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PRODUCTS OF THE PEROXIDASE-CATALYSED OXIDATION OF 4,2′,4′-TRIHYDROXYCHALCONE

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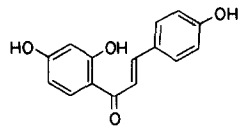
Key Word Index—*Cicer arietinum*; Leguminosae peroxidase; 4,2′,4′-trihydroxychalcone; 1,2-dioxetane; benzoxepinone; spirocyclohexadienone; 7,4′-dihydroxyflavonol.

Abstract—The enzyme responsible for the previously reported oxidation of 4,2′,4′-trihydroxychalcone to the corresponding dihydroflavonol and "hydrated aurone" in cell-free extracts of garbanzo seedlings (*Cicer arietinum*) has been identified as peroxidase (EC 1.11.1.7). Studies with the partially purified enzyme and with commercial horse radish peroxidase have shown that the initial product of the enzymic reaction under these conditions was an unstable compound, characterised as the novel 4-membered cyclic peroxide (1,2-dioxetane) derivative of the chalcone. Various stereochemical modifications of the cyclic peroxide were isolated and these were found to readily convert chemically to a second novel compound, a benzoxepinone-spiro-cyclohexadienone isomeric with 7,4′-dihydroxyflavonol. This flavonol was also obtained as a chemical transformation product of the cyclic peroxide enzymic products.

INTRODUCTION

Previous work [1–3] from this laboratory has shown that cell-free extracts of garbanzo (*Cicer arietinum*) and soya (*Glycine max*) bean seedlings catalyse the oxidation of 4,2′,4′-trihydroxychalcone (isoliquiritigenin) (**1**) to the corresponding dihydroflavonol (**2**) and the "hydrated aurone" (**3**). The latter product was isolated in diastereoisomeric forms (Y_1, Y_2) [3] both of which dehydrate readily to the aurone (**4**). Studies recorded in this and the following paper [4] have developed from a further investigation of these transformations, in the course of which the enzyme responsible was purified from garbanzo seedlings and identified as peroxidase (EC 1.11.1.7).



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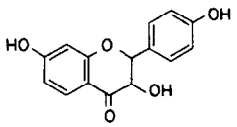

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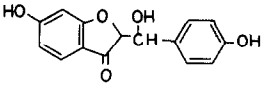

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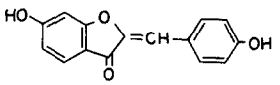

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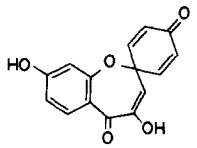
In contrast to the results with crude cell-free extracts, studies with the purified garbanzo enzyme or with horse radish peroxidase (HRP) showed that the main isolatable oxidation product of isoliquiritigenin under these conditions was an unstable compound (designated OC), characterised below as the benzoxepinone-spiro-cyclohexadienone (**5**). This novel compound is isomeric with 7,4′-dihydroxyflavonol (**6**) also found as a minor product under these conditions. Under certain experimental conditions the epoxide tautomer of OC, (ψOC) (**7**), was also isolated.



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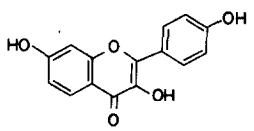

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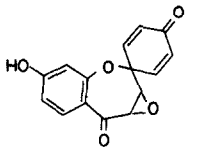

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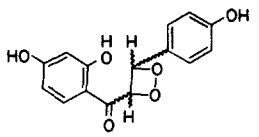

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The existence of compounds other than OC as initial products of the enzymic reaction was later discovered and compounds representing various stereochemical modifications of the 4-membered cyclic peroxide (1,2-dioxetane) structure (**8**) were characterised. These were shown to be the chemical precursors of OC, ψOC and 7,4′-dihydroxyflavonol, the relative proportions of the different artefacts formed being dependent on pH effects. This paper presents results of our studies on OC, ψOC and the cyclic peroxide isomers and on the nature of their chemical and stereochemical interrelationships. Biochemical details of the reactions leading to the various enzymic oxidation products will form the subject of the paper following [4]. A brief summary of this work has previously appeared [5].

RESULTS

Identification of the garbanzo enzyme as peroxidase

The initial enzymic activity in garbanzo cell-free extracts monitored was the formation of the "hydrated aurone" diastereoisomers, Y_1 and Y_2 (**3**) from isoliquiritigenin [1,3]. When the enzyme was partially purified, it

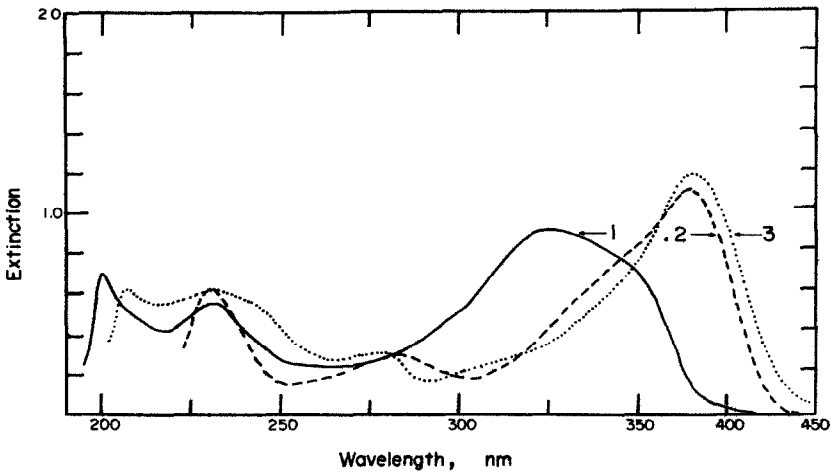


Fig. 1. UV spectra of OC. 1, 85% EtOH; 2, NaOAc or pH 8 buffer; 3, NaOH 0.003 N.

emerged that oxidation of the chalcone became much more extensive and the major product was OC. Under these conditions, Y_1 and Y_2 production persisted but was considerably reduced. The activities leading to these two classes of products furthermore could not be separated after a wide variety of enzyme purification treatments, which included [4] acid, ammonium sulphate and acetone precipitation, gel filtration and ion exchange chromatography. The same enzyme was thus responsible for both activities.

In the course of the preliminary studies, we suspected that the chalcone oxidising enzyme might be a peroxidase. Subsequently, enzymic activity at all stages of purification always coincided with peroxidase activity, as indicated by assay with guaiacol. On polyacrylamide gel electrophoresis, the activity coincided with three separate peroxidase isoenzymes as detected by benzidine staining reaction in the presence of H_2O_2. Finally, commercial HRP enzyme, when tested with isoliquiritigenin gave results closely similar throughout to those of the purified garbanzo enzyme. Comparative studies of the peroxidase enzymes from these two sources will be given in the following paper [4].

Detection and isolation of the oxidation products

Products of the reaction catalysed by purified peroxidase from garbanzo seedlings or commercial HRP were examined by two-dimensional PC [1]. OC appeared as a purple–brown spot in UV light and turned bright blue with ammonia. The UV spectrum of OC, as isolated by chromatography, is shown in Fig. 1. On addition of sodium acetate and of alkali, stable spectra of the ionised compound were observed, the original spectrum being obtained on reacidification.

The initial product spectrum of the enzymic oxidation of the chalcone (Fig. 2) observed *immediately* after a rapid (< 1 min) complete reaction of chalcone substrate was not that of OC at pH 8. The spectrum showed a complex pattern of changes in the first few min. Upon controlled addition of alkali however to a freshly prepared product solution, the spectrum was transformed cleanly after a few min into that of OC anion (Fig. 2). Acidification of the resulting alkaline solution then gave the spectrum of OC, the identity of which was confirmed by chromatography. Thus UV spectrophotometric evidence indicated that OC was not the initial product of the enzymic reaction, but was derivable from the initial

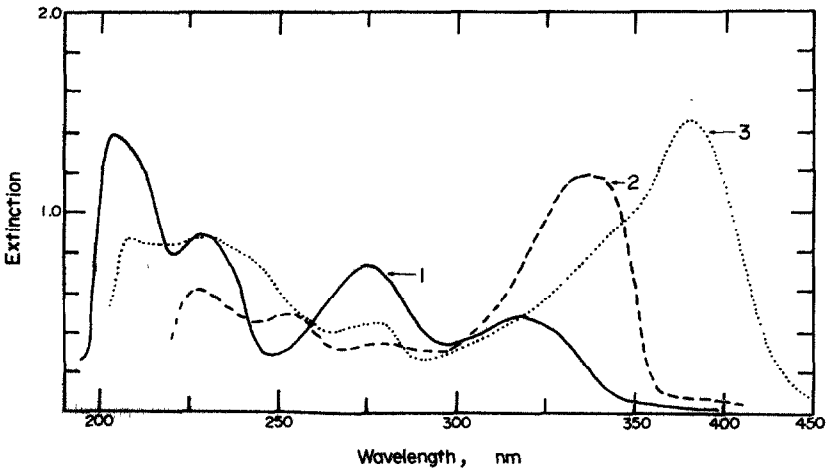


Fig. 2. UV spectral properties of enzyme products, EP_1 or EP_2. 1, 85% EtOH; 2, NaOAc or pH 8 buffer; 3, NaOH 0.003 N (spectrum is that of OC anion).

product, the transformation being catalysed irreversibly by alkali.

Because of the great lability of the initial product, special procedures involving mild acidification following rapid reaction of the chalcone were devised for its recovery in an unchanged stage. Careful, rapid 2D-chromatography showed that the recovered initial product consisted essentially of two compounds, designated EP_1 and EP_2 (Enzyme Product). These compounds overlapped the diastereoisomeric "hydrated aurone" Y_1 and Y_2 respectively on the chromatogram, and were detected in UV light as light blue-grey spots, the colours being due to artefacts produced inevitably by these compounds on keeping.

Elution of the chromatographically separated EP_1 and EP_2 spots showed that each had comparable UV spectral properties with those of the initial product (Fig. 2). Separate rechromatography of EP_1 and EP_2 after isolation showed that neither compound gave rise to detectable quantities of the other under the conditions of formation in the reaction and of subsequent handling. OC, however, was always obtained as artefact in these runs. By appropriate choice of the pH of the reaction medium at the solvent extraction step, the main product recovered can be predominantly either the EP compounds or OC. The other minor flavonoid products (**2**), (**3**) and (**4**), previously identified [1–3], were generally also present, albeit always in trace amounts.

For structural studies, mg amounts of EP_1 and EP_2 were successfully obtained by preparative PC at 0° with very dilute aqueous acetic acid as solvent, under which conditions the breakdown of these labile compounds was limited.

The EP_2 band obtained under normal preparative chromatographic conditions, when eluted as higher and lower R_f halves, gave eluates which were optically active. Further repeated chromatographic purification of the individual eluates resulted in increased specific rotation and values of $[\alpha]_D$ of (+) 241° and (−) 224° were finally recorded for the faster and slower moving components respectively. Within the accuracy of measurement with the small amounts of labile substances available, these values were taken to indicate that the resolved components had equal and opposite specific rotations. UV spectral properties of these two forms were identical, as were also the IR and PMR spectral data. The two components thus constitute enantiomeric forms of EP_2. No evidence was obtained for any inter-conversion of these two forms of EP_2 under the conditions of handling or

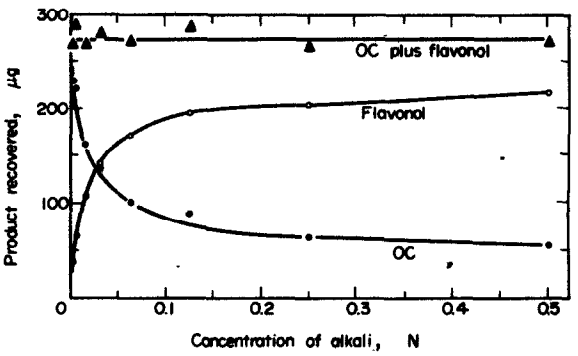


Fig. 3. Competitive formation of OC and 7,4′-dihydroxyflavonol from EP compounds as a function of the concentration of alkali.

incubation in pH 8 tris buffer. Transformation of these two forms to OC with alkali resulted in the loss of optical activity. No optical activity was detectable in the original reaction product mixture of EP_1 and EP_2, or in the chromatographically pure EP_1 component alone.

When chromatographically prepared samples of EP_1 or EP_2 were held as moist solids for 1–2 hr at room temperature in the presence of small amounts of acetic acid from the chromatographic solvent, small quantities of yet another OC-producing intermediate product was formed. This compound (ψOC) appeared as a bright blue fluorescent spot running just ahead of OC in the C_6H_6–HOAc–H_2O solvent system. The recovered ψOC compound had a UV spectrum essentially similar to that of the EP compounds in the region 270–400 nm (Fig. 2), and, again like these compounds, gave rise to OC irreversibly on treatment with alkali.

Flavonol formation

Under usual experimental conditions 7,4′-dihydroxyflavonol was generally noted in small amounts on chromatograms of reaction products. Preliminary work showed that this product also arose from alkali labile precursors formed in the enzymic reaction and that these were present on 2D-chromatograms in the spots containing the precursors of OC. Further studies on the nature of these flavonol precursors revealed that they were the same EP compounds which give rise to OC. The relative proportion of OC and flavonol products formed from the precursors was found to be dependent on pH effects. Under normal conditions of reaction, isolation and chro-

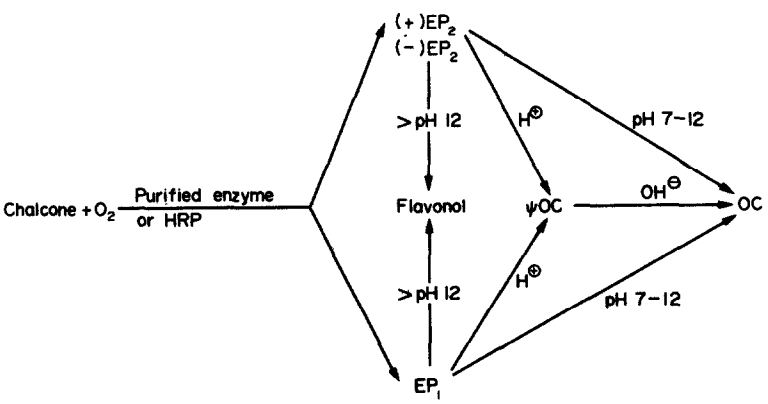


Fig. 4. Interrelationships of products of the main reaction.

matography, and upon treatment with a low concentration of alkali (<pH 12), the EP compounds were transformed predominantly to OC. It was found, however, that if added alkali concentration was increased, OC gradually gave way to the flavonol as the chief product. The quantitative relationship between these mutually exclusive transformation products as a function of the concentration of the aqueous alkali is shown in Fig. 3.

The interrelationships of the various compounds isolated as chemical and stereochemical variants of the main product of the enzymic reaction of peroxidase and isoliquiritigenin are summarised in Fig. 4.

Structural studies on OC and ψOC

OC was not sufficiently stable to permit purification by normal recrystallisation procedures. Methylation with diazomethane yielded a crystalline stable product, OC-Me ether. Other attempts at chemical manipulation of OC (e.g. acetylation) resulted in degradation. Hydrogenolysis of OC and OC-Me ether at room temperature in the presence of Pd–C catalyst yielded hydroquinone, together with a number of other products. The formation of hydroquinone as an artefact in the course of other manipulations of OC was noted frequently. It appeared, for example, in OC which had been recovered from chromatography in 10% acetic acid, and was formed during chromatography of OC on polyamide columns eluted with aqueous ethanol.

In the MS of OC [270 M^+ (100), 177 (68), 137 (11), 120 (12), 110 (64), 94 (42), 81 (17), 65 (18), 63 (18). Probe, 70 eV] and OC-Me ether [284 M^+ (100), 191 (71), 151 (10), 134 (21), 121 (14), 119 (22), 110 (52), 94 (39), 81 (14), 65 (18), 64 (22). Probe, 70 eV], the molecular ion at *m/e* 270.0513 (OC) and *m/e* 284.0675 (OC-Me ether), corresponds respectively to $C_{15}H_{10}O_5$ and $C_{16}H_{12}O_5$. OC-Me ether is thus a mono-Me ether of OC and OC is isomeric with 7,4′-dihydroxyflavonol (**6**). The major fragmentation paths for OC and OC-methyl ether were similar but significantly different from those of conventional flavonoids [6]. Both compounds gave a strong M-93 peak, with the peak at *m/e* 94 also being prominent. A scheme to rationalise the formation of these fragments in terms of the structure (**5**) proposed for OC is given in Fig. 5. Fragmentation of deuterium labelled OC (D_2O + OC at source) was consistent with the above scheme. The M^+ ion incorporated a maximum of two D atoms and both were retained in the ion corresponding to ion A and one in that corresponding to ion B (Fig. 5). The MS of the TMSi derivative of OC with M^+ 414, showed this compound contained two TMSi groups, in accord with the deuterium labelling results. The peak at *m/e* 110.0377 ($C_6H_6O_2$) present in the MS of both OC and OC-Me ether was considered to be that of the M^+ of hydroquinone formed by pyrolysis. Its relative intensity varied greatly with inlet temperature and a peak at *m/e* 112 was present in the spectrum for the deuterated OC sample.

The PMR data for OC are summarised in Table 1. The three aromatic protons of OC gave signals similar in pattern to those of flavonoid compounds similarly substituted in the A ring [7]. A one-proton singlet at δ7.33, downfield to all but the C-5 proton doublet was assigned to the conjugated olefinic C-2 proton. The four remaining protons absorbed as an AA′BB′ system symmetrical about its mean chemical shift (δ7.07). These signals were assigned to the protons of the substituted *p*-cyclohexadienone ring [8].

The IR spectrum of OC strongly supports the cyclohexadienone structure. Bands attributed to the splitting of the dienone carbonyl absorption [8] were present at 1655 and 1625 cm^{-1}. For the carbonyl in the heterocyclic ring, the prominent band was located at 1715 cm^{-1}, consistent with frequencies recorded for other benzoxepinone systems [9,10]. These significant features were also prominent in the IR spectrum of OC-Me ether.

The formulation of ψOC as the epoxide tautomer (**7**) of OC was again based largely on spectral evidence. The MS of ψOC established the compound as a structural isomer of OC. As the inlet temperature was raised, the MS changed with time becoming increasingly more like that of OC. The PMR spectrum of ψOC was similarly complicated by the gradual transformation of the sample to OC which occurred in the course of manipulation. However, the initial spectrum recorded revealed clearly

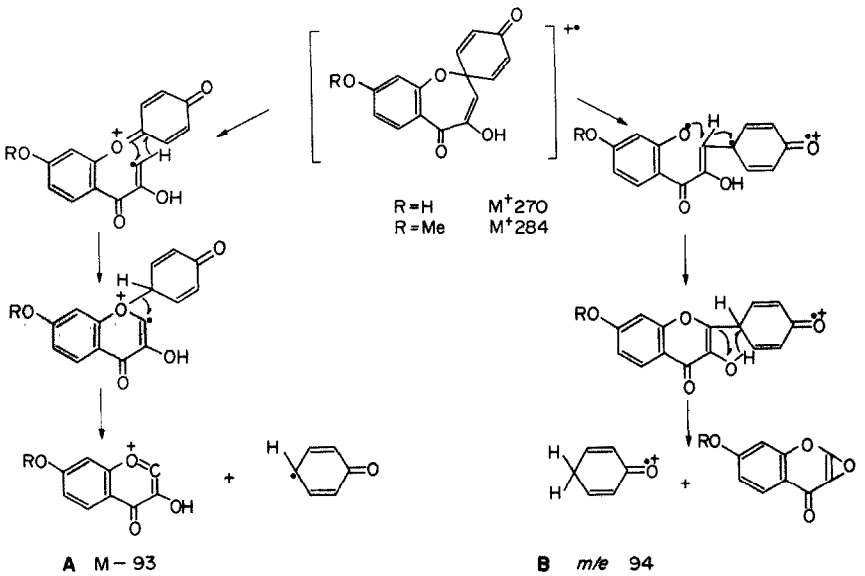


Fig. 5. MS fragmentation scheme for OC and OC-Me ether.

Table 1. PMR data for the enzymic products, OC and cognate compounds (60 MHz, deuteroacetone)

Compounds	Chemical shifts* (ppm δ) and coupling constants (J, Hz) H-2†	H-5	H-6	H-8	H-2',6'	H-3',5'	5-OH‡	7-OH‡	4'-OH‡
OC (5)	7 33	7.60*d* $J_{5,6}$ = 8.6	6.82*dd*	6.77*d* $J_{6,8}$ = 2.1	7.21*d* $J_{2',3'(5',6')}$ = 9	6.93*d*			
OC-Me ether	7 24	7 55*d* $J_{5,6}$ = 8 2	6.76*dd*	6.81 $J_{6,8}$ = 2.4	7.11*d* $J_{2',3'(5',6')}$ = 10	6.80*d*			
	H-2, H-3¶								
ψOC (7)	5 05, 3.89 $J_{2,3}$ = 6.0								
EP_1 (8)	5.32*d*, 5 15*d* $J_{2,3}$ = 2.3	7.16*d* $J_{5,6}$ = 8.8	6 52*dd*	6 37*d* $J_{6,8}$ = 2.0	7 02*d* $J_{2',3'(5',6')}$ = 8.4	6.53*d*	12.82 11.70	10.92 10.87	9 47 9.37
EP_2 (8)	5.19*d*, 4.65*d* $J_{2,3}$ = 2.0	7 27*d* $J_{5,6}$ = 9.3	6.43*dd*	6.37 $J_{6,8}$ = 2.0	7.22*d* $J_{2',3'(5',6')}$ = 9.0	6.66*d*			

* Relative to TMS internal standard. Multiplicities: *d* = doublet, *dd* = double doublet. Analysis of all spin systems was made by first order approximation.

† For simplicity, numbering of the carbon atoms in the different structures follows those given to the equivalent atoms in a conventional flavonoid structure.

‡ In DMSO as solvent.

¶ No separate assignments of chemical shift to H-2 and H-3 is made.

the significant PMR feature of ψOC, that of the AX pair of doublet (δ5.05, 3.89 and J 6.0 Hz) upfield of the main spectrum in the aromatic region consistent with the presence of the *cis* epoxide ring protons [11] in the proposed structure (7). The IR spectrum of ψOC closely resembles that of OC in the carbonyl/aromatic region (1720–1600 cm^{-1}) and in enhanced bands at 870 and 1277 cm^{-1}, consistent with formation of epoxide ring [12] at the expense of enolic OH.

Characterisation of the enzymic products, EP_1 and EP_2

The EP compounds were extremely labile. Spectral data were in general obtained only with difficulty because of the pronounced tendency of these compounds to convert to OC and other products under ordinary conditions. Great care had to be exercised in handling only freshly prepared material and carrying out measurements as quickly as possible.

The EP compounds could not be successfully methylated with diazomethane or subjected directly to MS analysis. Specifically for the latter purpose, formation of TMSi derivatives of EP_1 and EP_2 was attempted. The product mixture obtained for each isomer was subjected to GC–MS analysis, resulting in each case in one or more major component being obtained with GLC and MS properties closely similar to those of the $(TMSi)_3$ derivative of 7,4'-dihydroxyflavonol (486 M^+, 471, 414, 399).

The PMR spectra of EP_1 and EP_2 were similar and showed a pair of non-exchangeable one-proton doublets upfield of the aromatic region, assignable to the vicinal C-H protons in the dioxetane ring (Table 1). No separate assignment of chemical shift to these two protons is made, but the values obtained ($\delta \simeq 5.2$) are in good agreement with those reported for other dioxetane protons [13–15]. With DMSO as solvent, an enzymic reaction product mixture of EP_1 and EP_2 showed 6 exchangeable -OH protons downfield of the aromatic region in the PMR spectrum (Table 1), assignable to the three phenolic OH groups (with or without additional H bonding) in each of the two compounds.

The IR spectra of EP_1 and EP_2 differed significantly from those of the OC series (OC, OC–Me ether, ψOC) in the carbonyl absorption region. The C=O band at $\sim$1710 cm^{-1}, common to all compounds in the OC series was no longer present. Instead carbonyl frequencies at 1685 (EP_1) and 1645 cm^{-1} (EP_2) only were discernible. The carbonyl/aromatic spectral region differs for the two EP compounds in a way closely paralleling that for the previously studied pair of diastereoisomers, Y_1, Y_2 [3].

In the course of enzyme inhibition studies [4], it was found that in the presence of Cu^{2+}, the enzymic reaction product EP underwent extensive chemical degradation. *p*-Hydroxybenzaldehyde was identified as a product under these conditions. This compound, together with hydroquinone, was also found as a degradation product on treatment of EP compounds with mineral acids.

DISCUSSION

The novel structures (5) and (8) proposed for OC and the enzymic product EP satisfactorily accommodate the mass of spectral, chemical, stereochemical and biochemical [4] evidence associated with these labile compounds. The formulation of OC as the benzoxepinone-spirocyclohexadienone (5) is entirely consistent with the significant structural features indicated by the spectral data, which include: isomerism with 7,4'-dihydroxyflavonol but with one –OH less (MS), abnormal B ring MS fragmentation (Fig. 5), presence of cyclohexadienone ring (IR), and olefinic proton (PMR). The UV spectrum of OC is similar to that of 7,4'-dihydroxyflavone (λ_{max} 329, NaOH 392) [16] reflecting the presence of the Ring A-heterocyclic ring conjugation in OC which bears a resemblence to the cinnamoyl chromophore present in flavones. The little information available in the literature on spectral properties of benzoxepinones [9,10] compares well with the data found for OC. The IR evidence for ψOC requires that it be formulated as (7), the epoxide rather than the keto tautomer of OC. The PMR evidence for ψOC also supports this view.

Formulation of the enzymic products, EP_1 and EP_2, as stereoisomers of the 1,2-dioxetane (8) satisfactorily explains the very labile character of these compounds. The reactive 1,2-dioxetane structure [17] has in recent years been implicated as transient intermediates in photooxygenation of double bonds [18,19], in chemi- and bioluminescence [20–22] and in ozonolysis [15]. Since

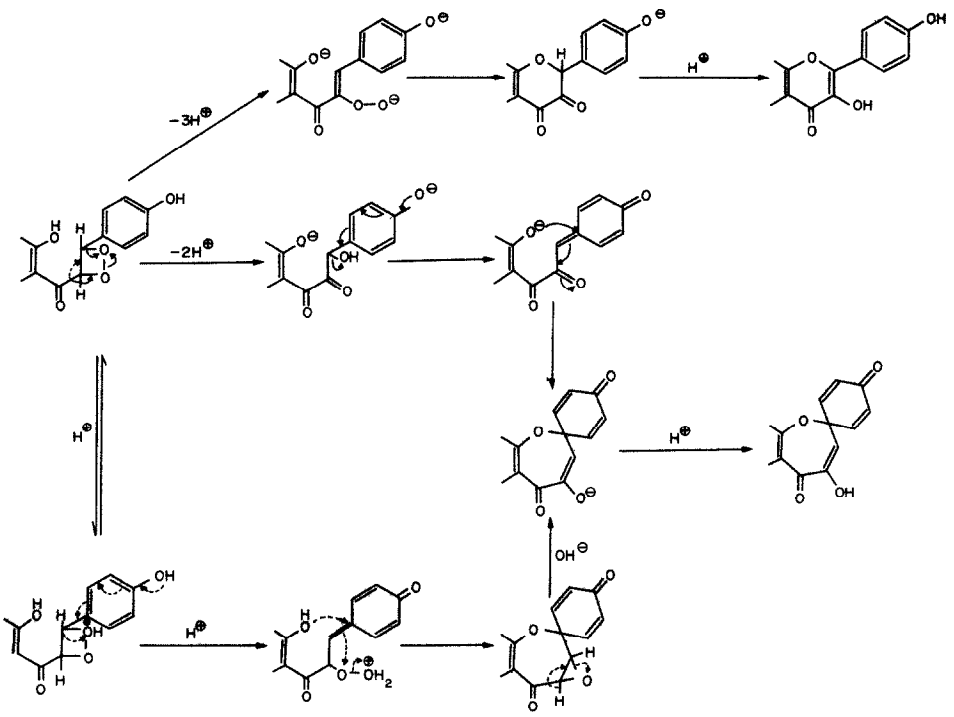


Fig. 6. Scheme for formation of OC, ψOC and 7,4'-dihydroxyflavonol from enzymic product.

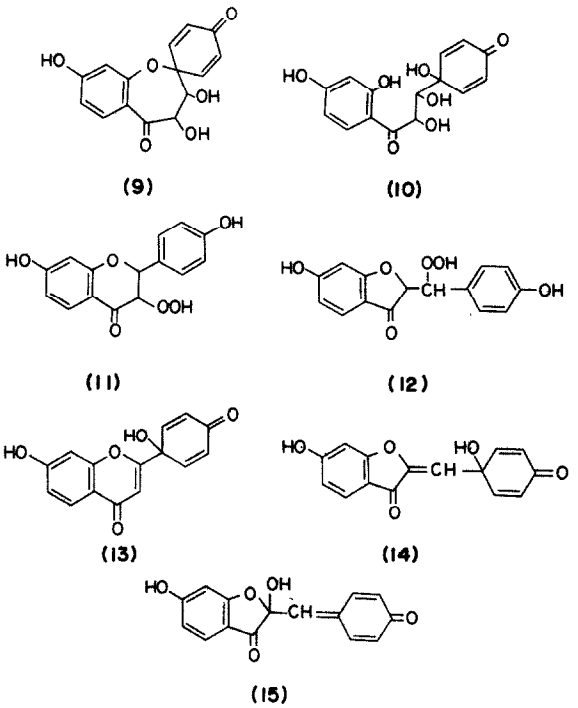
1969 several alkyl- [14,15,23], aryalkyl- [13] and alkoxy-substituted [18,19] 1,2-dioxetanes have been isolated, as well as a cannabinoid 1,2-dioxetane derivative [24]. Such 4-ring peroxides have also been postulated recently as intermediates in the enzyme catalysed oxygenation of aromatic compounds in micro-organisms [25].

In contrast to the structure (**5**) for OC, the chalcone peroxide structure (**8**) for the EP compounds allows retention of three phenolic OH groups as required by the PMR and IR data. The formation of $(TMSi)_3$ substituted artefacts from the silylation of the EP compounds followed by GC–MS is consistent only with such a structure. The UV spectra of the EP compounds in EtOH and EtOH–NaOAc are typically those of resacetophonene congenors.

The cyclic peroxide structure (**8**) also allows rationalisation of the various transformations of the EP compounds to OC, 7,4'-dihydroxyflavonol and ψOC. Suggested mechanisms for the formation of these products under basic or acidic conditions are presented in Fig. 6. The formation of *p*-hydroxybenzaldehyde as degradation product of the dioxetane (**8**) is in accord with expectation [14,15,18].

All available evidence is consistent with the view that EP_1 and EP_2 constitute the *cis* and *trans* forms of structure (**8**). The two chromatographically separated components of EP_2 are the enantiomeric forms of one diastereoisomer, and EP_1 though optically non-resolvable is by analogy a similar mixture of the corresponding (+) and (−) forms of the other. These findings, although further complicating an already complex products pattern, have important implications in the interpretation of the biochemical mechanism of the reaction [4]. The structures (**8**), (**6**) and (**5**) are all at the same oxidation level of 4-equivalents higher than chalcone (**1**). This oxidation level is supported by the stoichiometry observed for the enzymic reaction (chalcone + O_2 → product) [4].

In a preliminary report [5] of this work, structure (**9**) was proposed for the EP compounds. This structure, however, is not compatible with all of the evidence now available, as is also the case for the 5 other possible anhydro forms of structure (**10**) and for the hydroperoxy structures (**11**) and (**12**). Similarly the other possible structures (**13**)–(**15**) were considered for OC and rejected as being not compatible with the total evidence.



An independent study of the reaction forming the subject of this and the following paper has been reported by Rathmell and Bendall [26,27] who isolated flavonol (6) and aurone (4) from the oxidation of chalcone (1) with HRP or partially purified enzyme extracts of *Phaseolus vulgaris*. These workers were unaware of the existence of the EP compounds which in the light of present knowledge, must undoubtedly have been present also in their systems. Their assumption that stereoisomers of 7,4'-dihydroxyflavanonol (2) represent the unidentified intermediate compounds detected cannot therefore be justified.

EXPERIMENTAL

Garbanzo seedlings. Seeds of garbanzo bean (*Cicer arietinum*) were germinated in a continuous mist of tap H_2O at 15–20° in the dark for 5–8 days. Seedlings were rinsed in H_2O at harvest.

Chromatography. The standard solvent pair for 2-D work were BeAW (C_6H_6–HOAc–H_2O, 125:72:3) followed by 10% HOAc.

Enzymes. Peroxidase was partially purified from seedlings of garbanzo bean (*Cicer arietinum*) as described in the following paper [4]. Horse radish peroxidase (HRP), was obtained from Worthington (RZ 1.73). Its activity by the guaiacol assay [4] was 80 units/μg.

Reaction mixtures, conditions and product recovery. Analytical reaction mixtures generally contained in 4 ml of 0.05 M Tris–HCl buffer pH 8, enzyme (either garbanzo or HRP, 1–100 units), H_2O (0–10 μmol) and chalcone (0.2–2.0 μmol). For spectrophotometric observations the reaction vol was 2 ml. Chalcone was dissolved in EtOH (1–5 mg/ml); the EtOH in the reaction mixture had no effect on reaction rate or product composition. Addition of H_2O_2 started the reaction. Incubation lasted a few min at room temp (20–25°) and was normally restricted to the time taken for decolorization of the chalcone. EP compounds and/or OC were quantitatively extracted into Et_2O (2 extractions) from buffer acidified (HCl) to pH 7.5 or below. Isolation of products predominantly as EP compounds required buffer acidified to pH 3 or lower since mild acid conditions, contributed by H_2O present in the Et_2O extracts, stabilized the compounds. Solvent was removed *in vacuo* close to 0° to minimise decomposition. Extraction against pH 7.5 buffer resulted in recovery of product as OC; the transformation of the EP compounds initially present being remarkably rapid and was virtually complete during solvent removal. Extraction at pH 6–7 gave a mixture of EP compounds and OC.

Preparation of EP compounds and OC. Chalcone (50 mg—182 μmol in 5 ml of EtOH), HRP (800 units in 10 μl 0.05 M tris buffer pH 7.5) and H_2O_2 (430 μmol in 5 ml H_2O) were reacted in 0.05 M Tris buffer pH 8 (190 ml) in a separatory funnel at 20–25°. Chalcone was added to enzyme and buffer followed by H_2O_2 to start the reaction. The soln was aerated during the time (3.5–4 min) for complete reaction (indicated by decolorization). EP compounds were recovered by Et_2O extraction (2 × 200 ml) of the acidified (3.55 ml 1.44 N HCl) medium immediately the reaction was complete. Yield 85–90% of chalcone consumed. To prepare OC, EP compounds were transformed quantitatively prior to product extraction. Alakali (2.5 ml of 2.5 N NaOH) was added immediately the chalcone was consumed and 2 min later the medium was acidified prior to ether extraction of OC. Yields corresponded to those of EP compounds.

Purification of OC. OC was unstable in hot solvents and could not be purified efficiently by recrystallization. Purified OC was obtained as a granular light yellow solid from aq Me_2CO soln concentrated at room temp and held at 0°. This material melted at 223–234°. OC isolated from chromatograms was impure unless the time for which the chromatogram was dried in a cool air stream was minimized.

Separation and purification of EP compounds. The EP compounds separated by PC in aq HOAc but their instability complicated subsequent recovery and handling. Individual compounds were best prepared as follows. EP compounds from 10 mg of chalcone were banded across a sheet, the Me_2CO solvent removed with cool air and the chromatogram run immediately in 0.5% HOAc for 5–8 hr in darkness or dim light at 4°. Bands corresponding to EP_1 and EP_2 were detected and excised on the wet chromatogram adjacent to a blue fluorescence induced by irradiation of a guide strip with long wavelength UV light for about 1 min. Breakdown of EP compounds occurred if the chromatogram was dried. The compounds were stable in bands held at −10° for several days and were eluted with Me_2CO. The folded band, saturated with solvent, was squeezed tightly and the expressed liquid collected in a receiver held in ice. This elution cycle was repeated twice to achieve rapid efficient recovery of the EP compound. The Me_2CO in the total eluate was removed *in vacuo* close to 0°. The residual aqueous HOAc soln was extracted 2× with Et_2O, the Et_2O extract washed with H_2O and then taken to dryness. Traces of residual HOAc in the recovered sample were essential to stabilize the EP compound which otherwise underwent transformation to OC and general breakdown.

Transformation of EP compounds quantitatively to OC. Controlled addition of alkali was essential to achieve quantitative conversion of EP compounds to OC since this occurred effectively in 1–2 min only in the pH range 9–11. With insufficient alkali, conversion was slow and substantial general breakdown of the EP compounds occurred. Excessive alkali caused significant contamination of the OC with 7,4'-dihydroxyflavonol which was formed competitively under such conditions. Thus to convert EP compounds in 0.05 M Tris buffer pH 8 to OC, 25 μl 2.5 N NaOH per 2 ml buffer was added and in EtOH soln 5 μl 1.25 N NaOH was used. Empirical addition was made with eluates containing variable amounts of HOAc from paper chromatograms.

Isolation of ψOC. ψOC arose when samples of EP compounds (Et_2O extracted from pH 3 soln) were held as moist solid for a few hr at room temp prior to chromatography. The compound was also formed when reaction product mixtures of EP compounds in aq Me_2CO or EtOH were conc at room temp and placed in ice. ψOC separated as a white solid sparingly soluble in Me_2CO or EtOH. UV: (EtOH) 236, 273, 320; (NaOH) 381 nm (=OC anion).

Competitive formation of OC and flavonol from EP compounds as a function of alkali concentration. An aliquot of an enzyme product mixture of EP compounds, dissolved in 85% EtOH (100 μl, equivalent to 350 μg of OC), was added with rapid mixing to 3.9 ml of freshly prepared NaOH, (final concn given in Fig. 3) at room temp. After 30 sec the soln was acidified with a min of HCl and the product extracted into Et_2O (2 × 5 ml). OC and flavonol, separated by 1-D chromatography of a portion of each product mixture in BeAW, were eluted in 85% EtOH and estimated by *A* at 356 nm. Flavonol, $E^{1\%}_{1cm} = 0.78 \times 10^3$.

Preparation and isolation of TMSi ethers. TMSi ethers of EP compounds, OC and 7,4'-dihydroxyflavonol, were prepared and purified by GLC for MS. A sub-mg amount of each dry compound was combined with 50–100 μl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide in a reactivial. The mixture was allowed to react for at least 1–2 hr at room temp and was then analysed by GLC or GC–MS. Glass columns (4 mm ID × 2 m) packed with silylated 80–100 mesh Gas Chrom P and coated with 10% SE-30 were used, with temp programming from 200–240° at 1°/min. Inlet temp. was 230°, and He was at 50 ml/min.

Preparation of OC–Me ether. Dry OC (90 mg) was dissolved in 100 ml of dry Et_2O containing 5% MeOH and the soln cooled to 0°. CH_2N_2 (300 mg) was added in Et_2O and the mixture was left for 1 hr at 0° and then a further 1 hr at room temp. Solvent evaporation afforded OC-Me ether as a light yellow solid. Crystallization from Me_2CO–EtOH gave

fine needles. TLC on Si gel (C_6H_6–EtOH, 10:1) revealed only one spot, brown–purple in UV light, colour unaffected by exposure to NH_3, R_f 0.48 compared with OC R_f 0.35. The compound melted at 205–225° (decomp.). UV: (EtOH) 232, 322; (NaOAc) 322 nm; (NaOH) decomp. $E^{1\%}_{1cm} = 860$ at 322 nm. IR: 3150, 1705, 1650, 1625, 1600, 1280, 1020, 870 cm^{-1}.

Hydrogenolysis of OC and OC-methyl ether. Both compounds were hydrogenated in 95% EtOH over 10% Pd/C catalyst at room temp. and pres. Uptake of H_2 was very rapid (first mol equivalent in 3 min). OC (120 mg) or OC-Me ether (90 mg) in 60 ml EtOH was added to catalyst (200 mg) in 40 ml EtOH. When either 1 or 2 mol equivalents of H_2 had been taken up the catalyst was quickly filtered off, the filtrate dried and products examined by 2-D chromatography. Hydroquinone was identified as a product by chromatographic and UV comparison with an authentic sample.

Effect of Cu^{2+} on the enzymic product. Reaction mixtures containing chalcone (1.8 μmol), HRP enzyme (6 units), Cu^{2+} (2–4 mM) and H_2O_2 (4 μmol) in 4 ml 0.05 M tris buffer pH 8.0 were incubated for 15 min at room temp. Alkali (50 μl 2.5 N NaOH) was then added followed 2 min later by acid (150 μl 1.44 N HCl). The products were analysed by 2-D chromatography and *p*-hydroxybenzaldehyde (yield 30–36 μg) was identified by comparison with authentic material.

Degradation of OC and EP compounds by 2 N HCl. OC or EP compounds (1 mg in 0.1 ml EtOH) was added to 4 ml 2 N HCl and after 1 min the aq mixture was extracted with Et_2O and the products examined by chromatography. Hydroquinone was obtained in 30–40% yield under these conditions. *p*-Hydroxybenzaldehyde was also found as a product from runs with the EP compounds.

Optical rotations. Measurements were made with an automatic polarimeter, cell path length 2 cm, at room temp. Compounds were dissolved in 85% EtOH, concentrations of EP compounds 0.1–0.6 g/100 ml.

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