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AURONE BIOSYNTHESIS—II.

FORMATION OF 4',6-DIHYDROXY-2-(α-HYDROXYBENZYL)COUMARANONE FROM 2',4,4'-TRIHYDROXYCHALCONE BY CELL-FREE EXTRACTS OF SOYBEAN[1]

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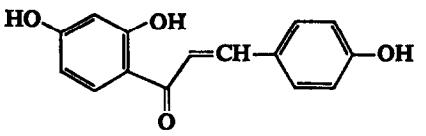
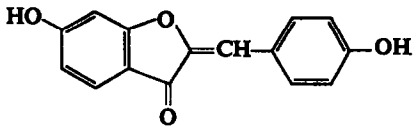
Abstract—Two-dimensional paper chromatographic analysis of the products of incubation of 2',4,4'-trihydroxychalcone with cell-free extracts of soybean revealed the presence of two compounds, which on the basis of u.v. spectral and chromatographic evidence were postulated to be diastereoisomeric forms of the hitherto unknown 4',6-dihydroxy-2-(α-hydroxybenzyl)coumaranone. This has now been confirmed by synthesis. Two diastereoisomers were obtained and found to be identical in chromatographic and spectra properties with the incubation products. The α-hydroxybenzylcoumaranone very readily dehydrates to 4',6-dihydroxyaurone, and is very likely an intermediate in the biosynthesis of the aurone from the chalcone.

INTRODUCTION

IN A previous paper[1] the enzymic conversion of 2',4,4'-trihydroxychalcone (I) (isoliquiritigenin) to the corresponding 4',6-dihydroxyaurone (II) (hispidol) has been reported. Further examination by means of two-dimensional paper chromatography of the products of incubation of isoliquiritigenin with cell-free extracts of soybean seedlings has revealed the presence of other products. Two of these, designated Y_1 and Y_2, were found to be very readily converted into the aurone (II). On the basis of chromatographic and u.v. spectral evidence these two products were postulated as being stereoisomeric modifications of the hitherto unknown compound 4',6-dihydroxy-2-(α-hydroxybenzyl)coumaranone (III). A preliminary note on these findings has been published.[2]



(I)



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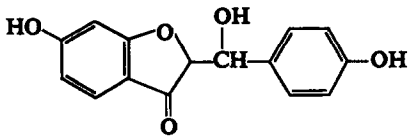


(III)

[1] Part I: E. WONG, *Phytochem.* **5**, 463 (1966).
[2] E. WONG, *Chem. Ind.* (*London*) 598 (1966).

The postulated α-hydroxybenzylcoumaranone structure (III) has now been synthesized. Two isomers were again produced and were found to be identical with Y_1 and Y_2 in chromatographic and spectral properties. All evidence is consistent with the view that Y_1 or Y_2 is a biosynthetic intermediate between isoliquiritigenin and hispidol.

RESULTS

Formation and Properties of Y_1 *and* Y_2

Cell-free extracts of soybean seedlings in tris buffer, pH 7·5, were incubated with isoliquiritigenin at 37° and phenolic products were examined by two-dimensional chromatography. 4',7-Dihydroxyflavanone (liquiritigenin)[3] and hispidol (II)[1] have previously been found to be the main products of the enzymic transformations. Hispidol exhibits characteristic yellow–green fluorescence under u.v. light, changing to yellow when fumed with ammonia. Two other spots which separated on the chromatogram were designated Y_1 and Y_2 and found to have the same characteristic fluorescent colours as hispidol. These colours, however, were observed for Y_1 and Y_2 only on chromatograms kept for a day or longer, indicating that the colours were probably due to artefact formation. The unknown compounds however could be

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF Y_1 AND Y_2

Compound	R_f		
	BeAW	30% HOAc	5% HOAc
Y_1	0·22	0·86	0·74
Y_2	0·22	0·76	0·54

Both had a bright yellow–green fluorescence in u.v. light on standing (see text) changing to yellow with NH_3. This gave an orange–brown with diazotized sulphanilic acid.

detected immediately by means of their colour reactions with diazotized sulphanilic acid and ferricyanide–ferric chloride sprays (Table 1). On elution and rechromatography, both Y_1 and Y_2 yielded some hispidol as an artefact; thus both Y_1 and Y_2 are capable of spontaneous conversion to the aurone under chromatographic conditions.

Microgram amounts of Y_1 and Y_2 were isolated and purified by means of preparative paper chromatography. The u.v. spectral properties of these two compounds were found to be almost identical. The spectra (Fig. 1) are typically those of hydroxyacetophenone congeners and are almost identical with those of 3,4',7-trihydroxyflavanone.[4] Reduction tests with magnesium–hydrochloric acid, sodium borohydride and sodium amalgam–hydrochloric acid however gave negative results, indicating that the compounds could not be flavanones. Alkaline silver nitrate oxidized these compounds only very slowly.

Since the properties of Y_1 and Y_2 are closely similar, differing significantly only in R_f in aqueous solvents, they were thought to be isomeric forms of the same enzymic product, with one being an artefact derived from the other. This view was confirmed by experiments in which either Y_1 or Y_2 was incubated at 37° variously with fresh and boiled enzyme extract and

[3] E. WONG and E. MOUSTAFA, *Tetrahedron Letters* 3021 (1966); E. MOUSTAFA and E. WONG, *Phytochem.* **6**, 625 (1967).
[4] E. WONG, P. I. MORTIMER and T. A. GEISSMAN, *Phytochem.* 4, 89 (1965).

with buffer (pH 7·5). In all cases a mixture of Y_1 and Y_2 and hispidol was obtained, as revealed by paper chromatography, showing that Y_1 and Y_2 are readily interconverted under non-enzymic conditions. The spontaneous conversion of Y_1 and Y_2 to hispidol during chromatography has previously been mentioned; no conversion of Y_1 to Y_2 or of Y_2 to Y_1 however appears to take place under these conditions.

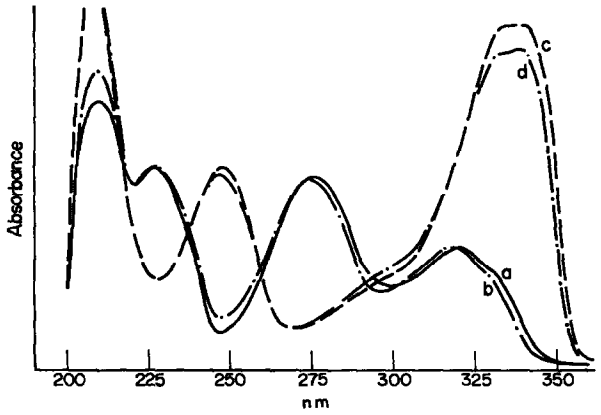


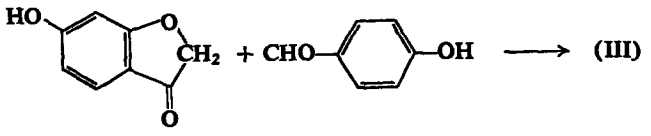
FIG. 1. U.V. ABSORPTION SPECTRA OF Y_1 AND Y_2.
(a) Y_1; (b) Y_2, in 85% ethanol; (c) Y_1; (d) Y_2, with added sodium hydroxide (0·003 N).

On the basis of the available evidence it was postulated that Y_1 and Y_2 are diastereoiscmerides of 4',6-dihydroxy-2-(α-hydroxybenzyl)coumaranone (III). This β-hydroxyketone structure would be expected to dehydrate very readily to the aurone. Compounds of this type have not previously been described but have been generally assumed to be reactive chemical intermediates.[5]

Because of the extremely low yield obtainable from enzymic incubation experiments, proof of the proposed structure was sought in chemical synthesis.

Chemical Synthesis of Y_1 *and* Y_2

Two compounds identical with Y_1 and Y_2 in chromatographic behaviour and u.v. spectral properties were synthesized in very low yield by the mild alkaline condensation of 6-hydroxycoumaranone (IV) with *p*-hydroxybenzaldehyde (V). The condensation of a



(IV) (V)

coumaranone with a benzaldehyde is a general reaction for the synthesis of aurones.[6] Such reactions are assumed to proceed via the α-hydroxybenzylcoumaranone as the aldol intermediate[7] although isolation of such an intermediate in aurone synthesis has hitherto not been

[5] F. M. DEAN and V. J. DODIMUANG, *J. Chem. Soc.* 3978 (1965).
[6] T. A. GEISSMAN and J. B. HARBORNE, *J. Am. Chem. Soc.* 77, 4624 (1955).
[7] H. O. HOUSE, *Modern Synthetic Reactions*, p. 216. Benjamin, New York (1965).

reported. By carrying out the reaction at low temperatures in dilute alkali, and working up the reaction mixture under very mild conditions, two products identical with Y_1 and Y_2 were obtained. These were detected chromatographically and were isolated by means of preparative paper chromatography.

The two compounds were almost colourless when freshly isolated but very readily became yellow owing to partial conversion to the aurone. This dehydration to hispidol is catalysed by dilute alkali and dilute acids. Under both conditions Y_1 was found to dehydrate at a faster rate than Y_2 (see Experimental). The greater instability of Y_1 was noticed also during isolation. On standing, both in the solid state or in solution, purified samples of Y_1 or Y_2 were found to revert gradually to a mixture of Y_1 and Y_2. The unstable nature of Y_1 and Y_2 is also reflected in their lack of definite melting points. Both compounds soften at about 190°, then gradually sinter to yellow needles which eventually melt with decomposition at about 280–290°. This is again most likely due to dehydration to the aurone on heating.

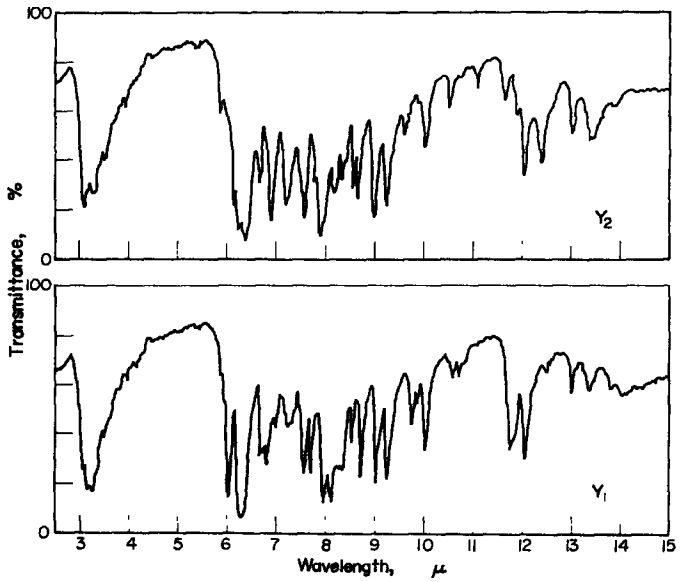


FIG. 2. I.R. SPECTRA OF SYNTHETIC Y_1 AND Y_2 (KBr DISC).

The i.r. spectra (KBr disc) of synthetic Y_1 and Y_2 are shown in Fig. 2. The two spectra are similar but not identical, as is expected of diastereoisomers. The NMR spectra[8] of synthetic Y_1 and Y_2 (in deuterated dimethyl sulphoxide, 60 Mc/s) are consistent with their being the α-hydroxybenzylcoumaranone (III). The aromatic signals could be assigned to patterns analogous to those found for similarly substituted flavonoid compounds,[9] although in the spectrum of Y_2 considerable overlapping of some of the peaks occurs. The signals for the two non-aromatic C—H protons of Y_2 appear as a broad multiplet centred at about $\delta = 5{\cdot}0$ and a doublet ($J = 2{\cdot}5$ c/s) at $\delta = 4{\cdot}74$. The former was assigned to the α-proton, with couplings to the protons at C_2 and the α-hydroxyl group. The proton of the alcoholic α-hydroxyl group was manifest as a broad doublet ($J = 5$ c/s) centred at $\delta = 5{\cdot}38$. In the case of Y_1 the peaks for

[8] The author thanks Dr. B. R. Thomas (D.S.I.R., N.Z.) and Dr. T. J. Batterham (A.N.U., Canberra) for measurements of NMR spectra.

[9] T. J. BATTERHAM and R. J. HIGHET, *Australian J. Chem.* **17**, 428 (1964).

the two non-aromatic protons overlap to give a multiplet centred at about $\delta = 4{\cdot}9$. A broad peak centred at about $\delta = 5{\cdot}7$ was assigned to the α-hydroxyl proton. Chemical shift assignments are summarized in Table 2.

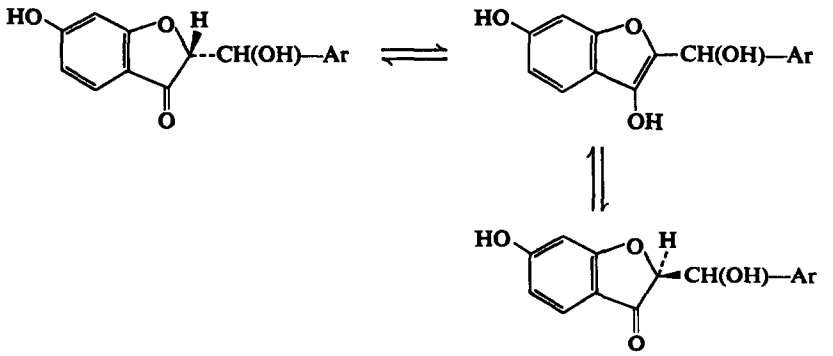
TABLE 2. CHEMICAL SHIFTS OF SYNTHETIC Y_1 AND Y_2 (δ, PPM)*

Compound	H_4	$H_{2',6'}$	$H_{3',5'}$	H_5	H_7	α-OH	H_α	H_2
Y_1	7·27	7·09	6·58	6·44	6·38	5·67	4·90	
Y_2	7·43	7·29	6·73	6·55	6·47	5·38	4·99	4·74

* NMR spectra were taken in deuterated dimethyl sulphoxide.

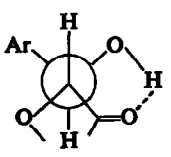
DISCUSSION

The proposed structure for Y_1 and Y_2, that of 4',6-dihydroxy-2-(α-hydroxybenzyl)-coumaranone (III) is consistent with all available evidence. Y_1 and Y_2 are thus the diastereoisomeric *threo-* and *erythro-* forms of this compound and their co-occurrence is readily explicable in terms of facile epimerization at C_2 via keto–enol tautomerism:

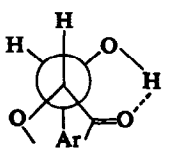


Ar = *p*-HO.C_6H_4-

The interesting question arises as to which of the diastereoisomeric forms is Y_1 and which is Y_2. A possible answer may be gained through consideration of the relative reactivities of the two forms.[10] In the *erythro-* isomer intramolecular hydrogen bonding can take place between the α-hydroxyl and the carbonyl groups with a sterically favourable conformational arrangement of substituent groups (VI). In the *threo-* isomer such a hydrogen-bonded conformation (VII) would result in the aryl group overlapping with the aromatic A-ring. Hydrogen bonding



erythro-
(VI)



threo-
(VII)

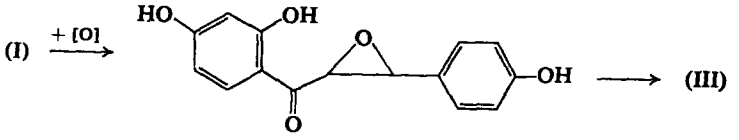
[10] E. L. ELIEL, *Stereochemistry of Carbon Compounds*, p. 124. McGraw-Hill, New York (1962).

would thus be less likely for steric reasons. The *erythro-* isomer is therefore expected to be more readily hydrogen bonded and thus the more stable isomer. If the relative reactivity of the two isomers is dependent only on the difference in ground state energies, as seems likely here in the case of their dehydration reactions, then since Y_2 is the isomer which is less readily dehydrated it is probable that it is the more stable *erythro-* isomer.

The chemical structures of Y_1 and Y_2 and their ready convertibility to aurone strongly suggest that one or other is an intermediate in the enzymic conversion of isoliquiritigenin to hispidol. The biosynthesis of aurone from chalcone thus proceeds via the sequence:

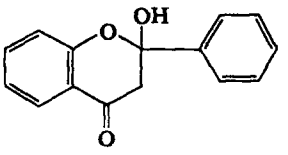
$$\text{Chalcone} \xrightarrow{+[O]} \alpha\text{-hydroxybenzylcoumaranone} \xrightarrow{-H_2O} \text{aurone}$$

The biochemical mechanism for the conversion of the chalcone to the α-hydroxybenzylcoumaranone is now of interest. This transformation involves a net gain of one oxygen atom and is likely to require at least two steps. One of several possible mechanisms for oxygenation is via the chalcone epoxide (VIII). Such a mechanism has been proposed[5] for the chemical conversion of chalcones to aurones by H_2O_2. Biochemically such transformations would involve enzymes of the hydroxylase and isomerase types. Although the dehydration of the α-hydroxybenzylcoumaranone to aurone can occur readily, it is probable that *in vivo* this step is also enzyme catalysed.

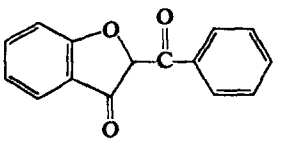


(VIII)

It is noteworthy that the α-hydroxybenzylcoumaranone structure is formally analogous to the unknown 2-hydroxyflavanone structure (IX). 3-Hydroxyflavanones are of course well known and the corresponding 2-hydroxy-2-benzylcoumaranone structure has in recent years been exemplified in several natural products.[11] In connexion with the present findings, the recent report[12] of the existence of an aroylcoumaranone (X) derivative in nature is of interest. It is likely that such a structure is also derived biosynthetically from an α-hydroxybenzylcoumaranone, in this case by a dehydrogenation reaction. Such a step would be formally analogous to the oxidation of a 3-hydroxylflavanone to a flavonol.



(IX)



(X)

EXPERIMENTAL

Enzymic Formation of Y_1 *and* Y_2

Batches of 80 g of commercial soybean were germinated for about a week at 25°. The seedlings (*ca.* 200 g) were macerated in a Waring blender with 100 ml of 0·05 M tris-HCl buffer (pH 7·5). After straining through

[11] A. J. BIRCH, E. RITCHIE and R. N. SPEAKE, *J. Chem. Soc.* 3593 (1960); H. G. C. KING, T. WHITE and R. B. HUGHES, *J. Chem. Soc.* 3234 (1961).

[12] R. HANSEL, H. RIMPLER and R. SCHWARZ, *Tetrahedron Letters* 1545 (1965).

muslin the mixture was centrifuged for 30 min at 27,000 *g* and the supernatant was dialysed against the same tris buffer for 48 hr at 2°. The crude dialysed extract (100 ml) was incubated with isoliquiritigenin (10 mg dissolved in 2 mole equivalent of 0·1 N NaOH) at 37°. After 1½ hr the mixture was boiled with an equal volume of ethanol and filtered. The alcoholic solution was concentrated *in vacuo* to a small volume and the aqueous residue extracted repeatedly with ether. The ether-soluble material after evaporation was taken up in 1 ml of ethanol and used for chromatography. Appropriate blank experiments were carried out using enzyme extracts inactivated by heating for 15 min in a boiling water bath.

Chromatography

General procedures and solvent systems were as previously described.[1,4] For two-dimensional chromatography 100–200 μl of the ethanolic solution was spotted on 3 MM paper and developed in the solvent system BeAW–30% HOAc. Y_1 and Y_2 could be located on chromatograms after keeping overnight by their yellow-green fluorescence under u.v. light. They can be located on fresh chromatograms by spraying with diazotized sulphanilic acid (Table 1).

Preparative paper chromatography for the isolation of Y_1 and Y_2 was carried out successively in BeAW and 5% HOAc on washed 3 MM paper. The amount of Y_1 and Y_2 that could be isolated by this means from an incubation experiment was about 0·5 mg, as measured spectrophotometrically ($E^{1\%}_{1cm}$, $Y_1 = 5{\cdot}52 \times 10^2$, $Y_2 = 5{\cdot}36 \times 10^2$, determined with synthetic material).

Synthesis of 4′,6-Dihydroxy-2-(α-hydroxybenzyl)coumaranone

To an ice-cold solution of 6-hydroxycoumaran-3-one (IV)[13] (4·56 g) in 300 ml 0·2 N NaOH (2 mole) 3·68 g of *p*-hydroxybenzaldehyde (V) was added with stirring. After standing at 2° for 3 days the solution was treated with solid CO_2 and the precipitate filtered off. This precipitate (4·6 g) consisted essentially of unchanged coumaranone. The aqueous filtrate was extracted with 3 × 400 ml ether and the combined ether extract concentrated *in vacuo* to dryness. Methanol (10 ml) was added to the solid residue and the excess solid, which consisted mainly of *p*-hydroxybenzaldehyde, was filtered off. The methanolic solution was chromatographed first in BeAW (16 papers) which separated the aldehyde, the coumaranone and the aurone. The Y_1, Y_2 band was then rechromatographed in 5% HOAc which separated the two isomers and the hispidol artefact. Bands were eluted in the cold with 85% ethanol and evaporated *in vacuo* below 40°. The products thus isolated (Y_1, 120 mg; Y_2, 30 mg) were essentially pure when freshly prepared and were used for the various spectral studies. Attempts to purify the products by crystallization from aq. EtOH were unsuccessful. The solubility of Y_2 in methanol and ethanol was found to be much less than that of Y_1. The lower yield after isolation of synthetic Y_2 is attributable to this fact. The synthetic compounds were found to be optically inactive. They are thus racemic modifications of the diastereoisomers.

Dehydration Experiments

Relative rates of dehydration were followed with a Beckman DK-2 recording spectrophotometer. To 2 ml of a standard solution of Y_1 or Y_2 (2 mg% in ethanol) in a cuvette was added 10 μl of 5% NaOH (final OH^- concentration approx. 0·006 N) and the change of E_{max} at 455 nm due to the anion of the hispidol[1] formed was followed over a 2-hr period at room temperature. For acid dehydration 50 μl of conc. sulphuric acid was added to 2 ml (final H^+ concentration approx. 0·9 N) and the change of E_{max} at 380 nm for hispidol[1] was followed. Initial rates were calculated graphically from the plots of E_{max} vs. time. Relative rates (Y_1/Y_2) of dehydration found were 2·6 (alkaline) and 1·7 (acid).

[13] R. L. SHRINER and M. WHITE, *J. Am. Chem. Soc.* **61**, 2328 (1933).