The Biosynthesis of Tropic Acid. Part 6. Enantioselective, Intact Incorporation of (*R*)-(+)-3-Phenyllactic Acid into the Tropic Acid Ester Alkaloids of *Datura*†

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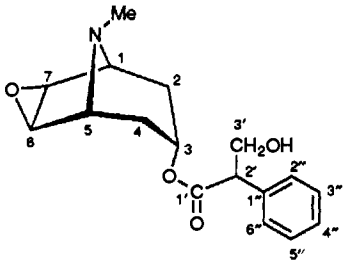
(*R*)-(+)- **11** and (*S*)-(−)-3-Phenyl[1,3-$^{13}C_2$; 1-^{14}C]lactic acid **13** were fed separately to *Datura stramonium* (Solanaceae) *via* the wick method. In a second feeding experiment (*R*)-(+)- and (*S*)-(−)-3-phenyl[1,3-$^{13}C_2$; 2-^{14}C]lactic acid were administered *via* the roots. In both cases hyoscine (scopolamine) and hyoscyamine **1** and **2**, respectively were isolated separately from the roots and aerial parts. The (*R*)-enantiomer **11** was more efficiently incorporated into the (*S*)-(−)-tropic acid **8** moiety of both bases. Examination of the ^{13}C NMR spectra of the bases and of tropic acid, obtained by hydrolysis of hyoscyamine, showed ^{13}C–^{13}C spin–spin coupling indicating that the tropoyl ester (−)-hyoscyamine **9** is formed by direct rearrangement of the (*R*)-(+)-3-phenyllactic acid **11** ester of tropine (littorine).

Studies on the biosynthesis of (*S*)-(−)-tropic acid **8**, the acid moiety of the important medicinal tropane alkaloids hyoscine **1** and hyoscyamine **2**, have shown that L-phenylalanine **7** serves



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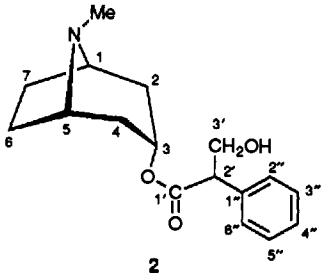

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as a precursor for all the carbons.[1,2] The exact sequence of events involved in the biosynthesis of this comparatively simple molecule has remained a puzzle, partly because of conflicting evidence which has been critically reviewed.[3–6] It has been shown that the carboxy group of phenylalanine **7** migrates in an intramolecular manner to C-3[6] with retention of configuration at that centre.[7] This 1,2-shift is accompanied by a transfer of the *pro*-(*S*) proton at C-3 to C-2 in the intermediary metabolites derived from phenylalanine.[8] Since the C-2 proton of phenylalanine **7** itself is lost during the process[9] it was considered most likely[4] that phenylpyruvate **10** was the major intermediate in the biosynthesis and a mechanism for its rearrangement has been proposed.[4,10] However, recent work[11] has demonstrated that phenylpyruvate is a poor, but specific precursor, and much inferior to 3-phenyllactate (3-phenyl-2-hydroxypropionate) when fed in parallel. The latter acid is found naturally as the (*R*)-(+) form **11**[12] in the Solanaceae where it provides the acid moiety of the alkaloid littorine **12** obtained from *Brugmansia sanguinea*[13] and *Anthocercis littorea*.[14] In fact, the co-occurrence of littorine and hyoscyamine is widespread in daturas.[15] Feeding experiments have recently indicated that the C-2 proton of 3-phenyllactic acid **11** is retained and transferred to the hydroxymethyl group of tropic acid **8** during biosynthesis, a discovery which eliminates phenylpyruvate as the primary intermediate.[16] Furthermore, this latter finding has been nicely confirmed by feeding experiments with 3-phenyl[2-^{3}H; 2-^{13}C]lactic acid.[17] This evidence strongly suggests that 3-phenyllactic acid, in an activated form, is the immediate precursor of tropic acid. We have recently demonstrated that this activated form is as the tropine ester, littorine **12**.[18] In our current investigation we comment on the stereochemistry of the process.

Results and Discussion

3-Phenyllactic acid was resolved into its enantiomers by fractional crystallisation of the morphine salts[19] and in our hands this is preferable to the use of strychnine.[20] The incorporations of ^{14}C label from each enantiomer in two separate feeding experiments are listed in Table 1. The ^{13}C NMR signals of (*R*)-(+)-3-phenyl[1,3-$^{13}C_2$]lactate **11** and its metabolites obtained by bathing the roots of *Datura* in tracer solution are presented in Table 2. Incorporations calculated[21] from the intensities of the satellite signals in the ^{13}C NMR spectra of labelled products show good correlation with figures gained from uptake of radioactivity. In all cases the (*R*)-isomer is the superior precursor and this is most marked in the root alkaloids, especially when the roots were bathed in precursor. The root is considered to be the primary site of synthesis of these alkaloids[22] and the results would support this conclusion. We had initially considered that phenyllactate probably represented a branch point in the pathway, the (*R*)-(+)-enantiomer **11** giving littorine **12** by esterification with tropine, leaving the (*S*)-acid **13** to form (*S*)-(−)-tropic acid **8** by rearrangement, but it is clear that only the (*R*)-form is utilised in the formation of both. On the other hand atropine, the racemate of (*S*)-(−)-hyoscyamine **9**, the naturally occurring base, is sometimes isolated from plants and it is by no means certain that this racemisation is the result of the extraction process. It is possible to isolate consistently one or the other by the same extraction

† For Part 5, see M. Ansarin and J. G. Woolley, *Phytochemistry*, 1994, **35**, 935.

Table 1 Summary of feeding experiments

	(*R*)-(+)-3-Phenyl[1,3-$^{13}C_2$;1-^{14}C]lactic acid **10** Sp. Act. 1.7 × 10^7 dpm mmol^{-1}			(*S*)-(−)-3-Phenyl[1,3-$^{13}C_2$;1-^{14}C]lactic acid **11**. Sp. Act. 1.7 × 10^7 dpm mmol^{-1}		
	Wt. Base (mg)	Sp. Act. (dpm mmol^{-1})	Sp. Incorp.[a,b] (%)	Wt. Base (mg)	Sp. Act. (dpm mmol^{-1})	Sp. Incorp.[a] (%)
Aerial parts						0.06
Hyoscine **1**	22.3	1.5 × 10^4	0.09	10.5	1.0 × 10^4	0.12
Hyoscyamine **2**	104.4	2.9 × 10^4	0.17	75.0	2.0 × 10^4	
Tropic acid **8**		3.0 × 10^4			2.0 × 10^4	
Roots						
Hoscine **1**	4.5			2.3		0.13
Hyoscyamine **2**	23.7	1.6 × 10^5	0.94 (0.93)	25.2	3.5 × 10^4	
	(*R*)-(+)-3-Phenyl[1,3-$^{13}C_2$;1-^{14}C]lactic acid **10** Sp. Act. 1.7 × 10^7 dpm mmol^{-1}			(*S*)-(−)-3-Phenyl[1,3-$^{13}C_2$;1-^{14}C]lactic acid **11**. Sp. Act. 1.7 × 10^7 dpm mmol^{-1}		
Aerial parts						
Hyoscine **1**	18.2	1.8 × 10^5	0.24 (0.22)	16.2	4.0 × 10^4	0.05
Hyoscyamine **2**	46.8	2.05 × 10^5	0.28 (0.30)	52.4	4.2 × 10^4	0.05
Tropic acid **8**		2.0 × 10^5			3.8 × 10^4	
Roots						
Hyoscine **1**	12.4	2.2 × 10^5	0.30 (0.30)	15.4	2.4 × 10^4	0.03
Hyoscyamine **2**	28.6	6.8 × 10^5	0.93 (0.95)	32.9	6.5 × 10^4	0.08
Tropic acid **8**		2.1 × 10^5				

[a] Calculated as sp. act. product, dpm mmol^{-1} × 100/sp. act. precursor, dpm mmol^{-1}. [b] Specific incorporations calculated[19] from the area of the satellite signals from the ^{13}C NMR spectra show good agreement and are in parentheses.

Table 2 NMR Signals

C	Hyoscine **1** δ_C	Hyoscyamine **2** δ_C	Tropic acid **8** δ_C	3-Phenyllactic acid **11** δ_C
1′	171.7*	172.2*	175.0*	175.6*
1″	135.8	135.9	137.8	138.5
2″ and 6″	128.9	128.8	129.6	130.2
3″ and 5″	128.5	128.1	129.2	129.8
4″	128.0	127.6	128.3	128.7
3	66.8	68.0		
3′	63.9	64.1	64.9	41.6*
1 and 5	58.0 and 57.8	59.8 and 59.6		
6 and 7	56.3 and 55.9	25.4 and 25.0		
2′	54.7*	54.6*	55.1*	71.9
8	42.2	40.2		
2 and 4	31.1 and 30.9	36.2 and 36.0		

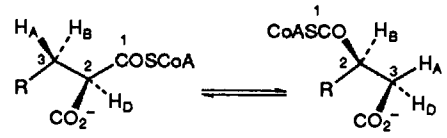
* Indicates ^{13}C labelled carbons, **1**, **2** and **8** displaying satellite signals.

process employed depending on the species used and the plant part. It is conceivable, therefore, that (*S*)-(−)-3-phenyllactate may have a role in some solanaceous plants. Although lignin is generally considered to be derived in part from phenylalanine *via* cinnamate without the involvement of phenyllactate,[23] it is noteworthy that (*S*)-(−)-phenyllactate **13** specifically is a precursor of lignin in dicotyledenous plants.[20] It is, therefore, reasonable to suggest that the (*S*)-(−)-enantiomer is a metabolite of phenylalanine.

In the aerial parts the situation is less clear-cut irrespective of the mode of feeding and it is possible that there is a reversible oxidation–reduction *via* phenylpyruvate **10** which converts (*S*)-(−)- **13** to the (*R*)-(+)-enantiomer **11** of phenyllactate. Nevertheless, the former incorporates 1.5–5.6 times better than the latter. This potential interconversion is likely to result in the partial loss of the C-2 proton ($H_{D/E}$ in **13**) and in his interesting paper[17] Dr. O'Hagan comments on this possibility. In our initial report of feeding experiments using C(2)-^{3}H labelled phenyllactate[16] some loss of tritium was observed but it was not considered significant and was not as high as the wash-out mentioned using deuteriated precursor.

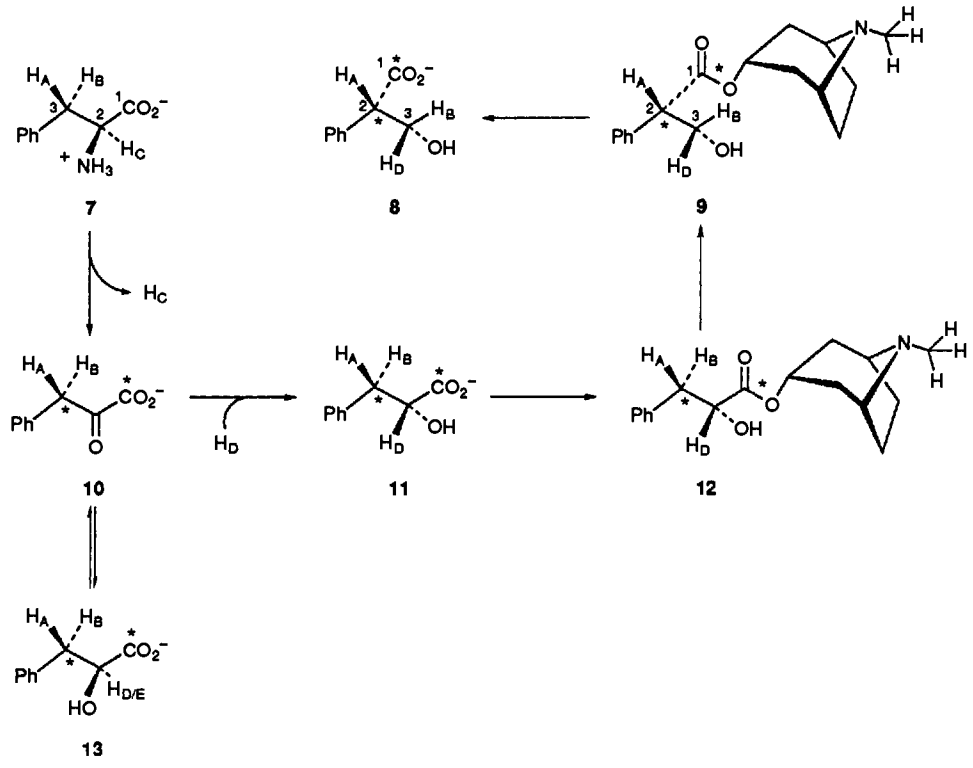
The ^{13}C NMR spectra of hyoscine **1** and hyoscyamine **2** reveal the presence of satellites on the signals near δ 172 (C-1′) and 55 (C-2′), *J ca.* 57 Hz (Table 2) and this indicates that phenyllactate is incorporated intact into tropic acid in these bases. Hydrolysis of some of the labelled bases confirmed that all the ^{14}C-label was confined to the tropic acid portion.

It has been suggested[8,24] that the rearrangement resembles that of methylmalonate **3** into succinate **4**. This 1,2-shift is



3 R = H_C **4** R = H_C
5 R = Me **6** R = Me

intramolecular as well[25,26] and is brought about at the CoA level by the enzyme (2*R*)-methylmalonyl-CoA mutase[27] (EC 5.4.99.2) which is dependent on adenosylcobalamin (AdoCbl) as its co-enzyme. Mutases generally appear to be dependent on AdoCbl to transport migrating protons, but it is very controversial as to whether vitamin B_{12} is present in plants.[28] The C(O)SCoA ester group migrates to the adjacent carbon[29] and the overall stereochemistry of the methylmalonate conversion is represented as **3**→**6**[30,31] based on experiments



Scheme 1 The biosynthesis of tropic acid **8**

using ethylmalonyl-CoA **5** which is an acceptable substrate too. The transcarboxylation proceeds with retention of configuration at both centres. In the process the *pro*-(*R*) proton at C-3 migrates to C-2. In comparison (Scheme 1) Leete has shown that during the formation of (*S*)-tropic acid **8** from phenylalanine **7** the C-3 *pro*-(*R*) proton (H_A) is retained and the *pro*-(*S*) proton migrates to C-2,[7,8] (and with the change in numbering) which becomes C-3 of tropic acid **8**. The processes are very similar (**7**→**12** in Scheme 1) but in contrast to the events which lead to succinate **4**, in tropic acid biosynthesis the carboxylate group approaches the *Si* face of the C-3 prochiral atom of phenyllactate as shown. These results and others[7,8,16,17] imply that the diastereotopic hydrogens of the hydroxymethyl group of **8** may be differentially labelled, perhaps as shown, but an inversion of configuration at C-3 cannot be ruled out. The rearrangement involves a 1,2-shift of the tropanoxycarbonyl group.

Recent competitive feeding experiments with 3-phenyl[1,3-$^{13}C_2$]lactic acid and putative intermediates in hairy root cultures of *D. stramonium* and a *B. candida x B. aurea* hybrid have shown that tropic acid is ineffective in inhibiting the incorporation of the tracer into hyoscine, hyoscyamine and other alkaloids.[32] More recently we have presented unambiguous evidence that 3-phenyllactic acid is esterified with tropine to give littorine prior to the rearrangement process[18] demonstrating that free tropic acid is not an intermediate.

Experimental

General.—Melting points are uncorrected. Optical rotations were measured on a Bendix NPL model 143D polarimeter standardised against aqueous sucrose or fructose and are given in units of 10^{-1} deg cm^2 g^{-1}. Mass spectrometry was carried out using a VG Trio-3 triple quadrupole mass spectrometer. NMR spectra were determined in $CDCl_3$ using a Varian EM 300X or a Bruker model AC 250 spectrometer. For the determination of radioactivity duplicate samples were counted in Packard Tri-Carb models 2002 or 3255 using dioxane based NE 240 scintillator (Nuclear Enterprises, Edinburgh).

Tracer Compound.—The synthesis of (*RS*)-3-phenyl[1,3-$^{13}C_2$]lactic acid (81.7 ± 2 atom% $^{13}C_2$) has been reported.[33-36] (*RS*)-3-Phenyl[1-^{14}C]lactic acid, sp. act. 1.5 × 10^8 and (*RS*)-3-phenyl[2-^{14}C]lactic acid, sp. act. 7.3 × 10^8 dpm mmol^{-1} were synthesised as described previously from sodium [^{14}-C]cyanide[16] and DL-phenyl[2-^{14}C]alanine[37] respectively, purchased from Amersham International, plc, Amersham, UK.

Thin-layer Chromatography.—Silica gel G plates (Merck) were developed using chloroform–ethanol–aqueous ammonia (conc.) (100:20:1)[38] as solvent and iodine in carbon tetrachloride or hexane for detection.

Plant Material.—Datura stramonium plants were grown under glass in commercially available peat–John Innes No. 2 compost mix from seeds obtained from the Zentralinstitut fur Genetik und Kulturpflanzenforschung, Gatersleben, Germany, and potted-on regularly to ensure rapid growth.

Feeding Experiment 1.—(*RS*)-3-Phenyl[1,3-$^{13}C_2$]lactic acid (81.7 atom% $^{13}C_2$, 222 mg) and (*RS*)-3-phenyl[1-^{14}C]lactic acid, sp. act. 1.5 × 10^8 dpm mmol^{-1} (28 mg) were dissolved in water (7 cm^3) at 90 °C giving an enrichment of 72.6 atom% $^{13}C_2$ and a sp. act. 1.7 × 10^7 dpm mmol^{-1}. Morphine (450 mg) was dissolved in the stirred solution which was then chilled overnight at 5 °C, when the (+)-acid morphine salt (PLAMO) (400 mg) crystallised out. After a second recrystallisation the PLAMO (mp 116 °C decomp.) was acidified by the addition of dilute hydrochloric acid (20 cm^3) and then extracted with diethyl ether (5 × 10 cm^3). Evaporation of the solvent gave a residue which was recrystallised from carbon tetrachloride twice to give the (*R*)-(+)-acid (90 mg), mp 124 °C (lit.,[9] 124–125 °C), $[\alpha]_D^{24}$ +18 (*l* 0.1; *c* 0.5, EtOH) (lit.,[9] $[\alpha]_D^{12}$ +18.5). The (*S*)-(−)-acid (85 mg) mp 124 °C (lit.,[19] 124 °C), $[\alpha]_D^{24}$ −19.5 (lit.,[12] $[\alpha]_D^{24}$ −19.8) was recovered from the PLAMO mother liquor in an identical manner.

Absorbent cotton wicks were sewn through the stems of 12 × 6 month old *D. stramonium* plants and dipped into small glass

vials taped to the stems. A solution of tracer in water (30 cm^3) containing labelled (*R*)-(+)-3-phenyllactic acid (50 mg), carefully neutralised with sodium hydrogen carbonate, was distributed equally between six plants. An equal weight of the (*S*)-(−)-acid was administered likewise to the six remaining plants. Plants were watered through the wicks for two days, then normally for another six days when they were harvested, keeping the roots and aerial parts separate, and dried at 60 °C for 18 h.

Powdered plant tissue was extracted and the alkaloids separated on Kieselguhr columns[39] containing phosphate buffer (0.5 mol dm^{-3}; pH 6.8) and checked for homogeneity by TLC[38] (R_f hyoscine 0.73, hyoscyamine 0.26). The neutralised bases (0.05 mol dm^{-3} sulfuric acid) were converted into their picrates by addition of saturated aqueous sodium picrate, and crystallised from water–ethanol to constant sp. act. and satisfactory mps (lit.,[40] hyoscine 187–188 °C, lit.,[41] hyoscyamine 165 °C).

Feeding Experiment 2.—(*RS*)-3-Phenyl[1,3-$^{13}C_2$,2-^{14}C]lactic acid, sp. act. 7.3 × 10^7 dpm $mmol^{-1}$, 73.5 atom% $^{13}C_2$ was resolved into (*R*)-(+)- and (*S*)-(−)-PLAMO salts. Four × 4 month old *D. stramonium* plants were uprooted and washed free of soil and suspended in commercial hydroponic solution (Phostrogen) (*ca.* 250 cm^3 each). The (*R*)-(+)- and (*S*)-(−)-PLAMO salts (equivalent to 25 mg of free acid per plant) were dissolved in water and added to the nutrient solutions. During the first 2 days exposed roots were regularly bathed in nutrient–tracer using a pipette and the plants were collected 3 days later. Aerial parts and roots were separately blended in a mix of chloroform–diethyl ether–aqueous ammonia (conc.) as described previously.[16] Bases were separated and isolated as the picrates as described above.

Hydrolyses. Selected bases were hydrolysed in baryta as described previously[37] and (*RS*)-tropic acid, mp 118 °C (lit.,[42] 116–117 °C) isolated.

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