyophilisate was adjusted to pH8.5 with 0.01M sodium hydroxide solution and the water was evaporated under reduced pressure to give a white solid (50mg) containing sodium acetate (41%) contaminated with sodium formate (59%) (calculated from NMR integration): yield of acetate 16%. $\delta_H(D_2O)$: 1.8 (3H, s, acetate), 8.3 (1.4H, s, formate). $\delta_C(D_2O)$ 26.16 (C-2), 173.96 (C-1).

7.56.1 *p*-Phenylphenacyl Acetate (67)

2-Bromo-4'-phenylacetophenone (12µg 42µmol) in warm ethanol (500µl) was added to a stirred solution of the product (3.5mg) in water (100µl). The solution was heated under reflux for 1h then cooled to 0°C. The white precipitate was collected by filtration,

re-dissolved in dichloromethane (1ml) and washed with water (1ml). The organic layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The crude product was chromatographed over silica gel eluting with dichloromethane (100%) and the product was analysed by MS: acetate derivative: *m/z*(CI+): 273 (M^++18, 2.32%), 255 (M^++1, 5.95%), formate derivative: *m/z*(CI+): 259 (M^++18, 3.18%), 241 (M^+, 4.36%).

7.57 Hydrolysis of Methyl [2-^{2}H]-Tropate : Exchange of Deuterium from C-2

A stirred solution of methyl [2-^{2}H]-tropate (93mg, 0.51mmol) in saturated barium hydroxide solution was heated under reflux for 30min. The reaction mixture was then poured into 50% HCl (20ml) and extracted into ether (3 x 20ml). The combined ethereal extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give [2-^{2}H]-tropic acid (68mg, 80%) containing 8% [2-^{1}H]-tropic acid as determined by ^{1}H NMR.

This material was then heated under reflux in 10% sodium hydroxide solution (25ml), removing aliquots (6ml) after 5, 10 20 and 30min. The solution removed was poured into 50% HCl (20ml) and the tropic acid extracted as described above. Spectroscopic data for tropic acid are given in section 7.41 and the ^{1}H NMR of the methine region is shown in fig. 85.

PART II

7.58 Growth of *Poronia piliformis* and the Isolation of Piliformic Acid[13]

Poronia piliformis was cultured on malt extract solution (3%, Oxoid) in Thompson bottles (300ml per bottle) at 24°C. Substrates were administered in sterile neutral solution 2-4 days after subculturing, when new growth was apparent. The supernatant was harvested after 6 weeks and separated from the mycelium by filtration. The filtrate was acidified (conc. HCl) and extracted into ethyl acetate (3x300ml per bottle). The combined organic extracts were then dried, filtered and evaporated under reduced pressure to give a brown gum. This was resuspended in ether and filtered to remove any insoluble material. The filtrate was then evaporated under reduced pressure and the residue chromatographed over silica gel, eluting with toluene : ethyl formate : formic acid 50: 49 : 1. The oily residue after solvent evaporation was recrystallised from nitromethane to give piliformic acid as an amorphous solid, typically about 55mg per bottle. $\delta_H(CDCl_3)$: 0.90 (3H, t, $-CH_3$), 1.28 (7H, m, $-(CH_2)_2$ and $-CH_3-$), 1.34 (2H, m, $-CH_2$), 2.24 (2H, m, $-CH_2$), 3.63 (1H, q, -CH), 7.02 (1H, d, -CH), 10.80 (2H, s, $-CO_2H$). $\delta_C(CDCl_3)$: 14.41 (C-9), 15.14 (C-11), 22.91 (C-8), 28.58 (C-6), 29.34 (C-5), 31.98 (C-7), 37.95 (C-2), 131.54 (C-3), 147.91 (C-4), 172.53 (C-10), 180.65 (C-1). $[\alpha]_D$ -86° (c = 0.067 in MeOH), lit -89° (c = 1.0 in MeOH)[1]

7.58.1 Sodium [1,2-$^{13}C_2$]-Acetate

Sodium [1,2-$^{13}C_2$]-acetate was administered to *P. piliformis* cultures (2x200ml) at a concentration of 15mmoldm^{-3}. Piliformic acid (36mg) was isolated with an incorporation of 5.7% into the C_8 fragment and 0.48% into the C_3 fragment (fig. 131).

7.58.2 Sodium [1-^{13}C, 2-$^{2}H_3$]-Acetate

Sodium [1-^{13}C, 2-$^{2}H_3$]-acetate was administered to *P. piliformis* cultures (2x200ml) at a concentration of 15mmoldm^{-3}. Piliformic acid (96mg) was isolated with an incorporation of 4.6% into the C_8 fragment (fig. 134, fig. 135, fig. 137).

7.58.3 Sodium [2-^{13}C, $^{2}H_3$]-Acetate

Sodium [2-^{13}C, $^{2}H_3$]-acetate was administered to *P. piliformis* cultures (2x300ml) at a concentration of 4.8mmoldm^{-3}. Piliformic acid (108mg) was isolated with an incorporation of 2.4% into the C_8 fragment (fig. 139).

7.58.4 Sodium [1-^{13}C]-Octanoate

Sodium [1-^{13}C]-octanoate was administered to *P. piliformis* cultures (2x300ml) at a concentration of 2.87mmoldm^{-3}. Piliformic acid (54mg) was isolated with an intact incorporation of 5.5% into the C_8 fragment and 2% incorporation into the C8 fragment *via* [1-^{13}C]-acetate (fig. 140).

7.58.5 Sodium [2-$^{2}H_3$]-Acetate

Sodium [2-$^{2}H_3$]-acetate was administered to *P. piliformis* cultures (21x300ml) at a concentration of 15mmoldm^{-3}. Piliformic acid (1.16g) was isolated and degraded to hexanoic acid as described in section 7.60.

7.58.6 Sodium 16-Fluoropalmitate

Sodium 16-fluoropalmitate was administered to *P. piliformis* cultures (2x300ml) at a concentration of 1.13mmoldm^{-3}. The piliformic acid (30mg) isolated showed no incorporation of fluorine.

7.58.7 Sodium [$^{2}H_7$]-Butyrate

Sodium [$^{2}H_7$]-butyrate was administered to *P. piliformis* cultures (1x300ml) at a concentration of 4.2mmoldm^{-3}. Piliformic acid (23mg) was isolated (fig. 177).

7.58.8 Sodium [$^{2}H_{11}$]-Hexanoate

Sodium [$^{2}H_{11}$]-hexanoate was administered to *P. piliformis* cultures (1x300ml) at a concentration of 2.6mmoldm^{-3}. Piliformic acid (47mg) was isolated (fig. 176).

7.58.9 Sodium [$^{2}H_{15}$]-Octanoate

Sodium [$^{2}H_{15}$]-octanoate was administered to *P. piliformis* cultures (1x300ml) at a concentration of 2.6mmoldm^{-3}. Piliformic acid (50mg) was isolated (fig. 175).

7.58.10 Sodium [$^{2}H_{15}$]-Octanoate

Sodium [$^{2}H_{15}$]-octanoate was administered to *X, mali* cultures (3x300ml) at a concentration of 1.84mmoldm^{-3}. This strain was cultured in an identical manner to *P. piliformis* as described in section 7.58 Piliformic acid (16mg) was isolated (fig. 182).

7.58.11 Sodium [1-^{13}C, 2-^{2}H]-Octanoate

Sodium [1-^{13}C, 2-^{2}H]-octanoate was administered to *P. piliformis* cultures (2x300ml) at a concentration of 2.51mmoldm^{-3} and piliformic acid (54mg) was isolated (fig) with an intact incorporation of 4.9% and 1.4% as [1-^{13}C]-acetate (fig. 180, fig. 181).

7.58.12 DL-[1-^{13}C]-Alanine

DL-[1-^{13}C]-Alanine was administered to *P. piliformis* cultures (2x300ml) at a concentration of 4.6mmoldm^{-3}. Piliformic acid (79mg) was isolated with an incorporation of 0.09% at C-1 (fig. 169).

7.58.13 DL-[3-^{13}C, $^{2}H_{3}$]-Alanine

DL-[3-^{13}C, $^{2}H_{3}$]-Alanine was administered to *P. piliformis* cultures (1x300ml) at a concentration of 4.5mmoldm^{-3}. Piliformic acid (42mg) was isolated (fig. 171).

7.58.14 Sodium [2,3-$^{2}H_{4}$]-Succinate

Sodium [2,3-$^{2}H_{4}$]-succinate was administered to *P. piliformis* cultures (2x300ml) at a concentration of 6.8mmoldm^{-3} Piliformic acid (34mg) was isolated (fig. 173).

7.59 Degradation of Piliformic Acid to Hexanoic Acid (126)[5]

Piliformic acid (300mg, 1.4mmol) was added to a vigorously stirred solution of potassium periodate (12,5g, 50mmol) and ruthenium trichloride (32mg, 0.154mmol) in water : acetonitrile : tetrachloromethane (3:1:1, 100ml). The reaction mixture was left to stir at 18°C for 48 hours and then filtered through a celite pad. The organic phase was separated, and the hexanoic acid, extracted into ether (3x60ml) and evaporated under reduced pressure. The residue was suspended in chloroform (2ml), covered with water (20ml) and then the aqueous layer was adjusted to pH8 with 0.1M sodium hydroxide solution. The aqueous phase was then separated, re-acidified with 50% hydrochloric acid and re-extracted into ether (3x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give hexanoic acid as a yellow oil, which was used without further purification (135mg, 83%). ν_{max}: 3013br, 2966, 2942, 2872, 2672, 1706, 1418. *m/z*(EI+) 117 (M^++1, 0.24%), 60 (M^+-56, 100%) $\delta_H(CDCl_3)$: 0.83 (3H, t, J_{vic} 6.6Hz, $-CH_3$), 0.125 (4H, m, $-(CH_2)_2-$), 1.57 (2H, m, $-CH_2$), 2.28 (2H, t, J_{vic} 7.5Hz, $-CH_2$), 9.36 (1H, s, $-CO_2H$), $\delta_C(CDCl_3)$: 14.33 (C-6), 22.77 (C-5), 24.84 (C-4), 31.68 (C-3), 34.54 (C-2), 180.69 (C-1).

7.60 Degradation of Piliformic Acid from Sodium [2-$^2H_3$]-Acetate fed *P. piliformis* (126a)

Piliformic acid (900mg, 4.4 mmol) was degraded according to the procedure described in section 7.59 to give hexanoic acid (371mg 77%). ν_{max}: 3013br, 2958, 2932, 2873, 1713, 1414. *m/z*(EI+) 118 (M^++1 (CHD), 60 (M+-56, 100%), 0.32%), 117 (M^++1 (CH_2) 0.04%), $\delta_H(CDCl_3)$; 0.89 (3H, t J_{vic} 6.6Hz, $-CH_3$), 1.32 (4H, m, $-(CH_2)_2-$), 1.63 (2H, m, $-CH_2$), 2.33 (2H, t, J_{vic} 7.5Hz, $-CH_2$), 11.0 (1H, s, $-CO_2H$), $\delta_C(CDCl_3)$: 14.31 (C-6), 22.75 (C-5), 24.82 (C-4), 31.66 (C-3), 34.56 (C-2), 181.18 (C-1).

7.61 Methyl (RS)-[2-^{2}H]-Hexanoate (128)

Methyl hexanoate (1.0g, 1.13ml, 7.68mmol) was added dropwise to a stirred solution of LDA (23.0mmol), generated from diisopropylamine (2.33g, 3.22ml, 23.0mmol) and *n*-butyllithium (14.38ml, 23.0mmol, 1.6M in hexanes), in THF (20ml) at -78°C. The solution was allowed to warm slowly to 18°C and was left to stir at this temperature for 1h. The reaction was then quenched by the addition of MeOD (2ml, excess) and the solution left to stir for a further 1h. The reaction mixture was then poured into saturated ammonium chloride solution (30ml) and the aqueous phase separated and extracted into ether (2x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue was purified by bulb to bulb distillation (0.03mbar, furnace temperature 50°C) to give methyl (RS)-[2-^{2}H]-hexanoate as a colourless oil (249mg, 25%). ν_{max}: 2958, 2934, 2870, 1714, 1462, 1435, 1218. *m/z*(EI+) 131 (M+ 11.86%) 87 (M^+-44) $\delta_H(CDCl_3)$: 0.89 (3H, t, J_{vic} 6.7Hz, $-CH_3$), 1.30 (4H, m, $-(CH_2)_2$), 1.63 (2H, m, $-CH_2$), 2.29, (1H, m, -CHD), 3.65, (3H, s, -OMe). $\delta_C(CDCl_3)$: 13.88 (C-6), 22.30 (C-5), 24.56 (C-4), 31.28 (C-3), 33.37 (t, $J_{^{13}C^2H}$ 19.3Hz, -CHD, C-2), 34.06 $-CH_2$, C-2), 51.42 (-OMe), 174.35 (C-1).

7.62 [2-^{2}H]-Hexanoic Acid (126b)

(RS)-Methyl [2-^{2}H]-hexanoate (150mg, 1.15mmol) was covered with a 1M solution of KOD in D_2O (10ml) and the stirred solution heated under reflux for 12h. The solution was left to cool to 18°C, and was then washed with ether (2x20ml). The aqueous solution was acidified with 50% HCl and extracted into ether (3x20ml) and the combined organic extracts from the second extraction were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give [2-^{2}H]-hexanoic acid as a colourless oil (99mg, 74%). ν_{max}: 3068br, 2958, 2932, 2873, 1704, 1413, 1290. *m/z*(EI+) 118 (M^++1, 0.26%) $\delta_H(CDCl_3)$: 0.89 (3H, t, J_{vic} 6.7Hz, $-CH_3$), 1.33 (4H, m, $-(CH_2)_2$), 1.63 (2H, m, $-CH_2$), 2.34 (1H, m, -CHD). $\delta_C(CDCl_3)$: 13.86 (C-6), 22.28 (C-5), 24.34 (C-4), 31.35 (C-3), 33.75 (t, $J_{^{13}C^2H}$ 19.5Hz, -CHD, C-2), 34.04 (-CH2, C-2), 180.38 (C-1).

7.63 Methyl O-Hexanoylmandelate (127)[14]

(RS)-Methyl mandelate (332mg, 2.00mmol) and dicyclohexylcarbodiimide (412mg, 2.00mmol), were added to a stirred solution of hexanoic acid (232mg, 0.215ml, 2.00mmol) and a catalytic quantity of 4-dimethylaminopyridine (5mg, 0.04mmol) in dichloromethane (10ml) at -10°C. The reaction mixture was left to stir at this temperature for 3h and the precipitated urea removed by filtration. The solvent was re-evaporated under reduced pressure and the residue suspended in dichloromethane (4ml) and re-filtered. The solvent was evaporated under reduced pressure and the residue chromatographed over silica gel eluting with dichloromethane (100%) to give methyl O-hexanoylmandelate as a colourless oil (412mg, 78%). ν_{max}: 2255, 2232, 1744, 1456, 1216, 1160, *m/z*(EI+) 264 (M^+ 0.69%), 99 (M^+-165, 100%) $\delta_H(CDCl_3)$: 0.89 (3H, t, J_{vic} 6.8Hz, $-CH_3$), 1.31 (4H, m, $-(CH_2)_2-$), 1.72 (2H, m, $-CH_2$), 2.45 (2H, m, $-CH_2$), 3.71 (3H, s, $-CH_3$) 5.94 (1H, s, -CH), 7.40 (5H, m, -Ar), $\delta_C(CDCl_3)$: 14.38 (C-12), 22.77 (C-11), 29.94 (C-10), 31.66 (C-9), 34.11 (C-8), 53.05 (-OMe), 74.73 (C-2), 128.09 (C-4), 129.26 (C-5), 129.68 (C-6), 134.39 (C-3), 169.88 (C-7 or C-1), 173.65 (C-1 or C-7). Found 265.14260, $C_{15}H_{21}O_4$ (M^++1) requires 265.14398

7.64 Methyl O-Hexanoylmandelate from Deuterated Piliformic Acid Derived Hexanoic Acid (127a)

Hexanoic acid from the [2-$^2H_3$]-acetate feeding experiment on piliformic acid was coupled with (R)-methyl mandelate according to the procedure described in section 7.63, to generate deuterated methyl O-hexanoylmandelate (227mg, 65%).ν_{max}: 2955, 2932, 2861, 1745, 1455, 1438, 1216, 1160, *m/z*(CI+) 285 ($(M+3)^+$+18, 3.1%), 284 ($(M+2)^+$+18, 3.71%), 283 ($(M+1)^+$+ 18, 13.95%), 282 (M^+ +18, 40.88%), 166 (M^+ -99, 100%). $\delta_H(CDCl_3)$: 0.90 (3H, t, J_{vic} 6.8Hz), 1.33 (4H, m, $-(CH_2)_2$), 1.70 (2H, m, $-CH_2$), 2.47 (2H, m, $-CH_2$), 3.72 (3H, s, -OMe), 5.95 (1H, s, -CH) 7.40 (5H, m, -Ar), $\delta_C(CDCl_3)$: 14.39, (C-12), 22.78 (C-11), 24.96 (C-10), 31.67 (C-9), 34.42 9C-8), 53.06 (-OMe), 74.70 (C-2), 128.10 (C-4), 129.27 (C-5), 129.60 (C-6), 134.38 (C-3), 169.80 (C-7 or C-1), 173.65 (C-1 or C-7), $\delta_{2H}(CCl_4)$:0.92 (-CD3), 1.39 (-CHD), 2.46 (-CHD).

7.65 Methyl (RS)-[2-^{2}H]-O-Hexanoyl-2'-(R)-mandelate (127b)

[2-^{2}H]-Hexanoic acid (130mg, 1.10mmol) was coupled to (R)-methyl mandelate according to the procedure described in section 7.63 to give methyl (RS)-[2-^{2}H]-O-hexanoyl-2-(R)-mandelate (250mg, 86%). ν_{max}: 2932, 2871, 1744, 1455, 1438, 1215, 1170, 733, 696. *m/z*(CI+) 284 ((M+2)$^+$ +18, 12.07%), 283 ((M+1)$^+$ +18, 19.99%), 282 (M$^+$+18, 10.19%), 166 (M$^+$ -99, 38 97%). δ_H($CDCl_3$): 0.90 (3H, t, J_{vic} 6.8Hz, -CH_3)-1.33 (4H, m, -$(CH_2)_2$-), 1.70 (2H, m, -CH_2), 2.46 (1H, m, CHD), 3.71 (3H, s, -OMe), 5.94 (1H, s, -CH), 7.37 (5H, m, -Ar), δ_C($CDCl_3$): 14.39 (C-12), 22.78 (C-11), 24.89 (C-10), 31.61 (C-9), 34.01 (C-8, t, $J_{^{13}C-^2H}$ 20Hz), 53.04 (-OMe), 74.73 (C-2), 128.10 (C-4), 129.27 (C-5), 129.69 (C-6), 134.40 (C-3), 169.87 (C-7 or C-1), 173.64 (C-1 or C-7), δ_{2H}(CCl_4): 2.50 (-CHD), 2.56 (-CHD).

2.66 Ethyl 2-Methyl-1,3-dithiane-2-carboxylate (134)

n-Butyllithium (4.22ml, 8.45mmol, 2M in pentane) was added dropwise to a stirred solution of ethyl 1,3-dithiane-2-carboxylate (1.49g, 7.75mmol) in THF at -78°C. The reaction mixture was allowed to warm to 0°C and was left to stir at this temperature for 1h. The solution was cooled again to -78°C and methyl iodide (1.0g, 7.04mmol) was added dropwise. The reaction mixture was allowed to warm to 0°C and left to stir at this temperature for 20min. The reaction was quenched by the careful addition of saturated ammonium chloride solution (20ml), the layers were separated and the aqueous layer extracted into ether (2x30ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give a yellow oil, which was chromatographed over silica gel, eluting with dichloromethane : petrol (1:1) to generate ethyl 2-methyl-1,3-dithiane-2-carboxylate as a colourless oil (1.46g, 91%).ν_{max}: 2969 1739, 1234, 1035, 701. *m/z*(EI+) 206 (21.21%, M$^+$). δ_H($CDCl_3$): 1.27 (3H, t, J_{vic} 7Hz, -CH_3), 1.59 (3H, s, -CH_3), 1.80 (m, 1H, -CH_2), 2.12 (1H, m, -CH_2), 2.58 (2H, dt -CH_2S), 3.32 (2H, td, -CH_2S), 4.17 (2H, q, J_{vic} 7Hz). $\delta_C\{^1H\}$($CDCl_3$): 13.94 (C-8), 28.88 (C-4 or C-5), 25.29 (C-4 or C-5), 27.84 (C-3) 45.86 (C-2), 62.44 (C-7) 171.18 (C-1). Found 206.04353, $C_8H_{14}O_2S_2$ requires 206.04352.

7.67 2-(*t*-Butyl)-3-methylimidazolidin-4-one (142)[15]

Glycine methyl ester hydrochloride (13.14g, 0.11mol)) was added to a stirred solution of 8M ethanolic methylamine (40ml), at 0°C and the solution was left to stir at 18°C for 12h. After this time the reaction mixture was concentrated under reduced pressure and the residue co-evaporated with dichloromethane (3x50ml). Pivalaldehyde (19.83g, 0.23mol), and triethylamine (30ml) were added to a stirred solution of the residue in dichloromethane (90ml) and the solution was heated under reflux for 12h. The cooled mixture was filtered through a celite pad, washed with ether (200ml) and the filtrate concentrated under reduced pressure. The residue was re-suspended in ether (60ml) and filtered again to remove further precipitated material and the solvent was then removed under reduced pressure. A saturated solution of HCl in methanol (60ml) was added to a stirred solution of the above residue in methanol (25ml) at 0°C and the reaction mixture was left to stir at this temperature for 30min, allowed to warm to 18°C and left to stir overnight. The solvent was removed under reduced pressure and the residue dissolved in dichloromethane (80ml). The solution was washed with 3M sodium hydroxide solution (60ml) which was then extracted again with dichloromethane (60ml). The combined organic extracts were filtered through a phase separating paper and concentrated under reduced pressure to give 2-(*t*-butyl)-3-methylimidazolidin-4-one (7.96g, 49%) an oil which formed a waxy solid upon cooling. ν_{max}: 2360 1684, 1466, *m/z*(CI+) 157 (M^++1, 100%), $\delta_H(CDCl_3)$: 0.91 (9H, s, -*t*-Bu), 2.04 (1H, -NH), 2.89 (3H, s, $-CH_3$), 3.41 (2H, d, J_{vic} 0.56Hz, $-CH_2$), 4.07 (1H, s, -CH), $\delta_C(CDCl_3)$: 26.00 ($-(CH_3)_3$), 31.58 ($-CMe_3$), 38.01 (-NMe), 49.62 (C-5), 83.53 (C-2), 175.23 (C-1). Found 157.13417, $C_8H_{17}ON_2$ (M^++1) requires 157.13408

7.68 1-Benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one (137)[16]

Benzoyl chloride (4.09g, 3.37ml, 29.07mmol) and 1M sodium hydroxide solution (16ml) were added to a stirred solution of 2-(*t*-butyl)-3-methylimidazolidin-4-one (4.13g, 26.4mmol) in dichloromethane (40ml) and the mixture was left to stir vigorously for 12h. The organic phase was separated and filtered through a phase

separating filter paper and the filtrate concentrated under reduced pressure. The residue was chromatographed over silica gel eluting with ether : petrol : methanol (60:30:5) to give, after evaporation, a white solid which was recrystallised from dichloromethane/petrol, 1-benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one (6.49g,94%). m.p. 141-143°C, lit: 143-144°C[16] ν_{max}: 3516, 3387, 3275, 2984, 1687, 1647. *m/z*(CI+) 261 (M^++1, 100%). $\delta_H(CDCl_3)$: 1.10 (9H, s, -*t*-Bu), 3.05, (3H, s, NMe), 3.83 (1H, d, J_{gem} 15.4Hz, $-CH_2$), 4.12 (1H, d, J_{gem} 15.4Hz, $-CH_2$), 5.59 (1H, s, -CH), 7.50 (5H, m, -Ar). $\delta_C(CDCl_3)$: 26.54 ($-(CH_3)_3$), 32.11 ($-CMe_3$), 40.25 (-NMe), 53.50 (C-5), 81.36 (C-2), 128.54 (C-3'), 129.10 (C-4'), 132.05 (C-5'), 134.84 (C-2'), 169.73 (C-1 or C-1') 172.14 (C-1' or C-1).

7.69 1-Benzyl-2-(*t*-butyl)-3,5-dimethylimidazolidin-4-one (138)[17]

A solution of LDA (14.79mmol), generated from diisopropylamine (1.5g, 2.07ml, 14.79mmol) and *n*-butyllithium (9.24ml, 14.79mmol, 1.6M in hexanes) in THF (10ml) was transferred into a stirred solution of 1-benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one (3.5g, 13.4mmol) in THF (135ml) at -78°C. The reaction mixture was left to stir at this temperature for 1h and then methyl iodide (1.91g, 0.84ml, 13.4mmol) was added dropwise. The solution was left to stir at -78°C for a further 45min, allowed to warm gradually to 18°C and then stirred for 12h at this temperature. The reaction was quenched by the addition of half saturated ammonium chloride solution (135ml) and then the layers were separated. The aqueous layer was extracted into ether (2x150ml) and the combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The crude product was chromatographed over silica gel eluting with ether : petrol : methanol (60:35:5) to give 1-benzyl-2(*t*-butyl)-3,5-dimethylimidazolidin-4-one as a white solid (2.08g 56%). mp: found 145.5-146.5°C lit: 145.6-146.2°C[17] ν_{max}: 2954, 2867, 1704, 1629. *m/z*(CI+): 275 (M^++1, 76.85%). $\delta_H(CDCl_3)$: 0.97 (3H, d, J_{vic} 6.6Hz, $-CH_3$), 1.06 (9H, s, -*t*-Bu), 3.07 (3H, s, -NMe), 4.25 (1H, q, Jvic 6.6Hz -CH), 5.66 (1H, s, -CH), 7.50 (5H, m, -Ar), $\delta_C(CDCl_3)$: 19.91 (-Me), 26.78 ($-(CH_3)_3$), 32.57 ($-CMe_3$), 41.23 (-NMe), 57.83 (C-5), 80.34 (C-2), 128.11

(C-3'), 129.39 (C-4'), 132.00 (C-5'), 137.46 (C-2'), 171.59 (C-1 or C-1'), 172.77 (C-1' or C-1), CHN Found C 69.90% H 8.15% N 10.01%, $C_{15}H_{20}O_2N_2$ requires C 70.04%, H 8.08% N 10.21%.

7.70 1-Benzyl-2-(*t*-butyl)-3-methyl-[5-^{13}C, $^{2}H_3$-*methyl*]-imidazolidin-4-one (139a)

1-Benzyl-2-(*t*-butyl)-3-methylimidazolidin-4-one (1.62g, 6.23mmol) was added to LDA (6.85mmol) in THF solution and then [1-^{13}C, $^{2}H_3$]-methyl iodide (1.0g, 6.85mmol) was added according to the procedure described in section 7.69 to give 1-benzyl-2-(*t*-butyl)-3-methyl-[5-^{13}C, $^{2}H_3$]-imidazolidin-4-one (977mg, 56%). ν_{max}: 2974, 2869, 1699, 1633. *m/z*(CI+) 279 (M^++1, 100%) $\delta_H(CDCl_3)$: 1.06 (9H, s, -*t*-Bu), 3.07 (3H, s, -NMe), 4.24 (1H, s, -CH), 5.66 (1H, 2, -CH), 7.50 (5H, m, -Ar). $\delta_C(CDCl_3)$:19.16 (septet $J_{^{13}C^{2}H}$ 19.5Hz -CD_3), 26.79 (-$(CH_3)_3$), 32.57 (CMe_3), 41.24 (-NMe), 57.68 (d, $J_{^{13}C^{13}C}$, C-5), 80.37 (C-2), 128.11 (C-3'), 129.39 (C-4'), 132.00 (C-5'), 127.47 (C-2'), 171.59 (C-1' or C-4), 172.80 (C-1' or C-4).

7.71 DL-Alanine (105)[18]

1-Benzyl-2(*t*-butyl)-3,5-dimethylimidazolidin-4-one (500mg, 1.82mmol) was heated with 6N HCl (6ml) to 180°C in a sealed tube for 12h. The solution was then removed from the tube by washing out with water (10ml) and then with dichloromethane (15ml). The aqueous solution was concentrated under reduced pressure to give DL-alanine hydrochloride contaminated with methylamine hydrochloride. The mixture was then purified by ion exchange chromatography (Dowex 50X2-400) to give DL-alanine as a white solid (146mg, 90%). m.p. 288-289°C (dec) lit. 295-296°C (dec)[19] ν_{max}: 3421, 3094, 2756, 2306, 2174, 2094, 1595, 1408, 1340. *m/z*(CI+) 90 (M^++1 24.16%). $\delta_H(D_2O)$: 1.31 (3H, d, J_{vic} 7.2Hz, -CH_3), 5.43 (1H, q, J_{vic} 7.2Hz, -CH), $\delta_C(D_2O)$: 18.98, 53.53 (C-2), 178.65 (C-1).

7.72 DL-[3-^{13}C, $^{2}H_3$]-Alanine (105a)

1-Benzyl-2-(*t*-butyl)-3-methyl-[5-^{13}C,$^{2}H_3$]-imidazolidin-4-one (972mg, 3.50mmol) was hydrolysed according to the procedure described in section 7.71 to give DL-[3-^{13}C, $^{2}H_3$]-alanine (254mg, 79%.) m.p. 289-290(dec), ν_{max}: 3550br, 3091, 2774, 2708, 2106, 1626, 1592. *m/z*(CI+) 94 (M^++1, 100%), $\delta_H(D_2O)$: 3.62 (1H, m, -CH). $\delta_C(D_2O)$: 18.20 (septet, $J_{^{13}C^{2}H}$ 19.6Hz, C-3), 53.13 (d, $J_{^{13}C^{13}C}$ 34.7Hz, C-2), 178.63 (C-1).

7.73 Methyl Octanoate (143)

A solution of diazomethane (50mmol) in ether (400ml) was added to a solution of octanoic acid (5.5g, 38mmol) in ether (50ml) such that the yellow colour persisted. The excess diazomethane was then removed by the addition of a few drops of glacial acetic acid to generate a colourless solution. The solution was then washed with 0.1M sodium hydroxide (2x150ml), dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by bulb to bulb distillation (0.005mbar, furnace temperature 50°C) to give methyl octanoate (5.19g, 98%). ν_{max}: 2954, 2926, 2857, 1740, 1461, 1436, 1168. *m/z*(EI+) 159 (M^++1, 5.27%) $\delta_H(CDCl_3)$: 0.88 (3H, t, J_{vic} 6.5Hz, -CH_3), 1.28 (8H, m, -$(CH_2)_4$), 1.62 (2H, m, -CH_2), 2.31 (3H, t, J_{vic} 7.5Hz, -CH_2), 3.67 (3H, s, -OMe). $\delta_C(CDCl_3)$: 14.07 (C-8), 22.61 (C-7), 24.98 (C-6), 28.94 (C-5), 29.13 (C-4), 31.67 (C-3), 34.13 (C-4), 54.44 (-OMe), 174.35 (C-1). Found 159.13772, $C_9H_{19}O_2$ (M^++1) requires 159.13850

7.74 Methyl [1-^{13}C]-Octanoate (143a)

A solution of diazomethane (20mmol), in ether (50ml) was added to a solution of [1-^{13}C]-octanoic acid (1.0g, 6.89mmol) in ether (50ml) such that the yellow colour persisted. The excess diazomethane was removed by bubbling a slow stream of N_2 through the solution until the yellow colour disappeared. The solution was then dried ($MgSO_4$), filtered and evaporated under reduced pressure and the residue purified by bulb to bulb distillation (0.15mbar, furnace temperature 50°C) to give methyl [1-^{13}C]-octanoate (964mg, 88%). ν_{max}: 2955, 2921, 2851, 1701, 1461, 1152. *m/z*(CI+): 160

(M^++1, 100%). $\delta_H(CDCl_3)$: 0.87 (3H, t, J_{vic} 6.5Hz, $-CH_3$), 1.27 (8H, m, $-(CH_2)_4$), 1.61 (2H, m, $-CH_2$), 2.30 (2H, dt, J_{1H13C} 7.3Hz, J_{vic} 7.3Hz, $-CH_2$), 3.66 (3H, d, J_{1H13C} 3.8Hz -OMe). $\delta_C(CDCl_3)$: 14.04 (C-8), 22.58 (C-7), 24.96 (C-5), 28.73 (C-4), 29.13 (C-3), 34.09 (d, J_{13C13C} 57.2Hz, C-2), 51.45 (-OMe), 174.36 (C-1).

7.75 Methyl [2-^{2}H]-Octanoate (143b)

Methyl octanoate (1.0g, 1.14ml, 6.32mmol) was added dropwise to a stirred solution of LDA (12.64mmol), generated from diisopropylamine (1.28g, 1.65ml, 12.64mmol), and *n*-butyllithium (7.89ml, 1.6M solution in hexanes) in THF (50ml) at -70°C. After 5min the reaction mixture was allowed to warm to 18°C and was left to stir at this temperature for 1h. MeOD (5ml, excess) was added and the solution left to stir for a further 1h. The reaction mixture was then poured into a saturated solution of ammonium chloride (50ml). The aqueous layer was extracted into ether (2x50ml) and the combined organic extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by bulb to bulb distillation (0.01mbar, furnace temperature 50°C) to give methyl [2-^{2}H]-octanoate. Deuterium incorporation at the α-carbon was ≈35%, as determined by ^{1}H NMR, (735mg, 73%). ν_{max}: 2954, 2929, 2857, 1741, 1461, 1255. *m/z*(EI+) 159 (M+, -CHD, 24.09%), 158 (M+, $-CH_2$, 40.55%). $\delta_H(CDCl_3)$: 0.88 (3H, t, J_{vic} 6.5Hz, $-CH_3$), 1.28 (8H, m, $-(CH_2)_4$), 1.62 (2H, m, $-CH_2$), 2.31 (1.3H, m, -CHD), 3.67 (3H, s, -OMe). $\delta_C(CDCl_3)$: 14.03 (C-8), 22.57 (C-7), 24.93 (C-6), 28.89 (C-5), 29.08 (C-4), 31.63 (C-3), 33.79 (t, J_{13C2H} 19.7Hz, -CHD, C-2), 34.09 ($-CH_2$, C-2), 51.40 (-OMe), 174.32 (C-1).

7.76 Methyl [1-^{13}C, 2-^{2}H]-Octanoate (143c)

Methyl [1-^{13}C]-octanoate (942mg, 5.91mmol) was deprotonated with LDA (11.84mmol) and quenched with MeOD (5ml) according to the procedure described in section 7.75 to give methyl [1-^{13}C, 2-^{2}H]-octanoate (501mg, 53%). ν_{max}: 2955, 2926, 2855, 1695, 1458, 1177. *m/z* (EI+) 160 (M+, -CHD, 19.07%), 159 (M+, $-CH_2$, 35.45%). $\delta_H(CDCl_3)$: 0.87 (3H, t, J_{vic} 6.6Hz, $-CH_3$), 1.27 (8H, m, $-(CH_2)_4$), 1.61 (2H,

m, -CH_2), 2.30 (0.9H, m, -CHD), 3.65 (3H, d, J_{1H13C} 3.8Hz, -OMe). $\delta C(CDCl_3)$: 14.06 (C-8), 22.58 (C-7), 24.90 (C-6), 28.91 (C-5), 29.10 (C-4), 31.64 (C-3), 33.80 (dt, J_{13C13C} 57.1Hz, J_{13C2H}, 19.1Hz, -CHD, C-2), 34.09 (d, J_{13C13C} 57.1Hz, -CH_2, C-2), 51.44 (-OMe), 174.41 (C-1).

7.77 [2-^{2}H]-Octanoic Acid (106a)

Methyl [2-^{2}H]-octanoate (735mg, 4.59mmol) was covered with a 1M solution of KOD in D_2O (20ml) and heated under reflux for 12h. The solution was allowed to cool and was then washed with ether (2x20ml). The aqueous solution was acidified with 50% HCl and was then extracted into ether (3x20ml). The combined ethereal extracts were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give [2-^{2}H]-octanoic acid as a clear oil (590mg, 88%). ν_{max}: 3044br, 2955, 2926, 2857, 1709, 1414, 1290. *m/z*(EI+), 145 (M+, -CHD, 1.78%), 144 (M+, -CH_2, 0.79%). $\delta H(CDCl_3)$: 0.86 (3H, t, J_{vic} 6.6Hz, -CH_3), 1.28 (8H, m, -$(CH_2)_4$), 1.62 (2H, m, -CH_2), 2.33 (1.1H, m, -CHD). $\delta C(CDCl_3)$: 13.99 (C-8), 22.57 (C-7), 24.58 (C-6), 28.89 (C-5), 28.98 (C-4), 31.62 (C-3), 33.38 (t, J_{13C2H} 19.1Hz, -CHD, C-2), 24.08 (-CH_2, C-2), 180.51 (C-1).

7.78 1-^{13}C, 2-^{2}H]-Octanoic Acid (106b)

Methyl [1-^{13}C, 2-^{2}H]-octanoate was hydrolysed according to the procedure described in section 7.77 to give [1-^{13}C, 2-^{2}H]-octanoic acid (287mg, 65%). ν_{max}: 3049br, 2956, 2928, 2857, 1668, 1395, 1272. *m/z*(EI+) 146 (M+, -CHD, 0.99%), 145 (M+, -CH_2, 0.51%). $\delta H(CDCl_3)$: 0.88 (3H, t, J_{vic} 6.5Hz, -CH_3), 1.28 (8H, m, -$(CH_2)_4$), 1.61 (2H, m, -CH_2), 2.34 (1.1H, m, -CHD). $\delta C(CDCl_3)$: 14.05 (C-8), 22.58 (C-7), 24.61 (C-6), 28.89 (C-5), 29.00 (C-4), 31.61 (C-3), 33.75 (dt, J_{13C13C} 54.6Hz, J_{13C2H} 19.3Hz, -CHD, C-2), 34.04 (d, J_{13C13C} 54.6Hz, -CH_2, C-2), 180.28 (C-1).

7.79 References

1. R. J. Robins, A. J. Parr, E. G. Bent and M. C. J. Rhodes, *Planta*, 1991, **183**, 185
2. E. Wenkert, J. S. Bindra, C-J. Chang, D. W. Cochran and F. M. Schell, *Acc. Chem. Res.*, 1974, **7**, 46
3. E. Leete, N. Kowanko and R. A. Newmark, *J. Am. Chem. Soc.*, 1975, **97**, 6826
4. J. Herscovici and K. Antonakis, *J. Chem. Soc., Chem. Commun.*, 1980, 561
5. P. H. J. Carlsen, T. Kasuki and K. B. Sharpless, *J. Org. Chem.*, 1981, **40**, 3936
6. E. Pierson, M. Giella and M. Tishler, *J. Am. Chem. Soc.*, 1948, **70**, 1450
7. A. McKenzie and H. Wren, *J. Chem. Soc.*, 1910, **97**, 1355
8. K. Saigo, H. Muira, K. Ishizaki and H. Nohira, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 1188
9. J. R. Cannon, K. R. Joshi, G. V. Meehan and J. R. Williams, *Aust. J. Chem.*, 1969, **22**, 221
10. E. Leete, *Can. J. Chem.*, 1987, **65**, 226
11. M. B. Watson and G. W. Youngson *J. Chem. Soc., Perkin Trans. I*, 1972, 1597,
12. N. L. Drake and J. Bronitsky, *J. Am. Chem. Soc.*, 1930, **52**, 3715
13. D. Parker, *J. Chem. Soc., Perkin Trans. I*, 1983, 83
14. R. Fitzi and D. Seebach, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 345
15. R. Naef and D. Seebach, *Helv. Chim. Acta.*, 1985, **68**, 135
16. D. Seebach, E. Juaristi, D. Miller, C. Schickli, T. Weber, *Helv. Chim. Acta.*, 1987, **70**, 237
17. D. Seebach, J. D. Aebi, R. Naef and T. Weber, *Helv. Chim. Acta.*, 1985, **68**, 144
18. *Handbook of Physics and Chemistry*, Ed. R. C. West and M. J. Astle, CRC Press Inc. Boca Raton, Florida, 63rd Edition, 1982

APPENDIX

Papers Published

"The Biosynthesis of Tropic Acid in Plants: Evidence for the Direct Rearrangement of 3-Phenyllactate" N. C. J. E. Chesters, D. O'Hagan, R.J. Robins, *J. Chem. Soc., Perkin Trans, I,* 1159, 1994"

"The Biosynthesis of Tropic Acid: The (2R)-D-Phenyllactyl Moiety is Processed by the Mutase Enzyme Involved in Hyoscyamine Biosynthesis in *Datura stramonium*" N. C. J. E. Chesters, D. O'Hagan, R.J. Robins, *J. Chem. Soc., Chem. Commun.,* 127, 1995

"The Biosynthesis of Tropic Acid: The Stereochemical Course of the Mutase Involved in Hyoscyamine Biosynthesis in *Datura stramonium*" N. C. J. E. Chesters, D. O'Hagan, R. J. Robins, A. Kastelle and H. G. Floss, *J. Chem. Soc., Chem. Commun.,* 1995, 129

"The Biosynthesis of Hyoscyamine: The Process by which Littorine Rearranges to Hyoscyamine" R.J. Robins, N. C. J. E. Chesters, D. O'Hagan, A. J. Parr, N. J. Walton, J. G. Woolley, *J. Chem. Soc., Perkin Trans. I,* 481, 1995

"The Biosynthesis of Tropic Acid: A Reevaluation of the Stereochemical Course of the Conversion of Phenyllactate to Tropate in *Datura Stramonium*" N. C. J. E. Chesters, K. Walker, D. O'Hagan and H. G. Floss, *J. Am. Chem. Soc.*, in press

Research Conferences Attended

Biological challenges for Organic Chemistry, St Andrews, 9th-13th July 1995
(poster presentation)

Symposium on Recent Progress in Polyketide Biosynthesis, Cambridge, 4th April 1995, (oral presentation).

SCI Novel Organic Chemistry: 6th Graduate Symposium, York, 1st March 1995, (oral presentation).

Recent Developments in Stereochemistry, Sheffield, December 20th 1994

Bio-Organic Group Postgraduate Symposium, Durham, 12th December 1994, (poster presentation).

Pfizer Organic Chemistry Poster Symposium 8th December 1994,
(poster presentation, runner-up prize).

Symposium on Recent Progress in Polyketide Biosynthesis, Cambridge, 17th March 1994

Bio-Organic Group Postgraduate Symposium Exeter, 13th December 1993

Recent Developments in Stereochemistry, Sheffield, December 16th 1992

Colloquia, Lectures and Seminars from Invited Speakers

October 1992 - September 1993

11.11.92	Prof. D. Robins, University of Glasgow
	Pyrrolizidine Alkaloids: Biological Activity, Biosynthesis and Benifits
25.11.92	Prof. Y. Vallee, University of Caen
	Reactive Thiocarbonyl Compounds
26.11.92	Dr D. Humber, Glaxo, Greenford
	AIDS: The Development of a Novel Series of Inhibitors of HIV
2.12.92	Prof. A. F. Hegarty, University College, Dublin
	Highly Reactive Enols Stabilised by Steric Protection
9.12.92	Dr A. N. Burgess, ICI Runcorn
	The Structure of Perfluorinated Ionomer Membranes
21.1.93	Prof. L. Hall, University of Cambridge
	NMR: Window to the Human Body
27.1.93	Dr W. Kerr, University of Strathclyde
	Development of the Pauson-Khand Annulation Reaction: Organocobalt
	Mediated Synthesis of Natural and Unnatural Products
28.2.93	Prof. J. Mann, University of Reading
	Murder, Magic and Medicine
11.2.93	Prof. S. Knox, University of Bristol
	Organic Chemistry at Polynuclear Metal Centres
17.3.92	Dr R. J. K. Taylor, University of East Anglia
	Adventures in Natural Product Synthesis
24.3.93	Prof. I. O. Sutherland, University of Liverpool
	Chromogenic Reagent for Cations
1.6.93	Prof. J. P. Konopelski, University of California, Santa Cruz
	Synthetic Adventures with Enantiomerically Pure Acetals
28.9.93	Prof. H. Ila, Norht Eastern Hill University, India
	Synthetic Strategies for Cyclopentanoids *via* Oxoketene Dithioacetals

October 1993 - September 1994

14.10.93	Dr P. Hubberstey, University of Nottingham
	Alkali Metals, Alchemist's Nightmare, Biochemist's Puzzle and Technologist's Dream
20.10.93	Dr P. Quayle, University of Manchester
	Aspects of Aqueous ROMP Chemistry
21.10.93	Prof. B. Caddy, University of Strathclyde
	Forensic Scientists: Do We Mean What We Say?
27.10.93	Dr R. A. L. Jones, Cavendish Laboratory, University of Cambridge
	Perambulating Polymers
4.10.93	Dr. C. J. Ludman, University of Durham
	Explosions
25.10.93	Dr R. P. Wayne, University of Oxford
	The Origin and Evolution of the Atmosphere
1.12.93	Prof. M. A. McKervey Queen's University, Belfast
	Synthesis and Applications of Chemically Modified Calixarenes
20.1.94	Dr A. M. Donald, University of Cambridge
	The Story of the Starch Granule, From Harvest to Table
26.1.94	Prof. J. Evans, University of Southampton
	Shining Light on Catalysis
27.1.94	Prof. R. D. Williams, University of Wales, Cardiff College
	Metals in Man and Medicine
3.2.94	Dr R. Alder, University of Bristol
	Bridgeheads Bicyclic and Bonding
17.2.94	Prof. R. K. Harris, University of Durham
	Chemistry (and Medicine) in a Spin: Towards 50 Years of NMR
3.3.94	Dr. M. Bamford, Glaxo, Greenford
	The Squalestatin Story
10.3.94	Prof. S. V. Ley, University of Cambridge
	New Methods for Organic Synthesis

October 1994 - September 1995

10.11.94	Dr M. Block, Zeneca Pharmaceuticals, Macclesfield
	Large Scale Manufacture of ZD 1542, a Thromboxane Antagonist Synthase Inhibitor
16.11.94	Prof. M. Page, University of Huddersfield
	Four Membered Rings and β-Lactamase
17.11.94	Dr Cairns-Smith, University of Glasgow
	Clay Minerals and the Origin of Life
23.11.94	Dr J. M. J. Williams, University of Loughborough
	New Approaches to Asymmetric Catalysis
11.1.95	Prof. P. Parsons, University of Reading
	Applications of Tandem Reactions in Organic Synthesis
16.2.95	Prof. H. Kroto
	C_{60} - The Cellestial Sphere that Fell to Earth
19.1.95	Prof. R. Bonnett, Queen Mary and Westfield College, London
	Chemical Aspects of Photodynamic Therapy
25.1.95	Dr D. A. Roberts, Zeneca Pharmaceuticals
	The Design and Synthesis of Inhibitors of the Renin-Angiotensin System
26.1.95	Mrs S. Owen, Northumberland Water
	Trace Organics in the Environment
22.2.95	Prof. E. Schaumann, University of Clausthal
	Silicon and Sulphur Mediated Ring Opening of Epoxides

The Biosynthesis of Hyoscyamine: the Process by which Littorine Rearranges to Hyoscyamine

Richard J. Robins,*,†,[a] Nicola C. J. E. Chesters,[b] David O'Hagan,[b] Adrian J. Parr,[a] Nicholas J. Walton[a] and Jack G. Woolley[c]

[a] *Institute of Food Research (Norwich Laboratory), Norwich Research Park, Colney, Norwich NR4 7UA, UK*
[b] *Department of Chemistry, University of Durham, Science Laboratories, South Road, Durham DH1 3LE, UK*
[c] *Natural Products Research, School of Applied Sciences, De Montfort University, The Gateway, Leicester LE1 9BH, UK*

The incorporation of isotope from specifically-labelled 3-phenyllactic acid **4** or littorine **7** into 3α-phenylacetoxytropane **10**, 3α-phenylacetoxy-6β,7β-epoxytropane and 3α-(2'-hydroxyacetoxy)-tropane **9** has been demonstrated. Transformed root cultures of *Datura stramonium* or *Brugmansia* (*Datura*) *candida x B. aurea* incorporated fed (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** into 3α-phenylacetoxytropane **10** and 3α-phenylacetoxy-6β,7β-epoxytropane with the efficient retention of both ^{13}C nuclei. In contrast, no label was incorporated into these two compounds from (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate **4**. From this evidence it can be deduced that 3-phenyllactic acid **4** is not incorporated into 3α-phenylacetoxytropane **10** *via* free phenylacetic acid **6**, a route which would result in the loss of the C-1 of 3-phenyllactic acid **4**. Furthermore, (*RS*)-(3'-phenyl[1',3'-$^{13}C_2$]lactoyl)[*methyl*-$^{2}H_3$]tropine (littorine **7**) was incorporated into 3α-phenylacetoxytropane **10**, at up to 4% specific incorporation, with the retention of all the ^{13}C and ^{2}H nuclei. Label was also incorporated into 3α-(2'-hydroxyacetoxy)tropane **9** from (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** and (*RS*)-(3'-phenyl[1',3'-$^{13}C_2$]lactoyl)[*methyl*-$^{2}H_3$]tropine **7**. We propose, on the basis of these observations, a putative process for the rearrangement of littorine **7** to hyoscyamine **8** and suggest that both 3α-phenylacetoxytropane **10** and 3α-(2'-hydroxyacetoxy)tropane **9** arise as by-products of the rearrangement process.

The aromatic moiety of the tropane alkaloids, hyoscyamine **8** and hyoscine, is (*S*)-tropic acid. During the biosynthesis of the tropoyl moiety from (*R*)-phenylalanine, a carbon skeletal rearrangement of the linear propanoid side chain occurs, forming the isopropanoid side chain characteristic of tropic acid.[1] It was clearly demonstrated that this rearrangement is intramolecular, since hyoscyamine **8** isolated from plants of *Datura innoxia* fed (*RS*)-phenyl[1,3-$^{13}C_2$]alanine had contiguous C–C coupling at the C-1' and C-2' positions in the NMR spectrum. Furthermore, it was shown[2,3] that a 1,2-vicinal interchange occurs, the carboxy residue migrating to C-3 with retention of configuration and the 3-*pro*-(*S*) proton of (*R*)-phenylalanine migrating in the counter direction.

Aspects of the route by which phenylalanine is incorporated into hyoscyamine **8** have been established by a series of recent studies. It has been demonstrated that 3-phenyllactic acid **4** is an obligatory intermediate;[4,5] alternative putative routes, *via* such intermediates as cinnamic acid,[6] 3-hydroxy-3-phenylpropanoic acid,[6] or 3-amino-2-phenylpropanoic acid[7] have now been discarded as improbable. The intermediacy of 3-phenyllactic acid was effectively shown by feeding (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** both to whole plants of *D. stramonium*[8] and to transformed root cultures of *D. stramonium* or *Brugmansia* (*Datura*) *candida x B. aurea*.[9] In these experiments, contiguity of the C-1' and C-2' in the derived hyoscyamine **8** was observed, mimicking the incorporation seen previously from phenyl[1,3-$^{13}C_2$]alanine.[1] That 3-phenyllactic acid **4** is incorporated without the intermediacy of phenylpyruvic acid **5** has also been shown, firstly by the unaltered $^{3}H:^{14}C$ ratio in the derived hyoscyamine **8** when (*RS*)-3-phenyl[1-^{14}C,2-^{3}H]lactic acid **4** was fed[4] and, unequivocally, by the incorporation from (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactic acid **4** of the intact ^{13}C–^{2}H bond into the C-3' of the derived hyoscyamine **8**.[5]

From these data it may be deduced that the carbon skeletal rearrangement occurs subsequent to the intermediacy of 3-phenyllactic acid **4**. One possibility is that free 3-phenyllactic acid **4** could be rearranged to form free tropic acid. However, we have recently presented evidence[9,10] that this is not the case. Firstly, the effects of tropic acid on the relative extents to which (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** is incorporated into hyoscyamine **8** and littorine **7** by transformed root cultures of *D. stramonium* and *B. candida x B. aurea* are incompatible with free tropic acid being an intermediate in this pathway.[9] Secondly, littorine **7** has been shown to be a direct precursor for hyoscyamine **8**, as (*RS*)-(3'-phenyl[1',3'-$^{13}C_2$]lactoyl)[*methyl*-$^{2}H_3$]tropine **7** is efficiently converted into hyoscyamine **8** without loss of label in transformed root cultures of *D. stramonium*.[10] Littorine **7** is, therefore, rearranged directly to form hyoscyamine **8**.

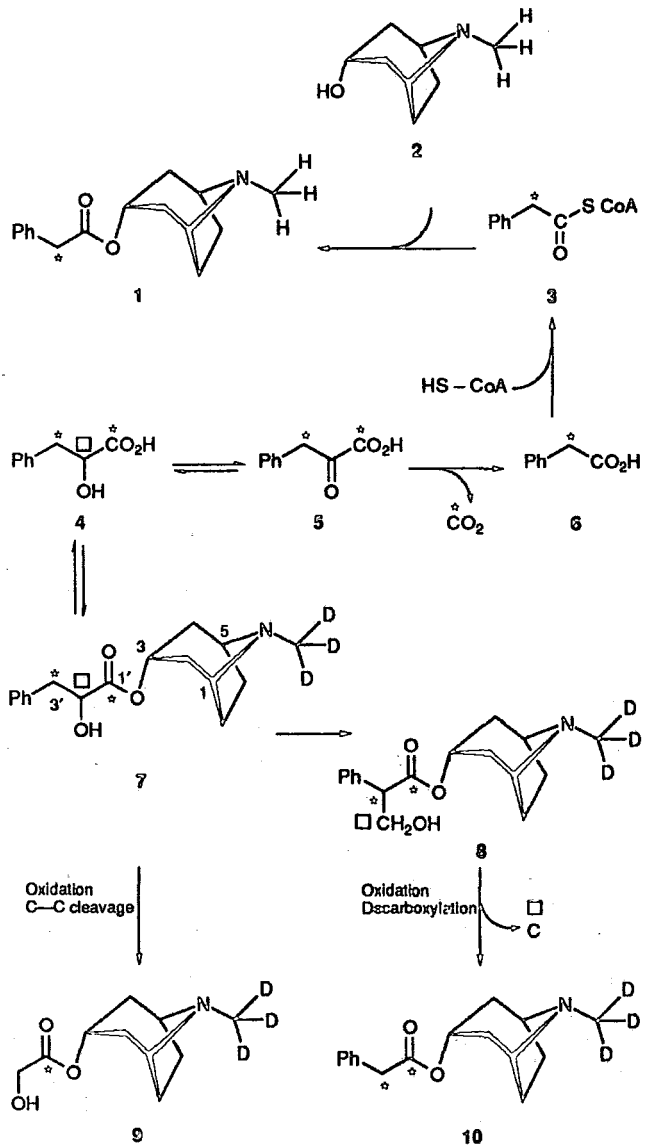
We now report the incorporations from (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4**, (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactic acid **4** and (*RS*)-(3'-phenyl[1',3'-$^{13}C_2$]lactoyl)[*methyl*-$^{2}H_3$]tropine **7** into 3α-phenylacetoxytropane **10**, 3α-phenylacetoxy-6β,7β-epoxytropane and 3α-(2'-hydroxyacetoxy)tropane **9**, as determined by GC–MS, and discuss the biosynthetic implications of the labelling patterns found. These bases all occur as minor alkaloids in extracts from transformed root cultures of *D. stramonium* or *B. candida x B. aurea*, as well as from other tropane-alkaloid-producing species.[11] 3α-Phenylacetoxytropane **10** could arise from the esterification of tropine **2** with phenylacetyl-coenzyme A **3** (Scheme 1). An enzyme capable of carrying out this esterification has been found[12] at low levels in a number of solanaceous species but has so far proved intransigent to purification. Similarly, 3α-(2'-hydroxyacetoxy)-tropane **9** could arise by the esterification of tropine **2** with 2-hydroxyacetyl-coenzyme A in a manner analogous to that

† *Present address:* Laboratoire de RMN-RC, URA-CNRS 472, Faculté des Sciences, 2 rue de la Houssinière, F-44072 Nantes, France.

Table 1 The incorporations of (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid into tropane alkaloids in transformed root cultures of *D. stramonium*

Additional acid fed (mmol dm^{-3})[b]	Specific incorporation (%)[a]				
	3α-(2′-Hydroxyacetoxy)-tropane **9**	3α-Phenylacetoxy-tropane **10**		Hyoscyamine **8**	
	M + 1	M + 1	M + 2	M + 1	M + 2
None	43.3	16.4	37.2	6.1	41.0
(*S*)-3-Phenyllactic acid **4** (1.0)	35.7	8.8	33.2	5.0	38.9
(*RS*)-3-Phenyllactic acid **4** (1.0)	45.1	10.2	44.3	7.2	45.4
(*RS*)-Tropic acid (1.0)	48.8	16.0	32.8	7.6	48.5

[a] Specific incorporation (%) = 100 × (Excess of isotope in the isolated product)/(Excess of isotope in the precursor). [b] (*RS*)-3-Phenyl[1,3-$^{13}C_2$]lactic acid **4** was fed to all cultures at 0.25 mmol dm^{-3}.



Scheme 1 Alternative putative pathways for the biosynthesis of 3α-phenylacetoxytropane

shown for the formation of 3α-acetoxytropane, 3β-acetoxytropane and 3β-tigloyloxytropane.[13,14] However, the incorporation patterns observed in 3α-phenylacetoxytropane **10**, 3α-phenylacetoxy-6β,7β-epoxytropane and 3α-(2′-hydroxyacetoxy)tropane **9** are incompatible with the alkaloids arising by such routes. Rather, all compounds contain ^{13}C at levels that indicate that they have arisen as by-products of the rearrangement of littorine to hyoscyamine.

Results and Discussion

(*RS*)-3-Phenyl[1,3-$^{13}C_2$]lactic acid **4** (0.25 mmol dm^{-3}) was fed to 4 day-old transformed root cultures of *D. stramonium* (7 mg per flask; 2 flasks; grown on to 14 days) or *B. candida x B. aurea* (7 mg per flask; 4 flasks; grown on to 7 days). The crude alkaloidal fractions (3.8 mg from 9.6 g fresh mass and 15.4 mg from 19.4 g fresh mass, respectively) were isolated and examined by GC–MS.[9,15] The mass spectra obtained from several replicate feedings to *D. stramonium* all showed a high specific incorporation of the label into 3α-phenylacetoxytropane **10** with the retention of both ^{13}C nuclei (Table 1). The specific incorporation observed was not diminished by diluting the fed phenyllactic acid to only 16% isotopic excess. Nor was the specific incorporation reduced by feeding the culture tropic acid, the acidic moiety of hyoscyamine **8**. In the *Brugmansia* hybrid, both 3α-phenylacetoxytropane **10** and 3α-phenylacetoxy-6β,7β-epoxytropane were labelled (Table 2). Again, there was no substantial diminution of specific incorporation by added tropic acid. This is the first demonstration that 3-phenyllactic acid **4** can act as a precursor for 3α-phenylacetoxytropane **10** and 3α-phenylacetoxy-6β,7β-epoxytropane. The slightly lower incorporation into 3α-phenylacetoxy-6β,7β-epoxytropane is probably indicative of this compound being a metabolite of 3α-phenylacetoxytropane **10**.[16]

The specific incorporations seen in the M + 2 ions of these compounds are comparable with those observed in hyoscyamine **8** and hyoscine (Tables 1 and 2). Therefore, the fed (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** cannot have been metabolised to 3α-phenylacetoxytropane **10** and 3α-phenylacetoxy-6β,7β-epoxytropane *via* free phenylacetic acid **6** (Scheme 1) because decarboxylation at C-1 of (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** would lead to the loss of the enriched nucleus at this position and a consequent lack of a M + 2 ion in any subsequent metabolites; only an M + 1 ion would be observed. In fact, there is a small enhancement over the natural abundance of the M + 1. However, this is much lower than the incorporation at the M + 2 level and some enhancement of the M + 1 is to be expected, as the (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** used contains about 9% (*RS*)-3-phenyl[1-^{13}C]lactic acid and (*RS*)-3-phenyl[3-^{13}C]lactic acid as a result of the synthetic method used in its preparation.[8] Incorporation from this source is strongly suggested by the comparable M + 1 incorporations into hyoscyamine **8** and hyoscine. In *D. stramonium*, the 3α-phenylacetoxytropane **10** does show a higher M + 1 than the hyoscyamine **8**, making it feasible that a route *via* free phenylacetic acid **6** might have made a small contribution in this species. Nevertheless, the data can only imply that it is primarily C-2 of 3-phenyllactic acid **4** that is lost, not

Table 2 The specific incorporations of (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** into tropane alkaloids in transformed root cultures of a *Brugmansia* (*Datura*) *candida* x *B. aurea* hybrid

Additional acid fed (mmol dm^{-3})[b]	Specific incorporation (%)[a]								
	3α-(2′-Hydroxyacetoxy)tropane **9**	3α-Phenylacetoxytropane **10**		Hyoscyamine **8**		3α-Phenylacetoxy-6β,7β-epoxytropane		Hyoscine	
	M + 1	M + 1	M + 2	M + 1	M + 2	M + 1	M + 2	M + 1	M + 2
None	41.7	9.5	40.9	7.5	41.3	8.5	32.3	6.8	33.3
(*RS*)-Tropic acid (0.25)	30.4	7.6	34.1	8.8	35.0	7.5	27.3	6.0	27.9
(*RS*)-Tropic acid (0.75)	30.3	5.4	34.8	9.4	36.9	3.9	14.3	3.6	15.2

[a] See Table 1. [b] (*RS*)-Phenyl[1,3-$^{13}C_2$]lactic acid **4** was fed to cultures at 0.25 mmol dm^{-3}.

Table 3 The percentage isotopic excess in tropane alkaloids extracted from transformed root cultures of *D. stramonium* fed with (*RS*)-(3′-phenyl[1′,3′-$^{13}C_2$]lactoyl)[*methyl*-$^2H_3$]tropine (littorine **7**)

Additional precursor fed (mmol dm^{-3})[b]	Percent isotopic excess[a]									
	3α-(2′-Hydroxyacetoxy)tropane **9**		3α-Phenylacetoxytropane **10**				Hyoscyamine **8**			
	M + 3	M + 4	M + 2	M + 3	M + 4	M + 5	M + 2	M + 3	M + 4	M + 5
None	5.1	13.0[c]	n.d.[d]	n.d.	n.d.	n.d.	3.3	4.8	1.4	4.5
Tropine **2** (0.25)	4.9	18.9	2.2	1.9	0.0	4.0	4.3	3.8	2.0	6.5
(*RS*)-3-Phenyllactic acid **4** (0.25)	4.1	16.0	1.8	1.6	0.0	3.3	4.0	3.5	1.8	6.1

[a] 100 × Enhancement of the isotopic abundance (corrected for natural abundance) relative to the M ion. [b] (*RS*)-(3′-Phenyl[1,3-$^{13}C_2$]lactoyl)-[*methyl*-$^2H_3$]tropine (littorine **7**) was fed to cultures at 0.125 mmol dm^{-3}. [c] Areas under the M + 5 peaks of 3α-(2′-hydroxyacetoxy)tropane **9** are very small. [d] n.d. = insufficient area under the peak for quantitation.

C-1 as would be the case were metabolism to take place *via* **6**.

Confirmatory evidence that the C-2 of 3-phenyllactic acid **4** is not incorporated into 3α-phenylacetoxytropane **10** was obtained in an experiment in which (*RS*)-3-phenyl[2-^{13}C, 2-^2H]lactic acid **4** (0.4 mmol dm^{-3}) was fed to 4 day-old root cultures of *D. stramonium* (1.2 mg, per flask; 9 flasks, pulse-fed; grown on to 19 days).[5] The alkaloid extracted from these cultures (40.5 mg) had no mass enhancement of the extracted 3α-phenylacetoxytropane **10**, even though some oxidation of the fed (*RS*)-3-phenyl[2-^{13}C,2-^2H]lactic acid **4** to phenylpyruvic acid **5** had apparently occurred. In contrast, high incorporations into hyoscyamine **8**, littorine **7** and apoatropine were observed by GC–MS and NMR.[5] If C-1 were lost, as in the route *via* **5** and **6**, then the derived 3α-phenylacetoxytropane **1** would have shown a strong M + 1 enrichment. Thus, it is demonstrated directly that during incorporation C-2 of phenyllactic acid **4** is lost.

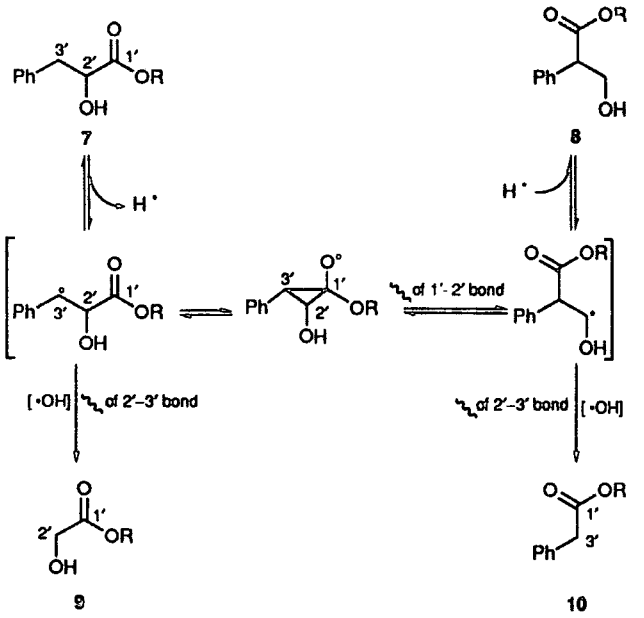
It is therefore indicated that the biosynthesis of 3α-phenylacetoxytropane **10** proceeds *via* a mechanism which does not involve free phenylpyruvic acid **6**. Rather, the close correlations of the levels of incorporation observed into hyoscyamine **8**/3α-phenylacetoxytropane **10** and hyoscine/3α-phenylacetoxy-6β,7β-epoxytropane (Tables 1 and 2) strongly suggest that the mechanism of biosynthesis of these alkaloids is closely linked.

Evidence that hyoscyamine **8** and 3α-phenylacetoxytropane **10** have a common biosynthetic origin is provided by the incorporation patterns seen after feeding (*RS*)-(3′-phenyl[1′,3′-$^{13}C_2$]lactoyl)[*methyl*-$^2H_3$]tropine **7** to cultures of *D. stramonium*.[10] This quintuply-labelled littorine is incorporated effectively into hyoscyamine **8** (Table 3), indicating that littorine **7** has rearranged directly. 3α-Phenylacetoxytropane **10** extracted from these cultures is also found to have a significant simultaneous incorporation of two ^{13}C and three ^2H nuclei to give a M + 5 mass ion (Table 3). The level of incorporation of the quintuply-labelled littorine **7** into the M + 5 ion of 3α-phenylacetoxytropane **10** is lower than into the M + 5 of hyoscyamine **8**, but still much higher than could have occurred *via* hydrolysis and partial re-use of the labelled material. If label from (*RS*)-(3′-phenyl[1′,3′-$^{13}C_2$]lactoyl)[*methyl*-$^2H_3$]tropine **7** only enters tropane alkaloids following hydrolysis and the separate reincorporation of the two parts of the precursor molecule, then products with M + 2 and M + 3 mass spectral peaks should be much more prominent than those with M + 5.[10] Incorporations into the M + 2 and M + 3 of 3α-phenylacetoxytropane **10** are, however, much smaller than into the M + 5 (Table 3) and, as a proportion of the total incorporation observed, are comparable with those determined for the M + 2 and M + 3 of hyoscyamine **8**. Therefore, it can be concluded that littorine **7** acts not only as a direct precursor for hyoscyamine **8**, as shown previously,[10] but also as a precursor for 3α-phenylacetoxytropane **10**.

In addition, we have observed that label from (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid **4** (Tables 1 and 2) or (*RS*)-(3′-phenyl[1′,3′-$^{13}C_2$]lactoyl)[*methyl*-$^2H_3$]tropine **7** (Table 3) is incorporated effectively into a M + 1 or M + 4 ion, respectively, of 3α-(2′-hydroxyacetoxy)tropane **9**. As can be seen, the level to which this base is labelled is comparable with the labelling of both hyoscyamine **8** and 3α-phenylacetoxytropane **10**. This would be extremely improbable were it to be derived by the esterification of tropine **2** with 2-hydroxyacetyl-coenzyme A. Furthermore, since no M + 5 ion was seen in 3α-(2′-hydroxyacetoxy)tropane **9** it cannot be derived by the degradation of hyoscyamine **8**, either endogenously or during extraction. Moreover, were 3α-(2′-hydroxyacetoxy)tropane **9** to be derived by the degradation of hyoscyamine, it is likely that 3α-(2′-hydroxyacetoxy)-6β,7β-epoxytropane, the equivalent degradation product of hyoscine, would simultaneously be detected. The absence of 3α-(2′-hydroxyacetoxy)-6β,7β-epoxytropane from the alkaloidal extract of *Brugmansia* supports the argument that 3α-(2′-hydroxyacetoxy)tropane **9** is a natural metabolite.

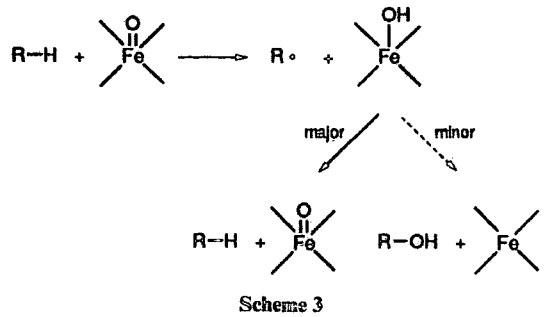
We deduce from these observations that the rearrangement

of littorine **7** to hyoscyamine **8** involves a reaction mechanism which, rarely, results in the loss of the C-2′ of littorine **7**, yielding 3α-phenylacetoxytropane **10** or, again rarely, the loss of the {C-3′ + phenyl}, giving 3α-(2′-hydroxyacetoxy)tropane **9**. A putative process is indicated in Scheme 2. Retention of the C-1′, C-2′ and C-3′ of littorine **7** after direct rearrangement to hyoscyamine **8** is the predominant outcome.



Scheme 2 A putative mechanism for the rearrangement of littorine to hyoscyamine (R = tropine; numbering refers to littorine)

It has been demonstrated [1–3] that the rearrangement involves the migration of the C-1 carboxy to C-3 and the simultaneous counter-migration of the 3-*pro*-(*S*)-H of phenylalanine (and therefore implicitly of 3-phenyllactate **4**) to the C-2′ of hyoscyamine **8**. We suggest, therefore, that the rearrangement is initiated by the abstraction of the 3′-*pro*-(*S*)-H from littorine **7**. This process may be anionic or radical in nature. Anionic carbon skeletal rearrangements are rare in biochemistry, whereas radical processes have precedent.[17] The potential similarity of this vicinal interchange process to methylmalonyl-CoA mutase[18] has been widely discussed.[3,19] Coenzyme B_{12}, however, is not associated with *Datura* plants,[19] which clearly limits development of an hypothesis involving this co-factor. More reasonably, we suggest that the radical process might be initiated by a haem-thiolate enzyme (cytochrome P450) as described for the flavanone–isoflavanone isomerisation.[20] In the light of the evidence discussed by Hakamatsuka *et al.*[20] that such processes may account for several other rearrangements in plants, we favour a free-radical process as outlined in Scheme 2 and summarised in Scheme 3. Homolytic abstraction of the 3′-*pro*-(*S*)-H of littorine **7** would generate a radical at C-3′, which may find stability by transient formation of a cyclopropyl alkoxyl radical. Cleavage of the bond between C-1′ and C-2′ would result in a rearranged free radical at the C-2′ position. Return of the sequestered 3′-*pro*-(*S*)-H proton from the enzyme (or cofactor) to this centre, as demonstrated by Leete,[3] would then generate hyoscyamine **8**. This is the major reaction. Supporting evidence for the involvement of a haem-thiolate protein is provided by the presence of **9** and **10** as minor co-metabolites. In two minor pathways, if the pre- and post-rearranged radicals are quenched by a hydroxyl radical, rather than by a hydrogen radical, then further oxidative processing would account for **9** and **10**, respectively as the end products of these oxidative pathways.



Scheme 3

The putative intermediacy of 2-formylphenylacetic acid between hyoscyamine **8** and **10** has also been suggested[21] and is consistent with our hypothesis. The extreme lability of this compound in neutral aqueous solution, however, renders it impossible to use in feeding experiments.

The loss of the hydroxymethyl group of the tropate moiety could in principle occur subsequent to hyoscyamine **8** formation. Small amounts of 3α-phenylacetoxytropane **10** and 3α-phenylacetoxy-6β,7β-epoxytropane can be generated from hyoscyamine and hyoscine, respectively, by thermal degradation in the heated split/splitless injector during analysis by GC–MS.[11] Several lines of evidence, however, indicate that this is not the cause of the isotopic incorporations seen here. Firstly, 3α-phenylacetoxytropane **10** is detected as a natural product in these extracts in GC profiles obtained using a cold on-column injector. Standard hyoscyamine **8** and hyoscine do not degrade under these conditions. Secondly, no 3α-(2′-hydroxyacetoxy)-tropane **9** occurs in standard hyoscyamine analysed by GC–MS. Thirdly, the M + 4 of 3α-(2′-hydroxyacetoxy)tropane **9** isolated following the feeding of quintuply-labelled littorine **7** has a higher level of incorporation than found in the recovered littorine **7** or hyoscyamine **8**: if it were an artifact this could not occur. Thus, it can be concluded that the labelling patterns seen are not due to post-extraction artifacts but arise from the metabolic processes involved in hyoscyamine **8** formation.

In conclusion, we propose that the unique rearrangement by which hyoscyamine **8** is formed from littorine **7** is a free radical process initiated by a haem-thiolate enzyme. Additionally, the pre- and post-rearranged radicals are adventitiously quenched by hydroxyl radicals to generate **9** and **10** after further oxidative processes.

Experimental

General.—(*RS*)-3-Phenyl[1,3-$^{13}C_2$]lactic acid (atom excess: 81 ± 2% at M + 2 ion)[8] (*RS*)-phenyl[2-^{13}C,2-^{2}H]lactic acid (atom excess: 99% at M + 2 ion)[5] and (*RS*)-(3′-phenyl[1′,3′-$^{13}C_2$]lactoyl)[*methyl*-$^{2}H_3$]tropine (atom excess: 0% at M ion, 0% at M + 1 ion, 0.3% at M + 2 ion, 1.8% at M + 3 ion, 18.7% at M + 4 ion, 81 ± 2% at M + 5 ion)[10] were prepared as described. (*RS*)-3-Phenyllactic acid and tropine were from Sigma Chemical Company (Poole, Dorset, UK) and Aldrich Chemical Company (Gillingham, Kent, UK), respectively.

Cultures.—Root cultures of *Datura stramonium* L. D15/5[22] and *Brugmansia* (*Datura*) *candida* x *B. aurea*[23] were grown as described. Feeding experiments were performed as described previously.

Alkaloid Separation and Identification.—Alkaloids were extracted and analysed by GC–MS essentially as described previously.[9,15]

Acknowledgements

We are grateful to Dr. Peter Bachmann for assistance with

the (*RS*)-(3'-phenyl[1',3'-$^{13}C_2$]lactoyl)[*methyl*-$^{2}H_3$]tropine-feeding experiment; to John Eagles (IFR) and Louise Tatton (IFR) for assistance with running the GC–MS analyses; to Birgit Dräger for valuable discussions; to Abbi Peerless for maintaining the cultures; to Morteza Ansarin for the synthesis of (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid; to the Nuffield Foundation, London, for financial support to J. G. W. and to the SERC, and the University of Durham for support to N. C. J. E. C.

Note in Proof.—The stereochemistry of this process is now fully established.[24–26]

References

1 E. Leete, N. Kowanko and R. A. Newark, *J. Am. Chem. Soc.*, 1975, **97**, 6826.
2 E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271.
3 E. Leete, *Can. J. Chem.*, 1987, **65**, 226.
4 M. Ansarin and J. G. Woolley, *Phytochemistry*, 1993, **32**, 1183.
5 N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1994, 1159.
6 E. Leete, *Phytochemistry*, 1983, **22**, 933.
7 R. J. Cox and D. O'Hagan, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2537.
8 M. Ansarin and J. G. Woolley, *Phytochemistry*, 1994, **35**, 935.
9 R. J. Robins, J. G. Woolley, M. Ansarin, J. Eagles and B. J. Goodfellow, *Planta*, 1994, **194**, 86.
10 R. J. Robins, P. Bachmann and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, 1994, 615.
11 A. J. Parr, J. Payne, J. Eagles, B. T. Chapman, R. J. Robins and M. J. C. Rhodes, *Phytochemistry*, 1990, **29**, 2545.
12 R. J. Robins, P. Bachmann, A. C. J. Peerless and S. Rabot, *Plant Cell Tissue Org. Cult.*, 1995, in the press.
13 R. J. Robins, P. Bachmann, T. Robinson, Y. Yamada and M. J. C. Rhodes, *FEBS Lett.*, 1991, **292**, 293.
14 S. Rabot, A. C. J. Peerless and R. J. Robins, *Phytochemistry*, 1995, in the press.
15 B. Dräger, A. Portsteffen, A. Schaal, P. McCabe, A. C. J. Peerless and R. J. Robins, *Planta*, 1992, **188**, 581.
16 T. Hashimoto and Y. Yamada, *Eur. J. Biochem.*, 1987, **164**, 277.
17 R. G. Finke, in *Molecular Mechanisms in Bioorganic Processes*, eds. C. Bleasdale and B. T. Golding, Royal Society of Chemistry, 1990, p. 245.
18 M. I. Page and A. Williams, *Enzyme Mechanisms*, Royal Society of Chemistry, 1987, p. 404.
19 E. Leete, *Planta Med.*, 1990, **56**, 339.
20 T. Hakamatsuka, M. F. Hashim, Y. Ebizuka and U. Sankawa, *Tetrahedron*, 1991, **47**, 5969.
21 G. G. Gross, K. J. Koelen and A. Müller, *Z. Naturforsch., C Biosci.*, 1981, **36**, 611.
22 R. J. Robins, A. J. Parr, E. G. Bent and M. J. C. Rhodes, *Planta*, 1991, **183**, 185.
23 R. J. Robins, A. J. Parr, J. Payne, N. J. Walton and M. J. C. Rhodes, *Planta*, 1990, **181**, 414.
24 M. Ansarin and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, 1995, 487.
25 N. C. J. E. Chesters, D. O'Hagan, R. J. Robins, A. Kastelle and H. G. Floss, *J. Chem. Soc., Chem. Commun.*, 1995, 129.
26 N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, *J. Chem. Soc., Chem. Commun.*, 1995, 127.

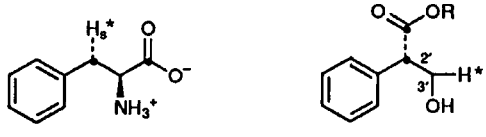
Paper 4/05057F
Received 17*th August* 1994
Accepted 8*th November* 1994

The Biosynthesis of Tropic Acid In Plants: Evidence for the Direct Rearrangement of 3-Phenyllactate to Tropate

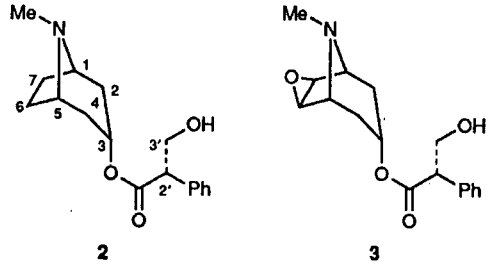
Nicola C. J. E. Chesters,[a] David O'Hagan *[,a] and Richard J. Robins *[,b]
[a] *University of Durham, Department of Chemistry, Science Laboratories, South Road, Durham DH1 3LE, UK*
[b] *Plant Biotechnology Group, Department of Genetics and Microbiology, Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK*

Dual labelled sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate was incubated with transformed root cultures of *Datura stramonium* and the incorporation of the label into the tropic acid ester moiety of hyoscyamine was assessed by $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ NMR spectroscopy and by GC–MS analysis. It is demonstrated that the ^{13}C–^{2}H bond of sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate is incorporated (GC–MS, M + 2, 17%) intact into the hydroxymethyl group at C-3′ of the (*S*)-tropoyl moiety. This result demonstrates unambiguously that 3-phenyllactate is a closer precursor to the tropate ester than phenylpyruvate or phenylalanine.

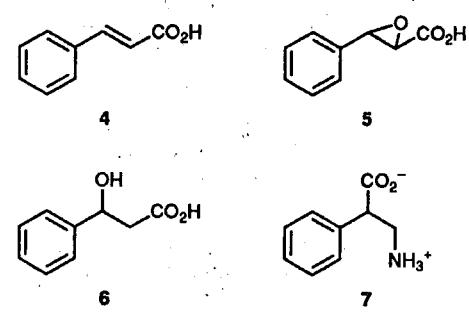
There has been considerable interest over many years in the biosynthesis of (*S*)-tropic acid **1**, the ester moiety of the tropane alkaloids hyoscyamine **2** and scopolamine **3**.[1,2] It is now well established that tropic acid has an origin in (*S*)-phenylalanine.[3] Perhaps the most intriguing feature of tropic acid biosynthesis is a carbon skeletal rearrangement of (*S*)-phenylanine to give tropic acid (Scheme 1). This was established in a definitive study[4] by Leete *et al.*, who fed (*RS*)-phenyl[1,3-$^{13}C_2$]alanine to *Datura innoxia* plants, and demonstrated that the two labelled carbons became contiguous, generating the [1,2-$^{13}C_2$]-tropic acid ester in the alkaloids. Subsequent studies[5,6] have established that the carboxylate group migrates with retention of configuration at C-3 and that the 3-*pro*-*S* hydrogen of (*S*)-phenylalanine migrates in the reverse direction. This 1,2-vicinal interchange process is illustrated in Scheme 1.



Scheme 1

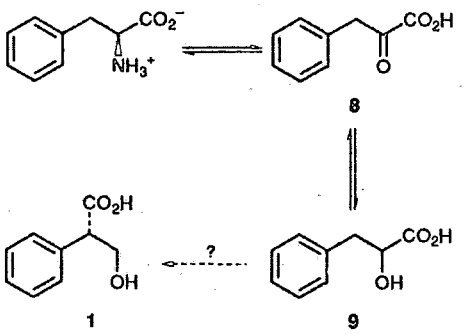


Considerable effort has been expended on identifying the intermediates between phenylalanine and the tropoyl moiety of hyoscyamine, scopolamine and other aromatic esters of tropine. It was demonstrated some time ago that cinnamic acid[7] **4**, its epoxide[8] **5** and 3-hydroxy-3-phenylpropanoic acid[9] **6** were not precursors and we have established[10] that (*RS*)-3-amino-2-phenylpropionic acid **7**, the rearranged product of phenylalanine, is not involved in the process. In contrast, feeding experiments with both whole plants[11–13] and root cultures[14] have established that phenylpyruvic and 3-phenyllactic acids

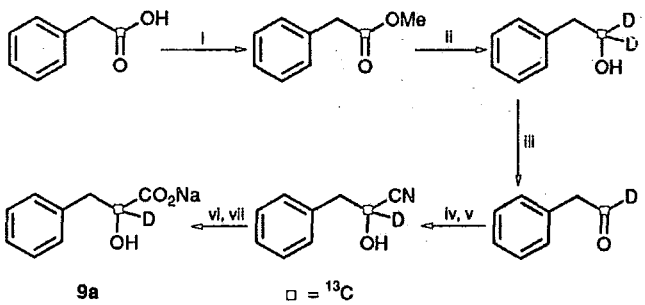


are readily incorporated into a range of tropane alkaloids. The rearrangement of 3-phenyllactic acid (or an ester) would give tropate directly. That this occurs was clearly demonstrated by feeding 3-phenyl[1,3-$^{13}C_2$]lactic acid to whole plants[13] or root cultures[14] of *D. stramonium* and showing the contiguity of the ^{13}C nuclei in the derived hyoscyamine and scopolamine. Furthermore, Ansarin and Woolley have demonstrated[11] with *D. stramonium* plants that ^{3}H from (*RS*)-3-phenyl[1-^{14}C,2-^{3}H]lactate is incorporated into the hydroxymethyl group at C-3′ of the tropoyl moiety of hyoscyamine, albeit with a low specific incorporation (0.1%). Over a series of experiments, the ^{3}H:^{14}C ratio of the recovered tropic acid remained similar to that of the 3-phenyl[1-^{14}C,2-^{3}H]lactate administered to the plants. This result suggests that 3-phenyllactic acid **9** is an obligatory precursor to this group and that it is not first oxidised to phenylpyruvic acid **8**. Additional evidence from competitive feeding experiments in which phenyl[1-^{14}C]-alanine, phenyl[2-^{14}C]pyruvate or 3-phenyl[1-^{14}C]lactate were fed with added unlabelled precursors,[12] indicates that phenyllactate is the closest precursor to the tropoyl moiety of hyoscyamine and scopolomine.

We are prompted now to report our results on the incorporation of (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate **9a** into the tropic acid ester moiety of hyoscyamine **2**. A dual isotope [^{13}C–^{2}H] labelling strategy was employed to allow an unambiguous assessment of the fate of the C–2–H bond of phenyllactate during tropic acid biosynthesis. Oxidation to phenylpyruvic acid **8**, with or without subsequent transamination to (*S*)-phenylalanine, would result in the loss of the deuterium atom (Scheme 2). On the other hand direct rearrangement would result in retention of the ^{13}C–^{2}H bond at C-3′ in the tropoyl moiety of hyoscyamine. The extent of incorporation of ^{13}C



Scheme 2

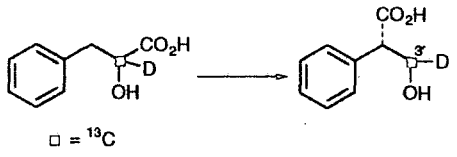


Scheme 3 *Reagents and conditions:* i, CH_2N_2; ii, $LiAlD_4$; iii, pyridinium chlorochromate (PCC), 3 Å mol sieves; iv, $NaHSO_3$; v, NaCN; vi, 50% HCl; vii, dil. NaOH

alone will indicate the degree to which phenylpyruvate has acted as an intermediary in the incorporation. To this end, sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate **9a** was prepared by the synthetic route outlined in Scheme 3. This double-labelled precursor was fed to transformed root cultures of *D. stramonium*. These cultures produce hyoscyamine as the predominant alkaloid[15] with lesser amounts of other alkaloids, including littorine. Examination of the crude alkaloidal extract by GC–MS showed both labels to have been incorporated into littorine, hyoscyamine and apoatropine as indicated by enhancements of the M + 2 ions by 18, 17 and 12% respectively, indicating ^{13}C–^{2}H incorporations. Preparative TLC gave hyoscyamine **2** containing a small amount (4.6%) of littorine **10**. These two alkaloids have similar chromatographic characteristics but distinct ^{13}C NMR resonances for the C-1′, C-2′ and C-3′ nuclei. Thus, the presence of a small amount of littorine did not interfere when the mixture was examined by ^{13}C{^{1}H} and ^{13}C{^{1}H,^{2}H} NMR spectroscopy. In the ^{13}C{^{1}H} spectrum [spectrum (*a*) in Fig. 1] the incorporation of ^{13}C from **9a** into the C-3′ of the tropate moiety of **2** was evident by an enrichment at δ 63.9.[4] Of greater significance however, was a triplet ($J_{^{13}C,^{2}H}$ 22 Hz) shifted further upfield by 0.35 ppm (α-shift) diagnostic[16] of the intact incorporation of ^{13}C–^{2}H at C-3′ retained from the 3-phenyl[2-^{13}C,2-^{2}H]lactate precursor. As expected, this triplet collapsed to a singlet in the ^{13}C{^{1}H,^{2}H} NMR experiment [spectrum (*b*), Fig. 1].

A similar pattern was also evident for the minor peaks at δ 71.5 (singlet) and 71.1 (triplet) in spectrum (*a*) in Fig. 1, arising from ^{13}C- and ^{13}C–^{2}H-enrichments, respectively, at C-2′ of the 3-phenyllactoyl moiety of littorine **10**. The triplet at δ 71.1 again collapsed to a singlet in the ^{13}C{^{1}H,^{2}H} NMR experiment [spectrum (*b*), Fig. 1]. These isotopic enrichments clearly arise as a result of direct esterification of the labelled 3-phenyllactate during littorine biosynthesis.

It is clear from this study that the C-2–H bond of 3-phenyllactic acid **9** can remain intact during the rearrangement process to become one of the C-3′–H bonds of the tropoyl moiety of hyoscyamine (Scheme 4). This experiment demonstrates unambiguously that 3-phenyllactic acid is a more direct precursor than phenylpyruvic acid **8** on the pathway to the tropate ester, confirming evidence obtained previously by inference.[12] It is noteworthy that there was significant washout of deuterium in both the resultant hyoscyamine **2** and littorine **3**. The mass enhancements, determined by GC–MS, of the M + 1 ions of littorine and hyoscyamine were 5.1 and 5.7% respectively, approximately one-third that of the M + 2 ions. This finding indicates that significant loss of the ^{2}H has occurred *in vivo* during the experiment. The most likely cause of this is the interconversion of 3-phenyllactate with phenylpyruvate. This contrasts with the previous report that negligible loss of ^{3}H relative to ^{14}C occurred.[11] However the operation of a kinetic isotope effect and the different biological systems used (plants *versus* root cultures) may invalidate too critical a comparison between these two experiments. Littorine has recently been demonstrated to be converted directly into hyoscyamine.[17] This evidence, coupled with the physiological evidence that free topic acid is not incorporated into hyoscyamine,[3,14] indicates that the putative mutase enzyme involved in hyoscyamine biosynthesis probably acts on littorine as a substrate. The similarity of this rearrangement to coenzyme B_{12}-mediated vicinal interchange processes, such as methylmalonyl-CoA mutase, has been discussed previously,[2,6] and is becoming increasingly striking. The occurrence of vitamin-B_{12} in higher plants is, however, not well documented and the



Scheme 4

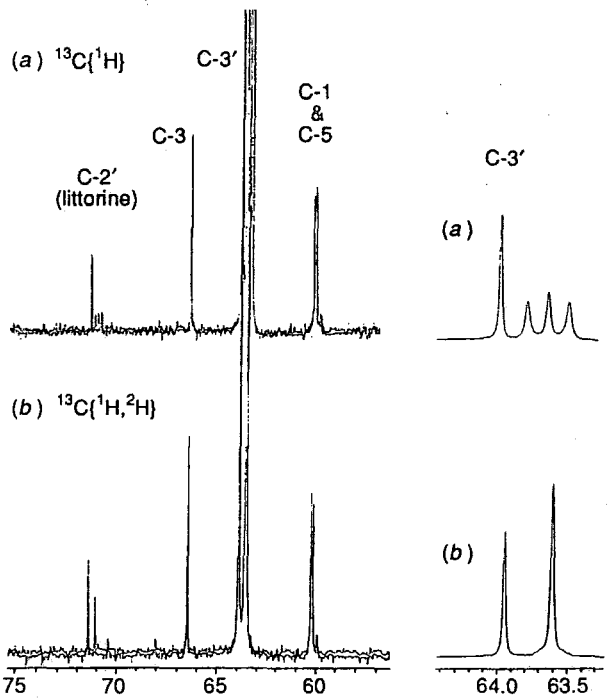
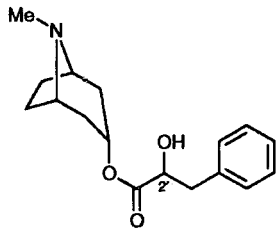


Fig. 1 (*a*) ^{13}C{^{1}H} and (*b*) ^{13}C{^{1}H,^{2}H} NMR spectra of hyoscyamine after incorporation of sodium DL-3-phenyl[2-^{13}C,2-^{2}H]lactate. The ^{13}C–^{2}H incorporation is evident by a triplet in spectrum (*a*) at δ 63.6, upfield from the ^{13}C-signal assigned to C-3′ of tropate at δ 63.9. The triplet collapses to a singlet in spectrum (*b*) (see inset for clarification). The triplet at δ 71.5 in spectrum (*a*) also collapses to a singlet in spectrum (*b*), 0.35 ppm upfield from the enriched uncoupled signal corresponding to C-2′ of littorine. The signals from C-1, C-3 and C-5 of hyoscyamine are also shown.

involvement of this co-factor must remain speculative. Indeed a study[6] by Leete failed to detect any vitamin-B_{12} from tropane-alkaloid-producing *Datura* plants.



10

Studies to determine the absolute stereochemistry of the 3-phenyllactate employed in the rearrangement process, and the stereochemical location of the hydrogen migrating to C-3′ in tropate, are ongoing.

Experimental

General Details.—IR spectra were recorded on a Perkin-Elmer F.T. 1720X or 1600 spectrometer. Mass spectra were recorded on a VG Analytical 7070E Organic mass spectrometer. NMR spectra were recorded on Varian Gemini 200 MHz (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz), Varian XL-200 (^{1}H at 200.057 MHz), Varian VXR 400(S) (^{1}H at 399.952 ^{13}C at 100.577 MHz) and 600 MHz Edinburgh spectrometers. Chemical shifts are quoted relative to TMS (Me_4Si) in $CDCl_3$ and H_2O in D_2O, all coupling constants are in Hz. GC–MS were recorded on a VG TRIO-1S mass spectrometer (VG Masslab Ltd., Manchester) fitted with a Hewlett Packard 5890 series II gas chromatograph (Hewlett Packard Inc., Fort Collins, USA) and a DB-17 column (J&W Scientific, Folsom, USA) was used for separation.[18] Flash chromatography was carried out using silica gel-60 (35–70 μm) (Fluka) or Sorbsil-C60-H (40–60 μm). All solvents were dried and distilled prior to use and ether refers to diethyl ether.

2-Phenyl[1-^{13}C,1-$^2H_2$]*ethanol.*—Phenyl[1-^{13}C]acetic acid (1.0 g, 7.34 mmol, 99 atom% ^{13}C, Aldrich Chem. Co. Ltd.) was converted directly into its methyl ester by the addition of an excess of an ethereal solution of diazomethane. The excess of CH_2N_2 was destroyed after 5 min by addition of a few drops of glacial acetic acid. After the solution had been dried ($MgSO_4$) the solvent was removed under reduced pressure to give methyl phenyl[1-^{13}C]acetate, which was then dissolved in ether (10 cm^3) and added to a stirred suspension of lithium aluminium-deuteride (1.3 g, 35 mmol) in ether (40 cm^3) and the reaction mixture was heated under reflux for 2 h under N_2. The reaction was then quenched by the addition of wet ether (30 cm^3) and then 5% H_2SO_4 (30 cm^3). The aqueous layer was extracted into ether (2 × 30 cm^3) and the combined organic extracts dried ($MgSO_4$), and evaporated under reduced pressure to give a yellow oil. Purification by chromatography over silica gel (CH_2Cl_2–ether, 9:1), afforded 2-phenyl[1-^{13}C,1-$^2H_2$]ethanol as a clear oil (0.65 g, 5.2 mmol, 71%); ν_{max}(neat)/cm^{-1} 3346br, 3027, 2931, 2190, 2090, 1603, 1496 and 1453; *m/z* (EI+) 125 (M^+ 38.60%), 91 (100); δ_H($CDCl_3$) 2.15 (1 H, s, OH), 2.81 (2 H, d, $J_{^{13}C,^1H}$ 5.3, CH_2) and 7.25 (5 H, m, ArH); δ_C($CDCl_3$) 39.5 (d, $J_{^{13}C,^{13}C}$ 35, C-2), 63.4 (quintet, $J_{^{13}C,^2H}$ 21.9, C-1), 126.9 (C-4′), 129.1 (C-2′, -6′), 129.5 (C-3′, -5′) and 139.1 (C-1′).

Phenyl[1-^{13}C,1-^{2}H]*acetaldehyde.*—To a stirred suspension of pyridinium chlorochromate (2.9 g, 13.4 mmol) and dry powdered 3 Å molecular sieves (35 g) in dichloromethane (60 cm^3) was added 2-phenyl[1-^{13}C,1-$^2H_2$]ethanol (0.65 g, 5.2 mmol) and the reaction mixture was stirred vigorously for 2 h. The reaction mixture was filtered through a silica gel pad, washing with dichloromethane (400 cm^3). The solvent was removed under reduced pressure to give phenyl[1-^{13}C,1-^{2}H]-acetaldehyde (0.533 g, 4.53 mmol, 87%) which was used directly, without further purification.

Selected spectroscopic data from an unlabelled synthesis: ν_{max}(neat)/cm^{-1} 3020, 1725, 1500, 1455, 750 and 700; δ_H($CDCl_3$) 3.66 (2 H, d, J_{vic} 2.3, CH_2), 7.30 (5 H, m, Ar-H) and 9.72 (1 H, d, 2.3, CHO); δ_C($CDCl_3$) 50.54 (C-2), 127.4 (C-4′), 129.9 (C-2′, -6′), 129.6 (C-3′, -5′), 131.9 (C-1′) and 199.4 (C-1).

Sodium (RS)-3-*phenyl*[2-^{13}C,2-^{2}H]*lactate* **9a**.—A solution of sodium metabisulfite (1.04 g, 5.47 mmol) in water (5 cm^3) was added to phenyl[1-^{13}C,1-^{2}H]acetaldehyde (0.533 g, 4.53 mmol) and the mixture was shaken vigorously for 10 min. Sodium cyanide (0.54 g, 11.02 mmol) was then added in several portions to the reaction mixture, and the cyanohydrin could be seen forming as a clear oil after a few minutes. This was then extracted into benzene (3 × 15 cm^3) and the organic extracts were combined, dried ($MgSO_4$) and evaporated to afford a clear oil. 50% HCl (20 cm^3) was added to the oil and the reaction heated under reflux for 2 h. The solution was allowed to cool and the product was extracted into ether (3 × 30 cm^3). The organic extracts were combined, dried ($MgSO_4$) and evaporated to afford (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactic acid, which was recrystallised from chloroform, m.p. 96–98 °C (racemate).

Selected analytical and spectroscopic data from an unlabelled synthesis (Found: C, 64.7; H, 5.9. $C_9H_{10}O_2$ requires C, 65.0; H, 6.02%); δ_H($CDCl_3$) 2.98 (1 H, dd, *J* 14, 6.9, C*H*H), 3.19 (1 H, dd, *J* 14, 4.2, CH*H*), 4.50 (1 H, dd, *J* 6.9, 4.2, CH) and 7.30 (5 H, m, Ar-H); δ_C($CDCl_3$) 40.1 (C-3), 71.0 (C-2), 127.1 (C-4′), 129.6 (C-2′, -6′), 129.5 (C-3′, -5′), 135.8 (C-1′) and 178.6 (C-1).

The 3-phenyllactic acid was then dissolved in chloroform (10 cm^3) and covered with water (10 cm^3) and the aqueous layer was adjusted to pH 8 with aqueous NaOH (0.1 mol dm^{-3}). Separation of the aqueous layer and evaporation of the water under reduced pressure afforded sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate (128 mg, 0.673 mmol, 15%) as a white amorphous solid; δ_H(D_2O) 2.74 (1 H, dd, J_{gem} 14.0, $J_{^{13}C,^1H}$ 5.1, CH_2), 2.97 (1 H, dd, J_{gem} 14.1, $J_{^{13}C,^1H}$ 3.8, CH_2) and 7.22 (5 H, m, Ar-H); δ_C(D_2O), 43.1 (d, $J_{^{13}C,^{13}C}$ 35.4, C-3). 75.9 (t, $J_{^{13}C,^2H}$ 22.4, C-2), 129.5 (C-4′), 131.4 (C-2′, -6′), 132.3 (C-3′, -5′) and 141.1 (C-1′). An additional signal in the ^{13}C NMR spectrum at δ 183.9 was apparent, only in the isotopically enriched synthesis, and was estimated to constitute 3.6% of the label. This was assigned to the carbonyl resonance of sodium phenyl[1-^{13}C]acetic acid after analysis of the ^{1}H NMR spectrum which showed a small doublet ($J_{^{13}C,^1H}$ 6.8) in the base line at δ 3.41. Reanalysis of ^{1}H NMR spectra from an unlabelled synthesis showed a minor signal (singlet) at δ 3.41, consistent with this interpretation.

Feeding Experiment and Alkaloid Extraction.—Transformed root cultures of *D. stramonium* D15/5 were maintained and grown in B50 medium as previously described.[15] Nine subcultured flasks each containing an initial inoculum of 0.5 g fresh mass of roots in 50 cm^3 of medium were pulse-fed with a sterile neutral solution of sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate (21 mmol dm^{-3}) on days 5, 7 and 9 to a final concentration of 0.4 mmol dm^{-3} in the medium. The roots were harvested after 17 days. The freeze-dried roots (2.72 g) were ground with acid-washed sand and extracted into H_2SO_4 (0.05 mol dm^{-3}; 60 cm^3) by stirring for 15 min. The aqueous extract was made basic with 35% NH_3 solution (10 cm^3) and the solution filtered through Kieselguhr (Varian Bondelut*) and eluted with $CHCl_3$–MeOH (20:1). The eluent was

evaporated to give a brown oil (40.5 mg), which was purified by preparative TLC ($CHCl_3$–Et_2NH, 9:1) to afford hyoscyamine (18.1 mg) contaminated with a trace amount of littorine (0.83 mg).

Acknowledgements

We thank Dr. John Parkinson of the Edinburgh University Ultra High Field NMR Service for recording the $^{13}C\{^1H,^2H\}$ spectra, Dr. Brian J. Goodfellow (IFR) for obtaining a 100.4 MHz ^{13}C NMR spectrum of littorine and the sample, John Eagles for GC–MS and Abbi Peerless and Dr. Peter Bachmann for help with the cultures and feeding experiments. Also we are most grateful to Dr. J. G. Woolley (De Montford University, Leicester) for providing an authentic sample of littorine and to the SERC (studentship to N. C. J. E. C.) and the University of Durham for financial support.

References

1 E. Leete, *Planta Med.*, 1979, **36**, 97.
2 E. Leete, *Planta Med.*, 1990, **56**, 339.
3 Y. Kitamura, A. Taura, Y. Kajiya and H. Miura, *J. Plant Physiol.*, 1992, **142**, 141.
4 E. Leete, N. Kowanko and R. A. Newmark, *J. Am. Chem. Soc.*, 1975, **97**, 6826.
5 E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271.
6 E. Leete, *Can. J. Chem.*, 1987, **65**, 226.
7 W. C. Evans and J. G. Woolley, *Phytochemistry*, 1976, **15**, 287.
8 E. Leete and E. P. Kirven, *Phytochemistry*, 1974, **13**, 1501.
9 E. Leete, *Phytochemistry*, 1983, **22**, 933.
10 R. J. Cox and D. O'Hagan, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2537.
11 M. Ansarin and J. G. Woolley, *Phytochemistry*, 1993, **32**, 1183.
12 M. Ansarin and J. G. Woolley, *J. Nat. Prod.*, 1993, **56**, 1211.
13 M. Ansarin and J. G. Woolley, *Phytochemistry*, in the press.
14 R. J. Robins, J. G. Woolley, M. Ansarin, J. Eagles, B. J. Goodfellow, *Planta*, in the press.
15 R. J. Robins, A. J. Parr, E. G. Bent and M. J. C. Rhodes, *Planta*, 1991, **183**, 185.
16 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, 539.
17 R. J. Robins, P. Bachmann and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, 1994, 615.
18 B. Dräger, A. Portsteffen, A. Schaal, P. McCabe, A. C. J. Peerless and R. J. Robins, *Planta*, 1992, **188**, 581.

Paper 3/07460I
Received 20*th December* 1993
Accepted 19*th January* 1994

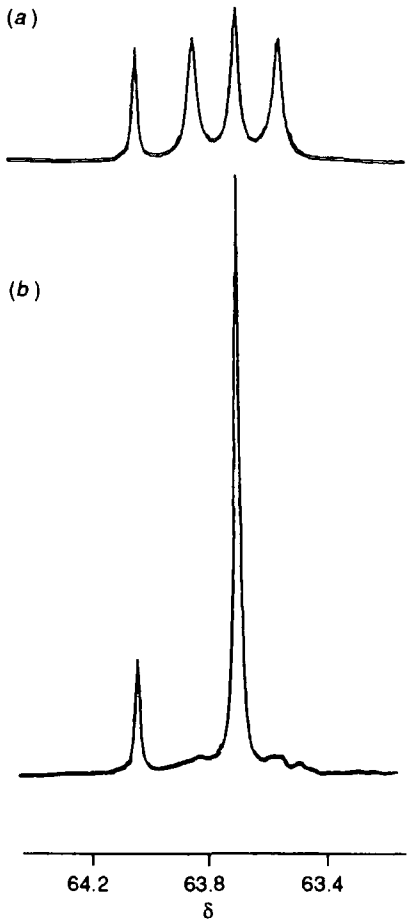


Fig. 1 The C-3′ resonance at δ 64.05 of the tropate moiety of **1** after incorporation of (*R*)-D-phenyl[2-^{13}C,^{2}H]lactic acid **5a**. The intact ^{13}C–^{2}H incorporation is evident by the triplet at δ 63.7 in (*a*) corresponding to the deuterium-induced α-shift, and ^{13}C–^{2}H coupling. This triplet collapses to a singlet in (*b*), the ^{13}C{^{1}H,^{2}H} spectrum.

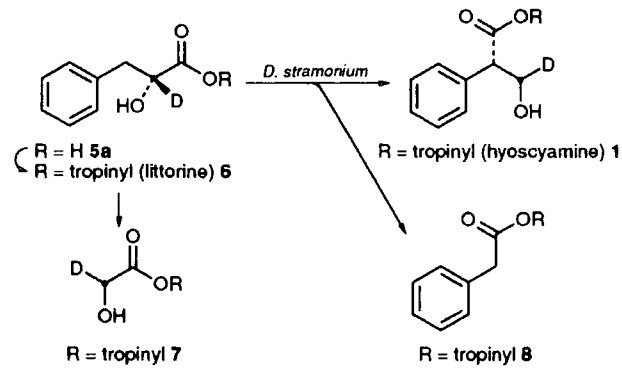
Table 1 Isotope enrichments of alkaloids determined by GCMS analysis after feeding experiments with **5a** and **5b**. Values represent the total isotope excess, corrected for natural abundance.[7]

	Phenyl[2-^{13}C,^{2}H]lactate			
	(*R*)-D	**5a**	(*S*)-L	**5b**
	M + 1	M + 2 (%)	M + 1	M + 2 (%)
1 (hyoscyamine)	0.8	21.7	5.1	2.4
6 (littorine)	1.5	28.9	10.3	3.3
7	1.4	18.4	9.8	1.0
8	−1.5[a]	−0.4	−0.6	−0.4

[a] Negative values represent an error in determination of ≈ 2%.

tropane **8**, from either enantiomer, consistent with our previous evaluation[14] that the phenylacetyl ester moiety originates from hyoscyamine *after* rearrangement of littorine. Adventitious oxidative removal of the C-3′ carbon of the tropate moiety during hyoscyamine formation emerges as the most likely pathway to **8**.

These experiments demonstrate unambigiously that (*R*)-D-phenyllactate **5a** is processed more directly that (*S*)-L-phenyllactate **5b**‡ and are consistent with (*R*)-D-littorine **6** as the true substrate for the mutase as shown in Scheme 2. (*R*)-D-Littorine is shown to be a precursor of hydroxyacetyl tropane and hyoscyamine and by implication[14] of phenylacetoxy tropane. It is already established[16] that littorine **6** has the (*R*)-D-configuration and clearly this stereochemical result is consistent with the direct interconversion of littorine and hyoscyamine in *Datura stramonium*. Implicit in this conclusion is the role of a (*R*)-D-phenyllactate dehydrogenase operating at a pivotal point between phenylalanine metabolism and alkaloid biosynthesis.



Scheme 2 The metabolic relationship between co-produced alkaloids in *Datura stramonium*. The (*R*)-D-phenyllactate moiety of littorine **6** is converted directly to hyoscyamine **1** and also labels the hydroxyacetyl moiety of **7**. No isotope is incorporated into the phenylacetyl moiety of **8**.

We thank Dr John Parkinson of the Edinburgh University ultra high field NMR Service for recording ^{13}C NMR spectra and Mrs Louise Tatton for recording GCMS spectra. The EPSRC is gratefully acknowledged for a studentship (N. C. J. E. C.) and the University of Durham is thanked for additional financial support.

Received, 25th October 1994; Com. 4/06541G

Footnotes

† (*R*)-D-Phenyl[2-^{13}C,^{2}H]lactate **5a**: mp 119–121 °C (lit.[14] 124–125 °C), $[\alpha]_D^{23}$ = +17.28 (*c* 4.6, EtOH); (*S*)-L-phenyl[2-^{13}C,^{2}H]lactate **5b**: mp 120–121 °C, $[\alpha]_D^{23}$ = −16.0 (*c* 10, EtOH). In both cases unlabelled preparations of **5a** and **5b** gave the higher optical rotation values of +22.5 (*c* 4.4, EtOH) [lit.[14] +19 (*c* 3.1, EtOH)] for **5a** and −21.27 (*c* 23.5, EtOH) for **5b**. We attribute this anomaly to the presence of the deuterium atom at the chiral centre. The optical purity of our samples was found to be *at least* >95% ee in each case after conversion of **5a** and **5b** to their methyl ester acetates, by ^{1}H NMR using the chiral shift reagent tris[3-heptafluoropropylhydroxymethylene)-(+)-camphoratol, europium(III) derivative [Eu(hfc)$_3$].

‡ During the preparation of this manuscript we became aware that Dr J. G. Woolley, De Montfort University, Leicester, has drawn a complementary conclusion after incorporation studies of (*R*)-D- and (*S*)-L-phenyl[1,3-^{13}C$_2$;1-^{14}C]lactate into scopolamine and hyoscyamine from *Datura stramonium* plants.[15]

References

1 E. Leete, *Planta Med.*, 1990, **56**, 97; 1979, **36**, 97.
2 E. Leete, N. Kowanko and R. A. Newmark, *J. Am. Chem. Soc.*, 1975, **97**, 6826.
3 E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271.
4 E. Leete, *Can. J. Chem.*, 1987, **65**, 226.
5 M. Ansarin and J. G. Woolley, *Phytochemistry*, 1993, **32**, 1183.
6 N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, *J. Chem. Soc., Perkin Trans. 1*, 1994, 1159.
7 R. J. Robins, P. Bachmann and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, 1994, 615.
8 W. C. Evans and J. G. Woolley, *Phytochemistry*, 1976, **15**, 287.
9 E. Leete and E. P. Kirven, *Phytochemistry*, 1974, **13**, 1501.
10 E. Leete, *Phytochemistry*, 1983, **22**, 933.
11 R. J. Cox and D. O'Hagan, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2537.
12 K. Saigo, H. Miura, K. Ishizaki and H. Nohira, *Bull. Chem. Soc., Jpn.*, 1982, **55**, 1188.
13 E. Leete and E. P. Kirven, *Phytochemistry*, 1974, **13**, 1501.
14 R. J. Robins, N. C. J. E. Chesters, D. O'Hagan, A. J. Parr, N. J. Walton and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, in the press.
15 M. Ansarin and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, submitted.
16 J. R. Cannon, K. R. Joshi, G. V. Meehan and J. R. Williams, *Aust. J. Chem.*, 1969, **22**, 221.

The Biosynthesis of Tropic Acid: The (*R*)-D-Phenyllactyl moiety is processed by the Mutase involved in Hyoscyamine Biosynthesis in *Datura stramonium*

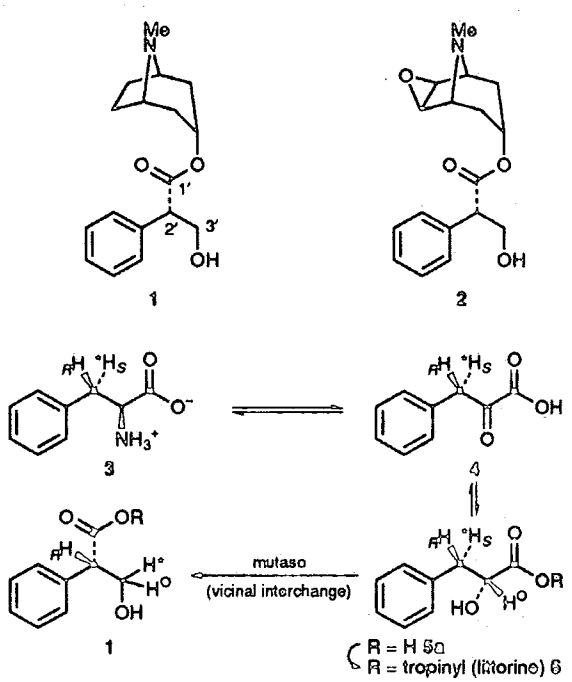
Nicola C. J. E. Chesters,[a] David O'Hagan*[a] and Richard J. Robins[b]

[a] *The Department of Chemistry, University of Durham, Science Laboratories, South Road, Durham, UK DH1 3LE*
[b] *Plant Biotechnology Group, Department of Genetics and Microbiology, Institute of Food Research, Norwich Research Park, Colney, Norwich, UK NR4 7UA*

Incubations of (*R*)-D-phenyl[2-^{13}C,^{2}H]lactic acid **5a** and (*S*)-L-phenyl[2-^{13}C,^{2}H]lactic acid **5b** with transformed root cultures of *Datura stramonium* have revealed that the ^{13}C–^{2}H bond is retained, intact at C-3 of the tropate moiety of hyoscyamine, only in the case of the (*R*)-D enantiomer **5a** (the deuterium is lost from the (*S*)-L-enantiomer **5b**); therefore, it is the (*R*)-D-phenyllactate moiety of littorine which is the enantiomer processed by the mutase in hyoscyamine biosynthesis.

The (*S*)-tropate ester moiety is found in the alkaloids hyoscyamine **1** and scopolamine **2** and its biosynthetic origin has been the focus of much interest for many years.[1] In 1975 Leete *et al.* showed[2] that the tropate moiety of hyoscyamine **1** originates from an intramolecular rearrangement of the L-phenylalanine **3** skeleton. Feeding of L-phenyl[1,3-$^{13}C_2$]alanine **3** to *Datura* plants resulted in hyoscyamine **1** with a [1,2-$^{13}C_2$]-labelled tropate moiety. The resultant contiguous arrangement of isotopes established the intramolecular nature of the rearrangement. Leete also demonstrated[3,4] that the carboxylate group of phenylalanine **3** migrates to C-3 with retention of configuration and that the 3-*pro*-*S* hydrogen of L-phenylalanine is abstracted during the rearrangement and is delivered to the hydroxymethyl carbon, C-3′ of **1** (Scheme 1).

Recently, phenyllactate has been identified[5,6] as an obligatory intermediate in tropate biosynthesis in *Datura stramonium*. A radiolabelled study[5] showed that the ^{3}H : ^{14}C ratio from fed (*R,S*)-DL-phenyl[1-^{14}C,2-^{3}H]lactate remained essentially the same in the recovered hyoscyamine **1** and scopolamine **2**. In a stable isotope study[6] we have demonstrated that the ^{13}C–^{2}H bond of (*R,S*)-DL-phenyl[2-^{13}C,^{2}H]lactate **5** is incorporated intact into C-3′ of **1**. Further, in an exciting development, Robins *et al.* have demonstrated[7] that hyoscyamine **1** is generated by an *intramolecular* rearrangement of littorine **6**, the phenyllactate ester of tropine. The study involved the incorporation of racemic phenyl[1,3-$^{13}C_2$]lactyl-[*N*-methyl-$^{2}H_3$]tropine (littorine) and it was shown that the quintuply-labelled precursor was predominantly incorporated intact, suggesting no requirement for tropate ester hydrolysis. These studies lay to rest speculation[8–11] on the nature of intermediates after L-phenylalanine and establish littorine **6** as the true substrate for the mutase enzyme. The pathway between L-phenylalanine and hyoscyamine is summarised in Scheme 1. In this and the following Communication we report the resolution of the remaining stereochemical questions concerning the rearrangement of the phenyllactate moiety of littorine **6** to the tropate moiety of hyoscyamine **1**.



Scheme 1 Biosynthetic intermediates and stereochemical summary between L-phenylalanine and hyoscyamine in *Datura stramonium*

It became relevant to establish whether (*R*)-D- or (*S*)-L-phenyllactate is the true substrate for the mutase enzyme. To this end we have prepared both sodium (*R*)-D-phenyl[2-^{13}C,^{2}H]lactic acid **5a** and (*S*)-L-phenyl[2-^{13}C,^{2}H]lactic acid **5b**. To achieve this, the dual-labelled racemate was synthesised as previously described[6] and was then resolved into its component enantiomers **5a** and **5b**† by the method of Saigo *et al.*[12] Due to the *in vivo* interconversion of these enantiomers, *via* phenylpyruvate **4**,[13] it was necessary to incorporate deuterium at C-2. Clearly any equilibrium with, or processing *via* phenylpyruvate **4**, will result in loss of the deuterium atom. Also, the double-labelling strategy provides a sensitive probe for deuterium incorporation as demonstrated previously with the dual-labelled racemate of phenyl[2-^{13}C,^{2}H]lactic acid.[6]

The hyoscyamine **1**, which was isolated after separate feeding experiments of **3** and **4** to transformed root cultures of *Datura stramonium*, was analysed by ^{13}C{^{1}H} and ^{13}C{^{1}H,^{2}H} NMR experiments. From the relevant sections of the ^{13}C NMR spectra shown in Fig. 1 it is apparent that the greater part of (*R*)-D-phenyl[2-^{13}C,^{2}H]lactic acid **5a** is incorporated into hyoscyamine with its ^{13}C–^{2}H bond intact. There is a clear α-shift associated with the enriched resonance at δ 64.05 corresponding to C-3′, the hydroxymethyl carbon of the tropate moiety of **1**. Conversely for (*S*)-L-phenyl[2-^{13}C,^{2}H]lactic acid **5b**, there was no α-shift component associated with the enriched C-3′ signal at δ 64, indicating that all of the deuterium had been washed out.

GCMS analysis of the crude alkaloidal extracts, conducted after each experiment, confirmed this isotopic distribution and allowed quantification of the enrichments. The percentage enrichments of the M + 1 and M + 2 ions are shown in Table 1. Such an analysis also allowed isotopic enrichments into the co-produced tropane alkaloids 3α-(2′-hydroxyacetoxy)tropane **7** and 3α-phenylacetoxytropane **8** to be evaluated. These alkaloids are produced at much lower levels and were not observable by ^{13}C NMR. Of some significance was the M + 2 ion (18.4%) evaluated for **7** after the (*R*)-D-phenyl[2-^{13}C,^{2}H]lactic acid **5a** feeding experiment. This demonstrates that one of the C–H bonds of the hydroxymethyl group of **7** derives intact from C-2 of (*R*)-phenyllactate, presumably after C-2/C-3 sission of littorine, and lends further support to a recent proposal[14] from our laboratories defining the metabolic relationship between these alkaloids. Also, in these experiments there was no significant incorporation into 3α-phenylacetoxy-

(N. C. J. E. C.) and the University of Durham and the US Public Health Service (NIH Grant GM 32333) for financial support.

Received, 25th October 1994; Com. 4/06542E

References

1 N. C. J. E. Chesters, D. O'Hagan and R. J. Robins, preceding paper.
2 E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271.
3 E. Leete, *Can. J. Chem.*, 1987, **65**, 226.
4 J. T. Kealey, S. Lee, H. G. Floss and D. V. Santi, *Nucleic Acids Res.*, 1991, **19**, 6465.
5 H. G. Floss and M. D. Tsai, *Adv. Enzymol.*, 1979, **50**, 243.
6 M. Sprecher, M. Y. Clark and D. B. Sprinson, *Biochem. Biophys. Res. Commun.*, 1964, **15**, 581; *Biol. Chem.*, 1966, **241**, 872.
7 K. A. Reynolds, D. O'Hagan, D. Gani and J. A. Robinson, *J. Chem. Soc., Perkin Trans. 1*, 1988, 3195.
8 G. Brendelberger, J. Retey, D. M. Ashworth, K. A. Reynolds, F. Willenbrock and J. A. Robinson, *Angew Chem., Int. Ed. Engl.*, 1988, **27**, 1089.

The Biosynthesis of Tropic Acid: The Stereochemical Course of the Mutase involved in Hyoscyamine Biosynthesis in *Datura stramonium*

Nicola C. J. E. Chesters,[a] David O'Hagan,*[a] Richard J. Robins,[b] Allen Kastelle[c] and Heinz G. Floss[c]

[a] *The Department of Chemistry, University of Durham, Science Laboratories, South Road, Durham, UK DH1 3LE*
[b] *Plant Biotechnology Group, Department of Genetics and Microbiology, Institute of Food Research, Norwich Research Park, Colney, Norwich, UK NR4 7UA*
[c] *Department of Chemistry, University of Washington, Seattle, Washington, 98195, USA*

Incubation of (*R,S*)-DL-phenyl[2-^{3}H]lactic acid with *Datura stramonium* generates hyoscyamine **2** with the tritium isotope located at C-3′ of the tropic acid ester moiety; the C-3′ hydroxymethyl group of **2** is converted into a chiral methyl group and is oxidised to generate chiral sodium acetate, with the (*R*) configuration (96% ee); the tritium is therefore located at the 3′-*pro*-S site of **2**; it follows that the 3′-*pro*-*R* hydrogen is introduced with *inversion* of configuration by the mutase operating during hyoscyamine biosynthesis.

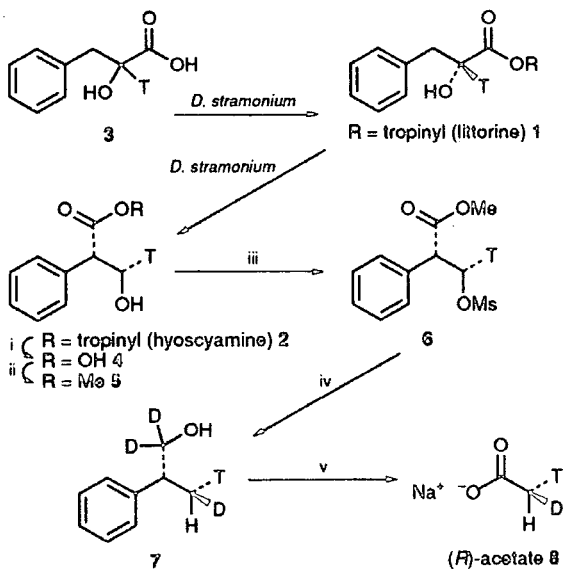
In the preceding communication[1] we report that (*R*)-D-phenyllactate is the stereoisomer processed by the mutase involved in the interconversion of littorine **1** and hyoscyamine **2**. During the rearrangement, the 3′-*pro*-*S* hydrogen of the phenyllactate moiety of littorine migrates in a vicinal interchange process, with the carbonyl group, to generate tropate.[2] The carbon hydrogen bond at C-3′ of the phenyllactate is broken and the new carbon–carbon bond is formed with retention of configuration.[3] The stereochemical course of the delivery and the location of the 3′-*pro*-*S* hydrogen after its delivery, to C-3′ of the tropate moiety have not previously been evaluated and are now reported.

To solve this problem we deployed chiral methyl group methodology and our approach is summarised in Scheme 1. (*R,S*)-DL-Phenyl[2-^{3}H]lactic acid **3** [specific activity 204 μCi mmol^{-1}] was incubated with transformed root cultures of *Datura stramonium*. The isolated hyoscyamine (50 mg) was diluted tenfold with cold hyoscyamine (500 mg) and then subjected to barium hydroxide hydrolysis to release the tropate moiety. Treatment of an acidic ether extract with diazomethane allowed recovery of the tropic acid **4** as its methyl ester **5**. Conversion of the alcohol moiety of **5** to a mesylate generated **6**, which was then reduced with $LiAlD_4$ (98 atom%). The resultant 2-phenyl[1-$^2H_2$, 3-^{3}H, ^{2}H]propan-1-ol **7** now posessed a chiral methyl group at C-3 of **7**, which was chiral by virtue of the presence of three isotopes of hydrogen. Oxidation of **7** with KIO_4–$KMnO_4$, and then steam distillation, allowed chiral acetic acid to be isolated.[4] After neutralisation with dilute NaOH followed by lyophilisation, sodium acetate **8** and sodium formate (ratio, acetate : formate 2 : 1, as determined by ^{1}H NMR) were recovered as a mixture. ^{1}H NMR analysis of this mixture [specific activity 6.0 μCi mmol^{-1}] distinguished two populations of acetate, singly deuteriated and unlabelled, in a 3 : 4 ratio. We have deduced that the unlabelled acetate arose from another source during the oxidation reaction. Reanalysis of the ^{1}H NMR spectrum recorded of **7** prior to its oxidation, revealed a trace of diethyl ether, the solvent used in the $LiAlD_4$ reduction. We therefore attribute the resultant unlabelled acetate to oxidation of this material. In the event, it was determined that the acetate molecules containing three different isotopes of hydrogen had predominantly a single configuration. The chiral purity of the acetate sample was assayed in the usual manner[5] by the coupled malate synthase/fumarase assay, and indicated the (*R*)-configuration for the acetic acid, with an enantiomeric excess of 96% (*F* value = 77.9).

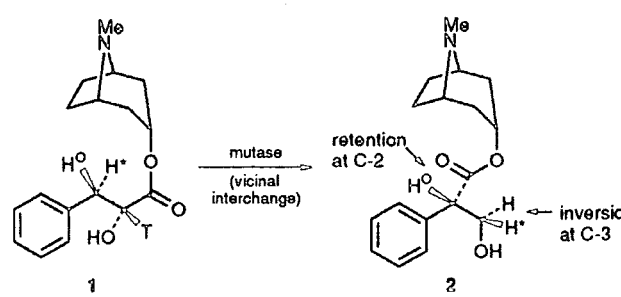
Taking into account the stereochemical inversion at carbon during the $LiAlD_4$ reduction, it is deduced that the tritium isotope occupied the 3′-*pro*-*S* site in the tropate moiety of hyoscyamine **2**. Consequently the migrating hydrogen from the 3′-*pro*-*S* site of phenyllactate must rest in the 3′-*pro*-*R* site of tropate after the vicinal interchange process. It can be further deduced, from a knowledge that (*R*)-D-phenyllactate is processed,[1] that the new C–H bond at C-3′ of the tropate moiety, replaces the old C^1–C^2 bond of phenyllactate with *inversion* of configuration.

All of the stereochemical features of the rearrangement of littorine to hyoscyamine are now evaluated and are summarised in Scheme 2. The vicinal interchange process has obvious similarities to the coenzyme-B_{12}-mediated rearrangements of methylmalonyl–CoA mutase[6] and isobutyryl–CoA mutase.[7,8] In both of these cases, however, the migrating hydrogen atom replaces the COSCoA group with retention of configuration. Therefore the steric course in this respect is opposite.

We thank Dr Peter Bachmann for assistance with the feeding experiments and are grateful to the EPSRC for a studentship

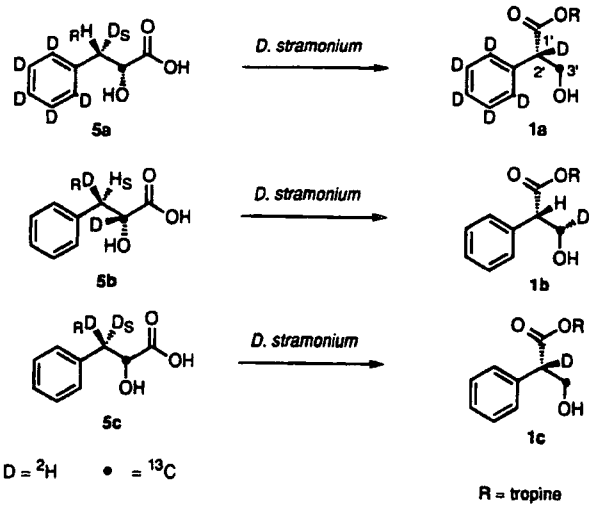


Scheme 1 *Reagents and conditions*: i, $Ba(OH)_2$; ii, CH_2N_2, Et_2O, 67% from **2**; iii, methanesulfonyl chloride, DMAP, pyridine, 0.5 h, 78%; iv, $LiAlD_4$, Et_2O, 2 h, 82%; v, KIO_4–$KMnO_4$, 2 h, steam distillation then lyophilisation, 16%



Scheme 2 Summary of the stereochemical course of the rearrangement of littorine **1** and hyoscyamine **2** in *Datura stramonium*

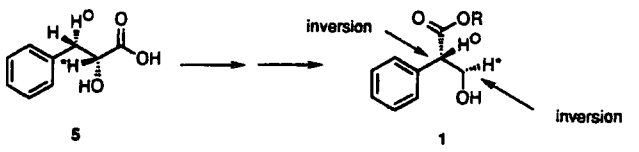
Scheme 2



of hyoscyamine is indicative of **1a** as a component (42%) of the alkaloids where deuterium occurs only on the adjacent C-2′ carbon. Thus, from this experiment it is deduced that the 3-*pro-S* hydrogen to carbon bond remains intact during the rearrangement. In the case of **5b** the isolated alkaloids contained **1b** (20.8%). The enriched ^{13}C-NMR signal has a single α-shift (Δppm = 0.35 ppm) associated with it, consistent with the retention of the ^{13}C—^{2}H bond from **5b**. Significantly there was no evidence of an additional β-shift, and thus there was no indication of deuterium retention at C-2′ of **1b**. Thus, the 3-*pro-R* hydrogen is lost during the rearrangement process; it does not migrate to the adjacent carbon.

The results of the feeding experiments with **5a** and **5b** are unambiguous and demonstrate that the 3-*pro-R* hydrogen is removed and the 3-*pro-S* hydrogen retained during the rearrangement of (*R*)-phenyllactate to (*S*)-tropate. The rearrangement proceeds with stereochemical *inversion* at this migration terminus. Additionally there is no evidence of a vicinal interchange process. The 3-*pro-R* deuterium of **5b** was not returned at a detectable level to C-3′ of the tropate skeleton. In order to further reinforce this conclusion we challenged the system with (*R*,*S*)-[2-^{13}C,3-^{2}H$_2$]phenyllactate (**5c**), in which both of the C-3 hydrogen atoms are replaced by deuterium. This compound was prepared by a modification of our previous method[3], and the result of the feeding experiment is summarized in Scheme 2. The ^{13}C-NMR spectrum of the resultant hyoscyamine showed a β-shift (Δppm = 0.063 ppm) associated with the enriched C-3′ carbon atom (64 ppm) indicative of the presence of **1c** (45%). In the light of the above results this observation is consistent with the loss of the 3-*pro-R*, and

Scheme 3



retention of the 3-*pro-S*, deuterium atom. There was again no evidence of an intact ^{13}C—^{2}H bond (α-shift) at C-3′ of the tropate ester; thus we can exclude the operation of a vicinal interchange process.

We can only speculate why Leete reached a different conclusion in his work.[8] Both previous studies[7,8] were carried out with appropriately labeled (*S*)-phenylalanines rather than the more immediate precursor, (*R*)-phenyllactate. Some stereospecific loss of tritium during the transamination of (2*S*,3*R*(or 3*S*))-[2-^{14}C,3-^{3}H$_1$]phenylalanines cannot be excluded and could complicate the interpretation of the previous results. Furthermore, the enantiomeric purity of the labeled (2*S*,3*R*(or 3*S*))-[2-^{14}C,3-^{3}H$_1$]phenylalanines at the C-3 stereogenic center in both of the previous studies was only about 90% ee and the incorporation of the precursors into the alkaloids was very low (<1%). Perhaps more importantly, the previous analyses[7,8] relied on ^{3}H/^{14}C ratios and the definitive conclusion was dependent on the complete postbiosynthetic washout of tritium at C-2 of the tropate ester moiety of hyoscyamine. If a kinetic isotope effect resulted in only partial washout during the relatively short (30 min) hydrolysis treatments, then this would validate Haslam's original conclusions.[7]

In summary, all of the stereochemical aspects of the D-phenyllactate to tropate rearrangement have been evaluated and are summarized in Scheme 3. During carboxylate migration an inversion of configuration occurs at both migration termini, and we can exclude the back migration of a hydrogen atom in a vicinal interchange process, as previously suggested.[8a]

Acknowledgment. We thank Dr. John Parkinson of the University of Edinburgh for recording ^{13}C{^{1}H,^{2}H}-NMR spectra, the U.S. Public Health Service (NIH Grant GM 32333) and the University of Durham for financial support, and the EPSRC for funding a studentship (N.C.J.E.C.).

Supporting Information Available: ^{13}C- and ^{1}H-NMR spectra for the synthesized compounds described in the text and the relevant sections of the ^{13}C-NMR spectra of the isotopically enriched hyoscyamines showing α- and β-shifts (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA9531485

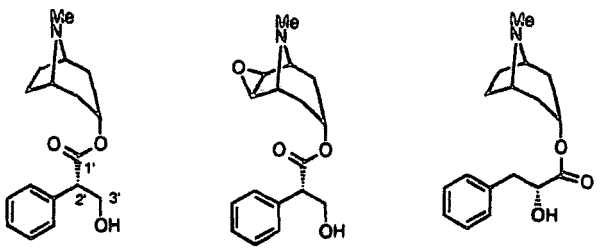
The Biosynthesis of Tropic Acid: A Reevaluation of the Stereochemical Course of the Conversion of Phenyllactate to Tropate in *Datura stramonium*

Nicola C. J. E. Chesters,[†] Kevin Walker,[‡] David O'Hagan,*[,†] and Heinz G. Floss[‡]

Departments of Chemistry, Science Laboratories University of Durham, South Road, Durham, DH1 3LE, U.K. University of Washington, Seattle, Washington 98195

Received September 13, 1995

The biosynthetic origin of the tropate ester moiety of hyoscyamine (**1**) and scopolamine (**2**) has attracted a lot of interest over many years,[1] yet many details of the process remain obscure. In 1975 Leete *et al.* showed[2] that the tropate moiety of **1** originates by an intramolecular rearrangement of the (*S*)-phenylalanine (**4**) skeleton. Incorporation studies with (*S*)-[1,3-$^{13}C_2$]phenylalanine (**4**) in *Datura* plants resulted in **1** with a 1′,2′-$^{13}C_2$-labeled tropate moiety as shown in Scheme 1. The resultant contiguous arrangement of isotopes established the intramolecular nature of the rearrangement.

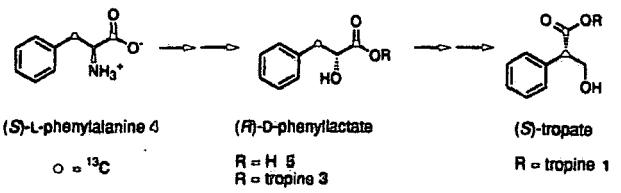


1 **2** **3**

We have recently demonstrated[3] that (*R*)-phenyllactate **5** is a closer precursor than (*S*)-phenylalanine (**4**) or (*S*)-phenyllactate to the tropate ester moiety, and in an illuminating study Robins *et al.*[4] have presented evidence which implicates the alkaloid littorine (**3**), the (*R*)-phenyllactate ester of tropine, as the substrate for the rearrangement. This counters the long-held contention that the rearrangement occurs at the coenzyme A ester level, which extended the analogy from methylmalonyl-CoA mutase which catalyzes a coenzyme B_{12} mediated rearrangement.[5]

Using chiral methyl group methodology we have shown[6] that the carboxylate group at C-2 of (*R*)-phenyllactate, which migrates in the process, is replaced by a hydrogen atom at C-3 of the (*S*)-tropate ester moiety of **1**, with *inversion of configuration*. The stereochemistry at the other migration terminus has been probed in two previous but contradictory studies[7,8] utilizing appropriately labeled (2*S*,3*R*(or 3*S*))-[2-^{14}C,3-$^{3}H_1$]-phenylalanines. Haslam *et al.*[7] concluded that the 3-*pro*-*R* hydrogen was lost during the rearrangement and thus, the new

Scheme 1



C−C bond was formed with inversion of configuration. However, in a reevaluation of this issue, Leete[8] concluded that the 3-*pro*-*S* hydrogen was replaced with retention of configuration. Leete argued that tritium at C-2′ in the resultant tropate ester was susceptible to washout, during the base hydrolysis required to release free tropic acid, prior to scintillation counting. The hyoscyamine was stirred in either NaOH or $Ba(OH)_2$ solution for 30 min prior to workup. Thus, if this is taken into account in the Haslam study,[7] then the absence of tritium at this site was not a consequence of the stereochemical course of the reaction, but of chemical manipulation. A further conclusion of Leete's study[8] was that the 3-*pro*-*S* hydrogen underwent an intra- or intermolecular vicinal interchange with the migrating carboxylate group and was returned to C-3′ of the tropate ester moiety. This further strengthened the analogy between this rearrangement and methylmalonyl-CoA mutase, where such a vicinal interchange is well established.[5]

Since our recent stereochemical study has established an inversion of configuration at the other migration terminus, the enzyme appeared to mediate a retention/inversion process, which is difficult to reconcile with a vicinal interchange process. Such a process would require the putative mutase to remove and deliver the migrating hydrogen to opposite faces of the substrate. We therefore decided to reinvestigate this issue. The knowledge that (*R*)-phenyllactate is a more proximate precursor of **1** than is **4**, and the availability of transformed root cultures which give much higher levels of precursor incorporation (20−45% for **5** into **1**),[3] allowed a more definitive examination of the reaction stereochemistry than was possible at the time of the earlier studies. Using dual labeled (^{2}H and ^{13}C) samples of **5**, the regiospecific location of the deuterium atoms in the resultant samples of **1** could be determined directly by ^{13}C-NMR spectroscopy by employing a ^{13}C label at C-2 of **5** as a reporter atom, without recourse to hydrolysis and isolation of tropic acid. Deuterium directly attached to or one bond removed from a carbon-13 atom induces a detectable α- or β-upfield-shifted signal, respectively, in the $^{13}C\{^1H,^2H\}$-NMR spectrum.[9]

(2*R*,3*S*)-[2-^{13}C,3-$^{2}H_1$,*phenyl*-$^{2}H_5$]-Phenyllactate (**5a**) carrying deuterium at the 3-*pro*-*S* site and (2*R*,3*R*)-[2-^{13}C,2,3-$^{2}H_2$]-phenyllactate (**5b**) carrying deuterium at the 3-*pro*-*R* site were prepared by appropriate modifications to the route described by Fryzuk and Bosnich.[10] In each case [2-^{13}C]glycine was used as a starting material. For **5a** [$^2H_6$]benzaldehyde[11] replaced benzaldehyde, and for **5b** deuterium gas was used in place of hydrogen gas. The stereochemical integrity of this synthetic protocol is already demonstrated, and as expected, the ee's of compounds **5a** and **5b** were judged to be 96% by GC analysis of the Mosher's ester derivatives of the methyl esters of the phenyllactates. Compounds **5a** and **5b** were then introduced into submerged cultures of *D. stramonium*, and the resultant crude hyoscyamine samples were purified by chromatography[3b] and analyzed by $^{13}C\{^1H,^2H\}$-NMR. The results are summarized in Scheme 2. In the case of **5a** a β-shift (Δppm = 0.064 ppm) associated with the carbon-13 signal (δ_C = 64 ppm) for C-3′

[†] University of Durham.
[‡] University of Seattle.

(1) (a) Leete, E. *Planta Med.* **1990**, *56*, 339; (b) **1979**, *36*, 97.
(2) Leete, E.; Kowanko, N.; Newmark, R. A. *J. Am. Chem. Soc.* **1975**, *97*, 6826.
(3) (a) Chesters, N. C. J. E.; O'Hagan, D.; Robins, R. J. *J. Chem. Soc., Chem. Commun.* **1995**, 127. (b) Chesters, N. C. J. E.; O'Hagan, D.; Robins, R. J. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1159.
(4) Robins, R. J.; Bachmann, P.; Woolley, J. G. *J. Chem. Soc., Perkin Trans. 1* **1994**, 615.
(5) (a) Rétey, J.; Robinson, J. A. *Stereospecificity in Organic Chemistry and Enzymology*; Verlag Chemie: Weinheim, 1982. (b) Overath, P.; Stadtman, E. R.; Kellerman, G. M.; Lynen, F. *Biochem. Z.* **1962**, *77*, 336.
(6) Chesters, N. C. J. E.; O'Hagan, D.; Robins, R. J.; Kastelle, A.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1995**, 129.
(7) Platt, R. V.; Opie, C. T.; Haslam, E. *Phytochemistry* **1984**, *23*, 2211.
(8) (a) Leete, E. *Can. J. Chem.* **1987**, *65*, 226. (b) Leete, E. *J. Am. Chem. Soc.* **1984**, *106*, 7271.
(9) Garson, M. J.; Staunton, J. *Chem. Soc. Rev.* **1979**, 539.
(10) Fryzuk, M. D.; Bosnich, B. *J. Am. Chem. Soc.* **1977**, *99*, 6262.
(11) The first sample synthesized, the (2*R*,3*S*) isomer **5a**, also carried five atoms of deuterium in the aromatic ring to facilitate analysis of the product by mass spectroscopy, should this become necessary.

0002-7863/96/1518-0925$12.00/0