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Resistance factors to grey mould in grape berries: identification of some phenolics inhibitors of *Botrytis cinerea* stilbene oxidase

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Abstract

Grey mould caused by *Botrytis cinerea* is one of the most important diseases of grapes. Between bloom and veraison, grape berries are resistant to *B. cinerea*, although they can harbour the pathogen without any visible signs of disease development. After veraison, *B. cinerea* can produce disease in susceptible grape varieties (e.g. *Gamay*), but remains quiescent in resistant varieties (e.g. *Gamaret*). Pathogen resistance in the quiescent stage is not yet fully understood, but is thought to involve multiple parameters including chemical and mechanical factors. The pathogenesis of *B. cinerea* is essentially linked to excretion of lytic enzymes such as polyphenoloxidases or laccases. One lytic enzyme, stilbene oxidase, can detoxify grape stilbenic phytoalexins, destroying the grapes' defence mechanisms and allowing the fungus to grow. Some constitutive grape berry phenolic compounds, however, strongly inhibit stilbene oxidase activity. Constitutive berry phenolic compounds were isolated from *Gamay* and *Gamaret* varieties and their biological activities, concentrations and chemical structures were comparatively analysed. Catechin, epicatechin-3-*O*-gallate, *trans*-caftaric, *trans*- and *cis*-coutaric and *trans*-coumaric acids, taxifoline-3-*O*-rhamnoside and quercetine-3-*O*-glucuronide were identified as potent stilbene oxidase inhibitors. High concentrations of some of those compounds could be closely involved in the persistence of the quiescent stage of *B. cinerea,* between bloom and veraison in all grape varieties and after veraison in resistant varieties.

Keywords: *Vitis vinifera*; Grapevine; *Botrytis cinerea*; Stilben oxydase; Caftaric acid; Coutaric *trans* acid; Coutaric *cis* acid; *p* trans Coumaric acid; Taxifolin-3-*O*-rhamnoside; Quercetin-3-*O*-glucuronide; Catechin; Epicatechin-3-*O*-gallate

1. Introduction

Grey mould is one of the most important diseases of grapes. Before bloom and after veraison, grape clusters can be infected and destroyed by *Botrytis cinerea,* depending on climatic conditions and the sensitivity of the grape variety. Between these two developmental stages, young clusters are resistant to *B. cinerea*. This natural resistance period is called the quiescent stage (McClellan & Hewitt, 1973; Pezet & Pont, 1986). Pezet and Pont (1984) have shown that crude extracts of healthy berries collected during this stage are able to strongly inhibit germination of *B. cinerea* conidia. The fungicidal activity of these extracts can be explained by the presence of low concentrations of pterostilbene (*trans*-3,5-dimethoxy-4-hydroxystilbene) (Pezet & Pont, 1988a), a known *vitaceae* phytoalexin (Langcake, Cornford & Ryce, 1979), and glycolic acid, which enhances the biocidal activity of pterostilbene (Pezet & Pont, 1988b). However, other chemical and mechanical processes could explain the resistance of the immature berries: polygalacturonase inhibiting protein (PGIP) (Grassin, 1987), pathogenesis-related protein (β-1,3-glucanase) (Renault, Deloire & Bierne, 1996), anthocyanins and phenolic compounds (Jersch, Scherer, Huth & Schlösser, 1989), as well as the cuticle mechan-

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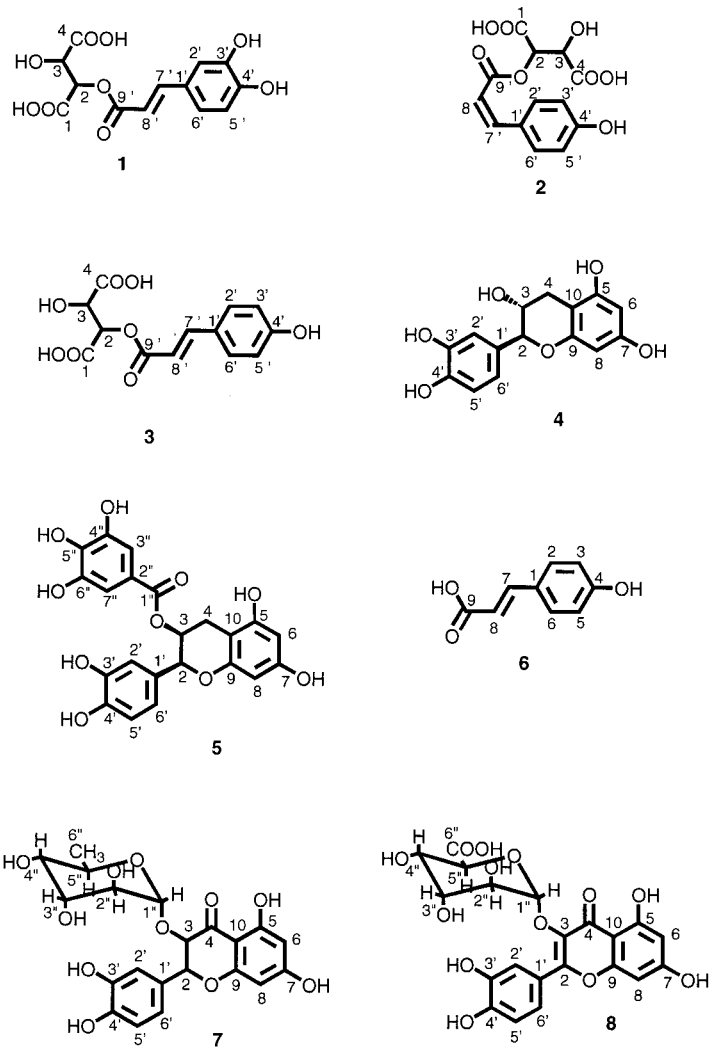


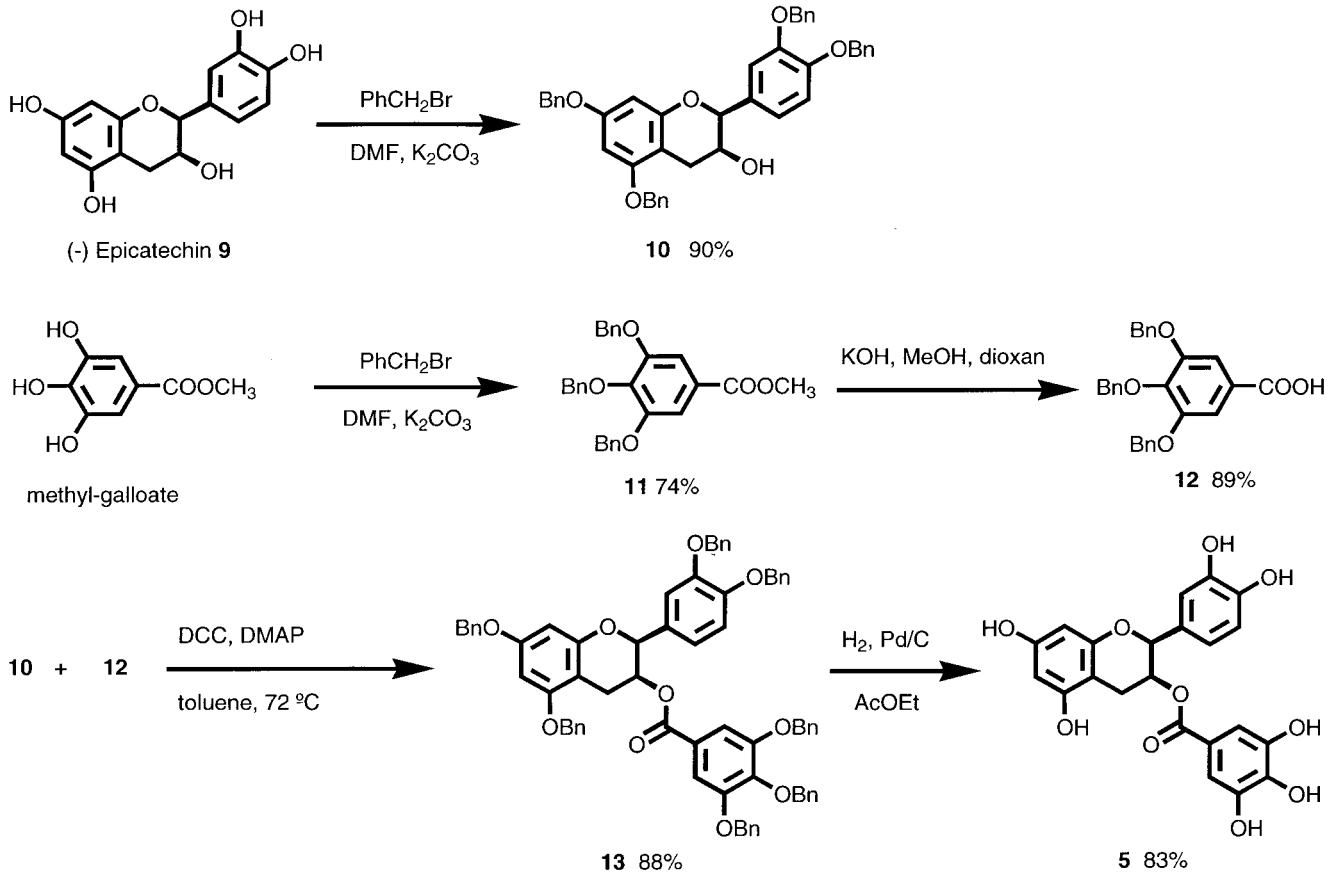
Fig. 1. Structures of isolated compounds.

ical defence (Kolattukudy, 1985). Although it can be concluded that the resistance of grape berries to *B. cinerea* is the result of several conjugated processes, these processes are not yet completely understood.

Exoenzymes excreted by *B. cinerea* are strongly implicated in the pathogenesis of this parasite (Grassin, 1987). Compounds from grape berries that inhibit the activities of these enzymes may be implicated in the defence against grey mould. The aim of this research project was to isolate metabolites from the crude extracts of young berries. In addition to the mechanisms described previously, these metabolites could be implicated in maintaining *B. cinerea* quiescence and inhibiting laccases. One of the laccases excreted by *B. cinerea* oxidizes stilbenes (Pezet, 1998), detoxifying the compound (Pezet, Pont & Hoang-Van, 1991) and breaking the grapes' induced defence. In this study, free phenolic compounds were isolated from young grape berries. Their potent inhibition of *B. cinerea* stilbene oxidase may contribute to the protection of stilbenic phytoalexins and their fungicidal properties.

2. Results and discussion

Immature grape berries, variety Pinot Noir, were harvested from June to September in vineyards situated in Epernay (France). Free phenolic compounds were extracted from the berries and purified by semi-preparative HPLC. Eight peaks, numbered 1 to 8 according to their retention time, were identified by means of UV, DCI-NH_3 or electrospray-mass spectroscopy, or ^1H and ^{13}C NMR (Fig. 1). All the resulting compounds were very polar, soluble only in water or methanol, with high molecular weights. In further biological studies we avoided, when possible, chemical



Scheme 1. Synthesis of (−) epicatechin-3-*O*-gallate.

modifications and use of solvents such as THF or DMSO.

Except for different retention times, the UV spectrum of **1** was identical to the spectrum of caffeic acid and the spectrums of **2** and **3** were identical to coumaric acid. At −40°C, **1**, **2** and **3** showed ^{1}H and ^{13}C NMR spectra similar to those of caffeic or coumaric acid (Satake, Murakami, Saiki & Chen, 1980). The extra signals observed at 5.65 and 4.85 ppm in the ^{1}H-NMR, and four more signals in the ^{13}C-NMR, could be assigned to a tartaric acid moiety. ^{1}H-^{13}C short- and long-range correlations at −40°C, as well as MS, confirmed compounds **1**, **2**, and **3** to be E-caftaric acid, Z-coutaric acid and E-coutaric acid, respectively (Cheminat, Zawatzky, Becker & Brouillard, 1988; Strack, Hartfeld, Austenfeld, Grotjahn & Wray, 1985; Weber, Hoesch & Rast, 1995).

The ^{1}H, ^{13}C-NMR data for compounds **4**–**8** was compared with previously published data for **4** (Mendez, Bilia & Morelli, 1995; Seto, Nakamura, Nanjo & Hara, 1997), **5** (Sakar, Petereit & Nahrstedt, 1993), **6** (Satake et al., 1980), **7** (De Britto, Manickam, Gopalakrishnan, Ushioda & Tanaka, 1995; Trousdale & Singleton, 1983) and **8** (Möhle, Heller & Wellmann, 1985; Weber, Hoesch & Rast, 1995). The data comparison indicated compounds **4**–**8** were catechin, epicatechin-3-*O*-gallate, *p*-coumaric acid, taxifolin-3-rhamnosyl and quercetin-3-glucuronid, respectively. ^{13}C-NMR of quercetin-3-glucuronid (**8**) had to be recorded at −40°C to enable visualization of the signals corresponding to the glucuronic acid moiety. Acid hydrolysis and TLC analysis confirmed the sugar moiety to be glucuronic acid by comparison with a pure sample. Although all the compounds identified in this study have been previously described in grapes (Ricardo da Silva, Rigaud, Cheynier, Cheminat & Moutounet, 1991; Singleton, Zaya & Trousdale, 1986; Souquet, Cheynier, Brossaud & Moutounet, 1996; Trousdale & Singleton, 1983; Weber et al., 1995), some of the published data is incomplete or outdated. Therefore, we chose to describe compounds **1**–**8** extensively in this experiment.

Compound **5** was synthesized to obtain sufficient amounts of the sample biological testing. This synthesis is illustrated in Scheme 1. The key step of this synthesis was the esterification of the hindered alcohol using dicyclohexyl carbodiimid (DCC). The hydroxyl groups of (−) epicatechin (**9**) and methyl gallate were

Table 1
Stilbene oxidase inhibition (and affinity) of *Botrytis cinerea* laccase

Inhibitors	nmoles of pterostilbene oxidized/min[a]				IC_{50} (mM)[b]	K_m (mM)
	Inhibitor concentrations (μg/ml)					
	0.66	6.6	20	33		
(*E*) Caffeoyl tartaric acid (**1**)	40.24	18.98	3.23	7.89	0.0150	0.11
(*Z*) Coumaroyl tartaric acid (**2**)	45.38	43.21	24.47	30.93	0.1110	ND
(*E*) Coumaroyl tartaric acid (**3**)	38.07	40.19	18.61	15.10	0.0537	0.64
Catechin (**4**)	41.84	15.63	4.86	2.07	0.0114	0.58
Epicatechin-3-*O*-gallate (**5**)	31.57	2.49	1.82	1.24	0.0022	ND
(*E*) *p*-Coumaric acid (**6**)	40.99	25.62	21.30	21.75	0.0540	0.25
Taxifoline-3-*O*-rhamnoside (**7**)	38.64	27.99	16.54	0.00	0.0222	ND
Quercetine-3-*O*-glucuronide (**8**)	47.55	41.42	15.21	7.03	0.0297	ND
Epicatechin (**9**)	20.00	4.45	3.41	0.77	0.0021	ND

[a] Without inhibitor pterostilbene is degraded at the rate of 50.84 nmoles/min; $K_m = 0.66$. Activity is determined in 0.1 M phosphate buffer pH 5.2 with 0.054 mM of pterostilbene as substrate.
[b] Calculated with a single exponential decay function (Grafit Erithacus software).

protected with benzyl groups, using standard protocols, to generate **10** and **11**, respectively. The ester (**11**) was hydrolysed into the acid derivative, **12**. Then, condensation of **10** and **12** yielded the fully protected (−) epicatechin-3-*O*-gallate (**13**). After esterification the proton in position 3 shifted from 4.18 to 5.7 ppm. Catalytic hydrogenation of **13** on Pd-charcoal to remove the protecting benzyl groups yielded **5**. The spectral data for the synthetic compound **5** were identical to those of the natural compound, confirming its structure. This synthetic route could be used as a general method for the preparation of other epicatechin or catechin ester derivatives.

All isolated phenols and catechols inhibited *B. cinerea* stilbene oxidase activity in vitro using pterostilbene as substrate, with IC_{50} values ranging from 0.0021 to 0.111 mM (Table 1). The highest inhibition was observed with epicatechin-3-*O*-gallate (**5**). This compound gave the same level of inhibition as the positive control, epicatechin. (*Z*) and (*E*) coumaroyl tartaric acid (**2** and **3**) and (*E*)-*p*-coumaric acid (**6**) were the least active phenols of the extracts. With *B. cinerea* laccase, (*E*)-coumaroyl tartaric acid (**3**) has a K_m of 0.64 mM, close to that of pterostilbene ($K_m = 0.66$ mM), while (*E*)-caffeoyl tartaric acid (**1**), one of the most active inhibitors, has a K_m of 0.11 mM (Gunata, Sapis & Moutounet, 1987; Pezet & Pont, 1992). These results suggest that these phenolic compounds competitively inhibit stilbene oxidase activity. However, further studies would be necessary to confirm this hypothesis.

To compare these compounds quantitatively, we analysed the phenolic extracts from *Gamay* and *Gamaret*; two grape varieties with different sensitivities to grey mould. The former variety develops a high level of grey mould after veraison, if the climatic conditions are favourable. The latter variety is considered to be resistant to *B. cinerea* in all conditions (Pezet & Pont, 1992). Young clusters and berries of those two varieties were harvested in the vineyards of the Swiss Federal Agricultural Research Station of Changins in 1991. Similar HPLC peaks were detected in the two varieties and the phenolic compounds identified in Pinot Noir were identical to those separated in *Gamay* and *Gamaret*; only quantitative differences were detected.

Fig. 2a and b shows the concentrations of each compound (determined by analytical HPLC) from grapes collected (two or three times per month from 19 June to 25 September) at every stage of development of the two varieties. Both profiles were very similar. Before blooming, when the grapes are most sensitive, *p*-coumaric acid (**6**) was the major phenol detected in both varieties; at the very start of blooming (27 June) it represented more than 90% of the extract, and almost disappeared after blooming (9 July). This can be explained by the fact that **6** (under its coenzyme A form) is the biosynthetic precursor of other shikimic acid metabolism components of the extract (Rupprich & Kindl, 1978; Smith & Banks, 1986). As the level of **6** decreased, the level of catechin (**4**) increased, up to a maximum observed on 23 July. Within 10 days (2 August), it dropped to a minimum, corresponding to the bunch closure period during which many berries dehydrate. This normal process sheds from the cluster the nascent fruit for which fertilization did not occur or the embryo aborted (Jackson, 1994). The catechin level then rose again to a maximum immediately prior to veraison (4 September), and a final setting level of **4** accompanied the later sensitive stage. The level of catechin (**4**) in the resistant *Gamaret* was twice as high as the level in the sensitive *Gamay* variety. At a lower

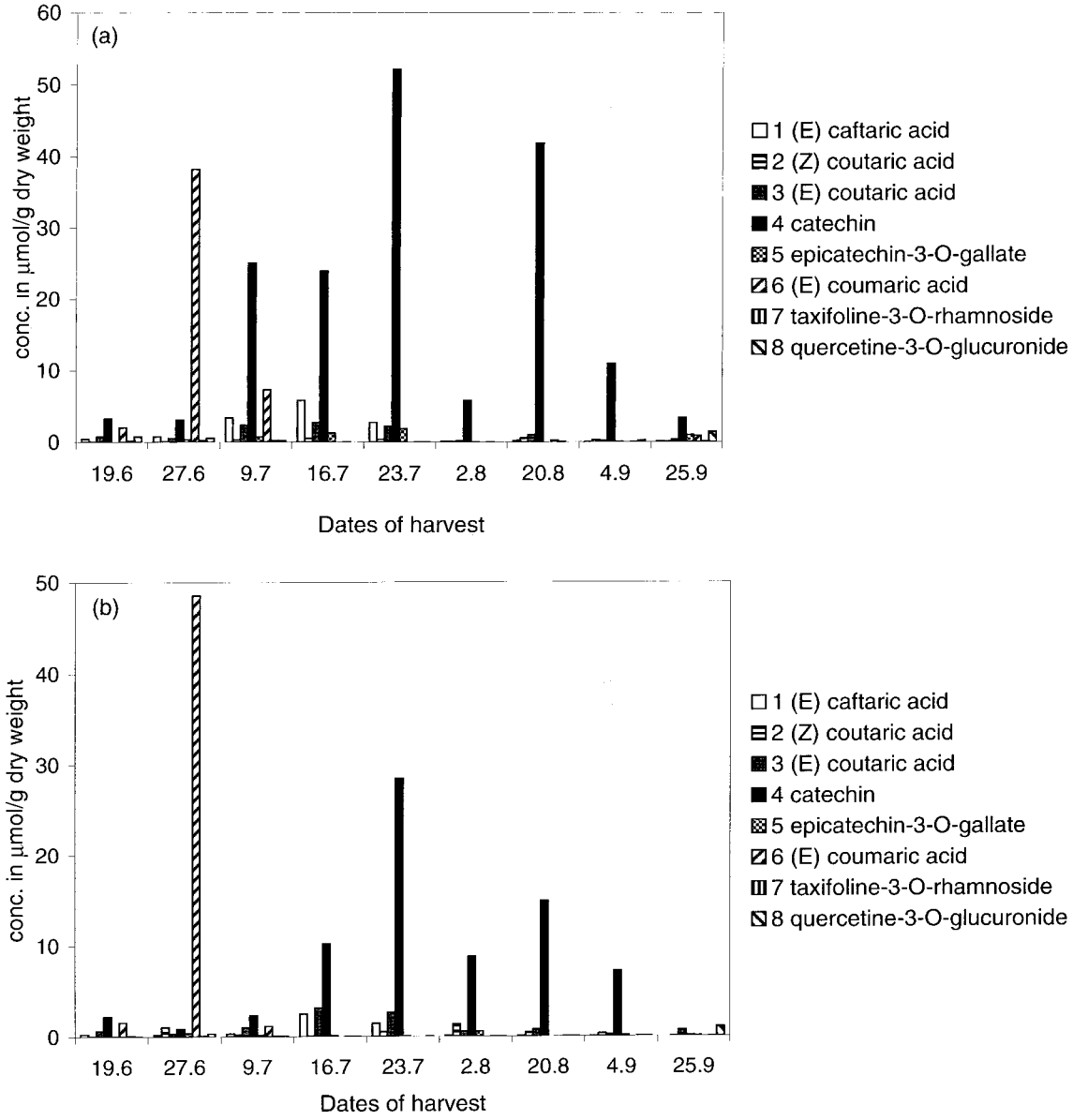


Fig. 2. Evolution over a growing season of the concentration of compounds extracted from (a) *Gamaret* Grapes and (b) *Gamay* grapes.

order of magnitude, quiescence for both varieties seems to be correlated with increased levels of *E* caftaric acid (**1**) and *E* coutaric acid (**3**), with epicatechin-3-*O*-gallate (**5**) produced only by *Gamaret*. Here again, levels of *E* caftaric acid (**1**) in *Gamaret* are twice as high as in *Gamay*. *Z* Coutaric acid (**2**), in the *Gamay* variety, shows two maxima, one during bloom, the other during bunch closure.

3. Conclusion

This study showed all isolated phenols and catechols were active against *B. cinerea* laccases. One can assume that by inhibiting the stilbene oxidase they may attenuate the *Botrytis* defence, leaving 'safer ground' for the classical phythoalexins, resveratrol and pterostilbene.

The resistance of young berries results from several conjugated processes; inhibition of the stilbene oxidase activity of *B. cinerea* laccases is one of them. Along with glycolic acid, low concentration of pterostilbene, a polygalacturonase inhibiting glycoprotein, a pathogenesis-related protein, the mechanical barrier of the epidermal complex, etc., high levels of the catechin (**4**) contribute to the quiescent stage. The fact that the catechin level in the resistant *Gamaret* is twice as high as in the sensitive *Gamay* variety can help to explain, but is not sufficient proof by itself, why differences in sensitivity to *B. cinerea* are observed in the two varieties.

4. Experimental

4.1. Extraction

Free phenolic acids were extracted according to Southerton & Deverall (1990). Grapes (*Vitis vinifera L.*) were harvested at different growth stages. Freeze-dried and finely powdered plant materiel (10 g) was extracted with MeOH (800 ml) at room temperature in the dark for 45 min. The solution was centrifuged at 4500 rpm for 45 min. The supernatant was kept; the residue was dissolved in MeOH (400 ml) and centrifuged at 4500 rpm for 10 min. The supernatants were combined and MeOH was evaporated at 40° under vacuum. The residue was dissolved in H_2O (800 ml) and the aq. soln was extracted with Et_2O (4 × 200 ml). Organic layers were combined, dried over Na_2SO_4, filtered and Et_2O evaporated. The residue was dissolved in MeOH–H_2O (60:40). The soluble part was pre-purified by elution over RP-18 column (length 4 cm × id 4 cm) using MeOH–H_2O (60:40). Extracts were then concentrated and ready for HPLC.

4.2. Isolation

Analytical HPLC experiments (RP 18, 10 μm, length 250 mm, id 4.6 mm) were performed with a Perkin–Elmer Liquid Chromatograph (series 3B) connected to a DAD detector coupled to an HP 9000 series 300 computer. The chromatographic elution condition was a linear gradient MeOH–aq.HCOOH 50 mM from (20:80) to (60:40) within 40 min, then from (60:40) to (100:0) within 10 min. 10 μl of extract were injected at a 1.5 ml/min flow rate and detected at 254, 300 and 360 nm. The detector used for preparative HPLC (RP 18, 5 μm, length 250 mm, id 8 mm) was a Perkin–Elmer LC-75 Spectrophotometric Detector. Elution conditions: a non-linear gradient (curve 2) MeOH–aq.HCOOH 50 mM from (20:80) to (40:60) within 20 min, followed by a linear gradient from (40:60) to (60:40) for 20 min and finally isocratically at (60:40) for 10 min. 100 μl of extract were injected at a 2.5 ml/min flow rate and detected at 300 nm. Each compound was purified once again using the same conditions but without formic acid in order to avoid traces of acid. Isolated compounds were dried and stored at +4°. All the analysis of peaks **1**, **2**, **3** and **4** were carried out with compounds extracted from grapes collected during both periods (cf Fig. 2a and b) that proved to be identical.

4.3. Enzyme assays

Enzymatic degradation of pterostilbene was assayed by measuring the change in absorbance of the reaction mixture at 300 nm using a Shimadzu UV-160 spectrophotometer, with a kinetic program (lag period 10 s, absorbance recorded every 30 s over a 3 min period). The reaction mixture for the enzyme assay contained 2.940 ml of phosphate buffer (0.1 M, pH 5.2), with 30 μl of ethanolic stock solution of pterostilbene (final conc. 0.054 mM) and 30 μl of protein solution. In each case 2, 20, 60 and 100 μg of inhibitor were added in the 3 ml quartz cuvettes.

4.4. NMR and MS

^{1}H–NMR spectra (400 MHz), ^{13}C (100.6 MHz), COSY, DEPT, ^{1}H-^{13}C inverse correlation (125 Hz, 10 Hz and 5 Hz) were measured on a Bruker AMX400 with CD_3OD as solvent. Chemical shifts (δ) are given in ppm using TMS as reference. Mass spectra obtained by electronic impact (EIMS) and chemical ionization (CIMS) with ammonia (positive) were measured on a Delsi–Nermag R-30-10. Electrospray mass spectra (ESMS positive) were performed on a Hewlett Packard 59987A.

4.4.1. (*E*)-Caffeoyl tartaric acid (**1**)

UV: λ_{max} (MeOH + formic acid 50 mM) nm: 218, 235, 242, 297, 326. ^{1}H NMR (400 MHz, CD_3OD, 25°): δ 7.83 (1H, *d, J* = 15.9 Hz, H-7′), 7.18 (1H, *d, J* = 1.8 Hz, H-2′), 7.06 (1H, *dd, J* = 1.8 Hz and 8.17 Hz, H-6′), 6.88 (1 H, *d, J* = 8.17 Hz, H-5′), 6.39 (1H, *d, J* = 15.9 Hz, H-8′), 5.65 (1H, broad, H-2), 4.85 (1H, broad, H-3). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 174.4 (C-4), 171.0 (C-1), 168.3 (C-9′), 150.1 (C-4′), 148.5 (C-7′), 146.5 (C-3′), 128.0 (C-1′), 123.5 (C-6′), 116.4 (C-5′), 115.6 (C-2′), 114.4 (C-8′), 74.9 (C-2), 71.6 (C-3). ESMS (positive) *m/z*: 335 [M + Na^+], 351 [M + K^+].

4.4.2. (*Z*)-p-Coumaroyl tartaric acid (**2**)

UV: λ_{max} (MeOH + formic acid 50 mM) nm: 210, 226, 308. ^{1}H NMR (400 MHz, CD_3OD, 25°): δ 7.77 (2H, *d, J* = 8.0 Hz, H-2′, 6′), 6.99 (1H, *d, J* = 12.0 Hz, H-7′), 6.86 (2H, *d, J* = 8.2 Hz, H-3′, 5′), 5.95 (1H, *d, J* = 12.0 Hz, H-8′), 5.62 (1H, broad, H-2), 4.82 (1H, broad, H-3). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 174.6 (C-4), 171.2 (C-1), 167.2 (C-9′) 160.6 (C-7′), 146.9 (C-4′), 134.3 (C-1′), 127.7 (C-2′, 6′), 116.1 (C-3′, 5′), 115.6 (C-8′), 74.8 (C-2), 71.8 (C-3). ESMS (positive): *m/z* 319 [M + Na^+], 335 [M + K^+].

4.4.3. (*E*)-p-Coumaroyl tartaric acid (**3**)

UV: λ_{max} (MeOH + formic acid 50 mM) nm: 210, 226, 311. ^{1} H NMR (400 MHz, CD_3OD, 25°): δ 7.83 (1H, *d, J* = 15.9 Hz, H-7′), 7.57 (2H, *d, J* = 8.5 Hz, H-2′, 6′), 6.90 (2H, *d, J* = 8.5 Hz, H-3′, 5′), 6.47 (1H, *d, J* = 15.9 Hz, H-8′), 5.65 (1H, broad, H-2), 4.86 (1H, broad, H-3). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 174.0 (C-4), 170.7 (C-1), 169.3 (C-9′), 162.7 (C-7′),

149.0 (C-4′), 132.4 (C-1′), 128.2 (C-2′, 6′), 117.8 (C-3′, 5′), 115.1 (C-8′), 75.0 (C-2), 71.6 (C-3). ESMS (positive) *m/z*: 319 [M + Na^+], 335 [M + K^+].

*4.4.4. Catechin (**4**)*

^{1}H NMR (400 MHz, CD_3OD, 25°): δ 6.93 (1H, *d*, *J* = 1.6 Hz, C-2′H), 6.80–6.88 (2H, *m*, H-6′, H-5′), 6.03 (1H, *d*, *J* = 2.2 Hz, H-6), 5.96 (1H, *d*, *J* = 2.2 Hz, H-8), 4.66 (1H, *d*, *J* = 7.5 Hz, H-2), 4.07 (1H, *m*, H-3), 2.94 (1H, *dd*, *J* = 5.4 Hz, *J* = 16.1 Hz, H-4ax.), 2.60 (1H, *dd*, *J* = 8.1 Hz, *J* = 16.1 Hz, H-4eq.). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 158.1, 157.8, 157.2, 146.5, 146.5, 132.5, 120.3, 116.4, 115.5, 101.1, 96.6, 95.8, 83.1, 69.1, 28.7.

*4.4.5. (E)-p-Coumaric acid (**6**)*

UV: λ_{max} (MeOH + formic acid 50 mM) nm: 227, 310. ^{1}H NMR (400 MHz, CD_3OD, 25°): δ 7.69 (1H, *d*, *J* = 15.9 Hz, H-7), 7.54 (2H, *d*, *J* = 8.6 Hz, H-2, 6), 6.89 (2H, *d*, *J* = 8.6 Hz, H-3, 5), 6.37 (1H, *d*, *J* = 15.9 Hz, H-8). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 171.3 (C-9), 161.2 (C-7), 146.9 (C-4), 131.3 (C-1), 127.5 (C-2, 6), 117.1 (C-3, 5), 115.8 (C-8). CIMS (NH_3, positive) *m/z*: 182 [M + NH_4^+], 165 [M + H^+], 147 [(M−OH)$^+$].

4.4.6. Taxifolin-3-O-rhamnoside (7)

UV: λ_{max} (MeOH + formic acid 50 mM) nm: 230, 290. ^{1}H NMR (400 MHz, CD_3OD, 25°): δ 7.06 (1H, *d*, *J* = 1.81 Hz, H-2′), 6.92 (1H, *dd*, *J* = 8.2 Hz, *J* = 1.86 Hz, H-6′), 6.89 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.01 (1H, *d*, *J* = 2.2 Hz, H-6), 5.99 (1H, *d*, *J* = 2.2 Hz, H-8), 5.16 (1H, *d*, *J* = 10.7 Hz, H-2), 4.67 (1H, *d*, *J* = 10.7 Hz, H-3), 4.14 (1H, *d*, *J* = 1.27 Hz, H-1″), 4.34 (1H, *dq*, *J* = 9.6 Hz, *J* = 6.0 Hz, H-5″), 3.74 (1H, *dd*, *J* = 9.6 Hz, *J* = 3.2 Hz, H-4″), 3.62 (1H, *dd*, *J* = 3.0 Hz, *J* = 1.5 Hz, H-3″), 3.4 (under the MeOH, H-2″), 1.28 (3H, *d*, *J* = 6.14 Hz, 3H-6″). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 196.2 (C-4), 168.9 (C-9), 165.8 (C-5), 164.4 (C-7), 147.7 (C-3′), 146.9 (C-4′), 129.5 (C-1′), 120.6 (C-6′), 116.2 (C-5′), 115.5 (C-2), 102.8 (C-10), 102.5 (C-1″), 97.8 (C-6), 96.2 (C-8), 84.0 (C-2), 79.5 (C-3), 74.3 (C-2″), 72.5 (C-4″) 71.8 (C-3″), 71.1 (C-5″), 18.1 (C-6″). ESMS (positive) *m/z*: 473 [M + Na^+], 923 [2 M + Na^+].

*4.4.7. Quercetin-3-O-glucuronide (**8**)*

UV: λ_{max} (MeOH + formic acid 50 mμ) nm: 205, 256, 354. ^{1}H NMR (400 MHz, CD_3OD, 25°): δ 7.72 (1H, *d*, *J* = 8.4 Hz), 7.71 (1H, *s*), 6.94 (1H, *d*, *J* = 8.2 Hz), 6.47 (1H, *s*), 6.28 (1H, *s*), 5.4 (1H, *d*, *J* = 7.3 Hz), 3.85 (1H, *m*), 3.62 (3H, *m*), 3.43 (1H, under the MeOH). ^{13}C NMR (100 MHz, CD_3OD, 25°): δ 179.5, 166.3, 163.3, 159.3, 158.7, 150.2, 146.2, 135.7, 123.8, 123.1, 117.5, 116.3, 105.9, 104.6, 100.2, 95.1, 77.9, 75.6, 73.2. ESMS (positive) *m/z*: 479 [M + H^+], 501 [M + Na^+], 979 [2 M + Na^+].

*4.4.8. Tetrabenzyloxy-5,7,3′,4′-epicatechin (**10**)*

A mixture of epicatechin (**9**) (116 mg, 0.4 mmol), K_2CO_3 (414 mg, 3 mmol) and benzyl bromide (240 μl, 2 mmol) in DMF (5 ml) was stirred at room temperature for 18 h. Water was added and extracted with Et_2O. The organic layer was washed with aq. HCl 10%, NaCl satd and dried with $MgSO_4$. The solvent was evaporated under reduced pressure and the oily residue was purified by column chromatography on silica gel (hexane–EtOAc, 9:1) to afford the desired product **10** (240 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (20H, *m*, Ar–H), 7.16 (1H, *d*, H-2′), 6.99 (2H, *d*, H-5′, H-6′), 6.28 (2H, *d*, H-6, H-8), 5.20 (2H, s, CH_2), 5.18 (2H, *s*, CH_2), 5.03 (2H, *s*, CH_2), 5.02 (2H, *s*, CH_2), 4.92 (1H, *s*, H-5), 4.20 (1H, *m*, H-3), 2.98 (2H, *m*, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.4, 159.0, 155.9, 149.7, 149.5, 137.9, 137.8, 137.6, 137.6, 132.1, 129.2, 129.2, 129.1, 129.1, 129.1, 128.7, 128.6, 128.5, 128.5, 128.4, 128.4, 128.2, 128.2, 127.9, 127.8, 127.8, 127.6, 120.2, 115.7, 114.2, 101.7, 95.4, 94.7, 79.0, 72.0, 72.0, 70.8, 70.6, 67.0, 28.9.

*4.4.9. Methyl tribenzyloxy-3,4,5-benzoate (**11**)*

The reaction was performed in the same conditions as above. The compound was obtained as a white solid (74% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (15H, *m*, Ar–H), 7.27 (2H, *m*, Ar–H), 5.15 (4H, *s*, 2 CH_2), 5.12 (2H, *s*, CH_2), 3.90 (3H, *s*, CH_3). ^{13}C-NMR ($CDCl_3$), 167.3, 153.2, 143.1, 138.1, 137.3, 129.2, 129.2, 128.8, 128.7, 128.6, 128.2, 125.9, 109.8, 75.8, 71.9, 52.9.

*4.4.10. Tribenzyloxy-3,4,5-benzoic acid (**12**)*

Methyl tribenzyloxy-3, 4, 5-benzoate **11** (2 g, 4.4 mmol), KOH (3.15 g, 40 mmol) in dioxan (50 ml) and MeOH (50 ml) were heated at reflux for 18 h. The solvent was removed under reduced pressure; the residue was diluted in water and extracted with EtOAc. The organic layer was washed with aq. HCl 10%, NaCl satd and dried with $MgSO_4$. The solvent was evaporated under reduced pressure to obtain the desired compound **12** as a white solid (1.7 g, 89%).^{1}H NMR (400 MHz, $CDCl_3$): δ 7.44 (15H, *m*, Ar–H), 7.27 (2H, *m*, Ar–H), 5.17 (6H, *m*, 3 CH_2).

*4.4.11. Tribenzyloxy-3,4,5-benzoate-3-tetrabenzyloxy-5,7,3′,4′-epicatechin (**13**)*

The acid **12** (63 mg, 0.13 mmol) and DCC (36 mg, 0.17 mmol) were dissolved in dry toluene (1 ml) under Argon and stirred for 5 min. The alcohol **10** (32 mg, 0.05 mmol) and DMAP (cat.) were added and the mixture was heated at 72° for 3 days. The solvent was evaporated under reduced pressure and the residue

was diluted with water and extracted with EtOAc. The organic layer was washed with aq. NaOH 1N, HCl 10%, NaCl satd and dried with $MgSO_4$. The solvent was evaporated under reduced pressure; the residue was purified by column chromatography on silica gel (hexane–EtOAc, 95:5) to afford the ester **13** as a white solid (48 mg, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (32H, *m*, Ar–H), 7.04 (1H, *d*, H-2′), 6.85 (2H, *m*, H-5′, H-6′), 6.35 (2H, 2 *d*, H-6, H-8), 5.62 (1H, *m*, H-3), 5.05 (12H, *m*, $6CH_2$), 4.97 (1H, *d*, H-2), 4.74 (2H, 2 *d*, CH_2), 3.10 (2H, m, H-4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.6, 159.5, 158.7, 156.4, 153.0, 153.0, 149.6, 149.6, 143.2, 138.1, 137.8, 137.7, 137.5, 137.5, 137.2, 131.7, 129.3, 129.2, 129.2, 129.1, 129.0, 129.0, 128.8, 128.8, 128.7, 128.6, 128.6, 128.5, 128.3, 128.1, 128.0, 128.0, 127.8, 125.6, 120.7, 115.4, 114.3, 109.7, 101.6, 95.3, 94.6, 78.3, 77.9, 75.7, 71.8, 71.8, 71.8, 71.7, 70.8, 70.6, 69.2, 26.8.

*4.4.12. Epicatechin-3-O-gallate (**5**)*

A suspension of tribenzyloxy-3,4,5-benzoate-3-tetrabenzyloxy-5,7,3′,4′-epicatechin **13** (2.1 mg, 0.002 mmol) and 10% Pd–charcoal in EtOAc (2 ml) were stirred under pressure of H_2 (40 bar) for 5 h. The catalyst was filtered off, washed with MeOH and the solvent was evaporated under reduced pressure. The residue was purified by prep. TLC (silica gel) to afford the desired compound **5**. ^{1}H NMR (400 MHz, CD_3OD): δ 7.04 (2H, *s*, H-2″, H-6″), 7.02 (1H, *d*, $J = 2$ Hz, H-2′), 6.91 (1H, *dd*, $J = 8.5$ Hz, $J = 2$ Hz, H-6′), 6.78 (1H, *d*, $J = 8$ Hz, H-5′), 6.05 (2H, 2 *d*, $J = 2.5$ Hz, H-6, H-8), 5.61 (1H, *m*, H-3), 5.12 (1H, *s*, H-2), 3.08 (1H, *dd*, $J = 4.7$ Hz, $J = 17$ Hz, H-4ax.), 2.93 (1H, *dd*, $J = 2.4$ Hz, $J = 17$ Hz, H-4eq.). ^{13}C NMR (100 MHz, CD_3OD): δ 167.9 (CO), 158.2, 157.6 (C-5,7,9), 146.6 (C-3″,5″), 146.3 (C-3′), 146.2 (C-4′), 140.1 (C-6′), 131.7 (C-1′), 121.8 (C-1″), 119.7 (C-6′), 116.3 (C-5′), 115.4 (C-2′), 110.5 (C-2″,6″), 99.7 (C-10), 96.8 (C-6), 96.2 (C-8), 78.9 (C-2), 70.3 (C-3), 27.2 (C-4). EIMS *m*/*z*: 442 [M^+], 273 [M-gallate$^+$], 170 [gallate$^+$]

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