Biosynthesis of Phenylpropanoid Compounds. Part II.[1] Incorporation of Specifically Labelled Cinnamic Acids into Eugenol

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Experiments on the incorporation in *Ocimum basilicum* L. of specifically labelled cinnamic acids have shown that in the biosynthesis of eugenol (i) cinnamic acid is a necessary intermediate, (ii) the carboxylic carbon atom of cinnamic acid is lost and an extra one-carbon unit is introduced into the allyl group, and (iii) both the olefinic hydrogen atoms of the cinnamic acid side-chain remain in their original position. These results can be explained on the basis of a new biogenetic hypothesis.

THE finding that L-phenylalanine is incorporated into allylphenols with loss of the carboxy-group and its replacement by a new C_1 unit [1] (see Scheme 1) prompted us to investigate more deeply this unusual biosynthetic pathway.

Preliminary tracer experiments were performed by administering various labelled cinnamic acids to *Ocimum basilicum* 'Genovese'.† The results (Table), together with those previously reported,[1] confirmed the role of cinnamic acid as a necessary intermediate in the biosynthesis of eugenol (2). The loss of the carboxylic carbon atom (experiment 5) is in agreement with the assumption of a pathway in which the first step is the conversion of L-phenylalanine into cinnamic acid ‡

† This is an Italian ecotype especially rich in eugenol when harvested in September.[1]

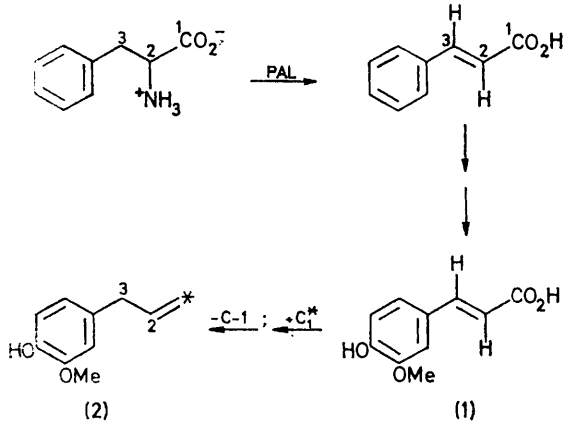
‡ This reaction is mediated by a well known [2] and widely distributed [3] enzyme, L-phenylalanine ammonia-lyase (PAL).

[1] Part I, P. Manitto, D. Monti, and P. Gramatica, *J.C.S. Perkin I*, 1974, 1727.

[2] R. H. Wightman, J. Stauton, A. R. Battersby, and K. R. Hanson, *J.C.S. Perkin I*, 1972, 2355.

[3] K. R. Hanson and E. A. Havir, 'Recent Advances in Phytochemistry, 4,' Appleton-Century-Crofts, New York, 1972, p. 45.

and the last is the transformation of the acrylic side-chain of a ring-substituted cinnamic acid into the allyl group. Ferulic acid (1) seems the most likely substrate for the last transformation (Scheme 1): this is supported,



(2) (1)

SCHEME 1 Suggested [1] pathway to eugenol in *O. basilicum*

inter alia, by appreciable incorporation (*ca.* 0.06%) of non-specifically tritiated ferulic acid into eugenol.

TABLE

Incorporation of various labelled (*E*)-cinnamic acids into eugenol

Expt.[a]	Labelling pattern	Total ^{14}C activity [b] (μCi)	Specific ^{14}C activity (mCi mmol^{-1})	^{3}H : ^{14}C Ratios [c] Precursor	Eugenol
1	[2-^{14}C; 2-^{3}H]	62.5	0.72	13.21 : 1	13.97 : 1
2	[3-^{14}C; 3-^{3}H]	80.0	2.16	2.39 : 1	2.04 : 1
3	[2-^{14}C; 3-^{3}H]	50.0	1.45	5.60 : 1	5.65 : 1
4	[3-^{14}C; 2-^{3}H]	20.0	1.05	18.13 : 1	17.28 : 1
5	[1-^{14}C; 2-^{3}H]	20.0	0.64	19.49 : 1	138.00 : 1

[a] Harvesting after 1 h was adopted in all experiments. [b] The labelled precursor was divided among a number of vigorous shoots of *O. basilicum*, each shoot receiving *ca.* 5 μCi of ^{14}C. [c] % Incorporations of ^{14}C were between 5 × 10^{-2} and 5 × 10^{-3} in all cases, except for experiment 5 (practically nil).

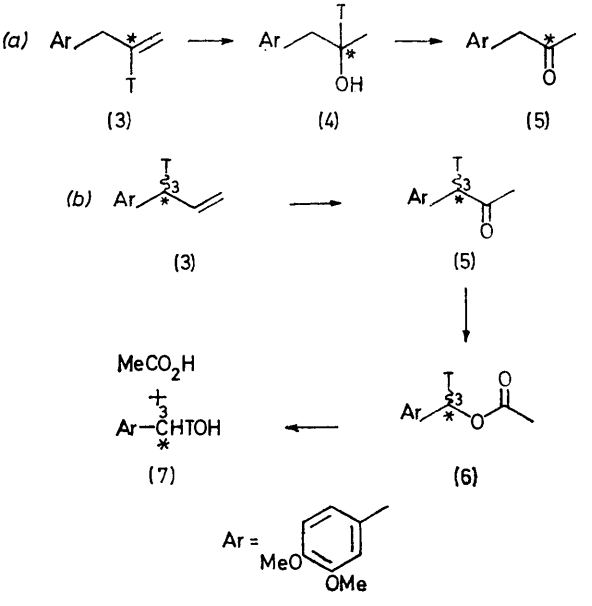
The data from experiments 1—4 indicate that eugenol from [2-^{3}H]- as well as from [3-^{3}H]-cinnamic acid retains all the original tritium, thus raising the question of the positions of both olefinic hydrogen atoms of cinnamic acid in the side-chain of eugenol; furthermore, knowledge of these positions could give valuable information about the mechanism by which the allyl group is formed *in vivo*.

The routes of Scheme 2 were developed as unambiguous methods for localizing the tritium label in methyleugenol (3), which is easily obtainable by methylation of eugenol with dimethyl sulphate. These routes were found satisfactory when applied to the three deuteriated estragoles (8), each specifically labelled in one position of the allyl group.[4]

The radioactive methyleugenol, obtained from incorporation experiment 1, was taken through sequence (*a*): the alcohol (4) showed a ^{3}H : ^{14}C ratio (13.55 : 1) almost identical with that of the starting eugenol (13.97 : 1), whereas the ketone (5) retained its ^{14}C but carried no tritium (the specific ^{14}C activity was constant in all the compounds of the sequence).

Degradation of methyleugenol arising from experiment 2 by sequence (*b*) gave 3,4-dimethoxybenzyl alcohol (7) whose ^{3}H : ^{14}C ratio (2.25 : 1 when measured on the corresponding α-naphthylurethane derivative) indicated the presence of tritium at position 3 of eugenol [the ^{3}H : ^{14}C ratios of the intermediates (5) and (6) were 2.11 : 1 and 2.19 : 1, respectively].

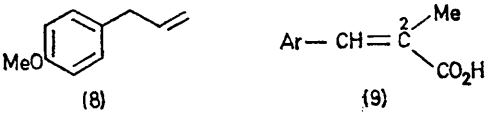
The above results demonstrate that both olefinic hydrogen atoms of the cinnamic acid side-chain remain in their original position in eugenol. Such a conclusion allows α-methylcinnamic acids (9) to be ruled out as intermediates in the biosynthesis of allylphenols.



(3) (4) (5) (3) (5) (7) (6)

SCHEME 2 Degradation of methyleugenol

The previous suggestion [1] that allylphenols arise from addition of a C_1 unit to styrenes [Scheme 3; route (*a*)] appears consistent with our results, provided that enzymic stereocontrol with respect to the hydrogen atoms



(8) (9)

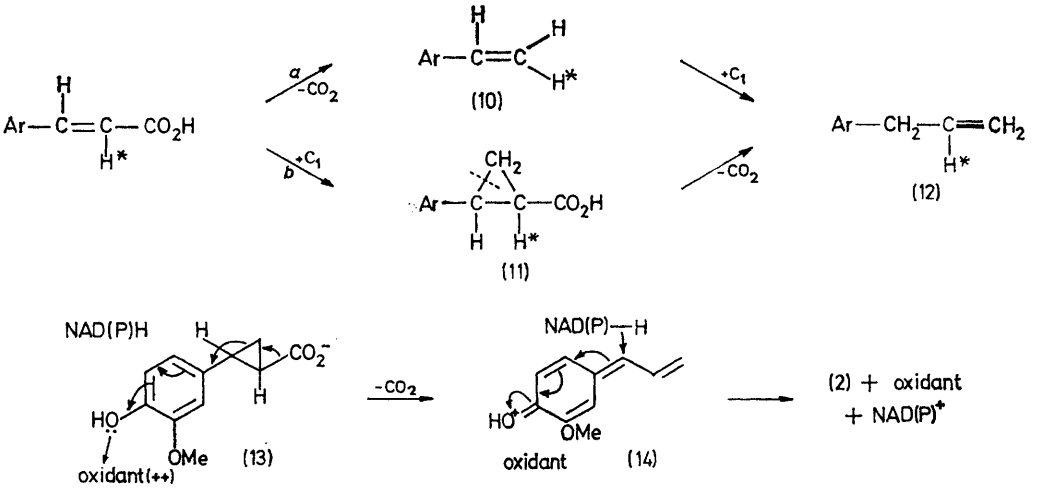
at C-2 is assumed in the decarboxylation step,* as well as in the C_1 addition reaction. However, we suggest here, as an alternative, the pathway (*b*) of Scheme 3: this requires no steric control and involves cyclopropanecarboxylic acids (11) formed by the attack of a C_1 unit donor (*S*-adenosylmethionine or an equivalent) on the double bond of the cinnamic acid side-chain (*in vivo* conversion of a double bond into a cyclopropane ring is a

* Microbiological decarboxylation of cinnamic acids has been shown to be stereospecific: *e.g.* (*E*)-[2-^{2}H]dimethoxycinnamic acid is converted into (*Z*)-[α-^{2}H]dimethoxystyrene by *Saccharomyces cerevisiae* (P. Manito, P. Gramatica, and B. M. Ranzi, *J.C.S. Chem. Comm.*, 1975, 442).

[4] D. Monti, P. Gramatica, and P. Manitto, in the press.

well documented process [5]). Cyclopropane ring cleavage and simultaneous decarboxylation would be induced by phenolic oxidation (13) ⟶ (14): the quinone methide (14) so formed might then undergo a regiospecific reduction by attack of hydride ion [*e.g.* from NAD(P)H] at C-3. There is a mechanistic analogy between this reduction and that proposed by Birch [6] for the conversion of cinnamyl alcohols into the corresponding allyl and propenyl derivatives. However, Birch's hypothesis has been recently rejected [1] with regard to the sequence cinnamic acid ⟶ cinnamyl alcohol ⟶ allyl derivative. The quinone methide (14) has been reported to be a long-lived transient in the enzymic dehydrogenation (with peroxidase) of eugenol; [7] it is also formed from coniferyl alcohol by flash photolysis [8] as well as by treatment with hydrogen bromide–sodium hydrogen carbonate.[9] Ring cleavage with decarboxylation by C-3 protonation of (11) cannot be ruled out, but seems mechanistically unlikely.

In accord with the oxidation–reduction process of Scheme 3, all the naturally occurring phenylpropanoid compounds carry an allyl group only when there is an oxygen atom *para* to the side chain.[10]



SCHEME 3 Suggested mechanisms for the one-carbon displacement involved in the biosynthesis of allylphenols

EXPERIMENTAL

General directions are given in Part I.[1]

Labelled phenylalanines were obtained from The Radiochemical Centre, Amersham; [3-^{14}C] cinnamic acid was purchased from Sorin (Saluggia, Italy).

Preparation of Labelled Precursors.—(*E*)-[2-^{3}H]*Cinnamic acid.*[11] *NN*-Dimethylphenylalanine [12] (100 mg) was treated with methyl iodide (0.8 mmol) in alkaline tritiated water (1 ml; activity 5 Ci; 30% sodium hydroxide) and kept at room temperature for 24 h and at 100 °C for 2 h. After acidification with concentrated hydrochloric acid (pH 1) the resultant solution was extracted with ether (3 × 5 ml). Evaporation of extracts afforded a residue (75 mg), which, when recrystallised twice from aqueous ethanol, gave cinnamic acid (m.p. and mixed m.p. 133—134°; constant specific activity 7.5 mCi mmol^{-1}).

(*E*)-[3-^{3}H]*Cinnamic acid.* This was prepared by procedure (A) of ref. 11. A solution of L-[2,3-$^{3}H_2$]phenylalanine (2 mCi; specific activity 1 Ci mmol^{-1}) and unlabelled phenylalanine (10 mg) in 0.02N-hydrochloric acid (4 ml) was heated with an excess of methyl iodide and dry silver(I) oxide (0.5 g) under reflux for 2 h; this mixture was filtered, acidified, with concentrated hydrochloric acid, filtered again, made alkaline with solid sodium hydroxide (30%), and kept at room temperature for 24 h and at 100 °C for 2 h. Ethereal extracts (3 × 10 ml) of the reaction mixture, made acidic with concentrated hydrochloric acid, afforded cinnamic acid (5 mg), which, after recrystallisation from aqueous ethanol, showed constant specific activity 6.2 mCi mmol^{-1}.

To check for labelling specificity, part of the cinnamic acid so prepared was mixed with [3-^{14}C]cinnamic acid and the resultant mixture (^{3}H : ^{14}C 2.39 : 1), after dilution with inactive material (1 g) and esterification with diazomethane, was degraded as follows. Methyl cinnamate (*ca.* 1 g) was reduced [13] to cinnamyl alcohol (0.72 g) which was then ozonized to give 3,4-dimethoxybenzyl alcohol, after reductive cleavage of the ozonide with sodium borohydride.[14] The alcohol so obtained (0.45 g) was converted into its α-naphthylurethane derivative (see later under 3,4-dimethoxybenzyl alcohol), which showed, after recrystallisation from light petroleum, constant label ratio (^{3}H : ^{14}C 2.21 : 1).

[1-^{14}C]- *and* [2-^{14}C]-*Cinnamic acids.* The synthesis was carried out exactly as described for [3-^{3}H]cinnamic acid, starting from [1-^{14}C]- and [2-^{14}C]-phenylalanine respectively, each diluted with the radio-inactive amino-acid (5 mg).

Doubly labelled specimens for incorporation experiments

[5] E. Lederer, *Quart. Rev.*, 1969, **23**, 453; T. Cohen, G. Herman, T. M. Chapman, and D. Kuhn, *J. Amer. Chem. Soc.*, 1974, **96**, 5627.

[6] A. J. Birch, in 'Chemical Plant Taxonomy,' ed. T. Swain, Academic Press, London, 1963, p. 141.

[7] J. C. Pew, W. J. Connors, and A. Kunishi, in 'Chimie et Biochimie de la Lignine, de la Cellulose, et des Hémicelluloses. Actes du Symposium International de Grenoble, Juillet, 1964,' Imprimeries Réunies, Chambéry, 1965, pp. 229—245.

[8] G. Leary, *Chem. Comm.*, 1971, 688.

[9] E. Adler and B. Stenemur, *Chem. Ber.*, 1956, **89**, 291.

[10] A. J. Birch and M. Slaytor, *Chem. and Ind.*, 1956, 1524.

[11] P. Manitto, D. Monti, P. Gramatica, and E. Sabbioni, *J.C.S. Chem. Comm.*, 1973, 563.

[12] R. E. Bowman and H. H. Stroud, *J. Chem. Soc.*, 1950, 1342.

[13] M. J. Jorgenson, *Tetrahedron Letters*, 1962, 559.

[14] J. A. Sousa and A. L. Bluhm, *J. Org. Chem.*, 1960, **25**, 108.

were obtained by mixing the appropriate specifically labelled cinnamic acids.

[*G*-^{3}H]*Ferulic acid*. Non-specifically tritiated L-phenylalanine (1 mCi) was administered to *O. basilicum* 'Genovese'.[1] After 0.5 h, the plant material was finely ground, transferred to a Soxhlet thimble and extracted with ether for 48 h. The ether was evaporated off and the residue separated by t.l.c. (Merck silica gel G; 1 mm; AcOEt–HCO_2H, 6 : 4). Ferulic acid thus isolated was crystallised from water, after addition of unlabelled material (30 mg), to constant specific activity (0.05 mCi mmol^{-1}).

Administration of Labelled Precursors to O. basilicum *'Genovese' and Isolation of Eugenol*.—Cinnamic acids were dissolved in N-sodium hydroxide (0.5—0.7 ml) and mixed with 0.05M-phosphate buffer (5 ml; pH 6.9). Administration of these solutions to shoots of *O. basilicum* (5—10 for each experiment) and isolation of radioactive eugenol were carried out as described in Part I.[1] In all cases radio-inactive eugenol (160 mg) was added as carrier to the essential oil before the chromatographic separation. Eugenol was checked for its chemical and radiochemical purity by t.l.c. and analytical g.l.c., and by partial conversion into its crystalline α-naphthylurethane derivative.[1]

Methylation of Eugenol.—See ref. 1.

1-(3,4-*Dimethoxyphenyl*)*propan-2-ol* (4).—Methyleugenol (133 mg) dissolved in tetrahydrofuran (3 ml) was added to a solution of mercury(II) acetate (400 mg) in tetrahydrofuran–water (1 : 2; 4.5 ml) and the mixture was stirred at room temperature for 24 h.[15] After addition of 3M-sodium hydroxide (4 ml) followed by 0.5M-sodium borohydride in 3M-sodium hydroxide (4 ml), mercury was allowed to settle and filtered off; solid sodium chloride was then added to saturate the water layer. The upper layer and ethereal extracts (3 × 10 ml) of the lower one were pooled, dried (Na_2SO_4), and evaporated. The residue (123 mg), homogeneous by g.l.c., was crystallised from ether–light petroleum (b.p. 40—60°) and shown to be 1-(3,4-dimethoxyphenyl)propan-2-ol (4), m.p. 44—45° (lit.,[16a] 43—45°; lit.,[16b] 46—47.5°) (Found: C, 67.7; H, 8.4. Calc. for $C_{11}H_{16}O_3$: C, 67.4; H, 8.2%); *m/e* (g.l.c.–mass spectrum) 196(M^+, 32%), 151(100), 137(37), 121(11), 107(11), and 91(7); $\nu_{max.}$ 3 400 cm^{-1}; τ ($CDCl_3$) 3.2 (3H, complex, aromatic), 6.0 (1H, complex, CH·OH), 6.22 (6H, s, OMe), 7.3 (2H, d, *J* 6.8 Hz, CH_2Ar), 8.22br (1H, s, exchanged with D_2O, OH), and 8.75 (3H, d, *J* 7.5 Hz, CH_3·C).

Radioactive 1-(3,4-dimethoxyphenyl)propan-2-ol (70 mg) was obtained similarly (from eugenol of experiment 1); one-fifth of this product was diluted with unlabelled compound (100 mg) and crystallised to constant label ratio.

3,4-*Dimethoxyphenylacetone* (5).—*Route* (a). 1-(3,4-dimethoxyphenyl)propan-2-ol (30 mg), prepared as just described, was dissolved in acetone (1 ml; refluxed over $KMnO_4$ and dried over Na_2SO_4) and oxidised with an excess of Jones reagent[17] at 0 °C for 5 min. After addition of isopropyl alcohol (0.5 ml) followed by saturated aqueous sodium hydrogen carbonate (2 ml), the mixture was extracted with chloroform (5 × 10 ml). The residue (25 mg) left by evaporation of the solvent was 3,4-dimethoxyphenylacetone,[1] homogeneous by g.l.c.

Route (b). 3,4-Dimethoxyphenylacetone (95 mg) was obtained from methyleugenol (138 mg) by one-step conversion [methoxymercuriation and *in situ* oxidation with palladium(II) chloride–copper(II) chloride] as described in Part I.[1]

Specific activity and ^{3}H : ^{14}C ratio of labelled 3,4-dimethoxyphenylacetone (from experiments 1 and 2 of the Table) were confirmed by counting the *thiosemicarbazone* prepared as follows. A solution of the ketone (38 mg) and thiosemicarbazide (18 mg) was stirred in 50% aqueous ethanol (1 ml) for 3 h at room temperature. After addition of water (2 ml), the mixture was extracted with ethyl acetate (3 × 5 ml). Evaporation of the extract gave a residue which was crystallised from ether (yield 42 mg); m.p. 118—120° (Found for radio-inactive material: C, 53.2; H, 6.55; N, 15.95. $C_{12}H_{17}N_3O_2S$ requires C, 53.9; H, 6.4; N, 15.7%).

3,4-*Dimethoxybenzyl Acetate* (6).—A solution of *m*-chloroperbenzoic acid (200 mg) in chloroform (10 ml) was added to a solution of 3,4-dimethoxyphenylacetone (90 mg) in chloroform (5 ml) and the mixture was stirred at 40 °C for 20 h. After washing with aqueous sodium thiosulphate (10 ml), saturated aqueous sodium hydrogen carbonate, and finally water, the chloroform solution was dried (Na_2SO_4) and evaporated under vacuum. The residue (75 mg) was shown to be 3,4-dimethoxybenzyl acetate on the basis of its spectroscopic properties [τ ($CDCl_3$) 3.13 (3H, complex, aromatic), 4.99 (2H, s, CH_2), 6.16 (6H, s, OMe), and 7.97 (3H, s, CH_3); $\nu_{max.}$ 1 740 cm^{-1}] and by comparison (g.l.c.) with an authentic sample.

Labelled 3,4-dimethoxybenzyl acetate was checked by g.l.c. for its chemical purity before conversion into 3,4-dimethoxybenzyl alcohol as described below.

3,4-*Dimethoxybenzyl Alcohol* (7).—3,4-Dimethoxybenzyl acetate (75 mg) was treated with N-potassium hydroxide (1 ml) and methanol (7 ml) and kept at room temperature overnight. After addition of water (4 ml) and evaporation of the methanol under vacuum, the resultant solution was extracted with ether (3 × 10 ml) and the extract was dried and evaporated to give 3,4-dimethoxybenzyl alcohol (60 mg), homogeneous by g.l.c.; $\nu_{max.}$ 3 500 cm^{-1}; τ ($CDCl_3$) 3.23 (3H, complex, aromatic), 5.50 (2H, s, CH_2), 6.22 (6H, s, OMe), and 6.90 (1H, s, exchanged with D_2O, OH).

Radioactive 3,4-dimethoxybenzyl acetate was treated similarly; in this case the resultant alcohol was converted into its α-naphthylurethane to obtain an accurate value for the ^{3}H : ^{14}C ratio. For the formation of the α-naphthylurethane, 3,4-dimethoxybenzyl alcohol (20 mg) in light petroleum (b.p. 100—120°) (1 ml) was treated at room temperature with α-naphthyl isocyanate (0.25 ml). The flask was stoppered and kept at 20 °C for 10 h, then at 0 °C for 24 h, and finally the suspension was heated at 90 °C. Filtration removed di-α-naphthylurea; the α-*naphthylurethane* crystallised from the filtrate; yield 29 mg; m.p. 146—148° (Found for radio-inactive material: C, 71.3; H, 5.8; N, 4.4. $C_{20}H_{19}NO_4$ requires C, 71.2; H, 5.7; N, 4.15%).

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[15] H. C. Brown and P. Geoghegan, jun., *J. Org. Chem.*, 1970, **35**, 1844.

[16] (*a*) G. R. Clemo and J. H. Turnbull, *J. Chem. Soc.*, 1947, 124. (*b*) S. Winstein and R. Heck, *J. Amer. Chem. Soc.*, 1956, **78**, 4801.

[17] L. F. Fieser and M. Fieser, 'Reagents for Organic Synthesis,' Wiley, New York, 1967, p. 142.