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COMPETITIVE FEEDING EXPERIMENTS WITH TROPIC ACID PRECURSORS IN *DATURA*[1,2]

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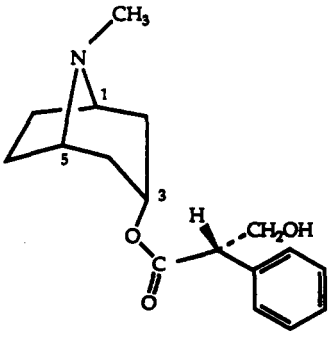
ABSTRACT.—Phenyl[1-^{14}C]alanine [**3**], phenyl[2-^{14}C]pyruvate [**4**], and phenyl[1-^{14}C]lactate [**5**] were fed singly and in competition with unlabeled phenylalanine, phenyllactate, and phenylpyruvate to *Datura stramonium*. The activities recorded in the alkaloids hyoscyamine [**1**] and hyoscine (scopolamine) [**2**] suggested that phenyllactate [**5**] was the closest precursor for the tropic acid moiety of these alkaloids.

Hyoscyamine [**1**], from plants fed with phenyl[2-^{14}C]pyruvate [**4**], was hydrolyzed, but the resultant tropic acid **7** contained only 21% of the activity, the remainder being located in the tropine. However, degradation of the tropic acid showed that the phenylpyruvate incorporated in a specific manner, although it is clearly not an efficient precursor.

(−)-*S*-Tropic acid is the aromatic acid moiety of the important tropane ester alkaloids hyoscyamine [**1**] and hyoscine (scopolamine) [**2**], which are constituents of *Datura* spp. Its biosynthesis, which has aroused much interest, was first investigated in 1960 when Leete (1) demonstrated that the label from phenyl[3-^{14}C]alanine infiltrated into *Datura stramonium* L. (Solanaceae) incorporated into C-2 of tropic acid [**6**]. Underhill and Youngken (2) and Gross and Schutte (3) were able to confirm this result using intact *D. stramonium* and *Datura metel* isolated root cultures, respectively. The former workers reported that phenylacetic acid was also a good precursor but that the usual one-carbon sources (formate and serine), capable of extending the two-carbon side chain to three carbons, only labeled the tropane part of the ester alkaloids. Methionine and formaldehyde (1) also failed as additional one-carbon sources, but bicarbonate (4), although labeling other carbons, predominantly labeled the hydroxymethyl group of tropic acid, a result that favored the intermediacy of phenylacetate. However, it was later (5) demonstrated that all three carbons in the side chain of tropic acid [**6**] were derived from the side-chain carbons of phenylalanine [**3**] by means of a rearrangement (Scheme 1). The primary role of phenylalanine was confirmed when it was shown that after very careful purification, the alkaloids derived from phenylacetate feedings were devoid of activity



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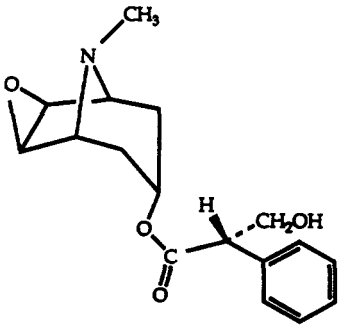

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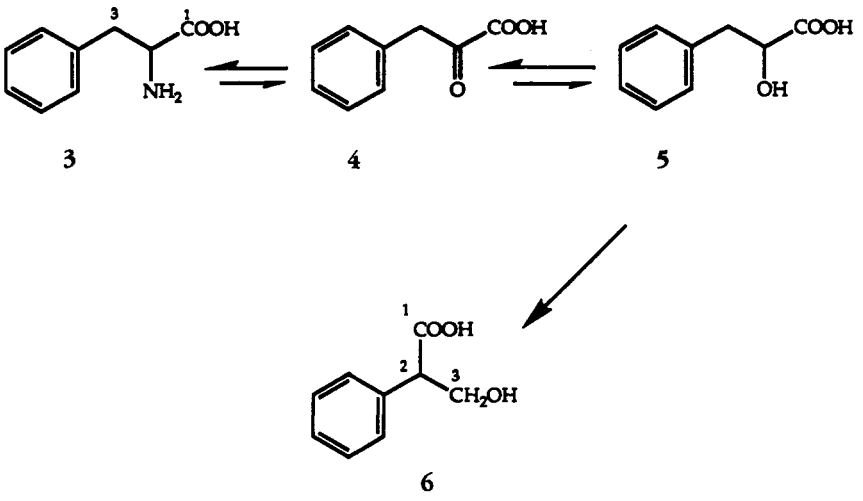
[1]Part 3 in the series "The Biosynthesis of Tropic Acid.' For Part 2, see Evans and Woolley (14).
[2]Dedicated to the memory of Professor Edward Leete.



SCHEME 1

(6). Likewise, suggestions that tryptophan can serve as a precursor (7,8) have been largely discounted (9,10). It has also been claimed (11) that cinnamate [derived from phenylalanine via the enzyme PAL (12)] is an intermediate but repeated feeding experiments (13–16) have failed to confirm this finding.

By using phenyl[1,3-^{14}C]alanine, Gibson and Youngken (17) demonstrated that the side-chain rearrangement was an intramolecular event, a result elegantly confirmed by Leete using a doubly-labeled ^{13}C tracer (6) which resulted in hyoscine and hyoscyamine having satellite signals in the ^{13}C-nmr spectrum from the newly formed contiguously labeled centers. The rearrangment is accompanied by a 1,2 shift of the *pro*-R H-3 to the hydroxymethyl group of tropic acid (18,19). However, little is known about the intermediate steps leading up to tropic acid, although a clue to its biosynthesis came in 1968 when the alkaloid littorine was independently isolated from *Datura sanguinea* (20) and *Anthocercis littorea* (21). Littorine is an ester of tropine and phenyllactic acid, and it was shown that phenyllactate [**5**] was a precursor of tropic acid [**6**] and at least as efficiently incorporated (13,14). It has also been reported that phenylpyruvate [**4**] can act as a precursor (22), but we have been unable to find complete published details of this observation. Because of the rapid interconversion of phenylalanine [**3**], phenylpyruvate [**4**] and phenyllactate [**5**] (23), it has been difficult to decide which acid undergoes the rearrangement process, although a mechanism involving phenylpyruvate has gained support (16,24). In order to solve the priority problem, the series of competitive feeding experiments reported here were conceived.

RESULTS AND DISCUSSION

Tables 1 and 2 show data gathered over two years. All three acids apparently serve as precursors for the tropic acid moieties of hyoscyamine [**1**] and hyoscine [**2**]. Phenylalanine [**3**] proved a better precursor in one instance, phenyllactate [**5**] being superior in the second group of feedings in agreement with our earlier findings (4). Surprisingly, phenylpyruvate [**4**] was poorly incorporated.

In parallel comparisons (Tables 1 and 2), phenyllactate consistently interfered with the incorporation of phenylalanine precursor, whereas phenylpyruvate had no impact. The reason for the apparent anomalous behavior of phenylpyruvate is unknown at present.

TABLE 1. Competitive Feeding Experiments, Year 1.

Precursor, phenyl [(1-^{14}C)]lactic acid, specific activity 1.5×10^{8} dpm/mmol

	Fed alone			Fed with phenylalanine			Fed with phenylpyruvate		
	Specific activity dpm/mmol $\times10^{-4}$	% Specific incorporation[a]	Relative incorporation[b] %	Specific activity dpm/mmol $\times10^{-4}$	% Specific incorporation[a]	Relative incorporation[b] %	Specific activity dpm/mmol $\times10^{-4}$	% Specific incorporation[a]	Relative incorporation[b] %
Hyoscyamine [1]	3.4	0.02	100	3.2	0.02	94	2.6	0.016	77
Hyoscine [2]	0[c]	0	0	1.1	0.007	32	0[c]	0	0

Precursor, phenyl [(1-^{14}C)]alanine, specific activity 7.3×10^{8} dpm/mmol

	Fed alone			Fed with phenyllactate			Fed with phenylpyruvate		
Hyoscyamine [1]	9	0.012	100	3.6	0.005	40	11	0.014	111
Hyoscine [2]	5.3	0.007	59	3.5	0.005	39	8.6	0.001	96

[a]Calculated as specific activity product (dpm/mmol)×100/specific activity precursor (dpm/mmol).
[b]Compared with hyoscyamine isolated from plants fed with precursor alone and calculated as specific activity (dpm/mmol)×100/specific activity hyoscyamine (dpm/mmol).
[c]Isolated but inactive.

TABLE 2. Competitive Feeding Experiments, Year 2.

Precursor, phenyl [(1-^{14}C)]lactic acid, specific activity 1.5×10^{8} dpm/mmol

	Fed alone			Fed with phenylalanine			Fed with phenylpyruvate		
	Specific activity dpm/mmol $\times10^{-4}$	% Specific incorporation[a]	Relative incorporation[b] %	Specific activity dpm/mmol $\times10^{-4}$	% Specific incorporation[a]	Relative incorporation[b] %	Specific activity dpm/mmol $\times10^{-4}$	% Specific incorporation[a]	Relative incorporation[b] %
Hyoscyamine [1]	5	0.03	100	3	0.02	60	20	0.12	400
Hyoscine [2]	0[c]	0	0	5	0.03	100	10	0.06	200

Precursor, phenyl [(1-^{14}C)]alanine, specific activity 4.5×10^{8} dpm/mmol

	Fed alone			Fed with phenyllactate			Fed with phenylpyruvate		
Hyoscyamine [1]	53	0.11	100	30	0.07	57	56	0.12	106
Hyoscine [2]	55	0.12	104	4	0.008	8	46	0.1	86

Precursor, phenyl [(2-^{14}C)]pyruvate, specific activity 8.2×10^{7} dpm/mmol

		Fed alone		Fed with phenylalanine			Fed with phenyllactate		
Hyoscyamine [1]	0.6	0.007	100	1.5	0.01	250	0.6	0.008	100
Hyoscine [2]	0.5	0.006	83	0.06	0.008	4	0.1	0.001	17

[a]Calculated as specific activity product (dpm/mmol)×100/specific activity precursor (dpm/mmol).

[b]Compared with hyoscyamine isolated from plants fed with precursor alone and calculated as specific activity (dpm/mmol)×100/specific activity hyoscyamine (dpm/mmol).

[c]Isolated but inactive.

When phenyllactate was used as a precursor, phenylalanine had no effect in year 1, but there was a significant depression in the activity observed in year 2. In the latter case, however, it is noteworthy that the activity in hyoscine matched that of the hyoscyamine when the precursor was fed alone. Since hyoscine [**2**] is produced from hyoscyamine [**1**] (25–28), we interpret this as an indication that the phenylalanine is not interfering with the incorporation of the labeled phenyllactate. Had it been so, then the relative incorporation into the hyoscine would have been lower than that of the hyoscyamine. Since the specific activity of the hyoscine is the higher, then hyoscyamine is being made (and diluted) with endogenous unlabeled precursor at this point after the feed. Phenyllactate fed in the presence of phenylpyruvate caused a dramatic increase in the incorporation of the former, and we interpret this as a sign that phenylpyruvate precedes phenyllactate in the biosynthetic pathway. It is probable that the exogenous phenylpyruvate is affecting the equilibrium of the reversible interconversion of phenylpyruvate to phenyllactate, thereby driving the overall sequence in the direction of tropic acid formation.

The results for the labeled phenylpyruvate feedings are less clear, except that it was poorly incorporated. Feeding in the presence of exogenous phenylalanine appeared to promote its incorporation, again perhaps by preventing the reversible step, phenylpyruvate to phenylalanine, and in doing so driving the labeled substrate through to phenyllactate and tropic acid. Phenyllactate has little effect on the incorporation of phenylpyruvate, except in the case of hyoscine which shows a marked depression of activity, a result one would expect if phenyllactate were the rearranging acid.

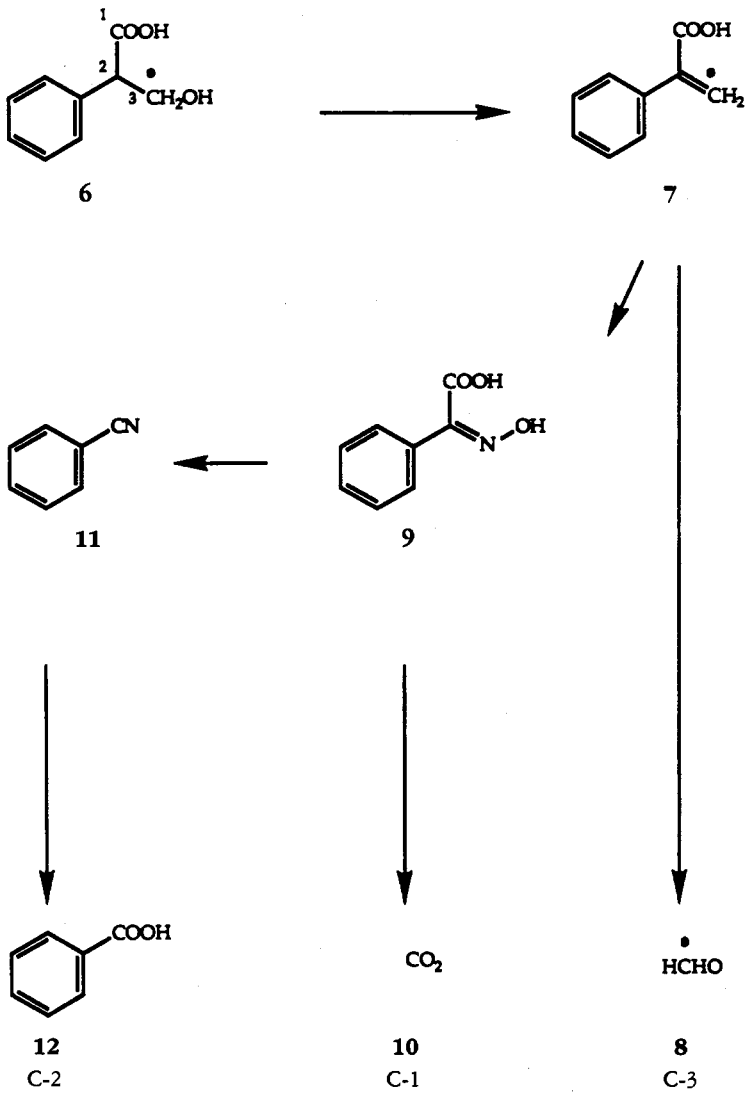
Broadly we believe that the results, within the limits of the methodology, favor phenyllactate as the immediate precursor of tropic acid. Recent work (29,30) using a very consistent plant system, the D15/5 hairy root cultures of *D. stramonium*, shows an obviously superior methodology which eliminates possible biological variation. Work here (31) and that recently reported elsewhere (32) supports our view that phenyllactate is the rearranging acid in the biosynthesis of tropic acid.

Because we have been unable to trace details of the systematic degradation of hyoscyamine [**1**] or hyoscine [**2**] labeled by phenylpyruvate [**4**], hyoscyamine from the phenylalanine competitive feed was degraded using the excellent method of Leete (5). To our surprise, hydrolysis of the base gave tropic acid [**6**] containing only 21% of the total activity, the bulk of the activity being located in the resultant tropine (which was not degraded). Degradation of the tropic acid [**6**] (Scheme 2) via atropic acid [**7**] and cleavage of the double bond with OsO_4 /sodium metaperiodate gave formaldehyde [**8**], collected as the dimedone derivative, which contained all the activity of the initial acid, indicating that phenylpyruvate [**4**] does incorporate in a specific manner. It is probable that phenyl[2-^{14}C] pyruvate is converted to phenyl [1-^{14}C] acetate, and decarboxylation of this provides a labeled one-carbon source to be refixed and incorporated into tropine via the glutamate, arginine, ornithine pathway. It is also possible, but unlikely, that the phenylacetate could be incorporated into tropic acid directly by utilizing an additional one-carbon source to provide the carboxyl group of the latter. Although exogenous phenylacetate has been shown not to be a precursor, phenylpyruvate here is supplying an endogenous substrate which may be bound in some way and treated differently.

EXPERIMENTAL

PLANT MATERIAL.—Plants were grown under glass in commercially available peat/John Innes No. 2 compost mix. Seeds were obtained from the Zentralinstitut fur Genetik und Kulturpflanzenforschung, Gatersleben, Germany.

RADIOACTIVE COMPOUNDS.—Phenyl[1-^{14}C]alanine, NaCN-^{14}C, and glycine-[2-^{14}C] were purchased from Amersham International, Amersham, UK.



SCHEME 2. Degradation of tropic acid [6].

COUNTING PROCEDURES.—Duplicate samples were counted (Packard Tri-carb model 2002 or 3255) in commercially available dioxane-based scintillators (Nuclear Enterprises NE 240), making appropriate correction for quenching. Alternatively, for samples of low activity (degradation of tropic acid), duplicate samples (10–15 mg) were mixed with glucose (30 mg) and burned (Packard Tri-carb sample oxidiser model 306) in the presence of O_2, the CO_2 being absorbed into a toluene-based scintillator (Carbo-sorb plus Permafluor V).

SYNTHESES.—Phenyl[1-^{14}C]lactic acid was synthesized by the condensation of phenylacetaldehyde with NaCN-^{14}C followed by hydrolysis of the resultant cyanohydrin as reported fully elsewhere (32).

For phenyl[2-^{14}C]pyruvic acid, glycine-[2-^{14}C] (250 μCi) in H_2O (1 ml) was mixed with carrier (500 mg) in H_2O (4 ml) plus Ac_2O (5 ml) and stirred at room temperature for 1 h. The mixture was diluted with H_2O (10 ml), adjusted to pH 4 (NaOH), and evaporated to dryness under reduced pressure. The residual sodium aceturate was dissolved in Ac_2O (10 ml) and freshly distilled benzaldehyde (2.5 ml) and refluxed for 1 h under N_2. The product, 4-benzylidine-2-methyl-Δ^2-oxazolin-5-one, crystallized on cooling and was washed with cold H_2O and a little Et_2O, dried over P_2O_5, and then refluxed under N_2 in 2 N HCl (45 ml) for 2 h. The cooled solution was extracted with Et_2O (4×10 ml), and the bulked organic layer was washed with a little NaCl solution prior to evaporation under reduced pressure. The residue was crystallized twice

from C_6H_6, giving phenyl[2-^{14}C]pyruvic acid mp 153°, 370 mg, specific activity 8.2×10^7 dpm/mmol. The acid was stored in a desiccator over P_2O_5 and used in feeding experiments within 5 days of synthesis.

FEEDING EXPERIMENTS.—*Year 1, December.*—Thirty 4-month-old plants were divided into 6 groups. Each plant had absorbent cotton thread sewn through the outer part of the stem about 3 inches from the soil, and the trimmed ends were placed in a small vial strapped to the stem. Each plant received 8.2 mg of tracer, and when appropriate the same weight of potential inhibitor in H_2O (5 ml) carefully neutralized with $NaHCO_3$. After 20 days the plants were sliced and dried at 60° for 24 h.

Year 2, August.—Eighteen 6-month-old plants were divided into 9 groups and fed as above with 13.2 mg tracer plus an equal weight of inactive competitor, as appropriate. Plants were harvested after 10 days and dried.

ISOLATION OF ALKALOIDS.—Alkaloids were extracted with lime and Et_2O as detailed previously (14) and resolved on Kieselguhr columns (10 g) containing 0.5 M phosphate buffer pH 6.8. The columns were developed with petroleum ether, Et_2O, and $CHCl_3$ (75 ml each), the latter two being collected in 5-ml fractions. Et_2O fractions yielded hyoscine [R_f 0.73 on Si gel 60 plates (Merck) run in $CHCl_3$-EtOH-concentrated NH_4OH (100:20:1) and detected with either Dragendorff's reagent or a saturated solution of I_2 in CCl_4], isolated as the picrate. $CHCl_3$ fractions gave hyoscyamine (R_f 0.26) which was similarly converted to the picrate. All picrates were recrytallized to constant specific activity from EtOH/H_2O.

HYDROLYSIS OF HYOSCYAMINE.—Labeled hyoscyamine was diluted with carrier during the shake-out process to a calculated specific activity 1.09×10^3 dpm/mmol. The base was dissolved in hot EtOH (2 ml), diluted with 5% $Ba(OH)_2$ solution (100 ml), and heated under reflux for 3.5 h. The cooled hydrolysate was acidified (50% H_2SO_4) and extracted with Et_2O (7×10 ml). Evaporation of the Et_2O gave a colorless oil which crystallized from C_6H_6/petroleum ether at 4° to give tropic acid [**7**] mp 116° (169 mg). The remaining aqueous phase was neutralized with excess $BaCO_3$ and centrifuged. The supernatant was evaporated to dryness under reduced pressure, the residue was redissolved in H_2O (2 ml), and a saturated solution of sodium picrate was added dropwise until no more crystals formed. Tropine picrate crystallized easily from aqueous EtOH mp 293° [lit. (2) 290–293°], 241 mg, specific activity 814 dpm/mmol.

DEGRADATION OF TROPIC ACID.—Tropic acid [**6**] (169 mg) was dissolved in KOH (0.8 g) in H_2O (2 ml) and refluxed under an N_2 stream for 45 min. The cooled acidified (dilute HCl) solution gave atropic acid [**7**] as long colorless needles: mp 105° [lit. (5) 102.5–107.5°], (99 mg).

Atropic acid (99 mg) in H_2O (5 ml) was neutralized by the addition of $NaHCO_3$, and OsO_4 (ca. 10 mg) was added. After 45 min, $NaIO_4$ (449 mg) in H_2O (10 ml) was added to the purple solution which was kept at 4° for 18 h when it was shaken with Et_2O to remove excess OsO_4. The acidified (1 N HCl) solution in ice was extracted with Et_2O (6×10 ml), and the combined Et_2O washings were stored in ice. Meanwhile, the aqueous phase was distilled. The distillate (ca. 15 ml) was poured into a solution of dimedone (200 mg) in H_2O (90 ml), which had been stored at 4° overnight, and allowed to crystallize at 4° for 2 days. The formaldehyde [**8**]-dimedone derivative formed as long white needles, yield 90 mg (46%) mp 189° [lit. (13) 190°] specific activity 175 dpm/mmol. The Et_2O solution of the benzoylformic acid, which had been cooled in ice, was evaporated under N_2 at room temperature, and a solution of hydroxylamine (70 mg) in freshly boiled H_2O (10 ml) was added to the residue to convert it to the oxime **9**. The solution was refluxed under a stream of N_2 for 50 min, the N_2 effluent passing through a freshly prepared 5% $Ba(OH)_2$ solution to collect any liberated CO_2 [**10**]. The precipitated $BaCO_3$ was washed with H_2O (2×5 ml), Me_2CO (2×5 ml) and finally Et_2O before drying, yield 86 mg (43%), inactive.

The remaining aqueous phase from which the CO_2 had been released was extracted with Et_2O (6×10 ml), and the residue left after evaporation of the solvent at room temperature was covered with 50% H_2SO_4 (20 ml) and refluxed (2 h). Benzoic acid [**12**] mp 120° [lit. (5) 121–122°] was extracted with Et_2O and the residue sublimed on to a cold-finger condenser, yield 21 mg (26%), inactive.

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LITERATURE CITED

1. E. Leete, *J. Am. Chem. Soc.*, **82**, 612 (1960).
2. E.W. Underhill and H.W. Youngken, *J. Pharm. Sci.*, **51**, 121 (1962).
3. D. Gross and H.R. Schutte, *Arch. Pharm.*, **296**, 1 (1963).

4. M.L. Louden and E. Leete, *J. Am. Chem. Soc.*, **84**, 1510 (1962).
5. M.L. Louden and E. Leete, *J. Am. Chem. Soc.*, **84**, 4507 (1962).
6. E. Leete, N. Kowanko, and R.A. Newmark, *J. Am. Chem. Soc.*, **97**, 6826 (1975).
7. A.M. Goodeve and E. Ramstad, *Experientia*, **17**, 124 (1961).
8. N.W. Hamon and J.L. Eyolfson, *J. Pharm. Sci.*, **61**, 2006 (1972).
9. E. Leete, *Abhandlungen der Deutschen Akademie der Wissenschaften zu Berlin*, 538 (1966).
10. E. Leete, in: "Biosynthesis, Specialist Periodical Report." Ed. by T.A. Geissman, Chemical Society, London, **2**, 115 (1973).
11. V. Prabhu, C.A. Gibson, and L.C. Schramm, *Lloydia*, **39**, 79 (1976).
12. R.H. Wightman, J. Staunton, A.R. Battersby, K.R. Hanson, *J. Chem. Soc., Perkin Trans. 1*, 2355 (1972).
13. W.C. Evans, J.G. Woolley, and V.A. Woolley, *Abhandlungen der Deutschen Akademie der Wissenschaften zu Berlin*, 227 (1971).
14. W.C. Evans and J.G. Woolley, *Phytochemistry*, **15**, 287 (1976).
15. H.W. Liebisch, 7th IUPAC Congress Report, Chemistry of Natural Products, Riga 557 (1970).
16. E. Leete, *Planta Med.*, **36**, 97 (1979).
17. C.A. Gibson and H.W. Youngken, *J. Pharm. Sci.*, **56**, 854 (1967).
18. E. Leete, *J. Am. Chem. Soc.*, **106**, 7271 (1984).
19. E. Leete, *Can. J. Chem.*, **65**, 226 (1987).
20. W.C. Evans and V.A. Major, *J. Chem. Soc. C*, 2775 (1968).
21. J.R. Cannon, K.R. Joshi, G.V. Meehan, and J.R. Williams, *Aust. J. Chem.*, **22**, 221 (1969).
22. H.W. Liebisch, G.C. Bhavsar, and H.J. Schaller, *Abhandlungen der Deutschen Akademie der Wissenschaften zu Berlin*, 233 (1971).
23. A.C. Neish, *Ann. Rev. Plant Physiol.*, **11**, 55 (1960).
24. D. Dalton, "An Alkaloid Primer," Marcel Dekker, New York, 1978, p. 57.
25. T. Hashimoto and Y. Yamada, *Plant Physiol.*, **81**, 619 (1986).
26. T. Hashimoto and Y. Yamada, *Eur. J. Biochem.*, **164**, 277 (1987).
27. T. Hashimoto, J. Kohno, and Y. Yamada, *Plant Physiol.*, **84**, 144 (1987).
28. T. Hashimoto, J. Kohno, and Y. Yamada, *Phytochemistry*, **28**, 1077 (1989).
29. R.J. Robins, A.J. Parr, J. Payne, N.J. Walton, and M.J.C. Rhodes, *Planta*, **181**, 414 (1990).
30. R.J. Robins, A.J. Parr, E.G. Bent, and M.J.C. Rhodes, *Planta*, **183**, 185 (1991).
31. R.J. Robins, J. Eagles, I. Colquhoun, J.G. Woolley, and M. Ansarin, Phytochemistry in Agriculture, Poster; Phytochemical Society of Europe/Royal Netherlands Chemical Society Symposium, Wageningen, Netherlands, 22–24 April 1992.
32. M. Ansarin and J.G. Woolley, *Phytochemistry* **32**, 1182 (1993).

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