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A joint experimental and theoretical investigation on the oxidative coupling of resveratrol induced by copper and iron ions

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ABSTRACT

Currently, a mounting interest exists on the biological activity of polyphenolic compounds, which have been suggested to exert positive effects on the human health. In this paper we report the first electrospray ionization mass spectrometry (ESI-MS) study on the gas-phase production of isomeric δ -viniferin and ε -viniferin dimers in racemic form, starting from acetonitrile/water solutions containing resveratrol and CuSO_4 or FeCl_3 , respectively. Interestingly, the formation of racemic δ -viniferin dehydrodimer is observed in ESI-MS experiments carried out on resveratrol-copper mixtures, while the analogous resveratrol-iron reaction affords the racemic ε -viniferin dehydrodimer. The use of gas-phase techniques and of ab initio calculations, at BHandHLYP/LACV3P++** level of theory, allowed us to elucidate some important aspects of these reaction mechanisms. In particular, a different stability for the resveratrol radicals involved in the oxidative coupling has been obtained in the presence of copper ion, favoring the formation of δ -viniferin, as proposed for the in vivo mechanism where copper is able to switch the resveratrol from an antioxidant to a prooxidant agent. Finally, the structure-reactivity relationship has been investigated for synthetic analogues of resveratrol, showing the crucial role of the OH group in *para* position.

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1. Introduction

Resveratrol ($= (E)-5-[2-(4\text{-hydroxyphenyl})\text{ethenyl}]$ benzene-1, 3-diol, **1** in Scheme 1) is a polyphenolic compound present in various plants, including grapevines, legumes, pines, berries and peanuts. Recent studies have renewed interest in this natural compound, which seems to be responsible for a variety of biological effects, including anti-inflammatory, anti-platelet, and anti-carcinogenic activities [1–3].

The biological properties of resveratrol are linked to its capacity to act both as an antioxidant as well as a prooxidant agent [4]. Theoretical studies on the antioxidant action have shown that hydrogen abstraction from the OH group in *para* position is more favored than from *meta* positions, leading to the formation of the phenoxide radical **A₁** [5–7]. This reactive species undergoes a coupling reaction producing **2**, followed by an intramolecular nucleophilic attack, to obtain the racemic dehydrodimer **3**, having structure like the natural product δ -viniferin (Scheme 1) [8]. However, also hydrogen abstraction from the OH group in *meta* position can occur, to

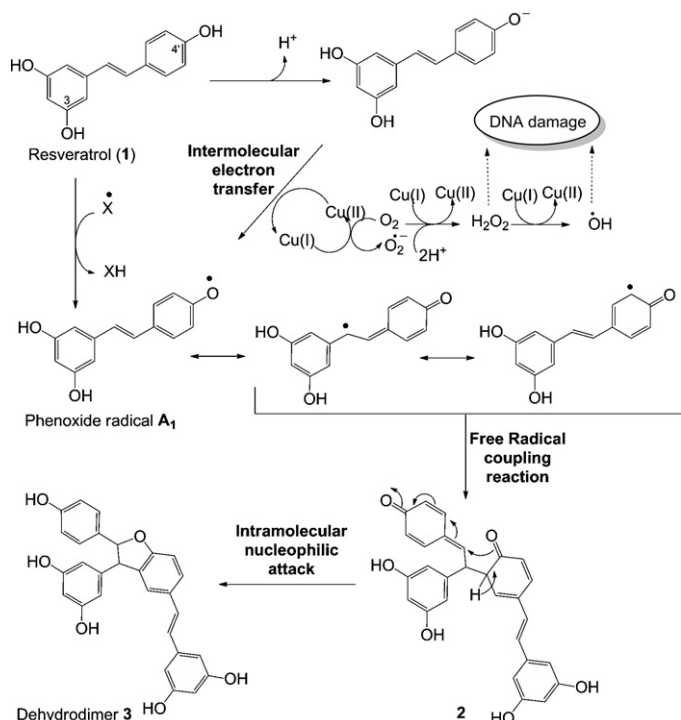
produce the racemic dehydrodimer **4** with the structure of the natural product ε -viniferin (Fig. 1) [8].

Copper is one of the most redox-active metal ions present in living cells and is able to switch the resveratrol from an antioxidant to a prooxidant agent. Cu(II) ions are reduced by resveratrol to Cu(I) , which then generate reactive oxygen species (ROS) [9–12]. The prooxidant mechanism of resveratrol is based on the acidity of the phenol group, involving a proton loss from the OH in *para* position, to give a phenoxide anion which participates in a redox reaction with Cu(II) ion. The product of the last reaction is again the phenoxide radical **A₁** (as in the antioxidant process), which eventually gives the same final dehydrodimer **3** [13]. The latter species has been isolated from plants where it is produced through a metabolic sequence induced in response to biotic or abiotic stress [14–16]. Beside copper, also other metal ions, such as iron (III) have similar redox reaction with resveratrol leading to either **3** or **4**, or both [17–19].

Aim of the present study is to investigate by ESI mass spectrometry the oxidation of resveratrol induced by metal ions to give products **3** and **4**, also compared with the reactivity of synthetic analogues **5–8** (Fig. 1). A simplified modeling considering only ab initio calculations on the thermodynamics of the possible reaction intermediates and products have been carried out to gain insights into the reaction mechanism.

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Scheme 1. Chemical structure of resveratrol (**1**) and proposed antioxidant and prooxidant mechanisms.

2. Material and methods

2.1. Material

Resveratrol and laccase, from *Trametes versicolor* (Activity Measures >20 U/mg) were supplied by Sigma–Aldrich Co. (St. Louis, USA). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and FeCl_3 were purchased from Carlo Erba (Milano, Italy). High purity grade methanol and acetonitrile solvents were purchased from Merck (Darmstadt, Germany).

2.2. Synthesis of resveratrol dehydrodimers and analogues

All the reagents were used without any further purification. Synthetic products were purified by liquid column chromatography performed on Merck silica gel (Si60 F₂₅₄, 15–25 μm) and characterized by mono and bi-dimensional $^1\text{H}/^{13}\text{C}$ NMR analysis, recording spectra in acetone- d_6 by a Bruker 400 MHz spectrometer.

Dehydrodimer **3** was synthesized by the reaction of resveratrol with anhydrous copper sulfate in acetonitrile. The laccase catalyzed dimerization was carried out according to Nicotra et al. [20], obtaining an optically active product ($[\alpha]_D = -28^\circ$). Dehydrodimer **4** was produced by the reaction of resveratrol with FeCl_3 in methanol [21].

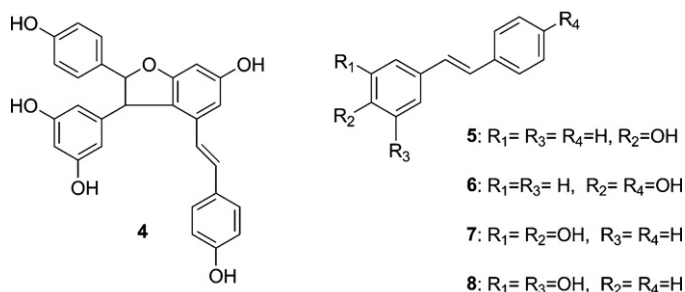


Fig. 1. Molecular structures of dehydrodimer **4** and resveratrol analogues **5–8**.

cis-Resveratrol was obtained by the photoisomerization reaction of natural resveratrol using UV light at 365 nm in ethanol [22].

Resveratrol analogues **5**, **7** and **8** were synthesized by the Wittig reaction of the corresponding substituted hydroxyl benzaldehyde protected as trimethyl tert-butyl silyl derivative and benzyltriphenylphosphonium bromide using potassium carbonate in refluxing isopropanol [23]. 4,4'-Dihydroxy-*trans*-stilbene (**6**) was prepared by the reaction of phenol with chloral hydrate [24].

2.3. MS measurements

ESI-MS and tandem fragmentation (MS/MS) experiments were performed by using a Bruker Esquire LCTM ion trap mass spectrometer (Bruker Daltonics, Germany) equipped with an electrospray ionization (ESI) source. The temperature of the source region was typically kept at 300 °C, while the capillary voltage was 4.0 kV. Nitrogen was used both as nebulizing and drying gas (pressure 20 psi, flow rate 6 L/min). Acetonitrile/water (1:1) solutions of resveratrol/ CuSO_4 and resveratrol/ FeCl_3 with 10^{-4} M concentration were mixed at room temperature and immediately introduced at a flow rate of 5 $\mu\text{L}/\text{min}$ in the mass spectrometer. The ion trap was operated with a scan rate of 10,000 u/s in the range m/z 100–600. Collision induced dissociation (CID) MS/MS experiments were carried out by using helium.

2.4. Computational methods

All calculations were performed with the Jaguar 7.5 quantum chemistry package [25] using density functional theory (DFT), unrestricted for open shell systems, with the BHandHLYP hybrid functional [26,27] which has been shown to give a good description of energy profiles for reactions involving radicals [28,29].

The 1s–3p core electrons of the copper and iron atoms were described with the Hay and Wadt core-valence relativistic effective core-potential (ECP) leaving the outer electrons to be treated explicitly by a basis set of double- ζ quality [30] whereas all electrons were considered for the remaining atoms with the 6–31G** basis set (denoted as LACVP** in Jaguar) [31]. For all the considered species, a preliminary conformational search was carried out by using a Monte Carlo Multiple Minimum approach at MM level (using the MMFFs force field). The most stable conformers for each molecule were optimized in gas phase with this basis set and the BHandHLYP functional. Frequency calculations were performed to verify the correct nature of the minima and to estimate zero-point energy (ZPE) and thermal corrections to thermodynamic properties.

The Poisson–Boltzmann (PB) continuum solvation model, as implemented in Jaguar [32], was used to estimate the energetic and structural changes in aqueous solution, addressed by performing single point calculations on the gas phase optimized geometries with a larger 6–311 + G** basis set for the main group elements and LACVP3P+, consisting of Hay and Wadt core-valence ECP basis set of triple- ζ quality plus one diffuse d function, for the metal atom [30,31].

Thermodynamic properties in solution were calculated as follows. Solvation free energies were taken as the difference between the solution and the gas phase free energies. The calculation of entropies in solution is a somewhat more delicate issue, especially when entropies (and free energies) need to be evaluated for bimolecular reactions involving separated reactants or products. Indeed in this case, the translational degree of freedom in the reactants/products becomes a loose vibration for adducts, leading to a loss/gain of entropy, much larger in the gas-phase than in the confined condensed phase. For this reason, the use of gas phase entropies in the simulation of reactions in solution often leads to artificially overestimated entropic corrections. A way to overcome

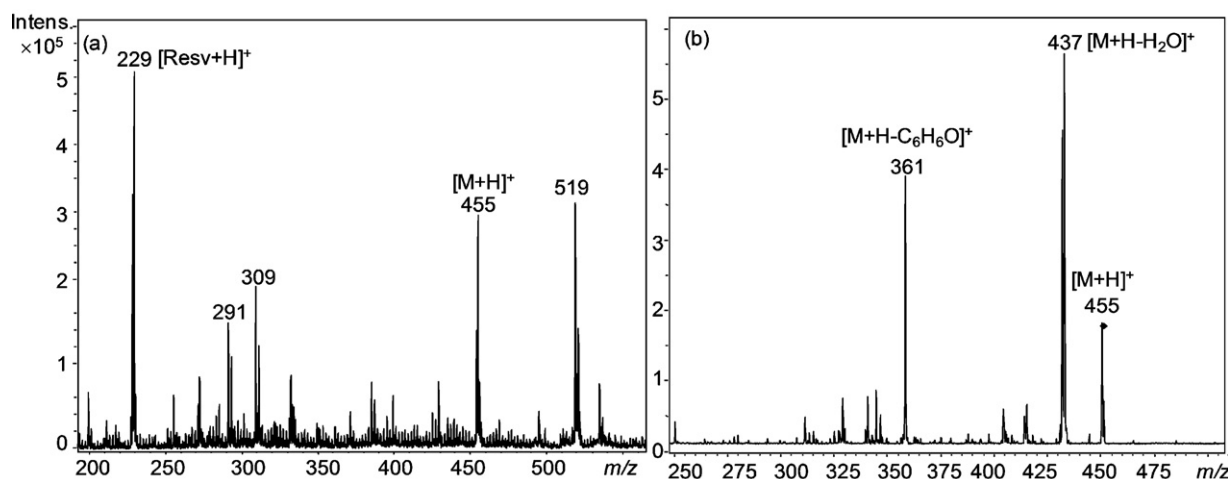


Fig. 2. (a) ESI(+)MS spectrum obtained from a 1:1 resveratrol/CuSO₄ solution in CH₃CN/H₂O (1:1); (b) MS/MS spectrum of [M+H]⁺ ion at *m/z* 455, M is the dehydromer **3**.

this problem was proposed by Wertz [33] and proved to yield solution entropies and free energies in excellent agreement with experimental values. Following this approach, a solute dissolved in a solvent loses a constant fraction of its entropy in vacuo. This loss, for water, amounts to [33,34]:

$\Delta S_{\text{solvation}} = -0.46 (S^\circ - 14.3) \text{ cal mol}^{-1} \text{ K}^{-1} - 6.32 \text{ cal mol}^{-1} \text{ K}^{-1}$ which, for a qualitative estimate, is often approximated as 50% of the gas phase entropy changed of sign.

3. Results and discussion

Recently we reported an investigation on resveratrol-copper metal complexes by ESI-MS/MS experiments and DFT calculations, suggesting geometries and energies of the detected complexes [35]. The positive ion mode ESI spectrum of resveratrol and copper(II)sulfate in acetonitrile/water (1:1) solution is reported in Fig. 2. In addition to [Resv+H]⁺ ion at *m/z* 229 and to the signals related to three Cu(I) complexes, a peak at *m/z* 455 is observed, tentatively assigned to product **3**. MS/MS fragmentation experiments on this ion yield intense signals at *m/z* 437 and *m/z* 361, corresponding to the loss of water and phenol respectively (Fig. 2).

In the negative ion mode there is no evidence for resveratrol-copper complexes, while signals appear for [Resv-H]⁻ ion at *m/z* 227, and at *m/z* 453 (product **3**). MS/MS fragmentation of *m/z* 453 (Fig. 3) produces various peaks: *m/z* 435 due to the loss of water, *m/z* 359 due to the loss of phenol, *m/z* 347 due to the loss of 4-methylenecyclohexa-2,5-dienone (C₇H₆O), *m/z* 333 corresponding

to [M-H-C₇H₄O₂]⁻, *m/z* 411 and *m/z* 369 related to the loss of one or two C₂H₂O, respectively. The loss of two C₂H₂O groups suggests that structure **3** has two hydroxyphenol (resorcinol) moieties [36,37].

In order to confirm the structure of the dehydromer, we have synthesized and characterized by NMR analysis the species **3** [20]. Successively we have performed MS/MS experiments on this compound obtaining the same fragments as those observed after injection of resveratrol/copper(II)sulfate solution (Fig. 3). We thus conclude that the product formed in the ESI/MS of a resveratrol-copper(II)sulfate solution is likely to own the structure **3** (Fig. 1). Interestingly the same compound is formed by resveratrol in presence of laccase, an oxidoreductase enzyme containing four copper ions [38]. Indeed, we observed the same MS and MS/MS spectra obtained in the case of a resveratrol-copper(II)sulfate solution as with an ethyl acetate solution of resveratrol and laccase in buffer solution. It is noteworthy that the dehydromer **3** produced in the reaction with laccase is optically active, as expected in the case of a chiral catalyst.

By replacing Cu(II) with Fe(III), we obtained the spectrum reported in Fig. 4. Differently from the copper case, the positive ion mode ESI produces the radical cation at *m/z* 228 in addition to [Resv+H]⁺ ion at *m/z* 229. Moreover a peak at *m/z* 455 is detected. MS/MS experiments on the latter ion produce intense fragments at *m/z* 437 and *m/z* 361, as in the case of copper(II)sulfate; however a minor fragment at *m/z* 349, attributable to the loss of 4-methylenecyclohexa-2,5-dienone, suggests a dehydromer structure different from **3**. Another peculiarity of the iron system is that no metal complexes could be detected. This is in line with previous reports on the inability of resveratrol, differently from other polyphenols [39], to chelate Fe(III) or Fe(II) ions, so that this molecule does not interfere with iron absorption in vivo [40].

The negative ion mode ESI-MS spectrum shows the expected peaks at *m/z* 227 for resveratrol, and at *m/z* 453 for the dehydromer. MS/MS experiments on the latter ion produce the same fragments of the copper(II)sulfate case, though the signal at *m/z* 359 is much more intense. Again this suggests a structure different from **3**.

For assigning the structure of the dehydromer produced in presence of iron ions, we carried out the reaction of resveratrol and FeCl₃ and characterized the product by NMR analysis [21]. The comparison between MS/MS spectra of this isolated product with those obtained for the FeCl₃ case allows us to establish the structure **4** corresponding to the racemic structure known for the natural product ϵ -viniferin (Fig. 1) [8]. We therefore conclude that Cu(II) and Fe(III) ions produce different dehydromer isomers.

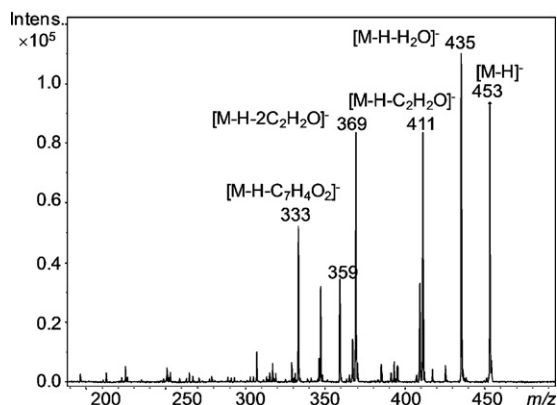


Fig. 3. ESI(-) MS/MS spectrum of [M-H]⁻ ion at *m/z* 453, obtained from a 1:1 resveratrol/CuSO₄ solution in CH₃CN/H₂O (1:1); M is the dehydromer **3**.

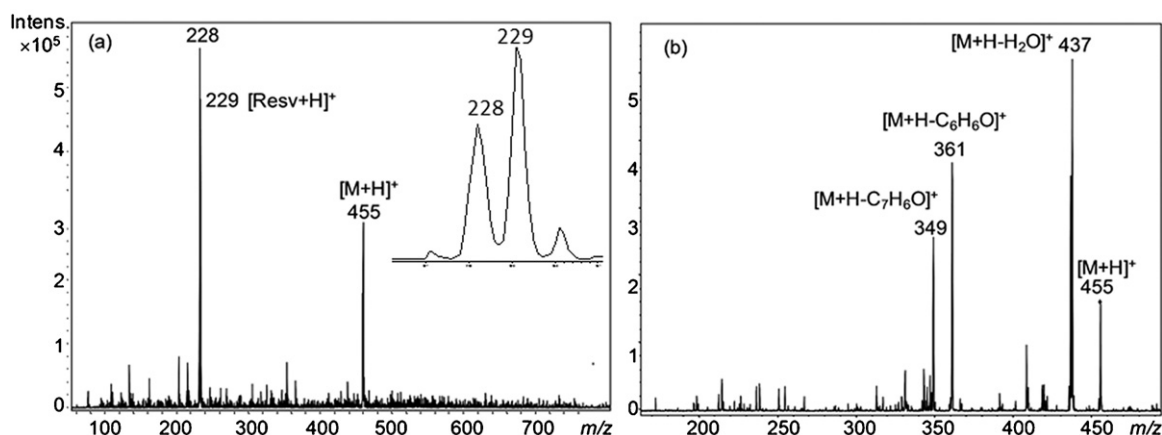


Fig. 4. (a) ESI(+) MS spectrum obtained from a 1:1 resveratrol/ FeCl_3 solution in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (1:1); (b) MS/MS spectrum of $[\text{M}+\text{H}]^+$ ion at m/z 455, M is the dehydromer **4**.

3.1. Theoretical investigations

In order to gain insights on the formation of dehydromers **3** and **4** in the presence of Cu(II) or Fe(III) ions, we have carried out DFT calculations on the thermodynamics of the possible resveratrol dimerization pathways. We assumed a reaction pathway consisting of: (i) the coordination of resveratrol to the metal ion through the 4'-O or 3-O atom and its deprotonation; (ii) an intramolecular electron transfer from the coordinated phenolate anion to the metal center leading to a phenoxyl radical coordinated to the reduced metal ion; (iii) the coupling of two metal coordinated radicals; and (iv) their rearrangement to the final dehydromers **3** and **4** with loss of the metal fragments, see Scheme 2a–b. We also considered a simplified scheme, where the phenoxyl radical detaches from the reduced metal ion prior of their coupling, which therefore involves only resveratrol free radical species, see Scheme 2c.

It is well known that two different radicals may be originated by the oxidation of resveratrol, corresponding to the loss of a hydrogen atom in the 4'-O or 3-O position, and both of them show several possible resonance structures, the most significant ones (**A**₁–**A**₅ and **B**₁–**B**₄ respectively) being reported in Scheme 3.

The importance of these structures for each of the two types of radicals can be estimated from the calculated spin densities reported in Fig. 5. We see that the most important structures are those with the unpaired electron localized on the oxygen atom

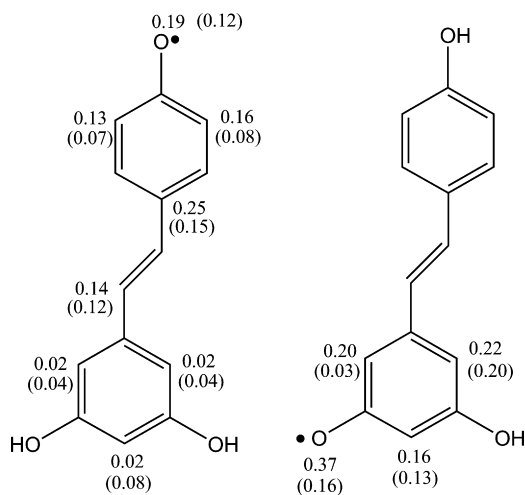


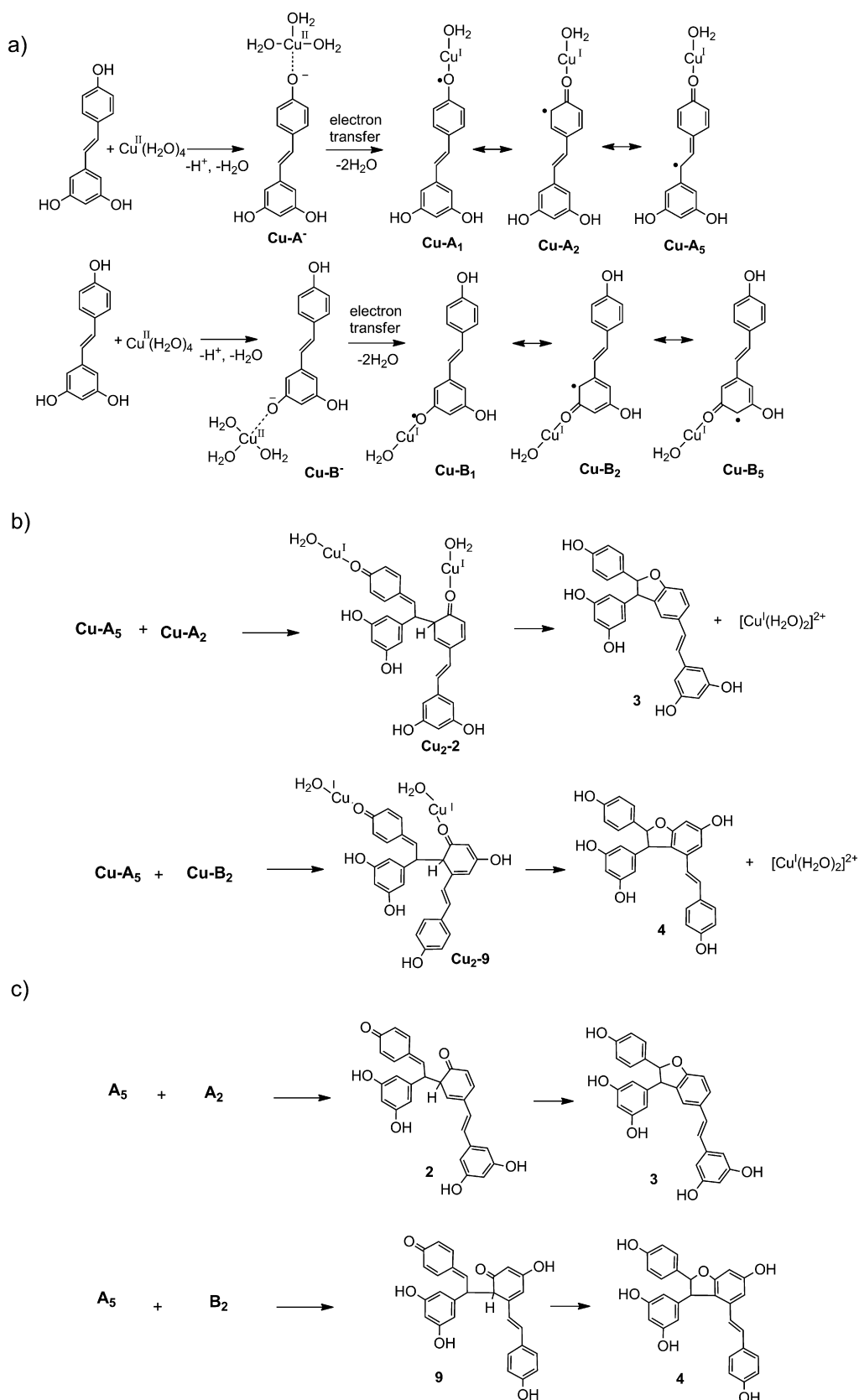
Fig. 5. Spin densities calculated for the two possible phenoxyl radicals **A**₁ and **B**₁ of resveratrol. In parentheses the values for the Cu(I) coordinated phenoxyl radicals, **Cu-A**₁ and **Cu-B**₁.

involved in the H-abstraction and on the *ortho* and *para* carbon atoms of the corresponding phenyl ring, **A**₁–**A**₄ and **B**₁–**B**₄, and, only for the 4'-O radical, on the distal carbon atom of the ethylene unit, **A**₅.

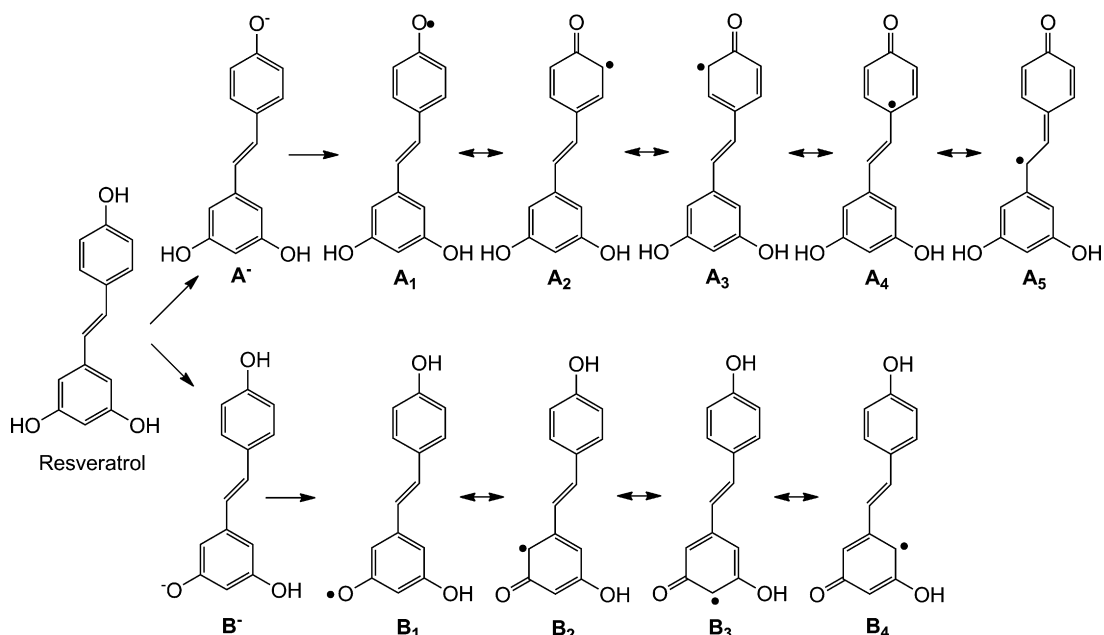
Preliminary calculations indicate that the 4'-O radical, type **A** is more stable than type **B** by $6.7 \text{ kcal mol}^{-1}$ in enthalpy and $6.6 \text{ kcal mol}^{-1}$ in free energy, in agreement with previous theoretical studies [5–7].

In the absence of metal ions, radicals of types **A** and **B** can form through one-electron oxidation of the corresponding anions (**A**[−] and **B**[−] in Scheme 3), present in aqueous solution due to the acid dissociation of resveratrol. However, the formation of **A**_n and **B**_n radicals from **A**[−] and **B**[−] is a difficult process and has been calculated to require 89.0 and $95.0 \text{ kcal mol}^{-1}$, respectively. This oxidation step is, however, made feasible by the coordination and subsequent intramolecular electron transfer to a Cu(II) or Fe(III) ion.

The Cu(II) or Fe(III) ions are expected to exist in aqueous solution as the $[\text{Cu}(\text{H}_2\text{O})_4]^{2+}$ or $[\text{Fe}(\text{H}_2\text{O})_6]^{3+}$ forms: our calculations indicate that the displacement of a water molecule from aquo-complexes by replacing with a resveratrol moiety is an essentially thermoneutral process. The coordination to a positively charged ion causes a significant increase in acidity of the metal bound phenol moiety: for instance, for the Cu(II) ion we calculated a decrease of ca. 10 units in pK_a and of ca. 20 kcal mol^{-1} in free energy, indicating that deprotonation of the metal bound resveratrol is a slightly exoergic process. Our calculations also indicate that deprotonation at the 4'-O position is easier than that at 3-O, with a lower reaction free energy, by ca. 5 kcal mol^{-1} , and a lower pK_a , by ca. 4 units, similarly to what observed for the free resveratrol molecule. Once the deprotonation of the 4'-O or 3-O atoms has occurred, the resulting Cu(II) or Fe(III) phenolate complex can undergo an electron transfer leading to the corresponding Cu(I) phenoxyl radical complex (Scheme 2), or similar Fe(II) radical complex. The “valence tautomerism” of phenolate complexes between these two possible limit forms is a long standing and debated problem in inorganic chemistry, which has been faced by both experimental and theoretical points of view [41,42]. Indeed, it is involved in important processes in catalysis and biochemistry, such as phenol oxidation, coupling or polymerization catalyzed by Cu(II) or Fe(III) ions, [43–45] or the important biological oxidation processes catalyzed by the galactose oxidase, glyoxal oxidase or tyrosinase copper enzymes [46–48]. Unfortunately, an accurate calculation of the intermediate geometries and energies and of possible energy barriers involved in such an intramolecular electron transfer from the coordinated phenolate anion to the metal ion, is a difficult task and should be treated by rigorous wave function methods, such as CASSCF and CASPT2, which are too computationally demanding for



Scheme 2. Proposed reaction paths leading to the dehydrodimers **3** and **4** in the presence of Cu(II) ions: (a) formation of coordinated phenoxyl radicals (some resonance forms are reported), and (b) their coupling and rearrangement, (c) coupling and rearrangement of the phenoxyl free radicals after detachment from the Cu(I) ion.



Scheme 3. Formation of the two possible phenoxyl radicals of resveratrol and their main resonance structures.

the size of the considered systems. We employed an approximate approach in which we limit ourselves to estimate the geometries and the relative stabilities at DFT level of the initial Cu(II) or Fe(III) phenolate species and the final products of the oxidation process, i.e. the corresponding complexes between the oxidized phenoxyl radical and the reduced Cu(I) or Fe(II) metal ions. We assumed the coordination of only one phenolate anion, and the metal coordination was saturated with water molecules to four, for the Cu(II) ion giving $[\text{Cu-A}^-]^+$ and $[\text{Cu-B}^-]^+$ (Scheme 2), and six, for the Fe(III) ion, (Fig. 6). Both 3-O and 4'-O phenolate anions (A^- and B^- respectively, in Scheme 3) were considered, and we performed geometry optimization starting from the geometrical parameters expected for both limiting forms.

The analysis of the final optimized geometries and spin densities shows that all considered complexes assume phenoxyl radical form, indicating the electron transfer has spontaneously occurred without any barrier. In particular (i) the calculated C(4')–O, or C(3)–O bond lengths (ca 1.26 Å and 1.30 Å in Cu and Fe complexes

respectively) are significantly lower than those expected for a C–O single bond in a phenol or phenolate anion (1.35–1.40 Å) and closer to the C=O double bond of a semiquinone species (1.25–1.30 Å); (ii) the spin density is almost completely localized on the resveratrol moiety and only negligibly (lower than 2%) on the metal ion for the Cu complex (Fig. 5); (iii) the simulated IR spectra reveal C(4')–O, or C(3)–O stretching modes around 1600 cm^{-1} , ca. 300 cm^{-1} higher than the value expected for a phenolate anion, a feature which has been shown to be diagnostic for the extent of electron transfer between a phenolate anion and a copper(II) ion [49].

It is also worth noting that the optimization of both 4'-O and 3-O Cu(II) complexes spontaneously leads to the detaching of two water molecules, giving a di-coordinated species, as expected for a Cu(I) ion, and confirming once more that the electron transfer has indeed occurred. This effect could not be observed for the Fe(III) complexes, as both Fe(III) and Fe(II) ions prefer hexa-coordination.

Moreover, our results show that while for the Cu(II) ion the final 4'-O phenoxyl radical complex is lower in enthalpy than the 3-O phenoxyl radical complex, by 6.6 kcal mol^{-1} in enthalpy (7.3 kcal mol^{-1} in free energy), for the Fe(III) ion the final 3-O phenoxyl radical complex is lower than the 4'-O phenoxyl radical complex, although by only 1.6 kcal mol^{-1} in enthalpy (0.4 kcal mol^{-1} in free energy). Once the intramolecular electron transfer has occurred, the resveratrol radicals can easily detach from the reduced metal center: indeed, a slightly endothermic and exoergic process has been calculated for the detachment from the metal ions with an enthalpy change of $+6.8\text{ kcal mol}^{-1}$ ($-0.1\text{ kcal mol}^{-1}$ in free energy) and $+7.1\text{ kcal mol}^{-1}$ ($-0.4\text{ kcal mol}^{-1}$ in free energy), respectively for the 4'-O and 3-O radicals, for Cu(II), and an enthalpy change of $+2.9\text{ kcal mol}^{-1}$ ($-4.1\text{ kcal mol}^{-1}$ in free energy) and $+2.6\text{ kcal mol}^{-1}$ ($-6.3\text{ kcal mol}^{-1}$ in free energy), respectively for the 4'-O and 3-O radicals, for Fe(III).

We therefore assumed that the phenoxyl radicals spontaneously uncoordinate from the reduced metal ions, so that we considered only the coupling of free resveratrol radicals to investigate the formation of the dehydrodimers **3** and **4** (Scheme 2c). Dehydrodimer **3** can be obtained from the coupling reaction of the radical species A_2 (or A_3) and A_5 (Scheme 3), to give an intermediate dimer **2**, which then undergoes an intramolecular nucleophilic attack. On the other

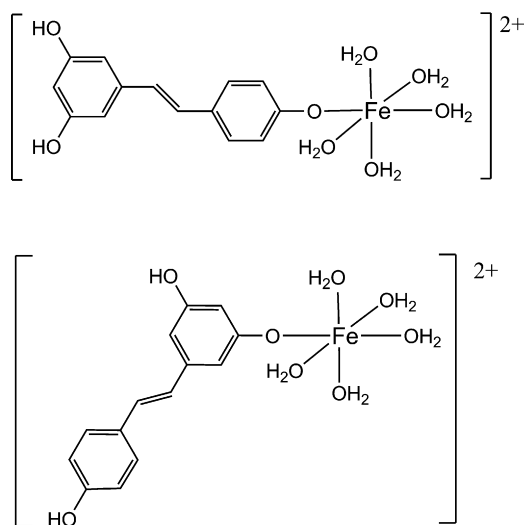


Fig. 6. Structures for the Fe(III) aquo-complexes with phenolate A^- and B^- .

Table 1

Reaction enthalpies and free energies (kcal mol⁻¹) at 298 K for the paths leading to the dehydrodimers **3** and **4**.

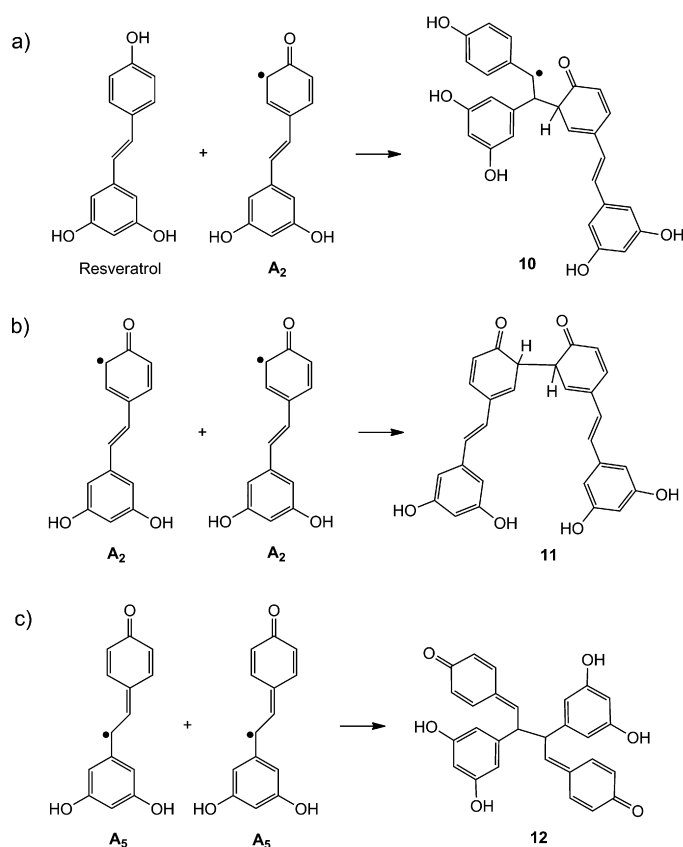
Reaction	ΔH_{gas}	ΔH_{sol}	ΔG_{gas}	ΔG_{sol}
A ₂ + A ₅ → 2	-12.8	-11.2	+6.1	-1.8
2 → 3	-47.7	-43.6	-48.0	-43.8
B ₂ + A ₅ → 9	-25.9	-20.8	-9.8	-12.8
9 → 4	-40.7	-40.5	-37.8	-39.1
Cu- A ₂ + Cu- A ₅ → Cu- 2	+26.6	-8.8	+41.8	-0.7
Cu- B ₂ + Cu- A ₅ → Cu- 9	+1.2	-25.8	+19.0	-17.1
A ₂ + Resv. → 10	+7.7	+16.7	+22.4	+24.0
A ₂ + A ₂ → 11	-10.5	-9.8	+5.6	-1.6
A ₅ + A ₅ → 12	-14.8	-17.9	+2.2	-9.4
A ₁ + A ₂ → 13	-16.6	-12.0	-0.2	-3.8
A ₁ + A ₅ → 14	-23.3	-17.2	-6.8	-8.4

hand, dehydrodimer **4** can be obtained from the coupling reaction of the radical species **B**₂ (or **B**₄) and **A**₅ to give an intermediate dimer **9** (Scheme 2c) which then also undergoes a similar intramolecular nucleophilic attack.

We considered only the thermodynamics of these processes and the calculated enthalpies and free energies for both reaction paths are reported in Table 1. Values in gas phase and in water solution are reported and show that the solution environment does not significantly change the qualitative picture, as expected for radical reactions, and suggest that the ESI-mass spectrometry approach is an adequate technique to investigate the reaction in solution.

The results in Table 1 indicate that both steps, radical coupling and subsequent intramolecular nucleophilic attack, are exothermic for both considered pathways. Indeed, the formation of the intermediate dimers **2** and **9** from the corresponding free radicals is an exothermic and exoergic process, with reaction free energies of -1.8 and -12.8 kcal mol⁻¹, respectively. Also the subsequent intramolecular nucleophilic attack, **2** → **3** and **9** → **4**, are both highly exothermic and exoergic with higher free reaction energies, respectively -43.8 and -39.1 kcal mol⁻¹, indicating that **3** and **4** are the thermodynamic products.

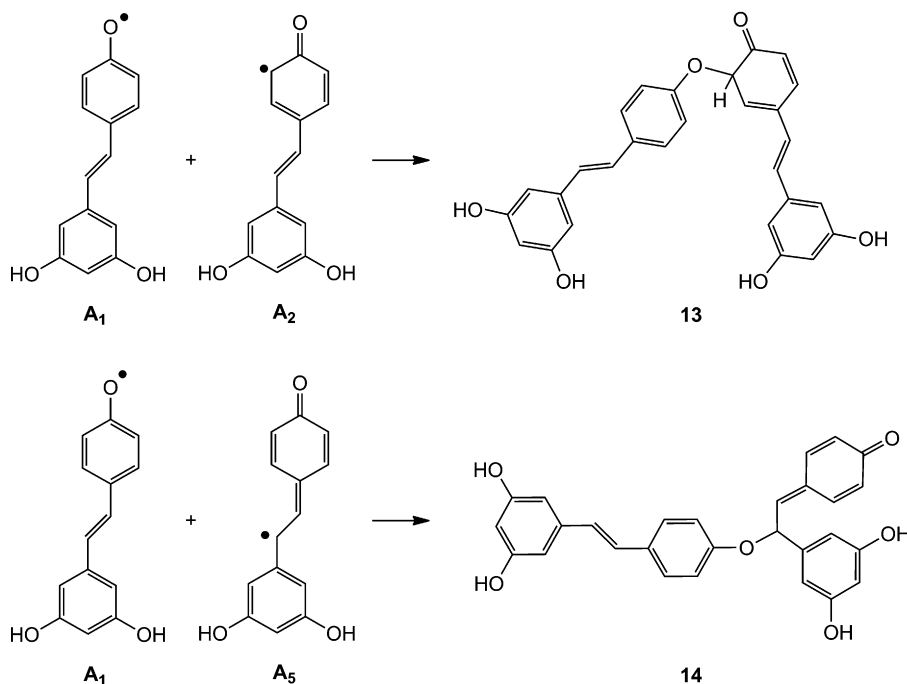
Together with the results for relative stabilities of the 4'-O and 3-O phenoxyl complexes, these results allow to explain the different regioselectivity of the formation of **3** and **4** when using Cu(II) or Fe(III) ions, i.e. the presence of Cu(II) ions leads only to the dehydrodimer **3**, while the use of Fe(III) ions preferentially, but not exclusively, leads to the dehydrodimer **4**. Indeed, the Cu(I) 4'-O phenoxyl complex (Cu-**A**₁) is significantly more stable than the corresponding 3-O complex (Cu-**B**₁), 7.3 kcal mol⁻¹ in free energy, so that the use of Cu(II) ions leads only to the formation of 4'-O radicals **A**_n whose coupling gives exclusively the dehydrodimer **3**. On the other hand, the Fe(II) 3-O phenoxyl complexes are barely more stable than the corresponding 4'-O complex, 0.4 kcal mol⁻¹ in free energy, so that the use of Fe(III) ion leads to the formation of both 3-O and 4'-O radicals **A**_n and **B**_n, whose coupling may give both dehydrodimers **3** and **4**. However, as shown in Table 1, the coupling of **A**₂ and **A**₅ has a reaction free energy of -1.8 kcal mol⁻¹, while that between **B**₂ and **A**₅ has a higher reaction free energy of -12.8 kcal mol⁻¹, and **4** is therefore expected to form preferentially. The interplay between the different stabilities of the **A**_n and **B**_n radicals, their dependence on the metal ion used as catalyst, and the different reaction energies of their coupling explain the contradictory experimental data existing in literature according to which either dimer **3** and **4**, or both, may form from the oxidation of resveratrol, depending on the experimental procedure [17–19]. In order to check the validity of our simplified approach based on the coupling of free resveratrol radicals, we considered the coupling of two metal coordinated phenoxyl radicals, limiting to the Cu(I) ion, i.e. (i) the coupling of two 4'-O-phenoxyl complexes in the resonance structures **A**₂ (or **A**₃) and **A**₅, to give the metal coordinated intermediate dehydrodimer Cu₂-**2**; (ii) the coupling



Scheme 4. Possible alternative resveratrol radical couplings leading to the formation of C—C bond.

of a 4'-O-phenoxyl complex in the resonance structure **A**₅ and a 3-O-phenoxyl complex in the resonance structure **B**₂ (or **B**₄), to give the metal coordinated intermediate dehydrodimer Cu₂-**9**. The results are reported in Table 1 and show that the reaction enthalpies and free energies calculated for the coupling of the metal coordinated radicals in solution are quite close (within 5 kcal mol⁻¹) to those calculated for the free radicals, confirming the validity of our simplified approach based on free resveratrol radicals.

We also considered other possible coupling reactions between different resonance forms of the 4'-O and 3-O resveratrol radicals. In particular we investigated (a) the attack of the **A**₂ radical to the central C=C bond of a neutral resveratrol molecule to form the initial dimer **10** with the same backbone of **2**; (b) the coupling of two radicals **A**₂ to form the dimer **11** featuring a C—C bond between two phenyl rings; and (c) the coupling of two radicals **A**₅ to form the dimer **12**, featuring a C—C bond between two ethylene units (Scheme 4). The thermodynamics of these reactions are reported in Table 1 and indicate: (i) an endothermic and endoergic reaction for the attack of the **A**₂ radical to the ethylene unit of the resveratrol molecule, by 22.4 kcal mol⁻¹ in enthalpy and 24.0 kcal mol⁻¹ in free energy; and (ii) an only slightly exothermic and exoergic reaction for the coupling of two radicals **A**₂ through their phenyl ring, by -9.8 kcal mol⁻¹ in enthalpy and -1.6 kcal mol⁻¹ in free energy, suggesting that these processes are not (case i) or less (case ii) plausible than the coupling of **A**₂ and **A**₅. The coupling reaction of two radicals **A**₅ through their ethylene units, leading to dimer **12**, is exothermic and exoergic with a reaction enthalpy of -17.9 kcal mol⁻¹ and free energy of -9.4 kcal mol⁻¹: such a result suggests that, although not observed in the present study, this is a plausible reaction, consistently with other experimental studies indicating the formation of different dehydrodimers, such as pallidol, when resveratrol is treated with peroxidases [18].

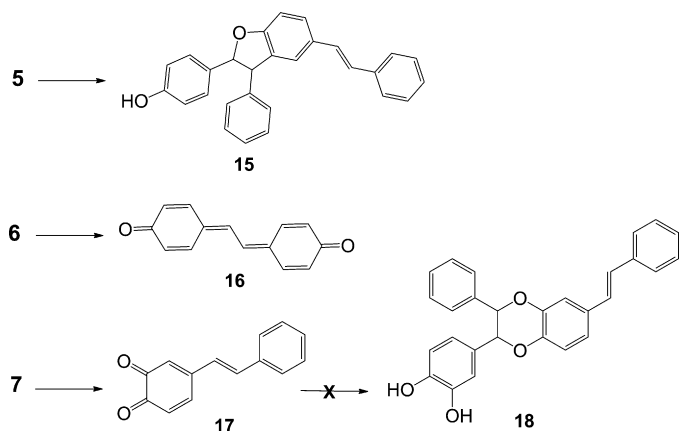


Scheme 5. Possible alternative resveratrol radical couplings leading to the formation of C–O bonds.

Finally, in order to evaluate the possible formation of dimers based on C–O bonds, we also investigated the coupling of radicals **A₁** and **A₂** or **A₁** and **A₅**, leading to dimers **13** and **14**, respectively (Scheme 5). The thermodynamics of both reactions are also reported in Table 1 and indicate free energies similar to those calculated for the formation of **2** and **9**. These results suggest that these are plausible reactions which are not observed probably because of the higher lability of the C–O with respect to the C–C bond, and/or of the lack of rearrangement paths leading to more stable dimers, such as **3** and **4**.

3.2. Resveratrol synthetic analogues

The structure-reactivity relationship can be investigated by studying synthetic analogues of resveratrol (Scheme 6). *cis*-Resveratrol, obtained by photoisomerization of the natural compound, shows the same behavior of resveratrol with copper(II) sulfate giving dehydrodimer **3**.



Scheme 6. Oxidative products from resveratrol analogues by ESI-MS studies in the presence of CuSO₄ solution.

Synthetic hydroxystilbenes **5–8** were also investigated in order to evaluate the role of the number and position of the OH groups. ESI(–)MS spectrum of an acetonitrile/water solution containing 4-hydroxy-*trans*-stilbene (**5**) and copper(II)sulfate indicated the pseudomolecular ion at m/z 195, together with the m/z 389 signal corresponding to $[M - H]^-$ ion of the dehydrodimer **15**. MS/MS fragmentation experiments on the latter ion yield ions at m/z 371 and m/z 295, due to the loss of water and phenol molecules, respectively.

To confirm the geometry, we have synthesized the dehydrodimer **15** by reaction with copper(II)sulfate and fully characterized its structure by NMR analysis, based also on reported data [14,50]. This product shows the same MS/MS spectrum of the dehydrodimer detected in the ESI-MS experiment.

4,4'-Dihydroxy-*trans*-stilbene (**6**) has a peculiar behavior, as the ESI-MS spectrum of its acetonitrile/water solution with copper(II)sulfate shows no clusters for copper complexes in positive ion mode, neither signals related to the dehydrodimer product in the negative ion mode. It is instead observed the oxidation product *para*-quinone **16**, responsible for the yellow color of the solution after addition of copper(II)sulfate to **6**. Its structure was confirmed by UV analysis under the same solvent conditions used in MS measurement 1:1 acetonitrile/water which provided $\lambda_{\max} = 430$ nm, in agreement with reported data for this compound [51].

Concerning the 3,4-dihydroxy-*trans*-stilbene (**7**), the negative ESI-MS spectrum of its solution with copper(II)sulfate does not contain signals attributable to the dehydrodimer. In ESI(+)-MS spectrum an intense isotopic cluster with the most intense signal at m/z 483 was detected, attributable to a Cu(I) ion complex involving two molecules of **17**, as deduced by the agreement with the simulated cluster for $[C_{28}H_{20}CuO_4]^+$ and MS/MS fragmentation experiments. A weak cluster at m/z 291 was also present, attributable to the Cu(I) complex containing **17** and one molecule of water, whereas no evidences were observed for the oxidative product **18**.

The relevance of the relative position of OH groups with respect to the oxidation process has been demonstrated by using 3,5-dihydroxy-*trans*-stilbene (**8**), having no OH groups in *para* position.

In this case we could not detect any signals corresponding to dehydrodimer products by ESI(–) MS analysis. In ESI(+)MS spectrum, weak signals for copper complexes were observed at m/z 487, 293 and 275 corresponding to $[2M + Cu]^+$, $[M + H_2O + Cu]^+$ and $[M + Cu]^+$.

4. Conclusions

In this work we have investigated the oxidative coupling of resveratrol induced by copper and iron ions. The formation of racemic δ -viniferin dehydrodimer **3** is observed in ESI-MS experiments carried out on resveratrol-copper mixtures, while the analogous resveratrol-iron reaction affords the racemic ε -viniferin dehydrodimer **4**. Computational results suggest that dehydrodimers **3** and **4** are formed by coupling reactions, and subsequent intramolecular nucleophilic attack, of phenoxide radicals. The latter are produced in the oxidation reaction of phenoxide anions by Cu(II) and Fe(III) ions. We find that Cu(II) preferentially produces the 4'-O resveratrol radical **A_n**, while Fe(III) produces more easily the 3-O resveratrol radical **B_n**. Given that the formation of dehydrodimer **3** requires **A_n** radicals as reactants, whereas that of dehydrodimer **4** also needs **B_n** radicals, these computational findings explain why reactions of resveratrol with Cu and Fe ions eventually result in the production of species **3** and **4**, respectively. Calculated enthalpies in gas phase and in water solution for the reaction paths leading to the two isomeric dehydrodimers suggest that the ESI-MS approach is an adequate technique to study the resveratrol reactivity in solution. In addition, we have investigated the structure-reactivity relationship by studying synthetic analogues of resveratrol. We find that the presence of the OH group in *para* position is crucial for the reactivity and biological behavior of resveratrol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijms.2012.05.010>.

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