The Biosynthesis of Tropic Acid In Plants: Evidence for the Direct Rearrangement of 3-Phenyllactate to Tropate

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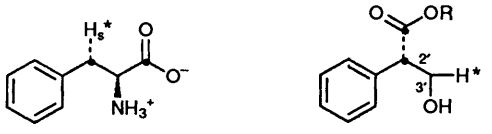
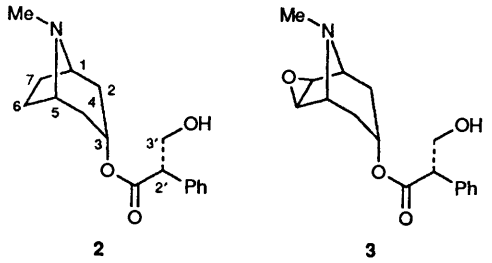
Dual labelled sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate was incubated with transformed root cultures of *Datura stramonium* and the incorporation of the label into the tropic acid ester moiety of hyoscyamine was assessed by $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ NMR spectroscopy and by GC–MS analysis. It is demonstrated that the ^{13}C–^{2}H bond of sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate is incorporated (GC–MS, M + 2, 17%) intact into the hydroxymethyl group at C-3′ of the (*S*)-tropoyl moiety. This result demonstrates unambiguously that 3-phenyllactate is a closer precursor to the tropate ester than phenylpyruvate or phenylalanine.

There has been considerable interest over many years in the biosynthesis of (*S*)-tropic acid **1**, the ester moiety of the tropane alkaloids hyoscyamine **2** and scopolamine **3**.[1,2] It is now well established that tropic acid has an origin in (*S*)-phenylalanine.[3] Perhaps the most intriguing feature of tropic acid biosynthesis is a carbon skeletal rearrangement of (*S*)-phenylanine to give tropic acid (Scheme 1). This was established in a definitive study[4] by Leete *et al.*, who fed (*RS*)-phenyl[1,3-$^{13}C_2$]alanine to *Datura innoxia* plants, and demonstrated that the two labelled carbons became contiguous, generating the [1,2-$^{13}C_2$]-tropic acid ester in the alkaloids. Subsequent studies[5,6] have established that the carboxylate group migrates with retention of configuration at C-3 and that the 3-*pro*-*S* hydrogen of (*S*)-phenylalanine migrates in the reverse direction. This 1,2-vicinal interchange process is illustrated in Scheme 1.



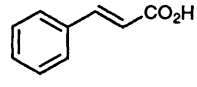
L-phenylalanine **1**

Scheme 1



2 **3**

Considerable effort has been expended on identifying the intermediates between phenylalanine and the tropoyl moiety of hyoscyamine, scopolamine and other aromatic esters of tropine. It was demonstrated some time ago that cinnamic acid[7] **4**, its epoxide[8] **5** and 3-hydroxy-3-phenylpropanoic acid[9] **6** were not precursors and we have established[10] that (*RS*)-3-amino-2-phenylpropionic acid **7**, the rearranged product of phenylalanine, is not involved in the process. In contrast, feeding experiments with both whole plants[11–13] and root cultures[14] have established that phenylpyruvic and 3-phenyllactic acids



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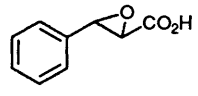

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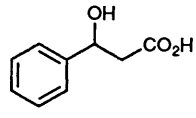

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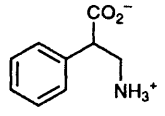

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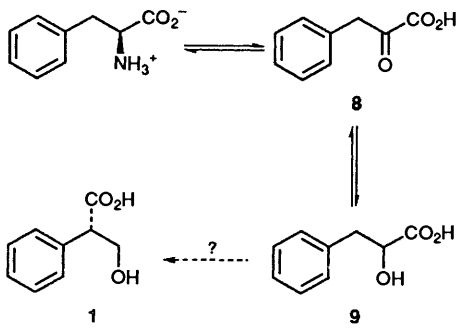
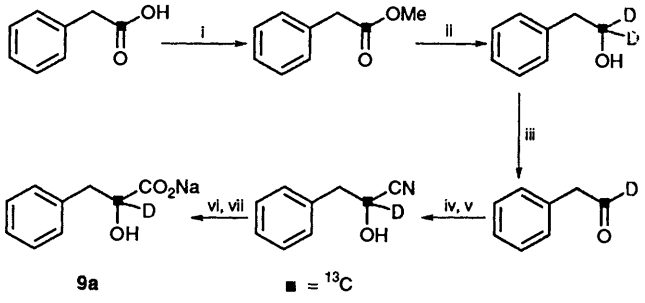
are readily incorporated into a range of tropane alkaloids. The rearrangement of 3-phenyllactic acid (or an ester) would give tropate directly. That this occurs was clearly demonstrated by feeding 3-phenyl[1,3-$^{13}C_2$]lactic acid to whole plants[13] or root cultures[14] of *D. stramonium* and showing the contiguity of the ^{13}C nuclei in the derived hyoscyamine and scopolamine. Furthermore, Ansarin and Woolley have demonstrated[11] with *D. stramonium* plants that ^{3}H from (*RS*)-3-phenyl[1-^{14}C,2-^{3}H]lactate is incorporated into the hydroxymethyl group at C-3′ of the tropoyl moiety of hyoscyamine, albeit with a low specific incorporation (0.1%). Over a series of experiments, the ^{3}H:^{14}C ratio of the recovered tropic acid remained similar to that of the 3-phenyl[1-^{14}C,2-^{3}H]lactate administered to the plants. This result suggests that 3-phenyllactic acid **9** is an obligatory precursor to this group and that it is not first oxidised to phenylpyruvic acid **8**. Additional evidence from competitive feeding experiments in which phenyl[1-^{14}C]-alanine, phenyl[2-^{14}C]pyruvate or 3-phenyl[1-^{14}C]lactate were fed with added unlabelled precursors,[12] indicates that phenyllactate is the closest precursor to the tropoyl moiety of hyoscyamine and scopolomine.

We are prompted now to report our results on the incorporation of (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate **9a** into the tropic acid ester moiety of hyoscyamine **2**. A dual isotope [^{13}C–^{2}H] labelling strategy was employed to allow an unambiguous assessment of the fate of the C–2–H bond of phenyllactate during tropic acid biosynthesis. Oxidation to phenylpyruvic acid **8**, with or without subsequent transamination to (*S*)-phenylalanine, would result in the loss of the deuterium atom (Scheme 2). On the other hand direct rearrangement would result in retention of the ^{13}C–^{2}H bond at C-3′ in the tropoyl moiety of hyoscyamine. The extent of incorporation of ^{13}C



Scheme 2

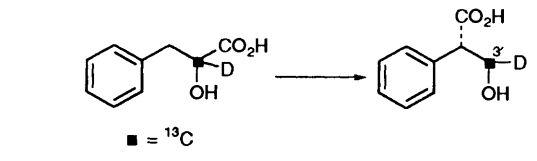


Scheme 3 *Reagents and conditions:* i, CH_2N_2; ii, $LiAlD_4$; iii, pyridinium chlorochromate (PCC), 3 Å mol sieves; iv, $NaHSO_3$; v, NaCN; vi, 50% HCl; vii, dil. NaOH

alone will indicate the degree to which phenylpyruvate has acted as an intermediary in the incorporation. To this end, sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate **9a** was prepared by the synthetic route outlined in Scheme 3. This double-labelled precursor was fed to transformed root cultures of *D. stramonium*. These cultures produce hyoscyamine as the predominant alkaloid[15] with lesser amounts of other alkaloids, including littorine. Examination of the crude alkaloidal extract by GC–MS showed both labels to have been incorporated into littorine, hyoscyamine and apoatropine as indicated by enhancements of the M + 2 ions by 18, 17 and 12% respectively, indicating ^{13}C–^{2}H incorporations. Preparative TLC gave hyoscyamine **2** containing a small amount (4.6%) of littorine **10**. These two alkaloids have similar chromatographic characteristics but distinct ^{13}C NMR resonances for the C-1′, C-2′ and C-3′ nuclei. Thus, the presence of a small amount of littorine did not interfere when the mixture was examined by ^{13}C{^{1}H} and ^{13}C{^{1}H,^{2}H} NMR spectroscopy. In the ^{13}C{^{1}H} spectrum [spectrum (*a*) in Fig. 1] the incorporation of ^{13}C from **9a** into the C-3′ of the tropate moiety of **2** was evident by an enrichment at δ 63.9.[4] Of greater significance however, was a triplet ($J_{^{13}C,^{2}H}$ 22 Hz) shifted further upfield by 0.35 ppm (α-shift) diagnostic[16] of the intact incorporation of ^{13}C–^{2}H at C-3′ retained from the 3-phenyl[2-^{13}C,2-^{2}H]lactate precursor. As expected, this triplet collapsed to a singlet in the ^{13}C{^{1}H,^{2}H} NMR experiment [spectrum (*b*), Fig. 1].

A similar pattern was also evident for the minor peaks at δ 71.5 (singlet) and 71.1 (triplet) in spectrum (*a*) in Fig. 1, arising from ^{13}C- and ^{13}C–^{2}H-enrichments, respectively, at C-2′ of the 3-phenyllactoyl moiety of littorine **10**. The triplet at δ 71.1 again collapsed to a singlet in the ^{13}C{^{1}H,^{2}H} NMR experiment [spectrum (*b*), Fig. 1]. These isotopic enrichments clearly arise as a result of direct esterification of the labelled 3-phenyllactate during littorine biosynthesis.

It is clear from this study that the C-2–H bond of 3-phenyllactic acid **9** can remain intact during the rearrangement process to become one of the C-3′–H bonds of the tropoyl moiety of hyoscyamine (Scheme 4). This experiment demonstrates unambiguously that 3-phenyllactic acid is a more direct precursor than phenylpyruvic acid **8** on the pathway to the tropate ester, confirming evidence obtained previously by inference.[12] It is noteworthy that there was significant washout of deuterium in both the resultant hyoscyamine **2** and littorine **3**. The mass enhancements, determined by GC–MS, of the M + 1 ions of littorine and hyoscyamine were 5.1 and 5.7% respectively, approximately one-third that of the M + 2 ions. This finding indicates that significant loss of the ^{2}H has occurred *in vivo* during the experiment. The most likely cause of this is the interconversion of 3-phenyllactate with phenylpyruvate. This contrasts with the previous report that negligible loss of ^{3}H relative to ^{14}C occurred.[11] However the operation of a kinetic isotope effect and the different biological systems used (plants *versus* root cultures) may invalidate too critical a comparison between these two experiments. Littorine has recently been demonstrated to be converted directly into hyoscyamine.[17] This evidence, coupled with the physiological evidence that free topic acid is not incorporated into hyoscyamine,[3,14] indicates that the putative mutase enzyme involved in hyoscyamine biosynthesis probably acts on littorine as a substrate. The similarity of this rearrangement to coenzyme B_{12}-mediated vicinal interchange processes, such as methylmalonyl-CoA mutase, has been discussed previously,[2,6] and is becoming increasingly striking. The occurrence of vitamin-B_{12} in higher plants is, however, not well documented and the



Scheme 4

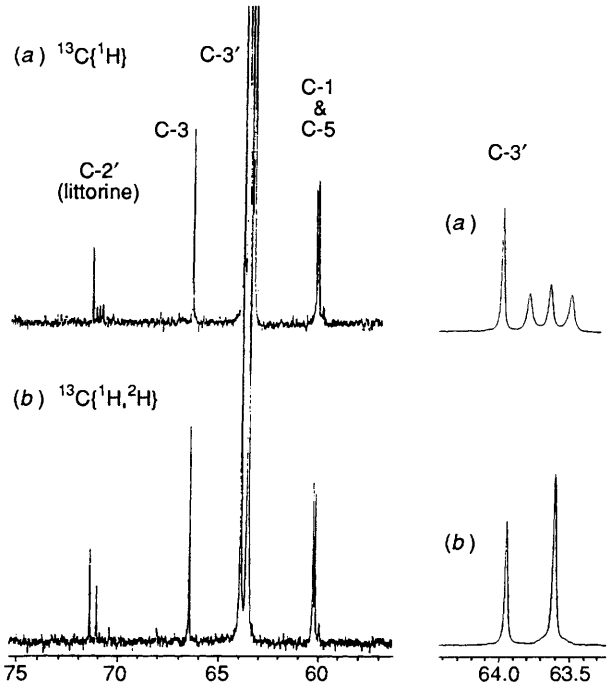
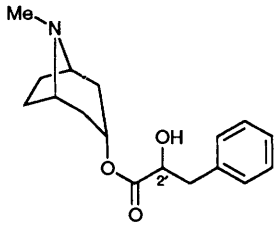


Fig. 1 (*a*) ^{13}C{^{1}H} and (*b*) ^{13}C{^{1}H,^{2}H} NMR spectra of hyoscyamine after incorporation of sodium DL-3-phenyl[2-^{13}C,2-^{2}H]lactate. The ^{13}C–^{2}H incorporation is evident by a triplet in spectrum (*a*) at δ 63.6, upfield from the ^{13}C-signal assigned to C-3′ of tropate at δ 63.9. The triplet collapses to a singlet in spectrum (*b*) (see inset for clarification). The triplet at δ 71.5 in spectrum (*a*) also collapses to a singlet in spectrum (*b*), 0.35 ppm upfield from the enriched uncoupled signal corresponding to C-2′ of littorine. The signals from C-1, C-3 and C-5 of hyoscyamine are also shown.

involvement of this co-factor must remain speculative. Indeed a study[6] by Leete failed to detect any vitamin-B_{12} from tropane-alkaloid-producing *Datura* plants.



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Studies to determine the absolute stereochemistry of the 3-phenyllactate employed in the rearrangement process, and the stereochemical location of the hydrogen migrating to C-3′ in tropate, are ongoing.

Experimental

General Details.—IR spectra were recorded on a Perkin-Elmer F.T. 1720X or 1600 spectrometer. Mass spectra were recorded on a VG Analytical 7070E Organic mass spectrometer. NMR spectra were recorded on Varian Gemini 200 MHz (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz), Varian XL-200 (^{1}H at 200.057 MHz), Varian VXR 400(S) (^{1}H at 399.952 ^{13}C at 100.577 MHz) and 600 MHz Edinburgh spectrometers. Chemical shifts are quoted relative to TMS (Me_4Si) in $CDCl_3$ and H_2O in D_2O, all coupling constants are in Hz. 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) and a DB-17 column (J&W Scientific, Folsom, USA) was used for separation.[18] Flash chromatography was carried out using silica gel-60 (35–70 μm) (Fluka) or Sorbsil-C60-H (40–60 μm). All solvents were dried and distilled prior to use and ether refers to diethyl ether.

2-Phenyl[1-^{13}C,1-$^2H_2$]*ethanol.*—Phenyl[1-^{13}C]acetic acid (1.0 g, 7.34 mmol, 99 atom% ^{13}C, Aldrich Chem. Co. Ltd.) was converted directly into its methyl ester by the addition of an excess of an ethereal solution of diazomethane. The excess of CH_2N_2 was destroyed after 5 min by addition of a few drops of glacial acetic acid. After the solution had been dried ($MgSO_4$) the solvent was removed under reduced pressure to give methyl phenyl[1-^{13}C]acetate, which was then dissolved in ether (10 cm^3) and added to a stirred suspension of lithium aluminium-deuteride (1.3 g, 35 mmol) in ether (40 cm^3) and the reaction mixture was heated under reflux for 2 h under N_2. The reaction was then quenched by the addition of wet ether (30 cm^3) and then 5% H_2SO_4 (30 cm^3). The aqueous layer was extracted into ether (2 × 30 cm^3) and the combined organic extracts dried ($MgSO_4$), and evaporated under reduced pressure to give a yellow oil. Purification by chromatography over silica gel (CH_2Cl_2–ether, 9:1), afforded 2-phenyl[1-^{13}C,1-$^2H_2$]ethanol as a clear oil (0.65 g, 5.2 mmol, 71%); ν_{max}(neat)/cm^{-1} 3346br, 3027, 2931, 2190, 2090, 1603, 1496 and 1453; *m/z* (EI+) 125 (M^+ 38.60%), 91 (100); δ_H($CDCl_3$) 2.15 (1 H, s, OH), 2.81 (2 H, d, $J_{^{13}C,^1H}$ 5.3, CH_2) and 7.25 (5 H, m, ArH); δ_C($CDCl_3$) 39.5 (d, $J_{^{13}C,^{13}C}$ 35, C-2), 63.4 (quintet, $J_{^{13}C,^2H}$ 21.9, C-1), 126.9 (C-4′), 129.1 (C-2′, -6′), 129.5 (C-3′, -5′) and 139.1 (C-1′).

Phenyl[1-^{13}C,1-^{2}H]*acetaldehyde.*—To a stirred suspension of pyridinium chlorochromate (2.9 g, 13.4 mmol) and dry powdered 3 Å molecular sieves (35 g) in dichloromethane (60 cm^3) was added 2-phenyl[1-^{13}C,1-$^2H_2$]ethanol (0.65 g, 5.2 mmol) and the reaction mixture was stirred vigorously for 2 h. The reaction mixture was filtered through a silica gel pad, washing with dichloromethane (400 cm^3). The solvent was removed under reduced pressure to give phenyl[1-^{13}C,1-^{2}H]-acetaldehyde (0.533 g, 4.53 mmol, 87%) which was used directly, without further purification.

Selected spectroscopic data from an unlabelled synthesis: ν_{max}(neat)/cm^{-1} 3020, 1725, 1500, 1455, 750 and 700; δ_H($CDCl_3$) 3.66 (2 H, d, J_{vic} 2.3, CH_2), 7.30 (5 H, m, Ar-H) and 9.72 (1 H, d, 2.3, CHO); δ_C($CDCl_3$) 50.54 (C-2), 127.4 (C-4′), 129.9 (C-2′, -6′), 129.6 (C-3′, -5′), 131.9 (C-1′) and 199.4 (C-1).

Sodium (RS)-3-*phenyl*[2-^{13}C,2-^{2}H]*lactate* **9a**.—A solution of sodium metabisulfite (1.04 g, 5.47 mmol) in water (5 cm^3) was added to phenyl[1-^{13}C,1-^{2}H]acetaldehyde (0.533 g, 4.53 mmol) and the mixture was shaken vigorously for 10 min. Sodium cyanide (0.54 g, 11.02 mmol) was then added in several portions to the reaction mixture, and the cyanohydrin could be seen forming as a clear oil after a few minutes. This was then extracted into benzene (3 × 15 cm^3) and the organic extracts were combined, dried ($MgSO_4$) and evaporated to afford a clear oil. 50% HCl (20 cm^3) was added to the oil and the reaction heated under reflux for 2 h. The solution was allowed to cool and the product was extracted into ether (3 × 30 cm^3). The organic extracts were combined, dried ($MgSO_4$) and evaporated to afford (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactic acid, which was recrystallised from chloroform, m.p. 96–98 °C (racemate).

Selected analytical and spectroscopic data from an unlabelled synthesis (Found: C, 64.7; H, 5.9. $C_9H_{10}O_2$ requires C, 65.0; H, 6.02%); δ_H($CDCl_3$) 2.98 (1 H, dd, *J* 14, 6.9, C*H*H), 3.19 (1 H, dd, *J* 14, 4.2, CH*H*), 4.50 (1 H, dd, *J* 6.9, 4.2, CH) and 7.30 (5 H, m, Ar-H); δ_C($CDCl_3$) 40.1 (C-3), 71.0 (C-2), 127.1 (C-4′), 129.6 (C-2′, -6′), 129.5 (C-3′, -5′), 135.8 (C-1′) and 178.6 (C-1).

The 3-phenyllactic acid was then dissolved in chloroform (10 cm^3) and covered with water (10 cm^3) and the aqueous layer was adjusted to pH 8 with aqueous NaOH (0.1 mol dm^{-3}). Separation of the aqueous layer and evaporation of the water under reduced pressure afforded sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate (128 mg, 0.673 mmol, 15%) as a white amorphous solid; δ_H(D_2O) 2.74 (1 H, dd, J_{gem} 14.0, $J_{^{13}C,^1H}$ 5.1, CH_2), 2.97 (1 H, dd, J_{gem} 14.1, $J_{^{13}C,^1H}$ 3.8, CH_2) and 7.22 (5 H, m, Ar-H); δ_C(D_2O), 43.1 (d, $J_{^{13}C,^{13}C}$ 35.4, C-3). 75.9 (t, $J_{^{13}C,^2H}$ 22.4, C-2), 129.5 (C-4′), 131.4 (C-2′, -6′), 132.3 (C-3′, -5′) and 141.1 (C-1′). An additional signal in the ^{13}C NMR spectrum at δ 183.9 was apparent, only in the isotopically enriched synthesis, and was estimated to constitute 3.6% of the label. This was assigned to the carbonyl resonance of sodium phenyl[1-^{13}C]acetic acid after analysis of the ^{1}H NMR spectrum which showed a small doublet ($J_{^{13}C,^1H}$ 6.8) in the base line at δ 3.41. Reanalysis of ^{1}H NMR spectra from an unlabelled synthesis showed a minor signal (singlet) at δ 3.41, consistent with this interpretation.

Feeding Experiment and Alkaloid Extraction.—Transformed root cultures of *D. stramonium* D15/5 were maintained and grown in B50 medium as previously described.[15] Nine subcultured flasks each containing an initial inoculum of 0.5 g fresh mass of roots in 50 cm^3 of medium were pulse-fed with a sterile neutral solution of sodium (*RS*)-3-phenyl[2-^{13}C,2-^{2}H]lactate (21 mmol dm^{-3}) on days 5, 7 and 9 to a final concentration of 0.4 mmol dm^{-3} in the medium. The roots were harvested after 17 days. The freeze-dried roots (2.72 g) were ground with acid-washed sand and extracted into H_2SO_4 (0.05 mol dm^{-3}; 60 cm^3) by stirring for 15 min. The aqueous extract was made basic with 35% NH_3 solution (10 cm^3) and the solution filtered through Kieselguhr (Varian Bondelut*) and eluted with $CHCl_3$–MeOH (20:1). The eluent was

evaporated to give a brown oil (40.5 mg), which was purified by preparative TLC ($CHCl_3$–Et_2NH, 9:1) to afford hyoscyamine (18.1 mg) contaminated with a trace amount of littorine (0.83 mg).

Acknowledgements

We thank Dr. John Parkinson of the Edinburgh University Ultra High Field NMR Service for recording the $^{13}C\{^1H,^2H\}$ spectra, Dr. Brian J. Goodfellow (IFR) for obtaining a 100.4 MHz ^{13}C NMR spectrum of littorine and the sample, John Eagles for GC–MS and Abbi Peerless and Dr. Peter Bachmann for help with the cultures and feeding experiments. Also we are most grateful to Dr. J. G. Woolley (De Montford University, Leicester) for providing an authentic sample of littorine and to the SERC (studentship to N. C. J. E. C.) and the University of Durham for financial support.

References

1 E. Leete, *Planta Med.*, 1979, **36**, 97.
2 E. Leete, *Planta Med.*, 1990, **56**, 339.
3 Y. Kitamura, A. Taura, Y. Kajiya and H. Miura, *J. Plant Physiol.*, 1992, **142**, 141.
4 E. Leete, N. Kowanko and R. A. Newmark, *J. Am. Chem. Soc.*, 1975, **97**, 6826.
5 E. Leete, *J. Am. Chem. Soc.*, 1984, **106**, 7271.
6 E. Leete, *Can. J. Chem.*, 1987, **65**, 226.
7 W. C. Evans and J. G. Woolley, *Phytochemistry*, 1976, **15**, 287.
8 E. Leete and E. P. Kirven, *Phytochemistry*, 1974, **13**, 1501.
9 E. Leete, *Phytochemistry*, 1983, **22**, 933.
10 R. J. Cox and D. O'Hagan, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2537.
11 M. Ansarin and J. G. Woolley, *Phytochemistry*, 1993, **32**, 1183.
12 M. Ansarin and J. G. Woolley, *J. Nat. Prod.*, 1993, **56**, 1211.
13 M. Ansarin and J. G. Woolley, *Phytochemistry*, in the press.
14 R. J. Robins, J. G. Woolley, M. Ansarin, J. Eagles, B. J. Goodfellow, *Planta*, in the press.
15 R. J. Robins, A. J. Parr, E. G. Bent and M. J. C. Rhodes, *Planta*, 1991, **183**, 185.
16 M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, 1979, 539.
17 R. J. Robins, P. Bachmann and J. G. Woolley, *J. Chem. Soc., Perkin Trans. 1*, 1994, 615.
18 B. Dräger, A. Portsteffen, A. Schaal, P. McCabe, A. C. J. Peerless and R. J. Robins, *Planta*, 1992, **188**, 581.

Paper 3/07460I
Received 20*th December* 1993
Accepted 19*th January* 1994