Phytochemistry, Vol. 28, No. 1, pp. 235–238, 1989.
Printed in Great Britain.

0031–9422/89 $3.00 + 0.00

OXYGENATION OF 4-HYDROXYCHALCONES CATALYSED BY PEROXIDASE AND BY LIGHT

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(*Received* 29 *February* 1988)

Key Word Index—Oxygenation; photooxygenation; peroxidase; 4-hydroxychalcones; 2′,4-dihydroxy-4′-methoxychalcone.

Abstract—Peroxidase or light catalysed oxygenation of 2′,4-dihydroxy-4′-methoxychalcone produced two labile initial products characterized as stereoisomers of 4′-hydroxy-6-methoxy-2-(α-hydroperoxybenzyl) coumaranone. By analogy, the products from similar oxygenation reactions of 2′,4,4′-trihydroxychalcone previously studied are now considered to be hydroperoxy-benzylcoumaranones rather than dioxetanes.

INTRODUCTION

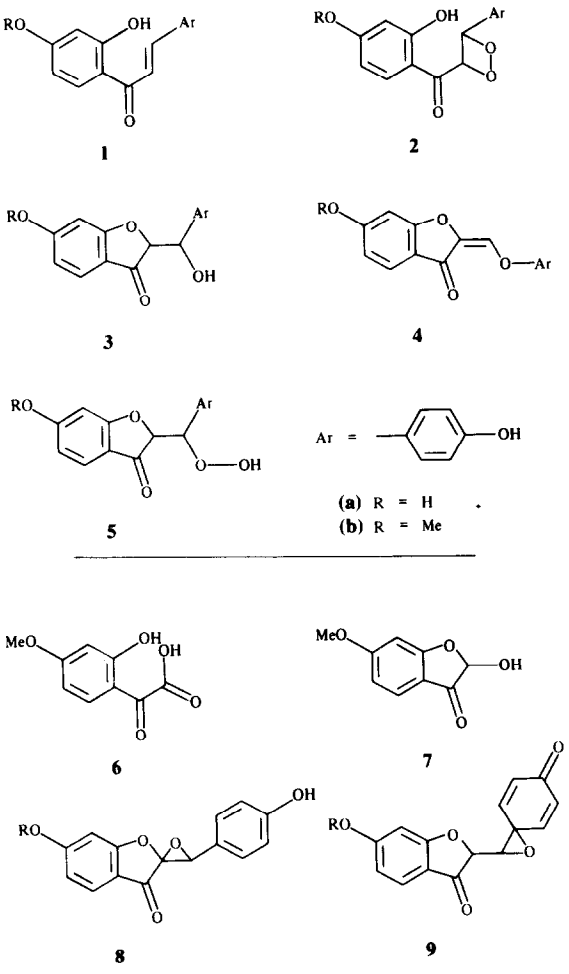
Our previous studies on the oxygenation of 2′,4,4′-trihydroxychalcone (**1a**) catalysed by peroxidase [1, 2] and by light [3] have led to the proposal that the dioxetane (**2a**) constitutes the initial product of oxygenation. The hydroperoxide tautomer (**5a**), has been postulated [2, 3] to be the probable intermediate in the facile transformation of this initial product to the end-products (**3a**) and (**4a**).

We have now carried out analogous enzymic and photochemical studies on the structurally related chalcone (**1b**). Two labile initial products have again been obtained and their spectral and chemical properties examined. The possibility that they represent diastereoisomeric forms of the hydroperoxide (**5b**), rather than those of the dioxetane (**2b**) expected on the basis of the earlier proposal, forms the subject of this paper. As before a whole series of subsequent transformation products was also obtained, paralleling closely those obtained from reactions with chalcone (**1a**).

RESULTS

By carrying out the enzymic reaction at a slightly lower pH (pH 7), and by selective final extraction of the reaction mixture with 40% aqueous methanol, it was found possible to obtain a clean mixture of the initial products accompanied by very little degradation material. This mixture, when freshly obtained, was used for the spectral and chemical studies without further separation. The use of HPLC has enabled these labile products to be monitored more quickly and more conveniently than was possible in the earlier studies [1]. On keeping, the initial product mixture invariably gives rise to other transformation products, the most prominent of which is generally 4-hydroxybenzaldehyde.

Reduction of the product mixture with tin(II)chloride cleanly converted it into a mixture of the two diastereoisomers of the hydroxybenzylcoumaranone (**3b**), identified by spectral comparisons (UV, NMR) with those of its previously known [4] congener (**3a**), and by the ready conversion of the individual isomers to 4′-hydroxy-6-methoxyaurone on treatment with alkali. The alcohol isomers (**3b**) are also common spontaneous products of the initial product mixture on standing, and on reactions



with other metal ions such as Fe^{2+} and Cu^{2+}. The initial product mixture is also readily reduced by potassium ioide.

On treatment with alkali to above pH 11, each of the two initial product forms showed UV spectral peaks about 425, 321 and 275 nm, with the first peak corresponding to that of 4′-hydroxy-7-methoxyflavonol anion. Reacidification of the mixture followed by TLC analysis revealed the presence of the flavonol, identified by its bright yellow fluorescence and by direct comparison with a synthetic sample.

Under milder alkaline conditions (*ca* pH 9–12) a product with properties analogous to those of the previously studied [1, 2, 5] quinol vinyl ether (**4a**) was obtained. Its ^{1}H NMR spectrum confirms its identity as the corresponding methyl derivative (**4b**), prepared previously [1] from methylation of (**4a**).

As with the earlier work, Cu^{2+} was found to degrade the product mixture very readily. Among degradation products identified were 4-hydroxybenzaldehyde, the keto-acid (**6**), and 4-hydroxy-, and 2-hydroxy-4-methoxybenzoic acid. The identity of the keto-acid (**6**) was confirmed by comparisons with the two synthetic products obtained from selenium dioxide oxidation of 4-*O*-methylresacetophenone, with properties in accord with structures (**6**) and (**7**). The hemiacetal (**7**) readily converts to the acid (**6**) in alkaline solution, and on reaction with Cu^{2+} under similar conditions as used for the initial product mixture. The alcohol isomers (**3b**) generally also show up as Cu^{2+} reaction products. In contrast to Cu^{2+}, addition of Ni^{2+} or Co^{2+} to the initial product mixture produced little change.

NMR spectral data for the initial product mixture and those for the hydroxy reduction products are summarized in Tables 1 and 2. Assignments of ^{13}C NMR chemical shifts are based on standard compilations for flavonoid compounds [6] or on selective decoupling experiments. The ^{13}C NMR spectrum of the initial product mixture was confined to the more intense signals only due to the low concentration of sample material available and its propensity to decompose over the time needed to accumulate the spectrum. Two peaks at δ 86.0 and 87.7 ppm were observed only at the early stages of the multiscan experiment, reflecting their association with the labile side chain carbon centres (2-C, α-C) of the initial product.

Dye-sensitized photochemical oxygenation experiments, carried out essentially as previously described [3], produced product mixtures consisting largely of the same two initial products as were produced in enzymic experiments.

DISCUSSION

The chemical properties of the labile initial product isomers studied in this work parallel exactly those of the enzymic product isomers (EP_1, EP_2) studied previously [1]. The properties are in general accord with those of dioxetanes [7–9], and this has earlier led to the dioxetane structural proposal (**2a**) for the EP compounds. On the basis of the additional chemical results presented in this work, particularly those relating to reactions with metal ions [10, 11], we now consider that the data are more consistent with the initial products being the hydroperoxides (**5a**) and (**5b**) respectively in the two studies. The close similarity in decomposition products patterns and the difficulty of differentiating between dioxetanes and their hydroperoxide analogues have been well emphasized by Frimer [12].

The spectral properties of the initial products for both series, again though not inconsistent with those of dioxetanes [7–9], are in fact strikingly similar to those of their respective reduction products, the hydroxybenzylcoumaranones (**3a**) and (**3b**). The ^{13}C NMR data from the present work, in particular, lend further support to the alternative hydroperoxy-benzylcoumaranone structures (**5**) for the initial products. The chemical shift assignments for 2-C and α-C of the hydroxy products (**3b**) are based on unambiguous selective decoupling experiments, with specific irradiation at the frequencies of signals in the ^{1}H NMR spectra, and the increase of 12–13 ppm in

Table 1. ^{1}H NMR spectral data for the initial-product mixture (**5b**), ($IP_1 + IP_2$); reduction products (**3b**) (RP_1, RP_2); and related coumaranone compounds.

Proton*	Mixture ($IP_1 + IP_2$)†	RP_1 (3b)	RP_2 (3b)	Vinylether (4b)	Hemiacetal‡ (7)	Ketoacid‡ (6)
4		7.34	7.48	7.61	7.49	7.67
5		6.54	6.65	6.83	6.64	6.45
7		6.57	6.66	6.91	6.56	6.39
α	5.36	5.07	5.16	7.31		
	(2.2)	(3.5)	(1.7)			
2	5.22, 4.81	4.87	4.73		5.49	
	(2.1) (2.1)	(3.6)	(2.0)			
2′6′		7.21	7.41	7.16		
3′5′		6.66	6.83	6.89		
O-Me	3.84, 3.91	3.86	3.91	3.96	3.87	3.78

Spectra were recorded at 80 MHz for solutions in d_6-acetone (or ‡ in d_4-methanol). Chemical shifts are shown in δ values relative to tetramethylsilane, and coupling constants (J in parentheses) in Hz.

*For simplicity, numbering of the carbon atoms in the different structures follows those given to the equivalent carbon atoms in structures **3** and **5**.

†Assignment of peaks for individual aromatic protons not made.

Table 2. ^{13}C NMR chemical shift data (80 MHz, d_6-acetone) for the initial product mixture (**5b**), reduction product mixture (**3b**) and compounds **6** and **7**.

Carbon *	Mixture † (IP_1+IP_2)	Mixture (RP_1+RP_2)	Hemiacetal ‡ (7)	Ketoacid ‡ (6)
α	86.0	74.3, 73.2		
2	87.0	90.0, 90.3	99.8	166.3
3	—	191.4, 198.3	198.1	191.2
4	125.4	125.4	126.8	134.9
5	112.0	111.8	112.6	109.3
6	—	169.0	170.9	167.3
7	97.1	96.9, 97.2	97.5	101.9
8	—	176.8	175.4	169.0
9	117.2	116.3, 116.8	113.6	111.4
1′	132.8	132.7, 133.2		
2′6′	130.3	129.3, 128.7		
3′5′	115.5	115.3, 115.7		
4′	—	157.7		
O–Me	56.4	56.3	56.7	56.3

*For simplicity, numbering of the carbon atoms in the different structures follows those given to the equivalent carbon atoms in structures **3** and **5**.

†Assignment of peak for some carbon atoms not possible due to low sample concentration and instability.

‡In d_4-methanol.

δ values for α-C going from the reduction products to the initial products is in accord with that expected for a change of the substituent group from –OH to –OOH [13].

The hydroperoxide tautomer **5** has previously [2, 3] been postulated by us to be the progenitor of the two stable end-products of the coumaranone series **3a** and **4a**. In a paper appearing just as the present manuscript is being prepared, Begley *et al.* [14] have modified their scheme for the derivation of the quinol ether **4a** studied by them independently, and have now opted for the hydroperoxide **5a** as the key intermediate. They have also anticipated our changed views by suggesting, on the basis of the ^1H NMR data presented in our earlier work [1], that the hydroperoxide **5a** rather than the dioxetane **2a** better constitutes our enzymic product isomers EP_1 and EP_2.

One strong argument in support of the earlier dioxetane proposal was that this structure, with oxygenation at both α and β-carbon atoms in the side chain, readily accounts for the initial product's role as progenator of flavonols as well as compounds of the coumaranone series. In terms of the alternative hydroperoxybenzylcoumaranone structure (**5**) now proposed, transformation to the flavonol would be possible via the epoxide intermediate (**8**). This is an alternative dehydration product to (**9**), which has already been proposed [5] as an intermediate to account for the formation of the rearrangement product (**4**). Aurone epoxide intermediates have previously been invoked to account for the formation of flavonols from aurone oxidation by hydrogen peroxide [15]. Recognition of the hydroperoxybenzylcoumaranone structure as an actual precursor of flavonols now adds to a more detailed understanding of this mechanism.

The hydroperoxide structure now being proposed for the isolatible initial products for both the enzymic and photochemical oxygenation of both of the 4-hydroxychalcones, could still be envisaged as being derived from an earlier dioxetane intermediate via a route such as that previously suggested [1, 3]. Alternatively, as seems more likely, the hydroperoxide could itself directly represent the oxygenation product. In which case, with oxygenation now taking place at the carbon atom β to the carbonyl, the attacking species would have to be a nucleophilic agent such as the superoxide anion, rather than just molecular oxygen.

EXPERIMENTAL

HPLC. Products from various reactions were analysed by HPLC on 5 μ RP-18 cartridge columns (13 cm × 4.6 mm) with 40% MeOH followed by 60% MeOH (20 ml each, at 1 ml/min) as solvent. Peaks were identified from RRts (=1 for 4-*O*-methylresacetophenone) and UV spectral data obtained either from on-line scanning under stop-flow conditions, or after peak collection.

Standard enzymic reaction. To a solution of the chalcone (**1b**) (50 mg) in EtOH (5 ml) was added 500 ml of 0.05 M Tris buffer, pH 7.1, containing EDTA (0.005 M). To the resulting turbid mixture was added 250 μl of peroxidase enzyme solution (3 mg/ml of NBC horseradish peroxidase in 0.05 M Tris buffer, pH 8.0) followed by 2.5 ml of H_2O_2 (1.5%) and the whole left to stand at room temp. for 5 min. The mixture was then extracted with an equal vol. of Et_2O and the solid residue obtained from the Et_2O phase was triturated with 10 ml of 40% MeOH to give a solution of the products largely free of unchanged chalcone starting material. An aliquot of this solution was used for HPLC. The bulk of the material after removal of the MeOH *in vacuo* was freeze-dried to yield a straw coloured solid (8 mg) consisting largely (*ca* 75%) of the two stereoisomeric forms (IP_1, IP_2) of the initial product (**5b**). This material could be stored at −20° for about a week with little change.

IP_1: λ_{max} (40% MeOH) 322, 276 nm, ($+OH^-$) 426, 322, 274 nm, ($+AlCl_3$) 322, 276 nm. IP_2: λ_{max} (40% MeOH) 318, 274 nm, ($+OH^-$) 424, 320, 276 nm, ($+AlCl_3$) 318, 274 nm. 4′-Hydroxy-7-methoxyflavonol: λ_{max} (60% MeOH) 358 nm, ($+OH^-$) 425, 277 nm.

Photooxygenation. A solution of the chalcone (40 mg) in MeOH (50 ml) containing Methylene Blue (10^{-5} M) was flushed with N_2 for 5 min, kept cool in an ice bath and irradiated with a 1 kW incandescent lamp for 4 hr. The mixture was then evapd *in vacuo* and the residue partitioned in Et_2O (40 ml) and H_2O (3 × 15 ml). The Et_2O extract was evapd and taken up in MeOH (800 μl) as a stock solution for chromatography. In contrast to previous procedure [3], air was not passed through the solution during the irradiation. It was found that with this modification and with the prior deaeration by N_2 flushing, higher chalcone consumption and initial product yields were obtained.

Reduction with $SnCl_2$. To a standard enzymic reaction mixture as above, after the 5 min reaction but before the ether extraction stage, was added 1 g of solid $SnCl_2$ and the mixture allowed to stand for a further 5 min. The mixture was then Et_2O extracted and worked-up as above described. HPLC analysis of the final 40% MeOH soluble material revealed a nearly pure (*ca* 75%) mixture of the two reduction products (RP_1, RP_2), (**3b**), indicating essentially complete reduction of the initial products. The RP isomers were isolated individually by means of HPLC for spectral studies.

RP_1: λ_{max} (40% MeOH) 322, 278 nm, ($+OH^-$) 466, 330, 274 nm. RP_2: λ_{max} (40% MeOH) 320, 276 nm, ($+OH^-$) 466, 330, 274 nm. 4′-Hydroxy-6-methoxyaurone: λ_{max} (60% MeOH) 389 nm, ($+OH^-$) 466 nm.

Reaction with Cu^{2+}. To a standard enzymic reaction mixture, after the initial 5 min reaction, was added 1 g solid $CuCl_2 \cdot 2H_2O$ and the mixture allowed to stand for a further 2 hr. After filtering the mixture was acidified to pH 4.5 and Et_2O extracted, with the final products taken up in MeOH for HPLC and PC analysis (PC in 5% HOAc). Chief products from this reaction were 4-hydroxybenzaldehyde and the keto-acid (**6**).

Reactions with Cu^{2+} were also carried out with the initial product mixture after isolation. To a stock solution of the initial product in 40% MeOH (10 ml) was added 340 mg of solid $CuCl_2 \cdot 2H_2O$ and the whole left for 2 hr. After removal of the MeOH *in vacuo* the residue was partitioned between H_2O and Et_2O and the Et_2O phase was worked-up and analysed by HPLC and PC as before. Products were as above, but with the keto-acid (**6**) now occurring in the lactone form (see following Section). Similar reactions were carried out with Fe^{2+}, Ni^{2+} and Co^{2+}.

SeO_2 *Oxidation of* 4-*O*-*methylresacetophenone.* A solution of the acetophenone (1.45 g) and SeO_2 (2.4 g) in 90% EtOH (22 ml) was refluxed for 20 hr. After cooling the mixture was filtered and the filtrate concd to a syrupy residue which was then triturated with H_2O (2 × 20 ml). The combined aq. phase was adjusted to pH 7.5 and extracted with Et_2O, from which the main product (7) λ_{max} (40% MeOH) 326, 280 nm, ($+OH^-$) 338, 278 nm, was isolated by prep. PC. The aqueous ML was then acidified to pH 2 and again extracted with Et_2O, yielding the keto-acid (**6**) λ_{max} (40% MeOH) 314sh, 282 nm, ($+OH^-$) 354, 278 nm, ($+AlCl_3$) 352, 296 nm. Under acidic conditions, the keto-acid (6) converts to its lactone form, λ_{max} (40% MeOH) 325, 285 nm, ($+OH^-$) 330, 278 nm, ($+OH^-$ + time) 352, 276 nm, ($+AlCl_3$) 282 nm.

Acknowledgement — I thank Dr Geoff Lane (Biotechnology Division, DSIR) for the NMR spectral measurements and for helpful discussions.

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