Tetrahedron Letters 52 (2011) 1165–1168

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Crude peroxidase from onion solid waste as a tool for organic synthesis. Part II: oxidative dimerization–cyclization of methyl *p*-coumarate, methyl caffeate and methyl ferulate

Sonia Moussouni [a,†], Maria-Liliana Saru [a,†], Efstathia Ioannou [b], Mohamed Mansour [a], Anastasia Detsi [c], Vassilios Roussis [b], Panagiotis Kefalas [a,*]

[a] Department of Food Quality and Chemistry of Natural Products, Mediterranean Agronomic Institute of Chania/Centre International de Hautes Etudes Agronomiques Méditerranéennes, 73100 Chania, Crete, Greece
[b] Department of Pharmacognosy & Chemistry of Natural Products, School of Pharmacy, University of Athens, Panepistimiopolis Zografou, Athens 15771, Greece
[c] Laboratory of Organic Chemistry, Department of Chemical Sciences, School of Chemical Engineering, National Technical University of Athens, Heroon Polytechniou 9, Zografou Campus, 15780 Athens, Greece

ARTICLE INFO

Article history:
Received 23 September 2010
Revised 15 December 2010
Accepted 4 January 2011
Available online 12 January 2011

Keywords:
Onion peroxidase
p-Coumaric acid
Caffeic acid
Ferulic acid
Dihydrobenzofuran lignans
Antioxidant activity

ABSTRACT

The ability of a crude onion peroxidase preparation to act as a biocatalyst for the oxidative dimerization–cyclization of methyl *p*-coumarate, methyl caffeate and methyl ferulate is presented. The products of the reaction have been fully characterized and were found to possess potent antioxidant activity in a ferric reducing antioxidant power (FRAP) assay.

Oxidative dimerization of phenolic compounds occurs in Nature and leads to the formation of dihydrodimeric products that play a significant role in the cross-linking of plant cell wall components, which in turn influences various important cell wall properties. This biotransformation is catalyzed by enzymes, such as peroxidases and polyphenol oxidases including laccases, or, in some cases, occurs via photochemical oxidation.[1–5] Moreover, oxidative cross-linking is very important for the properties and technological applications of biopolymers in which phenolics (feruroyl and coumaroyl residues) are covalently attached.[6,7]

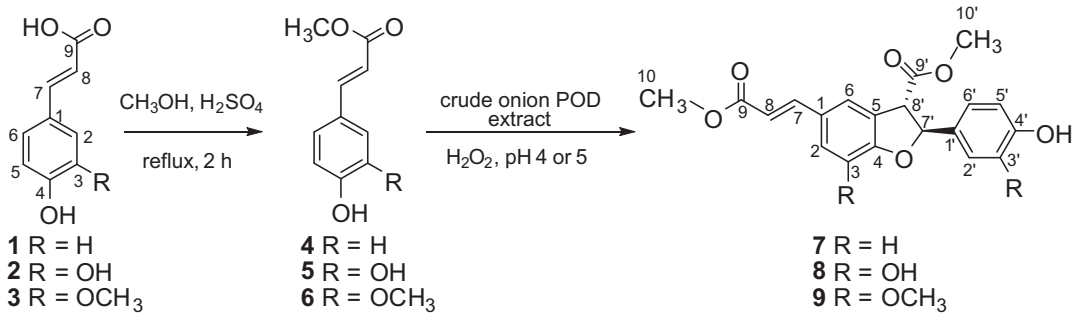
p-Coumaric acid (**1**), caffeic acid (**2**), and ferulic acid (**3**) (Scheme 1) are components of plant cell walls, and are subjected to oxidative dimerization to produce structurally diverse lignan-type natural products. Characteristic examples of common phenolic acid dihydrodimers isolated from plants are the ferulic acid dimers 8-*O*-4-diFA (compound **I**), 8,8-diFA (compound **II**), 5,5-diFA (compound **III**), and the dihydrobenzofuran lignans (compounds of type **IV**) (Fig. 1).[8–10] The latter display a wide range of biological activity including antitumor,[11] antiparasitic,[12] and antioxidant,[5] and as a result, have attracted considerable attention as potential synthetic targets with an important bioactivity.

The dimerization of phenolic acids to give lignans has been reported to occur efficiently mainly using the biomimetic peroxidase-H_2O_2 system, exploiting in most cases horseradish peroxidase,[4,13–16] but also potato peroxidase,[9] *Momordica charantia* peroxidase,[5] and a peroxidase from the leaves of *Bupleurum salicifolium*.[1] The dihydrobenzofuran dimer of *p*-coumaric acid has also been produced as a metabolic product of *Curvularia lunata*, as a result of the presence of peroxidase and laccase in the culture filtrates of this microorganism.[17] In addition, chemical single-electron oxidizing systems, such as Ag_2O or $K_3Fe(CN)_6$, are effective coupling agents for the dimerization of phenolic acids or their esters.[11,12,18]

The exploitation of food residuals as sources of crude enzymes can contribute not only to the reduction of the polluting load of food industry wastes, but also to the development of high added value products. As a continuation of our research on the potential biocatalytic activity of crude peroxidase (POD) from onion solid waste,[19] we herein describe the reactions of methyl *p*-coumarate (**4**), methyl caffeate (**5**), and methyl ferulate (**6**) using this enzymic preparation as a catalyst. The antioxidant activity of the reaction

* Corresponding author. Tel./fax: +30 2821035056.
E-mail address: panos@maich.gr (P. Kefalas).
† These two authors contributed equally to this research.

0040-4039/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2011.01.004



Scheme 1. Oxidative coupling of esters **4**–**6** catalyzed by crude onion POD extract.

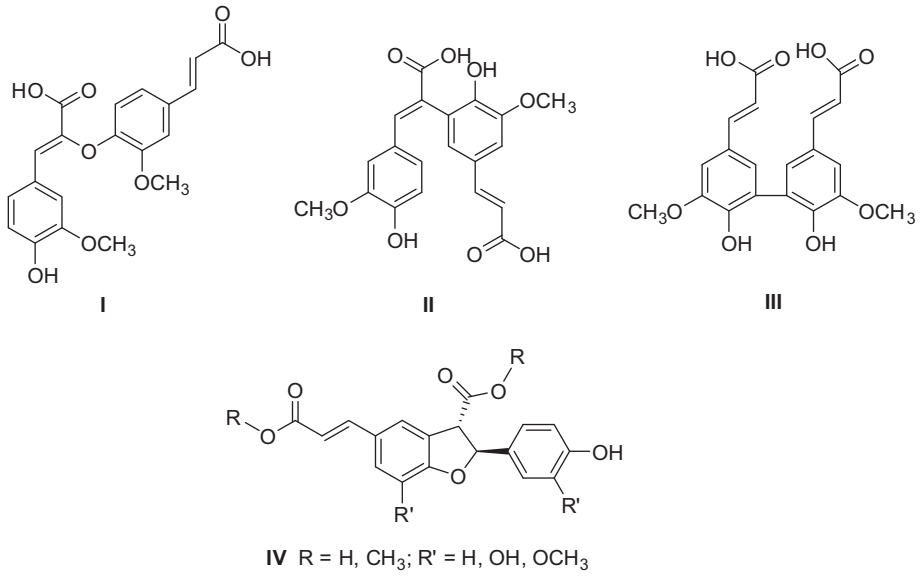


Figure 1. Chemical structures of common natural phenolic acid dihydrodimers.

products, as well as of their corresponding free acids and esters, was evaluated using the ferric reducing antioxidant power (FRAP) assay.

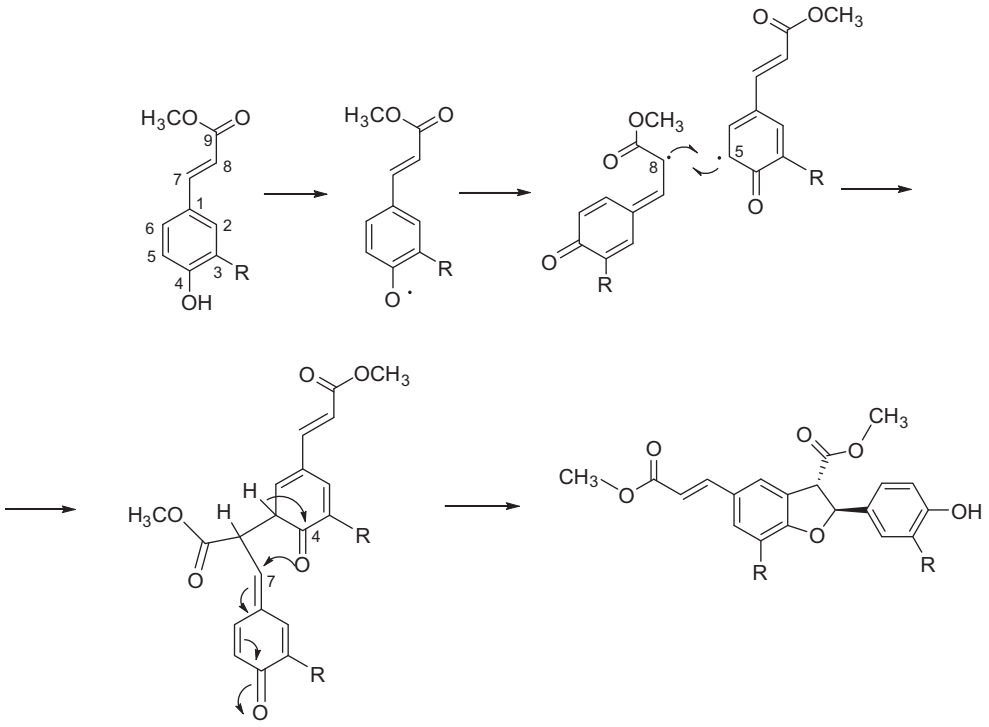
The starting esters **4–6** were synthesized via Fischer esterification of *p*-coumaric, caffeic, and ferulic acids **1**–**3**, respectively.[20] The onion solid waste used as the enzyme source in this study was obtained from a local catering facility (Chania, Crete, Greece) after processing of brown-skin onion bulbs (*Allium cepa*). This is the most widely cultivated horticultural crop in Europe, and the common onion variety that can be found in typical supermarkets in almost every region of Greece. The waste consisted of the apical trimmings of the bulbs, as well as the outer dry and semi-dry layers. The cell-free, POD-active extract was prepared as previously described.[19,21] In an effort to improve the enzyme activity we concentrated the POD-active extract by treatment with ammonium sulfate, stirring for one hour in an ice bath, and then centrifuging at 10,000 rpm at 4 °C for 15 min. The precipitate was dispersed in phosphate buffer (pH 4) and used as the crude enzyme source.[22]

The reaction conditions using methyl *p*-coumarate (**4**), methyl caffeate (**5**) and methyl ferulate (**6**) as substrates for the crude enzyme preparation were optimized in terms of pH and solvents. We performed the reactions at pH 4, 5 and 6, and optimum yields were observed at pH 5 for methyl *p*-coumarate (**4**), at pH 4 for methyl caffeate (**5**) and at pH 4 for methyl ferulate (**6**). In a typical experiment (Scheme 1), to the crude POD extract were added H_2O_2, as a solution in phosphate buffer, a solution of ester **4**, **5** or **6** in methanol and the appropriate co-solvent, and the mixture was stirred at room temperature for 30 minutes. The reaction was quenched by adding trichloroacetic acid and, after centrifugation, the aqueous supernatant was extracted with ethyl acetate.[23]

The reaction progress was monitored by RP-18 LC-DAD-MS at 278 and 340 nm.[24] In the case of methyl *p*-coumarate (**4**), a new compound appeared at λ_{max} 314 nm ($[M+H]^+$ at *m/z* 355), whereas in the case of methyl caffeate (**5**), a new compound appeared at λ_{max} 318 nm ($[M+H]^+$ at *m/z* 387). For methyl ferulate (**6**), a new compound appeared displaying a λ_{max} at 328 nm ($[M+H]^+$ at *m/z* 415). An LC-MS study indicated highly regiospecific dimerisation resulting from oxidative coupling of the starting esters. The crude products were purified using preparative TLC and HPLC to afford compounds **7–9**.[23] Dihydrodimer **8** was unstable under these conditions, and therefore was not isolated in pure form.

Based on 1D and 2D NMR data, the structures of the reaction products were assigned unambiguously as the dihydrobenzofuran lignans **7–9**.[25–27] Even though two different stereoisomers were possible for each reaction product, analysis of their spectroscopic characteristics suggested a single isomer, which was determined as having a 7′,8′-*trans* configuration, in accordance with data reported in the literature.[11] Pure compounds **7** and **9** did not show optical rotation. The ESI-MS spectra of pure compounds **7** and **9** included, apart from the molecular ion, several diagnostic fragments.

To the best of our knowledge, this is the first report describing the radical dihydrodimerisation of hydroxycinnamates involving crude onion POD as the biocatalyst. The procedure is straightfor-



Scheme 2. Proposed mechanism for the oxidative radical dihydrodimerisation of esters **4**–**6** catalyzed by crude onion POD extract.

ward and the compounds are obtained in good yields (70% and 90% for compounds **7** and **9**, respectively). The mechanism of the reaction, as shown in Scheme 2, involves initial formation of a new C–C bond arising from reaction between the C-8-centered semiquinone radical and the C-5-centered quinone methide radical. The product of this reaction is then cyclized regioselectively via nucleophilic attack of the carbonyl oxygen C-4 at C-7 to produce the 2,3-dihydrobenzofuran derivative.

The antioxidant activity of compounds **7** and **9**, and the corresponding starting phenolic acids and esters, was evaluated by the ferric reducing antioxidant power (FRAP) assay, which uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to the ferrous form (which has an intense blue color) can be monitored by measuring the change in absorption at 593 nm.[28,29] The reaction is non-specific, in that any half reaction that has a lower redox potential than that of the ferric-ferrous half reaction, will lead to ferrous ion formation. The change in absorbance is therefore, directly related to the combined or 'total' reducing power of the electron-donating antioxidants present in the reaction mixture. The results of the antioxidant activity evaluation (Table 1) show that the dihydrobenzofuran lignans **7** and **9** exhibit significantly higher activity in this assay than the corresponding phenolic acids, **1** and **3**, or the esters **4** and **6**. Esterification of *p*-coumaric and ferulic acids results in a decrease in the ferric reducing power activity. The most active compound of the series tested is dihydrobenzofuran **9**, derived from the oxidative dimerization–cyclization of methyl ferulate. This compound has also been reported as a potent inhibitor of cell-mediated LDL oxidation.[12]

Table 1
Antioxidant activity of compounds **1**, **3**, **4**, **6**, **7** and **9** as evaluated using the FRAP assay.

Compound	FRAP value (μM)
1	1.136
3	0.986
4	0.271
6	0.315
7	2.186
9	4.357

In conclusion, we have demonstrated the potential of using crude onion POD extract as a biocatalyst for the efficient oxidative dimerization–cyclization of methyl *p*-coumarate, methyl caffeate, and methyl ferulate leading to dihydrobenzofuran lignan-type compounds. The antioxidant activity of compounds **7** and **9** indicates that they are promising molecular scaffolds for the design and synthesis of more potent antioxidants derived from phenolic acids.

Acknowledgment

E.I. is grateful to PNG Gerolymatos for a Postdoctoral fellowship.

References and notes

1. Frias, I.; Siverio, J. M.; Gonzalez, C.; Trujillo, J. M.; Perez, J. A. *Biochem. J.* **1991**, *273*, 109–113.
2. Wallace, G.; Fry, S. C. *Phytochemistry* **1995**, *39*, 1293–1299.
3. Wallace, G.; Fry, S. C. *Phytochemistry* **1999**, *52*, 769–773.
4. Luis, J. C.; Gonzales, F. V.; Pérez, R. M.; Pérez, J. A.; Frias, I. *Prepar. Biochem. Biotech.* **2005**, *35*, 231–241.
5. Liu, H. L.; Huang, X. F.; Wan, X.; Kong, L. Y. *Helv. Chim. Acta* **2007**, *90*, 1117–1132.
6. Izydorczyk, M. S.; Biliaderis, C. G. *J. Agric. Food Chem.* **1992**, *40*, 561–568.
7. Izydorczyk, M. S.; Biliaderis, C. G. Arabinoxylans—Technologically and Nutritionally Functional Plant Polysaccharides. In *Functional Food Carbohydrates*; CRC Press: Boca Raton, USA, 2007; pp 249–290.
8. Ralph, J.; Bunzel, M.; Marita, J. M.; Hatfield, R. D.; Lu, F.; Kim, H.; Schatz, P. F.; Grabber, J. H.; Steinhart, H. *Phytochem. Rev.* **2004**, *3*, 79–96.
9. Arrieta-Baez, D.; Stark, R. E. *Phytochemistry* **2006**, *67*, 743–753.
10. Andreasen, M. F.; Christensen, P. L.; Meyer, A. S.; Hansen, A. *J. Agric. Food Chem.* **2000**, *48*, 2837–2842.
11. Pieters, L.; Van Dyck, S.; Gao, M.; Bai, R.; Hamel, E.; Vlietinck, A.; Lemiere, G. *J. Med. Chem.* **1999**, *42*, 5475–5481.
12. Rakotondramanana, D. L. A.; Delomenede, M.; Baltas, M.; Duran, H.; Bedos-Belval, F.; Rasoanaivo, P.; Negre-Salvayre, A.; Gornitzka, H. *Bioorg. Med. Chem.* **2007**, *15*, 6018–6026.

13. Chioccara, F.; Poli, S.; Rindone, B.; Pilati, T.; Brunow, G.; Pietikainen, P.; Setala, H. *Acta Chem. Scand.* **1993**, *47*, 610–616.
14. Ralph, J.; Garcia Conesa, M. T.; Williamson, G. *J. Agric. Food Chem.* **1998**, *46*, 2531–2532.
15. Larsen, E.; Andreasen, M. F.; Christensen, L. P. *J. Agric. Food Chem.* **2001**, *49*, 3471–3475.
16. Wakimoto, T.; Nitta, M.; Kasahara, K.; Chiba, T.; Yiping, Y.; Tsuji, K.; Kan, T.; Nukaya, H.; Ishiguro, M.; Koike, M.; Yokoo, Y.; Suwa, Y. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5905–5908.
17. Torres y Torres, J. L.; Rosazza, J. P. N. *J. Nat. Prod.* **2001**, *64*, 1408–1414.
18. Wasserman, H. H.; Brunner, R. K.; Buynak, J. D.; Carter, C. G.; Oku, T.; Robinson, R. P. *J. Am. Chem. Soc.* **1985**, *107*, 519–521.
19. Moussouni, S.; Detsi, A.; Majdalani, M.; Makris, D. P.; Kefalas, P. *Tetrahedron Lett.* **2010**, *51*, 4076–4078.
20. *Synthesis of methyl p-coumarate (**4**), methyl caffeate (**5**), and methyl ferulate (**6**)*: The appropriate phenolic acid (100 mg) was dissolved in MeOH (50 ml) to which 3 drops of H_2SO_4 had been added. The solution was refluxed for 2 h, $NaHCO_3$ (100 mg) was added, and the solvent was evaporated in vacuo at 40 °C. The residue was partitioned between Et_2O (15 mL) and H_2O (15 mL), the organic layer separated, dried (Na_2SO_4), and the solvent was evaporated in vacuo to afford the desired ester, as a white solid. The esters were used in the oxidation reaction without further purification.
21. Khiari, Z.; Makris, D. P.; Kefalas, P. *Food Bioprocess Technol.* **2009**, *2*, 337–343.
22. *Preparation of the crude onion peroxidase (POD) extract*: Parts of the onion bulb considered as waste (non edible) material, consisting of the apical trimmings, were used for preparing the crude POD extract. The material was transferred to the laboratory immediately after processing and ground in a domestic blender. An aliquot of the ground tissue (10 g) with solid polyvinylpyrrolidone (PVPP, 5 g) were suspended in phosphate buffer (100 ml, pH 4, containing 2 mM $CaCl_2$). The suspension was centrifuged at 3000 rpm for 10 min and filtered through filter paper to remove cell debris. The clear supernatant obtained was subjected to precipitation by the addition of solid NH_4SO_4, stirred for 1 h in an ice bath, and then centrifuged at 10,000 rpm at 4 °C for 15 min. The precipitate was dispersed in phosphate buffer (pH 4) and used as the crude enzyme source.
23. *Dimerization reaction of phenolic esters using crude onion POD*: A solution containing 2 mM of phenolic ester **4**, **5** or **6** dissolved in MeOH, DMF or glycerol (1 mL) was added to a mixture containing crude onion POD extract (10% of the total volume) and 8ml H_2O_2 (3 mM in phosphate/citrate buffer, pH 5 containing 2 mM $CaCl_2$) for 30 min at room temperature. The reaction was stopped by the addition of TCA (0.1 ml, 10% in EtOH) and then subjected to centrifugation at 5000*g* for 10 min. The mixture was extracted with EtOAc (3×20 mL) and the organic layer was dried over Na_2SO_4. After vacuum distillation of the solvent, the residue was redissolved in MeOH (1 mL), filtered through 0.45 μm syringe filters, and the filtrate was used for chromatographic analyses. The crude reaction products were purified using preparative silica gel TLC (developed with petroleum ether/EtOAc 7:3), followed by preparative normal phase HPLC using a CECIL 1100 Series liquid chromatography pump equipped with a GBC LC-1240 refractive index detector and a Supelcosil SPLC-Si (25 cm × 10 mm) column with cyclohexane/EtOAc (6:4) as eluent.
24. ESI+ conditions: probe voltage 4 kV; probe temperature 350 °C; collision induced fragmentation at 12 and 60 eV. The LC–MS carrier solvent was a gradient of 2.5% AcOH in H_2O and MeOH.
25. Methyl (*E*)-2-(4-hydroxyphenyl)-5-(3-methoxy-3-oxoprop-1-enyl)-2,3-dihydrobenzofuran-3-carboxylate (**7**): Yield: 70%; UV (CH_3OH) λ_{max} 314 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.65 (1H, d, *J* = 16.0 Hz, H-7), 7.53 (1H, br s, H-6), 7.41 (1H, dd, *J* = 8.4, 1.7 Hz, H-2), 7.25 (2H, dd, *J* = 8.9, 2.2 Hz, H-2′ and H-6′), 6.87 (1H, d, *J* = 8.4 Hz, H-3), 6.82 (2H, dd, *J* = 8.9, 2.2 Hz, H-3′ and H-5′), 6.30 (1H, d, *J* = 16.0 Hz, H-8), 6.08 (1H, d, *J* = 7.5 Hz, H-7′), 4.25 (1H, d, *J* = 7.5 Hz, H-8′), 3.82 (3H, s, H-10′), 3.78 (3H, s, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.8 (C-9′), 167.8 (C-9), 161.2 (C-4), 156.0 (C-4′), 144.6 (C-7), 132.2 (C-1′), 130.8 (C-2), 127.9 (C-1), 127.5 (C-2′ and C-6′), 125.1 (C-5), 125.0 (C-6), 115.7 (C-3′ and C-5′), 115.3 (C-8), 110.3 (C-3), 86.4 (C-7′), 55.1 (C-8′), 52.9 (C-10′), 51.7 (C-10); ESI-MS *m/z* 355 $[M+H]^+$, 323, 291, 263, 235, 207.
26. Methyl (*E*)-2-(3,4-dihydroxyphenyl)-7-hydroxy-5-(3-methoxy-3-oxoprop-1-enyl)-2,3-dihydrobenzofuran-3-carboxylate (**8**): UV (CH_3OH) λ_{max} 318 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.57 (1H, d, *J* = 15.9 Hz, H-7), 7.08 (1H, br s, H-6), 7.03 (1H, br s, H-2), 6.87 (1H, d, *J* = 1.4 Hz, H-2′), 6.84 (1H, d, *J* = 8.0 Hz, H-5′), 6.80 (1H, dd, *J* = 8.0, 1.4 Hz, H-6′), 6.26 (1H, d, *J* = 15.9 Hz, H-8), 6.05 (1H, d, *J* = 7.5 Hz, H-7′), 4.28 (1H, d, *J* = 7.5 Hz, H-8′), 3.81 (3H, s, H-10′), 3.78 (3H, s, H-10); ESI-MS *m/z* 387 $[M+H]^+$.
27. Methyl (*E*)-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-5-(3-methoxy-3-oxoprop-1-enyl)-2,3-dihydrobenzofuran-3-carboxylate (**9**): Yield: 90%; UV (CH_3OH) λ_{max} 328 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.63 (1H, d, *J* = 15.9 Hz, H-7), 7.16 (1H, d, *J* = 1.1 Hz, H-6), 7.00 (1H, d, *J* = 1.1 Hz, H-2), 6.89 (2H, m, H-5′ and H-6′), 6.88 (1H, m, H-2′), 6.30 (1H, d, *J* = 15.9 Hz, H-8), 6.09 (1H, d, *J* = 8.2 Hz, H-7′), 4.33 (1H, d, *J* = 8.2 Hz, H-8′), 3.89 (3H, s, H-11), 3.86 (3H, s, H-11′), 3.81 (3H, s, H-10′), 3.79 (3H, s, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.7 (C-9′), 167.6 (C-9), 149.9 (C-4), 146.7 (C-3′), 146.1 (C-4′), 144.8 (C-3), 144.7 (C-7), 131.4 (C-1′), 128.6 (C-1), 125.7 (C-5), 119.4 (C-6′), 117.9 (C-6), 115.6 (C-8), 114.5 (C-5′), 112.2 (C-2), 108.7 (C-2′), 87.5 (C-7′), 56.1 (C-11), 56.0 (C-11′), 55.5 (C-8′), 52.8 (C-10′), 51.6 (C-10); ESI-MS *m/z* 415 $[M+H]^+$, 383, 351, 323, 295, 267.
28. *FRAP assay*: the acetate buffer solution (a) 300 mM, pH 3.6 was prepared by weighing 3.1 g $NaOAc{\cdot}3H_2O$ and adding 16 ml of glacial AcOH and the volume was made up to 1 L with distilled H_2O. The second solution (b) contains 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and solution (c) contains 20 mM $FeCl_3{\cdot}6H_2O$ dissolved in distilled H_2O. The working FRAP reagent was prepared by mixing (a), (b) and (c) in the ratio of 10:1:1 at the time of use. The sample (100 μL, 1 mM) was mixed with 3 mL of working FRAP reagent and the absorbance (593 nm) was measured at 0 min after vortexing. Thereafter, samples were placed in a water bath (37 °C) and the absorption was measured after 4 min. The FRAP reagent was used as the blank solution, and ascorbic acid (1 mM) was used as the standard. Blank and ascorbic acid standards were processed in the same way. FRAP value of sample (μM) = (change in absorbance of sample from 0 to 4 min/change in absorbance of standard from 0 to 4 min) × FRAP value of the standard (1000 μM).
29. Benzie, F. F.; Strain, J. J. *Methods Enzymol.* **1999**, *299*, 15–23.