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Tetrahedron Letters 49 (2008) 619–623
Tetrahedron Letters

Mechanistic study on the enzymatic oxidation of flavonols

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Received 3 October 2007; revised 13 November 2007; accepted 23 November 2007

Abstract

Several flavonols have been transformed upon treatment by *Trametes versicolor* laccase. Most of the major oxidation products have been isolated by HPLC as pure compounds and their structures have been, when possible, investigated through spectral methods (HPLC–MS and NMR). The results are coherent with the predominance of a dismutation process, leading to cation formation, over direct radical–radical coupling.

Keywords: Polyphenols; Flavonoid; Enzymatic oxidation; Dismutation

The enzymatic oxidation of polyphenols[1,2] is highly implicated in numerous biological mechanisms in vegetal. As the radicals, formed through these oxidation processes, are relatively stable, they can undergo several coupling reactions, reoxidation or dismutation reactions. The coupling reactions of phenolic radicals are of major interest in lignin, tanins and melanin biosynthesis.

Out of flavonoids, flavonols (yellow pigments with chemical structure shown in Figure 1 and characterized by a fully conjugated flavan backbone, through a 3-hydroxy-pyranone C ring), often present in glycosidic forms, are one of the main sub-classes.

In a programme dedicated to the study of the total synthesis,[3–5] and the chemical[6–8] or enzymatic oxidation of polyphenols, we focused our interest, in this latter field, on the mechanistic features arising from the oxidation of flavonols by *Trametes versicolor* laccase. Indeed, the main transformations undergone by polyphenols in fruits and plants during food processing are caused by oxidative reactions catalyzed by phenol oxidases, such as laccases,[9,10] catechol oxidases, and tyrosinases, and by peroxidases. Oxidation proceeds in the presence of molecular oxygen for most polyphenol oxidases and with hydrogen peroxide for peroxidases.[11,12] Autoxidation and chemical oxidation processes may also occur.[13]

An important specificity of our study, compared to those already published, is also the use of a laccase as enzymatic oxidant. Indeed, other studies most often reported the oxidation of flavonoids either with chemical radical initiators like AIBN and DPPH or enzymatic systems like grape PPO (grape polyphenol oxidase; in this case, the flavonoids are not the primary substrate of the enzyme, the oxidation being mediated by the *o*-quinone from caffeoyl quinic acid, which is the true substrate of the enzyme). Therefore, in these cases, oxidation of the flavonols may occur through either a mono- or a di-electronic transfer. The possibility of a di-electronic transfer may indeed favour the oxidation of the B ring of flavonols through the formation of quinones, reactivity of which would thereafter favoured the formation of dimeric compounds through nucleophilic addition reactions, due to the high reactivity of aromatic rings derived from phloroglucinol. In the case of laccases, albeit the complete mechanism of the reaction, and mainly the pathway leading to oxygen reduction, is not clearly determined,[14] the mono-electronic transfer is the privileged mechanism. Use of a

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0040-4039/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2007.11.147

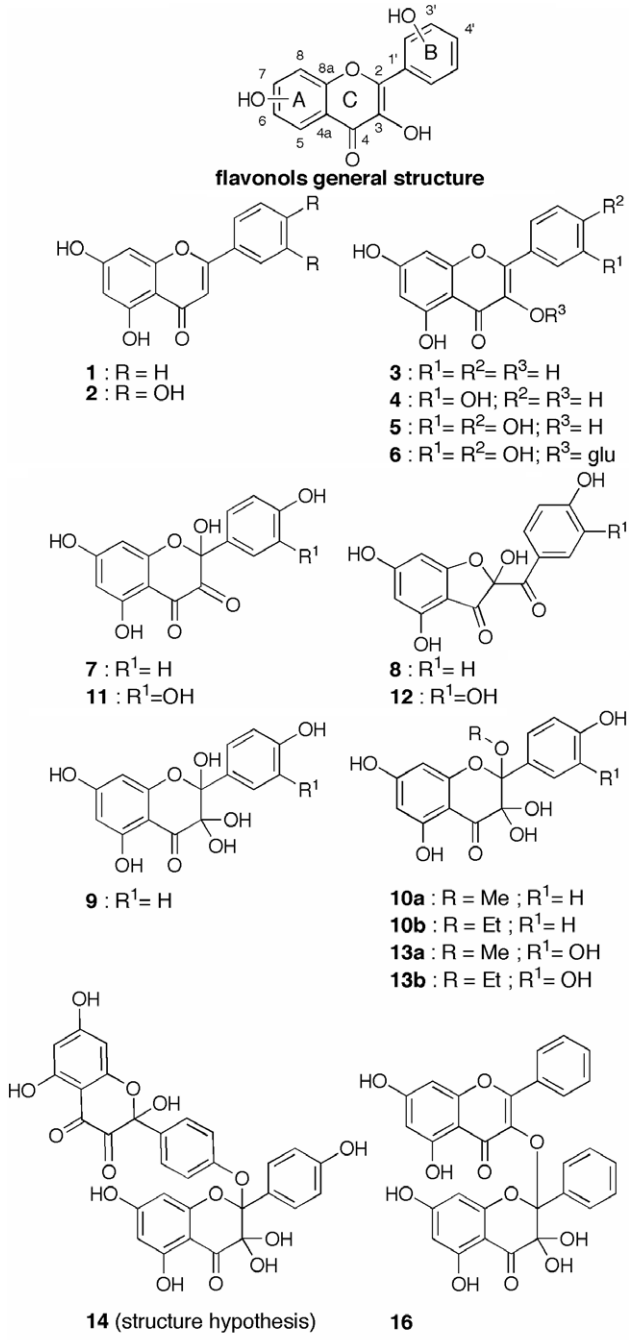

Fig. 1.

laccase as oxidizing enzyme would therefore allow us to investigate the stability and the reactivity of the various RO· potentially formed as primary products of the oxidation. Moreover, laccases are highly implicated in enzymatic evolution of flavonoids in *Arabidopsis thaliana* seeds. Assuming that the course of the laccase oxidation of polyphenols should be more influenced by the chemical structure of the starting polyphenol than by the nature of the enzyme used, we turned to the use of *T. versicolor* laccase, a reliable, easily available fungus enzyme with a low substrate specificity,[15,16] to study flavonol oxidations. We indeed preliminarily demonstrated[17] with kaempferol and quercetin that this enzyme gave rise to the same oxidation profiles than another laccase obtained from *A. thaliana* seeds.[18]

To allow chemical mechanism determination for this type of reaction, we decided to test our enzymatic system on several commercially available flavonoids with various substitution patterns either on their B or C rings. The choice of the starting materials **1**–**6** (Fig. 1) was made on account of several results published in the literature on the structure–antioxidant activity relationships of flavonoids. It has indeed been clearly established that the B-ring is the most important site for H-transfer and consequently antioxidant capacity. In contrast, the A-ring seems to be less important. On the other hand, the $\Delta^{2,3}$ double bond should also contribute to the antioxidant activity, as it ensures π-electron delocalization between the B- and C-rings, which contributes to the stabilization of the RO· radicals formed in the oxidation process after H-abstraction.[19] Moreover, an important issue that is still under debate is the role of the 3-OH group. In vitro studies demonstrated that this hydroxy group contributes to the antioxidant potential. Indeed, blocking the 3-OH group as in rutin or removing it as in luteolin significantly decreases the in vitro activity.[20]

Our starting compounds were therefore two flavones with no hydroxy groups at C3, namely chrysine **1** and luteolin **2**, three flavonols with an hydroxy group at C3 exhibiting various hydroxylation patterns on their B ring, namely galangin **3**, kaempferol **4** and quercetin **5**, and, finally, quercetin 3-*O*-glucoside **6**.

The first feature arising from our study[21] was the non-oxidation of both **1** and **2**, thereby confirming the crucial role of the 3-OH group in the oxidation process. Moreover, this first result also showed that the presence of an highly reactive catechol type B-ring is not sufficient to allow a one-electron oxidative process to occur, also reflecting the postulated important role (DFT study)[22,23] played by the hydroxy group at C3 in the first steps of oxidation of flavonols.

Turning thereafter to the enzymatic oxidation of flavonols **3**–**5**, we were not surprised to observe that these compounds, although not exhibiting the same hydroxylation pattern on the B-ring, presented similar behaviours under our oxidation conditions (although depsides[24,25] are often described as major compounds of flavonols oxidation, resulting from a rapid degradation of the first formed oxidation products, we were not able to detect them in our study). Indeed, the reaction mixture was reflecting in all cases the presence of only few compounds, MS analysis of which showed that the major ones were, for compounds **4** and **5**, isomeric products corresponding to the addition of one oxygen atom (M+16) at C-2 with possible rearrangement of the C-ring through trans acetalization of the highly reactive tri-keto system and hydrate formation at C-3 (compounds **7**–**9**, **11** and **12**). Depending on the solvent used for the reaction, the other products of the reactions were, in these two cases, thereafter shown to be either

dimeric compounds under aprotic conditions (acetonitrile, compounds **14** and **15**[26]) or products resulting from solvent addition in protic media (MeOH or EtOH, **10** and **13**). In this case, turning from methanol to ethanol was the key step allowing to unambiguously confirming this solvent addition. Later on, NMR analysis confirmed the structure of compounds **7**–**10** and **11**–**13** as oxidation products of kaempferol and quercetin, respectively, structure of which have already been more or less described in the literature.[24,25,27,28]

As an example, **13a** NMR spectra were used to determine its structure as follows. ^{1}H spectrum of **13a** exhibited the same resonance pattern for the aromatic protons than quercetin but also showed a supplementary resonance at 3.05 ppm, integrating for three protons in accordance with the presence of a methyl group attached to an oxygen atom. ^{13}C spectrum was also quite similar to quercetin's with two quaternary sp^2 carbon resonances less, which were replaced by two resonances at 91.7 and 100.9 ppm. HSQC and HMBC experiments were thereafter performed and allowed the complete assignment of all carbon resonances. Indeed, HMBC correlation (Fig. 2) were observed for the resonance at 100.9 ppm with protons of the methyl group as well as with protons H2′ and H6′. At the same time no HMBC correlations were observed for carbon resonance at 91.7, which thereby was attributed to C3 when the resonance at 100.9 ppm was assigned to C2, confirming the introduction of a methoxy group at C2.

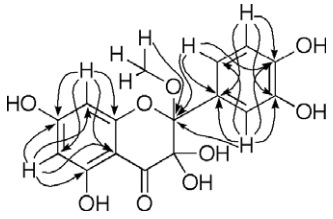

Fig. 2. ^{1}H–^{13}C long range correlations observed in **13a**.

The same reaction, performed with galangin **3**, however yielded under all the conditions tested (solvent and concentration changes) the exclusive formation of compound **16**, MS spectrum of which revealed its dimeric structure (*m*/*z* 556 [2M−2H+H_2O]). This result clearly shows that hydroxylation of ring B is not necessary for the oxidation process to take place and thereby strengthens the hypothesis of a kinetic oxidation of flavonols through the 3-OH group. Moreover, obtention of **16** as the unique oxidation product and the absence of adducts with solvent molecule (MeOH or EtOH) clearly shows that the radical formed at C-2 is not reactive enough to be directly quenched by an ROH molecule (solvent or substrate molecule) but should be first re-oxidized through the dismutation of two radicals (Fig. 3), leading the corresponding cation, which is thereafter trapped by a protic species; the proximity of the two radicals involved in the dismutation reaction, favoured by π-staking interactions, led to the formation of **16** rather than quenching by a solvent molecule.

The structure of the dimeric compounds obtained through oxidation of flavonols **4** and **5** in acetonitrile was however more difficult to establish because of their low abundance in the reaction mixture which did not allow us to get them as pure compounds for NMR spectroscopy. Kaempferol oxidation led to the formation of two dimeric compounds (*m*/*z* 604 [2M−2H+O+H_2O] and *m*/*z* 586 [2M−2H+O]) and quercetin allowed the formation of two pseudo dimeric compounds at *m*/*z* 498 and 618

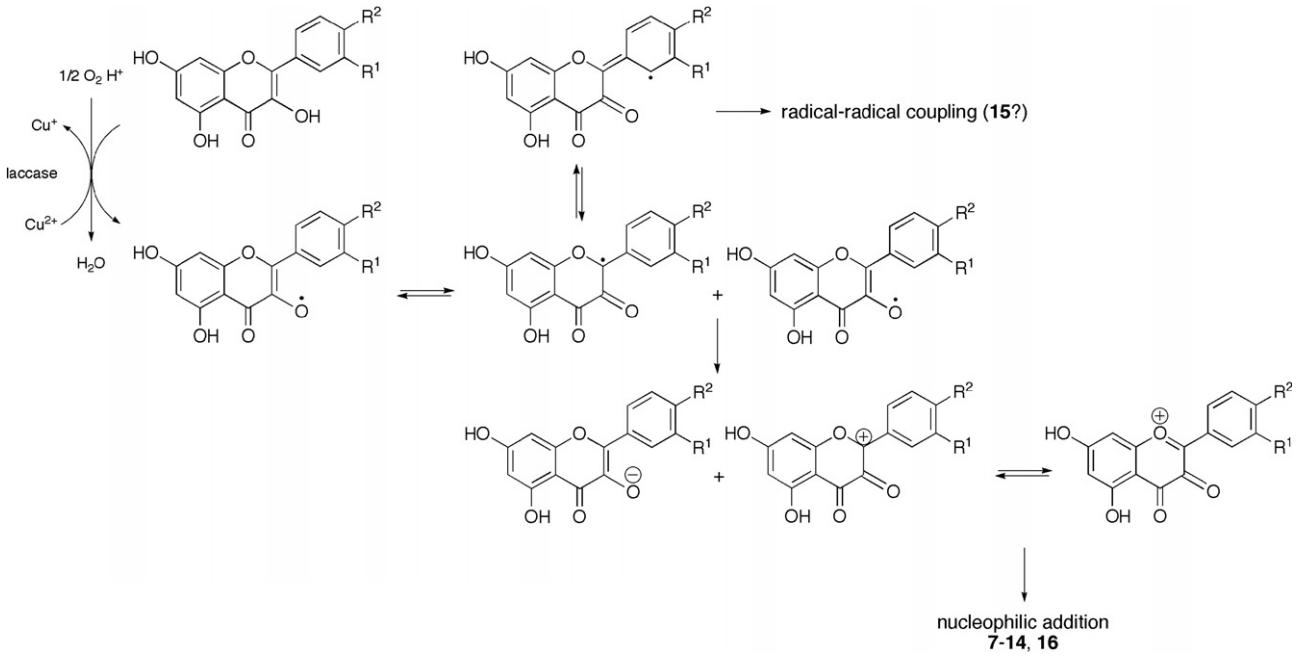

Fig. 3. Proposed mechanism for flavonols oxidation.

[2M−2H+O]. Careful examination of the MS spectra of compounds at *m*/*z* 604 (**14**) and 618 (**15**), obtained from **4** and **5**, respectively, however reflected structural differences between these two compounds (we were not able to isolate dimeric compound at *m*/*z* 586). Indeed, when the spectrum of **15** seemed to be compatible with the formation of either a carbon–carbon or a carbon–oxygen single bond between the two monomeric subunits (and re-oxidation of one of the subunit), formation of **14** is clearly only compatible with the formation of an ether linkage between two subunits. This assumption is supported by the observation in the MS spectrum of **14** of a major fragment at *m*/*z* 302 also observed in compounds **10a**,**b**, as a result of the easy cleavage of the OR bond after the loss of an H_2O molecule. On the other hand, MS spectrum of **15** mainly revealed classical fragmentations of the flavan skeleton.

Moreover, to allow incorporation of a H_2O molecule and a further oxygen atom, according to the molecular weight of **14**, the 'upper' subunit needs to be further oxidized; the most satisfactory structure hypothesis for **14** is therefore shown in Figure 1, involving the OH at C-4′ in the ether linkage between both subunits rather than the OH at C-3 as in **16**.

This discrepancy observed in the formation of **14** and **16** could be the consequence of a competition during the dismutation process, due, in the case of **4**, to the presence of the hydroxy group at C-4′, between π-stacking, dipole–dipole and steric interactions leading a different spatial arrangement of both subunits in the transition state than that observed with **3**. The hypothetical structure of **14**, however supports the hypothesis of a dismutation process rather than radical–radical coupling, since we did not have observed, in any cases, products resulting from a possible phenoxy radical formation.

A last interesting result is this obtained in the oxidation of quercetin 3-*O*-glucoside **6**. Indeed, even if the oxidation seemed to proceed very slowly, this compound, albeit exhibiting a protected hydroxy group at C-3, was partially oxidized and the major oxidation product (*m*/*z* 908) did not resulted from a loss of the glycosidic moiety. The fact that under the same conditions, luteolin **2** remained untouched may indicates that electro donating abilities of the oxygen atom at C-3 is sufficient to promote oxidation at C-2.

In conclusion, we have demonstrated the enzymatic oxidation of flavonols to be first promoted by the formation of the radical on the oxygen at C-3 through H abstraction even in the presence of phenolic hydroxy groups on the B ring. We have also demonstrated that most of the subsequent reactions either with solvent (water or alcohols) or with another substrate molecule were initiated by a dismutation reaction of two radicals, leading to the formation of a carbocation at C-2 that was thereafter quenched by a protic species. Therefore, intermolecular interactions are crucial factors for the reaction issue: increasing unfavourable interactions would allow radical isomerization and the formation of dimers with a diphenyl ether link. These evidences for carbocation formation during oxidation of phenolic species are of great importance and will help in a better understanding of the biosynthesis of phenolic oligomers and polymers in plants.

Acknowledgment

The authors thank L. Kerhoas (Inra) for LC–MS measurements.

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21. *Typical procedure*: A flavonol solution in methanol, ethanol or acetonitrile (10 ml, 10 mg/ml) and 60 ml citrate/phosphate buffer 0.1 M was incubated with 30 ml of laccase (purchased from Fluka; 23.3 U/mg) solution (2 U/ml) at pH 4 and 30 °C for 2 h with stirring, to allow oxygen dissolution in the reaction medium. Control reactions were also made in the absence of enzyme to ensure the products arising from enzymatic oxidations not being artefacts resulting from autooxidation processes. The suspension was centrifuged at 6000 rpm for 10 min; the supernatant was then filtered under vacuum through an ultra filtration membrane, ∅ 44.5 mm (Millipore). The filtrate was concentrated by evaporation. The oxidation products were separated

on a semi-preparative HPLC system including a Waters 600 pump, a manual U6K injector, a Uvicord S II UV detector (Pharmacia LKB) set at 254 nm, and a REC 102 recorder (Pharmacia Biotech). The column was a C18 Kromasil 250 × 20 mm (10 μm packing). Elution conditions: flow rate 18 ml/min, solvent A 0.5% acetic acid in H_2O; solvent B 0.5% in acetonitrile. Linear gradient from 15% to 50% B in 25 min followed by washing and reconditioning of the column. No yields are given for these transformations, since we focused on isolation of pure compounds out of a complex mixture. Products are dissolved in DMSO-d_6 for NMR spectroscopy.

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