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# Effect of Polymer Micelles on Antifungal Activity of Geranylorcinol Compounds against *Botrytis cinerea*

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Supporting Information

**ABSTRACT:** Herein, we explore the potential use of two micelle-forming block copolymers, i.e., Pluronic F-127 and poly(ethylene oxide)-*b*-poly(caprolactone), for application of fungicide agents. The polymer effect on the *in vitro* fungicide activity of a series of geranyl orcinol derivatives against *Botrytis cinerea* has been assessed. The results show that, for all test compounds, the incorporation into micelles, formed by Pluronic F-127, produces a great enhancement of the inhibitory effect on the growth of *B. cinerea*. For some compounds, at the lowest tested concentration (50 ppm), the percentage of inhibition increases significantly (from 0−10 to 80−90%) when the application is made using a polymer solution instead of an ethanol/water mixture. The synthesis and structural determination of a series of eight geranylphenols/diacetates, which were used as fungicide agents, are also discussed. These results suggest that polymer micelles are promising systems for application of crop-protecting agents.

**KEYWORDS:** *antifungal, Botrytis cinerea, polymer micelles, linear geranylphenols, structural determination*



## INTRODUCTION

*Botrytis cinerea* is a phytopathogenic fungus that causes diseases in different plant species, even though wine grapes are its more important host. This fungus has been controlled by dicarboximide fungicides, but after many years of continuous use, their effect on mycelial growth has been reduced.[1,2] A similar effect has been reported for two fungicides of the anilinopyrimidine group.[3] Therefore, much effort has been dedicated to the search of new compounds to control this fungus, and a number of natural, hemisynthetic, and synthetic products have been studied.[4−7] Recently, it has been shown that prenylated compounds exhibit antifungal activity against *B. cinerea*.[7] These compounds, which have been isolated predominantly from marine organisms, herbaceous plants (*Phacelia crenulata, Phacelia ixodes, Phacelia funereal*, and *Pyrola japonica*), and shrubs (*Parthenium argentatum* and *Simmondsia chinensis*),[8−11] often possess antimicrobial, antioxidant, anti-inflammatory, antiviral, and anticancer activities.[12−18] Thus, a complete series of geranyl compounds has been synthesized, and various attempts to relate the structural features of the metabolites with the observed activities have been made. Structure−activity relationships (SARs) of antioxidant, anticancer, antimicrobial, and antifungal activities for different groups of prenylated quinones have been proposed.[8,13]

The application of amphiphilic copolymers as container and/or drug delivery systems has received much attention in the past 2 decades.[19−21] It has been shown that, in aqueous solution, these polymers form aggregates by either collapse of a single polymer chain or self-aggregation of a number of chains in a process similar to micellization of normal surfactants.[22,23] In both cases, the amphiphilic polymer provides a hydrophobic microdomain, where non-water-soluble compounds can be incorporated.[24−26] From these, the most studied systems are block amphiphilic polymers because the nature and size of both polymer blocks can be modulated by synthesis. Thus, polymer micelles have been used to enhance the solubility and bioavailability of scarcely water-soluble drugs. On the other hand, the use of nanostructures for application of crop-protecting agents, such as fungicides, insecticides, and herbicides, is a very attracting field of study.[27,28] For example, nanoparticles has proven to be an efficient vehicle to apply pesticides.[28] The main aim of this work was to explore the possibility to use polymer micelles as vehicles of fungicide compounds in the treatment of *B. cinerea*. A series of eigth geranylphenols/diacetates were used as fungicide agents (see Figure 1). Two amphiphilic block copolymers containing a poly(ethylene oxide) block, i.e., poly(ethylene oxide)-*b*-poly(caprolactone) (PEO−PCL) and poly(propylene oxide)-*b*-poly(ethylene oxide)-*b*-poly(propylene oxide) (PPO−PEO−

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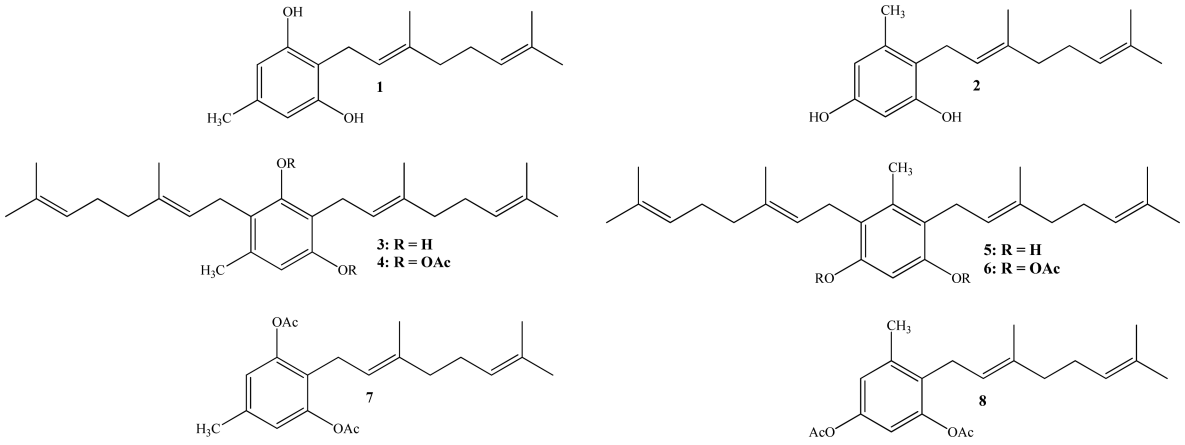


**Figure 1.** Structure of geranylphenols/diacetates synthesized in this work and tested against *B. cinerea*.

PPO), were used. The latter is a commercial polymer known as Pluronic F-127. In this work, we report the enhancement of antifungal activity of geranylphenols when they are solubilized in polymer micelles. In addition, the synthesis and structural determination of the studied compounds are discussed.

## MATERIALS AND METHODS

**Chemicals.** Unless otherwise stated, all chemical reagents purchased (Merck, Darmstadt, Germany, or Aldrich, St. Louis, MO) were of the highest commercially available purity and were used without previous purification. Silica gel (200−300 mesh, Merck) was used for column chromatography (CC) and silica gel plates $HF_{254}$ for thin-layer chromatography (TLC). TLC spots were detected by heating after spraying with 25% $H_2SO_4$ in $H_2O$.

**Spectroscopies.** Infrared (IR) spectra (32 scans per sample) were recorded as thin films in a Fourier transform infrared (FTIR) Nicolet 6700 spectrometer (Thermo Scientific, San Jose, CA), and wavelengths are reported in $cm^{-1}$. Low-resolution mass spectra were recorded on an Agilent 5973 spectrometer (Agilent Technologies, Santa Clara, CA) at 70 eV ionizing voltage in a DB-5 m, 30 m × 0.25 mm × 0.25 $\mu$m column, and data are given as $m/z$ (percent relative intensity). High-resolution mass spectra were recorded on a LTQ Orbitrap XL spectrometer (Thermo Scientific, San Jose, CA) by applying a voltage of 1.8 kV in the positive ionization mode and 1.9 kV in the negative ionization mode. The spectra were recorded using full-scan mode, covering a mass range from $m/z$ 100 to 1300. The resolution was set to 50 000, and maximum loading time for the ion cyclotron resonance (ICR) cell was set to 250 ms. Nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance 400 digital NMR spectrometer (Bruker, Rheinstetten, Germany), operating at 400.1 MHz for $^1H$ and 100.6 MHz for $^{13}C$. $^1H$, $^{13}C$, $^{13}C$ distortionless enhancement by polarization transfer (DEPT-135), selective gradient-selected one-dimensional (1D) $^1H$ nuclear Overhauser effect spectrometry (NOESY), gradient-selected two-dimensional (2D) heteronuclear single-quantum coherence (HSQC), and gradient-selected 2D heteronuclear multiple-bond correlation (HMBC) spectra were recorded in $CDCl_3$ solutions and are referenced to the residual peaks of $CHCl_3$ at $\delta$ = 7.26 and 77.0 ppm for $^1H$ and $^{13}C$, respectively. Chemical shifts are reported in $\delta$ parts per million (ppm), and coupling constants ($J$) are given in hertz.

**Dynamic Light Scattering (DLS).** Aqueous solutions of PEO−PCL and Pluronic F-127 (1 mM) in the absence and presence of geranyl derivatives (100 mM) were thermostated at 25 °C, and DLS measurements were performed using a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) equipped with a He−Ne laser (633 nm, 4 mW).

**Fluorescence Probing.** Steady-state fluorescence spectra of pyrene were recorded using a Horiba Jobin Yvon Fluoromax 4 fluorometer by exciting at 337 nm. All emission spectra were corrected for detector response using a correction curve supplied by the instrument manufacturer. The ratio $I_1/I_3$ corresponds to the ratio of intensities of peak one ($\lambda$ = 372 nm) to peak three ($\lambda$ = 384 nm), whereas the ratio $I_M/I_E$ corresponds to the ratio of intensities of the monomer band to the excimer band ($\lambda$ = 450 nm).

**Synthesis of Geranyl Derivatives.** *(E)-2-(3,7-Dimethylocta-2,6-dienyl)-5-methylbenzene-1,3-diol (**1**), (E)-4-(3,7-Dimethylocta-2,6-dienyl)-5-methylbenzene-1,3-diol (**2**), 2,4-Bis((E)-3,7-dimethylocta-2,6-dienyl)-5-methylbenzene-1,3-diol (**3**), and 4,6-Bis((E)-3,7-dimethylocta-2,6-dienyl)-5-methylbenzene-1,3-diol (**5**).* $BF_3{\cdot}OEt_2$ (0.46 g, 3.2 mmol) was slowly added dropwise, at room temperature and under a $N_2$ atmosphere, to a stirred solution of orcinol (6.1 g, 49 mmol) and geraniol (7.55 g, 49 mmol) in acetonitrile (2 mL) saturated with $AgNO_3$. After the addition was completed, the stirring was continued for 48 h. The end of the reaction was verified by TLC, and then the mixture was poured onto crushed ice (approximately 30 g) and salt (NaCl) vacuum filtration and extracted with EtOAc (3 × 20 mL). The organic layer was washed with $NaHCO_3$ (15 mL, 5%) and water (2 × 15 mL), dried over $Na_2SO_4$, and filtered. The solvent was evaporated under reduced pressure. The crude was redissolved in $CH_2Cl_2$ (5 mL) and chromatographed on silica gel with petroleum ether/EtOAc mixtures of increasing polarity (0:20 → 6:14). Five fractions were obtained: fraction I, 1554.8 mg of dark yellow viscous oil (9.9% yield, compound **3**); fraction II, 1281.8 mg of yellow viscous oil (12.5% yield, compound **1**); fraction III, 955.3 mg of dark yellow viscous oil (6.1% yield, compound **5**); fraction IV, 415.4 mg of dark yellow viscous oil (mixture of compounds **2** and **5**); and fraction V, 2816.2 mg of yellow viscous oil (27.6% yield, compound **2**). Unreacted orcinol (3.38 g) was recovered. Spectroscopic data of compounds **1** and **2** were consistent with those reported in the literature.[29−31] Spectroscopic data are given in the Supporting Information.

*Acetylation of Geranylated Phenols.* Geranylated phenols were acetylated by standard acetylation using $Ac_2O$ and 4-dimethylaminophenol (DMAP) in $CH_2Cl_2$. In a typical reaction, $Ac_2O$ (1.08 g, 10.6 mmol) is added to a solution of a geranylated phenol (0.284 mmol), DMAP (3.0 mg), and pyridine (1.0 mL) in dichloromethane (10−20 mL). The end of the reaction is verified by TLC (1.5 h), and then the mixture is extracted with EtOAc (2 × 20 mL). The organic layer is washed with 5% $KHSO_4$ (2 × 10 mL) and water (2 × 10 mL), dried over $Na_2SO_4$, and filtered. The solvent is evaporated under reduced pressure. The crude is redissolved in $CH_2Cl_2$ (5 mL) and chromatographed on silica gel with petroleum ether/EtOAc mixtures of increasing polarity (19.8:0.2 → 18.4:1.6).

*2,4-Bis((E)-3,7-dimethylocta-2,6-dienyl)-5-methyl-1,3-phenylene Diacetate (**4**).* Compound **4** was obtained by standard acetylation of compound **3** (170 mg, 0.429 mmol) with $Ac_2O$ (1.0 mL), DMAP (3.0 mg), and pyridine (1.0 mL) in dichloromethane (15 mL). Compound **4** was obtained as dark yellow viscous oil (185 mg, 90.5% yield). Spectroscopic data are given in the Supporting Information.

*4,6-Bis((E)-3,7-dimethylocta-2,6-dien-1-yl)-5-methyl-1,3-phenylene Diacetate (**6**).* Compound **6** was obtained by standard acetylation





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of compound **5** (245 mg, 0.618 mmol) with $Ac_2O$ (1.0 mL), DMAP (3.0 mg), and pyridine (1.0 mL) in dichloromethane (20 mL). Compound **6** was obtained as dark yellow viscous oil (267.5 mg, 89.8% yield). Spectroscopic data are given in the Supporting Information.

*(E)-2-(3,7-Dimethylocta-2,6-dienyl)-5-methyl-1,3-phenylene Diacetate (**7**).* Compound **7** was obtained by standard acetylation of compound **1** (74 mg, 0.284 mmol) with $Ac_2O$ (1.0 mL), DMAP (2.0 mg), and pyridine (1.0 mL) in dichloromethane (10 mL). Compound **7** was obtained as dark yellow viscous oil (93.3 mg, 96.4% yield). Spectroscopic data are given in the Supporting Information.

*(E)-4-(3,7-Dimethylocta-2,6-dienyl)-5-methyl-1,3-phenylene Diacetate (**8**).* Compound **8** was obtained by standard acetylation of compound **2** (362 mg, 1.39 mmol) with $Ac_2O$ (1.0 mL), DMAP (3.0 mg), and pyridine (1.0 mL) in dichloromethane (20 mL). Compound **8** was obtained as dark yellow viscous oil (444.6 mg, 92.8% yield). Spectroscopic data are given in the Supporting Information.

**Structure Determination.** Spectroscopic evidence of double substitution on the aromatic nucleus by geranyl chains in compound **3** was established from the $^1$H NMR spectrum by the following observations: a unique Ar−H signal at 6.30 ppm (s, 1H, H-6), four olefinic protons at 5.32 ppm (t, $J$ = 6.9 Hz, 1H, H-2′), 5.19 ppm (t, $J$ = 6.6 Hz, 1H, H-2″), 5.11 ppm (t, $J$ = 6.6 Hz, 2H, H-6′ and H-6″), and two doublet signals at 3.46 ppm ($J$ = 6.9 Hz, 2H, H-1′) and 3.35 ppm ($J$ = 6.6 Hz, 2H, H-1″). Finally, the complete determination of the asymmetrical structure was established by 2D HMBC and selective 1D NOESY experiments. The major 2D HMBC correlations confirming the structure of compound **3** are $^2J_{H-C}$ coupling between H-1′ and C-2 ($\delta_C$ = 111.5 ppm), $^3J_{H-C}$ coupling of H-1′ with C-3 and C-1 ($\delta_C$ = 153.4 and 152.2 ppm), respectively, $^2J_{H-C}$ coupling of H-1″ with C-4 ($\delta_C$ = 118.1 ppm), and $^3J_{H-C}$ coupling of H-1″ with C-5 ($\delta_C$ = 135.1 ppm) and C-3 ($\delta_C$ = 153.4 ppm) (Figure S1 of the Supporting Information). From selective 1D NOESY experiments, the *E* geometry in the C-2′−C-3′ and C-2″−C3″ double-bond position of the geranyl chain was established. In this case, the most important correlations are between H-1′ with the methyl group in the C-3′ position ($\delta_H$ = 1.86 ppm) and with both OH groups ($\delta_H$ = 5.50 and 5.44 ppm), whereas H-1″ correlates with $CH_3$−Ar ($\delta_H$ = 2.25 ppm), $CH_3$−C3″ ($\delta_H$ = 1.84 ppm), and OH group ($\delta_H$ = 5.50 ppm) (Figure S2 of the Supporting Information).

A similar spectroscopic analysis was performed to determine the structure of compound **5**. Spectroscopic evidence of double substitution on the aromatic nucleus by geranyl chains in compound **5** was obtained from the $^1$H NMR spectrum through the following observations: a unique Ar−H signal at 6.25 ppm (s, 1H, H-6), four olefinic protons at 5.11 ppm (t, $J$ = 6,4 Hz, 2H, H-2′), 5.05 ppm (t, $J$ = 6.6 Hz, 2H, H-6′), and a unique doublet signal at 3.33 ppm (d, $J$ = 6.6 Hz, 4H, H-1′). Finally, the complete determination of the symmetrical structure was established by 2D HMBC and selective 1D NOESY experiments. The main 2D HMBC correlations used to confirm the structure of compound **5** were a $^2J_{H-C}$ coupling of H-1′ ($\delta_H$ = 3.33 ppm) with C-4 and C-6 ($\delta_C$ = 118.5 ppm) and $^3J_{H-C}$ with C-1 and C-3 ($\delta_C$ = 152.9 ppm). The *E* geometry in the C-2′−C-3′ double-bond position of the geranyl chain was established by selective 1D NOESY experiments. The most important spatial correlations were observed between H-1′ with $CH_3$−Ar ($\delta_H$ = 2.07 ppm) and with the $CH_3$−C-3′ position ($\delta_H$ = 1.79 ppm). The main 2D HMBC and 1D NOESY correlations are shown in Figure 2.

The presence of diacetylated derivatives **4** and **6**, obtained by standard acetylation of compounds **3** and **5**, respectively, was confirmed by NMR spectroscopy. The $^1$H NMR spectrum of compound **4** shows two singlet signals at $\delta_H$ = 2.28 ppm (s, 3H, $CH_3CO$) and 2.26 ppm (s, 6H, $CH_3CO$ and $CH_3$−Ar), whereas $^1$H NMR of compound **6** show a singlet signal at $\delta_H$ = 2.28 ppm (s, 6H, 2× $CH_3CO$). Additionally, in the $^{13}$C NMR spectrum of compound **6**, two singlet signals at $\delta_C$ = 169.2 ppm (2× C═O) and 20.7 ppm (2× $CH_3CO$) were observed as a result of the symmetry of the molecule.

Finally, the $^1$H NMR spectrum of symmetric diacetylated derivative **7** shows a singlet signal at $\delta_H$ = 2.27 ppm (6H, 2× $CH_3CO$), whereas in the $^{13}$C NMR spectrum, the signal at $\delta_C$ = 169.2 ppm (2× C═O) confirms the presence of the diacetylated derivative. On the other hand, the $^1$H NMR spectrum of diacetylated derivative **8** shows a singlet signal at $\delta_H$ = 2.28 ppm (6H, 2× $CH_3CO$), whereas in the $^{13}$C NMR spectrum, two singlet signals are observed at $\delta_C$ = 169.3 ppm (C═O) and 169.2 ppm (C═O) as a result of the asymmetry of the molecule, confirming the presence of the diacetylated derivative.

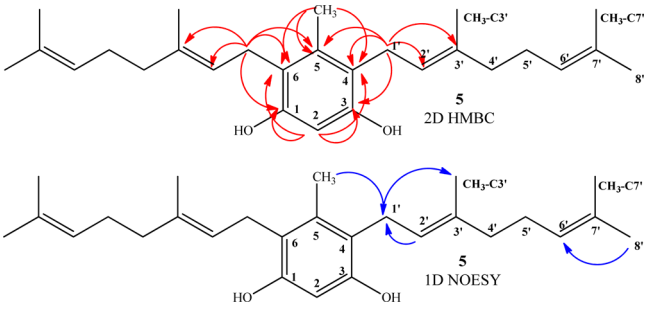


**Figure 2.** Most important 2D $^1$H−$^{13}$C HMBC and selective 1D NOESY correlations for compound **5**.

**Poly(ethylene oxide)-*b*-Poly(caprolactone) (PEO−PCL).** PEO−PCL was synthesized in bulk by ring-opening polymerization using stannous octanoate (SnOct) as a catalyst. Briefly, freshly distilled ε-caprolactone was added under a nitrogen atmosphere to poly(ethylene oxide) monomethyl ether (Aldrich, $M_n$ = 2000 g/mol). After complete dissolution of PEO, 0.01% of SnOct was added and the flask was heated at 135 °C with constant stirring. After 24 h, the resulting viscous liquid was cooled to room temperature, dissolved in 5 mL of tetrahydrofuran (THF), precipitated into a large excess of cold *n*-hexane, filtered, redissolved in THF, and precipitated in *n*-hexane. The white precipitate was collected by filtration and vacuum-dried at 35 °C for 2 days. Yield = 94%. Absolute molecular weight was measured using matrix-assisted laser desorption/ionization−time-of-flight (MALDI−TOF) (MW = 3409 g/mol). The data indicate that the degree of polymerization of ε-caprolactone is 14, and therefore, the resulting block copolymer composition is $PEO_{43}$-*b*-$PCL_{14}$.

**Preparation and Characterization of Polymer Micelles.** Polymer micelles were prepared by direct dissolution of block copolymers in water at a concentration well above the critical micelle concentration (cmc) (1 mM). The cmc was determined by a photophysical technique that consists of measuring the ratio $I_M/I_E$ of pyrene fluorescence as a function of the polymer concentration. The size distributions of polymeric micelles were determined by DLS.

**Fungal Isolate and Culture Condition.** In this study, a strain of *B. cinerea* isolated from a naturally infected grape (*Vitis vinifera*) was used in all experiments. This strain was maintained on potato dextrose agar (PDA) medium (Difco, Detroit, MI) at 4 °C. The inoculum of the pathogen was grown on PDA in a photoperiod of 16 h light/8 h dark at 23 °C for 5 days.

**Antifungal Activity.** To determine the antifungal activity of geranyl derivatives, two different stock solutions were prepared for each compound. Homogeneous stock solutions were formulated by dissolving 20−40 mg of each compound in 500 μL of ethanol and then diluting in water (5 mL) to obtain a 5000 ppm concentration. On the other hand, micro-heterogeneous solutions were obtained using the emulsion method. Briefly, aliquots (100 μL) of each compound dissolved in dichloromethane were added to 10 mL of an aqueous polymer solution (1 mM). After an emulsion has been formed, the organic solvent is eliminated by heating and sonication. In this process, compounds **1**−**8** are incorporated into the hydrophobic micelle core, reaching a concentration of 0.1 M. Before their application to Petri plates, these solutions are diluted to 650−1250 ppm but keeping the polymer concentration constant (1 mM).

The radial growth rate assay on PDA medium was used to determine the antifungal activity of test compounds.[32,33] Test compounds, in ethanol/water solution or aqueous polymer solution, were added to Petri plates containing PDA medium at 50 °C to give 50, 150, and 250 ppm. When the medium was almost solidified, 4 mm




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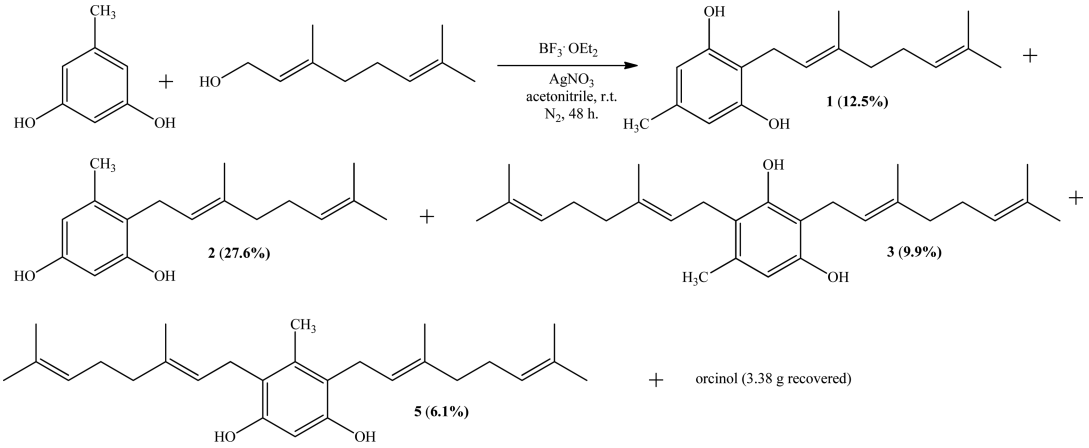
**Scheme 1. Synthesis of Compounds 1−3 and 5**




diameter disks of fungus cut were placed in the center of the plate. Negative controls (C−) are used in the corresponding stock solution with pure solvent (1% ethanol) or polymer solution (1 mM PEO−PCL or Pluronic F-127). Captan, a commercial fungicide, is used as the positive control (C+) at the same concentrations and conditions of test compounds. After incubation by 48 h (ethanol) and 72 h (polymer) at 23 °C under a photoperiod of 16 h light/8 h dark, diameters of colonies were measured with a ruler and the inhibition percentage was calculated as the average of three replicas compared to the negative control.

## RESULTS AND DISCUSSION

The direct geranylation of orcinol using the acetonitrile/$BF_3$·$Et_2O$/$AgNO_3$ system leads to compounds **1** and **2** with 12.5 and 27.6% yields, respectively, which are almost identical to those reported for the standard conditions, i.e., the dioxane/$BF_3$·$Et_2O$ system.[29] However, with the modified method, two additional digeranyl derivatives **3** and **5** were identified and isolated from the reaction products with 9.9 and 6.1% yields, respectively.

The synthesis of compounds **1**−**3** and **5** is described in Scheme 1.

It should be mentioned that incorporation of two geranyl chains to the aromatic ring has been previously described in the geranylation of methoxyphenols using ether as the solvent.[13] However, the reaction of 2,4,5-trimethoxyphenol with geraniol in dioxane follows a completely different reaction pathway, and no digeranylated phenols were obtained.[17] On the other hand, diprenyl phenols have also been obtained in the reaction between dihydroxybenzenes and 1,3,5-trihydroxybenzene with 3-methyl-2-buten-1-ol, using a mixture of ethyl ether/dichloromethane as the solvent.[18] Finally, digeranyl phenols have also been obtained in the geranylation of 1,3,5-trihydroxybenzene using the modified electrophilic aromatic substitution (EAS) reaction.[7] These results emphasize the important role played by solvent in this kind of reaction. Diacetylated derivatives **4** and **6** were obtained by standard acetylation of compounds **3** and **5** with 93.3 and 92.8% yields, respectively. Finally, derivatives **7** and **8** were obtained by standard acetylation of compounds **1** and **3** with 90.5 and 89.8% yields, respectively.

The physical and spectroscopic properties of compounds **1** and **2** were consistent with those previously reported.[11,29,31] The structural determination of new compounds **3**−**6** is described in detail in the Materials and Methods.

**Formation of Polymer Micelles.** In aqueous solution and at concentrations above the cmc, amphiphilic block copolymers self-aggregate forming spherical micelles. The values of cmc, determined by measuring the ratio $I_M/I_E$ of pyrene fluorescence as a function of the polymer concentration, are $2.7 \times 10^{-6}$ and $5.5 \times 10^{-5}$ M for Pluronic F-127 and PEO−PCL, respectively. Thus, to ensure the presence of micelles, a polymer concentration of 1 mM was used in all experiments. The formation of micelles has also been evidenced by DLS measurements, which give the size and size distribution of the formed aggregates. The results shown in Figures 3 and 4

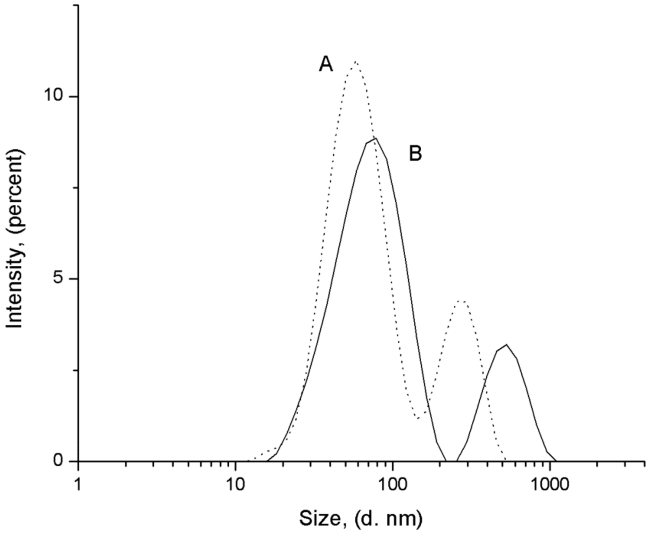



**Figure 3.** Distribution sizes of aggregates formed by PEO−PCL (1 mM) in the (A) absence and (B) presence of compound **1** (10 mM). DLS measurements were made at 25 °C.

indicate that small micelles and larger aggregates are formed at this polymer concentration. This agrees with previous studies, made with transmission electronic microscopy (TEM), where it has been shown that PEO−PCL forms spherical and elongated aggregates, and the sizes obtained from TEM were consistent with those obtained by DLS.[34,35] Additionally, the inclusion of geranylphenols into the micelles brings about a change in the size distribution. In both cases, the solute induces the formation of stable aggregates with a larger average diameter. This behavior is similar to that reported for solubilization of a family of alkylphenols in Pluronic F-127.[36]





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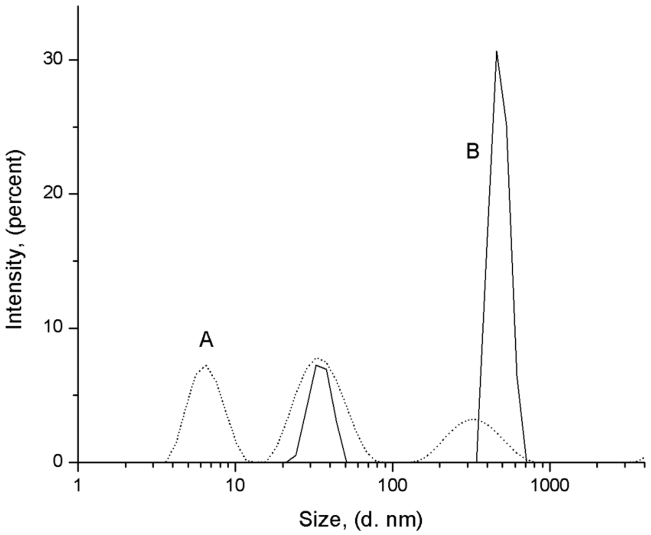


**Figure 4.** Distribution sizes of aggregates formed by Pluronic F-127 (1 mM) in the (A) absence and (B) presence of compound **1** (10 mM). DLS measurements were made at 25 °C.

**Table 1. Effect of Geranylphenols on Mycelial Growth of *B. cinerea* Measured as a Percentage of Inhibition**[a]

| | percentage of inhibition on mycelial growth of *B. cinerea* *in vitro*[b] | | |
|---|---|---|---|
| compound | 50 mg/L | 150 mg/L | 250 mg/L |
| **1** | 37 | 65 | 82 |
| **2** | 0 | 25 | 63 |
| **3** | 25 | 61 | 93 |
| **4** | 5 | 69 | 97 |
| **5** | 12 | 56 | 83 |
| **6** | 11 | 54 | 85 |
| **7** | 8 | 51 | 82 |
| **8** | 24 | 55 | 78 |
| C−[c] | 0 | 0 | 0 |
| C+[c] | 88 | 94 | 94 |

[a]Geranyl phenols were added to the incubation media from a stock solution using ethanol/water (1%) as the solvent. [b]The percentage of inhibition of mycelial growth is based on colony diameter measurements after 48 h of incubation. Each point represents the mean of at least three independent experiments. [c]C− refers to the negative control and corresponds to stock solution with pure solvent (1% ethanol), and C+ refers to the positive control (Captan).

The micropolarity of the hydrophobic micelle core was assessed by measuring the ratio $I_1/I_3$ in the pyrene fluorescence spectrum. It has been shown that this ratio strongly depends upon the polarity of the medium where pyrene is located,[37] and a Py polarity scale has been proposed.[38] The results indicate that the micelle formed by PEO−PCL ($I_1/I_3$ = 1.13) is much more hydrophobic than that formed by Pluronic F-127 ($I_1/I_3$ = 1.56). Consequently, nonpolar substrates should be more easily incorporated into the core of PEO−PCL micelles.

**Antifungal Activity against *B. cinerea*.** The effect of geranylphenols on the mycelial growth of plant pathogen *B. cinerea* was evaluated *in vitro* after 72 h of incubation using the radial growth rate assay with PDA as the medium. The test compounds were added to the incubation media in either homogeneous solution (using 1% ethanol in water as the solvent) or dissolved in an aqueous solution of polymer micelles (1 mM). The inhibitory effect is evaluated as a percentage of inhibition, which is calculated as the ratio of the area of *B. cinerea* in the presence and absence of geranylphenols. The means of three independent assays are summarized in Tables 1 and 2. For comparison, the values of the percentage of inhibition obtained with Captan, a molecule that is widely used to control the growth of *B. cinerea*, are included (measured at the same concentration used to determine the activity of geranylphenols).

Analysis of the results given in Table 1 shows that the inhibitory effect increases with increasing the compound concentration and that the antifungal activity depends upon the chemical structure of geranylphenol derivatives. Thus, monogeranyl phenols **1** and **2** exhibit inhibitory activity similar or slightly lower than that observed for diacetylated compounds (**7** and **8**) at 250 ppm. These results are in line with those reported previously for other series of geranylphenol derivatives[7] and, all together, suggest that the activity is mainly determined by the presence of the geranyl chain. On the other hand, the addition of a second geranyl chain brings about an increase on antifungal activity for all studied compounds (Table 1 compares compounds **3** and **1**, compounds **5** and **2**, compounds **4** and **7**, and compounds **6** and **8**). Compound **4** exhibits the highest inhibitory effect, i.e., 69 and 97% at 150 and 250 ppm, respectively. However, in previous work, it has been found that digeranyl derivatives exhibit lower activities than the corresponding monogeranyl compounds and that their activity strongly depends upon the presence of hydroxyl groups.[7] This structure effect on activity may be attributed to the higher solubility of digeranyl phenols, which would increase the biodisponibility of these compounds. To verify this hypothesis, a study of solubilization into polymer micelles was carried out.

The results given in Table 2 indicate that the inhibitory activity of all test compounds against mycelial growth of *B. cinerea* changes in the presence of micelle-forming polymers. In the case of PEO−PCL, the effect on mycelial growth depends upon the structure of geranyl compounds, i.e., at all studied concentrations, an increase is observed for compounds **2**, and **5**, whereas a decrease in activity was found for the other studied compounds. However, in the presence of Pluronic F-127, the increase in activity is very dramatic, changing from no effect to 80% of inhibition at a low concentration (compare data of compound **2** at 50 mg/L in Tables 1 and 2). This activity is independent of the geranylphenol structure and is almost the same obtained with Captan, the positive control. These results suggest that encapsulation into micelles formed by Pluronic F-127 might improve some properties of the molecules (stability or availability) or might provide new environmental conditions (homogeneous distribution), allowing for an inhibitory activity of test compounds on the growth of *B. cinerea*, even at a lower concentration, such as 50 mg/L.

The structure of the polymer micelle seems to be a determinant factor in the magnitude of the inhibitory effect exhibited by each compound. This effect could be attributed to differences in micellar sizes and polarity of the micellar core. Polymer micelles formed by Pluronic F-127 are smaller and the core is more polar than in micelles formed by PEO−PCL. These physical parameters might affect the distribution coefficients of these compounds between the aqueous phase and the pseudo-micellar phase provided by the block copolymers; i.e., these coefficients should be larger in the presence of micelles formed by PEO−PCL. On the other hand, the release rates of compounds solubilized in these micelles




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Table 2. Percentage of Inhibition of Geranylphenols on Mycelial Growth of *B. cinerea* Measured in the Presence of Polymer Micelles

| | percentage of inhibition on mycelial growth of *B. cinerea in vitro*[a] | | | | | |
|---|---|---|---|---|---|---|
| | PEO-*b*-PCL | | | Pluronic F-127 | | |
| compound | 50 mg/L | 150 mg/L | 250 mg/L | 50 mg/L | 150 mg/L | 250 mg/L |
| **1** | 9 | 32 | 42 | 82 | 96 | 96 |
| **2** | 37 | 61 | 73 | 76 | 90 | 88 |
| **3** | 16 | 36 | 37 | 85 | 91 | 96 |
| **4** | 10 | 31 | 50 | 93 | 99 | 99 |
| **5** | 93 | 94 | 97 | 82 | 92 | 96 |
| **6** | 0 | 36 | 36 | 93 | 99 | 99 |
| **7** | 32 | 35 | 59 | 77 | 88 | 94 |
| **8** | 17 | 39 | 80 | 81 | 88 | 93 |
| C−[b] | 0 | 0 | 0 | 0 | 0 | 0 |
| C+[b] | 93 | 99 | 99 | 98 | 99 | 99 |

[a]The percentage of inhibition of mycelial growth is based on colony diameter measurements after 72 h of incubation. Each point represents the mean of at least three independent experiments. [b]C− refers to the negative control and corresponds to aqueous polymer solution (1 mM PEO−PCL or Pluronic F-127), and C+ refers to the positive control (Captan).

should also be lower than in micelles of Pluronic F-127. The direct consequence of these two effects is that nonpolar compounds incorporated into PEO−PCL micelles are less available during the development of mycelial growth halo. A study where these physicochemical parameters are evaluated as a function of the substrate structure is currently being carried out. Additionally, the possibility that polymer micelles penetrate the fungus membrane and releasing of entrapped compounds occurs inside of a fungus cell should also be considered. The proposal of this alternative mechanism of action is based on results that have demonstrated that both polymer micelles can be internalized into cells by endocytosis.[34,39]

The results in Table 2 also show that the activity of all encapsulated test compounds is quite similar to that measured for Captan, a fungicide widely used to protect crops. In addition, an aqueous solution of polymer micelles can be easily applied *in vivo* by simple spraying. These interesting properties turn polymer micelles into very promising systems for control of *B. cinerea* in the field. Obviously, these results should be validated evaluating *in vivo* the biological effect of encapsulated test compounds on plant tissues infected by this fungus. Finally, it is worth mentioning that these polymers are biocompatible and Pluronic F-127 copolymers are listed in U.S. and British Pharmacopoeia as pharmaceutical excipients.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01920.

Spectroscopic information of compounds **1**−**8**, 2D $^{1}H-^{13}C$ HMBC spectrum of compound **3** (Figure S1), and main selective 1D NOESY correlations observed for compound **3** (Figure S2) (PDF)

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### Notes

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