Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion

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Plant polyphenols act as antioxidants mainly by trapping reactive oxygen species and by regenerating endogenous membrane-bound α-tocopherol (vitamin E). In both processes polyphenols are oxidized. Hence, knowledge of the oxidation mechanisms of polyphenols is important for an understanding of their antioxidant activity at the molecular level.

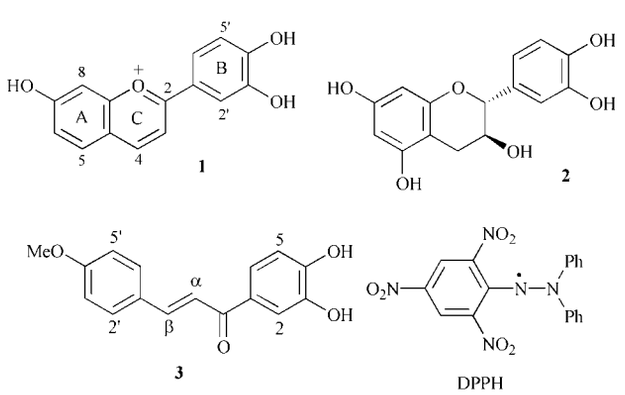
This work focuses on anthocyanins (pigments) and flavanols (tannins), two important classes of polyphenols which are both relatively abundant in human diet. The oxidation of the 3′,4′,7-trihydroxyflavylium ion (**1**) and catechin (**2**), respectively taken as models for anthocyanins and tannins, has been investigated. From kinetic data and partial product analysis, the mechanisms for the reactions of **1** and **2** with sodium periodate and DPPH, a H atom-abstracting radical, are proposed. Both polyphenols are shown to form *o*-quinone intermediates upon H atom abstraction and subsequent radical disproportionation. In the case of **2**, the quinone and a second molecule of antioxidant quickly couple to form dimers. By contrast, **1** is extensively degraded into coumarins by repeating sequences of oxidation–solvent addition, which consume several equivalents of oxidants.

In aqueous solutions, **1** is typically a mixture of coloured and colourless forms. The latter (chalcones) are also shown to take part in the antioxidant activity.

Introduction

Anthocyanins (pigments) and flavanols (tannins) are ubiquitous plant polyphenols which are relatively abundant in human diet, *e.g.* in vegetables, fruits, teas and red wines.[1] Recent epidemiological studies suggest that polyphenol-rich diets are correlated with a low risk of developing cardiovascular diseases, a major cause of mortality in occidental countries.[2] For instance, a moderate consumption of red wine could be beneficial (the so-called *french paradox*). In addition, a regular dietary intake of fruits and vegetables may reduce by a factor *ca*. 2 the occurrence of most types of cancer.[3] *In vitro* investigations strongly suggest that such protective effects are due to the well known antioxidant properties of polyphenols, which are particularly manifested by their ability to inhibit lipid peroxidation in plasma low-density lipoproteins (LDL),[4] an early cause of atherosclerosis. Flavonoids can exert their antioxidant properties *via* several mechanisms,[5] such as the trapping of reactive oxygen species (ROS, *e.g.*, hydroxyl, alkoxyl and peroxyl radicals) and the inhibition of enzymes involved in their production, the chelation of transition metal ions able to promote radical formation (Fenton reaction), and the regeneration of endogenous membrane-bound antioxidants such as α-tocopherol (vitamin E).

In the course of ROS trapping and vitamin E regeneration, the flavonoids are oxidized to short-lived radicals which quickly decay to nonradical oxidation–degradation products *via* complex pathways that may involve reactive quinones or quinonoid compounds as well as dimers and oligomers. In our opinion, the quantitative kinetic investigation of the chemical oxidation of flavonoids coupled to (partial) product analysis is an important complementary approach to investigations using fast techniques (*e.g.*, pulse radiolysis)[6] to obtain molecular information on the mechanism of antioxidant action. In particular, this approach not only allows us to compare the flavonoids in their ability to deliver H atoms (reaction with the DPPH radical) or electrons (autoxidation in alkaline conditions) but also provides a structural interpretation of the antioxidant stoichiometry (number of oxidant molecules reduced per molecule of antioxidant).[7] Finally, the reactivity, and eventual toxicity, of reactive intermediates such as flavonoid-derived quinones and quinonoid compounds may be assessed, particularly when the oxidation is conducted in the presence of biologically relevant molecules (*e.g.*, serum albumin) liable to be chemically modified by these electrophilic-oxidizing intermediates.[8] This approach has been recently applied to potent antioxidants from the flavonol family,[7,8] and is extended in this work to the 3′,4′,7-trihydroxyflavylium ion (**1**, Scheme 1) as a model for naturally occurring anthocyanins and to catechin (**2**), a ubiquitous monomeric flavanol. 3,4-Dihydroxy-4′-methoxychalcone (**3**)



Scheme 1

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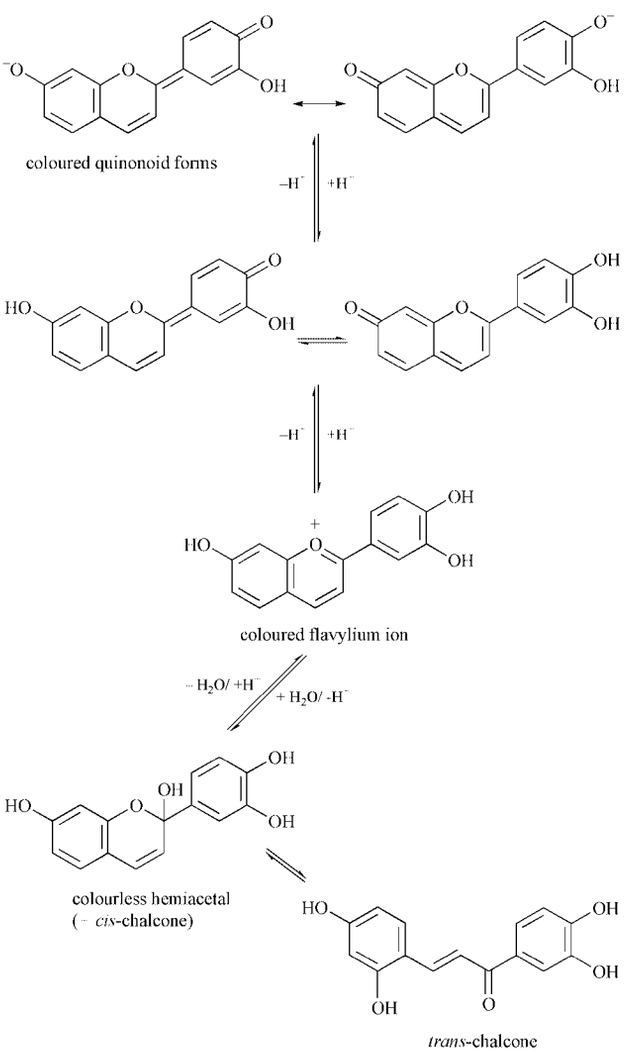
will also be considered as a model for the colourless forms of anthocyanin.

Results and discussion

Anthocyanins have been recently shown to efficiently trap lipid peroxyl radicals[4a,9] and other ROS such as superoxide[10] and nitric oxide.[11] The antioxidant efficiency of anthocyanins is, however, dependent on the anthocyanin structure.[9,12] For instance, a 1,2-dihydroxy substitution in the B-ring (catechol nucleus) is beneficial, probably because the *o*-semiquinone radical formed upon trapping of the ROS is more stable than simple aryloxyl radicals. In general, aglycons (anthocyanidins) are more powerful antioxidants than the corresponding 3-*O*-glycosides,[9b,c,12] although exceptions have been reported.[9a]

It must be emphasized that the interpretation of the antioxidant properties of anthocyanins is complicated by the relatively complex pathway of reversible structural transformations of anthocyanins in aqueous solution,[13] which not only includes proton transfer between coloured forms but also water addition to the pyrylium ring leading to colourless hemiacetal and chalcone forms (Scheme 2). Although the latter process is



Scheme 2

expected to be limited in natural media by the selective formation of stable π-stacking molecular complexes between the coloured forms and other (colourless) polyphenols called copigments,[14] it is very likely that both coloured and colourless forms contribute to the antioxidant properties of anthocyanins.[9a,e] Moreover, anthocyanidins are chemically unstable species except in strongly acidic solutions.[15] Hence, there is little doubt that investigations involving incubation of LDL or liposomes with anthocyanidins in neutral buffers for long periods of time[9b,c] (hours or even days) actually deal with the antioxidant properties of the degradation products.

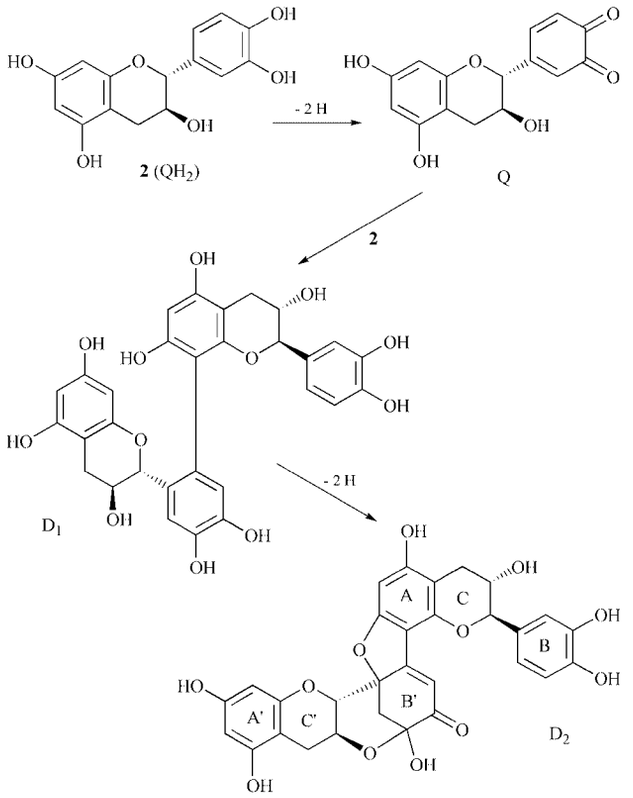
With the exception of a few of the most common pigments, anthocyanins are not commercially available and must be extracted from plants, *via* low yielding chromatographic procedures or chemically synthesized according to multistep pathways.[16] On the other hand, investigations dealing with simple hydroxylated flavylium ions, which can be easily synthesized on a gram scale, have already proved very helpful in understanding the pigmentation properties of anthocyanins.[16,17] Similarly, the antioxidant properties of **1** investigated in this work are expected to reflect those of naturally occurring anthocyanins which have a catechol group favourable to antioxidant activity.

Product analysis

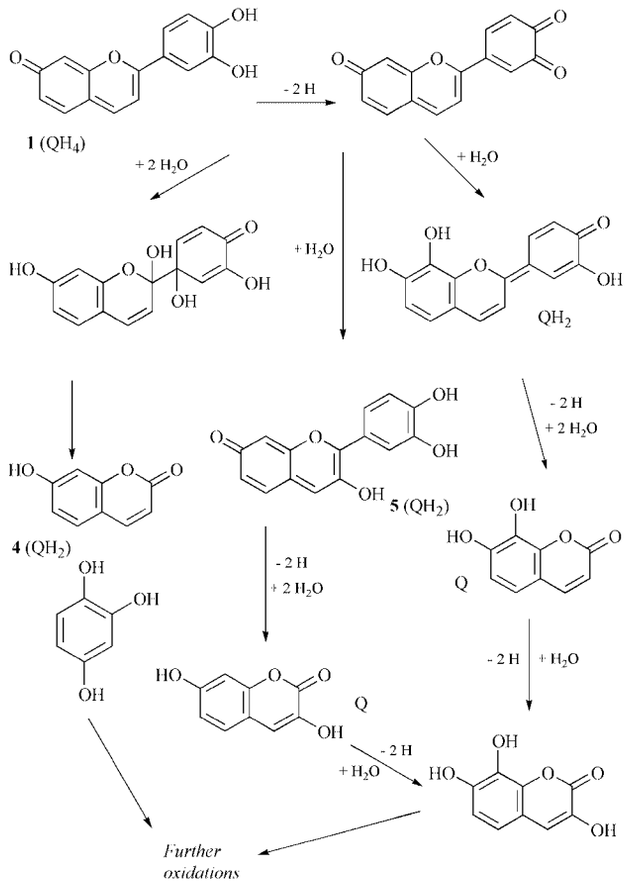
DPPH (2,2-diphenyl-1-picrylhydrazyl, Scheme 1) is a highly coloured commercially available radical widely used for estimating the ability of antioxidants to trap potentially damaging one-electron oxidants/H atom abstracting agents.[7,18] Addition of DPPH (2 equiv.) to a solution of **1** in CD_3OD followed by immediate recording of the ^{1}H-NMR spectrum did not allow us to detect oxidation products. In the case of **2** and under the same conditions, a significant amount of transient product builds up that may be the catechin quinone. For instance, the H-6′ signal is deshielded (*ca.* +0.5 ppm, masked by diphenylpicrylhydrazine signals) and the H-2′ and H-5′ signals are shielded (respectively, −0.41 and −0.33 ppm) with respect to the corresponding signals in **2** whereas the H-8 and H-6 signals are only weakly affected (less than 0.1 ppm). When treated with DPPH (2 equiv.) in the presence of sodium benzenesulfinate (1 equiv.) in MeOH, both **1** and **2** are rapidly converted into stable biarylsulfones. Such compounds result from regioselective Michael addition of the sulfinate anion on the 6′ position of quinone intermediates.

Methylation of the relatively complex mixture that was formed after the reaction of DPPH with **1** or **2** allowed product separation on silica. In the case of **2**, a yellow dimer could be isolated in low yield whose ^{1}H-NMR spectrum is consistent with the structures previously proposed for catechin dimers formed under mildly acidic enzymatic conditions[19] (polyphenol oxidase, pH 3.5) or mildly alkaline chemical conditions[20] (potassium ferricyanide, pH 9). Such dimers (D_2) could form *via* the following multistep procedure: nucleophilic attack of the electron-rich C-6 and C-8 atoms of the catechin A-ring on the electrophilic C-2′ and C-6′ atoms of the quinone B-ring to give colourless biphenyl-type dimers (D_1), oxidation of the electron-rich catechol moiety thus formed and subsequent intramolecular addition(s) to the *o*-quinone intermediate of suitably positioned OH groups. The D_2 dimer isolated in this work (Scheme 3) displays a ^{1}H-NMR spectrum which is very close to that of a yellow catechin dimer (isolated as its native non-methylated form) formed upon enzymatic oxidation of catechin and whose structure has been thoroughly characterized by ^{1}H- and ^{13}C-NMR and mass analyses.[19] The same structure, which results from an initial C-8–C-6′ coupling between catechin and its quinone, is thus proposed here, although C-6–C-6′ coupling may be equally probable. In particular, the structure of D_2 is consistent with the occurrence in the ^{1}H-NMR spectrum of two singlets at 6.49 and 6.26 ppm (H-5′ of the B′-ring, H-6 of the A-ring) and two doublets at 2.77 and 2.48 ppm with a coupling constant of 11 Hz (H-2′ of the B′-ring). The same dimer could be isolated after autoxidation of catechin in aerated alkaline solutions (saturated aqueous K_2CO_3) and subsequent methylation.

From the reaction of **1** with DPPH (2 equiv.) and subsequent methylation, 7-methoxycoumarin (**4**) and the quinonoid form



Scheme 3



Scheme 4

of the 7-hydroxy-3,3′,4′-trimethoxyflavylium ion (**5**) could be isolated after chromatography on silica gel. Alternatively, oxidation by sodium periodate (1 equiv.) in MeOH–0.2 mol dm^{-3} HCl (1 : 1) yielded 7-hydroxycoumarin, 7-hydroxy-3-iodocoumarin and 7,8-dihydroxy-3-iodocoumarin or 3,7-dihydroxy-8-iodocoumarin as the main products. The formation of iodinated compounds suggests that each of the four oxygen atoms of the periodate anion can be transferred to **1** and its oxidation products in the course of the oxidation. The iodide anion ultimately produced can then add to quinone or quinonoid intermediates (in competition with water molecules, see Scheme 4) to yield the iodinated compounds. When the periodate oxidation was conducted in MeOH–pH 3.5 acetate buffer (1 : 1) after previous conversion of **1** into the chalcone form (see below), only degradation products resulting from the B-ring and/or A-ring were evident by mass spectrometry, thus suggesting that an oxidative cleavage of the enone moiety took place.† Finally, under autoxidation conditions (0.2 mol dm^{-3} NaOH), only 7-hydroxycoumarin could be isolated in low yield.

Kinetic investigations

H atom transfer to DPPH. When monitoring the H transfer reactions from a flavonoid antioxidant to DPPH by UV-vis spectroscopy, two steps can be generally distinguished: a first step during which the DPPH visible absorbance decays quickly (typical time interval in MeOH: 1–3 min) and a second step during which the DPPH visible absorbance decays slowly over 15–60 min to a final constant value. The fast step essentially refers to abstractions of the most labile H atoms, whereas the slow step reflects the remaining activity in the oxidation–degradation products. A total stoichiometry can be determined in a static way from the overall amplitude of the kinetic run. A kinetic stoichiometry (n) can also be estimated from the curve-fitting of the kinetic traces featuring the decay of the DPPH band during the fast step according to a simple model in which the antioxidant is regarded as a source of n H atoms that are transferred to DPPH with the same rate constant.

Catechin. In the case of catechin, kinetic stoichiometries in the range 2.0–2.5 were repeatedly measured for the fast step. Such values are consistent with the formation of yellow dimers according to: 2 catechin + 4 DPPH→D_2 + 4 DPPHH. Higher total stoichiometries may reflect subsequent H abstractions from D_2, in particular on its catechol moiety (B-ring). A kinetic model can be used which takes into account the different steps in the reaction of catechin with DPPH (see Scheme 3):

– H abstraction from **2** (QH_2) by DPPH to form catechin aryloxyl radicals QH (rate constant k) that quickly disproportionate into QH_2 and its quinone (Q).

– Coupling between QH_2 and Q to give colourless dimers D_1 (rate constant k_c).

– H abstraction from D_1 by DPPH (rate constant k_1) and subsequent fast disproportionation of D_1 aryloxyl radicals into D_1 and yellow dimers D_2.

– H abstraction from D_2 by DPPH (rate constant k_2) and subsequent fast disproportionation of D_2 aryloxyl radicals into D_2 and the corresponding quinone.

In order to form quinones, the aryloxyl radicals may further react with DPPH or with themselves (disproportionation). The latter process is more probable since fast second-order decay of polyphenolic aryloxyl radicals in water ($2k$ in the range 10^6–10^7 dm^3 mol^{-1} s^{-1}) has already been reported in the case of flavonoids from pulse radiolysis experiments[6b–e] and in the case of caffeic acid from electrochemical investigations.[21]

Assuming a quasi-stationary state for the radicals, eqns. (1)–(5) were derived. The visible absorbance at λ_{max}(DPPH) is simply expressed as: $A = A_0$[DPPH]/c, A_0 and c being the initial

† From the mass and ^{1}H-NMR spectra of the mixture, the *o*-quinone of 3,4-dihydroxy-2-iodobenzoic acid (m/z = 279 in CI^+ mode, 2 doublets at 6.83 and 6.45 ppm with J = 8.1 Hz in CD_3OD) could be one of the most abundant products.

Table 1 Rate constants for H transfer reactions to DPPH in MeOH–aqueous buffer (1 : 1) at 25 °C. For a definition of the rate constants k_1 and k_2 see text. Values for the DPPH–antioxidant molar ratios (4, 6, 8) are specified in parentheses

Antioxidant	Buffer	n^a	k_1/dm^3 mol^{-1} s^{-1}	k_2/dm^3 mol^{-1} s^{-1}
1	Acetate (pH 3.5)	4.4 (200 s)–6.5 (1 h)	33.6 (±0.6) × 10^2 (8)	10.6 (±0.2) × 10^2 (8)
		3.0 (50 s)^b	29.4 (±0.4) × 10^2 (6)	10.9 (±0.2) × 10^2 (6)
		5.8 (900 s)^b	23.2 (±0.2) × 10^2 (8)^b	262 (±3) (8)^b
1	Phosphate (pH 7.4)	2.7–3.0 (50 s)	73.8 (±7.0) × 10^2 (8)	—
		4.2 (1 h)	64.9 (±1.8) × 10^2 (6)	
3	Acetate (pH 3.5)	2.2–2.8 (50 s)	1186 (±6) (4)	—
		6.2 (1 h)	1151 (±5) (6)	
			1193 (±11) (8)	
3	Phosphate (pH 7.4)	2.5–3.0 (50 s)	105.1 (±2.2) × 10^2 (4)	—
		3.1 (1 h)	68.5 (±3.6) × 10^2 (6)	
			105.8 (±6.4) × 10^2 (8)	

^{a} Stoichiometries (DPPH–antioxidant molar ratios = 6–8), values in parentheses are the durations of the kinetic runs. ^{b} After previous complete conversion of **1** into the chalcone form.

$$-\mathrm{d}[QH_2]/\mathrm{d}t = k[QH_2][DPPH]/2 + k_c[QH_2][Q] \quad (1)$$

$$\mathrm{d}[Q]/\mathrm{d}t = k[QH_2][DPPH]/2 - k_c[QH_2][Q] \quad (2)$$

$$\mathrm{d}[D_1]/\mathrm{d}t = k_c[QH_2][Q] - k_1[D_1][DPPH]/2 \quad (3)$$

$$\mathrm{d}[D_2]/\mathrm{d}t = k_1[D_1][DPPH]/2 - k_2[D_2][DPPH]/2 \quad (4)$$

$$-\mathrm{d}[DPPH]/\mathrm{d}t = k[QH_2][DPPH] + k_1[D_1][DPPH] + k_2[D_2][DPPH] \quad (5)$$

absorbance and total DPPH concentration, respectively. For the curve-fitting of the kinetic traces in MeOH, all H abstraction reactions were assumed to occur at the same rate ($k = k_1 = k_2$) so that the optimization procedure deals with 2 parameters only (k, k_c). An excellent curve-fitting was obtained for a DPPH–catechin molar ratio of 4 with $k = 488$ (±2) and $k_c = 1293$ (±38) dm^3 mol^{-1} s^{-1}.

3′,4′,7-Trihydroxyflavylium ion. H atom transfer from **1** to DPPH is in competition with the structural transformations of **1** (Scheme 2) which, in weakly acidic to neutral conditions, may be reduced to two overall equilibria:

– The slow conversion of the flavylium ion into a mixture of tautomeric (colourless) hemiacetal and *cis*- and *trans*-chalcones (thermodynamic constant K_h).

– The fast deprotonation of the flavylium ion into a mixture of tautomeric (coloured) quinonoid 4′-keto and 7-keto forms (thermodynamic constant K_a).

In aqueous solutions, the following values were measured at 25 °C: pK_h = 2.8, pK_a = 4.5.[17] Hence, **1** is stable as the flavylium form in strongly acidic conditions only (pH < 2). In the pH range 2.5–5.0 it is readily converted into colourless forms, mainly the *trans*-chalcone. However, above pH 5, this process becomes very slow because of the previous conversion of the flavylium ion into much less electrophilic quinonoid forms. From the decay of the flavylium absorption band, pseudo first-order rate constants for the conversion of **1** into the corresponding chalcone (2′,3,4,4′-tetrahydroxychalcone) have been measured in the hydroalcoholic mixtures‡ used in the investigation of H transfer reactions from **1** to DPPH: $k_{obs} = 16.9$ (±0.1) × 10^{-4} s^{-1} (flavylium half-life *ca*. 7 min) in MeOH–pH 3.5 acetate buffer (1 : 1), $k_{obs} = 25.8$ (±0.1) × 10^{-5} s^{-1} (flavylium half-life *ca*. 45 min) in MeOH–pH 5.5 acetate buffer (1 : 1). In MeOH–pH 7.4 phosphate buffer, no significant decay of the visible band could be observed over 1 h.

To investigate the ability of the coloured forms to transfer H atoms to DPPH, solvent addition to the flavylium ion must be kept at a minimum. Hence, aliquots of a concentrated solution of **1** in acidic MeOH (pure flavylium form) were added to a DPPH solution in MeOH–aqueous buffer (1 : 1) under varying conditions of acidity. In all cases, the decay of the DPPH absorption band (H transfer to **1**) is much faster than the decay of the flavylium band in control experiments carried out in the absence of DPPH (solvent addition). Alternatively, DPPH can be added in a solution of **1** after conversion of the flavylium ion into the *trans*-chalcone. In that case, the decay of the DPPH band essentially reflects H transfer from the *trans*-chalcone to DPPH.

Although the *trans*-chalcone reacts with DPPH significantly more slowly than do the coloured forms (Table 1), both display total stoichiometries of *ca*. 6 in MeOH–pH 3.5 acetate buffer. Such high values are evidence of complex multistep processes. Similar high stoichiometries were previously measured with quercetin (3,3′,4′,5,7-tetrahydroxyflavone).[7] Hence, electronic conjugation over the whole polyphenolic nucleus (*via* an unsaturated C-ring in the case of flavones, flavonols and anthocyanins or *via* an enone moiety in the case of chalcones) seems to favour extensive oxidative degradation. For instance, the quercetin quinone quickly adds solvent on C-2 with the simultaneous regeneration of a catechol moiety (B-ring) which can transfer additional H atoms to DPPH.[7] From the structure of the oxidation products of **1** (see above), a similar process seems to operate here, the preferential sites for solvent addition on the quinone being C-2 (C-1′), C-3 and C-8 (Scheme 4). Water addition to C-2 (C-1′) allows subsequent elimination of the B-ring, most probably as 1,2,4-trihydroxybenzene (undetected in our experiments probably because of its high oxidizability), to yield 7-hydroxycoumarin (**4**). Coumarins resulting from oxidative elimination of the B-ring have already been isolated after treatment of naturally occurring anthocyanins by hydrogen peroxide at pH 6–7.[22] Alternatively, water addition to C-3 leads to the 3,3′,4′,7-tetrahydroxyflavylium ion (**5**, isolated as its quinonoid form after methylation). Further oxidation–solvent addition steps from the latter compound would yield polyhydroxylated coumarins like those isolated after periodate oxidation of **1**. In Scheme 4, additions on *o*-quinone or *p*-quinonoid intermediates have been written with water as the nucleophile, although other nucleophiles present in the medium may also participate, for instance the iodide anion in the course of periodate oxidation.

The kinetic model used in the curve-fitting of the kinetic traces involves the following steps (see Scheme 4):

– H abstraction from **1** (QH_4) by DPPH to form aryloxyl radicals QH_3 (rate constant k_1).

‡ The H transfer kinetics in MeOH turned out to be very sensitive to the proton concentration in the initial solution of **1** (which is necessary to keep **1** in its pure flavylium form). Hence, H transfer reactions from **1** to DPPH were systematically investigated in hydroalcoholic buffered solutions.

– Fast disproportionation of QH_3 into QH_4 and its quinone and subsequent fast solvent addition to the quinone to give QH_2 (*e.g.*, compounds **4** and **5**).

– H abstraction from QH_2 by DPPH to form aryloxyl radicals QH (rate constant k_2).

– Fast disproportionation of QH into QH_2 and the corresponding quinones, which quickly add solvent molecules to yield Q (*e.g.*, polyhydroxylated coumarins).

Assuming a quasi-stationary state for the radicals, eqns. (6)–(9) were derived. In MeOH–pH 3.5 acetate buffer (1 : 1), curve-

$$-d[QH_4]/dt = k_1[QH_4][DPPH]/2 \quad (6)$$

$$d[QH_2]/dt = k_1[QH_4][DPPH]/2 - k_2[QH_2][DPPH]/2 \quad (7)$$

$$d[Q]/dt = k_2[QH_2][DPPH]/2 \quad (8)$$

$$-d[DPPH]/dt = k_1[QH_4][DPPH] + k_2[QH_2][DPPH] \quad (9)$$

fitting of the kinetic traces recorded over 1 min gave consistent values for the rate constants with different DPPH : **1** molar ratios (Table 1). Clearly, sequential H transfer reactions from **1** to DPPH take place with rates which are much faster than the competing water addition to **1**. Consequently, the latter process may be safely neglected in the kinetic scheme. In MeOH–pH 7.4 phosphate buffer (1 : 1), the first H transfer reaction is *ca.* twice as fast but the relatively low kinetic stoichiometry (*n ca.* 3)§ prevents the accurate determination of subsequent H transfer rate constants.

3,4-Dihydroxy-4′-methoxychalcone. For a more convenient investigation of H abstractions from the colourless forms chalcone **3**, which is devoid of an OH group on the 2′ position and is thus unable to cyclize into a flavylium ion, was synthesized. Although **3** has a smaller number of OH groups compared to the chalcone form of **1**, both chalcones display similar stoichiometries, thus indicating that the antioxidant activity of these flavonoids is also controlled by the catechol unit. The same theoretical treatment as that used for **1** affords consistent values for the rate constants of the first H abstraction with different DPPH–antioxidant molar ratios. As for **1**, the total stoichiometry is lower in neutral conditions (Table 1). Remarkably, H abstraction is accelerated to a larger extent (a factor of 6–9) when going from mildly acidic to neutral hydroalcoholic solutions. The fact that both **3** and the chalcone form of **1** display high stoichiometries and rate constants in their reaction with DPPH suggests that anthocyanins could exert part of their antioxidant activity *via* their colourless forms in agreement with previous observations.[9a,e]

Periodate oxidation in aqueous buffer. Periodate oxidation in protic solvents is a very convenient way of generating quinones from the parent catechols.[8] Unlike DPPH, sodium periodate does not absorb light in the typical absorption domain (300–500 nm) of **1**, **3** and the oxidation products of **2** (dimers). Hence, the oxidation kinetics should be easily monitored.

Catechin. Kinetic investigations of catechin oxidation by $NaIO_4$ have been carried out in a pH 7.4 phosphate buffer. Addition of $NaIO_4$ (1 equiv.) to a solution of **2** is followed by the appearence of two absorption bands with maxima at 335 and 440 nm. After a fast increase, the band at 335 nm slowly decays whereas the broad band at 440 nm (shoulder at 480 nm) rises monotonously over the whole kinetic run (Fig. 1). When larger $NaIO_4$ concentrations (3–4 equiv.) are used, the band at

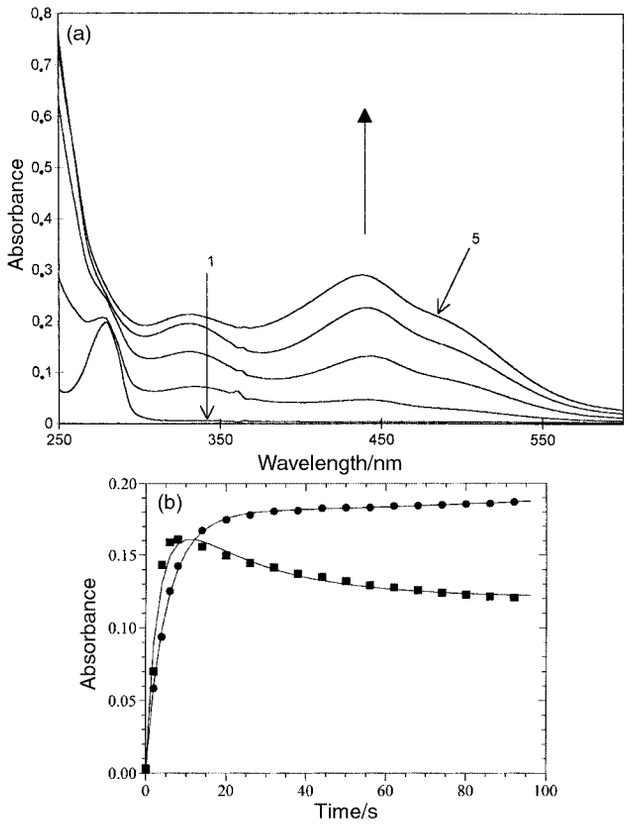


Fig. 1 (a) UV-Vis monitoring of catechin oxidation by $NaIO_4$ (1 equiv.). Catechin concentration: 5×10^{-5} mol dm^{-3}. pH 7.4 (0.05 mol dm^{-3} phosphate buffer). $T = 25$ °C. Spectrum 1: no oxidant. Spectra 2–5: 11, 14, 19 and 60 s after addition of $NaIO_4$. (b) Kinetic traces at 440 (circles) and 335 nm (squares).

335 nm also rises monotonously, although at a faster rate than the one at 440 nm. These observations seem consistent with the formation of a catechin quinone with an absorption maximum at 335 nm that then evolves into yellow dimers absorbing in the domain 400–500 nm. Hence, the kinetic scheme includes a bimolecular oxidation of **2** (QH_2) into the catechin quinone Q (rate constant k) that couples with a second molecule of **2** to form colourless dimers D_1 (rate constant k_c) which are further oxidized into yellow dimers D_2 (rate constant k_1). Consequently, eqns. (10)–(14) were used in the curve-fitting

$$-d[QH_2]/dt = k[QH_2][IO_4] + k_c[QH_2][Q] \quad (10)$$

$$d[Q]/dt = k[QH_2][IO_4] - k_c[QH_2][Q] \quad (11)$$

$$d[D_1]/dt = k_c[QH_2][Q] - k_1[D_1][IO_4] \quad (12)$$

$$d[D_2]/dt = k_1[D_1][IO_4] \quad (13)$$

$$-d[IO_4]/dt = k[QH_2][IO_4] + k_1[D_1][IO_4] \quad (14)$$

procedure (see Scheme 3). The visible absorbance in the domain of quinone and yellow dimer absorption (300–500 nm) can be expressed as: $A = \varepsilon[Q] + \varepsilon_2[D_2]$, ε and ε_2 being the molar absorption coefficients of Q and D_2, respectively. In the presence of an excess of $NaIO_4$, the increase of the band at 335 nm essentially reflects the build-up of Q and was thus used to estimate k (assuming $k_1 = 0$ and $k_c = 0$). The k value was then used in the curve-fitting of the kinetic trace at 440 nm (1 equiv. $NaIO_4$) with the additional assumption $k = k_1$ to obtain optimized values for k_c and the molar absorption coefficients. This procedure gave excellent curve-fittings and the following values for the rate constants: $k = 1433$ (±24), $k_c = 1737$ (±41) dm^3 mol^{-1} s^{-1}. The k_c value is close to that determined in MeOH

§ At the end of the kinetic runs following addition of **1** to the DPPH solution in MeOH–pH 7.4 phosphate buffer, a deeply coloured supernatant was invariably observed. This unexplained phenomenon probably indicates some inaccuracy in the stoichiometry and, to a lesser extent, in the rate constants. Manual stirring in the cell actually resulted in higher stoichiometries (*n ca.* 4).

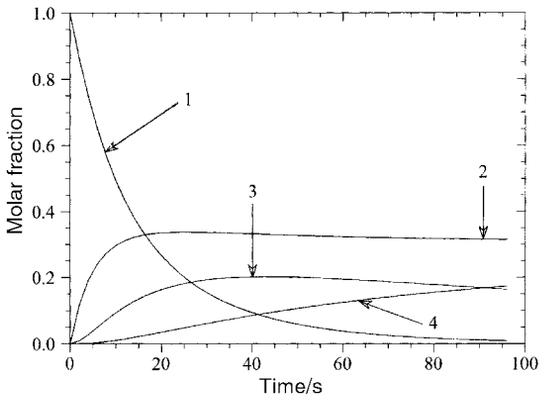


Fig. 2 Distribution diagram for the oxidation of catechin by $NaIO_4$ (1 equiv.). Catechin concentration: 5×10^{-5} mol dm^{-3}. pH 7.4 (0.05 mol dm^{-3} phosphate buffer). $T = 25$ °C. 1: catechin, 2: catechin quinone, 3: colourless dimers (D_1), 4: coloured dimers (D_2).

with DPPH as the oxidant. The distribution diagram (Fig. 2) shows that the catechin quinone rapidly reaches a quasi-stationary concentration because oxidation of **2** quenches the coupling reaction. A similar diagram was obtained with DPPH. The build-up of significant concentrations of catechin quinone is consistent with its easy detection by ^{1}H-NMR.

The propensity of catechin to oxidative dimerization may be traced to the high nucleophilicity of the catechin A-ring which allows its efficient coupling with the B-ring of the catechin quinone *via* Michael addition. Although it quantitatively fits the kinetic results, our scheme for catechin oxidation is clearly oversimplified. In particular, in addition to the C–C linked coloured and colourless dimers taken into account in Scheme 3, C–O–C linked colourless dimers (biaryl ethers) have been shown to form under enzymatic[19] or chemical[20] oxidative conditions. Such dimers probably result from the nucleophilic attack of the electrophilic catechin radicals on the electron-rich A-ring of a catechin molecule.

The A-ring of flavones, flavonols and anthocyanins is much less nucleophilic than that of catechin and other flavanols because of its conjugation with an electron-poor C-ring. In aqueous solution, the corresponding quinones quickly add solvent molecules[7,8] rather than couple with the parent flavonoids to form dimers.

3′,4′,7-Trihydroxyflavylium ion. The periodate oxidation of **1** has been carried out in hydroalcoholic mixtures of different acidities using a large excess of $NaIO_4$ (10 equiv.). In MeOH–pH 3.5 acetate buffer (1 : 1), the decay of the flavylium band (488 nm) was accompanied by an increase of an absorption band at lower wavelengths (λ_{max} *ca.* 380 nm). A hypothesis of two sequential oxidations was necessary to account for the kinetic traces at 488 and 380 nm. The curve-fittings were thus carried out according to eqns. (15)–(18) with the

$$-\mathrm{d}[QH_4]/\mathrm{d}t = k_1[QH_4][IO_4] \quad (15)$$

$$\mathrm{d}[QH_2]/\mathrm{d}t = k_1[QH_4][IO_4] - k_2[QH_2][IO_4] \quad (16)$$

$$\mathrm{d}[Q]/\mathrm{d}t = k_2[QH_2][IO_4] \quad (17)$$

$$-\mathrm{d}[IO_4]/\mathrm{d}t = k_1[QH_4][IO_4] + k_2[QH_2][IO_4] \quad (18)$$

additional relationship: $A = A_0[QH_4]/c + \varepsilon_1[QH_2] + \varepsilon_2[Q]$. A_0: initial absorbance, c: total concentration of **1** (QH_4), ε_1, ε_2: molar absorption coefficients of the primary (QH_2) and secondary (Q) oxidation products, respectively (see Scheme 4).

In MeOH–pH 5.5 acetate buffer (1 : 1), the two-step mechanism was more obvious since the absorbance at 502 nm (λ_{max} of **1**) first quickly increased and then more slowly decreased in the course of the oxidation, whereas a broad absorption band at

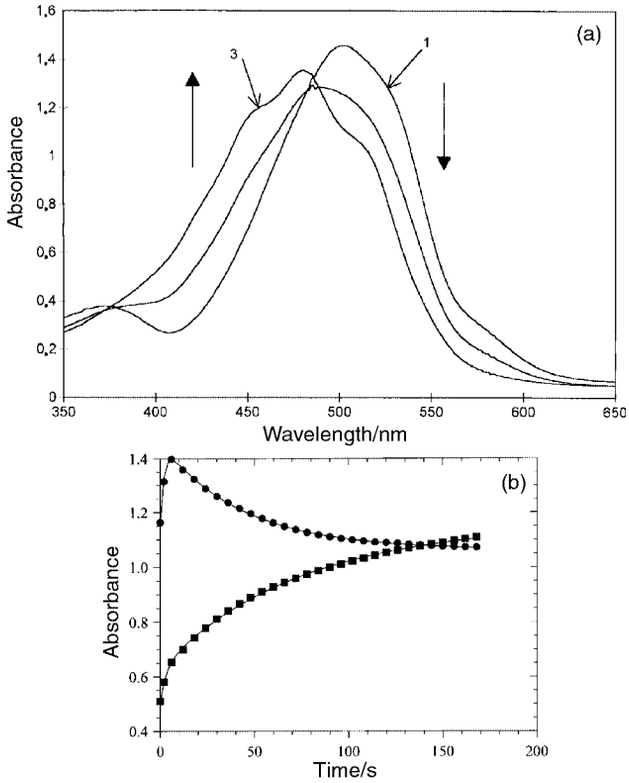


Fig. 3 (a) UV-Vis monitoring of the oxidation of **1** by $NaIO_4$ (10 equiv.) in MeOH–0.05 mol dm^{-3} acetate buffer (pH 5.5) (1 : 1). Pigment concentration: 5×10^{-5} mol dm^{-3}. $T = 25$ °C. Spectrum 1: no oxidant. Spectra 2 and 3: 40 and 170 s after addition of $NaIO_4$. (b) Kinetic traces at 502 (circles) and 450 nm (squares).

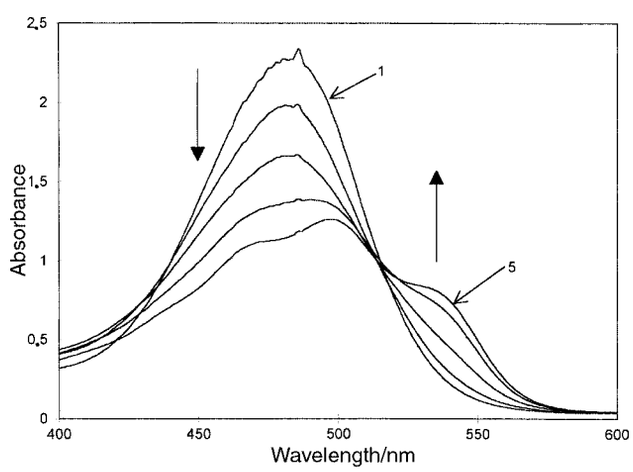


Fig. 4 UV-Vis monitoring of the oxidation of **1** by $NaIO_4$ (10 equiv.) in MeOH–0.2 mol dm^{-3} HCl (1 : 1). Pigment concentration: 5×10^{-5} mol dm^{-3}. $T = 25$ °C. Spectrum 1: no oxidant. Spectra 2–5: 1100, 2270, 4370 and 8240 s after addition of $NaIO_4$.

lower wavelengths (λ_{max} *ca.* 450 nm) steadily increased (Fig. 3). Periodate oxidation could also be conducted in strongly acidic conditions (MeOH–0.2 mol dm^{-3} HCl (1 : 1)) where **1** is as its pure flavylium form (Fig. 4).¶ In that case, the values for k_1 and k_2 (computed from the decay of the flavylium band ($\lambda_{max} = 481$ nm) as well as from the increase in a weak shoulder at higher wavelengths (*ca.* 530 nm)) were not significantly different and were then set to be equal in a second fitting. In all cases, consistent values for the rate constants (Table 2) were obtained from the curve-fittings at the two different wavelengths. Both k_1 and k_2 strongly increase when going from MeOH–0.2 mol dm^{-3}

¶ Similar experiments could not be run with DPPH because of the high instability of DPPH in MeOH–0.2 mol dm^{-3} HCl (1 : 1).

Table 2 Rate constants for periodate oxidation in MeOH–aqueous buffer (1 : 1) at 25 °C. For a definition of the rate constants k_1 and k_2, see text. The values in the second set of parentheses are the wavelengths of detection (in nm)

	Antioxidant	Buffer	k_1/dm^3 mol^{-1} s^{-1}	k_2/dm^3 mol^{-1} s^{-1}
	1	0.2 mol dm^{-3} HCl	1.413 (±0.009) (481) 1.50 (±0.02) (530)	1.413 (±0.009) (481)[a] 1.50 (±0.02) (530)[a]
	1	Acetate (pH 3.5)	174 (±13) (488) 164 (±20) (380)	6.36 (±0.07) (488) 5.17 (±0.07) (380)
	1	Acetate (pH 5.5)	979 (±27) (502) 695 (±47) (450)	61.4 (±1.0) (502) 31.7 (±0.5) (450)
	1	Phosphate (pH 7.4)	4980 (±620) (580)	681 (±15) (580)
	3	Acetate (pH 3.5)	45.4 (±1.5) (355) 39.8 (±2.9) (450)	3.64 (±0.06) (335) 3.13 (±0.04) (450)
	3	Phosphate (pH 7.4)	442 (±12) (520)	442 (±12) (520)[a]

[a] k_2 set equal to k_1 in the curve-fitting.

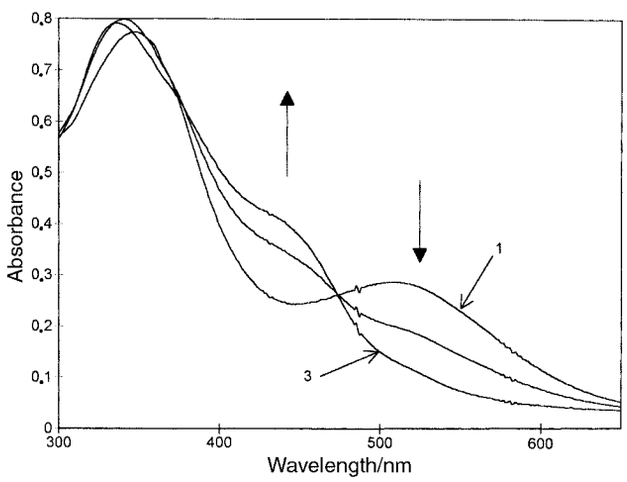


Fig. 5 UV-Vis monitoring of the oxidation of **3** by $NaIO_4$ (10 equiv.) in MeOH–0.05 mol dm^{-3} phosphate buffer (pH 7.4) (1 : 1). Chalcone concentration: 5×10^{-5} mol dm^{-3}. T = 25 °C. Spectrum 1: no oxidant. Spectra 2 and 3: 440 and 1700 s after addition of $NaIO_4$.

HCl (1 : 1) to MeOH–pH 7.4 phosphate buffer (1 : 1). This is in agreement with the increase in electron density in **1** brought about by the deprotonation of its acidic O4′-H and O7-H groups. The observation that **1** reacts with at least 2 equiv. $NaIO_4$ also shows that flavylium-derived quinones are highly unstable in aqueous media and quickly add solvent molecules, thereby regenerating catechol moieties which further react with the oxidant. This is consistent with the high stoichiometries measured in the trapping of the DPPH radical and with our failure to detect the quinone of **1** by NMR.

3,4-Dihydroxy-4′-methoxychalcone. The kinetic traces recorded during the periodate oxidation of **3** (Fig. 5) can be analyzed using the same model as for **1**. Two sequential oxidations could thus be evidenced, with a strong acceleration of both steps when going from MeOH–pH 3.5 buffer (1 : 1) to MeOH–pH 7.4 buffer (1 : 1) (Table 2).

Autoxidation. Autoxidation of flavonoids typically occurs in strongly alkaline aqueous or nonaqueous solutions and is accompanied by the production of flavonoid radical anions,[5h,23] superoxide and hydrogen peroxide.[24] With highly oxidizable flavonoids (*e.g.*, quercetin), the production of ROS can take place in physiological conditions and may be a cause of toxicity.

Autoxidation may be viewed as the simple transfer of one electron from the HOMO of electron-rich flavonoid polyanions to the half-occupied π* orbitals of dioxygen. However, catechol autoxidation is known to be very prone to catalysis by transition metal ions.[25] Hence, we cannot exclude the possibility that the apparently metal-independent autoxidation of flavonoids and other polyphenols is actually due to contaminating trace metals in water, bases or even solutes. Pulse radiolysis experiments have allowed the determination of the reduction potentials of flavonoid radicals at different pHs.[6g] When the pH rises, successive deprotonation of the most acidic OH groups in the parent flavonoids makes them more oxidizable and the corresponding potentials markedly decrease. In fairly alkaline conditions liable to promote extensive deprotonation of the polyphenolic nuclei (pH 13.5) and subsequent fast autoxidation,[5h,7,23] the reduction potentials of the quercetin and catechin radicals are −0.037 and 0.08 V, respectively. Although low, such values are 0.12–0.24 V higher than the potential for the one-electron reduction of aqueous dioxygen into superoxide (−0.16 V[26]). Hence, the metal-independent base-mediated autoxidation of common flavonoids would seem thermodynamically unfavourable,‖ although theoretical investigations have pointed out that catechol dianions display very low oxidation potentials (< −1.0 V) allowing complete one-electron transfer to dioxygen in polar solutions.[27] It is thus important to bear in mind that contaminating trace metals could play a key role in the relatively fast consumption of flavonoids in aerated alkaline solutions with concomitant formation of flavonoid radicals and ROS.

The kinetics of flavonoid autoxidation are critically dependent on the electron density on the polyphenolic nucleus and thus on the deprotonation of the OH groups, especially those born by an oxidizable catechol moiety, typically the B-ring. In the case of quercetin, deprotonation of O3-H (the third most acidic OH group after O7-H and O4′-H) allows autoxidation to proceed at an appreciable rate above pH 8.[7] However, the subsequent deprotonation of O3′-H (pH range 9–10) markedly accelerates the reaction. In the case of flavylium **1** and chalcone **3**, complete deprotonation of the catechol moiety seems to be required to reach significant autoxidation rates. Hence, autoxidation was monitored in strongly alkaline conditions (0.2 mol dm^{-3} NaOH) allowing deprotonation of the weakly acidic O3′-H group.

When monitoring autoxidation of chalcone **3** in 0.2 mol dm^{-3} NaOH over 30 min, a relatively fast increase in the absorbance at 340 nm followed by a slower decay may be observed. Simultaneously, the absorbance at 450 nm increases monotonously. Both kinetic traces can be fitted using modified versions of eqns. (6)–(9), k_1[DPPH] and k_2[DPPH] being respectively replaced by the apparent first-order rate constants of autoxidation, $k_1[O_2] = k'_1$ and $k_2[O_2] = k'_2$. The fitting at 340 nm gives $k'_1 = 5.3\ (\pm 0.1) \times 10^{-3}$ s^{-1} and $k'_2 = 6.2\ (\pm 0.3) \times 10^{-5}$ s^{-1}. Using the latter k'_2 value, the fitting at 450 nm yields $k'_1 =$

‖ Although strongly in favour of the flavonoid anions, the autoxidation equilibrium may be efficiently displaced toward the formation of the flavonoid radicals because of the subsequent fast disproportionation of the latter.

6.0 (±0.1) × 10^{-3} s^{-1}. Hence, **3** is autoxidized in an apparently two-step procedure that may reflect conversion of **3** into the corresponding quinone (one-electron oxidation/radical disproportionation) followed by fast addition of a hydroxide ion to form a hydroxylated analog of **3** that would be autoxidized by the same mechanism.** Similar mechanisms have already been postulated for the autoxidation and enzymatic oxidation of phenols to account for the EPR detection not only of the parent aryloxyl radicals but also of their hydroxylated analogs.[28]

When **1** is dissolved in 0.2 mol dm^{-3} NaOH, spectral changes very similar to those observed in MeOH–pH 3.5 acetate buffer (1 : 1) are observed, *i.e.* the apparent first-order decay of the flavylium absorption band (λ_{max} = 596 nm in 0.2 mol dm^{-3} NaOH) accompanied by the apparent first-order rise of an absorption band typical of the corresponding anionic chalcone form (λ_{max} = 493 nm). The fitting of both kinetic traces gave values for the rate constant in perfect agreement (k_{obs} = 153.0 (±0.9) × 10^{-4} s^{-1}, half-life of **1** *ca*. 45 s). Hence, addition of the hydroxide ion to the quinonoid forms of **1** to yield the corresponding chalcone (which would be faster than water addition on the flavylium ion in mildly acidic hydroalcoholic solutions) could be the dominant process, although competitive autoxidation of the quinonoid forms may also take place as evidenced by the isolation of small amounts of 7-hydroxycoumarin from the reaction mixture. When monitoring over 1 h, the absorption band at 493 nm slowly decays with an apparent rate constant k'_{obs} equal to 5.4 (±0.1) × 10^{-5} s^{-1}. The latter value is very similar to that for the second step in the autoxidation of chalcone **3**. It suggests that the first step could be a complex combination of addition of the hydroxide ion to **1** followed by autoxidation of the chalcone thus formed. Indeed, from the investigation of the autoxidation of **3**, both the chalcone and its primary oxidation products are expected to absorb in the range 450–500 nm.

Semi-empirical quantum mechanics calculations

Bond dissociation energy (BDE) values can be estimated from the difference in optimized energy values (AM1 parametrization, unrestricted Hartree–Fock method) between aryloxyl radicals and the corresponding phenols. The calculations were carried out on the flavylium, hemiacetal and *trans*-chalcone forms of **1** as well as on the forms resulting from the deprotonation of acidic OH groups of the flavylium ion (O4′-H, O7-H) and *trans*-chalcone (O4-H, O4′-H) (Table 3). For a given form and its radicals, intramolecular hydrogen bonding was taken into account with the following priorities: O4′-H···O3′ > O3′-H···O4′ (flavylium), O4-H···O3 > O3-H···O4 (*trans*-chalcone). As expected, the most labile H atoms in the flavylium, hemiacetal and chalcone forms are born by the catechol OH groups. The order of decreasing BDE values for the most reactive OH groups is as follows: flavylium ⩾ chalcone, neutral quinonoid form (4′-keto tautomer), hemiacetal > anionic quinonoid form, chalcone 4-and 4′-oxyanions > chalcone 4,4′-dioxyanion. The relatively low BDE values for the hemiacetal catechol group shows that labile phenolic H atoms are not inevitably associated with a large electron delocalization over the whole polyphenolic nucleus. Deprotonation typically lowers the BDE values of the remaining OH groups, thus indicating that the increase in electron delocalization promoted by deprotonation stabilizes the radical-anions more strongly than it does the parent phenolate anions. The sole deprotonation of the flavylium O4′-H group was considered since the 4′-keto quinonoid thus-formed turned out to be 10.8 kcal mol^{-1} more stable than the 7-keto tautomer (deprotonation of O7-H). Deprotonation of O4′-H lowers by 6.5 and 11.7 kcal mol^{-1} the BDE values of O3′-H and O7-H, respectively. The fact that the O7-H bond in the 4′-keto quinonoid is almost as labile as the O3′-H bond may reflect not only the strong electron conjugation between the A- and B-rings in the quinonoid form but also the probable involvement of O3′-H in a strong stabilizing hydrogen bond with O4′. Deprotonation of the 4′-keto quinonoid at O7-H is expected to increase the electron density at O4′ (Scheme 2) and thereby further strengthen the O3′-H···O4′ hydrogen bond. In this context, it may not be surprising that the BDE value of O3′-H in the anionic quinonoid is only 2.1 kcal mol^{-1} lower than in the neutral 4′-keto quinonoid. Similar combinations of electronic effects and intramolecular hydrogen bonding may operate with the chalcone.

Taken as a whole, the BDE values suggest that all anthocyanin forms may take part in the antioxidant activity in agreement with the experimental data. However, because of the deprotonation of their acidic OH groups at physiological pH, the quinonoid and chalcone forms are probably more potent hydrogen donors than the hemiacetal form.

Table 3 Dissociation energies of O–H bonds (in kcal mol^{-1}) in **1** deduced from semi-empirical quantum mechanics calculations *in vacuo* (HyperChem program, AM1 parametrization, unrestricted Hartree–Fock method)

Flavylium	4′-Keto quinonoid[a]	Anionic quinonoid	Hemiacetal ((*S*)-enantiomer)
82.1 (O3′-H)	75.6 (O3′-H)	73.5 (O3′-H)	74.3 (O3-H)
85.3 (O4′-H)	—	—	74.2 (O4-H)
88.0 (O7-H)	76.3 (O7-H)	—	76.6 (O7-H)
trans-Chalcone	*trans*-Chalcone 4-oxyanion	*trans*-Chalcone 4′-oxyanion[b]	*trans*-Chalcone 4,4′-dioxyanion
76.5 (O3-H)	73.8 (O3-H)	73.1 (O3-H)	71.2 (O3-H)
76.1 (O4-H)	—	72.8 (O4-H)	—
76.3 (O2′-H)	76.2 (O2′-H)	75.3 (O2′-H)	78.6 (O2′-H)
78.7 (O4′-H)	74.8 (O4′-H)	—	—

[a] The 7-keto quinonoid form is 10.8 kcal mol^{-1} less stable than the 4′-keto tautomer. [b] As stable (within 0.1 kcal mol^{-1}) as the tautomeric 4-oxyanion.

Experimental

Materials

Catechin and DPPH (Aldrich) were used as received.

3′,4′,7-Trihydroxyflavylium bromide (1). To a solution of 3,4-dihydroxyacetophenone (1 g, 6.5 mmol) and 2,4-dihydroxybenzaldehyde (908 mg, 6.5 mmol) in AcOEt (10 ml) cooled to 0 °C was added a 30% solution of HBr in acetic acid (2.5 ml, 2 equiv.). The mixture was placed at 4 °C overnight to ensure the complete precipitation of the pigment which was then collected by filtration, washed with AcOEt and dried under vacuum. Yield: 35%. ^{1}H-NMR (CD_3OD–TFA): δ(ppm) = 9.02 (d, J = 8.8 Hz, H-4), 8.27 (d, J = 8.8 Hz, H-3), 8.12 (d, J = 8.8 Hz, H-5), 8.00 (dd, J = 2.2, 8.8 Hz, H-6′), 7.85 (d, J = 2.2 Hz, H-2′), 7.50 (d, J = 2.2 Hz, H-8), 7.38 (dd, J = 2.2, 8.8 Hz, H-6), 7.07 (d, J = 8.8 Hz, H-5′). ^{13}C-NMR (CD_3OD–TFA): δ(ppm) = 173.0 (C-2), 168.9 (C-7), 159.1 (C-9), 156.3 (C-4′), 152.7 (C-4), 147.5 (C-3′), 132.7 (C-5), 125.3 (C-6′), 121.1 (C-6), 120.7 (C-1′), 118.8 (C-10), 117.0 (C-5′), 115.4 (C-2′), 112.6 (C-3), 102.6 (C-8). Mass (FAB, positive mode): m/z = 255 (M^+). UV-Vis (MeOH–HCl): λ_{max} = 486 nm.

3,4-Dihydroxy-4′-methoxychalcone (3). A solution of 3,4-dihydroxyacetophenone (1 g, 6.5 mmol) in DMF (30 ml) was

** A recent investigation of the autoxidation of simple 4-alkylcatechols[25] has convincingly demonstrated that water (or hydroxide ion) addition on *o*-quinone intermediates does not take place, the hydrogen peroxide formed during autoxidation being actually responsible for the production of hydroxyquinones.

dried by azeotropic distillation of water traces with toluene and placed under N_2. 4-Methoxybenzaldehyde (0.8 cm^3, 6.5 mmol) and potassium *tert*-butoxide (3.7 g, 5 equiv.) were added and the mixture was stirred at 50 °C for 24 h. The mixture was then cooled, acidified with 1 mol dm^{-3} HCl, extracted with AcOEt and washed with water. Pure **3** (0.7 g, 40%) was obtained by chromatography on silica gel (eluent: petroleum ether (bp 40–60 °C)–AcOEt 4:1 to 1:1). ^{1}H-NMR (CD_3OD): δ(ppm) = 7.69 (d, J = 15.5 Hz, H-β), 7.65 (d, J = 8.5 Hz, H-2′, H-6′), 7.57 (dd, J = 2.2, 8.1 Hz, H-6), 7.55 (d, J = 15.5 Hz, H-α), 7.51 (d, J = 2.2 Hz, H-2), 6.96 (d, J = 8.5 Hz, H-5′, H-3′), 6.87 (d, J = 8.5 Hz, H-5). ^{13}C NMR (CD_3OD): δ(ppm) = 190.0 (C=O), 162.2 (C-4′), 151.3 (C-4), 145.7 (C-3), 144.0 (C-β), 130.7 (C-1), 130.4 (C-2′, C-6′), 128.0 (C-1′), 122.6 (C-α), 119.3 (C-6), 115.4 (C-5), 114.9 (C-2), 114.4 (C-3′, C-5′), 54.9 (CH_3). Mass (FAB, positive mode): m/z = 271 ($M + H^+$). UV-Vis (MeOH): λ_{max} = 352 nm.

NMR spectra

These were recorded on 300 MHz and 500 MHz Bruker apparatus at 27 °C. ^{13}C signals were assigned from HMBC correlations (**1**, **5**) or by comparison with the literature.[29]

Absorption spectra

These were recorded on a Hewlett-Packard 8453 diode-array spectrometer equipped with a magnetically stirred quartz cell (optical pathlength: 1 cm) thermostated by a water bath.

Semi-empirical quantum mechanics calculations

Energy optimizations were run *in vacuo* on a Pentium 90 PC using the HyperChem program (Autodesk, Sausalito, California, USA) with the AM1 parametrization. The unrestricted Hartree–Fock method was selected for both the open-shell (flavonoid radicals and anion radicals) and closed-shell species (flavonoids and flavonoid anions). The bond dissociation energies were identified as the energy differences between the flavonoid radicals and the corresponding flavonoids.

Data analysis

The curve-fittings were carried out on a Pentium 120 PC (Scientist program, MicroMath, Salt Lake City, Utah, USA) through least-squares regression. Optimized values for the parameters and the corresponding standard deviations are reported.

Kinetic experiments

H abstraction by DPPH. To 2 cm^3 of a freshly prepared solution of DPPH in MeOH (DPPH concentration: 2×10^{-4} mol dm^{-3}) or in MeOH–aqueous buffer (1:1) (DPPH concentration: 10^{-4} mol dm^{-3}) placed in the spectrometer cell were added aliquots (20–100 mm^3) of a 10^{-3} mol dm^{-3} solution of flavonoid in MeOH (acidified with a few drops of concentrated HCl in the case of **1**).

Periodate oxidation. In a typical experiment, 50 mm^3 of a freshly prepared 2×10^{-3} mol dm^{-3} solution of flavonoid in MeOH (acidified with a few drops of concentrated HCl in the case of **1**) was diluted into 2 cm^3 of buffer placed in the spectrometer cell and thermostated at 25 °C. At time zero, 50 mm^3 of a solution of $NaIO_4$ in water (concentration: 2×10^{-3} to 2×10^{-2} mol dm^{-3}) was added. In the case of **1**, the flavonoid was added at time zero (after addition of $NaIO_4$) to avoid competitive formation of the colourless forms.

Autoxidation. In a typical experiment, 50 mm^3 of a freshly prepared 2×10^{-3} mol dm^{-3} solution of flavonoid in MeOH (acidified with a few drops of concentrated HCl in the case of **1**) was diluted into 2 cm^3 of 0.2 mol dm^{-3} NaOH placed in the spectrometer cell and thermostated at 25 °C.

Product analysis

Catechin quinone. DPPH (2 equiv.) was added to a 10^{-2} mol dm^{-3} solution of catechin in CD_3OD just before recording the ^{1}H-NMR spectrum. ^{1}H-NMR: δ(ppm) = 7.81 (br d, J = 10.3 Hz, H-6′), 6.48 (d, J = 10.3 Hz, H-5′), 6.40 (br s, H-8), 6.24 (br s, H-6), 5.48 (br s, H-2′).

Catechin quinone–benzenesulfinate adduct. $PhSO_2Na$ (1.5 equiv.) and DPPH (2 equiv.) were successively added to a 2×10^{-2} mol dm^{-3} solution of catechin in MeOH. After stirring for 30 min, the reaction mixture was concentrated and CH_2Cl_2 was added until a yellow solid precipitated, which contained the adduct and unreacted catechin. These were separated by chromatography on silica gel (eluent: petroleum ether (bp 40–60 °C)–AcOEt (4:1)). ^{1}H-NMR (CD_3OD): δ(ppm) = 7.82 (d, J = 7.4 Hz, Ph, H-*ortho*), 7.60 (t, J = 7.4 Hz, Ph, H-*para*), 7.56 (s, H-2′ or H-5′), 7.51 (t, J = 7.4 Hz, Ph, H-*meta*), 6.99 (s, H-2′ or H-5′), 5.92 (d, J = 2.2 Hz, H-8), 5.62 (d, J = 2.2 Hz, H-6), 5.57 (d, J = 6.6 Hz, H-2), 4.10–3.94 (*m*, H-3, H-4). Mass (FAB, negative mode): m/z = 429 ($M - H^+$). UV-Vis (MeOH): λ_{max} = 266 nm.

Flavylium quinone–benzenesulfinate adduct. 1 was quickly added to a freshly prepared solution of $PhSO_2Na$ (1.5 equiv.) and DPPH (2 equiv.) in MeOH to a final pigment concentration of 2×10^{-2} mol dm^{-3}. After stirring for 30 min, the reaction mixture was concentrated and CH_2Cl_2 was added until precipitation of a red solid containing the adduct and unreacted **1**. These were separated by chromatography on C18-silica gel (eluent: water to water–MeOH (9:1)). ^{1}H-NMR (CD_3OD–TFA): δ(ppm) = 9.34 (d, J = 8.8 Hz, H-4), 8.31 (d, J = 8.8 Hz, H-3), 8.10 (d, J = 7.8 Hz, H-5), 8.06 (s, H-2′), 7.92 (d, J = 8.1 Hz, Ph, H-*ortho*), 7.66 (d, J = 7.8 Hz, H-6), 7.55 (t, J = 8.1 Hz, Ph, H-*meta*), 7.55 (t, J = 8.1 Hz, Ph, H-*para*), 7.54 (s, H-5′), 7.38 (d, J = 2.2 Hz, H-8). Mass (FAB, positive mode): m/z = 395 (M^+). UV-Vis (MeOH–HCl): λ_{max} = 451 nm.

Catechin dimer. To a solution of catechin (145 mg, 0.5 mmol) in MeOH (50 ml) was added 197 mg (0.5 mmol) of DPPH. After stirring for 45 min, MeOH was removed under vacuum. The residue was washed with CH_2Cl_2, dissolved into acetone (30 ml) and placed under N_2. Me_2SO_4 (0.3 ml, 4 mmol) and K_2CO_3 (500 mg) were added and the mixture was brought to reflux for 36 h. After cooling and concentration, CH_2Cl_2 (40 ml) was added. The solution was filtered, washed with water, dried and concentrated. From the complex reaction mixture, a dimer (*ca*. 5 mg) was isolated by chromatography on silica gel (R_f (hexane–AcOEt (2:3)) = 0.21). From the NMR and mass analyses, it appears that only six of the seven OH groups have been methylated. The less reactive O3-H of the C-ring probably remains as its free form. ^{1}H-NMR ($CDCl_3$, assignments from ^{1}H–^{1}H COSY): δ(ppm) = 6.98–6.91 (m, 3H, B-ring), 6.49 (s, H-6 (A) or H-5′ (B′)), 6.26 (s, H-6 (A) or H-5′ (B′)), 6.04 (d, J = 2.2 Hz, H-8 (A′)), 5.81 (d, J = 2.2 Hz, H-6 (A′)), 4.83 (d, J = 8.8 Hz, H-2 (C)), 4.14–4.05 (m, 2 H-3), 4.00 (d, J = 8.8 Hz, H-2 (C′)), 3.94 (s, CH_3), 3.92 (s, CH_3), 3.85 (s, CH_3), 3.75 (s, CH_3), 3.69 (s, CH_3), 3.65 (s, CH_3), 3.12 (dd, J = 5.5, 16.3 Hz, H-4 (C)), 3.07 (dd, J = 5.9, 15.7 Hz, H-4 (C′)), 2.77 (d, J = 11.0 Hz, H-2′α (B′)), 2.65 (dd, J = 9.2, 16.3 Hz, H-4 (C)), 2.60 (dd, J = 10.3, 15.7 Hz, H-4 (C′)), 2.48 (d, J = 11.0 Hz, H-2′β (B′)). Mass (FAB, positive mode): m/z = 661 ($M + H^+$). UV-Vis (MeOH): λ_{max} = 280, 368 nm (shoulder at 340 nm).

Compound 5. 1 (167 mg, 0.5 mmol) was quickly added to a solution of DPPH (394 mg, 1 mmol) in MeOH (50 ml). After stirring for 45 min, the MeOH was removed under vacuum. The residue was washed with CH_2Cl_2, dissolved into acetone (30 ml)

and placed under N_2. Me_2SO_4 (0.6 ml, 7.5 mmol) and K_2CO_3 (620 mg) were added and the mixture was brought to reflux for 48 h. After cooling and concentration, CH_2Cl_2 was added. The solution was filtered, washed with water, dried and concentrated. From the complex reaction mixture, **5** (*ca*. 10 mg) was isolated by chromatography on silica gel. R_f (hexane–AcOEt (1 : 1)) = 0.30. ^{1}H-NMR ($CDCl_3$): δ(ppm) = 8.68 (s, H-4), 7.79 (d, J = 8.8 Hz, H-5), 7.58 (dd, J = 2.2, 8.8 Hz, H-6′), 7.41 (d, J = 2.2 Hz, H-2′), 6.95 (d, J = 8.8 Hz, H-5′), 6.89 (dd, J = 2.2, 8.8 Hz, H-6), 6.84 (d, J = 2.2 Hz, H-8), 4.01 (s, CH_3), 3.98 (s, CH_3), 3.92 (s, CH_3). ^{13}C-NMR (CD_3OD): δ(ppm) = 197.9 (C-7), 126.8 (C-3), 156.0 (C-2), 154.9 (C-9), 151.4 (C-4′), 149.4 (C-3′), 125.7 (C-1′), 124.8 (C-8), 119.8 (C-6′), 114.1 (C-6), 113.4 (C-10), 111.3 (C-5′), 108.9 (C-2′), 101.5 (C-4), 56.5–56.1 (CH_3), C-5 is not identified. Mass (EI): m/z = 312 (M^+). UV-Vis (MeOH–HCl): λ_{max} = 403, 456 nm.

Oxidation of 1 by sodium periodate. To a solution of **1** (150 mg, 0.45 mmol) in MeOH (45 ml), acidified by concentrated HCl to a final concentration of 0.2 mol dm^{-3}, was added a solution of $NaIO_4$ (96 mg, 0.45 mmol) in water (45 ml). After stirring for 2 h, the MeOH was removed under vacuum and the solution was extracted with AcOEt, dried and concentrated. The mixture was then purified by chromatography on silica gel (eluent: petroleum ether (bp 40–60 °C)–AcOEt (7 : 3) to pure AcOEt).

*7-Hydroxycoumarin (**4**). ca*. 8 mg. R_f (AcOEt) = 0.85. ^{1}H-NMR (CD_3OD): δ(ppm) = 7.83 (d, J = 9.6 Hz, H-4), 7.44 (d, J = 8.1 Hz, H-5), 6.78 (dd, J = 2.2, 8.1 Hz, H-6), 6.70 (d, J = 2.2 Hz, H-8), 6.17 (d, J = 9.6 Hz, H-3). ^{13}C-NMR (CD_3OD): δ(ppm) = 162.7 (C-7), 162.1 (C-9), 156.2 (C-2), 145.0 (C-4), 129.7 (C-5), 113.5 (C-6), 112.1 (C-10), 111.4 (C-3), 102.4 (C-8). Mass (EI): m/z = 162 (M^+). UV-Vis (MeOH): λ_{max} = 320, 490 nm.

7-Hydroxy-8-iodocoumarin. ca. 7 mg. R_f (AcOEt) = 0.79. ^{1}H-NMR (CD_3OD): δ(ppm) = 7.79 (d, J = 9.6 Hz, H-4), 7.44 (d, J = 8.1 Hz, H-5), 6.84 (d, J = 8.1 Hz, H-6), 6.20 (d, J = 9.6 Hz, H-3). Mass (FAB, positive mode): m/z = 289 (M + H^+). UV-Vis (MeOH): λ_{max} = 290, 495 nm.

7-Hydroxy-3-iodocoumarin. 21 mg as a mixture with the following compound. R_f (AcOEt) = 0.36. ^{1}H-NMR (CD_3OD): δ(ppm) = 8.48 (s, H-4), 7.42 (d, J = 8.1 Hz, H-5), 6.89 (d, J = 2.2 Hz, H-8), 6.83 (dd, J = 2.2, 8.1 Hz, H-6). Mass (FAB, positive mode): m/z = 289 (M + H^+).

7,8-Dihydroxy-3-iodocoumarin or 3,7-dihydroxy-8-iodocoumarin. R_f (AcOEt) = 0.36. ^{1}H-NMR (CD_3OD): δ(ppm) = 8.70 (s, H-4), 8.01 (d, J = 8.1 Hz, H-5), 7.69 (d, J = 8.1 Hz, H-6). Mass (FAB, positive mode): m/z = 397 (M + glycerol (matrix) + H^+).

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