Vitis vinifera Canes, a New Source of Antifungal Compounds against Plasmopara viticola, Erysiphe necator, and Botrytis cinerea

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Ⓢ Supporting Information

ABSTRACT: Methanolic and ethanolic crude extracts of *Vitis vinifera* canes exhibited significant antifungal activity against the three major fungal pathogens affecting grapevines, *Plasmopara viticola*, *Erysiphe necator* and *Botrytis cinerea*. The active extracts were analyzed by LC-PDA−ESI-MS, and selected compounds were identified. Efficient targeted isolation using medium-pressure liquid chromatography afforded six pure constituents in one step. The structures of the isolated compounds were elucidated by NMR and HRMS. Six identified compounds (ampelopsin A, hopeaphenol, *trans*-resveratrol, ampelopsin H, ε-viniferin, and *E*-vitisin B) presented antifungal activities against *P. viticola*. ε-Viniferin also exhibited a low antifungal activity against *B. cinerea*. None of the identified compounds inhibited the germination of *E. necator*. The potential to develop a novel natural fungicide against the three major fungal pathogens affecting *V. vinifera* from viticulture waste material is discussed.

KEYWORDS: *Vitis vinifera*, wood extract, LC-PDA−ESI-MS, antifungal activity, ampelopsin A, hopeaphenol, *trans*-resveratrol, ampelopsin H, ε-viniferin, E-vitisin B

■ INTRODUCTION

Worldwide, the vast majority of grapevine areas are planted with *Vitis vinifera* cultivars that are all sensitive to various fungal diseases, such as downy (*Plasmopara viticola*) and powdery (*Erysiphe necator*) mildews and gray mold (*Botrytis cinerea*). For this reason, numerous fungicide applications are required to ensure the production of high-quality wines. In Switzerland, between 6 and 12 fungicidal sprays are typically applied yearly depending on the disease pressure and the climatic conditions of the year. Even if used in accordance with good agronomical practices, these plant protection products have the potential to negatively impact both human health and the environment and can lead to the presence of residues on the grapes and in the wine.[1−3] Public concerns about these negative effects and especially the possible presence of residues have increased in recent years. Governments had to react, and different national action plans were developed to reduce both the use and the impact of plant protection products on the environment and health. In 2009, the reevaluation of all of the active ingredients authorized in the European Union led to a drastic reduction of the number of authorized plant protection products. Furthermore, most active ingredients are sensitive to the selection of resistant pathogen strains, reducing their efficacy in the field.[4] All of these reasons explain why the search for new active ingredients or alternative products with improved environmental and toxicological profiles is of paramount importance for sustainable viticulture in the future.

The exploitation of natural products of plant origin might be an alternative, as plant extracts have been shown to contain a wide variety of antifungal compounds.[5−7] Plant extracts are promising because of their antimicrobial activity, biodegrad-ability, and reduced toxicity to health and the environment. Many plant species remain to be screened for antimicrobial compounds, offering an interesting perspective for discovering new molecules. Molecules that can trigger the plant defense mechanisms, known as elicitors, have also been investigated, particularly in grapes, such as specific anthraquinones,[8] β-aminobutyric acid,[9] PS3 (sulfated laminarin),[10] botrycin and cinerein,[11] chitosan, and fosetyl-aluminum. However, this approach has only led to the generation of a few commercial products, such as Aliette (Bayer CropScience, Germany). Unfortunately, to date, the efficacies of alternative natural products as both antimicrobials and elicitors are significantly lower compared to reference synthetic molecules. This lack of efficacy is the main reason for the very limited number of commercial products derived from plants, for example, Semafort (Tribo Technologies, Soultz sous Forêts, France), containing among others extracts of *Ascophyllum nodosum*.

Grapes contain a wide variety of phenolic compounds, including phenolic acids, tannins, flavonoids, and stilbe-noids.[12−14] Among them, the health benefits of resveratrol have been well described and are known as the "French paradox".[15,16] The strong interest in resveratrol has resulted in numerous studies aimed to quantitate its levels in wine and grapes worldwide.[17] Grape-derived phenolic compounds have been shown to play a beneficial role in disorders including cardiovascular disease,[18] neurodegenerative disease and

aging,[19,20] and cancer.[21] A broader range of stilbenic phytoalexins are synthesized by *V. vinifera* in response to different stresses.[22−24] These stilbenes can be constitutively found in the grape canes and all woody parts.[25] Canes are traditionally eliminated by annual pruning from the vineyard, either deposited on the soil or burned. Nevertheless, this waste material constitutes a potential source of high value compounds.

In the present study, we investigated the antifungal activities of *V. vinifera* cane extracts of various polarity as well as a series of pure antifungal compounds isolated from the methanolic extract of *V. vinifera* grapevine canes that are active against *Plasmopara viticola*, *Erysiphe necator*, and *Botrytis cinerea*. The opportunity to obtain a novel natural fungicide against these three major pathogens affecting *V. vinifera* from the products of viticulture is discussed.

■ MATERIALS AND METHODS

General Experimental Procedures. NMR spectroscopic data were recorded on a 500 MHz Varian Inova spectrometer. Chemical shifts are reported in parts per million (δ) using the residual solvent signals (CD$_3$OD: δ_H 3.31, δ_C 49.0) (acetone-d_6; δ_H 2.05, δ_C 206.7) as the internal standards for ^{1}H and ^{13}C NMR and coupling constants (J) in Hz. The complete assignment was performed on the basis of 2D experiments (COSY, TOCSY, NOESY, edited-HSQC, and HMBC). The HRESI-MS data were obtained on a Micromass-LCT Premier time-of-flight mass spectrometer from Waters with an electrospray (ESI) interface. Analytical HPLC was carried out on a HP 1100 system equipped with a photodiode array detector. MPLC separation was performed using a Shimadzu LC10AD pump equipped with a Knauer UV detector and an MPLC glass column (460 × 70 mm i.d.) loaded with ZEOprep C18 as the stationary phase (15−25 μm, Zeochem AG, Uetikon am See, Switzerland). Melting points were determined using a Büchi melting point B-540 (Büchi, Flawil, Switzerland).

Plant Material. Grape canes of *Vitis vinifera* L. cultivars Pinot noir, Gamaret (Gamay × Reichensteiner), and Divico (Gamaret × Bronner, synonym IRAC 2091) were collected from the experimental untreated plots of Agroscope ACW (Nyon, Switzerland) in January 2011 and stored dry under a constant temperature (20 °C) in the dark for one month. Canes were cut into 2 cm pieces and dried at 30 °C for 72 h in an oven. The dried cane sections were ground in a hammer mill with <1 mm diameter mesh (Culatti AG, Zurich, Switzerland) and stored as a powder in sealed plastic boxes at 20 °C in the dark until use.

Extraction. Four extraction methods were carried out on 20% w/v Pinot noir cane powder, typically composed of 20 g plant powder in 100 mL solvent: (1) aqueous extraction in nanopure water by stirring for 3 h at room temperature (AE); (2) aqueous extraction at a temperature from 80 to 100 °C in reflux for 10 min (AER); (3) methanolic extraction by stirring for 3 h at room temperature (ME); and (4) ethanolic extraction by stirring for 3 h at room temperature (EE). Only steps 3 and 4 were applied to Divico and Gamaret cane powder. These initial extracts were then centrifuged (4,000 rpm, 10 min, 20 °C). The supernatants were filtered under vacuum through 9 cm diameter 589/3 cellulose filters (Schleicher & Schuell AG, Dassel, Germany). The filtrates were then freeze-dried in lyophilizator and weighed. The ethanolic and methanolic extracts were dried under vacuum with a rotary evaporator, and the resulting residues were solubilized in 100 mL of nanopure water using an ultrasonic bath before lyophilization. All of the resulting extracts were stored at room temperature in the dark. The extracts from the Pinot noir cultivar (20 g) afforded the following yields: AE (3.19 g) 15.95%, AER (2.09 g) 10.48%, EE (0.66 g) 3.33%, and ME (1.39 g) 6.95%. From the Divico and Gamaret cultivars (20 g each), the ME afforded 8.2% (1.64 g) and 5.4% (1.08 g) respectively.

SPE Fractionation. The fractionation by solid phase extraction (SPE) was performed using a 100 × 20 mm i.d., 5 μm, C-18 cartridge column (Waters, Milford, MA, USA). One hundred milligrams of extract was deposited in the cartridge. The solvent system used was a

mixture of H$_2$O (A) and MeOH (B) in a four step gradient: 80:20 (fraction 1), 60:40 (fraction 2), 40:60 (fraction 3), and 0:100 (fraction 4). The fractions were collected every 20 mL. After collection, each fraction was evaporated to dryness using N$_2$. The SPE separation yielded 4 fractions, F1 (60 mg), F2 (14.33 mg), F3 (7.86 mg), and F4 (8.45 mg). All fractions were analyzed by HPLC-PDA using the same conditions used for the analysis of the crude plant extracts. All fractions were used in the antifungal assays at 1 mg/mL.

LC-PDA−MS Analysis. LC-PDA−MS data were obtained with an Agilent 1100 series system consisting of an auto sampler, high-pressure mixing pump, and PAD detector connected to a Finnigan MAT LCQ ion trap mass spectrometer equipped with a Finnigan electrospray interface (ESI). The HPLC conditions were as follows: a column 250 × 4.6 mm i.d., 5 μm, X-Bridge C-18 (Waters, Milford, MA, USA); solvent system, (A) H$_2$O containing 0.002% formic acid, (B) MeOH with 0.002% formic acid; gradient mode, 10 to 60% of B in 50 min, 60 to 100% of B in 10 min, and 100% B during 10 min; flow rate, 1 mL/ min; injection volume, 20 μL; and sample concentration, 5 mg/mL in H$_2$O. The UV detection was at 217 and 254 nm, and the UV spectra (PDA) were recorded between 190 and 600 nm in 2 nm steps. The ESI-MS conditions were as follows: capillary voltage, 30 V; capillary temperature, 200 °C; source voltage, 4.5 kV; source current, 80 μA; nitrogen as the sheath gas flow; and positive and negative ion mode. The spectra (m/z 150−1600) were recorded every 3 s.

UPLC−TOF-HRMS analysis. The LC−MS metabolite profiling was performed on a Micromass-LCT Premier time of flight (TOF) mass spectrometer with an electrospray interface and coupled with an Acquity UPLC system. The ESI conditions were as follows: capillary voltage, 2800 V; cone voltage, 40 V; microchannel plate (MCP) detector voltage, 2400 V; source temperature, 150 °C; desolvation temperature, 300 °C; cone gas flow, 20 L/h; and desolvation gas flow, 800 L/h. The detection was performed in positive ion mode (PI) in the range m/z 100−1000 and a scan time of 0.25 s in W-mode. The MS was calibrated using sodium formate, and leucine enkephalin was used as an internal reference at 2 μg/mL and infused through the Lock Spray probe at a flow rate of 10 μL/min with the assistance of a second LC pump. The separation was carried out on a 50 mm × 1.0 mm i.d., 1.7 μm, Waters Acquity BEH C-18 UPLC column (Waters, Milford, MA, USA). The separation was performed using a flow rate of 0.3 mL/ min with the following solvent system: (A) 0.1% formic acid−water, (B) 0.1% formic acid−acetonitrile, with a gradient of 5−95% B over 4.0 min. The temperature was set at 40 °C. The injected volume was kept constant (1 μL).

Isolation. The methanol extract of *Vitis vinifera* cv. Pinot noir (10 g) was first fractionated using MPLC with a 460 × 70 mm i.d., 15−25 μm, ZEOprep C-18 (Zeochem AG, Uetikon am See, Switzerland) as the stationary phase with MeOH and H$_2$O containing 0.002% formic acid in a linear gradient mode from 5% to 100% MeOH (steps 2.5%). The flow rate was 4.5 mL/min, and the UV detection was at 220 nm. The MPLC separation yielded 89 fractions. All fractions were analyzed by HPLC-PDA. Fraction 3 yielded sucrose (224.2 mg), fraction 24−25 ampelopsin A (**1**) (250.4 mg), fraction 30−31 hopeaphenol (**2**) (148.1 mg), fraction 35−36 *trans*-resveratrol (**3**) (110.5 mg), fraction 43 ampelopsin H (**4**) (15.3 mg), fraction 48−50 ε-viniferin (**5**) (324.3 mg), and fraction 58−59 *E*-vitisin B (**6**) (56.2 mg).

Ampelopsin A (1). Amorphous brown powder: LC−ESI-MS m/z 469.02. [M − H]$^-$; HRESI-MS m/z 471.1439 [M + H]$^+$ (calculated for C$_{28}$H$_{23}$O$_7$, 471.1444).

Hopeaphenol (2). Amorphous brown powder: LC−ESI-MS m/z 905.18 [M − H]$^-$; HRESI-MS m/z 907.2776 [M + H]$^+$ (calculated for C$_{56}$H$_{43}$O$_{12}$, 907.2755).

trans-Resveratrol (3). Amorphous white powder: mp 255.2−255.8 °C; LC−ESI-MS m/z 227.06 [M − H]$^-$; HRESI-MS m/z 229.0859 [M + H]$^+$ (calculated for C$_{14}$H$_{13}$O$_3$, 229.0865).

Ampelopsin H (4). Amorphous brown powder: LC−ESI-MS m/z 905.13 [M − H]$^-$; HRESI-MS m/z 907.2768 [M + H]$^+$ (calculated for C$_{56}$H$_{43}$O$_{12}$, 907.2755).

ε-Viniferin (5). Amorphous brown powder: LC−ESI-MS m/z 453.18 [M − H]$^-$; HRESI-MS m/z 455.1502 [M + H]$^+$ (calculated for C$_{28}$H$_{23}$O$_6$, 455.1495).

E-Vitisin B (6). Amorphous brown powder: LC−ESI-MS *m/z* 905.15 [M − H]⁻; HRESI-MS *m/z* 907.2753 [M + H]⁺ (calculated for $C_{56}H_{43}O_{12}$, 907.2755).

Biological Assays. *Antifungal Assay against Plasmopara viticola.* *Plasmopara viticola* (Berk & Curt.) Berl. & de Toni sporangia were collected from sporulating lesions of artificially infected leaves of *Vitis vinifera* cv. Chasselas by vacuum aspiration using a filtered tip. A sporangia suspension, adjusted to 2 × 10⁵ cells/mL, was generated in a 15 mL Falcon tube containing sterile nanopure water at 4 °C and gently stirred at room temperature. As soon as the zoospores were released (5 × 10⁴ mobile zoospores/mL), this suspension was added to the target compounds (concentrations 1−10−100−1,000−10,000 μM) solubilized in 2% ethanolic aqueous solution, and the suspension was stirred in a VorTemp tube shaker at 20 °C and 60 rpm for 30 min (Labnet International, Woodbridge, NJ, USA). Controls were treated in the same way with water alone, with 2% (v/v) aqueous ethanolic solution, or with a commercial fungicide (Melody Combi, 9% iprovalicarb + 56% folpet, Bayer) used at 2 mg/mL. The number of mobile zoospores was counted in a volume of 0.3 mm³ under a light microscope at ×100 magnification in a fixed field of observation. In parallel, twenty 15 μL droplets of the resulting zoospore suspension were deposited on the abaxial leaf surface of *V. vinifera* cv. Chasselas (fifth and sixth fully expanded leaf of cuttings). The grafted *Vitis vinifera* L. cultivar Chasselas plants were cultivated in the greenhouse.[26] Three leaves for each treatment were incubated in a humidified chamber placed in phytotrons (80% relative humidity, constant temperature of 23 °C, under alternating 16 h day and 8 h night cycles). A negative control in each humidified chamber was prepared using an aqueous suspension of mobile zoospores. The number of sporulating spots was assessed 7 days postinoculation (dpi). All experiments were performed in triplicate. The IC₅₀ of each tested compound was calculated from a sigmoidal dose−response curve (variable slope) according to the following formula:

$$y = \min + (\max - \min)/(1 + 10^{(\log IC_{50}-x)\text{Hillslope}})$$

where *y* represents either the number of mobile zoospores or the disease development and *x* represents the logarithm of the tested concentration, using Sigmaplot Regression Wizard module (Systat Software Inc., Chicago, IL, USA).

Antifungal Assay against Erysiphe necator. Target compounds were solubilized in a 3.5% (v/v) aqueous ethanolic solution, and aqueous solutions of the pure compounds were prepared to obtain final concentrations of 1−10−100−1,000−10,000 μM. Controls were generated in the same way using water, a 3.5% (v/v) aqueous ethanolic solution, or the commercial fungicide Thiovit (wettable sulfur, Syngenta) at 4 mg/mL. A solution of Oxoid No. 3 technical agar (1.5% w/v) was added to the aqueous ethanolic solution, vortexed (30 s), and rapidly spread out on sterile glass slides equipped with frame-seal 300 μL incubation chambers (Bio-Rad, Hercules, CA, USA). The slides were randomly placed in Petri dishes (245 × 245 × 25 mm) and inoculated by blowing conidia from the sporulating leaves of *V. vinifera* cv. Chasselas 14 days after artificial infection. The Petri dishes were sealed and incubated in phytotrons (80% relative humidity, constant temperature of 23 °C, under 16 h day and 8 h night cycles). The conidia were observed under a light microscope at ×100 magnification, 48 h postinoculation (hpi), and the germination rate (ratio of germinated conidia out of 100 observed conidia) was calculated.

Antifungal Assay against Botrytis cinerea. A nutrient agar medium (PDA, potato dextrose broth, Difco) and a nutrient liquid medium (PDB, potato dextrose broth, Difco) were prepared for fungal development. Pure compounds were prepared to obtain final concentrations of 1−10−100−1,000−10,000−50,000 μM. The target compounds were solubilized in a 3.5% (v/v) aqueous ethanolic solution. Controls were generated in the same way using water, a 3.5% (v/v) aqueous ethanolic solution, or the commercial fungicide Switch (Cyprodinil 37.5% + Fludioxonil 25%, Syngenta) at 1 mg/mL. The ethanolic solutions were added to PDB, vortexed (30 s), and distributed into sterile 96-well microplates (Greiner, Frickenhausen, Germany). PDA maintained at 45 °C was added to each well (30% v/v). Conidia of *B. cinerea* were collected by vacuum aspiration from 10-day-old sporulating colonies using a filter tip and suspended in a sterile 50 mL Falcon tube containing nanopure water. The concentration was adjusted to 2 × 10⁶ conidia/mL. Ten microliters of this conidial suspension was added to each well. The plates were incubated in a growth chamber (60% relative humidity, constant temperature of 21 °C, under alternating 16 h day and 8 h night cycles). The mycelial development was observed a 5 dpi.

Elicitation Assays. To determine whether the crude cane extracts could elicit grapevine defense mechanisms, 20 μL droplets of 0.45 μm diameter filtered (Nalgene, Rochester, NY, USA) aqueous solutions at 1 mg/mL of each extract were applied onto half of the abaxial surface of the fifth detached leaf of Chasselas. Water droplets were applied onto the other half of the same leaf as a negative control. At 24 h post-treatment, three pieces of leaf corresponding to the droplet surface were cut from each treated leaf and stored at −20 °C until use. The enhancement of resistance to downy mildew on detached leaves using these plant extracts was tested by the application of droplets (20 μL) of the eliciting solution and of water (control) on the abaxial leaf surface. These droplets were then removed after 24 h using soft paper and were replaced by an aqueous suspension of 10⁵ mobile zoospores/mL, and the leaves were then placed in humid chambers. At 72 h postinoculation (hpi), three pieces of leaf corresponding to the droplet surface were cut from each inoculated leaf and stored at −20 °C until use. The elicitation was observed by HPLC quantitation of the levels of piceid, resveratrol, viniferins, and pterostilbene in the leaf extracts by LC-PDA according to Pezet et al.[22] The disease development was observed 7 dpi.

Electron Microscopy. Mobile zoospores of *P. viticola* or conidia of *B. cinerea* in aqueous suspensions were used to observe the cytotoxic effect of the methanolic cane extract. The methanolic dry extract (see above) was added to the *P. viticola* suspension at 1 mg/mL and gently stirred at room temperature for 6 and 24 h. The samples were centrifuged, and the resulting pellet was prepared according to Roland and Vian,[27] prefixed with a solution of 3% glutaraldehyde−2% paraformaldehyde in 0.07 M pH 7 phosphate buffer, embedded in 2% agarose, and postfixed with a solution of 1% OsO₄. They were then dehydrated in a graded series of ethanol solutions of 30−50−70−95−100% (v/v) and embedded in LR White resin (London Resin Company, West Chester, USA). After polymerization (24 h at 60 °C), thin (0.08 μm) sections were cut and stained with 2% uranyl acetate followed by lead citrate, according to Reynolds.[28] Thin sections were observed with a transmission electron microscope CM10 (Philips, Hillsboro, OR, USA) with a Mega View II camera. Controls were generated in the same way without the methanolic extract or using Melody Combi (9% iprovalicarb and 56% folpet, Bayer) against *P. viticola* or Switch (37.5% Cyprodinil and 25% Fludioxonil, Syngenta) against *B. cinerea.*

■ RESULTS AND DISCUSSION

Bioactivity of Cane Extracts. Berry fruits are known to be a rich source of antioxidants.[29] In particular, grapes contain a wide variety of phenolic compounds, including phenolic acids, tannins, flavonoids, and stilbenes, which are often linked to defense responses against fungal pathogens. Among these phenolic compounds, the health benefits of resveratrol have been well-known for centuries in Japanese and Chinese traditional medicine.[30] However, although stilbenes have been well studied with regard to health, little is known about their potential use as antifungal compounds.[31] *V. vinifera* canes represent an unexploited agronomical waste material. Their biological activity against the major grapevine pathogens and their chemical contents were investigated to determine whether extracts or products could be potentially used in a sustainable manner to protect grapevines. To this end, the canes from *Vitis vinifera* cv. Pinot noir, a widespread cultivar in Switzerland and in France, were selected as the starting material for our research. The choice of solvent extraction has focused on the

Table 1. Fungitoxic Activity of *Vitis vinifera* cv. Pinot noir, cv. Gamaret, and Divico Cane Extracts[a]

| | Pinot Noir | | | | Divico | Gamaret | | |
	AE[a]	AER	ME	EE	ME	ME	positive control	negative control
P. viticola							Melody Combi[b]	water
zoospore mobility	−[c]	−	+[d]	+	+	+	+	−
sporulation	−	−	+	+	+	+	+	−
E. necator							Thiovit[e]	water
conidia germination	−	−	+	+	+	+	+	−
B. cinerea							Switch[f]	PDA[g]
mycelium growth	−	−	+	+	+	+	+	−

[a]AE = aqueous extract, AER = aqueous extract obtained under reflux, ME = methanolic extract; EE = ethanolic extraction. The extracts were tested at the concentration of 1 mg/mL against *Plasmopara viticola* and *Erysiphe necator* and 5 mg/mL against *Botrytis cinerea*. [b]Tested at 2 mg/mL. [c]No fungitoxicity. [d]Fungitoxicity. [e]Tested at 4 mg/mL. [f]Tested at 1 mg/mL. [g]Potato dextrose agar.
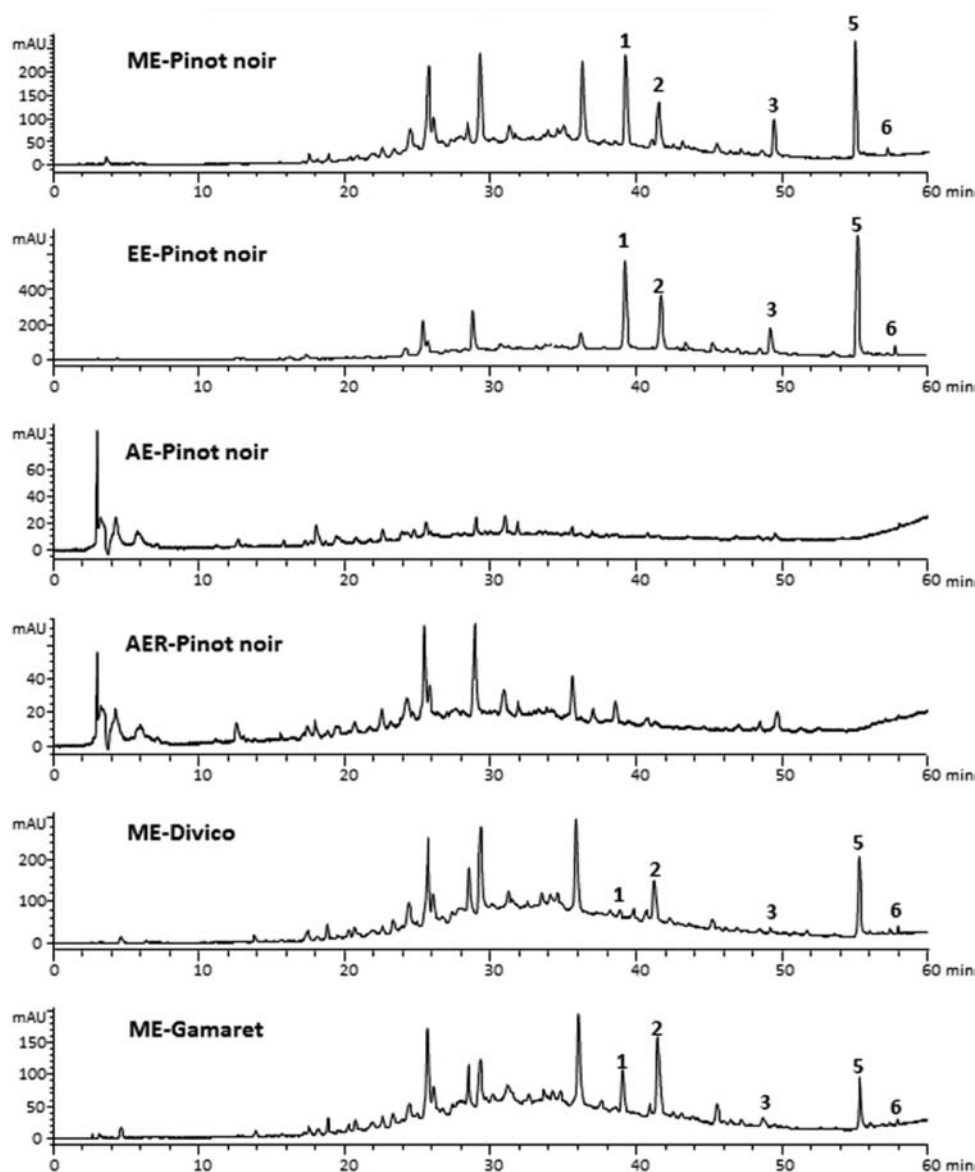

Figure 1. HPLC-PAD chromatograms at 217 nm of the methanolic, ethanolic and aqueous extracts of *V. vinifera* cv. Pinot noir canes and the methanolic cane extracts of *V. vinifera* cv. Divico and Gamaret. The following isolated compounds are highlighted: ampelopsin A (**1**), hopeaphenol (**2**), *trans*-resveratrol (**3**), ε-viniferin (**5**), and *E*-vitisin B (**6**).

desire to work with hydrophilic compounds, which are easier to use for vineyard protection. The Pinot noir canes were extracted using four different methods, affording four different extracts: aqueous (AE), aqueous by reflux (AER), methanolic

(ME), and ethanolic (EE). The different extracts were screened for their antifungal activities against the phytopathogens *Plasmopara viticola*, *Erysiphe necator*, and *Botrytis cinerea*. Among these extracts, the methanolic and ethanolic extracts
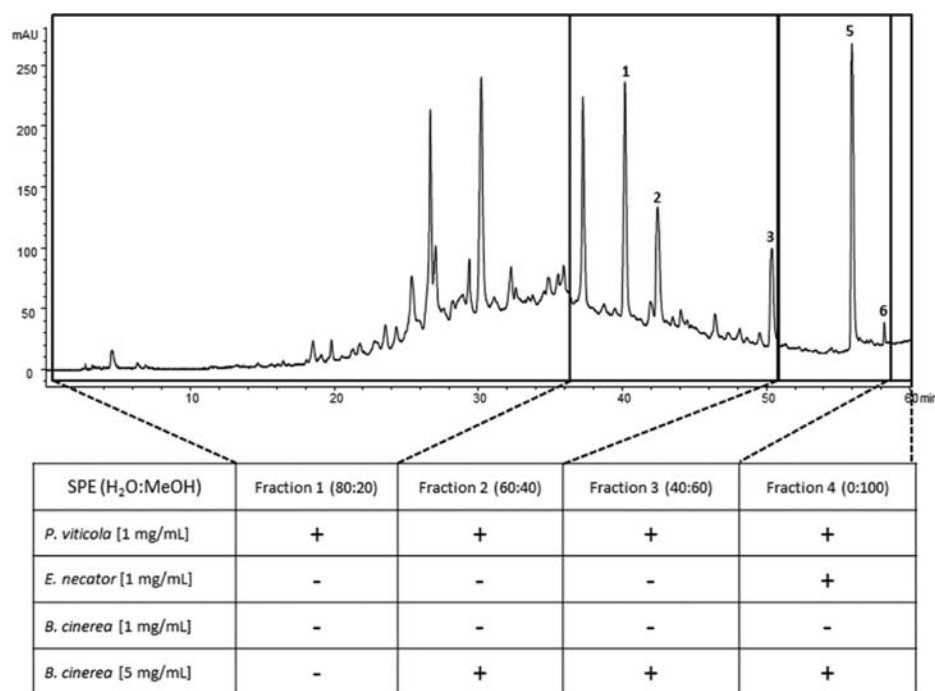
SPE (H₂O:MeOH)	Fraction 1 (80:20)	Fraction 2 (60:40)	Fraction 3 (40:60)	Fraction 4 (0:100)
P. viticola [1 mg/mL]	+	+	+	+
E. necator [1 mg/mL]	-	-	-	+
B. cinerea [1 mg/mL]	-	-	-	-
B. cinerea [5 mg/mL]	-	+	+	+

Figure 2. HPLC-PDA chromatogram at 217 nm of the methanolic extracts of *V. vinifera* cv. Pinot noir canes. The fractions obtained by SPE (F1–F4) are highlighted in the chromatogram after the HPLC control. The antifungal activity of each fraction was evaluated against *Plasmopara viticola* and *Erysiphe necator* (concentration of 1 mg/mL) and *Botrytis cinerea* (concentrations of 1 and 5 mg/mL).

presented antifungal activities against the three selected pathogens, while the aqueous extracts were inactive (Table 1).

In addition, the potential to induce "elicitation", or grapevine chemical defenses, against *P. viticola* was also investigated. Elicitation is indeed an alternative strategy for the control of phytopathogenic organisms by raising the basal defenses of the host during fungal infection.[32] None of the extracts significantly induced the synthesis of stilbenic phytoalexins. However, the methanolic extract was found to induce very small amounts of viniferins upon deposition on *Vitis* leaves, but the concentration was clearly below the IC$_{50}$ measured for these compounds.[33]

To investigate whether the antifungal activity was only found in the canes of the Pinot noir cultivars or was also present in other members of the *Vitis* species, two cultivars with different characteristics were selected: Gamaret, known for a high level of resistance to *B. cinerea*,[34,35] and Divico, known for its high resistance to *P. viticola*.[36] The extracts of these additional resistant cultivars displayed a similar antifungal activity against the three pathogens (Table 1). The comparison of the HPLC profiles of the three cultivars presented only slight differences in composition. This was in agreement with their similar biological activity profiles (Figure 1). This finding might indicate that most of the compounds found in *Vitis* canes are constitutive and present rather stable bioactivities and chemical profiles. It has been previously demonstrated that different active polyphenolic compounds can be induced upon fungal infection in plants that belong to the Vitaceae family.[37,38] These defense mechanisms are related to the susceptibility of each species.[36] However, some of these compounds are constitutively present in the woody parts of grapevine, even in susceptible *V. vinifera* cultivars.[39] Despite the difference in the agronomical behavior of the three cultivars analyzed against fungal pathogens, all extracts exhibited similar antifungal activity but differed slightly in their HPLC profiles. The biological activity did not appear to be related to the cultivar.

The woody parts of any *V. vinifera* cultivar could be used as a source of the active compounds. However, resistant hybrids, such as Divico, may be a better source to obtain high yields of fungitoxic compounds of interest.

Chemical Characterization of Cane Extract. Because no significant difference among the active extracts (ethanolic and methanolic) was observed, the methanolic extract of Pinot noir was chosen for further in depth chemical and biological investigation, as this type of extract exhibited a higher extraction yield. However, in view of the development of a commercial product, the ethanolic extract may be chosen for the industrial scaling up of the extraction process because of its more convenient use. The methanolic extract was analyzed by LC-PDA−ESI-MS for the first dereplication step (Figure 1). The UV-PDA spectra of the constituents revealed the presence of different phenolic compounds with the characteristic UV spectra of stilbenoids.[40] The MS data suggested the presence of simple and oligomeric stilbenoids. For example, the molecular weight of **3** (m/z 227.2 [M − H]$^-$) and its UV spectrum indicated the possible presence of resveratrol.[2] The spectra were compared with a standard of *trans*-resveratrol previously isolated from *V. vinifera* and were unambiguously identified by this means.[26,41] Attempts have been made to crystallize the isolated compounds, and only *trans*-resveratrol (**3**) was obtained in the crystal form. The melting point of *trans*-resveratrol obtained was 255.2−255.8 °C, in good agreement with the literature.[21] Using the same approach, peak **5** was identified as *ε*-viniferin.[42] For the other compounds, the online UV and MS data were insufficient for efficient dereplication. The purity of compounds **1**, **2**, **5**, and **6** has been estimated to be >95% based on HPLC−UV and ^{1}H NMR analysis. The purity of compound **4**, isolated as a minor constituent, was found less pure, but it also displayed not a very significant activity.
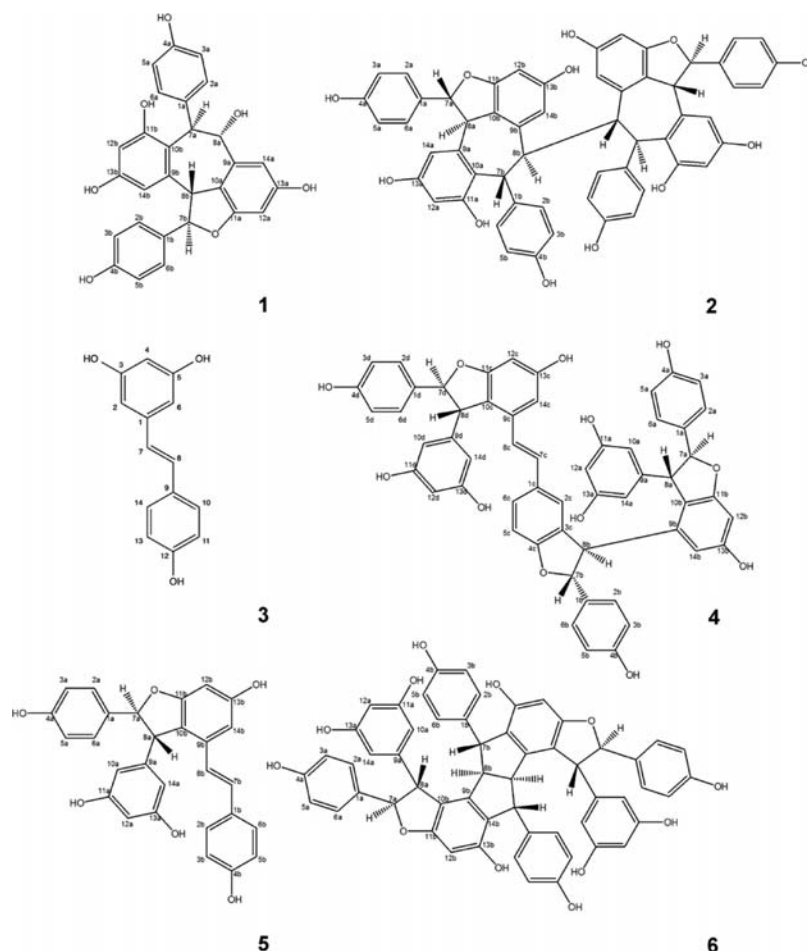
Figure 3. Compounds isolated from the methanolic extracts of *V. vinifera* cv. Pinot noir canes.

To rapidly localize the compounds responsible for the antifungal activity and to evaluate whether the bioactive compounds could be separated in reversed-phase conditions, the methanolic extract was fractionated by solid phase extraction (SPE) using a reversed-phase cartridge. The four SPE fractions obtained (F1 to F4) were analyzed by HPLC and tested for their antifungal activity against *P. viticola, E. necator,* and *B. cinerea.* All fractions presented an activity at 1 mg/mL against *P. viticola* (Figure 2). No activity against *B. cinerea* was found at 1 mg/mL, but fractions F2 to F4 were active at 5 mg/mL. Activity against *E. necator* was found only in fraction F4.

For the efficient isolation of the active compounds in large amounts, a transfer of the analytical HPLC conditions to medium pressure liquid chromatography (MPLC) was performed. The separation was made on 10 g of crude methanolic extract of the grapevine *V. vinifera* cv. Pinot noir. The MPLC separation resulted in the isolation of six pure compounds in one step (1−6). The structural elucidation of the isolated compounds was performed based on the NMR and HRMS analyses. The compounds were identified as ampelopsin A (1),[42] hopeaphenol (2),[43] *trans*-resveratrol (3),[44] ampelopsin H (4),[45] *ε*-viniferin (5),[42] and *E*-vitisin B (6)[46] (Figure 3). The very polar fraction was found to mainly consist of sucrose.

In Vitro Antifungal Assays. The identified stilbenoids exhibited different levels of fungitoxicity against *Plasmopara viticola.* For each compound, the IC_{50} was determined both on zoospore mobility and disease development by monitoring sporulation. According to the IC_{50} values (Table 2), *E*-vitisin B

Table 2. Concentration (μM) Causing 50% Inhibition (IC_{50}) of Zoospore Mobility and Disease Development Monitored by the Sporulation of *Plasmopara viticola*

compounds	zoospore mobility		sporulation	
	$IC_{50}{}^{a}$	R^2	$IC_{50}{}^{a}$	R^2
saccharose				
ampelopsin A (1)	124	0.961	282	1
hopeaphenol (2)	17	0.995	26	1
trans-resveratrol (3)	122	0.973	121	1
ampelopsin H (4)	92	0.927	282	1
ε-viniferin (5)	66	0.986	63	0.992
E-vitisin B (6)	13	0.983	12	0.997

[a]Calculated from a sigmoidal dose−response (variable slope) according to the formula $y = min + (max − min)/(1 + 10^{(logIC50−x)Hillslope})$ (Sigmaplot Regression Wizard module).

(6) was the most toxic compound, followed by hopeaphenol (2), *ε*-viniferin (5), *trans*-resveratrol (3), ampelopsin H (4), and ampelopsin A (1), with IC_{50} values ranging from 12 to 282 μM. The IC_{50} was not calculated for the positive control (Melody Combi), but no pathogen development was observed at 2 mg/mL, as expected. According to previous results,[33] *E*-vitisin B (6) (IC_{50} 13 μM) and hopeaphenol (2) (IC_{50} 17 μM) have similar toxicities against downy mildew as pterostilbene (IC_{50} 12 μM) and *δ*-viniferin (IC_{50} 14 μM); the latter two have been reported to be stress-induced stilbenes involved in grapevine defense reactions.[32] To our knowledge, this study
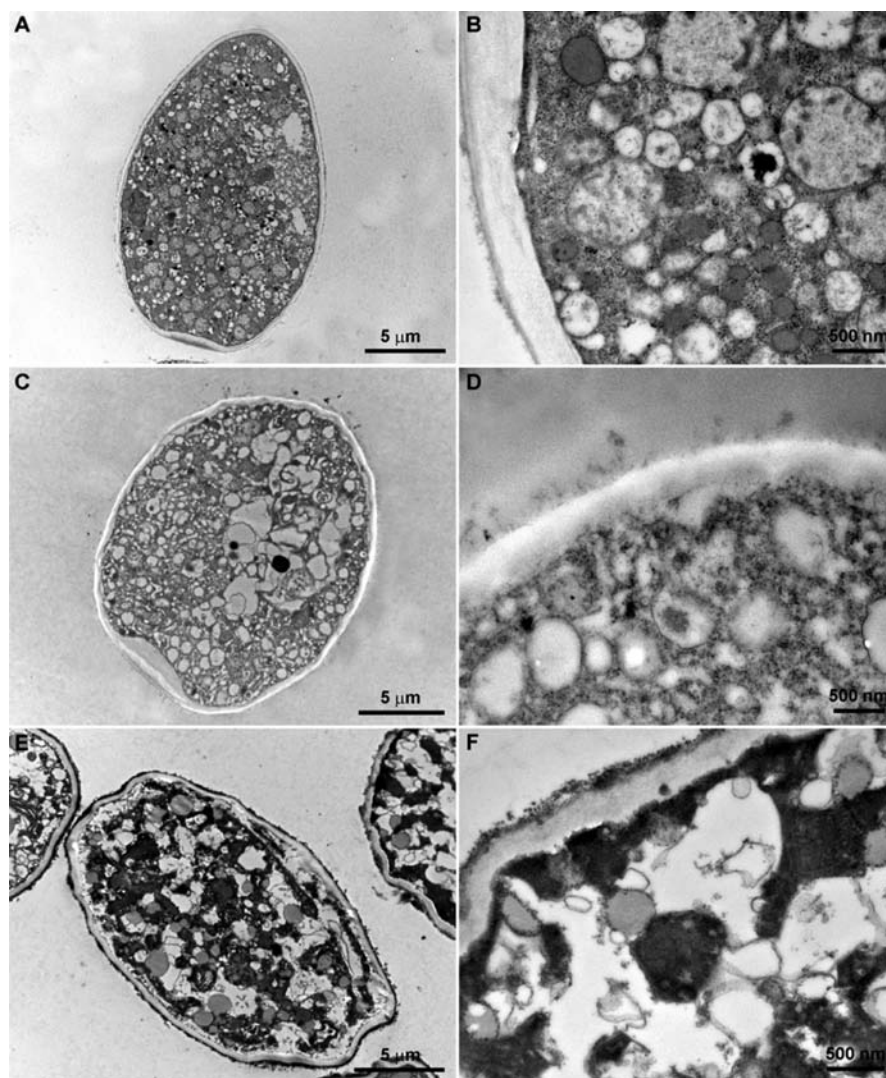
Figure 4. Ultrastructure of *Plasmopara viticola* sporangia by transmission electron microscopy after incubation with water (A and B), Melody Combi (9% iprovalicarb and 56% folpet, Bayer) (C and D), and methanolic cane extract from *V. vinifera* cv. Pinot noir (E and F).

is the first to report IC_{50} values for hopeaphenol (**2**) and *E*-vitisin B (**6**) against *P. viticola*. In the case of resveratrol, the IC_{50} value for *trans*-resveratrol has been reported (192 μM for zoospore mobility and 145 μM for sporulation).[33] This is consistent with the IC_{50} determined in this study with values in the same order of magnitude in both biological evaluations (122 μM for zoospore mobility and 121 μM for sporulation). None of the six identified compounds exhibited any toxic activity against *E. necator*. Previous studies have shown that viniferins may play a role in the defense mechanism of grapevines against powdery mildew.[47] However, this role was evaluated in in vivo tests to assess the production of stilbenic phytoalexins at the penetration site, which is very different from in vitro germination tests. Indeed, in vivo the plants produce other stilbenes against *E. necator*, such as δ-viniferin or pterostilbene.[47] In the case of *B. cinerea*, only ε-viniferin (**5**) at 5 mM inhibited the development of the fungus, confirming previous results (R. Pezet, personal communication).

Electron Microscopy. Examination of the ultrastructure of the fungal propagules was performed to evaluate the toxic effect of the methanolic extract of *V. vinifera* cv. Pinot noir. The treated sporangia of *P. viticola* importantly manifested cell shrinkage and the rapid disorganization of the cell membranes and organelles 6 h after treatment (Figure 4). In comparison to the untreated control, the presence of dark material on the outer surface of the cell wall was observed. The positive control (Melody Combi) provoked an alteration of the outer layer of the cell wall as well as an important vacuolization of the cellular content. The changes in the cell wall are consistent with the mode of action of iprovalicarb, which inhibits a cellulose synthase.[48] After 24 h, the cytoplasm was completely coagulated, and cell membranes were no longer visible in sporangia treated with both the methanolic extracts from *V. vinifera* and the positive control. The same results were obtained on conidia of *B. cinerea*, which exhibited complete disorganization of cell integrity after 24 h of treatment (data not shown). This is consistent with previous studies,[36,49,50] demonstrating that low concentrations of specific stilbenes are able to coagulate the cytoplasmic material and disorganize organelles and cellular membranes in both *B. cinerea* and *P. viticola*.

The present study has demonstrated that both crude methanolic and ethanolic extracts have potent antifungal properties against the three major fungal pathogens affecting

grapes, regardless of the grape cultivar. Six major molecules among all of the constitutive compounds have been characterized, which displayed varying levels of bioactivity against downy mildew. Current investigations are in progress to identify other compounds that are effective against other pathogenic fungi. Importantly, this work highlights the development of novel natural fungicides using grapevine extracts. By mixing molecules with antifungal properties with those that elicit plant defense mechanisms, the biological activity of the final product should have a unique complementary mechanism of action. However, efforts are also needed to formulate the final product to enhance the photostability and resistance to leaching. The use of grapevine waste constituents to protect the vine against fungal pathogens constitutes an elegant concept. Grapevine canes are available everywhere where grapes are produced, offering a unique opportunity to develop an innovative antifungal product and to control grape pathogens using grape extracts for a sustainable viticulture.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Tables S1, S2, and S3 and Figures S1 to S26. This material is available free of charge via the Internet at http://pubs.acs.org.

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