Biosynthesis of Phenylpropanoid Compounds. Part I.[1] Biosynthesis of Eugenol in *Ocimum basilicum* L.

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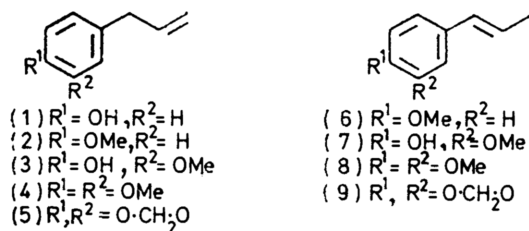
L-Phenylalanine and cinnamic acid, but not L-tyrosine, have been found to be precursors of eugenol (4-allyl-2-methoxyphenol) in *Ocimum basilicum*. Separate feeding experiments with [1-^{14}C,*G*-^{3}H]-, [2-^{14}C,*G*-^{3}H]-, and [3-^{14}C,*G*-^{3}H]-phenylalanine have shown that the carboxylic carbon atom of the amino-acid is lost during the biosynthesis of eugenol (probably at the ferulic acid level), and an extra one-carbon unit is introduced into the allyl group. [2-^{14}C]- and [3-^{14}C]Phenylalanine have been shown to be incorporated specifically into eugenol by unambiguous degradations of the isolated allylphenol; these results indicate that the foregoing decarboxylation and C_1 introduction involve no rearrangement of the side chain of phenylalanine (and, by implication, of cinnamic acid precursors). Evidence is given that the other allylphenols of *O. basilicum*, *i.e.* estragole and chavicol, are biosynthesized in the same manner as eugenol.

ALLYLPHENOLS [*e.g.* (1)—(5)] and propenylphenols [*e.g.* (6)—(9)] occur widely in higher plants [2-5] and are commonly found in the higher boiling (aromatic) fractions of essential oils.[6] The allyl compounds appear to be predominant over the propenyl derivatives in nature. In certain cases the allyl and propenyl isomers occur together in the same plant, *e.g.* safrole (5) and isosafrole (9) in *Cananga odorata* (Anonaceae), *etc.*[7]



The mode of biosynthesis of these substances is as yet obscure.[5,7,8] Their origin through the shikimic–prephenic–phenylalanine (or tyrosine)–cinnamic (or *p*-coumaric) pathway can be assumed, but the processes whereby the final structure of the side chain is reached require experimental investigations. However, an attractive biosynthetic hypothesis for both groups of phenylpropanoids has been suggested by Birch.[9] This hypothesis, formulated as in Scheme 1 (or in terms of the corresponding S_N2 mechanism), explained the above predominance by postulating preference of path (*b*) over path (*a*).[5] The only relevant precursor-incorporation data reported are concerned with the biosynthesis of anethole (6) in *Foeniculum vulgare*.[10] Although in agreement with Birch's hypothesis, this evidence does not appear conclusive. We are at present studying the biosynthesis of both allyl- and propenyl-phenols more deeply. The present paper deals with four allylphenols occurring in *Ocimum basilicum* L. (Labiatae), *viz.* eugenol (3), methyleugenol (4), estragole (2), and chavicol (1).

Since *O. basilicum* L. was reported [11,12] to contain various amounts of estragole (2) and eugenol (3) according to the ecotype, the steam-volatile oils from the available Italian forms of this plant were screened by g.l.c. for

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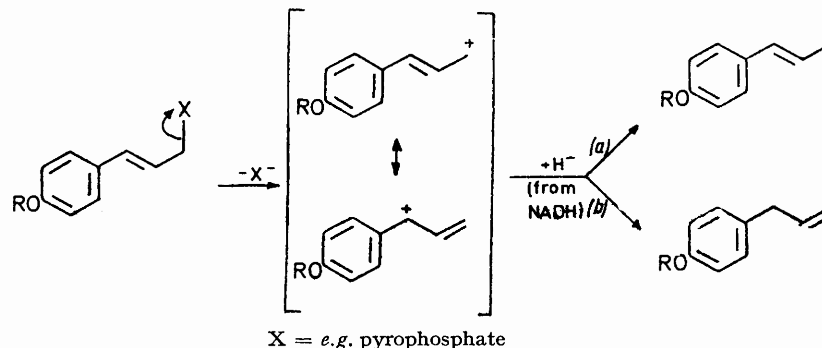
[9] A. J. Birch and M. Slaytor, *Chem. and Ind.*, 1956, 1524.
[10] K. Kaneko, *Chem. and Pharm. Bull.* (*Japan*), 1960, **8**, 611, 875; 1961, **9**, 108.
[11] Ref. 2, 1966, vol. IV, p. 314.
[12] H. J. Nicholas, *J. Biol. Chem.*, 1962, **237**, 1485.

allylphenols (Table 1). On the basis of the results we chose for our investigation *O. basilicum* ' Genovese ', because of its high content of eugenol (and methyleugenol) as well as its great genetic purity. Seasonal differences in the ratio of eugenol to methyleugenol in this plant appeared unimportant with regard to the projected precursor-incorporation experiments. Chemical degradations could be carried out conveniently after conversion of the mixture of the two allylphenols into methyleugenol (4).

Preliminary tracer experiments were performed by administering various possible precursors of eugenol, *i.e.* L-[*U*-^{14}C]phenylalanine, L-[*U*-^{14}C]tyrosine, and [3-^{14}C]-cinnamic acid to vigorous shoots of *O. basilicum* ' Genovese ' (corresponding to specimen 2 of Table 1). Batches of shoots were harvested at various times and steam distilled. The volatile oil, after an exploratory counting, was worked up for eugenol with addition of radio-inactive carrier. Eugenol obtained by column chromatography was checked for its chemical and radiochemical purity by t.l.c. and analytical g.l.c., and by conversion into its crystalline α-naphthylurethane. In all cases, the specific radioactivity of the eugenol was completely retained in its derivative, even after recrystallization. The results (Table 2) showed three noteworthy aspects: (i) isolation of eugenol from the crude oil caused a sharp fall in radioactivity; (ii) L-phenylalanine was satisfactorily incorporated only in short feeding times; and (iii) L-tyrosine did not appear a good precursor of eugenol. The first finding indicated that one might look for possible biosynthetic precursors of eugenol. The other two observations strongly support the L-phenylalanine ⟶ cinnamic ⟶ (*p*-coumaric ⟶ ferulic) ⟶ eugenol pathway without participation of L-tyrosine. This is in agreement with the lack of L-tyrosine ammonia-lyase in dicotyledonous plants.[13]



X = *e.g.* pyrophosphate

SCHEME 1 Proposed [5,7] pathways to allyl- and propenyl-phenols

TABLE 1

Examination of various forms of *O. basilicum* for allylphenols by g.l.c.

Specimen	Form	Eugenol (3)	Methyleugenol (4)	Estragole (2)	Chavicol (1)
1	' Genovese ' [a]	Minor	Major	N.D. [b]	N.D.
2	' Genovese ' [c]	Very large (0·1—0·2%) [d]	Trace	N.D.	N.D.
3	' Napoletano ' [c]	N.D.	N.D.	Large (0·07—0·1%) [d]	Trace

[a] Harvested in June. [b] N.D. = not detected. [c] Harvested in September. [d] Percentages refer to the weight of the fresh plant.

TABLE 2

Tracer experiments on *O. basilicum* ' Genovese '

Expt.	Precursor	Total activity (μCi)	Specific activity (mCi mmol^{-1})	Duration of of expt. (h)	% Incorp. into crude oil	% Incorp. into pure eugenol [a]
1	L-[*U*-^{14}C]Phenylalanine	20	400	0·25	1·01	0·20
2		20	400	0·5	1·56	0·41
3		20	400	1	1·06	0·12
4		20	400	3	0·44	0·054
5		20	400	24	0·11	0·034
6		20	400	96	0·12	0·041
7	L-[*U*-^{14}C]Tyrosine	20	400	0·5	$<10^{-3}$	
8		20	400	1	$<10^{-3}$	
9		20	400	24	$<10^{-3}$	
10	[3-^{14}C]Cinnamic acid	20	5	0·5		0·04
11		20	5	1		0·11

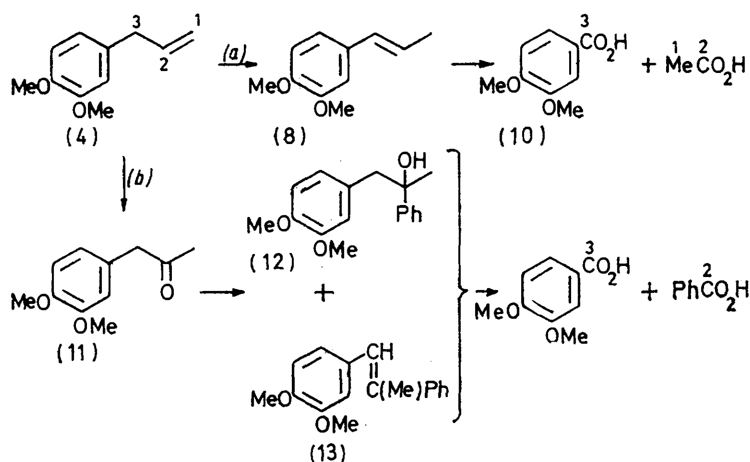
[a] Calculated on the samples arising from column chromatography.

[13] M. R. Young, G. H. N. Towers, and A. C. Neish, *Canad. J. Botany*, 1966, **44**, 341.

Moreover, the fact that maximum incorporation of L-phenylalanine was obtained after 0·5 h feeding indicates a rapid turnover for eugenol, which might be used by the plant for lignin [14] and lignan and neolignan [8] biosynthesis. Therefore harvesting after 0·5 h was adopted in further studies of incorporation of labelled phenylalanines.

To elucidate the mode of transformation of the L-phenylalanine (or cinnamic acid) side chain into the allyl group, DL-phenylalanines each carrying a ^{14}C-label at one of the three positions in the side chain were mixed with non-specifically tritiated L-phenylalanine; these precursors were administered to *O. basilicum* ' Genovese ' shoots and eugenol was isolated in the usual manner. The results are shown in Table 3. A decrease in the ^{3}H : ^{14}C ratio in the conversion of phenylalanines into

In order to isolate separately from the side chain of eugenol (3) the carbon atoms corresponding to C-2 and C-3 of L-phenylalanine, it became necessary to develop unambiguous methods for degrading the allyl group. The routes (*a*) and (*b*) of Scheme 2 were found satisfactory. Both start from methyleugenol (4), easily obtainable by methylation of eugenol with dimethyl sulphate. The radioactive eugenol obtained by feeding L-[3-^{14}C]phenylalanine to *O. basilicum* was taken through sequence (*a*); the veratric acid so obtained carried (on a molar basis) 93% of the original activity. Route (*b*) was followed to degrade eugenol arising from incorporation of L-[2-^{14}C]phenylalanine: the specific activity of the final benzoic acid corresponded to 85% of that of the eugenol (as its α-naphthylurethane derivative). In the latter case, the amount of benzoic acid obtained did not



SCHEME 2 Degradation of methyleugenol

eugenol in experiments 1 and 2 is understandable if we assume retention of C-3 and C-2 with the expected loss of tritium from the non-specifically tritiated amino-acid.† However, the very large rise in ^{3}H : ^{14}C ratio observed in experiments 3 and 4 led us to conclude that, contrary to expectation, unequivocal loss of C-1 occurs during the *in vivo* conversion.

TABLE 3

Incorporation of various labelled DL-phenylalanines into eugenol

Expt.	Labelling pattern [b]	^{3}H : ^{14}C Ratios [c] Precursor	Eugenol
1	[3-^{14}C,^{3}H]	10·3 : 1	7·90 : 1
2	[2-^{14}C,^{3}H]	10·4 : 1	7·71 : 1
3	[1-^{14}C,^{3}H]	9·05 : 1	200 : 1
4	[1-^{14}C,^{3}H]	19·4 : 1	252 : 1

[a] Ratios for eugenol were determined from the corresponding α-naphthylurethane. [b] Non-specifically tritiated L-phenylalanine was used as internal reference. [c] 45 min feeding period.

† If only the L-isomer of phenylalanine is utilized for eugenol biosynthesis (as seems likely in view of the stereospecificity of phenylalanine ammonia-lyase [15]), the actual loss of tritium is much more marked than appears from the ratios of Table 3 (cf. note *b*).

allow the counting of many replicate samples. However, an accuracy of ±10% seems probable for the activity measurements on the basis of our experience in counting ' low-level ' samples.

The foregoing results indicate that the biotransformation of the side chain of L-phenylalanine into the allyl group of eugenol involves the loss of the carboxylic carbon atom (probably at the ferulic acid level) and the introduction of an extra C_1 unit, without skeletal rearrangement. That such a biosynthetic mechanism is probably common to all natural allylphenols is supported by incorporation experiments with *O. basilicum* ' Napoletano ' (specimen 3 of Table 1). When we fed a mixture of DL-[1-^{14}C]phenylalanine and non-specifically tritiated L-phenylalanine (^{3}H : ^{14}C 9·05 : 1) to shoots of this plant and isolated the pure allylphenols by preparative t.l.c., the estragole (2) and chavicol (1) obtained showed ^{3}H : ^{14}C ratios of 213 : 1 and 194 : 1, respectively.

The exact mechanism of the one-carbon displacement remains to be clarified. Perhaps styrenes could be

[14] K. Freudenberg and A. C. Neish, ' Constitution and Biosynthesis of Lignin,' Springer Verlag, Berlin–Heidelberg–New York, 1968, p. 32.
[15] J. Koukol and E. E. Conn, *J. Biol. Chem.*, 1961, **236**, 2692.

intermediates. Such compounds appear to be uncommon in plants (styrene was found in balsamic species of *Liquidambar*, *Myroxylon*, and *Styrax*,[3,16,17] 4-hydroxystyrene in poppy straw,[18] and a glucoside of 4-hydroxystyrene in *Viburnum furculatum* leaves[19]). However, the extreme susceptibility of these materials to both polymerization and oxidation would explain their rareness. On the other hand, decarboxylation of hydroxy- and methoxy-cinnamic acids has been reported to occur in bacteria[20] and fungi,[21] and probably in many micro-organisms decomposing ligneous materials of forest.[20]

On the basis of our results, pathway (*b*) of Scheme 1 must be rejected (at least in the case of *O. basilicum*); however, pathway (*a*) for propenylphenols cannot be ruled out without direct experimental evidence.

EXPERIMENTAL

T.l.c. was carried out on plates coated with Merck silica gel GF_{254} (G; 0·5 mm thickness for preparative separations), with $CHCl_3$ as solvent, unless otherwise stated. Merck silica gel (70—325 mesh) was used for column chromatography. Analytical g.l.c. was carried out on a Carlo Erba Fractovap model 2400 V instrument with nitrogen as carrier gas and glass columns containing neopentyl glycol succinate (LAC 767) (3% w/w) on silanized Chromosorb W (80—100 mesh).

Radioactive samples were counted on a Packard Tricarb liquid scintillation spectrometer (model 3320) (internal standard [^{3}H]- and/or [^{14}C]-toluene). I.r. spectra were recorded for solutions in chloroform on a Perkin-Elmer 257 spectrometer, and n.m.r. spectra for solutions in deuteriochloroform on a 60 MHz Varian NV14 spectrometer (tetramethylsilane as standard). Mass spectra were determined with an LKB 9000 single-focusing gas chromatograph–mass spectrometer fitted with a 3 m $\times$ 4 mm column packed as for analytical g.l.c. M.p.s were determined with a silicon-bath apparatus.

Labelled phenylalanines were obtained from Amersham; [3-^{14}C]cinnamic acid was purchased from Sorin (Saluggia, Italy). Commercial samples of eugenol, methyleugenol, and estragole were employed; a sample of chavicol was kindly supplied by Firmenich (Geneva). The various forms of *O. basilicum* were obtained from Ingegnoli (Milan).

Screening of Plants for Allylphenols.—The plant material (20—30 g fresh wt.) was ground and steam distilled. Extraction of the distillate (100 ml) with ether (4 $\times$ 30 ml) and evaporation of the extract gave an oil which was analysed by g.l.c.–mass spectroscopy (Table 1).

Administration of Labelled Precursors and Isolation of Allylphenols.—The labelled phenylalanine (or L-tyrosine) was dissolved in distilled water (5—10 ml) and the clear solution was divided equally among 5—10 glass test tubes (1·2 $\times$ 10 cm). Cinnamic acid was dissolved in N-sodium hydroxide (0·5 ml) and mixed with 0·05M-phosphate buffer (5 ml; pH 6·9). Vigorous shoots (*ca.* 7—15 in long) were cut in September from specimens of *O. basilicum* and the cut ends were immediately immersed in the solutions in the feeding tubes. When the shoots had absorbed almost all the solution, the remainder was continuously 'washed in' with distilled water until the shoots were harvested 30 min later (unless otherwise stated). The feeding tubes finally contained 0·5—1% of the original activity. Batches of shoots treated equally were worked up as described in the previous paragraph.

The essential oil from *O. basilicum* 'Genovese' was chromatographed on a silica gel column after addition of radioinactive eugenol (30 mg in exploratory experiments and 160 mg in experiments for degradative work). The first fractions eluted with light petroleum (b.p. 40—70°)–benzene (2 : 3) afforded eugenol, which was shown to be pure by g.l.c. Small amounts of methyleugenol were obtained occasionally in later fractions eluted with the same solvent mixture. For formation of the naphthylurethane, eugenol (20 mg) in light petroleum (b.p. 100—120°) was treated at room temperature with α-naphthyl isocyanate (0·15 g). The flask was stoppered and kept at 20° for 10 h, then at 0° for 24 h, and finally the suspension was heated to 90°. Filtration removed di-α-naphthylurea and the α-naphthylurethane crystallized from the filtrate; it was recrystallized from light petroleum to constant specific activity; m.p. 121—122° (lit.,[22] 122°) (Found for radioinactive material: C, 75·15; H, 5·65; N, 4·25. Calc. for $C_{21}H_{19}NO_3$: C, 75·65; H, 5·75; N, 4·2%).

Estragole and chavicol were isolated from the essential oil of *O. basilicum* 'Napoletano' by preparative t.l.c. Final purification of both compounds was performed by t.l.c.

Methylation of Eugenol.—The mixture of radioactive eugenol and methyleugenol obtained from column chromatography (177 mg) was dissolved in dioxan (10 ml) containing sodium hydroxide (0·5 ml; 10%) and treated with dimethyl sulphate in dioxan (2·5 ml; 10%). After 5 h under reflux the mixture was poured in water (40 ml). Extraction with ether (4 $\times$ 20 ml) and evaporation of the extract under vacuum gave methyleugenol (4) (131 mg) of satisfactory purity (t.l.c.; g.l.c.) for the next degradative steps.

Isomerization of Methyleugenol.—Methyleugenol (131 mg) dissolved in pentyl alcohol (20 ml) containing potassium hydroxide (1·2 g) was refluxed under nitrogen until isomerization appeared (g.l.c.) to be complete (*ca.* 8 h). Water (20 ml) was added and the mixture neutralized with N-hydrochloric acid. The aqueous layer was extracted with chloroform (3 $\times$ 25 ml) and the extract combined with the pentyl alcohol solution. This solution was dried and freed from organic solvents by distillation, and the residue was chromatographed on a silica gel column. The fractions eluted with light petroleum (b.p. 40—70°)–benzene (2 : 3) were examined by g.l.c. and those containing *cis*- and *trans*-methylisoeugenol[23] as the only detected compounds were collected (yield 68 mg).

Ozonolysis of cis- *and* trans-*Methylisoeugenol.*—The mixture of *cis*- and *trans*-methylisoeugenol (68 mg) dissolved in chloroform (5 ml) was ozonized at −45° for 15 min. Ozone was removed in a stream of nitrogen and the solution was evaporated under vacuum. The residue was

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[22] H. E. French and A. F. Wirtel, *J. Amer. Chem. Soc.*, 1926, **48**, 1736.
[23] A. T. Shulgin, *J. Chromatog.*, 1967, **30**, 54.

treated with aqueous sodium hydroxide (2 ml; 5%) and hydrogen peroxide (2 ml; 30%) at 100° for 40 min. The solution was acidified with a few drops of conc. sulphuric acid and kept overnight at 4°. Veratric acid was isolated by filtration, dried, and sublimed (15 mg); one crystallization from benzene was sufficient to reach a constant specific activity of 34×10^3 disint. min^{-1} $mmol^{-1}$ (the specific activity of the α-naphthylurethane of the original eugenol was $36{\cdot}7 \times 10^3$). Acetic acid was recovered by distillation of the filtrate. Its sodium salt, obtained by accurate neutralization of the aqueous distillate (30 ml) with sodium hydroxide (0·1N; 3·1 ml) and evaporation was virtually radioinactive (600 counts min^{-1} $mmol^{-1}$).

3,4-*Dimethoxyphenylacetone* (11).[24]—Mercury(II) acetate (8·96 g) and methyleugenol (5 g) were stirred at 25° for 20 min in methanol (180 ml) and then added to a stirred solution of lithium chloride (238 mg), palladium chloride (493 mg), and copper(II) chloride (14·4 g) in methanol (250 ml). The mixture was heated under reflux for 1 h, then poured into saturated sodium hydrogen carbonate solution (500 ml); the solution was filtered and heated under reduced pressure to remove methanol. The residual solution was extracted with ether (6 × 100 ml) and the extract was dried and evaporated. Distillation of the oil so obtained (5·2 g) under vacuum afforded 3,4-dimethoxyphenylacetone (3·6 g), b.p. 120—123° at 0·6 mmHg (lit.,[25] 118° at 0·2 mmHg); homogeneous by g.l.c.; m/e 194 (M^+), 151, 107, 91, 78, and 43; $\nu_{max.}$ 1720 cm^{-1}; τ 7·82 (3H, s), 6·35 (2H, s), 6·1 (6H, s), and 3·21 (3H, centre of m, aromatic).

Radioactive methyleugenol (152 mg) was treated similarly; in this case, the crude ketone (154 mg) was purified by column chromatography (silica gel) with benzene as eluant, monitored by g.l.c. Evaporation under vacuum afforded 3,4-dimethoxyphenylacetone (108 mg), homogeneous by g.l.c. and t.l.c.

Cleavage of the Ketone (11).—Radioinactive 3,4-dimethoxyphenylacetone (2·5 g) in ether (50 ml) was added during 15 min to a solution of phenylmagnesium bromide [from magnesium (0·9 g) and bromobenzene (6 g)] in ether (60 ml). The mixture was refluxed under argon for 3 h, cooled to room temperature, and treated with ammonium chloride (4 g in 30 ml) in portions. The organic layer was separated and the aqueous solution extracted twice with ether (15 ml). The ethereal solution was washed with saturated sodium hydrogen carbonate solution (50 ml), dried ($MgSO_4$), and evaporated to leave an oil (2·9 g), which was divided into two parts.

A solution of potassium permanganate (4 g) and potassium carbonate (1·2 g) in water (200 ml) was added to one portion (0·9 g) of the oil and the mixture was heated under reflux for 3 h. The brown precipitate was removed and the filtrate was acidified with conc. sulphuric acid and extracted with chloroform (3 × 50 ml). The crude mixture of benzoic and veratric acids, obtained by evaporation of the extract, was chromatographed on a column of deactivated silica gel (refluxed in methanol for 3 h and dried at 130° for 48 h); fractions were monitored by t.l.c.[26] Elution with benzene–chloroform (4 : 1) gave benzoic acid, which was recrystallized from hot water (180 mg); elution with benzene–chloroform (3 : 2) then afforded veratric acid (250 mg, after sublimation).

The second portion (2 g) of the oil was chromatographed on a silica gel column. Elution with light petroleum (b.p. 40—70°)–benzene (1 : 4) gave an oily mixture (200 mg) of *trans*- and *cis*-3,4-dimethoxy-β-methylstilbene (13); τ (*trans*-isomer) 7·74 (3H, d, J 1·5 Hz), 6·16 (6H, s), 3·15 (1H, m), and 2·8 (8H, centre of m, aromatic); τ (*cis*-isomer) 8·07 (3H, d, J 1·5 Hz), 6·16 (6H, s), 3·28 (1H, m), and 2·7 (8H, centre of m, aromatic); this mixture, when oxidized as already described, afforded benzoic and veratric acids. Elution with benzene–chloroform (2 : 3) gave 1-(3,4-dimethoxyphenyl)-2-phenylpropan-2-ol (12) (970 mg), τ 8·43 (3H, s), 8 (1H, m), 7 (2H, m), 6·18 (6H, s), and 3·5—2·5 (8H, 2 m, aromatic). Its structure was confirmed by oxidation to benzoic and veratric acids.

In the ^{14}C-series the reaction was carried out by adding the ketone (108 mg) in ether (5 ml) to phenylmagnesium bromide [from magnesium (40 mg) and bromobenzene (0·27 g)] in ether (10 ml). The crude product was directly oxidized with potassium permanganate (500 mg) and potassium carbonate (200 mg) in water (20 ml). Benzoic acid (22 mg) and veratric acid (30 mg) so obtained were separated by column chromatography; after two crystallizations the former had reached a constant activity of $6{\cdot}4 \times 10^3$ disint. min^{-1} $mmol^{-1}$ (the activity of the α-naphthylurethane from the original eugenol was $7{\cdot}6 \times 10^3$). The sublimed veratric acid showed a specific activity of 270 counts min^{-1} $mmol^{-1}$.

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