# Biosynthesis of Hyoscyamine involves an Intramolecular Rearrangement of Littorine

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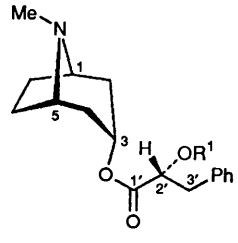
The direct biosynthesis of hyoscyamine **3** from littorine **1** has been demonstrated by feeding (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine (littorine **13**) to transformed root cultures of *Datura stramonium*. In tropane alkaloids isolated 7 days later and examined by GC–MS, the labelling patterns of hyoscyamine **3** and apoatropine **4** indicated that the quintuply labelled precursor had been incorporated intact. Incorporations into the M + 5 peaks of hyoscyamine **16** and apoatropine **4** of 4.4 and 3.8% isotopic excess were found respectively. The % isotopic excesses in the M + 2 and M + 3 ions of these alkaloids indicate that a biosynthetic route involving the hydrolysis of the ester followed by re-esterification could only account for 0.2% isotopic excess in the observed M + 5 incorporation into hyoscyamine **16**. Furthermore, the incorporation of (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl-[*methyl*-$^{2}H_3$]tropine **13** into hyoscyamine **16** and apoatropine was not diminished in the presence of added tropine **6** or (*RS*)-phenyllactic acid. The retention of a high isotopic excess within hyoscyamine **16**, coupled to the inability of either added tropine **6** or phenyllactic acid to dilute the extent of labelling, shows that hyoscyamine **3** is derived directly by the rearrangement of littorine **1**. That this rearrangement is intramolecular is shown by the high level of $^{13}C$ spin-spin coupling observed in the $^{13}C$ NMR spectrum of the derived hyoscyamine **16**.

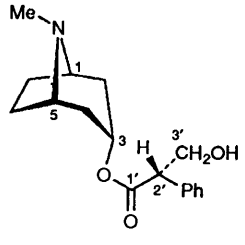
Littorine **1**, the (*R*)-(+)-phenyllactoyl ester of tropine **6**, was simultaneously and independently isolated some time ago as a minor base of *Brugmansia* (*Datura*) *sanguinea*[1] and as the major alkaloid of the indigenous Australian plant *Anthocercis littorea*.[2] Both plants are members of the Solanaceae. The co-occurrence in many *Datura* and related genera[3] of littorine **1** and hyoscyamine **3**, the (*S*)-(−)-tropoyl ester of tropine **6**, gave the clue to the biosynthetic origin of tropic acid **14**. It is now apparent that the two bases are always found together in *Datura*[3] and other tropane alkaloid-producing solanaceous plants.

The phenyllactoyl moiety of littorine **1** and the tropoyl moiety of hyoscyamine **3** have both been shown by label-incorporation studies[4–6] to be derived from the amino acid, L-phenylalanine **9**. Furthermore, it has been demonstrated that phenyllactic acid **10** is an efficient precursor[4,7] of the tropoyl moiety of hyoscyamine **3**. The exact biosynthetic pathway for the conversion of phenylalanine **9** into tropic acid **14** has been the subject of much debate. In particular, the mechanism by which the linear side-chain of phenylalanine **9** or its metabolites undergoes intramolecular rearrangement to form tropic acid **14** is undefined. Current knowledge of this process has been summarised.[8,9] Recently, it has been shown, by feeding phenyl-[2-$^{2}H$]lactic acid to various root cultures[10] or an admixture of phenyl[2-$^{3}H$]lactic and phenyl[1-$^{14}C$]lactic acids to plants of *Datura stranonium*,[11] that phenyllactic acid **10** is an obligatory intermediate in this process. Furthermore, evidence has been presented which indicates that the rearrangement takes place at the level of phenyllactate and not before.[11,12]

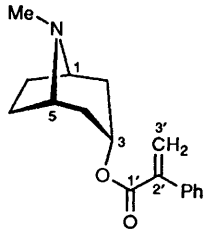
In feeding experiments[13] with phenyl[1,3-$^{13}C_2$]lactic acid introduced into transformed root cultures of *D. stramonium* or a *Brugmansia* hybrid, it was observed that tropic acid, when fed in competition, did not markedly inhibit the specific incorporation of the labelled precursor into hyoscyamine **3** and other aromatic bases. Moreover, competition from tropic acid **14** led to an increased incorporation of the tracer into littorine. In addition, it has been shown[14] that, when root cultures of



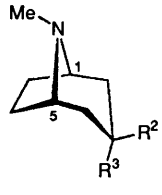

**1** $R^1$ = H
**2** $R^1$ = OAc




**3**

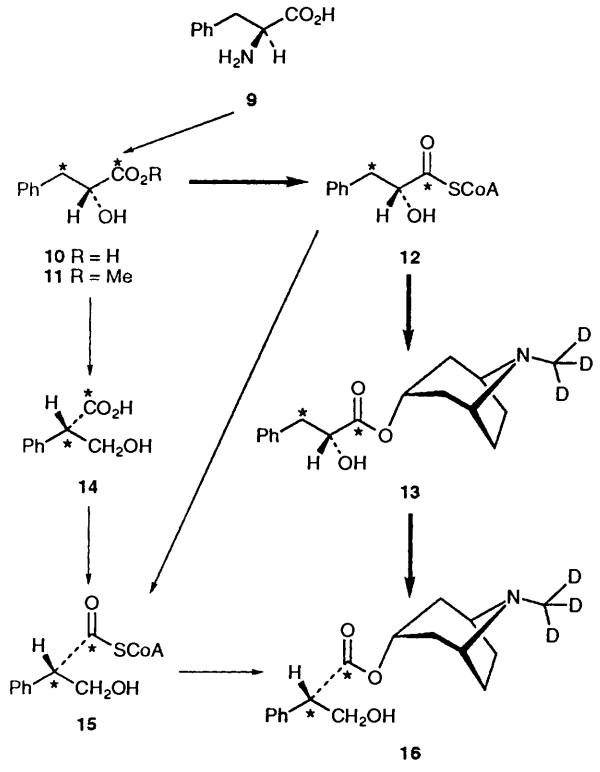



**4**




**5** $R^2R^3$ = O
**6** $R^2$ = H, $R^3$ = OH
**7** $R^2$ = H, $R^3$ = OAc
**8** $R^2$ = H, $R^3$ = MeCHC(Me)$CO_2$

*Duboisia leichhardtii* were fed phenyl[1-$^{14}C$]alanine, label could be trapped with phenyllactic acid **10** but not with tropic acid **14**. These findings imply that *free* tropic acid **14** is not involved in the biosynthesis of tropane esters. Therefore, it appears that the rearrangement process probably occurs with an esterified form of phenyllactic acid **10**, which is used directly to make hyoscyamine **3**. This might be an activated ester, such as the coenzyme A thioesters (**12**, **15**, Scheme 1), or an alkaloidal ester, such as littorine **1**.



Scheme 1

In an earlier attempt to test the potential intermediacy of littorine **1** in the biosynthesis of hyoscyamine **3**, [1'-$^{14}$C]-littorine and [3β-$^{3}$H]littorine were synthesised and fed as an admixture ($^{3}$H : $^{14}$C of 6.75 : 1) to plants of *D. stramonium*.[15] The $^{3}$H : $^{14}$C of the hyoscyamine **3** (33 : 1) isolated after the feed differed markedly from that in the precursor, indicating that considerable hydrolysis had occurred. The authors concluded that littorine **1** had not been rearranged to hyoscyamine **3**. Apparently, however, they did not consider that the direct incorporation of even quite a substantial proportion of the labelled precursor would be masked as a result of differential utilisation of the labelled alkamine and free acid produced by hydrolysis. Neither did they examine the extent of incorporation into the pools of these compounds. More recently, an indication that littorine **1** might be a precursor of hyoscyamine **3** has been suggested by the relative extents to which $C^2H_3{}^{13}CO_2Na$ is incorporated into these bases by root cultures of *Hyoscyamus albus*.[16]

These discrepancies prompted us to re-examine the possible intermediacy of littorine **1** in hyoscyamine **3** biosynthesis. For this purpose, we synthesised a quintuply labelled species, (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl-[*methyl*-$^{2}H_3$]tropine **13**. If directly metabolised to form hyoscyamine **16** and other aromatic esters, this process would yield products giving M + 5 ions which could be uniquely monitored by GC–MS in spite of limited hydrolysis. The extent of hydrolysis could be monitored by the proportion of M + 2- and M + 3-labelled alkaloids present.

## Results and Discussion

Transformed root cultures of a number of *Datura* species and of related genera have proved invaluable in helping to unravel the biochemical pathway by which tropane alkaloids are formed.[17] In particular, they are excellent material for examining the incorporation of labelled precursors, up to 55% specific incorporation having been obtained in previous experiments here.[13] *D. stramonium* transformed root cultures accumulate an alkaloid spectrum very similar to that of the root of the intact plant, dominated by hyoscyamine **3** with littorine **1**, apoatropine **4**, 3α-acetoxytropane **7** and 3α-tigloyloxytropane **8** as the major other constituents.[18]

In order to investigate the possibility that littorine **1** is directly converted into hyoscyamine **3**, highly enriched quintuply labelled (80.9 atom%) (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13** was synthesised (see Experimental section) and fed (0.125 mmol dm$^{-3}$) to 3-day-old transformed root cultures of *D. stramonium*. After a further 7 days' growth, the alkaloids were isolated and fractionated by capillary gas chromatography (GC) and mass analysis obtained by direct input mass spectrometry (MS). Peak identity was obtained by reference to authentic external standards.

The mass ion analysis of the alkaloids obtained is given in Table 1. Littorine **13** contained 8.7% isotopic excess in the M + 5 peak, indicating that a proportion of the fed (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13** was retained unmetabolised in the root tissue. This littorine **13** also contained isotopic excesses of 10.1 and 3.9% for the M + 2 and M + 3 peaks, indicating that some *de novo* synthesis re-utilising the phenyllactic acid **10** and, to a lesser extent, the tropine **6** produced by hydrolysis had occurred. Some *O*-acetyllittorine **2** formation had also occurred.

That hydrolysis of the fed (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl-[*methyl*-$^{2}H_3$]tropine **13** had taken place is indicated by the presence of M + 3 label in the free tropine **6** pool (12.2%) and M + 2 label in the phenyllactoylmethyl ester **11** pool (18.0%). Nevertheless, both hyoscyamine **16** and its derivative, apoatropine, were significantly labelled at the M + 5 level, 4.4 and 3.8%, respectively. Thus, these metabolic products contained, respectively, mass enhancements at the M + 5 level of 51 and 44% that obtained in the recovered littorine **13**. Incorporation could have occurred by hydrolysis of the fed (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13** followed by re-esterification from the free tropine **6** and phenyllactic acid **10** pools. A small contribution to these pools will be made by the free tropine **6** and phenyllactic acid **10** present in the fed littorine (see Experimental section). However, the % isotopic excesses seen in hyoscyamine **16** for the M + 2 and the M + 3 ions were 3.1 and 4.7%, respectively, from which it may be calculated that incorporation at the M + 5 level due to utilization of free, labelled tropine **6** and phenyllactic acid **10**, from whatever source, could not exceed 0.2%. Thus, the majority of the 4.4% isotopic excess seen in hyoscyamine **16** can only have been derived by direct incorporation from littorine **13**. It was confirmed that the alkamine moieties of the recovered littorine **13**, hyoscyamine **16**, apoatropine and *O*-acetyllittorine, contained an additional 3 mass units and the acidic moieties an additional 2 mass units from the % atomic excesses observed in the relevant fragments (data not shown).

Increasing the sizes of the free tropine **6** and phenyllactic acid **10** pools by the exogenous addition (0.25 mmol dm$^{-3}$) of one or the other of these precursors, did not lead to any dilution of the isotopic enrichment of the M + 5 ions of hyoscyamine **16** and apoatropine (Table 1). Indeed, both treatments led to an enhancement of the incorporation observed, possibly due to the presence of these products inhibiting the extent to which hydrolysis of (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13** occurred. Thus, in the presence of an excess of tropine **6**, the isotopic excess of isolated littorine **13** was 11.8%, while hyoscyamine **16** and apoatropine had isotopic excesses of 6.5 and 7.1%, respectively, or 56 and 60% the level of the recovered (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13**. Similarly, with an excess of phenyllactic acid **10**, isotopic excesses are seen in hyoscyamine **16** and apoatropine of 5.5 and 7.1%, respectively, or 52 and 67% the level of the recovered (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13**. Both tropine

**Table 1** Percentage isotopic excesses in littorine and littorine-derived metabolites

| Compound | Feeding regime: Littorine as label (mmol $dm^{-3}$) | Further additive | mmol $dm^{-3}$ | % Isotopic excess*: M + 2 | M + 3 | M + 5 |
|---|---|---|---|---|---|---|
| Littorine **1** | 0.125 | — | | 10.1 | 3.9 | 8.7 |
| | 0.125 | Tropine | 0.25 | 13.0 | 3.4 | 11.8 |
| | 0.125 | Phenyllactic acid | 0.25 | 11.9 | 5.2 | 10.6 |
| Hyoscyamine **3** | 0.125 | — | | 3.1 | 4.7 | 4.4 |
| | 0.125 | Tropine | 0.25 | 4.2 | 3.8 | 6.5 |
| | 0.125 | Phenyllactic acid | 0.25 | 3.6 | 6.5 | 5.5 |
| Apoatropine **4** | 0.125 | — | | 2.2 | 4.0 | 3.8 |
| | 0.125 | Tropine | 0.25 | 3.9 | 5.1 | 7.1 |
| | 0.125 | Phenyllactic acid | 0.25 | 4.0 | 6.4 | 7.1 |
| *O*-Acetyllittorine **2** | 0.125 | — | | 5.5 | 2.6 | 3.9 |
| | 0.125 | Tropine | 0.25 | 9.0 | 2.3 | 5.3 |
| | 0.125 | Phenyllactic acid | 0.25 | 8.2 | 2.6 | 5.8 |
| Phenyllactic acid methyl ester **11** | 0.125 | — | | 18.0 | 0.0 | 0.0 |
| | 0.125 | Tropine | 0.25 | 24.8 | 0.0 | 0.0 |
| | 0.125 | Phenyllactic acid | 0.25 | 19.7 | 0.0 | 0.0 |
| Tropine **6** | 0.125 | — | | 0.0 | 12.2 | 0.0 |
| | 0.125 | Tropine | 0.25 | 0.0 | 9.7 | 0.0 |
| | 0.125 | Phenyllactic acid | 0.25 | 0.0 | 16.5 | 0.0 |
| 3α-Acetoxytropane **7** | 0.125 | — | | 0.0 | 7.0 | 0.0 |
| | 0.125 | Tropine | 0.25 | 0.0 | 8.0 | 0.0 |
| | 0.125 | Phenyllactic acid | 0.25 | 0.0 | 3.7 | 0.0 |
| 3α-Tigloyloxytropane **8** | 0.125 | — | | 0.0 | 5.0 | 0.0 |
| | 0.125 | Tropine | 0.25 | 0.0 | 6.3 | 0.0 |
| | 0.125 | Phenyllactic acid | 0.25 | 0.0 | 6.6 | 0.0 |

* Corrected for the calculated contribution of natural abundance isotopes.

**6** and phenyllactic acid **10** have previously been demonstrated readily to be absorbed and metabolised by these root cultures.

Examination of the alkaloid extracts by $^{13}C$ NMR spectroscopy showed that the $^{13}C$ nuclei of the fed littorine had become contiguous in the derived hyoscyamine **16** (Fig. 1). A mean incorporation of 6.9% was determined from the integration of the C-1′ and C-2′ peak areas, comparable to the incorporations into M + 2 plus M + 5 determined by GC–MS (Table 1). Thus, the observed mass increment cannot be due to degradation of the phenyllactic acid moiety, followed by the independent re-incorporation of the $^{13}C$ nuclei. Previously,[13] we have shown that phenyllactate **10** in an activated form undergoes an intramolecular rearrangement during its incorporation into hyoscyamine **3**. It is now demonstrated that this activated from is littorine **1**, as rearrangement without differential loss of either C-1′ or C-3′ label occurs with this precursor.

In summary, it is apparent from these incorporations, that fed littorine **13** is being directly converted into hyoscyamine **16**. From this observation, it can be inferred that an intramolecular rearrangement of littorine **1** occurs *in vivo*, leading to the formation of the hydroxymethyl group of hyoscyamine from the C-2′ of the phenyllactoyl moiety of littorine **1**. While it might be argued that this has only occurred with exogenous littorine **1**, there is no reason to doubt the equivalence of the *de novo* pathway, based on previous experience with this experimental system. As it has been shown that (*R*)-(+)-phenyllactic acid is more efficiently incorporated into hyoscyamine **3** than the (*S*)-(−)-enantiomer (unpublished result, J. G. W.), it is reasonable to deduce that the (*R*)-(−)-littorine from the racemic mixture fed undergoes the rearrangement process (Scheme 1). Thus, the present results are entirely compatible with previous data on hyoscyamine biosynthesis. What cannot be excluded by these data is that the alternative mechanism (Scheme 1), namely the rearrangement of the phenyllactoyl-coenzyme A thioester **12**, does not also occur *in vivo*. However, it seems improbable that hyoscyamine is formed by two parallel routes in a single species.

## Experimental

*General.*—[*methyl*-$C^2H_3$]Methylamine hydrochloride and tropine were purchased from the Aldrich Chemical Company Ltd., Gillingham. (*RS*)-Phenyllactic acid was from Sigma Chemical Company (Poole, Dorset).

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). $^{13}C$ NMR spectra were recorded on a Bruker 400 MHz spectrometer (Bruker Spectrospin, Coventry) at 100.4 MHz. Melting points are uncorrected.

[methyl-$C^2H_3$]*Tropine.*[19,20]—2,5-Dimethoxytetrahydrofuran (840 mg, 7 mmol) was added to water (10 $cm^3$) containing 3 drops of concentrated hydrochloric acid and stirred until a single phase formed. [*methyl*-$C^2H_3$]Methylamine hydrochloride (493 mg, 7 mmol, nominally 98 atom% $^2H_3$), in water (10 $cm^3$) and freshly prepared acetone dicarboxylic acid (1.022 g, 7 mmol) were then added to the solution. With continuous stirring, molar di-sodium hydrogen phosphate solution was also added to the solution and, when final dissolution had been achieved, the solution was adjusted to pH 5 using 1 mol $dm^{-3}$ aqueous sodium di-hydrogen phosphate and diluted with water to 250 $cm^3$. After 3 days at room temperature, the solution was boiled for 10 min, cooled, made alkaline with aqueous ammonium hydroxide (50%) and continuously extracted with light petroleum for 6 h. Thin-layer chromatography of the extract on alumina (ether), using iodine in carbon tetrachloride as locating agent, revealed a single product ($R_F$ *ca.* 0.5). Complete evaporation of the solvent gave a pale brown gum which was neutralised with 0.05 mol $dm^{-3}$ sulfuric acid. Addition of a saturated aqueous sodium picrate to the solution immediately gave an amorphous precipitate, which was recrystallised twice from aqueous acetone to give [*methyl*-$^2H_3$]-tropinone **5** as glassy plates (643 mg, 25%), m.p. 217 °C. The mass spectrum[21] showed $M^+$ 142 (98.9 ± 1 atom% $^2H_3$) and *m/z* 113 (11), 99 (43), 85 (100), 84 (77), 71 (18), 62 (30) and 45 (79).

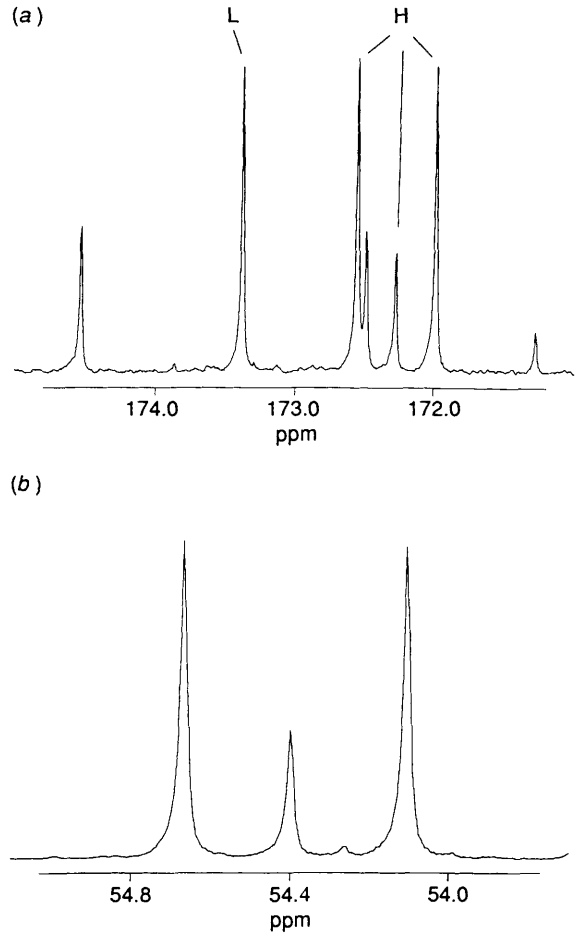



**Fig. 1** 100.4 MHz $^{13}C$ NMR spectrum of the alkaloid extract isolated from transformed root cultures of *D. stramonium* fed (*RS*)-phenyl-[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13**: (*a*), C-1′ of hyoscyamine at $\delta$ 172.3; (*b*), C-2′ of hyoscyamine at $\delta$ 54.4. H = hyoscyamine; L = littorine.

[*methyl*-$^{2}H_3$]Tropinone **5** base was recovered from the picrate (98 mg) by dissolution in aqueous ammonium hydroxide (10%) and extraction into chloroform (6 × 10 cm$^3$). The bulked chloroform extracts were washed once with water (10 cm$^3$), dried ($Na_2SO_4$) and evaporated to dryness. The base was dissolved in ethanol (5 cm$^3$) and hydrogenated at atmospheric pressure over freshly prepared Raney nickel (150 mg). Reduction, which was completed over 3 days, was monitored by thin-layer chromatography on silica gel (chloroform–ethanol–conc. aqueous ammonium hydroxide 7 : 7 : 1),[22] $R_F$ [*methyl*-$^{2}H_3$]tropinone **5** 0.9 and [*methyl*-$^{2}H_3$]tropine **6** 0.2. Removal of the solvent and catalyst gave [*methyl*-$^{2}H_3$]tropine **6** (35 mg) as a pale yellow gum which crystallised as transparent plates on storage.

(RS)-*Phenyl*[1,3-$^{13}C_2$]*lactoyl*[*methyl*-$^{2}H_3$]*tropine*(*Littorine*) **13**.—[*methyl*-$^{2}H_3$]Tropine (0.24 mmol) was dried for 24 h over sodium hydroxide pellets and (*RS*)-phenyl[1,3-$^{13}C_2$]lactic acid (81.7 atom% $^{13}C_2$; 45 mg, 0.24 mmol), previously prepared[23–26] and dried over phosphorus pentoxide, was added to it. The mixture was heated to 130 °C on an oil-bath and exposed to a current of dry hydrogen chloride for 5 h.[20,27] The cooled product was dissolved as completely as possible in 0.05 mol dm$^{-3}$ sulfuric acid (5 cm$^3$). The filtered aqueous solution was made alkaline with 10% aqueous ammonium hydroxide and extracted into chloroform (6 × 5 cm$^3$). Evaporation of the combined extracts gave a gum which was redissolved in chloroform (2 cm$^3$) and submitted to partition chromatography[28] on Kieselguhr (10 g) containing 0.5 mol dm$^{-3}$ phosphate buffer pH 6.8. Development of the column with successive 50 cm$^3$ volumes of light petroleum, ether and chloroform gave littorine **13** in the early chloroform fractions[1] (48 mg, 62%). GC and GC–MS[29] of the amalgamated fractions on a DB-17 capillary column (30 m × 0.32 mm, ramp 70–280 °C at 8 °C min$^{-1}$) indicated almost pure (91%) littorine **13**, $t_R$ 23.07 min, containing traces of tropine **6** (2%), $t_R$ 7.55 min, and phenyllactic acid methyl ester **11** (5%), $t_R$ 10.92 min. The mass spectrum showed M$^+$ 294 (80.9 atom% $^{2}H_3$ + $^{13}C_2$) and *m/z* 143 (3), 127 (100), 99 (11), 97 (29), 92 (28), 85 (31), 84 (5), 67 (11) and 45 (7).

*Cultures.*—Root cultures of *Datura stramonium* L. D15/5 transformed with *Agrobacterium rhizogenes* LBA9402 were maintained as described previously.[18] Feeding experiments were performed using roots growing in the absence of added antibiotics.

*Feeding.*—All solutions for feeding were prepared as neutral aqueous stocks at 6.25 mmol dm$^{-3}$ for (*RS*)-phenyl[1,3-$^{13}C_2$]lactoyl[*methyl*-$^{2}H_3$]tropine **13** and 12.5 mmol dm$^{-3}$ for tropine **6** and (*RS*)-phenyllactic acid. Stock solutions (0.5 cm$^3$) were filter-sterilised directly into flasks at 3 days following subculture. Cultures were harvested after a further 7 days of culture.

*Alkaloid Separation and Identification.*—Alkaloids were extracted by homogenisation of freeze-dried tissue with 0.05 mol dm$^{-3}$ sulfuric acid. Following removal of debris by filtration, the solution was basified (35% aqueous ammonium hydroxide) and applied to an Extrelute® column (Merck, Poole). Elution with dichloromethane–methanol (95 : 5) yielded a crude total alkaloid fraction. Alkaloids were separated by GC on a 30 m DB-17 capillary column and identified by GC–MS, essentially as described previously.[29] Identification was by reference to external standards of tropine **6**, 3α-acetoxytropane **7**, 3α-tigloyloxytropane **8**, littorine **1**, apoatropine **4** and hyoscyamine **3**.

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