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THE OBLIGATORY ROLE OF PHENYLLACTATE IN THE BIOSYNTHESIS OF TROPIC ACID*

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DEDICATED TO THE MEMORY OF PROF. EDWARD LEETE, 1928–1992

Key Word Index—*Datura stramonium*; Solanaceae; phenyllactic acid; tropane alkaloids; tropic acid biosynthesis.

Abstract—Phenyl[1-^{14}C] lactic acid and phenyl [2-^{3}H] lactic acid were synthesized by conventional routes and fed in admixture (^{3}H:^{14}C ratio 10:1) to *Datura stramonium* via the roots. Hyoscine (scopolamine) and hyoscyamine were isolated and found to have essentially the same ^{3}H:^{14}C ratios as the precursor (8.5 and 10.15, respectively). Hydrolysis of hyoscyamine showed that all the activity was present in the tropic acid moiety. The tropic acid was converted to atropic acid and then cleaved by means of osmium tetroxide and sodium metaperiodate to yield formaldehyde containing all the tritium and phenylglyoxylic acid containing all the ^{14}C, a result which shows that the tritium at C-2 of the phenyllactate precursor labelled tropic acid in the hydroxymethyl group. These findings show that phenyllactate is an obligatory intermediate in the biosynthesis of tropic acid and that the rearrangement of the side-chain takes place at the phenyllactate and not the phenylpyruvate level.

INTRODUCTION

(−)-*S*-Tropic acid is the aromatic acid moiety of the important medicinal tropane alkaloids hyoscine (**1**) (scopolamine) and hyoscyamine (**2**) which are constituents of *Datura* species. Its biosynthesis, and in particular the formation of the branched side-chain has aroused considerable interest. In 1960, Leete [1] demonstrated that phenylalanine (**3**) was a precursor, a result confirmed by Underhill and Youngken [2]. Later using an elegant degradation scheme Leete [3] was able to show that all the carbons in the side-chain of tropic acid (**8**) were derived from this amino acid by means of a rearrangement. Suggestions that tryptophan can serve as a precursor [4] have been largely discounted [5, 6] although as recently as 1981 [7] it was again implicated. It has also been reported that phenylacetic acid can incorporate into tropic acid [2, 8] deriving the third carbon from a one carbon source. Careful purification of initially radioactive bases derived from such feeds were later shown [9] to be contaminated with unidentified labelled material (perhaps phenylacetyl esters of tropanes). By means of feeding experiments with phenyl[1,3-^{13}C]alanine [9] it was shown that the rearrangement of the side-chain was intramolecular by the appearance of contiguously labelled centres located by satellite signals in the NMR spectra of the products. The rearrangement is accompanied by a 1,2 shift of the *pro R* C-3 proton to the hydroxymethyl group of tropic acid [10, 11]. However, little is known about the intermediate steps leading up to tropic acid. A clue to its biosynthesis came in 1968 when the alkaloid littorine was independently isolated from *Datura sanguinea* [12] and *Anthocercis littorea* [13]. Littorine is an ester of tropine and phenyllactic acid (**5**). It was shown that phenyllactate was a precursor of tropic acid and at least as efficiently incorporated [14, 15]. Cinnamic acid (**6**), in spite of several attempts [14–17] failed to serve as a precursor although Prabhu *et al.* [18] have claimed that it is. It has also been reported that phenylpyruvate (**4**) can act as a precursor [19] 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**) [20] it has been difficult to decide which acid undergoes the rearrangement process although a mechanism involving phenylpyruvate (**4**) has gained support [17, 21].

RESULTS AND DISCUSSION

Phenyl[1-^{14}C]lactic acid was prepared by condensation of phenylacetaldehyde [22, 23] with labelled sodium cyanide followed by hydrolysis of the resultant cyanohydrin. Phenyl[2-^{3}H]lactic acid was synthesized [24] by reduction of freshly prepared phenylpyruvate [25] with sodium borotritide and fed in admixture {as in (**9**)} with ^{14}C labelled acid to *Datura stramonium* via the roots. Hyoscine (**1**) and hyoscyamine (**2**) isolated from the feed

*Part 4 in the series 'The Biosynthesis of Tropic Acid'. For Part 3, see ref. [26].

Table 1. Specific activities and ^3H:^{14}C ratios of alkaloids and degradation products from plants fed with phenyl[1-^{14}C; 2-^3H]lactic acid

	Sp. act. ^{14}C (dpm mM^{-1})	Sp. act. ^3H (dmp mM^{-1})	^3H:^{14}C ratio	Sp. incorp.* (%)
Hyoscine	1.2×10^4	9.8×10^4	8.2	0.01
Hyoscyamine†	5.5×10^4	5.6×10^5	10.2	0.1
Tropic acid‡	554	5542	10.0	
Atropic acid	621	5855	9.4	
Formaldehyde (dimedone derivative)	—	5112		
Phenylglyoxylic acid (syn oxime)	521	—	10.0	

*Based on incorporation of ^{14}C and calculated as sp. act. product × 100/sp. act. precursor.
†Diluted with hyoscyamine picrate carrier.
‡Diluted with tropic acid carrier.

had essentially the same ^3H:^{14}C ratio as the precursor (Table 1). Degradation of the hyoscyamine (**2**) (Scheme 1) in the sequence tropic acid (**10**), atropic acid (**11**), phenylglyoxylic acid (**12**) plus formaldehyde (**13**) proved that the incorporation was specific, the tritium residing in the hydroxymethyl group (C-3) of tropic acid (**10**) and the ^{14}C was located in the carboxyl group.

Feeding experiments with labelled phenylalanine, phenyllactate and phenylpyruvate in competition with unlabelled species [26] broadly show the same picture but the data here are quite unambiguous. If the rearrangement is taking place at the phenylpyruvate level, as previously suspected, then the tropic acid produced would show no tritium label from the phenyllactate precursor. One could argue that it is still possible for cinnamate to be an intermediate if it is formed by dehydration of phenyllactate, but this is untenable on closer examination, apart from the many reports that labelled cinnamate fails to incorporate into tropic acid [14–17]. Leete has carried out feeding experiments with phenylalanine stereospecifically labelled with tritium in the C-3 *pro*-chiral centre [10, 11]. A route involving dehydration of phenyllactate would inevitably lead to the loss of one or other of the protons at C-3, whereas Leete has shown that they are both retained, the *pro-S* proton migrating to C-2 during the rearrangement process. This latter result is entirely consistent with our findings reported here. These results show that tropic acid is formed in Nature by a rearrangement of the side-chain of phenyllactic acid. We believe that this observation has important consequences for the biotechnological production of tropic acid esters.

EXPERIMENTAL

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 für Genetik und Kulturpflanzenforschung, Gatersleben, Germany.

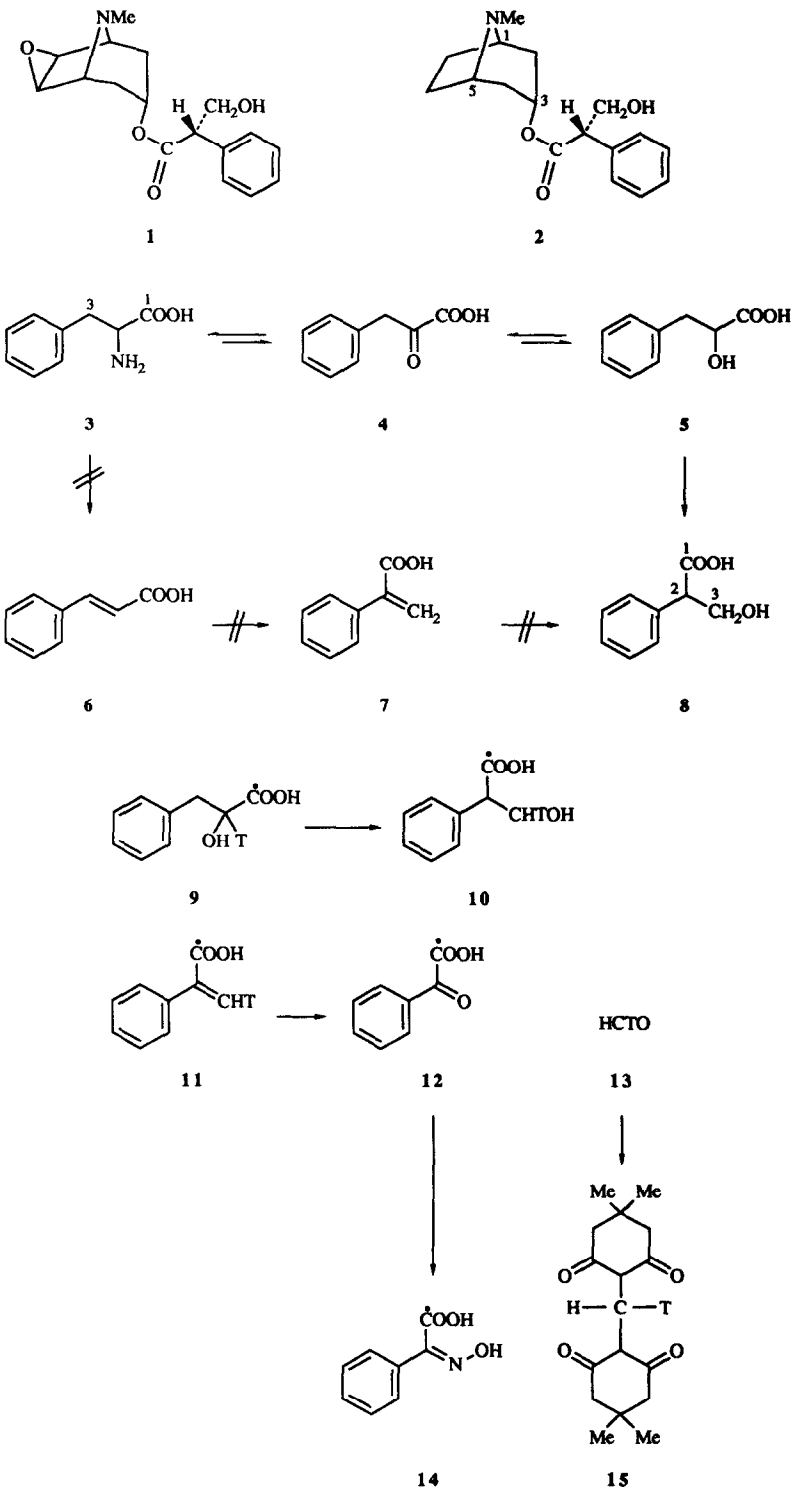
Radioactive materials. Sodium cyanide-^{14}C and sodjum borotritide were purchased from Amersham International (Amersham, U.K.).

Thin layer chromatography [27]. Samples were separated on silica gel plates (Merck) using $CHCl_3$–EtOH–conc NH_4OH (100:20:1) and detected with either Dragendorff's reagent or a satd soln of I_2 in CCl_4.

Counting procedures. Duplicate samples (15 mg) were mixed with glucose (30 mg) and burned in the presence of O_2, the $^{14}CO_2$ being absorbed into a toluene based scintillator, the ^3H_2O being collected in a xylene type scintillator fluid.

Synthesis of phenyl[1-^{14}C]*lactic acid.* Phenylacetaldehyde dimethylacetal (5 ml) was mixed with 50% MeOH (5 ml), 3 drops conc HCl and refluxed for 30 min. Evapn of the solvent under red. pres. gave, after dissolving in dry MeOH followed by evapn several times, a clear yellow sample of phenylacetaldehyde bp 192°. The oil was dissolved in Et_2O (20 ml) cooled (0°) in salt/ice. NaCN-^{14}C (500 μCi) in H_2O (2 ml) containing carrier (245 mg) was mixed with Na metabisulphite (475 mg) in H_2O (15 ml), cooled (0°) and added dropwise with vigorous stirring to the phenylacetaldehyde soln over 15 min. After allowing the mixture to warm up to room temp. with continuous stirring for 45 min, the crude phenylacetaldehyde cyanohydrin was extracted with fresh Et_2O (6 × 20 ml). Evapn of the Et_2O gave a yellow oil which was covered with conc HCl (15 ml) and H_2O (15 ml) and refluxed for 1 hr. After cooling, the hydrolysate was made alkaline (KOH) and washed several times with Et_2O. On reacidification (conc HCl), the aqueous phase was extracted with Et_2O (6 × 20 ml), and after drying (Na_2SO_4), the Et_2O was evapd leaving a semi-solid residue which was recrystallized twice from C_6H_6 to give phenyl[1-^{14}C]lactic acid mp 96° (lit. 95.5–96.5° [27, 28] IR (KBr) identical to authentic phenyllactic acid, yield 135 mg (16%), sp. act. 1.5×10^8 dpm mM^{-1}.

Synthesis of phenyl[2-^3H]*lactic acid.* Phenylpyruvic acid was freshly prepared by standard methods [25]. The



Scheme 1. Incorporation of phenyl[1-^{14}C; 2-^{3}H]lactic acid and degradation of tropic acid.

Na salt (228 mg) was dissolved in a mixture of satd Na_2CO_3 (1 ml) plus H_2O (6 ml) and cooled to 0° in salt/ice. Sodium borotritide (152 mg, 100 mCi) was added in small portions with stirring. After 2 hr the soln was acidified (dil HCl) and continuously extracted with CH_2Cl_2 for 17 hr. The residue obtained by evapn of the solvent was azeotroped with C_6H_6 to remove H_2O and crystallized twice from CCl_4 to give phenyl[2-^{3}H]lactic

acid as plates, mp 96°, yield 185 mg (81%), sp. act. 1.5×10^{10} dpm mM^{-1}.

Feeding of tracers. Phenyl[1-^{14}C]lactic acid (20 mg) sp. act. 1.5×10^8 dpm mM^{-1} and phenyl [2-^{3}H] lactic acid (2 mg) sp. act. 1.5×10^{10} were added to H_2O (20 ml) and neutralized by the addition of $NaHCO_3$ and made up to 100 ml with more H_2O. Four 4-month-old pot-grown *Datura stramonium* were carefully washed free of compost, supported in beakers containing the tracer soln. Periodically over the first day the roots were bathed in tracer by using a small pipette and then liberally supplied with Phostrogen fluid for 6 days.

Extraction and isolation of alkaloids [27]. Whole plants were blended in a food mixer using a mixture of $CHCl_3$–Et_2O (1:1, 1600 ml) and conc NH_4OH (40 ml). After standing for 2 days the extract was filtered through muslin, then filter paper and concd to a small vol. (*ca* 50 ml). The soln was extracted with dil HCl (6×10 ml) and basification of the bulked aq. phases (Na_2CO_3) followed by extraction with $CHCl_3$ gave, after evapn, a crude alkaloid fraction. The residue was redissolved in $CHCl_3$ (2 ml) and transferred to a kieselguhr (10 g) column containing 5 ml 0.5 M Pi buffer pH 6.8 [29]. Development of the column 50 ml each of petrol, Et_2O and $CHCl_3$, the latter two being collected in 5 ml fractions gave hyoscine (Et_2O) isolated as the picrate mp 188° (R_f 0.73), 2.9 mg, and hyoscyamine, picrate mp 163°, 42.7 mg.

Degradation of hyoscyamine. Hyoscyamine picrate (20 mg) ^{3}H:^{14}C ratio 10.2, was mixed with carrier picrate (60 mg), made alkaline with dil NH_4OH and extracted with $CHCl_3$ (6×10 ml). The residue remaining after removal of the solvent was redissolved in EtOH (2 ml) and diluted to 20 ml with 5% $Ba(OH)_2$ soln, sealed in an ampoule and heated in steam for 3 hr. The cooled and acidified (50% H_2SO_4) hydrolysate was extracted with Et_2O (6×2 ml) which gave on evapn and recrystallization from C_6H_6–petrol, tropic acid (15.2 mg), mp 118° (lit. 116.5–118° [3]). The tropic acid was diluted with carrier (210 mg) and recrystallized from C_6H_6 again giving 215 mg 3H:^{14}C 10.0.

Tropic acid (170 mg) was refluxed with 10 N KOH (4 ml) under N_2 for 40 min. The cooled, acidified (dil HCl) soln pptd atropic acid 109 mg (72%) mp 106° (lit. [3] 105–107.5°) ^{3}H:^{14}C 9.4.

Atropic acid (80 mg) was dissolved in Na_2CO_3 (25 mg) in H_2O (5 ml), cooled to 0° and a few crystals of OsO_4 (*ca* 5 mg) were added. The soln acquired a purple colour and $NaIO_4$ (250 mg) in H_2O (10 ml) was added dropwise over 45 min. After 20 hr at 4° the mixture was washed with Et_2O to remove residual OsO_4, acidified with HCl (3 N) and the phenylglyoxylic acid extracted with Et_2O. $NaHCO_3$ (0.5 g) in H_2O (5 ml) was added to the Et_2O soln cooled to 0°, followed by hydroxylamine HCl (70 mg) [29]. The Et_2O was evapd off, more $NaHCO_3$ (1 g) was added and the reaction mixture was left at room temp. for 48 hr. The oxime was extracted from the acidified (dil H_2SO_4) mixture with Et_2O. The residue obtained after evapn of the Et_2O was sublimed under red. pres. (0.01 mm Hg) giving glassy plates (20 mg), mp 129° (lit. [29] 130–131°) which were free of any ^{3}H activity. In contrast to the previous report cited [3], the *syn* isomer of phenylglyoxylic acid (**14**) was isolated here and this is in keeping with the original observations of Ahmed and Spenser [30].

The remaining aqueous phase from which the phenylglyoxylic acid had been extracted with Et_2O was distilled and the distillate (5 ml) mixed with a soln of dimedone (156 mg) in H_2O which had been stored at 4° for 24 hr. After standing at 4° for 24 hr the crystalline formaldehyde dimedone derivative (**15**) was collected (100 mg), mp 190° (lit. [3] 192–193°) which was free of any ^{14}C activity.

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