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Structural modification of phenylpropanoid-derived compounds and the effects on their participation in redox processes

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Abstract—Oxidation and reduction processes are fundamental to many of the proposed mechanisms by which dietary phytochemicals are thought to exert protective effects against cardiovascular disease and some cancers. An understanding of the redox chemistry of these compounds is essential in assessing their potential to participate in these processes. Phenylpropanoid-derived compounds were selected and synthesised where required to represent many of the structural features found in this important group of compounds. Using electron paramagnetic resonance spectroscopy and computational chemistry a structure–redox activity relationship was obtained. Good correlation of computational and experimental results was observed for the mono-hydroxylated compounds. This demonstrated the value of computational chemistry in obtaining information about compounds, not readily available and the effect of electron delocalisation on parent radical stability. For compounds containing more than one hydroxyl, the relationship was found to be more complex. The importance of quinone formation in compounds containing more than one hydroxyl substituent was highlighted, as this was found to have a significant effect on stabilisation and therefore, their participation in redox processes.

1. Introduction

Diet appears to be an important factor contributing to cancer aetiology and in particular to colorectal cancer.[1,2] Dietary phytochemicals have the potential to modulate many of the stages involved in the development of cancer.[3] As antioxidants they are postulated to protect biomolecules such as DNA, protein and lipids from oxidant species, or by direct scavenging of carcinogens.[4,5] Phytochemicals may also exert anticancer effects by modulating the signal transduction pathways that control proliferation and programmed cell death.[6–8] Other important targets are their ability to inhibit the mammalian metalloenzymes involved in the arachidonic acid cascade and other pro-oxidant enzymes such as xanthine oxidase.[9–11] Since oxidation and reduction are fundamental to many of the stages involved in carcinogenesis, an understanding of how the structure of these plant-derived compounds effects their redox chemistry is essential in assessing their potential to participate in these processes.

Although there are a large variety of plant secondary metabolites, approximately one fifth of all carbon fixed by plants is channelled through the shikimate pathway. This pathway is absent in mammals and therefore, the plant metabolites produced by this pathway are an essential component of our diet. The switch from primary to secondary metabolism occurs with the E2 elimination of ammonia from the amino acid, L-phenylalanine to form cinnamic acid. Cinnamic acid is the first metabolite of the phenylpropanoid pathway and the metabolites of this pathway produce the largest range of natural products considered potentially protective of health (Fig. 1). Cinnamic acid substitution follows an *ortho* oxygenation and subsequent methylation pattern and compounds representing both methylated and unmethylated, mono, di and tri-hydroxylated cinnamic acids are included in this study. Compounds representing further transformation reactions of the side-chain reactions have also been included. These compounds, along with the cinnamic acids are the building blocks to lignans and the lignan-like dilignols also selected for study. Substituted benzoic acids and their derivatives are also included, because although these compounds are formed directly from intermediates early in the shikimate pathway, in plants they are more usually formed by degradation of cinnamic acid derivatives. Also included are the cinnamic acid lactone derivatives;

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the coumarins and the structurally diverse and biologically important family of compounds known collectively as the flavonoids, which include representatives of the flavanones, flavones, flavanols, dihydroflavanols and catechins. In addition to all of these basic structural types, effects of hydroxylation, methylation and glycosylation have also been investigated.

Electron paramagnetic resonance (EPR) spectroscopy is the most powerful single technique for the detection and characterisation of radicals. Using EPR we have determined the ease with which the series of phenylpropanoid-derived compounds donated an electron, by measuring the extent and rate at which they reduced the synthetic radical, galvinoxyl.[12] Computational techniques can be used to estimate particular properties when compounds are not readily available and in this paper we have compared calculations made at the AM1 level of theory[13] to the results obtained experimentally. In an attempt to elucidate the more complex effects of structural change on the redox chemistry, we then compared results of spin density distributions for a selected set of structurally related compounds.

2. Results

2.1. C_6C_1 compounds

Mono-hydroxylated acids (2-, 3- and 4-hydroxybenzoic acids **1a**–**c**) were not oxidised and only when certain patterns of di- and trihydroxylation were present was the galvinoxyl radical reduced (Table 1). The trihydroxylated compound (3,4,5-trihydroxybenzoic acid **1j**) was the most easily oxidised, with the dihydroxyl substituents **1d**–**i** showing the following order of ease of oxidation: 2,5 > 2,3 > 3,4, > 2,6. Structures containing 2,4-, and 3,5-dihydroxyl substituents were not oxidised. From this observation, it appears that compounds with dihydroxyl substituents *para* to each other are more easily oxidised than those containing *ortho* substituents, which are in turn more easily oxidised than those containing *meta* substituents. The carboxylic acid side chain had the effect of increasing the ease of oxidation, when it was *meta* to the substituents. For the dihydroxylated compounds, it was observed that all compounds with a HOMO energy greater than −9.46 eV were oxidised. Modification of the structure to contain one methoxyl group at C3 **1k** resulted in the compound not being oxidised, whereas a compound containing two methoxyl groups at C3 and C5 **1l** was oxidised. Therefore, it appears that methoxyl substitution increases the ease of oxidation, but not to the same extent as when additional hydroxyl were substituents. To study the effect of varying the functional group at C1 in the C_6C_1 compounds, a series of compounds in which the aromatic substitution pattern (4-OH, 3-OMe) remained constant were selected (Table 1). For these compounds, only the alcohol **3** reduced the galvinoxyl radical. The acid **1k**, and aldehyde **2a** were not oxidised. When the pattern of substitution was reversed (3-OH, 4-OMe), as was the case for the iso-aldehyde **2b**, the compound still was not oxidised.

2.2. C_6C_3 compounds

To study the effect of increasing the chain length from C1 to C3, the aromatic substitution pattern (4-OH, 3-OMe) and the carboxylic acid functional group were kept constant (Table 2). Only the C_6C_3 acid **6h** reduced the galvinoxyl radical and neither the C_6C_2 **4** nor the C_6C_1 **1k** compounds were oxidised. For the C_6C_3 compounds the effects of both substitution and functional group were studied. Despite the C_6C_3 compounds being more easily oxidised than their C_6C_1 homologues, the same effect of increasing ease of oxidation with increas-

Table 1. Reaction stoichiometry for galvinoxyl radical reduction by C_6C_1 compounds and the frontier electron orbital energy values (eV) for the parent compounds and their radicals

Compound	Reaction stoichiometry	Parent compound		Parent radical		
		HOMO	LUMO	HOMO	SOMO	LUMO
1a 2-Hydroxybenzoic acid	Not reduced	−9.50954	−0.45878	−10.91551	−5.96784	−0.41643
1b 3-Hydroxybenzoic acid	Not reduced	−9.51455	−0.56945	−10.94828	−5.82593	−0.68828
1c 4-Hydroxybenzoic acid	Not reduced	−9.60871	−0.48173	−10.93326	−6.11756	−0.25208
1d 2,3-Dihydroxybenzoic acid	1.41 ± 0.03	−9.22076	−0.55975	−10.4717	−5.6075	−0.46471
				−10.45479	−5.59048	−0.61731
1e 2,4-Dihydroxybenzoic acid	Not reduced	−9.50396	−0.45423	−10.33417	−6.15759	−0.48044
				−10.35394	−6.20571	−0.16392
1f. 2,5-Dihydroxybenzoic acid	1.50 ± 0.03	−9.07923	−0.59993	−10.87503	−5.6193	−0.46485
				−10.88799	−5.47255	−0.59617
1g 2,6-Dihydroxybenzoic acid	0.80 ± 0.03	−9.46883	−0.22441	−10.37278	−6.09513	−0.28892
1h 3,4-Dihydroxybenzoic acid	1.30 ± 0.11	−9.22056	−0.58606	−10.50312	−5.50581	−0.66763
				−10.48061	−5.74064	−0.26935
1i 3,5-Dihydroxybenzoic acid	Not reduced	−9.4869	−0.65919	−10.33745	−6.00346	−0.77985
1j 3,4,5-Trihydroxybenzoic acid	2.13 ± 0.04	−9.22625	−0.66964	−9.86016	−5.73572	−0.77693
				−9.97177	−5.59501	−0.28485
1k. 4-Hydroxy-3-methoxybenzoic acid	Not reduced	−9.10837	−0.51922	−10.33483	−5.61908	−0.16121
1l 4-Hydroxy-3,5-dimethoxybenzoic acid	1.17 ± 0.02	−9.01434	−0.52645	−9.71723	−5.39653	−0.10019
2a 4-Hydroxy-3-methoxybenzaldehyde	Not reduced	−9.03822	−0.48898	−10.28841	−5.54942	−0.1956
2b 3-Hydroxy-4-methoxybenzaldehyde	Not reduced	−9.033	−0.48716	−10.26674	−5.34458	−0.58254
3 4-Hydroxy-3-methoxybenzyl alcohol	0.60 ± 0.04	−8.57475	0.39312	−9.88132	−4.96278	0.42639

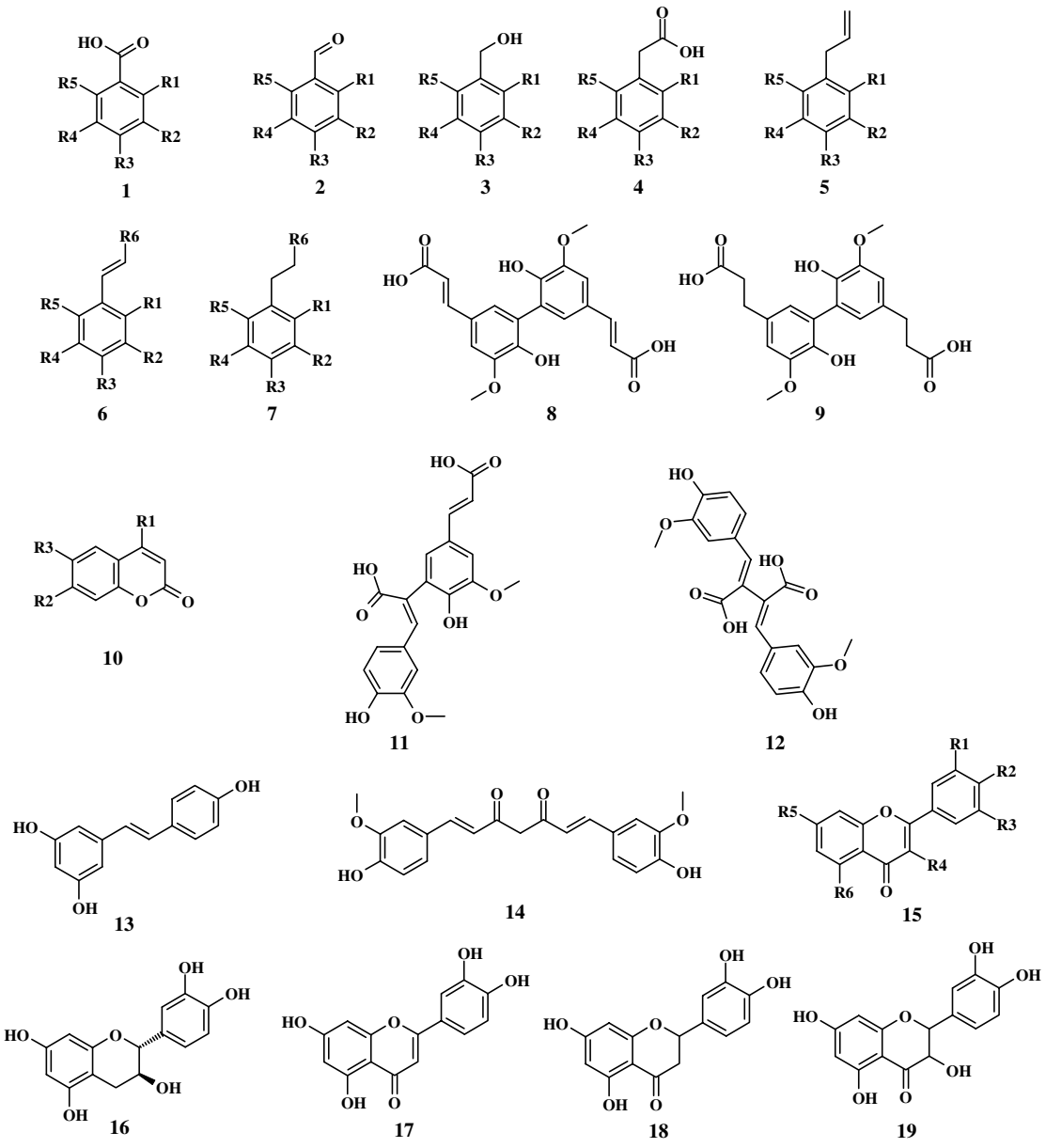

Figure 1. R1 = OH, R2–R5 = H (**1a**); R2 = OH, R1, R3–R5 = H (**1b**); R3 = OH, R1, R2, R4 and R5 = H (**1c**); R1 and R2 = OH, R3–R5 = H (**1d**); R1 and R3 = OH, R2, R4 and R5 = H (**1e**); R1 and R4 = OH, R2, R3 and R5 = H (**1f**); R1 and R5 = OH, R2–R4 = H (**1g**); R2 and R3 = OH, R1, R4 and R5 = H (**1h**); R2 and R4 = OH, R1, R3 and R5 = H (**1i**); R2–R4 = OH, R1 and R5 = H (**1j**); R3 = OH, R2 = OMe, R1, R4 and R5 = H (**1k**); R3 = OH, R2 and R4 = OMe, R1 and R5 = H (**1l**); R3 = OH, R2 = OMe, R1, R4 and R5 = H (**2a**); R4 = OH, R3 = OMe, R1, R4 and R5 = H (**2b**); R3 = OH, R2 = OMe, R1, R4 and R5 = H (**3**); R3 = OH, R2 = OMe, R1, R4 and R5 = H (**4**); R3 = OH, R2 = OMe, R1, R4 and R5 = H (**5**); R2 = OH, R1, R3–R5 = H, R6 = CO_2H (**6a**); R1 = OH, R2–R5 = H, R6 = CO_2H (**6b**); R3 = OH, R1, R2, R4 and R5 = H, R6 = CO_2H (**6c**); R3 = OH, R1, R2, R4 and R5 = H, R6 = CO_2Et (**6d**); R3 = OH, R2 = OMe, R1, R4 and R5 = H, R6 = CO_2Et (**6e**); R3 = OH, R1, R2, R4 and R5 = H, R6 = CH_2OH (**6f**); R3 = OH, R2 and R4 = OMe, R1 and R5 = H, R6 = CO_2Et (**6g**); R3 = OH, R2 = OMe, R1, R4 and R5 = H, R6 = CO_2H (**6h**); R3 = OH, R2 = OMe, R1, R4 and R5 = H, R6 = CH_2OH (**6i**); R3 = OH, R2 and R4 = OMe, R1 and R5 = H, R6 = CO_2H (**6j**); R3 = OH, R2 and R4 = OMe, R1 and R5 = H, R6 = CH_2OH (**6k**); R2 and R3 = OH, R1, R4 and R5 = H, R6 = CO_2H (**6l**); R3 = OH, R2 = OMe, R1, R4 and R5 = H, R6 = CO_2H (**7a**); R2 and R3 = OH, R1, R4 and R5 = H, R6 = CO_2H (**7b**); R3 = OH, R1, R2, R4 and R5 = H, R6 = CO_2H (**7c**); R2 = OH, R1, R3–R5 = H, R6 = CO_2H (**7d**); R2 = OH, R1 and R3 = H (**10a**); R1 = OH, R2 and R3 = H (**10b**); R2 and R3 = OH, R1 = H (**10c**); R1 = Me, R2 = OH and R3 = H (**10d**); R2 = Me, R1 = OH and R3 = H (**10e**); R2 = OH, R3 = OMe and R1 = H (**10f**); R2 = OH, R3 = O-glucose and R1 = H (**10g**); R2, R4–R6 = OH R1 and R3 = H, (**15a**); R1, R2, R4–R6 = OH, R3 = H, (**15b**); R1–R6 = OH (**15c**); R1, R2, R4 and R6 = OH, R3 = H, R5 = OMe (**15d**); R2, R4–R6 = OH, R3 = H, R1 = OMe (**15e**); R1, R4–R6 = OH, R3 = H, R2 = OMe (**15f**) and R1, R2, R5, R6 = OH, R3 = H, R4 = O-glucoside (**15g**).

ing ring hydroxylation and methoxylation was observed. For all substitution patterns, modification of the side chain from the carboxylic acid to the alcohol resulted in increasing the ease of oxidation, as seen for the C_6C_1 compounds and esterification decreased the compound ability to reduce the galvinoxyl radical. Again replacing the 3-methoxyl group with a hydroxyl group to give 3,4-dihydroxycinnamic acid **6l** resulted in increasing the ease of oxidation. With the standard 4-hydroxyl, 3-methoxyl and 3,4-dihydroxyl substitution

Table 2. Reaction stoichiometry for galvinoxyl radical reduction by C_6C_2 and C_6C_3 compounds and the frontier electron orbital energy values (eV) for the parent compounds and their radicals

Compound	Reaction stoichiometry	Parent compound		Parent radical		
		HOMO	LUMO	HOMO	SOMO	LUMO
4 Homovanilic acid	Not reduced	−8.88491	0.13174	−10.05663	−5.17296	0.25485
5 Eugenol	0.79 ± 0.06	−8.5759	0.36403	−9.88842	−4.96512	0.40289
6a 3-Hydroxycinnamic acid	Not reduced	−9.32604	−0.86748	−10.22476	−5.77831	−0.99105
6b 2-Hydroxycinnamic acid	Not reduced	−9.85363	−0.80141	−10.08041	−5.77815	−0.75452
6c 4-Hydroxycinnamic acid	Not reduced	−9.11988	−0.78821	−10.87332	−5.80599	−0.8232
6d Ethyl 4-hydroxycinnamate	Not reduced	−9.16465	−0.77723	−10.89863	−5.83169	−0.77034
6e Ethyl 4-hydroxy-3-methoxycinnamate	0.14 ± 0.05	−8.95258	−0.82792	−10.28084	−5.47885	−0.68911
6f 4-Hydroxycinnamyl alcohol	0.74 ± 0.02	−8.76362	−0.12819	−10.38905	−5.39648	−0.26929
6g Ethyl 4-hydroxy-3,5-dimethoxycinnamate	0.87 ± 0.17	−8.80779	−0.83035	−8.78757	−5.26116	−0.62532
6h 4-Hydroxy-3-methoxycinnamic acid	1.37 ± 0.05	−8.84913	−0.82485	−10.23216	−5.43796	−0.7103
6i 4-Hydroxy-3-methoxycinnamyl alcohol	1.49 ± 0.10	−8.45336	−0.11646	−9.72197	−5.00762	−0.06633
6j 4-Hydroxy-3,5-dimethoxycinnamic acid	1.67 ± 0.06	−8.76742	−0.83769	−9.77507	−5.24628	−0.68652
6k 4-Hydroxy-3,5-dimethoxycinnamyl alcohol	1.78 ± 0.06	−8.38945	−0.07973	−9.38175	−4.79961	−0.02136
6l 3,4-Dihydroxycinnamic acid	1.69 ± 0.02	−8.9397	−0.88366	−10.57365	−5.54341	−0.93819
				−9.98376	5.43439	−0.93819
7a 3-(4-Hydroxy-3-methoxy phenyl)propionic acid	0.17 ± 0.06	−8.79374	0.13826	−10.07926	−5.1014	0.22796
7b 3-(3,4-Dihydroxyphenyl)propionic acid	2.17 ± 0.10	−8.93147	0.09917	−10.17905	−5.34007	0.1038
				−10.09473	−5.26871	0.02141
7c 3-(4-Hydroxyphenyl)propionic acid	Not reduced	−9.104	0.2111	−10.67721	−5.51714	0.17081
7d 3-(3-Hydroxyphenyl)propionic acid	Not reduced	−9.22479	0.16044	−10.43876	−5.61166	0.0472

patterns, the effect of a fully saturated side chain was investigated. For the methoxylated compound **7a** this resulted in a decrease in the ease of oxidation, suggesting that for this pattern of substitution, the presence of the conjugated side chain, has an important role in stabilising the parent radical. However, for the dihydroxylated compound **6l** an increased ease of oxidation was achieved by reduction of the olefinic side chain **7b**, demonstrating that for this structure the ability of the compound to reduce the galvinoxyl radical was increased, not by extended delocalisation of the parent radical, but more likely by formation of a quinone. This stabilising property of the olefinic double bond was further investigated for a series of compounds, which showed an increased ease of oxidation with increased methoxyl substitution and also an increased ease of oxidation as the functional group was modified. The alcohol was found to be more easily oxidised than the acid, which in turn was more easily oxidised than the ester. For these compounds the distribution of the unpaired electron was calculated and compared (Fig. 2). For all compounds, it was observed that for the compounds which reduced the galvinoxyl radical, a greater proportion of the positive spin density was found at EPR-silent positions (i.e., >0.53). This value is most likely to reflect the amount of spin density residing on the phenolic oxygen and is in good correlation with the graphical distributions calculated computationally. This suggests that for the mono-hydroxylated compounds, extending the conjugation stabilises the parent radical formed and that the distribution patterns of the unpaired electron appears to also have an important effect. For all of the mono-hydroxylated compounds (with the exception homovanillic acid), there was good correlation of the HOMO energies with the ease of oxidation. Compounds with HOMO energies greater than −9.01 eV and SOMO energies of the parent radicals greater than −5.48 eV were oxidised, whereas the compounds with lower energy orbitals were not.

2.3. C_6C_3 coumarins and $(C_6C_3)_2$ compounds

For the coumarins, it is possible to delocalise the radical between both rings and so a series of structures, which represented both the standard 4-hydroxyl, 3-methoxyl and 3,4-dihydroxyl substitution patterns were selected (Table 3). Structures with hydroxyls on each separate ring, with **10d**,**e** and without methylation **10a**,**b** and where the hydroxyl at C3 was methoxylated **10f** and glycosylated **10g** were also included. Only the 3,4-dihydroxylated compound **10c** reduced the galvinoxyl radical. As for all the simple single ring phenols, monohydroxylated compounds were not oxidised. However, unlike the 4-hydroxy,3-methoxycinnamic acid **6h**, the 4-hydroxyl, 3-methoxyl pattern of substitution for the coumarin structure **10f** did not reduce the galvinoxyl radical. This demonstrates that formation of a second ring of this structure had an inhibitory effect on the ease of oxidation. To examine this further, compounds were synthesised to represent potential delocalisation of the radical from 4-hydroxy,3-methoxycinnamic acid into further ring systems by coupling at positions C5 and C8 (Table 3). Coupling at C5 **8** resulted in an increase in the ease of oxidation, whereas coupling at C8 to C5 **11** or C8 to C8 **12** both resulted in a decrease in the ease of oxidation, as did full saturation of the olefinic double bond **9**. It appeared that extended conjugation through the side chain caused a reduction in the ability of the compound to reduce the galvinoxyl radical whereas, when the compound was coupled through the aromatic rings alone, an increase in the ability of this compound to reduce the galvinoxyl radical was observed. To test whether the increased ease of oxidation with C5–C5 coupling was a result of the presence of an additional hydroxyl group

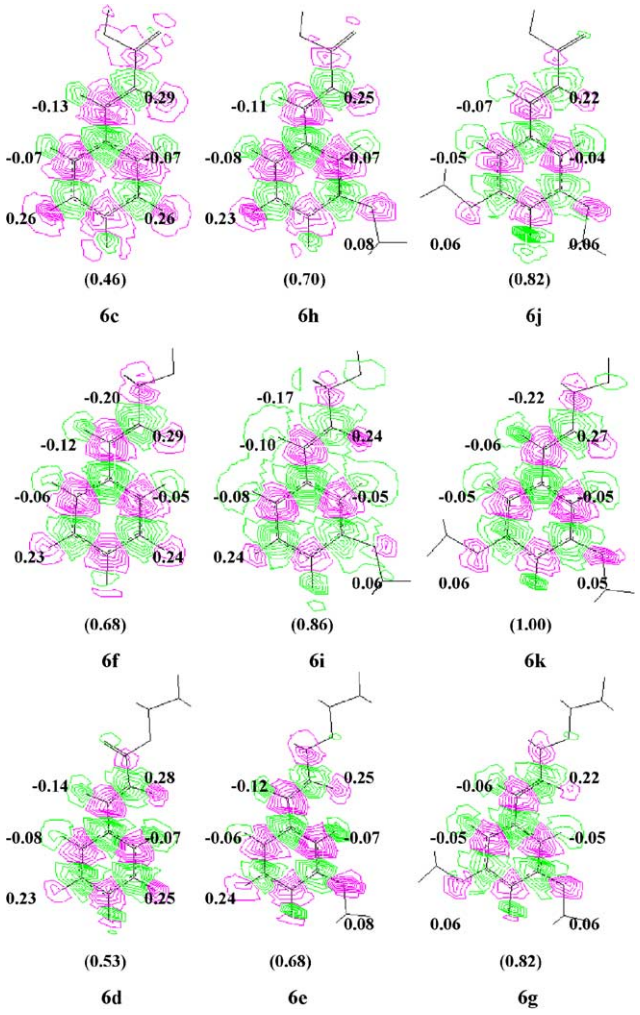

Figure 2. Graphical distribution of the unpaired electron for geometrically optimised structures calculated computationally with spin density distributions at the carbon nuclei calculated from EPR hyperfine constants (see experimental methods). Positive spin densities are shown in green and the calculated EPR-silent values are given in parentheses.

at C4, a compound representing coupling through the side chain without involvement and extension of conjugation of the olefinic double bond was tested **14**. This compound was also more easily oxidised and showed a very similar level of oxidation to the C5–C5 coupled compound, suggesting that the presence of the second hydroxyl substituent is most likely to be responsible.

2.4. $C_6C_3C_6$ compounds

Quercetin **15b** was chosen as the standard skeleton-type representative of the flavonoids, as this compound has the 5,7-dihydroxylation pattern on the A-ring most commonly observed in nature and the 3,4-dihydroxylation pattern on the B-ring investigated thoroughly for the simpler phenols. As for the simple phenols, when the extent of hydroxylation on the B-ring was varied, the results followed the same pattern with the trihydroxylated compound **15c** being more easily oxidised than the dihydroxylated compound **15b**, which in turn was more easily oxidised that the monohydroxylated compound **15a**. To determine whether the A-ring or B-ring had the most significant effect on ease of oxidation, a series of compounds, which were selectively, methoxylated on the A and B rings were examined **15d–f**. Methoxylation on both rings resulted in a decrease in the ease of oxidation and this followed the pattern: B4 > B3 > A7, showing the B-ring to have the most significant effect. However, when hydroxyl formation on the C-ring at position 3 was blocked by glycosylation **15g**, this had the most significant effect on the ability of this compound to reduce the galvinoxyl radical. Modifying the C-ring in this way not only removed the additional hydroxyl group from the structure, but will also have an effect on the delocalisation of the parent radical throughout the entire molecule. Keeping the 5,7-hydroxylation on the A-ring and 3,4-hydroxylation on the B-ring, the effect of C-ring structure modification was determined. When an olefinic double bond was present in the C-ring **15**,**17**, this resulted in an overall increased ease of oxidation, but this was slightly reduced if position 3 was hydroxylated **15**. This suggests that for these compounds the extended conjugation from the B-ring into the C-ring is important in stabilising the parent radical, as the presence of a substituent at C3 in the C-ring will block the formation of a radical at this site, therefore decreasing the stability of the parent radical formed. This is further confirmed by the structure containing a carbonyl group at position 4 and absence of an olefinic double bond in the C-ring **18**, which showed a dramatic decrease in the ability of this compound to reduce the galvinoxyl radical. For this structure, introduction of the hydroxyl at C3 **19** showed an increase in the ease of oxidation in contrast to when extended

Table 3. Reaction stoichiometry for galvinoxyl radical reduction by compounds containing more than one ring. Standard deviations are calculated for $n = 3$ (a), $n = 6$ (b) and $n = 5$ (c) values

Compound	Reaction stoichiometry
8 4-Hydroxy-3-methoxycinnamic acid, 5-5 linked	1.72 ± 0.03^a
9 3-(4-Hydroxy-3-methoxyphenyl)propionic acid, 5-5 linked	0.22 ± 0.01^a
10a 7-Hydroxycoumarin	Not reduced
10b 4-Hydroxycoumarin	Not reduced
10c 6,7-Dihydroxycoumarin	3.77 ± 0.01^a
10d 7-Hydroxy-4-methylcoumarin	Not reduced
10e 4-Hydroxy-7-methylcoumarin	Not reduced
10f 7-Hydroxy-6-methoxycoumarin	Not reduced
10g 7-Hydroxycoumarin-6-glucoside	Not reduced
11 4-Hydroxy-3-methoxycinnamic acid, 8-5 linked	0.97 ± 0.03^a
12 4-Hydroxy-3-methoxycinnamic acid, 8-8 linked	Not reduced
13 Resveratol	0.55 ± 0.12^b
14 Curcumin	1.75 ± 0.09^b
15a Kaempferol	2.68 ± 0.15^c
15b Quercetin	3.34 ± 0.40^c
15c Myricetin	4.80 ± 0.26^c
15d Rhamnetin	2.52 ± 0.22^b
15e Isorhamnetin	2.26 ± 0.21^b
15f Tamarixetin	1.07 ± 0.03^b
15g Isoquercetin	1.35 ± 0.05^b
16 Epicatachin	2.92 ± 0.18^c
17 Luteolin	3.53 ± 0.05^c
18 Taxifolin	2.98 ± 0.12^c
19 Eriodictyol	0.90 ± 0.17^c

conjugation was present. There is very little change in the ability of this compound to reduce the galvinoxyl radical whether the carbonyl group at C4 is present **19** or not **16**, suggesting that this effect is predominantly due to the presence of the additional hydroxyl group alone. Overall this suggests that, although the presence of an additional hydroxyl group increases the ease of oxidation, this has a less significant effect than the increased stability obtained through extended conjugation.

Stoichiometric studies are ideal for comparing the reducing capabilities of the various phenolic compounds studied. However, in a biological system kinetic factors may also have an important role in controlling the redox chemistry. This is likely to be particularly relevant in poly-hydroxylated compounds such as the flavanoids and for this group of compounds, studies have already demonstrated differences in reaction stoichiometry and kinetics.[12] To determine the kinetic effect of the C_6C_1 and C_6C_3 phenolic acids on the reduction of the galvinoxyl radical, the decay curves of this radical was observed in the presence of a selected subset of phenolic compounds (Fig. 3). For the hydroxylated C_6C_1 compounds (Fig. 3A), the 3,4,5-trihydroxylated compound, which had the highest effect in the stoichiometric measurements also had the greatest effect on the in situ reduction of the galvinoxyl radical. The 3,4-dihydroxylated and monhydroxylated compounds were all less effective and followed the order 3-OH > 2-OH > 3,4-diOH > 4-OH. The effect of methoxylation on the C_6C_3 hydroxycinnamyl alcohols also showed the same pattern as the stoichiometric measurements with the dimethoxylated compound having the greatest effect and the mono-methoxylated compound being more effective than the non-methoxylated compound. The effect of functional group in the C_6C_3 compounds showed that as for the stoichiometric measurements, that the cinnamyl alcohol was the most effective. However, unlike the stoichiometric results, the ester appeared to be slightly more effective than the acid, but both of these compounds were quite low in effect.

3. Discussion

For all compounds studied, the ease of phenolic oxidation was increased by introduction of methoxyl or hydroxyl substituents into the aromatic ring, hydroxylation having the greater effect. Keeping the aromatic substitution pattern constant and increasing the chain length also increased the ease of oxidation and this was additionally increased by introduction of an olefinic double bond in the C_6C_3 cinnamic acids. However, this effect can not be simply attributed to the effect of extending the conjugation of the system, as the ease of oxidation for the 3,4-dihydroxylated cinnamic acid **6l** increased on full saturation of the olefinic double bond **7b**. For the monohydroxylated phenols, this is due to the distribution of the unpaired electron within the parent radicals, as demonstrated for a series of phenylpropanoids varying in their ability to oxidise the galvinoxyl radical. However, for compounds containing 3,4-dihydroxylated substituents this is more likely to be attributed to quinone formation and for the simpler phenols this has been addressed in a separate study.[14]

When the effect of ring formation was investigated, again contrasting results were obtained for the 4-hydroxy-3-methoxyl and the 3,4-dihydroxyl patterns of substitution. The coumarin structure containing the 4-hydroxy-3-methoxyl substituents **10f** was not oxidised, demonstrating that ring formation of this type had an inhibitory effect, whereas the 3,4-dihydroxylated coumarin **10c** reduced the galvinoxyl radical more easily than 3,4-dihydroxycinnamic acid **6l**. Again, this is likely to be due to the fact the dihydroxylated does not give increased stabilisation through extended delocalisation of the parent radical, but through quinone formation. An inhibitory effect on ease of oxidation was also observed when 4-hydroxy-3-methoxycinnamic acid was coupled through C8 **12**. Again, this is most likely to be due to removal of extended conjugation, as the C8 position is not free to support a stable radical. This is further confirmed by an increased and similar ease of oxidation observed

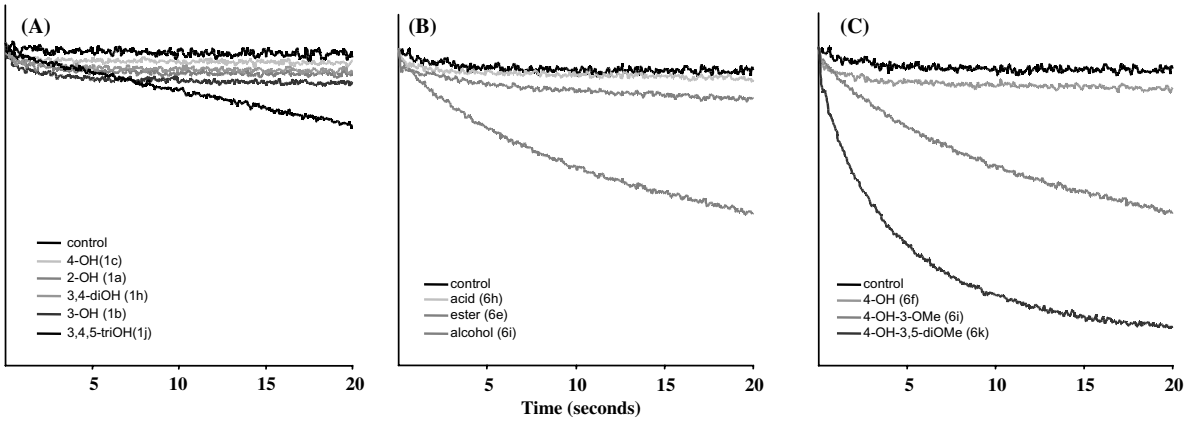

Figure 3. Decay curves of the galvinoxyl resonance obtained during in situ reduction by A. hydroxylated C_6C_1 compounds; B. 4-hydroxy-3-methoxycinnamyl alcohol, 4-hydroxy-3-methoxycinnamic acid and ethyl 4-hydroxy-3-methoxycinnamate and C. substituted C_6C_3 hydroxycinnamyl alcohols.

when 4-hydroxy-3-methoxycinnamic acid is coupled through C5 **8** and C9 **14**. The flavonoids exhibited the same results with regard to hydroxylation patterns as the simpler phenols. With the A-ring containing the commonly found 5,7-dihydroxylation pattern, it was observed that the *ortho*-hydroxyl substituents on the B-ring had a greater contribution to the ability of the compound to reduce the galvinoxyl radical. However, the most significant effect on ease of oxidation was observed when the C-ring structure was modified, as this will effect both stabilisation obtained through increased delocalisation of the radical and through quinone formation.

The HOMO and SOMO energies calculated for the mono- and dihydroxylated compounds and their radicals at the AM1 level of theory correlate well with the experimental data obtained and gave a good indication as to whether a compound reduce the galvinoxyl radical. However, it was observed that the dihydroxylated compounds, which were oxidised, had lower HOMO and SOMO energies than the mono-hydroxylated compounds and this in part may be due to different mechanism of stabilisation. Since the mechanisms of stabilisation in the $(C_6C_3)_2$ and $C_3C_6C_3$ compounds are more complex due to the extended conjugation, multiple hydroxyl substituents and the ability of some of these compounds to form quinones,[15] no attempt was made to correlate the frontier electron energies with the experimental data. However, rigorous computational calculations have been shown to be extremely useful in studies of particular flavonoids with regard to their radical scavenging ability.[16–18] Generally, semi-empirical calculations gave a good indication of the ease of oxidation within a series of compounds with similar structural properties. To explore the mechanisms of stabilisation further and to investigate the redox properties of more complex molecules, further work to improve the accuracy of these results using ab initio calculations would be desirable. Overall, these results highlight the importance of electron delocalisation and the effect this has on stability of the parent radicals. It also draws attention to the significance of quinone formation in compounds that contain more than one hydroxyl, which for the compounds of interest tend to be the more biologically important in terms of their redox potential. The kinetic data obtained for the C_6C_1 and C_6C_3 compounds showed similar effects for hydroxyl and methoxyl substitution to that of the stoichiometric data studied. However, it is likely that in a biological system where many of these compounds will be present as mixtures, the reaction rates will be an important aspect. Although, the data suggests that this may be less important in the simpler phenolics, where the trends are similar, it may be an overriding factor in the more complex poly-hydroxylated $C_6C_3C_6$ compounds. So, although the redox chemistry of phytochemicals is not the only factor to be considered in the interaction of these compounds with metalloenzymes and other redox systems, an understanding of these processes can serve as an extremely useful aid in interpreting results from further studies in this area.

4. Experimental procedures

Compounds **1b**, **1e–i**, **2a**, **2b**, **3**, **5**, **6a–c**, **6j**, **10c**, **10d** and **10f** were purchased from Aldrich, compounds **1a**, **1c**, **1j**, **6l**, **10a**, **10b**, **10e**, **10g**, **13**, **15**, **15a–c** and **16** were purchased from Sigma and compounds **1d**, **4** and **6h** were purchased from Fluka. Compounds **15d–g**, **17–19** were purchased from Apin. Compound **14** was purchased from Alexis. Compounds **6d–g**, **6i** and **6k** were synthesised as reported previously.[19] Compound **8** was prepared by the initial coupling of 4-hydroxy-3-methoxy-benzaldehyde. The 4-hydroxyl substituent was then protected by acetylation and the side chain extended by a malonic acid condensation, as detailed below. Compound **9** was then prepared by hydrogenation of compound **8**, as were compounds **7a–d** from their corresponding cinnamic acids. Compound **11** was prepared according to the procedure of Ralph et al.[20] and compound **12** by the procedures of Cartwright and Haworth[21] and Ralph et al.[20]

4.1. Synthesis: 5-5′ dehydrodi-(4-hydroxy-3-methoxy-cinnamic acid) 8

4-Hydroxy-3-methoxybenzaldehyde [Aldrich] (5.22 g; 6.6 mmol) was dissolved in citrate/phosphate buffer (0.1 mol dm^{-3}; pH 4.2). Hydrogen peroxide (2.82 cm^3; 27.5% w/v) and horseradish peroxidase [Sigma] (2025 units) were added and the reaction left stirring at 36.5 °C overnight.[22] The precipitate was filtered and washed with water and then chloroform to give the coupled product. Yield 97%; δ_H ($(CD_3)_2SO$) 3.92 (6H, s, OCH_3), 7.44 (4H, s, C(2 and 6)H) and (2H, s, C(7)H) ppm. δ_C ($(CD_3)_2SO$) 55.95 (OCH_3), 109.20 (C2), 124.63 (C3), 127.61 (C6), 127.97 (C1), 148.20 (C4), 150.66 (C5), 190.95 (CHO) ppm. The product (2 g, 6.6 mmol) was dissolved in a mixture of acetic anhydride (50 cm^3) and sodium acetate (2 g) and the temperature raised to 100 °C until dissolved and then maintained at 80 °C for 1 h. Diluted with ice water (100 cm^3) and left for 1 h. Extracted into chloroform (20 cm^3 × 3) and washed with sodium hydrogen carbonate (3% w/v). The organic layer was left to stand over sodium sulfate (anhydrous), filtered and the solvent removed in vacuo. Yield 79%; δ_C ($(CD_3)_2SO$) 19.86 ($COCH_3$), 56.34 (OCH_3), 111.87 (C2), 124.50 (C5), 130.60 (C6), 134.45 (C1), 141.84 (C4), 151.88 (C3), 167.38 ($COCH_3$), 191.67 (CHO) ppm. δ_H ($(CD_3)_2SO$), 2.06 (6H, s, ($COCH_3$)), 3.91 (6H, s, OCH_3), 7.45 (2H, d, *J* 1.90, C(2)H), 7.69 (2H, d, *J* 1.90, C(6)H), 9.99 (2H, s, CHO) ppm. The product (0.5 g, 1.3 mmol) and malonic acid [BDH], (1 g) were dissolved in pyridine (2 cm^3).[23] Piperidine and aniline (0.04 cm^3 of each; both freshly distilled) were added and the mixture warmed to 55 °C for 1 h under nitrogen. Left to stand for 16 h at room temperature and then precipitated with HCl (2 mol dm^{-3}). The precipitate was filtered, washed with water and crystallised from acetic acid to give a white solid. Yield 68%. δ_C ($(CD_3)_2SO$) 19.93 ($COCH_3$), 56.23 (OCH_3), 111.44 (C2), 119.98 (C5), 122.70 (C8), 130.66 (C6), 132.62 (C1), 138.46 (C4), 142.86 (C7), 151.33 (C3), 167.32 (C9), 167.61 ($COCH_3$) ppm. δ_H ($(CD_3)_2SO$), 2.02 (6H, s, $COCH_3$), 3.91 (6H, s, OCH_3),

6.59 (2H, d, *J* 16, C(8)H), 7.10 (2H, s, C(2)H), 7.52 (2H, s, C(6)H), 7.57 (2H, d, *J* 16, C(7)H) ppm. The product was dissolved in sodium hydroxide (5 cm^3; 20% w/v) and left at 55 °C for three hours. Crystallised from methanol to give 5-5′ dehydrodi-(4-hydroxy-3-methoxycinnamic acid) as an off-white solid. Yield 98%. δ_C ($(CD_3)_2SO$) 56.00 (OCH_3), 109.53 (C2), 115.84 (C5), 124.82 (C6), 125.16 (C8, C1), 144.37 (C7), 146.34 (C3), 147.88 (C4), 167.76 (C9) ppm. δ_H ($(CD_3)_2SO$), 3.88 (6H, s, OCH_3), 6.38 (2H, d, *J* 15.8, C(8)H), 7.03 (2H, d, *J* 2.0 C(2)H), 7.30 (2H, d, *J* 2.0 C(6)H), 7.50 (2H, d, *J* 15.8, C(7)H) ppm.

4.2. 5-5′ Dehydrodi-3-(4-hydroxy-3-methoxyphenyl)propionic acid 9

Compound **8** was dissolved in ethyl acetate (5 cm^3), palladium on activated carbon was added (5%; 1 mg) and the mixture was stirred at room temperature under hydrogen for 2 h. The mixture was then filtered and the solvent removed in vacuo to give 5-5′ dehydrodi-(4-hydroxy-3-methoxy- phenylpropionic acid) as a white solid. Yield 100%. δ_C ($(CD_3)_2SO$) 29.74 (C8), 35.27 (C7), 55.52 (OCH_3), 110.52 (C2), 122.29 (C5), 125.51 (C6), 130.63 (C1), 141.35 (C3), 147.27 (C4) and 173.46 (C9) ppm. δ_H ($(CD_3)_2SO$), 2.50 (4H, t, *J* 7.2, C(8)H), 2.74 (4H, t, *J* 7.2, C(7)H), 3.79 (6H, s, OCH_3), 6.57 (2H, d, *J* 1.9 C(2)H) and 6.79 (2H, d, *J* 1.9 C(6)H) ppm.

4.3. 3-(4-Hydroxy-3-methoxyphenyl)propionic acid 7a, 3-(3,4-dihydroxyphenyl)propionic acid 7b, 3-(4-hydroxyphenlyl)propionic acid 7c and 3-(3-hydroxyphenyl)-propionic acid 7d

Compounds **6h**, **6l**, **6c** and **6a** were hydrogenated as described for compound **9** to give the corresponding hydroxypropionic acids **7a**–**7d**.

4.4. 8-5 Dehydrodi-(4-hydroxy-3-methoxycinnamic acid) 11

Compound **6e** (2 g) was dissolved in acetate buffer (2 mol dm^{-3}; pH 4) by heating to 60 °C and then cooled to 40 °C. Hydrogen peroxide (0.76 cm^3) and horseradish peroxidase [Sigma] (10 mg in 2 cm^3 buffer) were added and the precipitate collected by filtration after 10 min. The filtrate was partitioned between ethyl acetate and saturated aq NaCl. The organic layer was then left to stand over Na_2SO_4 (anhydrous), filtered and the solvent removed in vacuo. Yield 99%. This compound (83.5 mg, 0.189 mmol) was dissolved in CH_2Cl_2 (2 cm^3), 1,8-diazabicyclo(5.4.0.)undec-7-ene (0.125 cm^3, 0.836 mmol) was added and the solution stirred at room temperature for 4 h. Diluted with CH_2Cl_2 and washed with HCl (3% v/v) and saturated aq NaCl. The organic layer was left to stand over Mg_2SO_4, filtered and evaporated to a residue. Purified by flash chromatography eluting with $CHCl_3$/EtOAc (5:1) and ethyl acetate to obtain a yellow solid. Yield 85%. This compound was dissolved in 1,4-dioxane (2 cm^3) under nitrogen, KOH (40% w/v; 5 cm^3) was added and the solution stirred at room temperature for 2 h. Acidified with HCl (2 mol dm^{-3}) and partitioned between ethyl acetate and saturated aq NaCl. The organic layer was then left to stand over Na_2SO_4 (anhydrous), filtered and the solvent removed in vacuo. Yield 84%. Purified by flash chromatography eluting with $CHCl_3$/ethyl acetate (5:1) and ethyl acetate to give 8-5 dehydrodi-(4-hydroxy-3-methoxycinnamic acid) as a yellow solid. Yield 45%; NMR δ_H (DMSO) 3.35 (3H, s, $AOCH_3$), 3.87 (3H, s, $BOCH_3$), 6.24 (1H, d, *J* 15.9 B(8)H), 6.57 (1H, d, *J* 2.1 B(2)H), 6.61 (1H, d, *J* 8.4 B(5)H), 6.74 (1H, dd, *J* 2.1, 8.4 B(6)H), 6.88 (1H, d, *J* 1.9 B(6)H), 7.18 (1H, d, *J* 1.9 B(2)H), 7.52 (1H, d, *J* 15.9 B(7)H) and 7.74 (1H, s, A(7)H) ppm.

4.5. 8-8′ Dehydrodi-(4-hydroxy-3-methoxycinnamic acid) 12

Initial coupling of compound **6h** (2 g) was as described for compound **6e**. Purified by flash chromatography eluting with ethyl acetate/$CHCl_3$ 3:1. Yield 10%; NMR δ_H (DMSO) 3.80 (6H, s, OCH_3), 4.17 (2H, br s, C(8)H), 5.83 (2H, br s, C(7)H), 6.1 (2H, d, *J* 9.1 C(5)H), 6.87 (2H, d, *J* 9.1 C(6)H) and 6.98 (2H, br s, C(2)H) ppm. NMR δ_C (DMSO) 47.96 (C8), 55.76 (OCH_3), 81.91 (C7), 110.64 (C2), 115.41 (C5), 119.07 (C6), 128.90 (C1), 147.31 (C4), 147.80 (C3) and 175.21 (C9) ppm. The product (100 mg) was dissolved in NaOH (10 cm^3; 2 mol dm^{-3}) and left stirring overnight under nitrogen. Acidified with HCl (2 mol dm^{-3}) and partitioned between ethyl acetate and saturated aq NaCl. The organic layer was left to stand over Na_2SO_4, filtered and removed solvent in vacuo. The product was dissolved in methanol (10 cm^3) and diazo(trimethylsilyl)methane (1.62 cm^3 of 2 mol dm^{-3} in hexane) was added in aliquots of 0.27 cm^3. The solvent was removed in vacuo, the product dissolved in CH_2Cl_2 (anhydrous, 10 cm^3) and DBU (0.15 cm^3) was added. Stirred at room temperature for 4 h, diluted with CH_2Cl_2 and washed with HCl (3% w/v) and saturated aq NaCl. The organic layer was left to stand over Na_2SO_4 and removed solvent in vacuo. The product was dissolved in 1,4-dioxane (2 cm^3) under nitrogen at room temperature and NaOH (2 mol dm^{-3}; 5 cm^3) was added. After 20 h, acidified with HCl (2 mol dm^{-3}) and partitioned between EtOAc and saturated aq. NaCl. The organic layer was left to stand over Na_2SO_4, filtered and the solvent removed in vacuo. Purified by flash chromatography eluting with ethyl acetate/$CHCl_3$ 3:1 and recrystallised from methanol to give 8-8′ dehydrodi-(4-hydroxy-3-methoxycinnamic acid) as a yellow solid. Yield 76%; NMR δ_H (DMSO) 3.80 (6H, s, OCH_3), 6.75 (2H, d, *J* 9.1 C(5)H), 7.02 (2H, d,d *J* 9.1, 2.1 C(6)H), 7.19 (2H, d, *J* 2.1 C(2)H) and 7.83 (2H, s, C(7)H), ppm.

4.6. Electron paramagnetic resonance spectroscopy

Aliquots (3 cm^3) of galvinoxyl [Aldrich] (0.5 mmol dm^{-3} in methanol) were mixed with substrates (measured at various concentrations to determine linearity, typically 0.1–0.5 mmol dm^{-3} in methanol) and transferred to an EPR quartz cell. Spectra (X-band) of unreacted galvinoxyl were recorded after 5 on a Bruker E106 spectrometer, equipped with a TM_{110} cavity. The following instrument settings were used: modulation frequency 100 kHz; centre field 3480.40 Gauss; sweep width

60 Gauss; time constant 40.96 ms; power, 1.01 mW and a suitable receiver gain setting, typically 1×10^4. The galvinoxyl concentrations remaining were calculated by integration of the signal and comparison with the control. From these concentrations, the stoichiometry of the reaction was calculated. Continuous-flow EPR spectroscopy was performed as reported previously.[19] Computer simulations of spectra, giving the hydrogen hyperfine coupling constants (a_H), were performed using the SIMEPR program,[24] which sequentially varies all the parameters for each radical species until a minimum in the error surface is located. Goodness-of-fit was determined by visual comparison and as a minimum in the sum of the squared residuals. The density of the unpaired electron was calculated from the hyperfine coupling constants using the McConnell relationship[25] in which the proportionality factor was 22.5 Gauss, the best estimate assuming the value to be constant for all C–H bonds.[26] The unpaired electron density on the EPR-silent positions was then calculated by difference. Kinetic measurements were obtained using solutions of galvinoxyl and the selected phenolics (0.2 mmol dm^{-3}) in methanol and de-oxygenating in a stream of nitrogen. Aliquots were transferred to gas-tight syringes, which were rapidly evacuated into a two-stream EPR cell. Decay curves were obtained by operating in time sweep mode with a static field set at the resonance maximum for the galvinoxyl radical signal and using a microwave power of 1.01 mW and time constants of 5.12 ms. Decay curves were obtained for each phenolic in triplicate and the mean curves are given in Figure 3.

4.7. Semi-empirical calculations

Calculation of the frontier electron orbitals and graphical representations of the spin densities were made using the AM1 theory on geometry optimised structures with the MOPAC program running on an SGI Indigo 2 workstation.

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