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Biosynthetic studies on the tropane ring system of the tropane alkaloids from *Datura stramonium*

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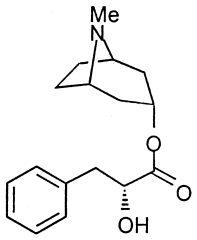
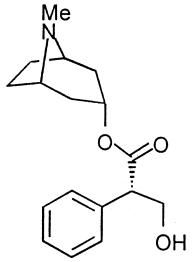
Abstract

Isotopic labelling experiments have been carried out in *Datura stramonium* root cultures with the following isotopically labelled precursors; [$^2H_3$]-, [2-^{13}C, $^2H_3$]-, [1-^{13}C, $^{18}O_2$]-acetates, ^2H_2O, [$^2H_3$-*methyl*]-methionine, [2-^{13}C]-phenyllactate, [3-^{2}H]-tropine and [2′-^{13}C, 3-^{2}H]-littorine. The study explored the incorporation of isotope into the tropane ring system of littorine **1** and hyoscyamine **2** and revealed that deuterium from acetate is incorporated only into C-6 and C-7, and not into C-2 and C-4 as previously reported. Oxygen-18 was not retained at a detectable level into the C(3)–O bond from [1-^{13}C, $^{18}O_2$]-acetate. The intramolecular nature of the rearrangement of littorine **1** to hyoscyamine **2** is revealed again by a labelling study using [2′-^{13}C, 3-^{2}H]-littorine, [2-^{13}C]-phenyllactate and [3-^{2}H]-tropine.

Keywords: *Datura stramonium*; Biosynthesis; Tropane alkaloids; Hyoscyamine; Littorine



littorine **1**



hyoscyamine **2**

1. Introduction

The biosynthesis of the tropane ring alkaloids such as littorine **1** and hyoscyamine **2** has attracted a lot of attention over many years (O'Hagan & Robins, 1998; Zabetakis, Edwards & O'Hagan, 1999). The origin of the tropane ring skeleton is recognised to arise from ornithine, acetate and methionine as illustrated in Scheme 1. The four carbon atoms C(1), C(5), C(6) and C(7) and the nitrogen atom derive from ornithine (Scheme 1) via the *N*-methyl-Δ^1-pyrrolinium ion **3** (Hemscheidt & Spenser, 1992). The *N*-methyl group derives from the methyl group of methionine via S-adenosylmethionine (SAM). The remaining three carbon atoms C(2), C(3) and C(4) are readily labelled by acetate (Sankawa, Noguchi, Hashimoto & Yamada, 1990), however, the details of their incorporation is unclear. The most advanced precursor to date, which has become successfully incorporated into the tropane ring skeleton, is **4** (Robins, Abraham, Parr, Eagles & Walton, 1997; Abraham & Leete, 1995). The ethyl ester of **4** was administered in the feeding experiment,

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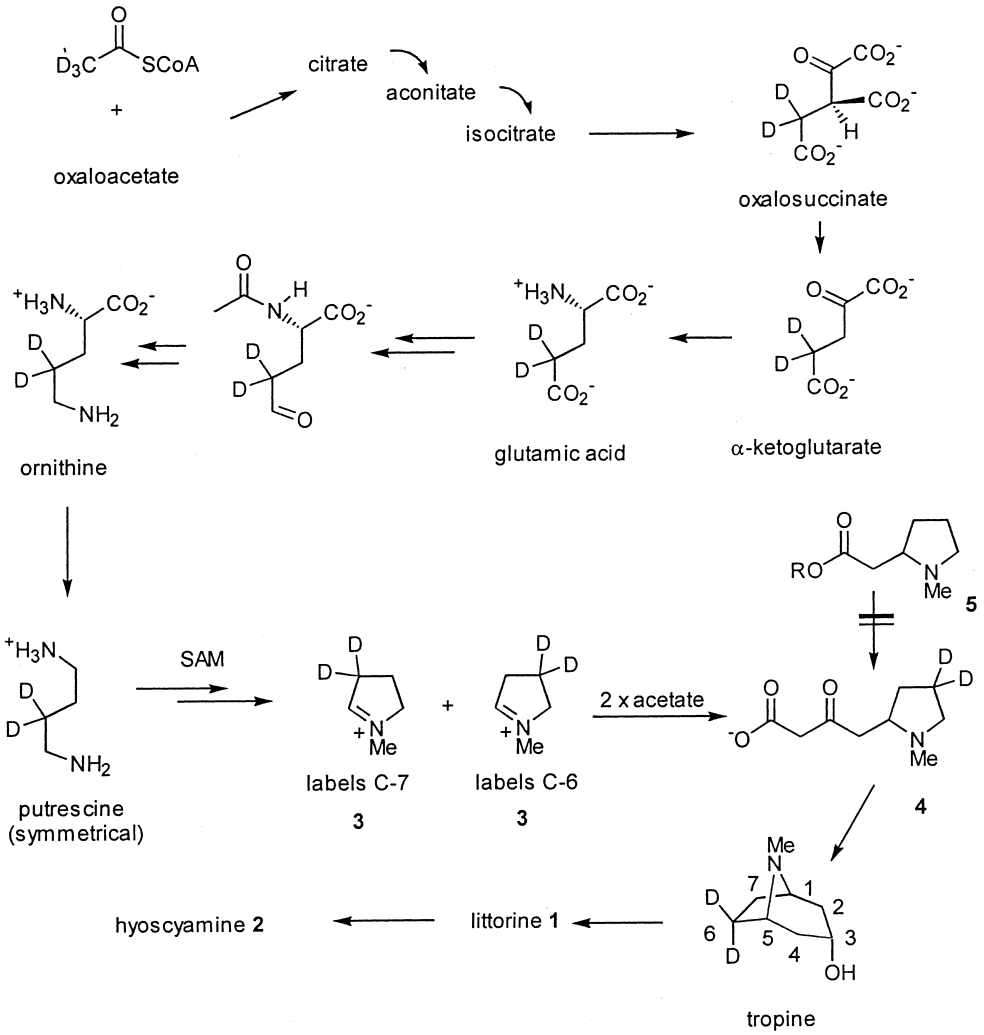
which was presumably hydrolysed in vivo to the corresponding carboxylate, prior to activation and inclusion on the biosynthetic pathway. Clearly, **4** could arise from condensation of acetoacetate with **3**, or alternatively by two sequential condensations of acetate/malonate with **3**, in a polyketide manner. There is no clear experimental evidence to support either of these hypotheses. [1,2,3,4-$^{13}C_4$]-Acetoacetate was not incorporated intact (Hemscheidt & Spenser, 1992) into the tropane ring skeleton, instead the labelling pattern mirrored that of a [1,2-$^{13}C_2$]-acetate feeding experiment, arguing against the intact incorporation of acetoacetate. On the other hand, the incorporation of **5** failed (whereas that of **4** succeeded), arguing against the sequential incorporation of acetate units. So, at present, the details by which the acetate derived carbons C(2), C(3) and C(4) are incorporated to generate **4** is unclear. In order to add more detail to the origin of these carbons, we have studied deuterium and oxygen-18 incorporations from appropriately labelled acetates. At the outset, we were particularly interested in exploring deuterium retention at C(2) and C(4) from [$^2H_3$]-acetate. This was stimulated by a report (Sauerwein, Shimomura & Wink, 1993) a few years ago, which indicated up to a 50% incorporation (as determined by ^{1}H-NMR integration) of deuterium from [1-^{13}C, $^2H_3$]-acetate into C(2) and (4), however, there was an internal contradiction as the same study reported only a 3% incorporation of carbon-13 (into C(3)).

In a final experiment, we have reconfirmed (Robins, Bachmann & Woolley, 1994) the intramolecular nature of the rearrangement of littorine **1** to hyoscyamine **2** by carrying out comparative incorporation studies into hyoscyamine **2** after feeding experiments with [2′-^{13}C, 3-^{2}H]-littorine versus [2-^{13}C]-phenyllactate and [3-^{2}H]-tropine.



Scheme 1. Biosynthetic pathway of the tropane ring skeleton.

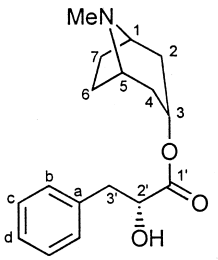
2. Results

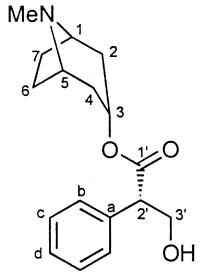
2.1. Feeding experiments with sodium [$^2H_3$]-, [2-^{13}C, $^2H_3$]-, and [1-^{13}C, $^{18}O_2$]-acetates, ^2H_2O and [$^2H_3$-methyl]-methionine

Sodium [$^2H_3$]-acetate was administered to 28 flasks of aseptic transformed root cultures of *Datura stramonium* on day 7 at a final concentration of 8 mM. The alkaloids were extracted and littorine **1** and hyoscyamine **2** were separately purified by preparative thin layer chromatography (TLC). The alkaloids were then analysed by ^{2}H-NMR and gas chromatography–mass spectrometry (GC–MS), to determine deuterium enrichments. It was important at this point to fully assign the hydrogen atoms around the tropane ring system in the ^{1}H-NMR of each of the alkaloids. This was achieved using a combination of ^{1}H-COSY and ^{1}H–^{13}C HETCOR analysis, which allowed assignment of both the ^{1}H- and ^{13}C-NMR spectra of the alkaloids. In this way, a secure assignment for each proton could be made and these are presented in Table 1, however, a limitation of the assignment was the inability to resolve the diastereotopic relationship between C(1)/C(2)/C(7) and C(5)/C(4)/C(6) and thus these carbons and the hydrogens attached to them are arbitrarily assigned in this respect.

The ^{1}H-NMR assignments were then used to assign the deuterium enrichments in the resultant ^{2}H-NMR spectra of the alkaloids isolated after a [$^2H_3$]-acetate feeding experiment. Deuterium had become incorporated only into C(6)Hα/Hβ and C(7)Hα/Hβ of the tropane ring system. These incorporations clearly arose as a consequence of acetate becoming involved in ornithine biosynthesis of **1** and **2** and our suggested rationale is highlighted in Scheme 1. GC–MS analysis

Table 1
^{1}H- and ^{13}C-NMR data of the alkaloids littorine **1** and hyoscyamine **2**[e]





Position	Littorine **1**		Hyoscyamine **2**	
	^{1}H-NMR (500 MHz, $CDCl_3$)	^{13}C-NMR (125 MHz,$CDCl_3$)	^{1}H-NMR (500 MHz, $CDCl_3$)	^{13}C-NMR (125MHz, $CDCl_3$)
1	3.18 (*br s*)	59.7 (*d*)	3.07 (*m*)	[c]61.0 (*d*)
2α	2.21 (*ddd*)	[a]35.6 (*t*)	2.09 (*ddd*)	[d]36.5 (*t*)
2β	1.57 (*d*)		1.61 (*d*)	
3	5.00 (*t*)	67.7 (*d*)	5.83 (*t*)	68.5 (*d*)
4α	2.21 (*ddd*)	[a]35.4 (*t*)	2.17 (*ddd*)	[d]36.7 (*t*)
4β	1.68 (*d*)		1.81 (*d*)	
5	3.18 (*br s*)	59.7 (*d*)	3.18 (*br s*)	[c]61.1 (*d*)
6	1.97 (*m*)	[b]25.0 (*t*)	2.00 (*m*)	25.8 (*t*)
7α	1.81 (*m*)	[b]24.9 (*t*)	1.85 (*m*)	26.1 (*t*)
7β	1.81 (*m*)		1.35 (*m*)	
NMe	2.30 (*s*)	40.5 (*q*)	2.25 (*s*)	40.2 (*q*)
1′		173.1 (*s*)		173.2 (*s*)
2′	4.36 (*dd*)	71.5 (*d*)	3.78-3.83 (*m*)	56.2 (*d*)
3′[a]	3.08 (*dd*)	40.5 (*t*)	4.18 (*dd*)	64.6 (*t*)
3′[b]	2.98 (*dd*)		3.78–3.83 (*m*)	
a		136.5 (*s*)		137.4 (*s*)
b		129.2 (*d*)		129.9 (*d*)
c	7.22–7.30 (*m*)	128.3 (*d*)	7.31–7.40 (*m*)	129.2 (*d*)
d		126.6 (*d*)		128.7 (*d*)

[a-d] a, b, c, d are interchangeable signals.

[e] Selected coupling constants (Hz): **1**: $J_{2\alpha\text{-}2\beta} = 15.5$ Hz; $J_{2\alpha\text{-}3} = 5.4$ Hz; $J_{3\text{-}4\alpha} = 5.4$ Hz; $J_{4\alpha\text{-}4\beta} = 15.5$ Hz; $J_{2'\text{-}3'a} = 5.5$ Hz; $J_{2'\text{-}3'b} = 7.0$ Hz; $J_{3'a\text{-}3'b} = 13.5$ Hz. **2**+: $J_{2\alpha\text{-}2\beta} = 15.5$ Hz; $J_{2\alpha\text{-}3} = 5.0$ Hz; $J_{3\text{-}4\alpha} = 5.0$ Hz; $J_{4\alpha\text{-}4\beta} = 15.5$ Hz; $J_{2'\text{-}3'a} = 11.1$ Hz; $J_{3'a\text{-}3'b} = 12.6$ Hz.

Table 2
Incorporation of label determined by GC–MS from various labelled compounds into littorine **1** and hyoscyamine **2**[a]

Labelled precursor	Incorporation into littorine **1** (%)						Incorporation into hyoscyamine **2** (%)					
	M	M + 1	M + 2	M + 3	M + 4	M + 5	M	M + 1	M + 2	M + 3	M + 4	M + 5
1. Sodium [$^2H_3$]-acetate	92.2	2.8	5.0	< 1	< 1	< 1	91.9	3.8	4.2	< 1	< 1	< 1
2. Sodium [2-^{13}C, $^2H_3$]-acetate	74.7	15.2	7.0	1.8	< 1	< 1	68.5	16.7	10.3	2.6	1.1	< 1
3. Sodium [1-^{13}C, $^{18}O_2$]-acetate	93.8	6.2	< 1	< 1	< 1	< 1	93.3	6.7	< 1	< 1	< 1	< 1
4. ^2H_2O	6.7	14.3	23.9	25.2	19.2	10.7	18.1	13.9	21.1	22.7	16.1	8.1
5. [$^2H_3$-*methyl*]-Methionine	75.7	< 1	< 1	23.8	< 1	< 1	84.5	< 1	< 1	15.5	< 1	< 1
6. [2′-^{13}C, 3-^{2}H]-Littorine	50.9	22.0	27.0	< 1	< 1	< 1	79.9	10.1	9.0	< 1	< 1	< 1
7. [3-^{2}H]-Tropine + [2′-^{13}C]- phenyllactate	79.8	18.8	1.4	< 1	< 1	< 1	80.2	18.2	1.4	< 1	< 1	< 1

[a] The data are presented following correction for natural abundance levels, which were determined experimentally using authentic standard compounds.

(Table 2, entry 1) of the resultant littorine **1** and hyoscyamine **2** revealed that the M + 2 ions were more intense than the M + 1 ions for both **1** and **2**, and thus, the greater proportion of incorporated acetate molecules carried two deuterium atoms on each of these carbons. The methylene group is retained throughout the biosynthesis, and exchange processes are clearly not so extensive. On the other hand, there was no evidence from ^{2}H-NMR for any deuterium incorporation into C(2) and C(4) of the tropane rings in **1** and **2**. In order to reinforce this conclusion, a second experiment was carried out in the same way but using sodium [2-^{13}C, $^2H_3$]-acetate. There is an advantage in directly bonding the deuterium to carbon-13 as any retained deuterium can be observed by an induced chemical shift in the resultant ^{13}C{^{1}H, ^{2}H}-NMR spectrum (Chesters, O'Hagan & Robins, 1995). Each deuterium atom induces a shift in the carbon-13 signal to lower frequency of ~0.3–0.35 ppm in magnitude. In the event, there was a clear carbon-13 enrichment (6.6%) of the signals corresponding to C(2) and C(4) in the ^{13}C-NMR spectra of **1** and **2**, but again there was no indication of any retained deuterium at these sites. By contrast, the signals corresponding to C(6) and C(7) were enriched by carbon-13 (1.8%), and they both had clearly discernible shifted signals in the ^{13}C{^{1}H, ^{2}H}-NMR spectra corresponding to the presence of populations of molecules carrying both one and two deuterium atoms at these sites (Fig. 1). This reinforced the earlier conclusion that up to two deuterium atoms are incorporated into carbon C(6)/C(7) from labelled acetate. At the end of this study, it is concluded that deuterium is retained only at C(6) and C(7), the ornithine derived carbons of the tropane ring. In contrast to a previous report (Sauerwein et al., 1993), there was no deuterium retention at C(2) and C(4), presumably as a consequence of facile exchange with the medium at these sites during biosynthesis.

In an effort to demonstrate that C(2) and C(4) can incorporate isotope from the medium, an experiment was conducted where *D. stramonium* root cultures were grown in a medium prepared with water enriched with ^2H_2O (24%). The resultant littorine **1** and hyoscyamine **2** were analysed both by ^{2}H-NMR and GC–MS analysis. The ^{2}H-NMR spectra showed that deuterium had become incorporated into all positions around the ring system, including C(2) and C(4). GC–MS analysis (Table 2, entry 4) revealed enrichments up to M + 5 (higher ions were not recorded). Clearly, there was significant incorporation of isotope from the enriched medium into the hydrogen atoms of the tropane ring system, but there was no evidence from the ^{2}H-NMR of a significantly higher level of incorporation into C(2)/C(4) over the other sites.

In order to explore the biosynthesis of the acetate derived moiety of the tropane ring further, a feed-

ing experiment was conducted with sodium [1-^{13}C, $^{18}O_2$]-acetate (99% ^{13}C, 48% ^{18}O). In the event there was a clear enrichment (~8%) in the signal corresponding to C(3) in the resultant ^{13}C-NMR spectrum, however, there was no indication of any induced shift in the carbon-13 signal for C(3) as a consequence of residual oxygen-18. Further analysis of this sample by GC–MS (Table 2, entry 3) did not reveal any enrichment of an M + 3 ion; thus, it is concluded that all the oxygen-18 was washed out, at least below the detectable threshold (~0.4% incorporation) during the biosynthesis of the tropane ring system.

A feeding experiment with [$^2H_3$-*methyl*]-methionine was then conducted and the resultant littorine **1** and hyoscyamine **2** were subjected to GC–MS analysis. As anticipated, this gave rise in both cases to significant enhancements of the M + 3 ions (23.8 and 15.5%, respectively) indicating incorporation of all three deuterium atoms into the *N*-methyl group. There was, however, no indication of any washout of these deuterium atoms as evidenced by the negligible M + 2 ion enhancements in each case (entry 5, Table 1). Thus, these atoms are not libel to exchange at any stage of the biosynthetic process.

2.2. Feeding experiments with [2′-^{13}C, 3-^{2}H]-littorine, [2-^{13}C]-phenyllactate and [3-^{2}H]-tropine

Robins et al. (1994) revealed, for the first time, that the tropate ester moiety of hyoscyamine **2** arose by an intramolecular isomerisation of littorine **1** to hyoscyamine **2**. This was an important observation as until then it was assumed that hyoscyamine **2** was generated by esterification of tropine and tropic acid. Two experiments have been carried out in this study, which reinforce the earlier observation. Firstly, [2′-^{13}C, 3-^{2}H]-littorine was prepared with an isotopic label on each side of the ester moiety, and was fed to *D. stramonium* root cultures. Clearly, if littorine **1** rearranges directly to hyoscyamine **2** then both labels will become incorporated into hyoscyamine **2** resulting in a significant M + 2 ion. Alternatively, if littorine **1** is hydrolysed and the resultant phenyllactate rearranges, to tropic acid, and recombines with tropine, both labelled and unlabelled, to generate hyoscyamine **2**, this process will lead to a significant dilution in the population of the resultant M + 2 labelled hyoscyamine **2**. In a control experiment, [2-^{13}C]-phenyllactate and [3-^{2}H]-tropine were added together to root cultures of *D. stramonium* and the single and double isotope incorporation levels

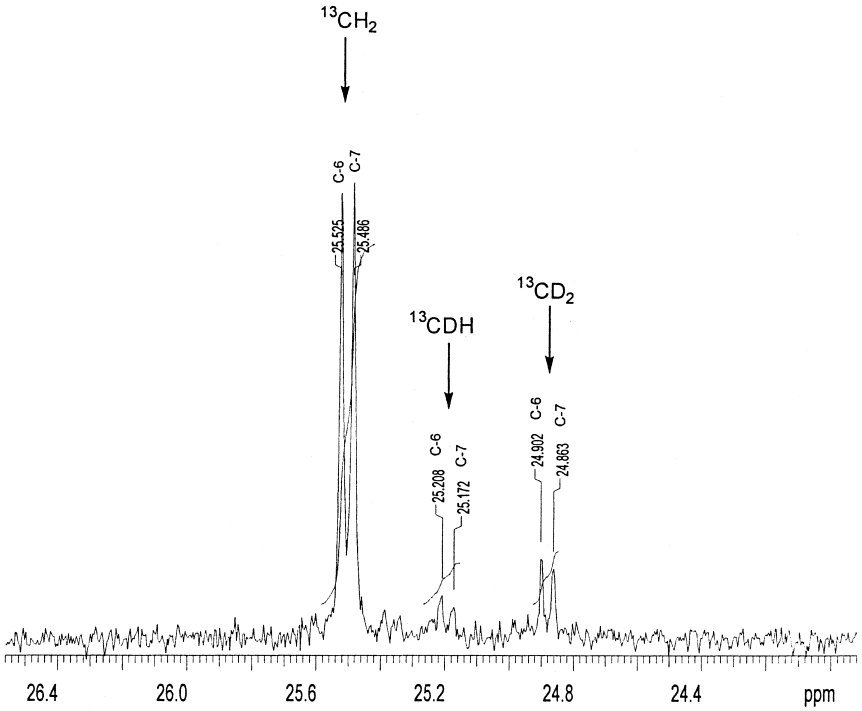


Fig. 1. Region of the $^{13}C\{^1H, ^2H\}$-NMR spectrum showing the signals for C(6) and C(7) of littorine **1** after a feeding experiment with sodium [2-^{13}C,$^2H_3$]-acetate. It is clear from the signals to lower frequency that there are populations of molecules for both C(6) and C(7) with $^{13}CD_2$, ^{13}CDH as well as $^{13}CH_2$ at these sites in the resultant littorine **1**.

evaluated in the resultant hyoscyamine **2** by GC–MS. The GC–MS data are presented in Table 2 (entries 6 and 7). Clearly, the level of double label (M + 2) found in hyoscyamine **2** after feeding with [2′-^{13}C, 3-^{2}H]-littorine (Table 2, entry 6) is significantly higher than that observed in the [2-^{13}C]-phenyllactate + [3-^{2}H]-tropine experiment (Table 2, entry 7) (9.0% versus 1.4%). This is consistent with the intramolecular rearrangement of littorine **1** to hyoscyamine **2** as previously reported. Clearly, if phenyllactate or a derivative (e.g. CoA ester) was the substrate for rearrangement to tropate, then the levels of incorporation should be similar in each experiment, or perhaps reversed as littorine **1** would require to be hydrolysed to deliver phenyllactate for tropate biosynthesis. These results are inconsistent with this scenario and support the direct rearrangement of littorine **1** to hyoscyamine **2** (Scheme 1).

3. Conclusions

Feeding experiments with [$^{2}H_3$]- and [2-^{13}C, $^{2}H_3$]-acetates have demonstrated that up to two of the C–H bonds of acetate become incorporated intact into C(6)/C(7), and that the predominant labelled species possesses two deuterium atoms at these sites. These carbons formally derive from ornithine, and clearly, acetate is entering into ornithine biosynthesis as illustrated in Scheme 1. On the other hand, there was no deuterium retention at all in these experiments at C(2)/C(4), carbons which also derive from C(2) of acetate. In experiments where [1-^{13}C]- and [2-^{13}C]-acetates were fed to *D. stramonium* root cultures, carbon-13 incorporations into carbons C(2)/C(3)/C(4) are always higher than carbons C(1)/C(5)/C(6)/C(7), suggesting a more direct involvement of acetate in the former case. However, the complete washout of deuterium from these sites after [$^{2}H_3$]- and [2-^{13}C-, $^{2}H_3$]-acetates experiments and the lack of retention of oxygen-18 at C(3)–O of the tropane ring after the [1-^{13}C, $^{18}O_2$]-acetate experiment clearly suggests that the biosynthetic intermediate(s) delivering this three carbon unit is highly enolisable and susceptible to complete exchange with the medium. There are several studies in polyketide biosynthesis (O'Hagan, Robinson & Turner, 1983; Cane, Liang & Hasler, 1982) where detectable levels of deuterium and oxygen-18 are retained in such sites in metabolites derived from the carbonyl of acetate/malonate. Thus, perhaps there is a fundamental difference which remains to be revealed in the biosynthetic origins of this system and polyketides in general.

Finally, the intramolecular nature of the rearrangement of littorine **1** to hyoscyamine **2** is demonstrated in a comparative experiment between double labelled [2′-^{13}C, 3-^{2}H]-littorine and after admixing [2-^{13}C]-phenyllactate and [3-^{2}H]-tropine. This confirms the conclusion reached by Robins et al. (1994). Despite considerable attention, the mechanism of this rearrangement remains to be elucidated (Ollagnier, Kervio & Retey, 1998; O'Hagan & Robins, 1998).

4. Experimental

4.1. General

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Unity 300 MHz (^{1}H at 299.908 MHz, ^{13}C at 75.412 MHz) and Varian Inova 500 MHz (^{1}H at 499.779 MHz, ^{13}C at 125.670 MHz) spectrometers, and ^{2}H-NMR spectra were recorded on a Varian Inova 500 MHz (^{2}H at 76.719 MHz) spectrometer. Chemical shifts are quoted relative to TMS (Me_4Si) in $CDCl_3$ or CD_3OD. GC–MS analyses were conducted using a Hewlett Packard 6890 gas chromatograph, equipped with a Hewlett Packard 7683 autosampler, directly linked to a Hewlett Packard 5973 Mass Selective Detector (MSD). Chromatographic separations were performed using a SGE fused-silica wall-coated open tubular capillary column (25 m × 0.22 mm) with Cydex-B (0.25 μm) as the bonded phase. The MSD was operated in the selected ion monitoring mode measuring ion currents at m/z 289 (M^+), 290, 291, 292, 293 and 294. Preparative TLC was performed on Whatman K6F silica gel 60 Å, 0.25 mm layer thickness and fluorescent indicator. Chemicals were obtained from Aldrich and D_2O from GOSS Scientific Instruments.

4.2. Organism and culture conditions

Transformed roots cultures of *D. stramonium* L. D15/5 were grown and maintained in B50 medium as described previously (Robins, Parr, Bent & Rhodes, 1991) and subcultured every 21 days.

4.3. Synthesis and determination of the isotopic composition of sodium [1-^{13}C, $^{18}O_2$]-acetate

Sodium [1-^{13}C, $^{18}O_2$]-acetate was prepared as previously described by Cane et al. (1982). Accurate isotopic composition was determined through GC–MS analysis after preparation of the *p*-phenylphenacyl derivative (Cane et al., 1982). MS, m/z 255–259, $^{13}C^{16}O_2$ (24.3%), $^{12}C^{18}O_1$ (1.8%), $^{13}C^{18}O_1$ (46.8%), $^{12}C^{18}O_2$ (1.0%), $^{13}C^{18}O_2$ (26.1%). This ratio has been corrected for natural abundance and demonstrates an isotope content of 100% ^{13}C, 46% ^{18}O and 26% $^{18}O_2$ in the sample.

4.4. Synthesis of (RS)-[2-^{13}C]-phenyllactic acid

(*RS*)-[2-^{13}C]-Phenyllactic acid was prepared from commercially available [1-^{13}C]-phenylacetic acid (99 at% ^{13}C) as described by Chesters, O'Hagan and Robins (1994). In the event, $LiAlH_4$ was used instead of $LiAlD_4$.

4.5. Synthesis of [3-^{2}H]-tropine

A solution of tropinone (527 mg, 3.8 mmol) in CH_3OH (43 ml) was treated with $NaBD_4$ (282 mg, 6.7 mmol) and stirred for 20 h at room temperature. Excess reducing reagent was then quenched with H_2O. The reaction mixture was made basic and the CH_3OH evaporated. The aqueous phase was extracted into CH_2Cl_2 and the solvent evaporated to give a mixture (500 mg) of the epimers [3-^{2}H]-tropan-3*endo*-ol (58%) and [3-^{2}H]-tropan-3*exo*-ol (42%). [3-^{2}H]-Tropan-3*endo*-ol was purified from [3-^{2}H]-tropan-3*exo*-ol by column chromatography on silica gel using $CHCl_3$/EtOH/NH_3 (7:7:1) as solvent system, mp 60°C (dec.) (lit (Berger, Jacobson & Kondritzer, 1957) mp 63–64.5°C). The isotopic composition was 92% (calculated by integration of the resultant ^{1}H-NMR spectrum).

4.6. Synthesis of (RS)-[2′-^{13}C, 3-^{2}H]-littorine

(*RS*)-[2′-^{13}C, 3-^{2}H]-Littorine was prepared from (*RS*)-[2-^{13}C]-phenyllactic acid and [3-^{2}H]-tropan-3*endo*-ol as previously described (Robins et al., 1994) and purified by column chromatography over silica gel using $CHCl_3$/EtOH/NH_3 (7:7:0.2) as the eluent. The isotopic composition was determined by GC–MS analysis, *m*/*z* 291 (M + 2) 95.1%.

4.7. Feeding experiments: extraction and isolation of the tropane alkaloids

4.7.1. General method

The subcultured flasks, containing an initial inoculum of fresh mass (0.5 g) of roots of *D. stramonium* in culture medium (50 ml), were fed with a filter-sterilised solution of the labelled precursor in H_2O or CH_3OH on day 7. Root cultures were harvested and freeze-dried after 17 days. The freeze-dried roots were then grounded with acid-washed sand and extracted into 50 mM H_2SO_4 by stirring for 20 min. The aqueous extract was made basic with 35% NH_3 solution and then filtered through Hydromatrix® and eluted with $CHCl_3$:CH_3OH (20:1). The solvent was evaporated under reduced pressure to give a brown oil containing the alkaloids. The extract was chromatographed on preparative TLC plates, using $CHCl_3$:Et_2NH (9:1) as eluent system, to afford littorine **1** and hyoscyamine **2**.

4.7.2. Feeding of sodium [$^{2}H_3$]-acetate to D. stramonium root cultures

Twenty-eight subcultured flasks with *D. stramonium* were fed with 0.9 ml of a filter-sterilised solution of [$^{2}H_3$]-acetic acid (28 mg/ml, pH = 5.8) in H_2O to a final concentration of 8 mM. Work up of the freeze-dried roots (6 g) yielded a crude alkaloid extract (54 mg), which was purified as described in the general method to afford **1** (4.4 mg) and **2** (15.8 mg).

4.7.3. Feeding of sodium [2-^{13}C, $^{2}H_3$]-acetate to D. stramonium root cultures

In accordance with the above procedure, eight subcultured flasks of *D. stramonium* were fed with a filter-sterilised solution (0.5 ml) of sodium [2-^{13}C, ^{2}H$_3$]-acetate (50 mg/ml) in H_2O to a final concentration of 8 mM. Work up of the freeze-dried roots (2.6 g) yielded a crude alkaloid extract (33.6 mg), which was purified as described in the general method to afford **1** (4.0 mg) and **2** (10.4 mg).

4.7.4. Feeding of sodium [1-^{13}C, $^{18}O_2$]-acetate to D. stramonium root cultures

To six subcultured flasks, a previously prepared sterile solution of sodium [1-^{13}C, ^{18}O$_2$]-acetate (99% ^{13}C, 48% ^{18}O, 59.8 mM) in H_2O was pulse fed on days 5, 7 and 9 to a final concentration of 4.43 mM in the medium. Work up of the freeze-dried roots (1.8 g) as described in the general method gave an alkaloid extract (30 mg), which was submitted for GC–MS analysis. Purification of hyoscyamine by preparative TLC provided a sample for ^{13}C-NMR analysis.

4.7.5. Feeding $^{2}H_2O$ to D. stramonium root cultures

Ten flasks, containing 50 ml of B50 culture medium in 24% of D_2O, were inoculated with 0.5 g of fresh mass of roots of *D. stramonium* and incubated for 17 days. Work up of the freeze-dried roots (2 g) yielded a crude alkaloid extract (23 mg), which was purified as described in the general method to afford **1** (2.4 mg) and **2** (7.4 mg).

4.7.6. Feeding of [$^{2}H_3$-methyl]-methionine to D. stramonium root cultures

Ten subcultured flasks with *D. stramonium* were fed with 0.5 ml of a filter-sterilised solution of [^{2}H$_3$-*methyl*]-methionine (23 mg/ml) in H_2O to a final concentration of 1.5 mM. Work up of the freeze-dried roots (2.2 g), as described in Section 4.7.1, gave an alkaloid extract (22.4 mg) which was submitted for GC–MS analysis.

4.7.7. Feeding of [2′-^{13}C, 3-^{2}H]-littorine to D. stramonium root cultures

In accordance with the general procedure, eight subcultured flasks of *D. stramonium* were fed with a sol-

ution (200 μl) of [2′-^{13}C, 3-^{2}H]-littorine (7.3 mg/ml) in CH_3OH to a final concentration of 0.1 mM. Work up of the freeze-dried roots (3.2 g) yielded a crude alkaloid extract (30 mg), which was purified as described in Section 4.7.1 to afford **1** (5.5 mg) and **2** (9.9 mg).

4.7.8. Feeding of [2-^{13}C]-phenyllactate and [3-^{2}H]-tropine to D. stramonium root cultures

Eleven subcultured flasks with *D. stramonium* were fed with a filter-sterilised solution (242 μl) of [2-^{13}C]-phenyllactic acid (3.5 mg/ml, pH = 6.4) in H_2O and a solution (200 μl) of [3-^{2}H]-tropine (3.6 mg/ml) in CH_3OH to a final concentration of 0.1 mM for each labelled precursor. Work up of the freeze-dried roots (3.6 g) yielded a crude alkaloid extract (45.5 mg), which was purified as described in Section 4.7.1 to afford **1** (3.5 mg) and **2** (14.1 mg).

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