Biosynthesis of Caffeic Acid in *Ocimum Basilicum* L.

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Summary During the transformation of L-phenylalanine into caffeic acid, a precursor of eugenol in *O. basilicum*, a partial C_1 exchange of the carboxy carbon with that of a C_1 donor occurs.

THE biosynthesis of allylphenols in plants is still a matter of controversy, in that results from precursor incorporation experiments have been interpreted in a contradictory manner. We first reported that the synthesis of eugenol (**8**) and methyleugenol (**9**) in *Ocimum basilicum* strain 'Genovese' [and of chavicol (**10**) and estragole (**11**) in *O. basilicum* strain 'Napoletano'] proceeds from phenylalanine[1,2] *via* (*E*)-cinnamic acid (**2**)[3] and involves an exchange of the γ-carbon, in the basic C_6–C_α–C_β–C_γ skeleton, with that from a donor molecule (*e.g.* methionine).[1] It was suggested that the carbon atom exchange could result from the decarboxylation of an oxygenated cinnamic acid (**3**)—(**5**) with simultaneous or later incorporation of an 'extra' C_1 -unit to rebuild the phenylpropanoid skeleton.[3] Our observations were claimed to be incorrect by Klischies *et al.*[4] since they observed no change of the ^3H: ^{14}C ratio from precursor to eugenol when [$^{14}CO_2H$; $OCH_2{}^3H$]glucoferulic acid, [$^{14}CH_2OH$; $OCH_2{}^3H$]-, and [$^{14}C^3H_2OH$]-glucoconiferyl alcohol were administered to *O. basilicum* 'Genovese'. More recently, however, Senanayake *et al.*[5] found that the methyl of methionine is incorporated specifically into the terminal position of the allyl side chain of eugenol in *Cinnamomum zeylanicum*, a result which agrees with our previous findings. We report here some results which might reconcile all experimental observations so far reported on allylphenols biosynthesis.

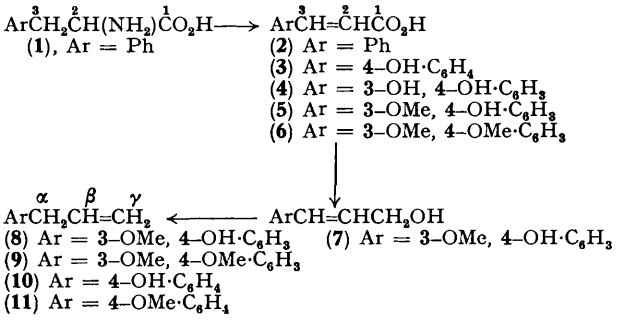
TABLE 1. Incorporation of labelled L-phenylalanines into caffeic acid in *O. basilicum* Genovese[a]

Expt.	Labelling pattern	Total ^{14}C μCi activity for each cutting	Duration of expt./h	Precursor $^{3}H:^{14}C$ ratio	Caffeic acid[b] $^{3}H:^{14}C$ ratio
1	[1-^{14}C; G-^{3}H][c]	1·8	0·5	22·5:1	20·7:1
2	,, ,,	1·8	2	22·5:1	22·9:1
3	[1-^{14}C; G-^{3}H][d]	12·5	1	7·74:1	7·3:1
4	[1-^{14}C; G-^{3}H][e]	2·5	0·5	10·4:1	10·7:1
5	,, ,,	2·5	2	10·4:1	10·9:1
6	[3-^{14}C; G-^{3}H][e]	5	0·5	7·0:1	3·6:1
7	,, ,,	5	2	7·0:1	3·1:1

[a] The precursors were fed through the cut ends of 10 plants (15—25 cm high) for each expt. All expts. were carried out in the sunlight. [b] Isolated by the following general procedure: ethanol extraction of the homogenised plant material after addition of 1% sodium dithionite; evaporation of the solvent at 40 °C and hydrolysis of the residue with dil. H_2SO_4 (5%, 100 ml); column chromatography (XAD2, 100 g) of the hydrolysed product, after addition of inactive carrier (10 mg), collecting fractions eluted with $CHCl_3$–AcOEt (3:1); preparative t.l.c. (silica gel, $CHCl_3$–AcOEt–HCO_2H 5:4:1) followed by repeated crystallisations (from H_2O) of the product to constant specific activity and $^{3}H:^{14}C$ ratio. Incorporations were estimated in the range 0·02—0·08%. [c] April, 1 month after germination. [d] July, 3 months after germination. [e] June, 2 months after germination. All expts. were carried out simultaneously, in the same environment conditions and with plants of the same batch; the same specimen of non-specifically tritiated phenylalanine was used.

Investigating the origin of oxygenated cinnamic acids in *O. basilicum*, we observed that the incorporation of L-[1-^{14}C; G-^{3}H]phenylalanine in caffeic acid (**4**) occurred with no appreciable decrease of the $^{3}H:^{14}C$ ratio (Table 1). By contrast, the conversion of L-[3-^{14}C; G-^{3}H]phenylalanine was found to bring about a marked decrease of the $^{3}H:^{14}C$ ratio (expts. 6, 7 of Table 1), as expected considering both the distribution of tritium in the starting amino-acid (26% of the label in the side chain, mostly at the 3 positions, and 74% in the ring, fairly equally distributed)[6] and the reactions involved in the path to caffeic acid.[7] The apparent tritium retention in expts. 1—5 could be explained assuming an exchange of the carboxy carbon with a C_1-donor during the conversion of phenylalanine into caffeic acid. Such an exchange should affect only a part of the caffeic acid present in the plant, the small variations in the $^{3}H:^{14}C$ ratio being a consequence of the concomitant tritium loss. It is well established that caffeic acid is biosynthesized through different routes[8] and occurs in different forms.[7]

When L-[Me-^{14}C]methionine and sodium [^{14}C]formate were tested as C_1-donors, ^{14}C-labelled caffeic acid was isolated in both cases. It was then converted into 3,4-dimethoxycinnamic acid (**6**) and decarboxylated by a strain of *Saccharomyces cerevisiae*.[9] The 3,4-dimethoxystyrene obtained from both the experiments retained (on a molar basis) only *ca.* 25% of the ^{14}C-activity present in the 3,4-dimethoxycinnamic acid (Table 2), thus demonstrating a strong predominance of ^{14}C incorporation at C(1) of caffeic acid.† These data appear consistent with those of Table 1 and reinforce the assumption that the incorporation of an 'extra' carbon in the terminal position of phenylpropanoid metabolites, in place of the original carboxyl carbon atom, takes place in *O. basilicum* as a side process with respect to the main route from phenylalanine to caffeic acid [(**1**)→(**2**)→(**3**)→(**4**)].



SCHEME

Presumably, *p*-coumarate (**3**) and/or caffeate (**4**), but not ferulate (**5**), are involved in the carboxy-carbon exchange.[1-3] This would explain why C-γ retention in

TABLE 2. Radioactivity of caffeic acid, labelled by methionine and formate, during degradation to 3,4-dimethoxystyrene

Precursor[a]	^{14}C μCi activity for each cutting	Duration of expt./h	Caffeic acid[b] % incorporation	3,4-dimethoxycinnamic acid (**6**)[c] specific activity dpm μmol^{-1}	3,4-dimethoxystyrene[d] specific activity dpm μmol^{-1}
L-[Me-^{14}C]-Methionine	10	2	0·03	167·5	40·6[e]
Sodium [^{14}C]-formate	12·5	3	0·018	231·1	70·7[e]

[a] Precursor, mixed with 0·7 mg of L-phenylalanine, was fed as described in note a of Table 1. [b] Isolated as in note b of Table 1 except for using a charcoal column (20 g; AcOEt–MeOH 9:1) instead of preparative t.l.c. [c] Caffeic acid was refluxed for 1 h in dry acetone with Me_2SO_4 and K_2CO_3; after column (SiO_2) purification, methyl 3,4-dimethoxycinnamate was saponified with KOH in Bu^{t}OH. [d] *Cf.* ref. 9; *ca.* 60% yield. [e] Average of 2 independent decarboxylation experiments.

† Similar activity distribution in the C_6–C_3 units of lignins has been reported using [3-^{14}C]pyruvate (S. N. Acerbo, W. J. Schubert, and F. F. Nord, *J. Amer. Chem. Soc.*, **1960**, **82**, 735) and [U-^{14}C]pyruvate (C. J. Coscia, M. I. Ramirez, W. J. Schubert, and F. F. Nord, *Biochemistry*, **1962**, **1**, 447) as precursors.

eugenol (**8**) was found in feeding labelled ferulic acid (**5**) and coniferyl alcohol (**7**),[4] *i.e.* intermediates following caffeate (Scheme). The incorporation differences between [1-^{14}C]- and [2-^{14}C]- or [3-^{14}C]phenylalanines in eugenol and related compounds[1-3,5] are higher than those in caffeic acid isolated from our experiments. This fact could be accounted for by the occurrence of the C(1) exchange in subcellular compartments or tissues wherein cinnamic acids are transformed into allylphenols.

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[1] L. Canonica, P. Manitto, D. Monti, and M. Sanchez A., *J.C.S. Chem. Comm.*, 1971, 1108.
[2] P. Manitto, D. Monti, and P. Gramatica, *J.C.S. Perkin I*, 1974, 1727.
[3] P. Manitto, P. Gramatica, and D. Monti, *J.C.S. Perkin I*, 1975, 1548.
[4] M. Klischies, J. Stöckigt, and M. H. Zenk, *J.C.S. Chem. Comm.*, 1975, 879.
[5] U. M. Senanayake, R. B. H. Wills, and T. H. Lee, *Phytochemistry*, 1977, **16**, 2032.
[6] M. C. Clifford, E. A. Evans, A. E. Kilner, and D. C. Warrell, *J. Labelled Compounds*, 1975, **11**, 435; J. M. A. Al-Rawi, J. A. Elvidge, J. R. Jones, V. M. A. Chambers, and E. A. Evans, *ibid.*, 1976, **12**, 265.
[7] E. Haslam, 'The Shikimate Pathway,' Butterworths, London, 1974, pp. 186—240.
[8] M. J. C. Rhodes and L. S. C. Wooltorton, *Phytochemistry*, 1976, **15**, 947; B. Ulbrich and M. H. Zenk, *ibid.*, 1979, **18**, 929.
[9] P. Manitto, P. Gramatica, and B. M. Ranzi, *J.C.S. Chem. Comm.*, 1975, 442.