Metabolism of 2',4,4'-Trihydroxychalcone in *Amorpha fruticosa* Seedlings; Structure and Role of Chalaurenol, a Novel Heterocyclic Enol Ether formed by Enzymic Oxidation

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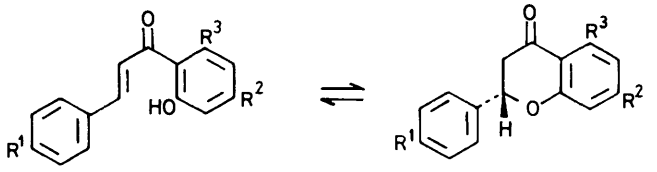
Amorpha fruticosa seedlings contain two enzymes capable of catalysing reactions of 2',4,4'-trihydroxychalcone. One is a chalcone-flavanone isomerase, which has been purified and occurs as two isozymes. More interestingly, the second is a peroxidase, which oxidises the chalcone (**1**; $R^1 = R^2 = OH$, $R^3 = H$) to a novel labile quinol enol ether, chalaurenol (**6**), whose structure is established by chemical studies and *X*-ray diffraction of the 6-*O*-methyl ether (**5**). The possible origins and role of chalaurenol in plant metabolism are discussed.

2'-Hydroxychalcones (**1**) hold a key position in the biogenesis of *O*-heterocycles. They enter into enzyme-catalysed equilibrium with the corresponding 2*S*-flavanones (**2**), with position of equilibrium determined by the hydroxylation pattern. Since modification processes commonly occur at a later stage the most important chalcones are 2',4,4'-trihydroxychalcone (**1**; ($R^1 = R^2 = OH$, $R^3 = H$) (isoliquiritigenin) and 2',4,4',6'-tetrahydroxychalcone (**1**; $R^1 = R^2 = R^3 = OH$), formed from *p*-coumaroyl CoA, malonyl CoA, and the appropriate synthase.[1] The chalcone-flavanone pair form a major point



(**1**) (**2**)

of biosynthetic diversification, to flavonoids, aurones, isoflavonoids, *etc.*[2] The *in vivo* mechanisms of a good deal of this plant biochemistry lying beyond chalcone synthase remains unclear, although there are ample hypotheses based on *in vitro* reactions. It can be difficult to determine whether chalcone or flavanone is the true substrate for a given reaction, as exemplified by investigations of isoflavone biosynthesis, where a labile isoflavone synthase has only recently been characterised and shown to act on a flavanone substrate.[3]

We have been interested for some time in the biosynthesis of rotenoids in *Amorpha fruticosa* seedlings and have extensively detailed a chalcone→isoflavanone→rotenoid pathway.[4] It now seemed important to an understanding of the overall metabolic situation with respect to *O*-heterocyclic synthesis, to investigate the chalcone-converting enzymes present in *A. fruticosa* seedlings. At the outset we expected to find a chalcone-flavanone isomerase and this was indeed detected, partly purified and characterised. However, in addition, a cell-free preparation was isolated capable of oxidising 2',4,4'-trihydroxychalcone to an apparently unique product, chalaurenol. In the event, this substance proved to have been reported in the literature from other sources, but to have been assigned an incorrect structure. We now present our work on the isolation and constitution of this compound, and discuss its origins and significance.

To obtain cell-free preparations, sterilised *A. fruticosa* seeds were germinated and seedlings of the appropriate age were homogenised with buffer and 2-mercaptoethanol. The homogenate was treated with Polyclar AT and centrifuged. The resulting supernatant was active in metabolising 2',4,4'-trihydroxychalcone as shown by the decrease in light absorption at 410 nm, relative to control experiments. After some initial experimentation it was found that two major chalcone-converting enzymes were present and that one of them could be effectively inhibited by potassium cyanide. Activities of the two proteins could be separated by assays with and without 10mM potassium cyanide; no reaction between cyanide and chalcone was observed on the time-scale of our experiments. The enzyme not inhibited by cyanide proved to be an isomerase, with maximal activity in 1—3 days old seedlings and the nature of the conversion was demonstrated by a preparative reaction; the major product obtained by t.l.c. was shown to be 4',7-dihydroxyflavanone (liquiritigenin) by i.r., u.v., ^1H n.m.r., m.s., and mixed m.p. comparison with an authentic specimen. Its o.r.d. curve was of the same sign as that reported for the 2*S*-enantiomer.[5] Purification of the enzyme was achieved by successive ammonium sulphate and pH precipitations. After application of a gel exclusion column using Sephadex G-150, the protein was further purified on a DEAE-cellulose ion-exchange column using a sodium chloride gradient. Finally, chromatography on Sephadex G-100 (superfine) separated two isoenzymes with approx. molecular weights, by the method of Andrews,[6] of 50 and 45.5 kdaltons. The latter was the major protein and was dominant in young seedlings; it had pH_{opt} 8.3, K_m 3.0μM, used no co-factors, and was not inhibited by product. The isomerase isolated from soya bean[7] had K_m 4.0μM and exhibited significant product inhibition. Overall 261-fold purification was achieved, and the specific activity of the final protein was 1 314 units mg^{-1} [*i.e.* it could convert 1.314μM chalcone (0.36 mg) per min per mg at 30 °C, pH 8.3]. Both isoenzymes afforded liquiritigenin as product by t.l.c. comparison with authentic material. Chalcone-flavanone isomerases (EC 5.5.1.6) have been isolated from a number of plants, *e.g. Phaseolus aureus*,[8] *Cicer arietinum* L.,[8] *Petroselinum hortense*,[8] *Soja hispida*,[7,9] and *Citrus aurantius*,[10] and isozymes have been reported in several cases.[7,8]

The second enzyme acting on 2',4,4'-trihydroxychalcone in the crude cell homogenates also required no added co-factors and gave an initial product $\lambda_{max.}$ 336 nm (pH 8.0) close to the maximum of 4',7-dihydroxyflavanone. However, this enzyme was inhibited by cyanide, permitting, by difference, its assay in the presence of isomerase. The enzyme was observed in the gel-excluded fraction from a Sephadex G-150 column, corresponding to protein of molecular weight >200 kdaltons, and maximal activity was observed in 6—7 day old seedlings (when

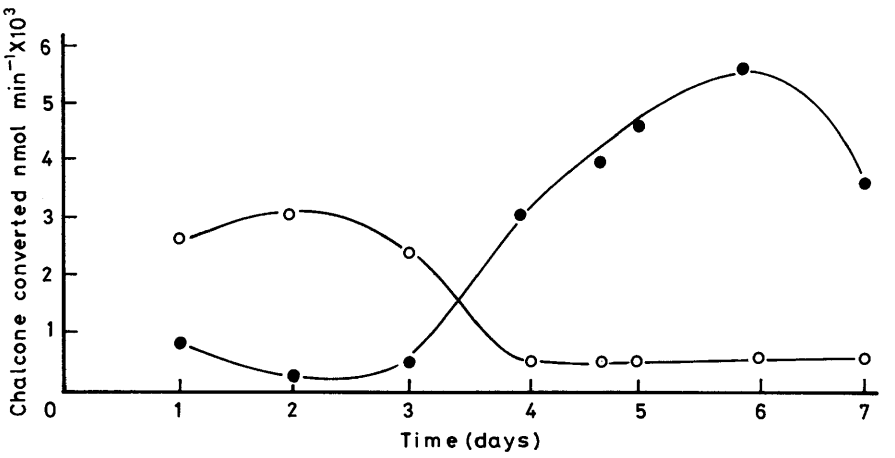
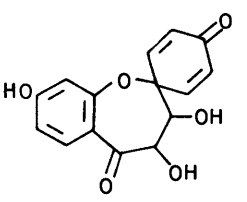


Figure 1. Utilisation of chalcone (**1**; $R^1 = R^2 = OH$, $R^3 = H$) by the peroxidase (●) and the isomerase (○) enzymes from *A. fruticosa*

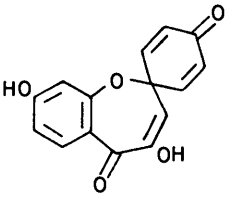
isomerase activity was reduced, see Figure 1). No significant difference was observed between light-grown or etiolated seedlings. The crude enzyme preparation showed pH_{opt} 8.0 and was inhibited by $NADP^+$ and by NADH, although detailed studies were not undertaken and further protein purification has not yet been pursued.

Following the enzyme-chalcone reaction by u.v. spectroscopy showed rapid formation of an initial product, $\lambda_{max.}$ 336 nm, which then changed slowly to a second product, $\lambda_{max.}$ 385 nm. Examination of the products revealed (i) some liquiritigenin, arising either from unseparated isomerase, or non-enzymic cyclisation of chalcone; (ii) 4-hydroxybenzaldehyde and 2,4-dihydroxyacetophenone, which could arise from the chalcone by retro-aldol reaction; (iii) some recovered chalcone; and (iv) a new, pale yellow, crystalline compound in *ca.* 25% isolated yield. The new product, named chalaurenol, proved labile and purification was attended by loss of material. However, we obtained i.r., u.v., m.s., and ^1H n.m.r. data, from which it was apparent that the compound had the formula $C_{15}H_{10}O_5$ and thus arose by mono-oxygenation of starting chalcone. A carbonyl group remained ($\nu_{max.}$ 1 695 cm^{-1}) and the ^1H n.m.r. data indicated that the aromatic rings were relatively little disturbed; one further olefinic/aromatic proton was present. At this stage it was recognised that chalaurenol might be similar to a substance isolated by Wong and Wilson[11] from the action of either horseradish peroxidase, or a peroxidase from Garbanzo seedlings, on the same chalcone: a similar substance had been obtained by Partridge and Keen[12] using soybean peroxidase. Comparison of the i.r. spectra of the original material, kindly supplied by Dr. E. Wong, with our product, supported the identity which was confirmed by further comparisons of u.v., m.s., and ^1H n.m.r. data. The enzyme present in *A. fruticosa* is thus apparently a peroxidase, though no added hydrogen peroxide was necessary in incubations with the *A. fruticosa* enzyme to obtain chalaurenol. Partridge and Keen[12] also conducted their experiments without added peroxide, whereas Wong and Wilson[11] included peroxide in all preparations and assays.

The spectral data obtained for chalaurenol, although apparently restrictive, still permitted a substantial number of structures to be considered. Structure (**3**) was reported by Wong in a preliminary communication,[13] but this was later revised to the benzoxepinone (**4**) after correction of the formula.[11] Three alternative structures were suggested by Wong and Wilson as possible fits to the evidence, two more were offered by Partridge and Keen,[12] and a further two were considered possibilities by us in the early stages of this work. As we doubted that a conclusive solution would arise from n.m.r. studies we chose



(**3**)

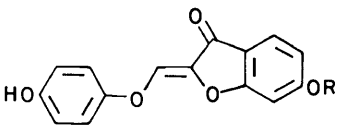


(**4**)

to attempt *X*-ray diffraction on a crystalline derivative of chalaurenol. To this end chalcone (**1**; $R^1 = R^2 = OH$, $R^3 = H$) (100 mg) was oxidised with horseradish peroxidase (1 600 units) and hydrogen peroxide. The organic products were chromatographed on Sephadex LH 20. Crude chalaurenol was purified by reverse phase h.p.l.c. and methylated with diazomethane to afford a relatively stable crystalline monomethyl ether. In a repeat incubation the total crude products were methylated and the methyl ether obtained directly by h.p.l.c. In this way 8 mg of the pure ether were accumulated from which was obtained, on controlled evaporation from ethanol, a single crystal suitable for *X*-ray study. After collection of the diffraction data the crystal was removed and ^1H n.m.r. and m.s. data collected from it to ensure its identity with the bulk specimen.

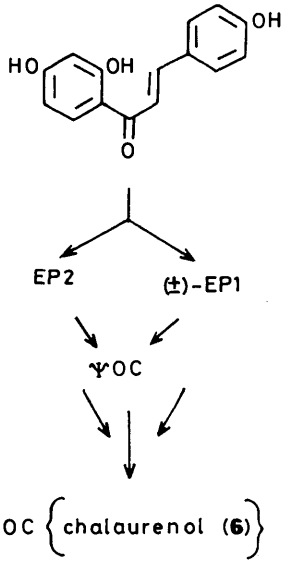
The *X*-ray structure of chalaurenol methyl ether was solved by direct methods using diffractometer data. Least-squares refinement converged to an *R* value of 5.55% over the 539 independent observed reflections. The structure of the chalaurenol molecule is as presented in Figure 2 which incorporates the *X*-ray numbering. Bond lengths and angles are listed in Tables 1 and 2 and are unexceptional. The whole of the coumaranone portion of the molecule is planar, including O(9) and methyl C(16), with the largest displacement from this plane of 0.05 Å. However, the quinol portion of the molecule is twisted out of this plane [C(9), O(9), C(10), C(11) torsion angle 38°] presumably to relieve the close contact between H(9) and H(11). A short intermolecular hydrogen bond was located in the crystal structure [O(13)–O(7) $(x, \frac{1}{2} - y, z - \frac{1}{2}) = 2.71$ Å]. The hydrogen atom H(13) was located in a difference map to be fairly central [O(13)–H(13) = 1.39 Å and H(13)–O(7)^I = 1.44 Å with the bond angle at H(13) of 147°].

The structure is thus revealed as the methyl ether (**5**), and natural chalaurenol is the quinol enol ether (**6**). It is notable that no such structure had been taken into consideration by ourselves or by others for chalaurenol, and that no other natural *O*-heterocycle of this type is known.



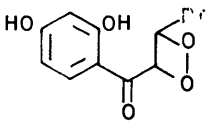
(5) R = Me
(6) R = H

In their careful study of the action of horseradish peroxidase on 2,4,4′-trihydroxychalcone Wong and Wilson [11] detected products intermediate between chalcone and chalaurenol (which is designated compound 'OC' in their work); Scheme 1

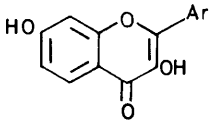


Scheme 1.

summarises their results. The first product was designated EP, obtained as two isomers (diastereoisomers?), both of which afforded chalaurenol at pH > 12. At lower pH a second intermediate compound 'ψ-OC' was recognised, also being converted into chalaurenol: 'ψ-OC' was allocated a benzoxepinone structure (4) similar to that proposed for compound 'OC'. Product EP was assigned the dioxetane structure (7).



(7)



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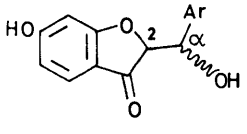
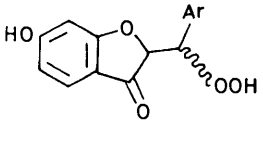


(9)



(10)

Ar = C_6H_4OH-p

Following a preliminary communication [14] of the present results, with an alternative mechanistic interpretation, Wong [15] has maintained his view that compound 'EP' is the dioxetane (7) and made further observations on its chemistry. The major features of 'EP' are (i) it is formed as two diastereoisomers, one of which appeared to be resolved on paper chromatography; hence at least two chiral centres are present; (ii) it contains three silylatable hydroxy groups, by m.s.; (iii) it gives flavonol (8) as a product in alkali; (iv) it decomposes to *p*-hydroxybenzaldehyde; and (v) on treatment with diphenyl sulphide it affords the diastereoisomers of the hydroxybenzylcoumaranone (9). I.r., u.v., and ^{1}H n.m.r. data were collected from compound 'EP'; the n.m.r. showed the resonances expected for the two aryl moieties, with two further C–H doublets *J* 2.3 Hz (EP1) and *J* 2.0 Hz (EP2) which were assigned to the dioxetane ring protons.

It seems to us that this evidence might be equally well interpreted in favour of the diastereoisomers of the hydroperoxides (10). Thus deoxygenation (10)→(9) becomes straightforward. The formation of two diastereoisomers of (10) (in a non-stereoselective radical reaction, see below) seems more acceptable than two diastereoisomers of (7) one form of which must be a relatively unstable *cis*-1,2 structure. Fragmentation of (10) to *p*-hydroxybenzaldehyde is understandable. The measured couplings between non-aryl C–H's seem small for cyclic structure (7) where the *cis* form must have a 2-H, 3-H dihedral angle close to zero. The chemical shifts and couplings quoted [11] for these protons in EP1 and EP2 {5.32d, 5.15d, *J* 2.3 Hz and 5.19, 4.65, *J* 2.0 Hz, both in [$^2H_6$]acetone} are com-

Table 1. Bond lengths (Å) for chalaurenol: all standard deviations are 0.01 Å

C(1)–C(2)	1.38
C(1)–C(6)	1.38
C(1)–O(1)	1.38
C(2)–C(3)	1.39
C(3)–C(4)	1.40
C(3)–O(3)	1.36
C(4)–C(5)	1.37
C(5)–C(6)	1.40
C(6)–C(7)	1.45
C(7)–C(8)	1.47
C(7)–O(7)	1.23
C(8)–C(9)	1.30
C(8)–O(1)	1.40
C(9)–O(9)	1.36
C(10)–C(11)	1.41
C(10)–C(15)	1.37
C(10)–O(9)	1.43
C(11)–C(12)	1.40
C(12)–C(13)	1.36
C(13)–C(14)	1.39
C(13)–O(13)	1.35
C(14)–C(15)	1.40
C(16)–O(3)	1.41

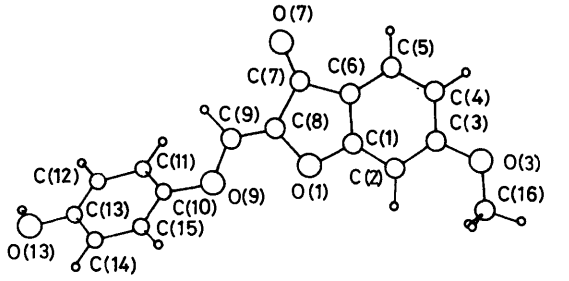
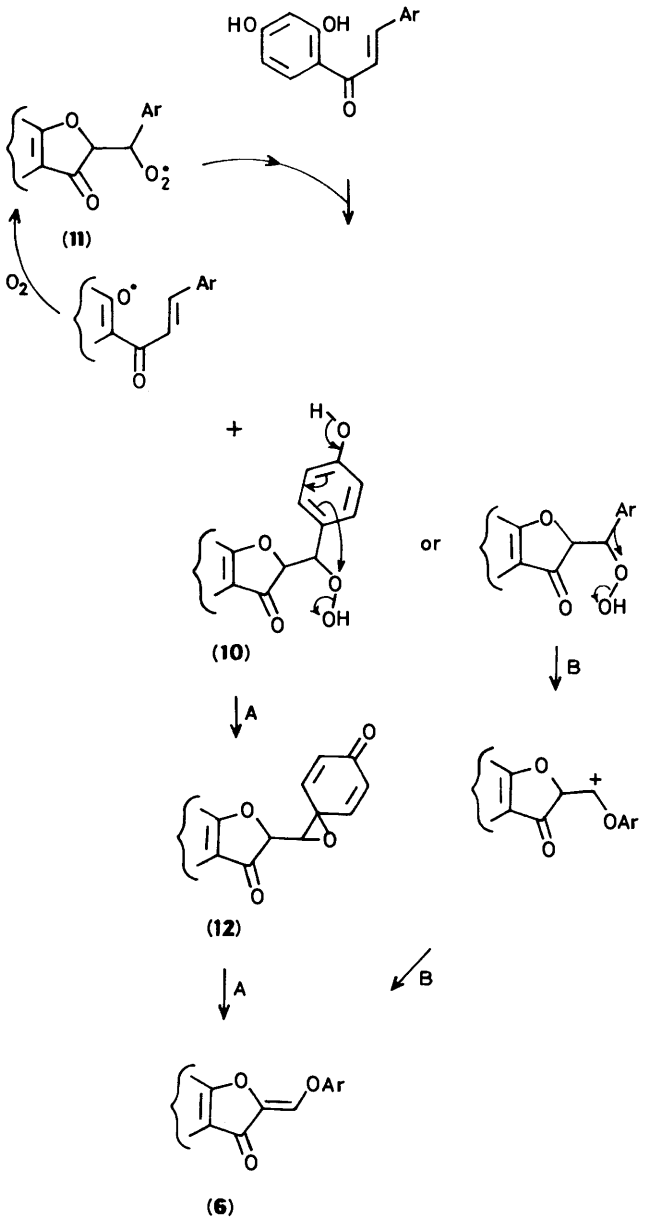


Figure 2. *X*-Ray crystal structure of chalaurenol with crystallographic numbering



Scheme 2.

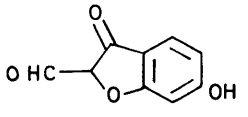
patible with structure (**10**); *cf.* 2-H and α-H in the related hydroxybenzyl coumaranones (**9**), [δ 4.99, 4.74, *J* 2.5 Hz and δ 4.9 'signals coincident' (both in [$^2H_6$]dimethyl sulphoxide)]. The conversion of compound 'EP' into 4′,7-dihydroxyflavonol (**8**) might be accommodated by, for example, base-catalysed isomerisation to a reactive dioxetane (**7**) followed by conversion into flavonol following the literature proposal.[11] A hydroperoxide structure for the first product of oxidation would also seem preferable to the chalcone epoxide possibility mentioned in our communication,[14] as, unlike P450 oxidases, peroxidases do not normally effect direct epoxidation. Our hypotheses are summarised in Scheme 2. After initiation by hydrogen abstraction from the chalcone the resulting radical reacts with dioxygen to form a peroxyradical (**11**): reaction of (**11**) with starting chalcone sets up a radical chain process, in accord with literature observations,[16] yielding the hydroperoxide (**10**). This may be converted into chalaurenol by route (A), through a spiroepoxide intermediate (**12**) (probably substance 'ψ-CO'), or by an aryl migration, path (**B**), reminiscent of the chemistry of

Table 2. Bond angles (°) for chalaurenol: all standard deviations are 1°

C(2)–C(1)–C(6)	125
C(2)–C(1)–O(1)	122
C(6)–C(1)–O(1)	113
C(1)–C(2)–C(3)	115
C(2)–C(3)–C(4)	122
C(2)–C(3)–O(3)	123
C(4)–C(3)–O(3)	115
C(3)–C(4)–C(5)	120
C(4)–C(5)–C(6)	119
C(1)–C(6)–C(5)	118
C(1)–C(6)–C(7)	107
C(5)–C(6)–C(7)	134
C(6)–C(7)–C(8)	104
C(6)–C(7)–O(7)	129
C(8)–C(7)–O(7)	127
C(7)–C(8)–C(9)	129
C(7)–C(8)–O(1)	110
C(9)–C(8)–O(1)	122
C(8)–C(9)–O(9)	121
C(11)–C(10)–C(15)	121
C(11)–C(10)–O(9)	122
C(15)–C(10)–O(9)	117
C(10)–C(11)–C(12)	118
C(11)–C(12)–C(13)	121
C(12)–C(13)–C(14)	121
C(12)–C(13)–O(13)	122
C(14)–C(13)–O(13)	117
C(13)–C(14)–C(15)	119
C(10)–C(15)–C(14)	120
C(1)–O(1)–C(8)	106
C(3)–O(3)–C(16)	119
C(9)–O(9)–C(10)	119

the cumene-phenol process: the route taken might be dependent on pH. It is of interest that the diastereoisomers of ketol (**9**) have been isolated from the oxidation of chalcone (**1**; $R^1 = R^2 =$ OH, $R^3 = H$) with a peroxidase from *Soja hispida*;[17] other products observed from similar oxidations include 3,4′,7-trihydroxyflavone[12,18,19] and 4′,6-dihydroxyaurone.[19,20] Treatment of the latter with horseradish peroxidase does not produce chalaurenol so a pathway to (**9**) involving this aurone is not supported. Further work on the interesting processes involved in the peroxidase oxidation of chalcones is clearly desirable.

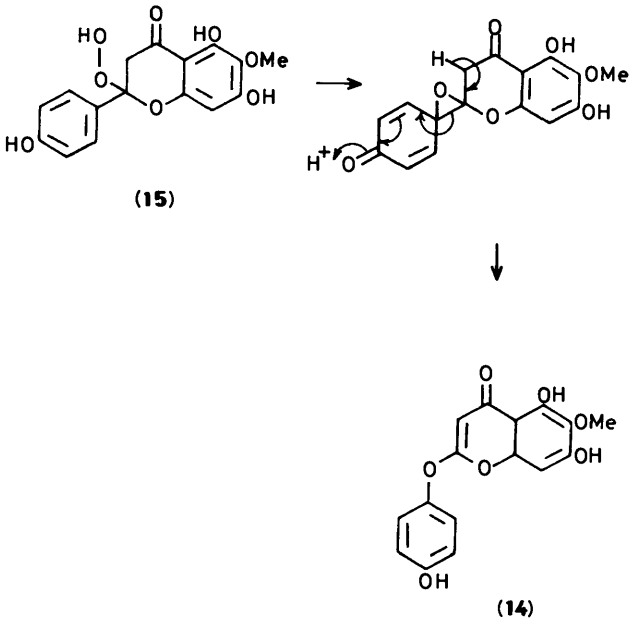
The status of chalaurenol itself is curious. It is formed by an enzyme from *A. fruticosa* on a substrate present in *A. fruticosa*, but has not yet been directly observed as a natural product—perhaps, not surprisingly, in view of its lability. Most likely chalaurenol is a normal intermediate of chalcone catabolism, although it could arise simply from supplying an unnatural substrate to the peroxidase. Chalaurenol is an obvious candidate as a source of hydroquinone in nature as the latter forms readily on hydrolysis: hydroquinone occurs widely in plants, both in the free state and as the mono-D-glucoside arbutin.[21] At present other recognised routes to hydroquinone include oxidative decarboxylation of *p*-hydroxybenzoic acid,[22,23] and phenolic oxidation of a 4′-hydroxyflavanone.[24,25] The second product from hydrolysis of chalaurenol would be a 2-formylcoumaranone (**13**) appearing at first glance to belong to the



(**13**)

phenylalanine-cinnamate group, although, in fact, possessing an acetate-derived aromatic ring with a three-carbon side-chain transferred from cinnamate.

A small group of phenol enol ethers, capillarisin[26] (**14**), and its relatives[27] have been isolated from *Artemisia capillaris* Herba and their biosynthesis may be related to the process discussed above. Thus, such compounds could arise from a flavanone hydroperoxide (**15**), in a pathway outlined in Scheme 3.



Scheme 3.

Conyzorigin,[28] claimed to be another flavonoid quinol ether, has recently been shown to be identical with eupalestrin, a known flavanone.[29]

Experimental

Growth of Amorpha fruticosa *Seedlings.*—Outer husks of *Amorpha fruticosa* seeds were removed by mechanically scarifying them with glasspaper, and sieving through 0.5 × 1.5 mm mesh to remove dust and husked seeds. Residual husks were separated by flotation and the seeds were sterilised by washing with ethanol (2 × 10 min) and 8% cupric sulphate (2 × 5 min). Germination was initiated by heating the seeds at 40 °C for 10 min. The seeds were grown on moist tissue paper in sterile trays covered with foil, at 28 °C, in the dark. For time course experiments, batches (3 g) of dry seed were set in Petri dishes.

Preparation of Homogenates.—Whole seedlings were homogenised in a Waring blender in 0.1M potassium phosphate buffer (pH 6.7) containing 1.0 mM 2-mercaptoethanol. The resulting pulp was filtered through glass wool and the filtrate was treated over 1 h with moist Polyclar AT (1 g dry polymer per 2 g wet seedlings). After filtration (glass wool) the filtrate suspension was centrifuged (10 000 g, 20 min). The pH of the clear supernatant was adjusted as appropriate to the enzyme to be studied and the extract was stored at −20 °C.

Assay of Chalcone-Flavanone Isomerase.—Assays were routinely conducted in Tris-HCl buffer (pH 8.3, 50 mM) at 30 °C. To determine the pH maximum, potassium phosphate buffer (50 mM) was used up to pH 7.5, and the Tris-HCl buffer up to pH 8.6. Incubations used enzyme preparation (0.1 cm^3) diluted to 3 cm^3 with buffer and 2′,4,4′-trihydroxychalcone in 2-methoxyethanol (0.1 cm^3) to give a final 20 μM concentration. Absorbance at 410 nm was measured. In assays of enzyme in early stages of purification, potassium cyanide (0.1 cm^3; 10 mM) was added.

Table 3. Fractional atomic co-ordinates for chalaurenol with standard deviations in parentheses

Atom	*x/a*	*y/b*	*z/c*
C(1)	0.276(2)	0.020 3(5)	0.651 7(12)
C(2)	0.346(2)	−0.023 8(4)	0.622 5(9)
C(3)	0.394(2)	−0.053 8(4)	0.703 9(11)
C(4)	0.376(2)	−0.039 8(4)	0.807 7(10)
C(5)	0.313(2)	0.005 4(5)	0.832 3(10)
C(6)	0.260(2)	0.036 4(4)	0.752 5(12)
C(7)	0.177(2)	0.083 7(4)	0.749 0(12)
C(8)	0.153(2)	0.093 4(5)	0.638 0(10)
C(9)	0.083(2)	0.131 0(4)	0.593 5(9)
C(10)	0.034(2)	0.179 2(4)	0.441 7(9)
C(11)	0.122(2)	0.221 0(5)	0.476 5(9)
C(12)	0.094(2)	0.261 1(4)	0.422 5(10)
C(13)	−0.013(2)	0.265 5(5)	0.337 6(10)
C(14)	−0.100(3)	0.224 0(4)	0.303 2(9)
C(15)	−0.074(2)	0.180 3(5)	0.355 5(10)
C(16)	0.489(2)	−0.116 0(4)	0.588 1(11)
O(1)	0.213(1)	0.053 4(3)	0.580 5(5)
O(3)	0.460(1)	−0.099 3(3)	0.689 9(6)
O(7)	0.139(1)	0.110 9(3)	0.821 4(7)
O(9)	0.063(1)	0.133 2(2)	0.488 8(6)
O(13)	−0.051(1)	0.307 0(3)	0.287 5(6)
H(2)	0.358	−0.0327	0.5441
H(4)	0.413	−0.0640	0.8647
H(5)	0.301	0.0160	0.9091
H(9)	0.037	0.1611	0.6372
H(11)	0.208	0.2195	0.5423
H(12)	0.155	0.2962	0.4502
H(14)	−0.185	0.2251	0.2395
H(15)	−0.140	0.1483	0.3344
H(16a)	0.540	−0.1504	0.5844
H(16b)	0.580	−0.0949	0.5477
H(16c)	0.376	−0.1163	0.5436
H(13)	0.086	0.3392	0.3111

Assay of Peroxidase.—The assay was run as above but at pH 8.0, monitoring at 400 nm.

Purification of Chalcone-Flavanone Isomerase.—In these experiments buffer A was 50 mM ammonium acetate, pH 6.8, containing 2-mercaptoethanol (1 mM); buffer T was 50 mM Tris-HCl buffer, pH 6.8.

Three-day old seedlings (300 g wet weight) were homogenised as above. The homogenate (diluted to 760 cm^3) was treated with solid ammonium sulphate (275 g) added slowly over 2 h. The solution was centrifuged for 20 min at 10 000 g, and more ammonium sulphate (259 g) was added over 4 h. The solution was stirred for 2 h and centrifuged for 20 min at 10 000 g. The supernatant was stirred with Celite (8 g) for 30 min and centrifuged for 5 min at 2 000 g. The pellets from the two centrifugations were resuspended in buffer A, and the pH was lowered to 4.0 using 5M hydrochloric acid. The turbid solution was centrifuged for 10 min at 5 000 g, and the pH of the supernatant was adjusted to 7.0 by adding Tris-base. The solution was dialysed against buffer A (4 × 1 l) for 36 h, and then lyophilised. The lyophilisate was redissolved in buffer B, (4 cm^3) at 4 °C, and chromatographed on a Sephadex G-150 column (2.6 × 50 cm^3), eluting with buffer T containing 0.2M potassium chloride. Active fractions were bulked, dialysed, and lyophilised as before, and dissolved in buffer T (10 cm^3). This

solution was chromatographed on a DEAE cellulose column and eluted with buffer T containing sodium chloride to form a 0—0.3M gradient. Active fractions were lyophilised, dissolved in buffer T (3 cm^3), and chromatographed on Sephadex G-100 superfine, eluting with buffer T incorporating potassium chloride (0.2M). Active fractions of each isoenzyme were bulked and desalted using a Sephadex G-25 column eluting with buffer A. Bovine serum albumin (200 mg) was added to each isoenzyme solution before lyophilisation and storage at −20 °C. Progress of the purification is charted in Table 4.

Action of Peroxidase from A. fruticosa *on 2',4,4'-Trihydroxychalcone.*—An homogenate from 7-day old seedlings was initially treated as above. The protein excluded from the Sephadex G-150 column, run in Tris-HCl buffer (50 mM, pH 8.0), was diluted with the same buffer (495 cm^3) and equilibrated at 30 °C. The title chalcone in 2-methoxyethanol was added in aliquots (2 mg) with u.v. monitoring; over 2 h, chalcone (30 mg) was supplied. After 1 h more, the mixture was acidified and extracted with ethyl acetate (1 × 200 cm^3, 3 × 100 cm^3). The extracts were washed, bulked, dried, and evaporated. T.l.c. analysis using (i) benzene–ethyl acetate–methanol–light petroleum (b.p. 60—80 °C) (a) 24:4:1:4, and (b) 24:4:1:8, and (ii) chloroform–isopropyl alcohol, 20:1, on silica loaded plates showed eight components. Four of these corresponded in all three solvent systems with 4-hydroxybenzaldehyde, 2,4-dihydroxyacetophenone, and 4',7-dihydroxyflavanone. Repeated p.l.c. in the second solvent system listed allowed isolation of the major unrecognised band which yielded on evaporation *chalaurenol* (**6**) (7.2 mg, 25%), m.p. 222—234 °C (Found: *M*, *m/z* 270.051. $C_{15}H_{10}O_5$ requires *M*, 270.053); $\lambda_{max.}$(EtOH) 200, 233, and 235 nm; $\lambda_{max.}$(EtOH–KOH) 206, 240i, 281, and 382 nm; $\nu_{max.}$(KBr) 3 400, 2 940, 1 695, 1 600, 1 510, 1 150, 1 110, 1 010, 980, 940, 880, 830, 770, and 750 cm^{-1}; $\delta_H(CD_3COCD_3)$ 7.58 (1 H, d, *J* 9.1 Hz, 4-H), 7.29 (1 H, s, 8-H), 7.17 and 6.91 (both 2 H, d, *J* 9.4 Hz, 2',6'- and 3',5'-H), 6.78 (1 H, dd, *J* 1.8, 9.1 Hz, 5-H), and 6.74 (1 H, d, *J* 1.8 Hz, 7-H); *m/z* 270 (50), 177 (32), 137 (8), 120 (13), 110 (100), and 94 (20).

Chalaurenol 6-O-*Methyl Ether.*—2',4,4'-Trihydroxychalcone (100 mg) in 2-methoxyethanol (20 cm^3) was stirred into Tris-HCl buffer (pH 8, 50 mM, 180 cm^3) and horseradish peroxidase (1 600 units, 14 mg) was added. Hydrogen peroxide (0.4 vol., 100 cm^3) was added dropwise to the mixture, with u.v. monitoring. When the chalcone absorption band (400 nm) had disappeared, peroxide addition was stopped, and the mixture was set aside for 30 min. The pH was then raised to 9.6 by adding 2M potassium hydroxide and then adjusted to pH 4.0 with 5M hydrochloric acid. The solution was extracted with ethyl acetate (1 × 100 cm^3, 2 × 50 cm^3) and the combined extracts were washed, dried, and evaporated. The residue was chromatographed on Sephadex LH20 eluting with methanol–water (5:1). An unidentified product {$\lambda_{max.}$(EtOH) 231 and 283 nm; $\lambda_{max.}$(EtOH–KOH) 251 and 344 nm; $\nu_{max.}$ 3 200 2 950, 1 680, 1 660, 1 580, 1 100, 860, and 830 cm^{-1}} was eluted first, followed by chalaurenol as the other major product identified by h.p.l.c. and u.v. comparison. The chalaurenol fraction was repurified by h.p.l.c. {C_{18}-reverse phase column, methanol–water (88:12)} and the major fraction was methylated in ether by treatment with an excess of ethereal diazomethane for 1 h at 0 °C and 1 h at ambient temperature, to yield the first sample of the title compound, used for h.p.l.c. calibration. More material was obtained by repeated oxidations, methylation with diazomethane of the residue from ethyl acetate extraction, and h.p.l.c. of this product (C_{18}-reverse phase). The third and major component eluted by this procedure was 6-O-*methylchalaurenol* (**5**), forming needles from ethanol, m.p. 204—221 °C (decomp.) (Found: *M*, *m/z* 284.068. $C_{16}H_{12}O_5$ requires *M*, 284.069); $\nu_{max.}$(KBr) 3 000, 1 695, 1 640, 1 620, 1 600, 1 180, 1 140, 1 100, 1 080, 1 020, 970, 940, 880, 870, 840, 820, 770, 740, and 700 cm^{-1}; $\delta_H(CD_3COCD_3)$ 7.63 (1 H, d, *J* 10.5 Hz, 4-H), 7.33 (1 H, s, 8-H), 7.18 and 6.92 (both 2 H, d, *J* 8.7 Hz, 2'-6'- and 3',5'-H), 6.92 (1 H, d, *J* 2 Hz, 7-H), 6.84 (1 H, dd, *J* 2, 10.5 Hz, 5-H), and 3.97 (3 H, s, OMe); *m/z* 284 (90), 191 (75), 151 (5), 134 (100), 119 (60), 110 (25), and 94 (75).

Table 4. Chalcone-flavanone isomerase purification

Fractionation step	Protein (mg)	Activity (units)	Specific activity (units mg^{-1})	Relative purity
(a) Homogenate	8 225.0	41 345	5.05	1.0
(b) $(NH_4)_2SO_4$ + Celite	1 380.0	17 610	12.75	2.5
(c) Acid pH	367.0	11 025	30.0	6.0
(d) G-150 Sephadex	46.0	9 310	203.0	40.0
(e) DEAE-cellulose	12.0	4 390	375.0	74.5
(f) G-100 Sephadex	3.7	4 200	1 313.5	261.0

Crystallographic Analysis of Chalaurenol Methyl Ether.—*Crystal data.* $C_{16}H_{12}O_5$, *M* = 284.27, orthorhombic, *a* = 7.473(1), *b* = 27.646(2), *c* = 12.911(1) Å, *V* = 2 667.47 $Å^3$, *Z* = 8, D_c = 1.42 g, cm^{-3}, *F*(000) = 1 184, space group *Pbca*, Cu-K_α radiation, λ = 1.541 78 Å, μ(Cu-K_α) = 9.0 cm^{-1}.

A crystal of approximate dimensions only 0.25 × 0.05 × 0.01 mm was mounted on an Enraf Nonius CAD4 diffractometer and 25 reflections (θ ~ 20°) were used to determine accurate lattice parameters. Intensity data were collected using an ω—θ scan for 1° ≤ θ ≤ 55°. A total of 1 664 independent reflections was measured of which only 539 had $I \geq 3\sigma(I)$ and were considered observed and used in the subsequent refinement. The data were corrected for Lorentz and polarisation factors but no absorption corrections were made. Crystallographic calculations were performed using the CRYSTALS system of programs.[30] The structure was solved by direct methods using the MULTAN program.[31] Following least-squares refinement including anisotropic thermal parameters for non-hydrogen atoms, a difference Fourier synthesis revealed approximate positions for all hydrogen atoms. These positions were then calculated from bond length and angle considerations and included in the structure factor calculations without refinement, with the exception of the hydroxy hydrogen [H(13)] which was induced at the position of the difference map peak. Refinement terminated at *R* 0.0555 (R_w 0.0462) after 15 cycles with maximum δ/σ 0.05. Final atomic co-ordinates are listed in Table 3.

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