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BIOSYNTHESIS OF PIPERLONGUMINE

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Key Word Index—*Piper longum*; Piperaceae; alkamides; piperlongumine; biosynthesis.

Abstract—The incorporation of L-[U-^{14}C]lysine and L-[U-^{14}C]phenylalanine into piperlongumine has been demonstrated in *Piper longum*. The subsequent stepwise degradation to methyl-(3,4,5-trimethoxyphenyl)-propanoate and δ-aminovaleric acid revealed that the C_6–C_3 moiety of the alkamide arises from phenylalanine; the heterocyclic ring is biosynthesised from lysine. It has also been shown that DL-[2-^{14}C]tyrosine and [2-^{14}C]sodium acetate are poor precursors of piperlongumine.

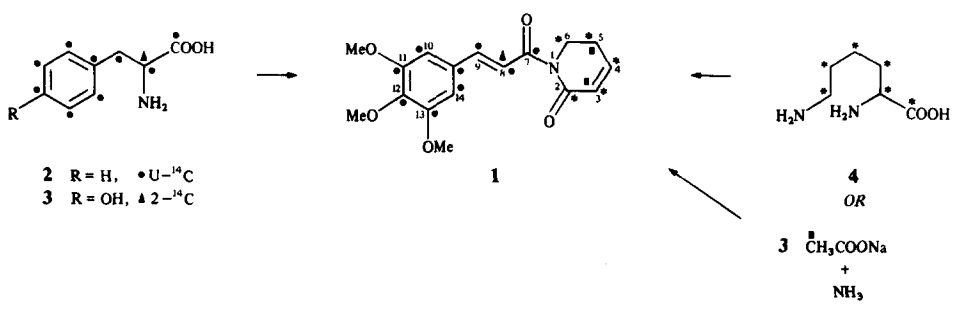
INTRODUCTION

The alkamides commonly encountered in the genus *Piper* are probably condensation products of aromatic or long chain aliphatic acids with pyrrolidine, piperidine or *iso*butylamine moieties. Piperlongumine (**1**), the major constituent of *Piper longum*, differs from the typical piperidine derived alkamides in that it has an α-carbonyl group and a 3,4-double bond in the piperidine ring [1]. This structure has been confirmed by its total synthesis [2].

The piperlongumine molecule (Scheme 1) can be looked upon as a condensation product of a cinnamic acid part arising possibly from phenylpropanoids like phenylalanine (**2**) or tyrosine (**3**) and a C_5N unit which could arise from either three molecules of acetate and ammonia or from one molecule of lysine (**4**). The piperidine ring of coniine type alkaloids has been shown to arise from acetate units [3]. On the other hand, lysine has been shown to be the precursor of the same heterocyclic moiety in sedamine and related alkaloids [4]. Therefore, biosynthesis of piperlongumine which contains α-carbonyl and a 3,4-double bond, assumes special significance. Accordingly, if L-[U-^{14}C]phenylalanine is the precursor, then the activity in piperlongumine would be expected to reside in the 3,4,5-trimethoxycinnamic acid moiety. If DL-[2-^{14}C]tyrosine is the precursor for this part of the molecule, then C-8 would be radiolabelled. Since the interconversion of phenylalanine into tyrosine is a relatively rare feature in dicotyledons, the incorporation of both these precursors into piperlongumine would not be expected. It was therefore thought worthwhile to study the biosynthesis of piperlongumine.

RESULTS AND DISCUSSION

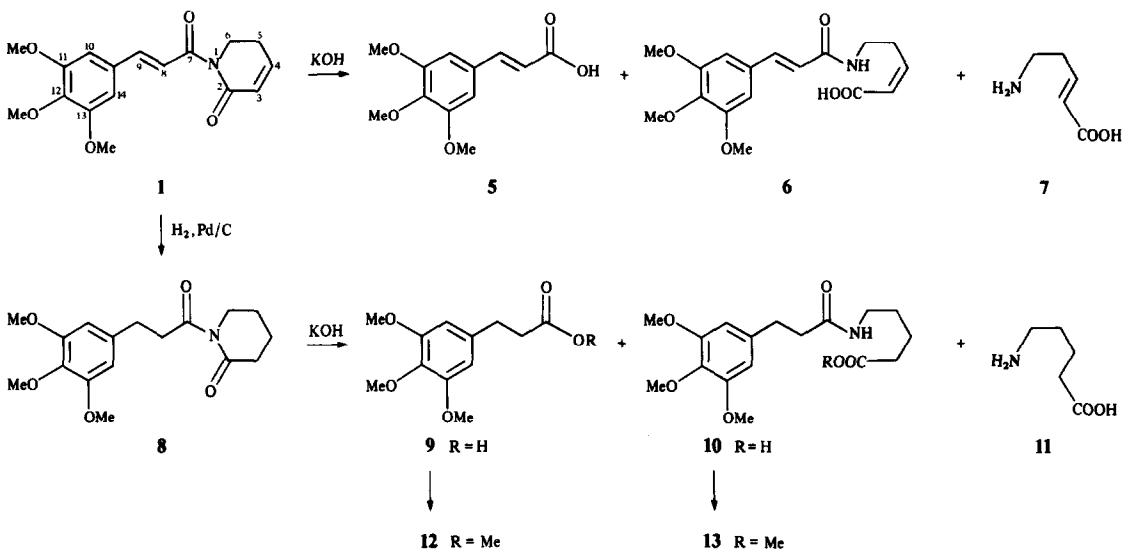
The present communication deals with the experiments in which L-[U-^{14}C]phenylalanine, L-[U-^{14}C]lysine, [2-^{14}C]sodium acetate and DL-[2-^{14}C]tyrosine were administered separately to four sets of one year old flowering *Piper longum* plants by the wick method (Table 1). The plants were harvested after 14 days and piperlongumine was isolated in each case. It was found that L-[U-^{14}C]lysine and L-[U-^{14}C]phenylalanine were incorporated efficiently into the alkamide (0.061% and 0.029%, respectively), whereas acetate and tyrosine showed negligible incorporation (< 0.001%) (Table 1). To locate the position of radioactivity in the labelled piperlongumine, degradative studies were carried out. As reported by Chatterjee and Dutta [5], alkaline hydrolysis of piperlongumine (**1**) yielded only two acids, viz. 3,4,5-trimethoxycinnamic acid (**5**) and piperlongumic acid (**6**) (Scheme 2). Our inability to isolate the third expected degradation



Scheme 1. Possible biosynthetic pathways for the formation of piperlongumine (**1**).

Table 1. Incorporation of precursors into piperlongumine

Precursors (sp. act.)	Activity administered (mCi)	Carrier (mg)	No. of plants (fr wt, kg)	Yield (mg)	Sp. act. (dpm/mM)	% Incorporation
L-[U-^{14}C]Lysine (230 mCi/mM)	0.30	8.8	3 (1.34)	340	3.77×10^5	0.061
L-[U-^{14}C]Phenylalanine (360 mCi/mM)	0.04	15.0	1 (0.30)	73	1.12×10^5	0.029
[2-^{14}C]Sodium acetate (47.8 mCi/mM)	1.00	10.0	1 (0.28)	80	3.00×10^4	<0.001
DL-[2-^{14}C]Tyrosine (50 mCi/mM)	0.10	10.0	1 (0.27)	65	1.20×10^4	<0.001



Scheme 2. Degradation of piperlongumine (1).

product viz. $\Delta^{\alpha,\beta}$-δ-aminovaleric acid (7) rendered this method unsuitable for precise degradative studies using radioactive compounds. We, therefore, followed an alternative procedure in which piperlongumine was first hydrogenated using Pd–C catalyst to give tetrahydropiperlongumine (8). This compound, on alkaline hydrolysis, yielded three products, viz. 3-(3,4,5-trimethoxyphenyl)propanoic acid (9), tetrahydropiperlongumic acid (10) and δ-aminovaleric acid (11) (Scheme 2). For better separation, the acids 9 and 10 were converted to their respective methyl esters 12 and 13, respectively. However, the formation of (9) and α-piperidone have been reported as the only degradation products of the alkaline hydrolysis of 8 [5].

Following the above degradative procedure, [^{14}C]piperlongumine obtained from L-[U-^{14}C]lysine was degraded to give the three compounds. Of these, the ester 13 and the amino acid 11 were found to contain the expected amount of radioactivity, while the ester 12 was found to be devoid of any activity. Likewise, [^{14}C]piperlongumine obtained from the L-[U-^{14}C]phenylalanine experiment was degraded. In this case, the aromatic esters 12 and 13 contained radioactivity, while the amino acid 11 was found to be inactive. These results, summarized in Table 2, indicate that the C_6–C_3 moiety of piperlongumine arises from phenylalanine and the piperidone ring is formed from lysine.

EXPERIMENTAL

Mps are uncorr. ^{1}H NMR (60 MHz): $CDCl_3$. MS (70 eV) direct insertion. UV: MeOH. IR: KBr or as film. CC separations were carried out on silica gel and Dowex 50W-X4 (H^+ form). TLC was performed on silica gel G using $CHCl_3$–MeOH. Spots were detected in UV (254 nm) using I_2 vapour and by heating to 100° after spraying with 10% H_2SO_4. PC (descending) was carried out on Whatman No. 1 paper; amino acid spots were detected by spraying with ninhydrin: 2,2-dihydroxy-1H-indene-1,3(2H)-dione (0.2% EtOH soln) and heating to 100°. One and a half year old *P. longum* plants cultivated at the Experimental Field Station, Bhabha Atomic Research Centre were used for the biosynthetic experiments.

[2-^{14}C]Sodium acetate (1 mCi, 1.72 mg, 47.8 mCi/mM), L-[U-^{14}C]lysine (0.3 mCi, 0.19 mg, 230 mCi/mM) and L-[U-^{14}C]phenylalanine (0.04 mCi, 0.02 mg, 360 mCi/mM) were obtained from the Isotope Division, Bhabha Atomic Research Centre, Trombay. DL-[2-^{14}C]Tyrosine (0.1 mCi, 0.36 mg, 50 mCi/mM) was obtained from the Radiochemical Centre, Amersham.

Radioactive samples were counted in a scintillation cocktail prepared by dissolving BBOT: 2,5-*bis*[2-(5-tertbutylbenzoxazolyl)]thiophene (4 g), in toluene (A.R. 1 l.). In each case 10 ml of the scintillation soln was used. Aq. radioactive samples were counted using Bray's soln viz. dimethyl POPOP: 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)]benzene (0.2 g), PPO: 2,5-diphenyl-

Table 2. Activities of [^{14}C]piperlongumine obtained from L-[U-^{14}C]lysine and L-[U-^{14}C]phenylalanine and its degradation products

Compounds	L-[U-^{14}C]Lysine (0.3 mCi, 0.19 mg, 230 mCi/mM)		L-[U-^{14}C]Phenylalanine (0.04 mCi, 0.02 mg, 360 mCi/mM)	
	Activity (10^3 dpm/mg)	(10^5 dpm/mM)	Activity (10^2 dpm/mg)	(10^5 dpm/mM)
Piperlongumine (**1**)	1.19	3.77	3.55	1.12
Tetrahydropiperlongumine (**8**)	1.15	3.69	3.49	1.12
3-(3,4,5-Trimethoxyphenyl) propanoic acid Me ester (**12**)	—	—	4.00	1.00
Tetrahydropiperlongumic acid Me ester (**13**)	1.03	3.60	2.71	0.95
δ-Aminovaleric acid (**11**)	3.03	3.55	—	—

oxazole (5 g) and naphthalene (80 g) in dioxane (A.R. 1 l.).

Isolation of piperlongumine. Fresh plants were homogenized in a Waring Blender with EtOH (*ca* 2 ml/g). The macerate was extracted with the same solvent (4 × 2 l.) in a percolator. The dark brown semisolid mass (*ca* 3%) obtained, was subjected to CC over silica gel. Elution was carried out with C_6H_6 and subsequently with more polar solvents using EtOAc and MeOH. The C_6H_6–EtOAc (4:1) fractions on concn gave a greenish semisolid mass. This, on trituration with Et_2O, deposited a colourless solid. Purification by repeated crystallization from C_6H_6–petrol gave colourless needles (*ca* 0.025%). The identity of this compound was established as piperlongumine by comparing its spectral data with that reported in the lit. [1].

Degradation of [^{14}C]*piperlongumine* (**1**) *obtained from* L-[U-^{14}C]*lysine. Hydrogenation of* [^{14}C]*piperlongumine.* The compound (51 mg) in EtOH (20 ml) was hydrogenated using Pd–C (10%, 15 mg) in a Parr apparatus (40 psi) for 4 hr. After usual work-up, crude tetrahydropiperlongumine (**8**, 48 mg) was obtained. It was repeatedly crystallized (dry Et_2O, 43 mg, mp 83–84°; lit. [1]) to constant activity (3.69×10^5 dpm/mM). *Alkaline hydrolysis of* [^{14}C]*tetrahydropiperlongumine* (**8**). The compound (43 mg, 3.69×10^5 dpm/mM) was dissolved in EtOH (10 ml) and hydrolysed by alkali (20% aq. KOH, 5 ml) at 100° for 4 hr under N_2 atm. The reaction mixture was then dil with H_2O (10 ml) and the EtOH removed *in vacuo*. The aq. phase was extracted with $CHCl_3$ (25 ml) and acidified with dil. HCl. The acids thus ppted were extracted with Et_2O (4 × 20 ml). The combined organic extracts were washed with brine and then dried (Na_2SO_4). Removal of Et_2O yielded a mixture (29 mg) of acids **9** and **10**. This mixture was dissolved in MeOH (10 ml) and esterified by excess CH_2N_2–Et_2O. Removal of solvents *in vacuo* yielded a mixture of esters (37 mg) which were separated by prep. TLC ($CHCl_3$–MeOH, 99:1) to give 3-(3,4,5-trimethoxyphenyl)propanoic acid Me ester **12** (22.64 mg, R_f 0.8) and tetrahydropiperlongumic acid Me ester **13** (3.7 mg, R_f 0.5, 3.60×10^5 dpm/mM). The former ester was found to be identical (TLC, co-TLC and superimposable IR) with an authentic sample prepared from 3,4,5-trimethoxycinnamic acid. Ester **13** had UV λ_{max}^{MeOH} nm (log ε): 265 (3.52). IR ν_{max}^{film} cm^{-1}: 3400 (NH), 1739 (C=O, aliphatic COOMe), 1653 (C=O, secondary amide), 1600, 1471 (C=C aromatic), 1250 and 1015 (OMe). ^{1}H NMR ($CDCl_3$): δ1.5 (4H, *m*, C-2′ and C-3′ methylene protons), 2.4 (4H, *m*, $-CH_2-COOMe$, $-CH_2-CONH$), 2.88 (2H, *m*, Ar–CH_2–), 3.23 (2H, *m*, $-CONH-CH_2$), 3.66 (3H, *s*, COOMe), 3.85 (9H, *s*, Ar–OMe), 5.92 (1H, *br*, NH, exchangeable), 6.46 (2H, *s*, Ar–H). MS *m/z* (%): 353 $[M]^+$ (30), 322 (5), 223 (19), 222 (12), 195 (45), 181 (100).

The aq. phase was evapd to dryness *in vacuo*. The residue was then refluxed in EtOH (3 × 25 ml) for 1 hr. The combined EtOH extracts were evapd to dryness to yield a residue (13 mg). This on passing through a cation exchange resin (Dowex 50W-X4, H^+ form, 50–100 mesh, 5 g, elution with 2 N NH_3 soln) yielded pure δ-aminovaleric acid (**11**) (mp 156°, 11.2 mg, 3.55×10^5 dpm/mM; PC, *n*-BuOH–HOAc–H_2O, 50:8:25, R_f 0.5). It was identical (mp, PC) with an authentic sample prepared from cyclopentanone oxime via α-piperidone, following the procedure reported for ε-aminocaproic acid [6, 7]. [^{14}C]Piperlongumine isolated from the L-[U-^{14}C]phenylalanine expt was degraded in a similar manner. Results are presented in Table 2.

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