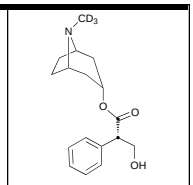
Tropic acid ester biosynthesis in *Datura stramonium* and related species



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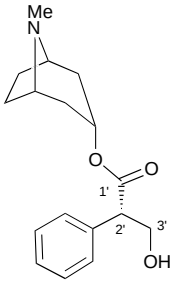
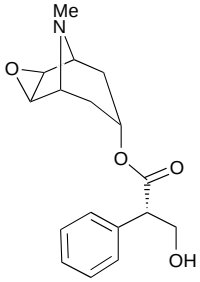
The origin of the tropic acid ester moiety, found in some of the tropane alkaloids, but particularly in hyoscyamine and scopolamine, has been a subject of discussion and investigation in biosynthesis for many years. Recently it has been shown in *Datura stramonium* root cultures that hyoscyamine arises by isomerisation of the tropane alkaloid littorine. The mechanism of this isomerisation process is not obvious and in this review we present our recent results and current thinking on this process.

1 Introduction

In the last few years new experimental evidence has forced a re-evaluation of the biosynthesis of the tropate ester moiety of the tropane alkaloids hyoscyamine and scopolamine. Robert Robinson recognised in 1928[1] that the tropate ester is an isomer of the phenylpropionoid skeleton, a structural motif common in plant alkaloids and other plant metabolites such as the flavanoids. The phenylpropionoid moiety derives from (*S*)-phenylalanine and Robinson later (1955) proposed[2] that tropic acid may originate by a rearrangement of the phenylpropanoid skeleton.

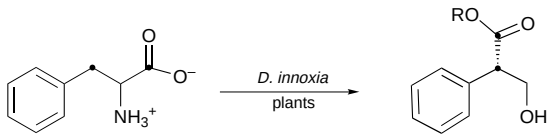


hyoscyamine



scopolamine

In a definitive experiment twenty years later (Scheme 1) Leete demonstrated[3] in *Datura innoxia* plants that (*R*,*S*)-phenyl[1,3-$^{13}C_2$]alanine was indeed incorporated intact into the tropate moiety of hyoscyamine. Importantly, this experiment demonstrated an intramolecular rearrangement where the two isotopes became contiguous in the resultant tropate. Thus, the origin of the tropate ester from phenylalanine was established. A number of intriguing questions remained, however, concerning this conversion. Although phenylalanine is a good precursor, the true substrate for the rearrangement and the stereochemistry remained ill-defined until recently. The rearrangement belongs to a small class of enzyme-mediated carbon skeletal isomerisations and the mechanism of these enzymes has attracted wide interest. The rearrangement has a



(*RS*)-phenyl[1,3-$^{13}C_2$] alanine

Scheme 1

David O'Hagan was born in Glasgow in 1961. He was an undergraduate at the University of Glasgow (1982) and carried out his doctoral research (1985) on antibiotic biosynthesis at the University of Southampton with Professor John A Robinson. After a postdoctoral year with Professor Heinz G Floss (Ohio State University) he took up a position at the University of Durham where he is now a Senior Lecturer. His main research interests are in the biosynthesis of secondary metabolites and in bio-organic fluorine chemistry.

David O'Hagan

Richard J. Robins read Biochemistry at the University of Oxford (1971–75) and carried out doctoral work on the digestive processes of carnivorous plants with Dr B. E. Juniper, University of Oxford. After a post-doctoral study of the mechanism of amino acid absorption with Professor D. D. Davies (University of East Anglia), he joined (1981) the AFRC Institute of Food Research to study the biosynthesis and control of secondary product formation in plant tissue cultures. In 1995 he moved to Nantes to become Director of the CNRS Unit of Isotopic and Electrochemical Studies of Metabolism. His current research interests include the biosynthesis of secondary metabolites and the use of natural-abundance isotopic techniques to study metabolism.

Richard J. Robins

superficial similarity to those mediated by some co-enzyme B_{12}-dependant mutases however the process does not appear to be B_{12}-dependent.

2 Plants and transformed roots of *Datura stramonium*

Much of the recent work on the biosynthetic origin of tropic acid has exploited transformed root cultures as experimental material. These cultures are generated by infecting the lightly-damaged surface of sterile leaf or stem from tropic acid-producing plants such as *D. stramonium* with a suspension of the pathogenic bacterium, *Agrobacterium rhizogenes*. The bacterium inserts into the plant cells a short section of DNA (the Ri-DNA) which stimulates cell division to cause root formation. The emergent roots can be removed, treated with antibiotic to kill the remaining bacteria, and cultured in perpetuity in a sterile liquid medium. A small part of the culture is transferred to fresh medium every two or three weeks (see ref 4 for a review of the properties of these cultures).

Figure 1

This system offers many advantages over the use of whole plants;

* large amounts of genetically identical material can be generated.
* the roots grow at a constant rate.
* the experiment is conducted under asceptic conditions.
* precursors can readily be added and their absorption by the tissue monitored directly and multiple additions can be made.
* experiments can be conducted on small amounts of material, allowing considerable savings on the quantity of isotopically labelled precursor required.
* very high specific incorporations can be obtained, rendering analysis by GCMS and NMR relatively straightforward.

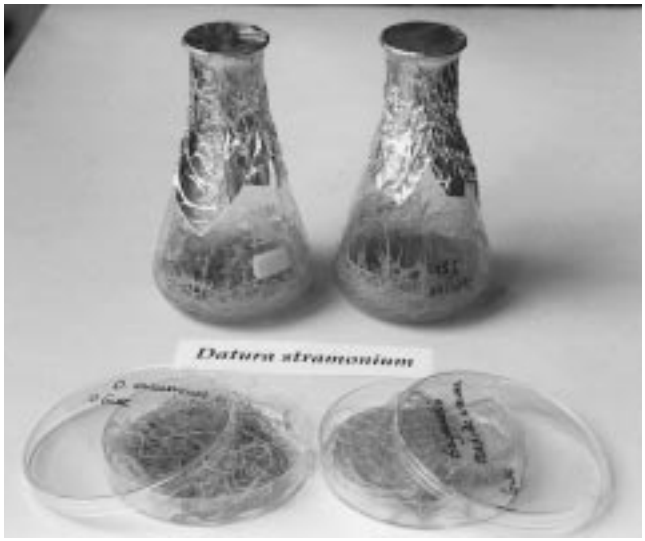


Figure 2

Root cultures are not, however, suitable for all studies. In *D. stramonium* plants, scopolamine is a major product in the leaves, yet only traces of this alkaloid are recovered from root cultures. In other species, however, such as *Hyoscyamus muticus* or a *Brugmansia* hybrid, scopolamine accumulates in the root cultures as a major alkaloid. A key requirement for using root cultures is that this tissue must be the site of biosynthesis in the plant. For example, terpenes in *Mentha* species are made in leaf glands and shoot cultures[5] are required in this case.

3 The role of (*R*)-phenyllactate

(*S*)-Phenylalanine is efficiently incorporated into the tropate ester moiety of hyoscyamine.[3] Other experiments[6] with ^{14}C-labelled phenylpyruvate and phenyllactate indicated that all of these compounds become similarly incorporated into the tropate ester moiety of the alklaloids. This observation is readily rationalised if these compounds interconvert *in vivo*. The importance of phenyllactic acid as an intermediate was confirmed by feeding (*R,S*)-phenyl[1,3-$^{13}C_2$]lactate to *D. stramonium* transformed root cultures[7] or plants.[8] High incorporation was obtained and the observed spin-spin coupling of the two ^{13}C nuclei in the extracted hyoscyamine[7] and scopolamine[8] confirmed both the intramolecular rearrangement shown by Leete[3] and the putative role of phenyllactic acid in the formation of hyoscyamine.

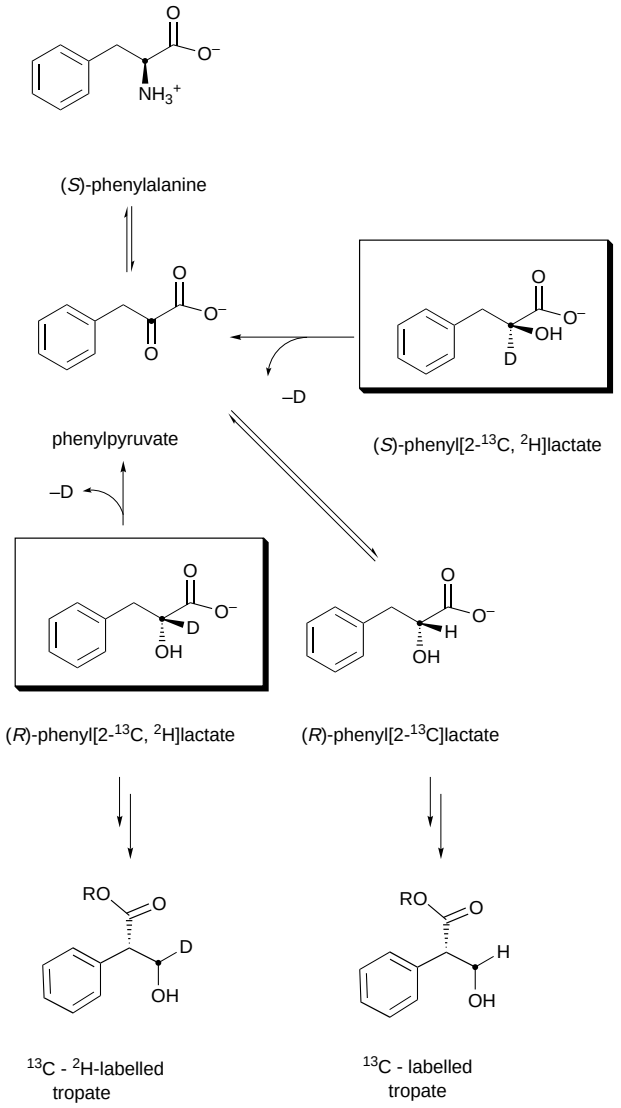
It remained, therefore, to identify the most direct precursor of the three. In an effort to resolve this issue, we studied the incorporation of deuterium from (*R,S*)-[2-^{13}C, ^{2}H]phenyllactate. If phenyllactate is oxidised to phenylpyruvate, then the deuterium atom will be lost prior to incorporation into tropic acid. If, however, phenyllactate is utilised directly then the deuterium atom will be retained.

An initial feeding experiment supplementing *D. stramonium* root cultures with (*R,S*)phenyl[2-^{13}C,^{2}H]alanine resulted[9a] in a substantial retention of deuterium isotope attached to carbon-13 (17%), at C-3′ of the tropate ester moiety of hyoscyamine. In a further experiment[9b] resolved (*R*)- and (*S*)-phenyl[2-^{13}C, ^{2}H]-alanines were added to *D. stramonium* cultures. Feeding the (*R*)-isomer resulted in hyoscyamine showing retention of the dual ^{13}C-^{2}H isotopes (28.9%) indicating that the bond had remained intact during the biosynthesis. For the (*S*)-isomer, there was a significant ^{13}C-enrichment but all of the deuterium was lost, indicating that this bond had been broken during hyoscyamine formation. These results demonstrate that (*R*)- and not (*S*)-phenyllactate is the stereoisomer used during the biosynthesis. (*S*)-Phenyllactate must be converted to the

(*R*)-isomer, presumably *via* phenylpyruvate, prior to incorporation into tropic acid. The experiment also established that the C-2 hydrogen atom of (*R*)-phenyllactate is retained during the rearrangement. Experiments using (*R*)- and (*S*)-phenyl[1,3-$^{13}C_2$]lactates fed to whole plants[10] have also indicated, on the basis of differential incorporation levels, that only the (*R*)-isomer is the precursor of hyoscyamine and scopolamine.

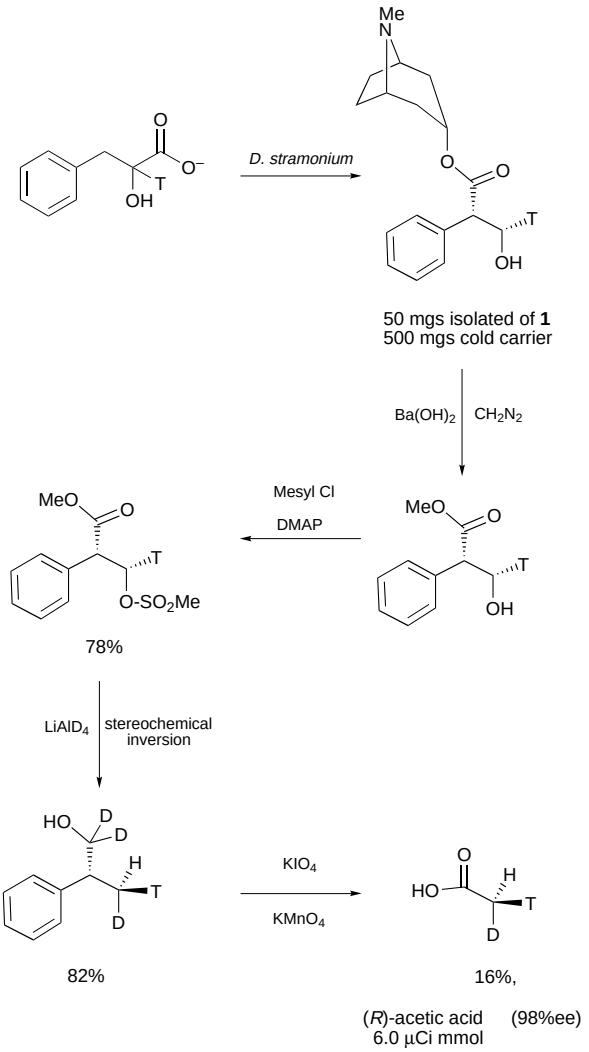
4 Stereochemistry of the rearrangement

During the rearrangement of phenyllactate to tropate, two bonds are broken and two are formed. In a given reaction bonds are normally broken/formed with either retention or inversion of configuration. A recent stereochemical analysis[11] on tropate biosynthesis has revealed that both of the bonds are broken/formed with inversion of configuration.



Scheme 2

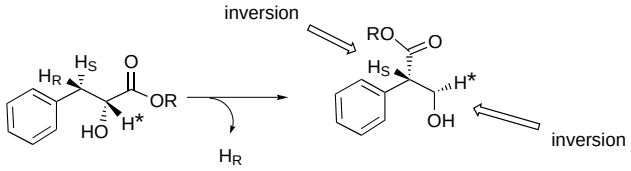
Firstly, radiolabelled phenyl[2-^{3}H]lactate was incubated with *D. stramonium* root cultures.[11*a*] The resultant hyoscyamine retained tritium at the 3-*pro*-*S* position of the tropate ester. This conclusion was drawn after diluting the isolated hyoscyamine with 'cold' unlabelled alkaloid and converting the carbon atom carrying the tritium into a chiral methyl group by introduction of deuterium in a stereospecific manner. The strategy is shown in Scheme 3. Oxidation of 2-phenylpropanol carrying the chiral methyl group generated a sample of chiral acetic acid. Although cold carrier was added such that sufficient material could be manipulated through the derivatisation protocol, it is important to note that all of the molecules carrying tritium had come through the biosynthetic experiment and that only these molecules give rise to chiral acetic acid. Enzymatic assay of the resultant chiral acetic acid indicated that the methyl group had an *R*-configuration (98% ee). Thus, by deduction, the tritium must have occupied the 3-*pro*-*S* site in the hyoscyamine isolated after the biosynthetic experiment. In view of the fact that (*R*)- and not (*S*)-phenyllactate is utilised, then it was concluded that there is an overall inversion of configuration at this centre during the rearrangement.



Scheme 3

The stereochemistry at the other migration terminus was established after feeding experiments with (2*R*,3*R*)- and (2*R*,3*S*)-phenyl[3-^{2}H]lactates. In the event only deuterium from 3-*pro*-*S* (2*R*)-phenyllactate was retained. However, this deuterium–carbon bond had become configurationally inverted in the resultant hyoscyamine. In the complementary experiment, the 3-*pro*-*R* hydrogen was lost during the rearrangement. With this information we concluded that the bond breaking/forming at this carbon atom proceeds with an inversion of configuration. The overall stereochemical course of the rearrangement is summarised in Scheme 4, inversion of configuration occurring at both migration termini.

This study has corrected a previous stereochemical analysis[12] of this system in plants and supported an even earlier study by Haslam[13] who also showed an inversion of configuration at C-2 of the tropate ester.

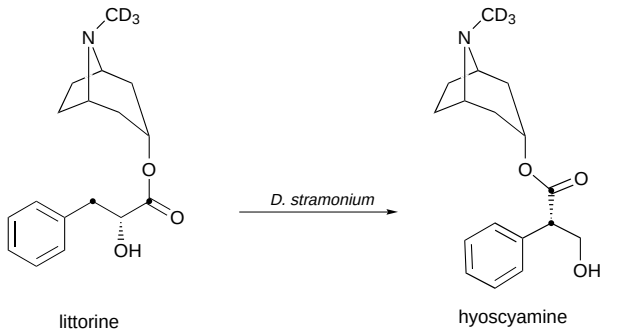


Scheme 4

5 Substrate for the rearrangement process

The above experiments proved a clearly defined role for (*R*)-phenyllactate in the biosynthesis of the tropate ester moiety. However, the true substrate for the putative isomerase is not free (*R*)-phenyllactate. It is poignant that the co-produced alkaloid littorine, the tropine ester of (*R*)-phenyllactate, is found widely in tropane-alkaloid forming species. So clearly, (*R*)-phenyllactate can couple to tropine in *D. stramonium*, raising the possibility that littorine is the substrate for the enzyme. Indirect evidence to support this conclusion was obtained from experiments[7] in which added unlabelled tropic acid failed to diminish the incorporation of label from (*R,S*)-phenyl[1,3-$^{13}C_2$]lactate. Thus, free tropic acid was found unlikely to be an intermediate of hyoscyamine formation. Similarly, ^{14}C-tropic acid was found[14] to be a very poor precursor for hyoscyamine compared with ^{14}C-phenylalanine in root cultures of *Duboisia leichhardtii*.

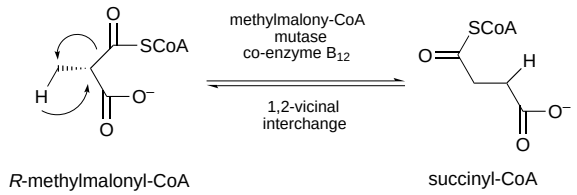
The role of littorine as a direct precursor of hyoscyamine was established in an experiment[14] using littorine isotopically labelled in both the tropane ring and the tropate ester moiety. This study demonstrated unequivocally that littorine can rearrange *in vivo* to hyoscyamine. The precursor littorine was labelled by incorporating three ^{2}H nuclei in the *N*-methyl of the tropine moiety and two ^{13}C nuclei in the phenyllactoyl moiety (Scheme 5). A specific incorporation into hyoscyamine of between 4.5 and 6.5% of the quintuply-labelled molecule was measured by GCMS analysis. Some hydrolysis of littorine occurred, resulting in labelling of both tropine (at the M + 3 ion) and phenlylactate methyl ester (at the M + 2 ion). From the percent isotopic excess in these products it could be estimated, however, that a route involving hydrolysis of the ester followed by reincorporation could only account for about 0.2% isotopic excess in the isolated hyoscyamine. Furthermore, neither added cold tropine nor phenyllactate diluted the percent isotopic incorporation. Confirmation that the rearrangement is intramolecular was shown by NMR, the isolated hyoscyamine showing a high level of ^{13}C spin-spin coupling due to the adjacent enriched nuclei at the C-1′ and C-2′ positions. Thus after many years of speculation the substrate for the isomerisation is now established as the tropane alkaloid, littorine.



Scheme 5

6 Some ideas on the mechanism of rearrangement

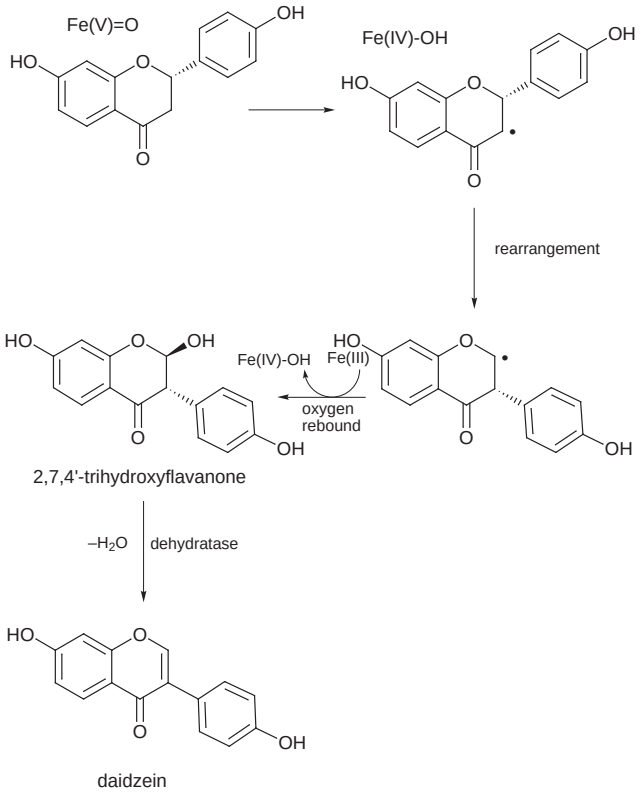
There is a superficial similarity between co-enzyme B_{12} processes and the rearrangement of littorine to hyoscyamine. However, in the related co-enzyme B_{12} processes a vicinal interchange process is apparent. This is illustrated typically for (*R*)-methylmalonyl-CoA mutase[15] in Scheme 6 where the thioester carboxylate migrates to the vicinal carbon, and the hydrogen that is removed from this carbon is relocated at the



Scheme 6

original carboxy site. It had been reported[12] that the hydrogen at C-3 (of phenyllactate), which is removed during the process, was relocated at the C-3′ position of hyoscyamine. This conclusion was drawn after a ^{3}H/^{14}C labelling study and the observation of an apparent vicinal interchange process, clearly implied a role for co-enzyme B_{12} in the rearrangement process. However, our stable isotope study[11b] did not reveal any evidence for a vicinal interchange process *i.e.* the 3-*pro-R* hydrogen of littorine is not relocated at the 3-*pro-R* site of hyoscyamine. This observation and the apparent lack of co-enzyme B_{12} in plants, lays to rest the putative involvement of this co-factor.

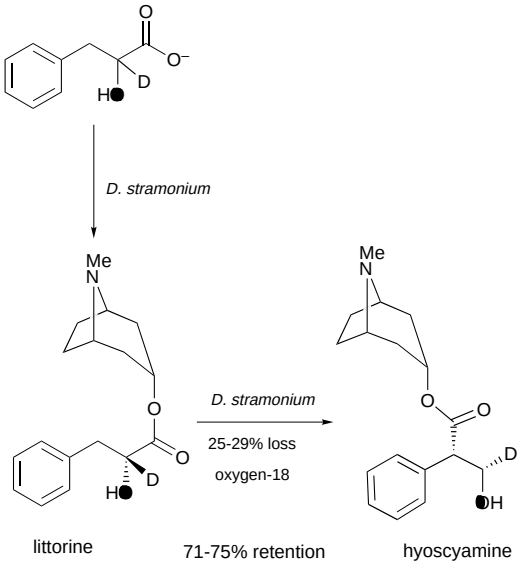
In many plant systems iron-oxo species operate to generate radicals. For example Sankawa has shown[16] that the isoflavone synthase of *Pueraria lobata* cell cultures is a cytochrome P_{450}-mediated reaction as illustrated in Scheme 7. An important



Scheme 7

observation in that system is the ‘oxygen rebound’ process where it is proposed that the rearranged radical is quenched by an hydroxyl radical from Fe(IV)-OH to generate 2,7,4′-trihydroxyisoflavone. In cell free extracts this intermediate was isolable and the new hydroxy group was labelled from $^{18}O_2$. A dehydratase then acts to generate the isoflavanone daidzein.

We have recently demonstrated that the P-450 inhibitor chlotrimazole appears to inhibit the conversion of littorine to



Scheme 8

hyoscyamine[17] in roots of *D. stramonium*. So it became relevant to explore the possibility of an oxygen rebound process operating in the rearrangement of littorine to hyoscyamine. Our most recent results[18] utilising (*R*,*S*)-phenyl[2-^{2}H,^{18}O]lactate have shed some light on this issue, but do not provide convincing evidence for an oxygen rebound process operating during the rearrangement of littorine to hyoscyamine. After supplementing *D. stramonium* cultures with (*R*,*S*)-phenyl[2-^{2}H,^{18}O]lactate as shown in Scheme 8, GCMS analysis demonstrated that both littorine and hyoscyamine had enriched M + 3 ions showing that the oxygen-18 and deuterium atoms were both incorporated, but to different extents in each of the metabolites. The relative ratio of M + 1 (^{2}H only) to M + 3 (^{18}O + ^{2}H) in the molecular ions of hyoscyamine and littorine demonstrated that ~71–75% of the oxygen-18 was retained and recriprocally that ~25–29% of the oxygen-18 was lost during the conversion from littorine to hyoscyamine. This can be accounted for by several mechanistic possibilities as illustrated in Schemes 9, 10 and 12.

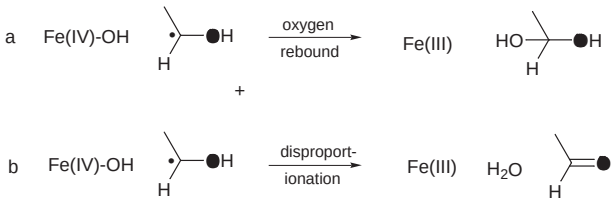
A process initiated by iron-oxo abstraction of hydrogen will generate a substrate radical. Rearrangement to a product radical followed by oxygen rebound in the classical manner [Scheme 9 and process (*a*) Scheme 10] would then generate an aldehyde hydrate as an intermediate. If this is the case then the collapse of this hydrate to an aldehyde, prior to reduction by a dehydrogenase, may be partially stereospecific, as only ~25–29% of the original C-2′ oxygen of littorine is lost, or fully stereospecific, forwarding retention of the labelled oxygen, with loss of isotope occurring by exchange of the aldehyde oxygen with the aqueous medium, prior to reduction. A non-stereospecific process would lead to 50% loss and this is not observed.

An alternative explanation [Scheme 9 and process (*b*) Scheme 10] invokes disproportionation of the putative Fe(IV)-OH intermediate and the product radical to generate an aldehyde directly. Such a conclusion has been discussed in a P-450 mediated oxidation operating during oestrogen biosynthesis[19,20] where a similar level (80%) of oxygen-18 retention was observed in the oxidation of a primary alcohol to an aldehyde as shown in Scheme 11.

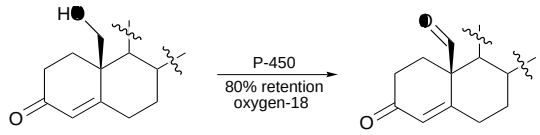
Again the high retention of oxygen-18 here either requires a collapse of a diol hydrate (generated after oxygen rebound) or disproportionation. In the latter case the ~20% loss of oxygen-18 can be accounted for by some exchange of the aldehyde carbonyl with the aqueous medium. In the light of the high level of retention of oxygen-18 in going from littorine to hyoscyamine, and also in the case in Scheme 11, the oxygen rebound process perhaps appears less likely than disproportionation as it requires both systems to display the same stereoselectivity and to favour retention of the original C–O bond.

Alternatively a two electron oxidation of littorine to generate a carbocation as illustrated in Scheme 12 offers an appealing mechanism. The generation of carbocations in iron-oxo systems is not judged so common but such intermediates are implicated for example during the biosynthesis of prostacylin and thromboxane,[21] in two closely related heme-thiolate enzymes. Also carbocations have been recently implicated[22] in the generation of minor side products in reactions of mechanistic probes in P-450 enzyme hydroxylations.

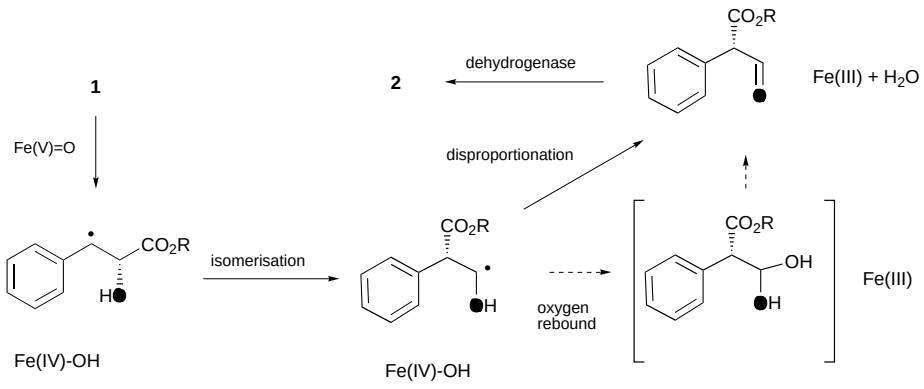
Scheme 12 illustrates a two electron oxidation of littorine **1** to a substrate carbocation. Rearrangement and the collapse of the product carbocation to an aldehyde would not require oxygen loss, and is consistent with the experimental observation with labelled oxygen, if accompanied by some exchange at the aldehyde level. An attractive feature here is that the substrate,



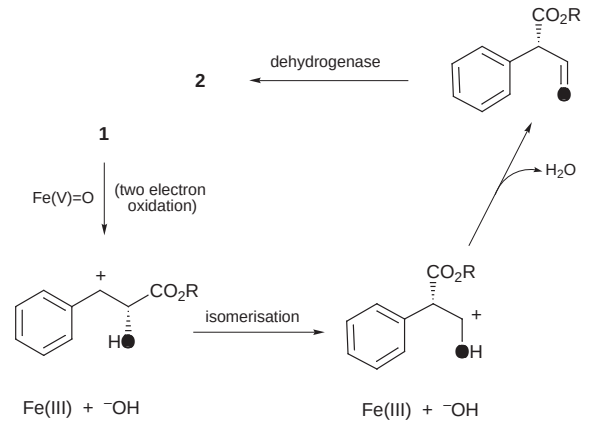
Scheme 10



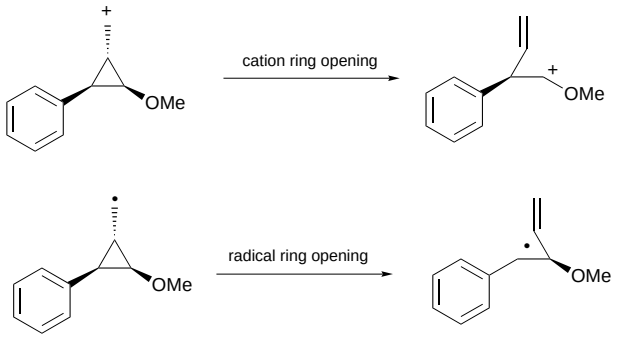
Scheme 11



Scheme 9



Scheme 12



Scheme 13

benzylic carbocation is predicted to rearrange to the more stable product carbocation,[22,23] an oxonium ion. This is most clearly illustrated by the methylcyclopropane ring opening reactions of Newcomb[23] shown in Scheme 13 which show a common cyclopropane ring being opened under radical and carbocation conditions. The stabilising substituents are aryl and oxygen and the system closely models the putative intermediates in the littorine to hyoscyamine rearrangement. It was demonstrated that the methylcyclopropane carbocation opens towards oxygen, in the same direction as the rearrangement of littorine to hyoscyamine, whereas the methylcyclopropane radical opens towards the aryl ring, the opposite direction to the rearrangement. So such models suggest a carbocation process.

In conclusion our working hypothesis proposes the involvement of two enzymes (mutase + dehydrogenase). The experimental evidence is consistent with an iron-oxo mutase, perhaps a heme-thiolate in view of the inhibition by the P-450 inhibitor chlotrimazole[17] and an analogy with carbocation generating heme-thiolate enzymes, however the mechanism of the rearrangement remains elusive and must await further evaluation and in particular enzyme isolation.

More generally little is known of the enzymes implicated on this biosynthetic pathway. Earlier claims made in the literature for enzyme activities that convert phenylalanine to phenylpyruvate and that esterify tropine with free tropic acid (see ref 24 for a review) have proved dubious. A transaminase for phenylalanine reported[25] from *Hyoscyamus albus* transformed roots shows only weak activity and poor kinetic properties. Despite the efforts of several laboratories, the conversion of phenylpyruvate to phenyllactate *in vitro* has not been demonstrated. Similarly, the putative CoA-thioligase for phenyllactate and phenyllactoyl-CoA : tropine acyltransferase activities have both proved ellusive. Yet these studies have used tissues from which other enzymes,[24] notably hyoscyamine 6β-hydroxylase,[26] have been readily extracted and purified. A clear definition *in vitro* of these activities is required to confirm that the pathway of tropic acid biosynthesis proposed on the evidence of chemical labelling is indeed that which functions *in planta*.

7 Acknowledgements

We are grateful to all of our co-authors but particularly to Dr Nicola C. J. E. Chesters and to Professor Heinz G. Floss, Dr Jack G. Woolley and Dr Nicholas J. Walton for their contributions to the project. We also thank the CIBA foundation for an ACE award.

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