PERGAMON

Phytochemistry 61 (2002) 323–329

PHYTOCHEMISTRY

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Biosynthetic studies on the tropane alkaloid hyoscyamine in *Datura stramonium*; hyoscyamine is stable to in vivo oxidation and is not derived from littorine via a vicinal interchange process

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Received 17 April 2002; received in revised form 29 May 2002

Abstract

The conversion of littorine to hyoscyamine has been investigated by feeding deuterium labelled (*RS*)-[2-^{2}H]-, [3, 3-$^{2}H_2$]-, [2, 3, 3-$^{2}H_3$]- phenyllactic acids to transformed root cultures of *Datura stramonium*. Isolation and GC–MS analyses of the isotope incorporation into the resultant hyoscyamine does not support the involvement of a vicinal interchange process operating during the isomerisation of littorine to hyoscyamine. Additionally a metabolism study with [1′-^{13}C, 3′, 3′-$^{2}H_2$]-hyoscyamine has established that the alkaloid is metabolically stable at C-3′ with no evidence for a reversible in vivo oxidation process to the corresponding aldehyde. The data do not support an *S*-adenosy-L-methionine (SAM **5**)/co-enzyme-B_{12} mediated process for the isomerisation of littorine to hyoscyamine.

Keywords: *Datura stramonium*; Solanaceae; Transformed root culture; Biosynthesis; Secondary metabolism; Tropane alkaloids; Hyoscyamine; Littorine; Tropic acid

1. Introduction

Littorine **1**, hyoscyamine **2** and scopolamine **3** constitute the major tropane alkaloids of *D. stramonium*. The biosynthesis of tropic acid, the ester moiety of the tropanes hyoscyamine **2** and scopolamine **3** has been a topic of interest for many years (Leete et al., 1975; Ansarin and Woolley, 1994; Chesters et al., 1995, 1996; Humphrey and O'Hagan, 2001). It has been demonstrated by isotopic labelling studies in transformed root cultures of *Datura stramonium* that littorine **1** [(*R*)-phenyllactoyl tropine] is the direct biosynthetic precursor of hyoscyamine **2** and that littorine **1** is converted to hyoscyamine **2** via an intramolecular rearrangement (Robins et al., 1994) (Scheme 1). Thus, the tropate ester moiety of hyoscyamine **2** derives by isomerisation of the (*R*)-phenyllactate ester of littorine **1**. This type of carbon skeletal rearrangement finds some precedence in co-enzyme B_{12} mediated isomerisations, e.g. methylmalonyl-CoA mutase (Eggerer et al., 1960; Aeberhard et al., 1983) (Scheme 2) and glutamate mutase, (Barker et al., 1958). It is well known that co-enzyme B_{12} mediated isomerisations are accompanied by a *vicinal interchange* process (Scheme 3), where a hydrogen atom and the migrating functional group apparently swap places on adjacent carbon atoms. This arises because the co-factor adenosyl radical abstracts a hydrogen atom to generate a substrate radical (Scheme 2). An isomerisation of the substrate radical to product radical then takes place on the surface of the enzyme and finally the product radical is quenched by abstracting back a hydrogen to regenerate the adenosyl radical. This offered an attractive mechanism for the littorine **1** to hyoscyamine **2** rearrangement. In 1987, Leete published data which indicated the 'back migration' of tritium from feeding (2*S*, 3*S*)-[1-^{14}C, 3-^{3}H]-phenylalanine into C-3′ of hyoscyamine **2**. Incorporations in this study were low (2%). In a more recent study investigating stereochemical aspects of hyoscyamine biosynthesis, data emerged pertinent to the putative vicinal interchange process. As part of a wider series of studies (Chesters et al., 1996), a feeding experiment involving (*R*/*S*)-[2-^{13}C, 3,3-$^{2}H_2$]-phe-

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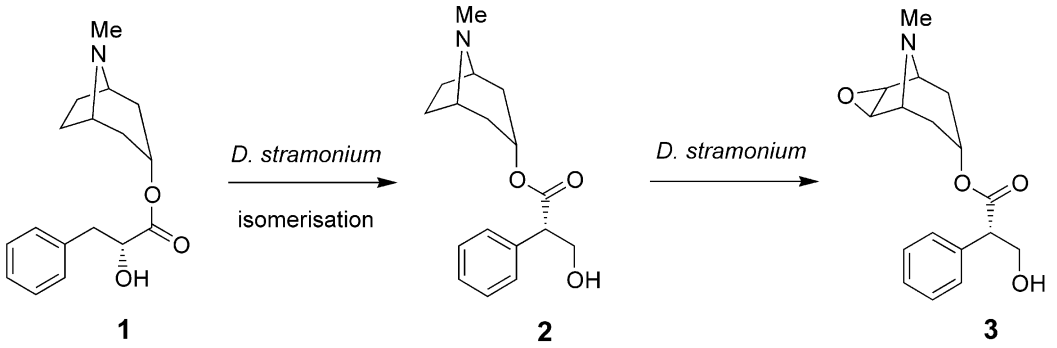
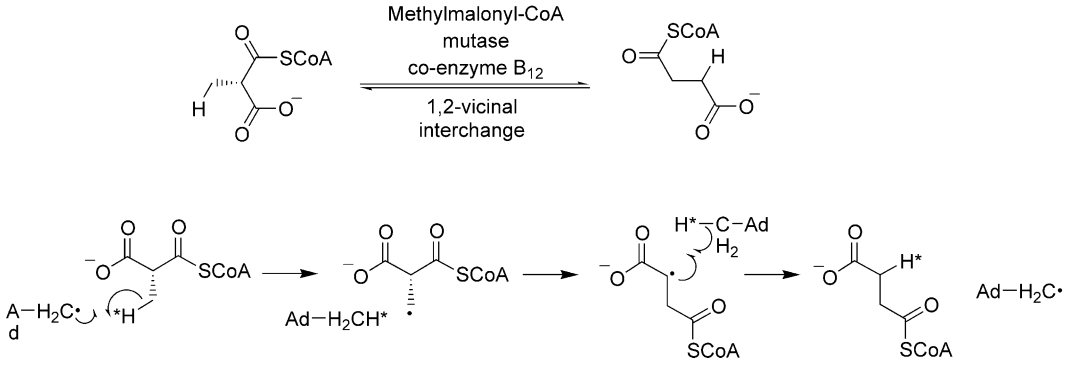
PII: S0031-9422(02)00200-5



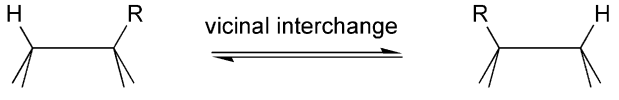
Scheme 1. The tropane alkaloids littorine **1**, hyoscyamine **2** and scopolamine **3**.



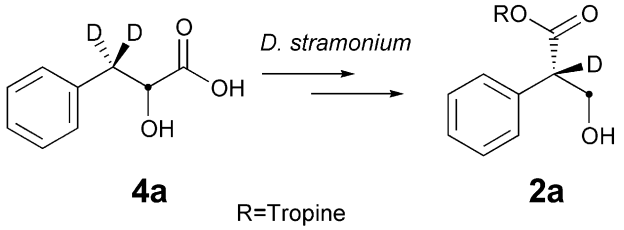
Scheme 2. The minimal mechanism of methylmalonyl-CoA mutase.

nyllactate **4a** followed by ^{13}C NMR analysis indicated removal of the *3′-pro-R* hydrogen of littorine **1**, but despite a 45% incorporation of isotope from the *3′-pro-S* site of littorine **1** into C-2′ of hyoscyamine **2**, there was no evidence for the redelivery of the original *3′-pro-R* hydrogen of littorine **1** to C-3′ of hyoscyamine **2** (Scheme 4). This observation contradicted the earlier tritium result both in mechanism and stereochemistry, i.e. it is the *3-pro-S* hydrogen of phenyllactate which is removed (Platt et al., 1984; Chesters et al., 1996). However, in view of the substrates fed where phenyllactate is a closer metabolite to littorine **1** than phenylalanine, and the levels of incorporation detected (20–45% versus 2%), it was concluded that the more recent result was the more reliable. The report 'excluded the possibility of a vicinal interchange process'.

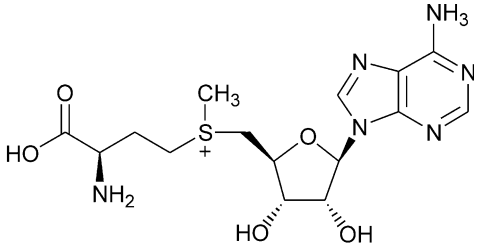
In the interim, a report has been published indicating that *S*-adenosyl-L-methionine **5** (SAM) is the co-factor mediating the isomerisation of littorine **1** to hyoscyamine **2** (Ollagnier et al., 1998). It was proposed that similar to co-enzyme-B_{12} an adenosyl radical, this time derived from SAM **5** (Kilgore and Aberhart, 1991), initiates the rearrangement. Implicit in this proposal would be the operation of a vicinal interchange process (Scheme 3). In the study, cell free extracts of *D. stramonium* root cultures were used and addition of littorine **1** and SAM **5** resulted in a 10–20 fold increase in the levels of hyoscyamine **2** in the assay. However, the assay has proven to be extremely erratic (Rétey, personal communication, 2002). The experiment used the unnatural (*S*)-isomer of littorine and this would clearly require an in vitro isomerisation with appropriate enzymes and co-factors in the cell free extract to generate some (*R*)-littorine **1** prior to isomerisation to hyoscyamine **2**. Additionally the experiment did not use isotopically labelled (*S*)-littorine and thus there was no direct evidence that the increase in hyoscyamine **2** was contributed from the administered (*S*)-littorine. Also when the experiment was carried out with [2,8,5′-$^{3}H_3$]-SAM there was no incorporation of tritium into hyoscyamine **2** as might be expected from the 5′ site, but



Scheme 3. A vicinal interchange process.

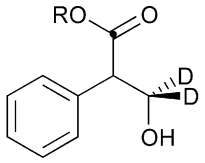


Scheme 4. Feeding (*R*/*S*)-[2-^{13}C, 3,3-$^{2}H_2$]-phenyllactate **4a** to *D. stramonium* resulting in hyoscyamine **2a** retaining the 3′-*pro-S* deuterium from **4a** (Chesters et al., 1996).



5

S-adenosyl-L-methionine **5**



2e

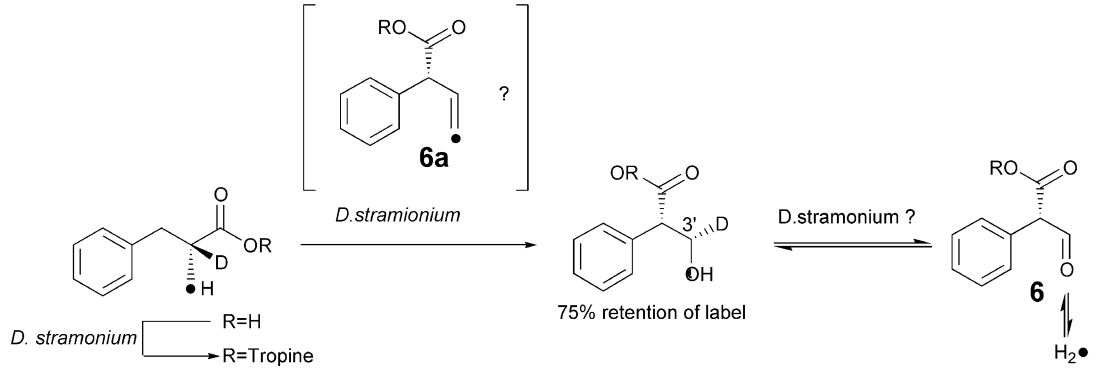
R=Tropine

(*R/S*)-[1'-^{13}C, 3',3'-$^{2}H_2$]-hyoscyamine **2e**

some loss of radioactivity into the bulk water. It was proposed (Ollagnier et al., 1998) that the failure to detect tritium in the resultant hyoscyamine **2** may be due to the activity of an aldehyde dehydrogenase acting reversibly on product hyoscyamine **2** to generate the corresponding aldehyde **6** with 'washout' of tritium from the 3′-*pro-S* site, rather than the absence of a vicinal interchange process a prerequisite of a SAM **5** mediated isomerisation.

Previous studies from our laboratory (Wong et al., 1998) have shed some light on the extent of washout of the C-3′ oxygen in going from littorine **1** to hyoscyamine **2**. The action of an oxido-reductase enzyme could result in the loss of the 3′-*pro-S* hydrogen, and at the aldehyde level **6** the oxygen may then be susceptible to some exchange with bulk water (Scheme 5). To explore the level of oxygen washout a feeding experiment with (*RS*)-[2-^{2}H, ^{18}O]-phenyllactic acid to *D. stramonium* root cultures was conducted. This showed a significant but only partial loss (25–29%) of oxygen-18 label during the isomerisation of littorine **1** to hyoscyamine **2** (Wong et al., 1998). Between 70 and 75% oxygen-18 was retained during the isomerisation. Clearly this level of isotope exchange at C-3′ of hyoscyamine **2** in a whole cell experiment over 11–17 days is at variance with the complete loss of tritium from the SAM study in a cell free extract over a few hours. Alternatively, this oxygen-18 at C-2′ of littorine **1** could have been liberated due to the intermediacy of an (enzyme bound? **6a**) aldehyde *during* isomerisation to hyoscyamine **2** which is then reduced prior to leaving the enzyme (a reducto-isomerase?).

It was important then to explore these issues again in the light of the recent report (Ollagnier et al., 1998) proposing SAM **5** as the co-factor in the isomerisation of littorine **1** to hyoscyamine **2**. In order to reinvestigate the operation of a vicinal interchange process we have carried out feeding experiments in transformed root cultures of *D. stramonium* with (*RS*)-[2-^{2}H]-**4b**, [3, 3-$^{2}H_2$]-**4c**, [2, 3, 3-$^{2}H_3$]-**4d** phenyllactic acids. Phenyllactic acids undergo enzymatic esterification to littorine **1** and thus these are good substrates for generating the corresponding labelled littorines **1b–d** in vivo. The labelled littorines **1b–d** are then converted to hyoscyamines **2b–d**. GC–MS analysis of the isotope incorporations into hyoscyamine offered an extremely sensitive method of analysis with a lower (<0.5%) detectable isotope incorporation than previous NMR methods. Additionally it appeared appropriate to design a probe to explore the level of post-biosynthetic in vivo oxidation at C-3′ of hyoscyamine **2** as this has a direct bearing on the observation of the vicinal interchange process by isotope labelling. In particular it was important to determine if the isotope that is delivered to position C-3′ of hyoscyamine **2** is rapidly washed out in vivo. Accordingly (*RS*)- [1′-^{13}C, 3′, 3′-$^{2}H_2$]- hyoscyamine **2e** was prepared and the level of isotope monitored over an 18 day period after administering this compound to transformed root cultures of *D. stramonium*.



Scheme 5. Feeding (*RS*)-[2-^{2}H, ^{18}O]-phenyllactic acid to *D. stramonium* resulted in a 70–75% retention of the label in oxygen at C-3′ of hyoscyamine. The loss of label could be due to washout at the aldehyde level from either post-biosynthetic oxidation **6** or an intermediate in the isomerisation **6a**.

2. Results and discussion

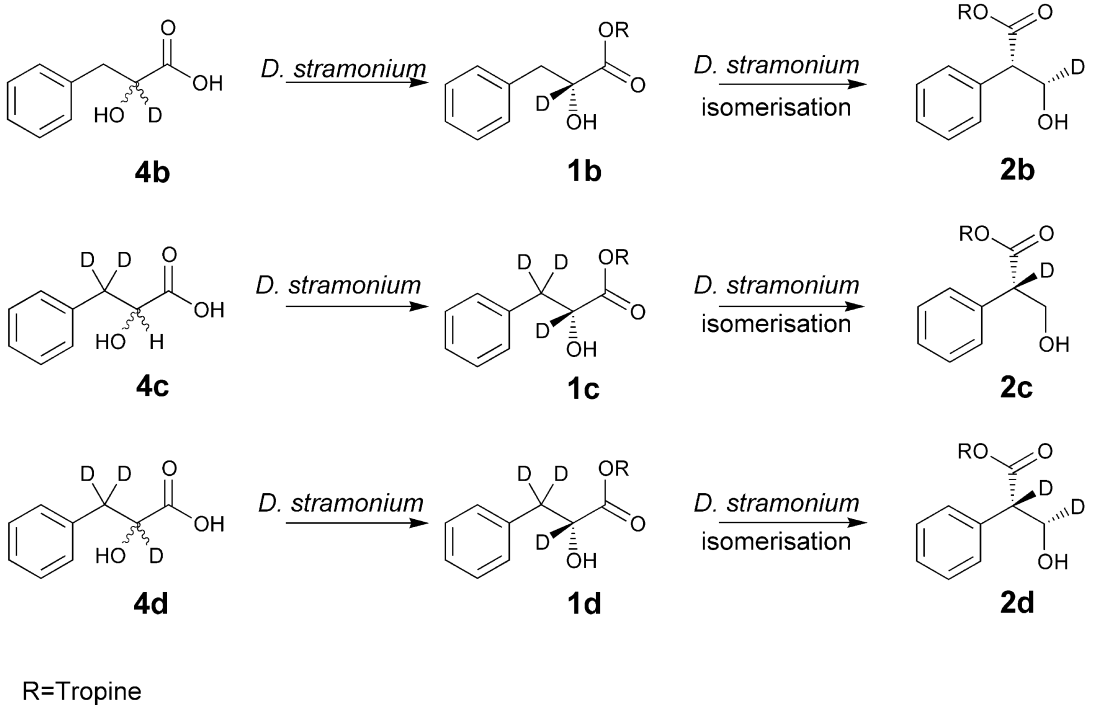
2.1. Synthesis and feeding experiments with labelled phenyllactic acids *4b–d*

Samples of (*RS*)-[2-^{2}H]-, [3,3-$^2H_2$]-, [2,3,3-$^2H_3$]- phenyllactic acids **4b–d** were prepared from unlabelled phenylpyruvic acid or [3,3-$^2H_2$]-phenylpyruvic acid by reduction with $NaBH_4$ or NaB^2H_4. The resultant deuterium labelled phenyllactic acids **4b–d** were administered to transformed root cultures of *D. stramonium* at a final concentration of 0.25 mmol dm^{-3} in the medium. Addition of (*R*)-phenyllactic acid at this concentration is known to stimulate the production of littorine **1** and hyoscyamine **2** (Zabetakis et al., 1998). The mass ion enrichments of isolated littorine **1** and hyoscyamine **2** were then determined by GC–MS (CI, CH_4) analysis (Wong et al., 1998) of the crude alkaloid extract and comparison with unlabelled alkaloids (Table 1).

The feeding experiment with (*RS*)-[2-^{2}H]-phenyllactic acid **4b** resulted in an M + 1 enrichment (3.6–3.9%) of both littorine **1b** and hyoscyamine **2b**, consistent with the earlier observation that the C-2′ hydrogen of littorine **1** is retained as the 3′-*pro-S* hydrogen of hyoscyamine **2** after isomerisation (Chesters et al., 1995). The feeding experiment with (*RS*)-[3, 3-$^2H_2$]-phenyllactic acid **4c** resulted in a M + 2 enrichment (8%) into littorine **1c**, but significantly there was no detectable M + 2 enrichment in the co-isolated hyoscyamine **2c**. Instead, only an M + 1 enrichment (4%) was observed in hyoscyamine **2c** indicating that one of the C-3′ deuterium atoms is lost during the isomerisation of littorine **1c** to hyoscyamine **2c**. Similarly the feeding experiment with (*RS*)-[2, 2, 3-$^2H_3$]-phenyllactic acid **4d** resulted in an M + 3 enrichment into littorine **1d**, but only an M + 2 enrichment in the resultant hyoscyamine **2d**, again indicating the loss of one deuterium atom during the isomerisation. The results are summarised in Scheme 6. These results reinforce the earlier study (Chesters et al., 1996) which indicated that one C-3′ hydrogen (3′-*pro-S*) of littorine **1** is retained as the C-2′ hydrogen of hyoscyamine **2** and one (the 3′-*pro-R*) is lost during iso-

Table 1
Percentage incorporations of deuterium labelled phenyllactic acids into littorine **1** and hyoscyamine **2**

	Lit **1**				Hyo **2**			
	M	M + 1	M + 2	M + 3	M	M + 1	M + 2	M + 3
[2-^{2}H]-Phlactate	96.07	3.67	0.20	0.07	96.15	3.82	0.02	0.02
[3, 3-$^2H_2$]-Phlactate	87.70	3.89	8.28	0.14	95.75	4.02	0.11	0.12
[2, 3, 3-$^2H_3$]-Phlactate	80.05	5.91	7.31	6.73	92.23	4.18	3.49	0.11



Scheme 6. Summary of feeding isotopically labelled phenyllactic acids **4b–d** to *D. stramonium*.

Table 2
Incorporation of (*RS*)-[1′-^{13}C, 3′,3′-$^{2}H_2$]- hyoscyamine into hyoscyamine **2**. The values are an average of three flasks, the greatest variance is given in parentheses

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

merisation. The complete loss of one deuterium atom from C-3′ of littorines **1c, d** during the isomerisation to hyoscyamine **2c, d** is inconsistent with a vicinal interchange process which by definition, requires relocation of the isotope onto the product.

2.2. Synthesis and metabolism study with (*R*/*S*)-[1′-^{13}C, 3′, 3′-$^{2}H_2$]- hyoscyamine **2e**

In order to explore the reversible redox metabolism at C-3′ of **2** to aldehyde **6** a sample of (*RS*)-[1-^{13}C, 3′, 3′-$^{2}H_2$]- hyoscyamine **2e** was prepared as described previously (Patterson and O'Hagan, 2002) and administered to root cultures of *D. stramonium* at a final concentration of 0.25 mmol dm^{-3}. This sample contains three isotopically enriched atoms and accordingly the ratio (M + 2/M + 3 × 100) was used as a measure of the loss of a single deuterium over time. The data in Table 2 were recorded every 3 days for 18 days after initial administration and each data point represents an average of three separate flasks. Although there is some variation in (M + 2/M + 3 × 100) the ratio remains approximately stable over this extended time period. In fact there is a tendency for the ratio to decrease a little through the experiment which is *opposite* to that expected for any loss of isotope at C-3′ due to a reversible oxidative metabolism of hyoscyamine **2**. The data does not support an in vivo redox process over an extended time period.

In a separate series of experiments cell free extracts of *D. stramonium* were prepared and assayed for dehydrogenase activity. The extracts had good dehydrogenase activity when incubated with pyruvate and NADH indicating active protein in the extract. However, in experiments where hyoscyamine **2** was incubated with the co-factors NAD or NADP, there was no evidence for any dehydrogenase activity in the extract capable of oxidising hyoscyamine **2**. Of course it may be that the level of activity of a relevant dehydrogenase is so low in the assay that it was undetectable. However, with this caveat in mind such an activity seems unlikely to account for the complete washout of isotope after a vicinal interchange process as observed in the previous cell free extract experiments (Ollagnier et al., 1998).

3. Concluding remarks

In conclusion the results presented indicate that the rearrangement of littorine **1** to hyoscyamine **2** does not occur with an observable vicinal interchange process and are inconsistent with a mechanism involving SAM **5**. The proposal that a reversible in vivo oxidation at C-3′ of hyoscyamine **2** is not supported by the data. Accordingly the mechanism and co-factor(s) for the isomerisation of littorine **1** to hyoscyamine **2** are currently unknown and remain to be determined.

4. Experimental

4.1. General experimental procedures

Sodium borodeuteride (98% atom D) was purchased from Across Organics, deuterium oxide (99.9% atom D) from Goss Scientific Instruments Ltd. and all other chemicals from the Aldrich Chemical Co. or Fisher Scientific unless otherwise stated.

NMR spectra were recorded on a Bruker Avance 300 MHz (^{1}H at 300.06 MHz, ^{13}C at 74.45 MHz) and Varian Unity Plus 300 MHz (^{1}H at 299.99 MHz, ^{13}C at 75.43 MHz) spectrometers. Chemical shifts are quoted relative to TMS in $CDCl_3$ (δ_H 0.00 ppm) or MeOH in CD_3OD (δ_C 49.15 ppm) and coupling constants are given in Hertz (Hz). ^{13}C NMR spectra are proton decoupled, with multiplicities resulting from ^{2}H–^{13}C coupling.

Infrared analyses were recorded using a Perkin-Elmer 2000 FT-IR. Melting points were recorded on a GallenKamp GRIFFIN MPA350.BM2.5 melting point apparatus and are uncorrected.

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

Mass enrichments were calculated by comparison with unlabelled synthetic samples. GC–MS analyses of the crude alkaloid extracts were performed as above except for the use of a chiral SGE column (fused silica, cydex-B, 25 m×0.22 mm with a film thickness of 0.25 μm), with a flow rate of 64.8 ml min^{-1} in splitless mode. For CI analysis the reagent gas was methane.

4.2. Plant material and maintenance

Transformed root cultures of *D. stramonium* D15/5 were grown and maintained in Gamborgs B50 medium as described previously (Robins et al., 1991). This root culture line is deficient in scopolamine biosynthesis and instead accumulates high concentrations of hyoscyamine.

4.3. Synthetic protocols

4.3.1. Synthesis of [3,3-$^2H_2$]-phenylpyruvic acid

An emulsion of phenylpyruvic acid (259 mg, 1.6 mmol) in deuterium oxide (30 ml) was stirred at rt for 48 h. The level of deuterium exchange was monitored directly by ^1H NMR of the filtered reaction mixture. The product was treated directly as described later.

4.3.2. Synthesis of (RS)-[2-^2H]-phenyllactic acid

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

^1H NMR (300 MHz) ($CDCl_3$): δ 7.35–7.24 (5 H, *m*, aromatics), 3.10 (2 H, *dd*, $^2J_{HH}$ = 14.1, C–$\underline{H}_2$). ^{13}C NMR (75 MHz) (CD_3OD): δ 41.6 (C-3), 72.5 (C-2, *t*, $^1J_{CD}$ = 23), 127.6, 129.3, 130.6, 139.0 (aromatics), 177.2 (C-1). ν^{KBr} cm^{-1}; 3446 (OH), 2927 (CO$\underline{O}$H), 1726 (C$\underline{O}$OH). mp. 95–97 °C (lit. 97–98 °C, McKenzie and Wren, 1910). GC–MS (EI, 70 eV), *m*/*z* (rel. int.): 195 $[M]^+$ (0.5), 177 $[M–H_2O]^+$ (65), 149 $[M–EtOH]^+$ (13), 132 $[M–H_2O–Et]^+$ (24), 122 $[M–73]^+$ (40), 104 $[M–PhCH_2]^+$ (33), 91 $[PhCH_2]^+$ (100), 77 $[Ph]^+$ (9), 65 $[M–130]^+$ (10).

4.4. Synthesis of (RS)-[3,3-$^2H_2$]-phenyllactic acid

The title compound was prepared following the method for (*RS*)-[2-^2H]-phenyllactic acid above instead using [3,3-$^2H_2$]-phenylpyruvic acid and sodium borohydride (118 mg, 2.8 mmol). Yield (172 mg, 64%). The methyl ester was prepared in dry MeOH (as Section 3.3.2) for GC–MS analysis.

^1H NMR (300 MHz) ($CDCl_3$): δ 7.34–7.23 (5 H, *m*, aromatics), 4.51 (1 H, *s*, C$\underline{H}$OH). ^{13}C NMR (75 MHz) (CD_3OD): δ 41.4 (*m*, C-3), 72.9 (C-2), 127.6, 129.3, 130.7, 140.0 (aromatics), 177.2 (C-1). ν^{KBr} cm^{-1}; 3448 (OH), 2962 (CO$\underline{O}$H), 1733 (C$\underline{O}$OH). mp. 96–97 °C (lit. 97–98 °C, McKenzie and Wren, 1910). GC–MS (EI, 70 eV), *m*/*z* (rel. int.): 182 $[M]^+$ (1.7), 163 $[M–HDO]^+$ (45), 132 $[M–HDO–MeO]^+$ (8), 123 $[M–COOMe]^+$ (15), 105 $[M–Ph]^+$ (13), 93 $[PhCD_2]^+$ (100), 78 $[M–104]^+$ (5), 66 $[M–116]^+$ (5).

4.4.1. Synthesis of (RS)-[2,3,3-$^2H_3$]-phenyllactic acid

The title compound was prepared following the method for (*RS*)-[3,3-$^2H_2$]-phenyllactic acid above using sodium borodeuteride (118 mg, 2.8 mmol). Yield (147 mg, 54%). The methyl ester was prepared (as Section 3.3.3) for GC–MS analysis.

^1H NMR (300 MHz) ($CDCl_3$): δ 7.34–7.24 (5 H, *m*, aromatics). ^{13}C NMR (75 MHz) (CD_3OD): δ 41.1 (*m*, C-3), 72.3 (*m*, C-2), 127.5, 129.2, 130.5, 138.7 (aromatics), 177.1 (C-1). ν^{KBr} cm^{-1}; 3451 (OH), 2930 (CO$\underline{O}$H), 1733 (C$\underline{O}$OH). GC–MS (EI, 70 eV), *m*/*z* (rel. int.): 183 [M] (1.5), 164 [M–HDO] (45), 133 [M–HDO–MeO] (8), 124 [M–COOMe] (15), 106 [M–Ph] (11), 93 $[PhCD_2]$ (100), 78 [M–105] (4), 66 [M–106] (5).

4.5. Feeding protocols

4.5.1. Feeding labelled phenyllactic acids

To four aseptic Erlenmeyer flasks (250 ml), each containing an initial innoculum (0.5 g) of fresh mass of roots in culture medium (50 ml), a filter-sterilised solution (1 ml) of labelled phenyllactic acid dissolved in methanol/water was administered on day 6 at a final concentration of 0.25 mmol dm^{-3}. Root cultures were then incubated at 27 °C as previously described (Robins et al., 1991) for a further 11 days. The cell mass was freeze-dried and the alkaloids extracted (as Section 3.5).

4.5.2. Feeding (RS)-[1′-^{13}C,3′,3′-$^2H_2$]-hyoscyamine

Fifteen subcultured flasks of roots were fed as described in Section 3.4.1. Root cultures were then incubated for a further 6, 9, 12, 15 or 18 days (three each) before alkaloid extraction. All root cultures were freeze-dried separately prior to individual alkaloid extraction.

4.6. Isolation and analysis of alkaloids

Alkaloids were isolated from freeze-dried root cultures as described previously (O'Hagan et al., 1999). The trimethylsilyl (TMSi) derivatives of the alkaloid samples were prepared using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Fluka;

Bakola-Christianopoulou et al., 1994). Mass enrichments of isolated littorine **1** and hyoscyamine **2** were calculated by comparison with unlabelled samples [commercial hyoscyamine, Aldrich Chemical Co., and littorine **1** prepared as previously described (Robins et al., 1994)].

4.7. Preparation of cell free extract and a dehydrogenase assay

All procedures were carried out at 4 °C. The cell masses from six root cultures of *D. stramonium* aged 11–17 days (fresh wt. 29.4 g) were harvested, washed with cold water and blotted dry. The cells were then frozen in liquid N_2 and ground in a pre-chilled pestle and mortar with sand and extraction buffer (60 ml, see later) for 20 min. The slurry was filtered through 3 layers of cheesecloth and clarified by centrifugation (10,000 × *g*, 30 min). The supernatant protein solution was then used as the crude extract for enzyme assays.

Extraction buffer: Phosphate buffer (100 mM) pH 7.5 containing; glycerol (20%), DTT (5 mM), cysteine (0.05%), β-mercaptoethanol (10 mM), FAD (5 μM), FMN (5 μM) and sodium ascorbate (50 mM).
Dehydrogenase assay. In a cuvette (1.5 ml) was added 0.2 M Tris–HCl buffer (1.4 ml, pH 7.3), NAD *or* NADP (50 μl of 6.6 mM) and hyoscyamine 2 (50 μl of 30 mM). This mixture was incubated for 5 min before the addition of 100 μl of cell free extract and the absorbance recorded at 340 nm over 10 min. No change in absorbance was observed.

Acknowledgements

The EPSRC is acknowledged for providing a studentship (S.P.).

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