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Antimicrobial Mechanism of Resveratrol-*trans*-Dihydrodimer Produced From Peroxidase-Catalyzed Oxidation of Resveratrol

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ABSTRACT: Plant polyphenols are known to have varying antimicrobial potencies, including direct antibacterial activity, synergism with antibiotics and suppression of bacterial virulence. We performed the in vitro oligomerization of resveratrol catalyzed by soybean peroxidase, and the two isomers (resveratrol-*trans*-dihydrodimer and pallidol) produced were tested for antimicrobial activity. The resveratrol-*trans*-dihydrodimer displayed antimicrobial activity against the Gram-positive bacteria *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* (minimum inhibitory concentration (MIC) = 15.0, 125, and 62.0 μM, respectively) and against Gram-negative *Escherichia coli* (MIC = 123 μM, upon addition of the efflux pump inhibitor Phe-Arg-β-naphthylamide). In contrast, pallidol had no observable antimicrobial activity against all tested strains. Transcriptomic analysis implied downregulation of ABC transporters, genes involved in cell division and DNA binding proteins. Flow cytometric analysis of treated cells revealed a rapid collapse in membrane potential and a substantial decrease in total DNA content. The active dimer showed >90% inhibition of DNA gyrase activity, in vitro, by blocking the ATP binding site of the enzyme. We thus propose that the resveratrol-*trans*-dihydrodimer acts to: (1) disrupt membrane potential; and (2) inhibit DNA synthesis. In summary, we introduce the mechanisms of action and the initial evaluation of an active bactericide, and a platform for the development of polyphenolic antimicrobials.

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KEYWORDS: resveratrol; peroxidase; antimicrobial; disrupted membrane potential; DNA gyrase inhibition

Introduction

The emergence of antibiotic resistant bacteria with increasing frequency in both hospitals and the general community is a worldwide public health problem (Fischbach and Walsh, 2009). The phenomenon of resistance in microbes has been associated with the intensive use of antimicrobial agents and interrupted treatments in humans and animals (Hammerum and Heuer, 2009). Bacteria develop resistance by horizontal gene transfer (through transposons,

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integrons, or plasmids) or occurrence of specific gene mutations, and involve a variety of complex mechanisms (i.e., impermeable cell wall barriers, multidrug resistance efflux pumps, and generation of antibiotic degrading or modifying enzymes) (Allen et al., 2010; Lee et al., 2010). Despite a constant annual growth of 4% in the market for antibiotics over the last 5 years (Hamad, 2010), the interest of pharmaceutical companies in antibiotics development has decreased due to the high associated costs, short-term use, quick development of resistance, and the tendency to reserve the novel formulations for treating multidrug resistant bacteria. Thus, identifying novel antimicrobials and understanding their mechanism of action are equally imperative.

Natural products have been used as antibiotics or as scaffolds for formulation of active semisynthetic derivatives (Butler and Buss, 2006; Hemaiswarya et al., 2008), including some well-known natural product classes, such as β-lactams (e.g., penicillins and cephalosporins), tetracyclines (e.g., glycylcyclines), macrolides (e.g., erythromycin and rifamycin analogues), spectinomycins, glycopeptides (e.g., vancomycin and teicoplanin analogues), flavonoids, alkaloids, and quinones, among others (Baquero, 1997; Clardy et al., 2006; Saleem et al., 2010). Among natural products, plant polyphenols possess some antimicrobial activities of varying potencies (Lewis and Ausubel, 2006). However, many of these active secondary metabolites are produced at very low levels in plants (El-Elimat et al., 2014), making it difficult to identify and test their bioactivities. As a result, the success rate of identifying potent plant-based antimicrobial polyphenols is relatively low (Lewis and Ausubel, 2006).

Attempts to circumvent these hurdles of low availability and varying antimicrobial potencies have centered on the metabolic engineering of plant-derived polyphenols in heterologous hosts (Bhan et al., 2013; Chemler and Koffas, 2008; Xu et al., 2013) or potentiation of their antibiotic activity via combinatorial synthesis (Fowler et al., 2011). In vitro oligomerization of phenols and polyphenols, catalyzed by peroxidases (Antoniotti et al., 2004), has been shown to be a practical method of generating molecular libraries with enhanced biological activity and potential applications against diseases like Alzheimer's and cardiovascular diseases (Ladiwala et al., 2011; Mora-Pale et al., 2009, 2012). In addition, this enzymatic approach avoids conventional multi-step chemical synthesis (Snyder et al., 2011). We hypothesize that enzymatic oligomerization of these polyphenols could be used as a tool to generate more efficient antimicrobials. Along these lines, we tested the antimicrobial activity of two isomers, resveratrol-*trans*-dihydrodimer and pallidol, obtained from the enzymatic oligomerization of the plant polyphenol resveratrol.

We report herein the identification of resveratrol-*trans*-dihydrodimer as an effective antimicrobial against Gram-positive and Gram-negative bacteria. In contrast, its isomer, pallidol, displayed no significant effect on the growth of either Gram-positive or Gram-negative strains. The differential activity of these isomeric dimers of resveratrol prompted us to study the mechanism of action of the active dimer. The results indicate that oligomerization of plant polyphenols can be an effective strategy to synthesize and activate natural products as antimicrobials.

Materials and Methods

Enzymatic Production of Resveratrol-*trans*-Dihydrodimer and Pallidol

Resveratrol oxidation products (ROP) were synthesized by soybean peroxidase (SBP)-catalyzed oxidation of resveratrol, as described by Antoniotti et al. (2004). Briefly, resveratrol (2.6 mmol, Sigma–Aldrich, St. Louis, MO) was dissolved in 5 mL of dimethylformamide (DMF) and transferred to 490 mL phosphate buffer (50 mM, pH 7). SBP (Sigma) (5 mL of a 1 mg/mL solution) was added and the reaction was initiated by using a syringe pump to introduce H_2O_2 (30% w/v) at 0.1 mL/min for 12 min to afford 12 mmol H_2O_2. Finally, the reaction was stopped after 2 h. Enzymatic products were soluble in aqueous buffer and were extracted with ethyl acetate, dried and stored at −20°C under argon. Conversion of resveratrol was 99% as determined by thin-layer chromatography (TLC) and LC-MS. TLC was carried out using Merck plates of silica gel 60 with fluorescent indicator and revealed with UV light (254 nm) and 5% H_2SO_4 in a solution of dichloromethane: EtOH, 9:1. ROP mixture was analyzed by LC-MS (Shimadzu LCMS-2010A) using an Agilent Zorbax 300SB-C_{18} column, 5 μm, 2.1 × 150 mm with isocratic elution (MeCN: H_2O, 3:7; 0.2 mL/min) (Supplementary Figure S1).

Fractionation of ROP-Isolation of Resveratrol Dimers

The ROP mixture (600 mg) was dissolved in the minimum amount of chloroform and loaded onto a silica gel column (flash chromatography, 50 g silica gel 230–400 mesh, Natland International Corporation, Morrisville, NC) and eluted with a gradient of ethyl acetate: petroleum ether 1:1 to 1:0 and ethyl acetate (10%, v/v, MeOH). Resveratrol-*trans*-dihydrodimer (454 m/z) was obtained as a white powder and characterized by HRMS, and ^{1}H and ^{13}C NMR (Supplementary Figure S2), which were in agreement with literature data (Huang et al., 2000; Li et al., 2012). Spectra were acquired at 25°C on a Bruker 800 MHz Avance II NMR spectrometer equipped with a cryoprobe with *z*-axis gradients. NMR samples were dissolved in d_6-acetone. The methyl resonances of tetramethylsilane (TMS) were used as an internal chemical shift reference standard with reported chemical shifts (δ) indicated in ppm and coupling constants (*J*) in Hz. ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature, in CD_3OD (Varian 500 MHz). Chemical shifts (δ) are indicated in ppm and coupling constants (*J*) in Hz. ^{1}H NMR (500 MHZ, CD_3OD) δ = 7.36 (1H, dd, J = 8.5 Hz, J = 2.0 Hz), 7.18 (3H, m), 6.98 (1H, d, J = 16.5 Hz), 6.85 (1H, d, J = 8.5 Hz), 6.80 (3H, m), 6.44 (2H, d, J = 2.0 Hz), 6.21 (1H, d, J = 2.0 Hz), 6.15 (3H, m), 5.39 (H, d, J = 8.5 Hz), 4.40 (1H, d, J = 8.5 Hz). ^{13}C NMR (125 MHZ, CD_3OD) δ = 159.6, 158.5, 158.2, 157.3, 144.0, 139.8, 131.4, 131.0, 128.0, 127.3, 126.0, 122.8, 114.9, 109.0, 106.4, 104.5, 101.3, 101.1, 93.5, 57.3.

A second fraction of ROP was obtained and MS analysis showed the molecular weight of a second dimer. The ROP fraction (∼20 mg) was dissolved in ethyl acetate with 10% methanol, and separated through preparative thin layer chromatography (TLC, silica gel with F254 indicator, 20 cm × 20 cm) developed in 100% diethyl ether for 1.5 h. Three fractions were obtained: a colorless (top band, 2.4 mg), a yellow fraction (middle band, 2.9 mg), and an orange fraction (bottom band, 8 mg). MS analysis of the orange

fraction showed a mass of 474 m/z that corresponds to the mass of a second resveratrol dimer (Pallidol, 454 m/z)) and was characterized by HRMS, and by ^{1}H and ^{13}C NMR (Supplementary Figure S3), which were in agreement with literature data (Li et al., 2012). ^{1}H NMR (800, d_6-acetone) $\delta = 8.05$ (2H, s), 8.03 (2H, s), 7.79 (2H, s), 6.98 (4H, d, $J = 8.5$ Hz), 6.71 (4H, d, $J = 8.5$ Hz), 6.62 (2H, d, $J = 1.7$ Hz), 6.19 (2H, d, $J = 1.6$ Hz), 4.57 (2H, s), and 3.82 (2H, s). ^{13}C NMR (201 MHz, d_6-acetone) 159.3, 156.2, 155.2, 150.3, 137.7, 129.0, 123.2, 115.7, 103.3, 102.4, 60.5, 54.0.

Determination of MIC and Half Maximal Inhibitory Concentration (IC_{50}) Values

Antibacterial activity of resveratrol-*trans*-dihydrodimer and pallidol was evaluated against Gram-positive *B. cereus*, *Listeria*, L. monocytogenes and *S. aureus* and Gram-negative *E. coli BL21** in presence and absence of efflux pump inhibitors. Briefly, after overnight growth of each strain (*B. cereus* was cultured in tripticase soy broth, L. monocytogenes in BH media, *S. aureus* in NB media, and *E. coli BL21** in LB media), the initial OD_{600} was adjusted to 0.1 ($\sim 10^8$ cells/mL) and incubated in 96-well plates with both resveratrol dimers in a range of concentration from 0 to 125 μM for 24 h by triplicates. Cell viability was calculated by normalizing the difference in OD_{600} at 24 h and 0 h; IC_{50} and MIC values were calculated using GraphPad Prism software (San Diego, CA). To validate the accuracy of OD_{600} measurements, agar plates were cultured, colonies were counted, and IC_{50} and MIC values were calculated and compared with those obtained through the OD_{600} readings. Potentiation of resveratrol dimers was evaluated by adding efflux pump inhibitors during incubation with bacterial strains; Phe-Arg-β-naphthylamide (PABN, 25 μg/mL) for *E. coli* (Fowler et al., 2011), 1(1-naphthylmethyl)-piperazine (NMP, 100 μg/mL) for *B. cereus* (Zechini and Versace, 2009), Reserpine (10 μg/mL) for *Listeria* (Godreuil et al., 2003; Jiang et al., 2012), piperine (100 μg/mL) for *S. aureus* (Sangwan et al., 2008).

Microarray Analysis

B. cereus 10987 was cultured overnight in tripticase soy broth and new cultures (initial $OD_{600} = 0.1$ in 50 mL) were started with and without the addition of the resveratrol-*trans*-dihydrodimer (0.5 IC_{50}) for 8 h at 30°C. Treated and untreated bacteria were collected by centrifuging samples (2600*g*, 4°C) for 10 min. Samples were washed subsequently with PBS buffer and RNAprotect® Bacteria Reagent (Qiagen, Germantown, MD), and frozen at −80°C. Samples were sent to the Microarray Core Facility at University of Albany, New York for the study of gene regulation of treated and untreated cells. The scans were imported into Nimblegen DEVA software, aligned and call files were generated. The call files were quantile normalized and baseline transformed to the median in Gene Spring 12. The data were filtered to remove any probe sets that may be in the bottom 20th percentile of signals across all conditions. The data were also filtered to include genes that were differentially expressed at a twofold cut off.

Validation of Microarray Analysis by qRT-PCR

B. cereus 10987 cultures (initial $OD_{600} = 0.1$ in 50 mL) were started with and without the resveratrol-*trans*-dihydrodimer (0.5 IC_{50}) for 8 h at 30°C. Treated and untreated bacteria were collected by centrifuging samples (2600*g*, 4°C) for 10 min. Samples were washed subsequently with sterile PBS buffer and RNAprotect® Bacteria Reagent (Qiagen), and frozen at −80°C. RNA isolation was carried out by disrupting the cells with Trizol reagent (Invitrogen, Carlsbad, CA). Isolated RNA was washed with ethanol and dried to eliminate Trizol. Finally, RNA was stored in ethanol and solubilized in water before the qRT-PCR reactions. Primers were designed using the free online primer design software Primer3. Before the comparison of gene regulation between treated and untreated samples, the standard curves for each gene were established for determining the efficiency (~100%) of the PCR reaction and the threshold line. To this aim, five different PCR reactions, per each gene evaluated, were performed using the isolated RNA (1, 5, 25, 125, 250 ng/μL) from untreated bacteria including controls without RNA; the concentration of primers was 20 nM, and all PCR products were then run on a 1.2% agarose gel to confirm the correct size of the fragment and sequence analyzed to determine correct amplification. PCR reactions using RNA extracted from treated and untreated bacteria (25 ng/μL) were performed in a total volume of 30 μL and carried out for 40 cycles using the CFX96 Touch™ Real-Time PCR Detection System by Bio-Rad with the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen). Fold changes were calculated using the $\Delta\Delta C_t$ method (Equation 1). All reactions were run in triplicates and there were two biological duplicates for the treated RNA extract.

$$\frac{x_{\text{test}}}{x_{\text{control}}} = 2^{\Delta\Delta C_t} = 2^{(C_{tx} - C_{tR})_{\text{control}}} - 2^{(C_{tx} - C_{tR})_{\text{test}}} \quad (1)$$

Measurement of Bacterial Membrane Potential

The effect of the resveratrol *trans*-dihydrodimer on the membrane potential of *B. cereus* was measured using the BacLight™ Bacterial Membrane Potential Kit (Invitrogen) following its experimental procedure. Cells (1×10^8 cells/mL) were incubated with the resveratrol-*trans*-dihydrodimer (30 μM) and fluorescent dye diethyloxacarbocyanine ($DiOC_2$, 30 μM) for 30 min at 30°C. The excess dye was removed after centrifugation of cells (9000*g*) and cells resuspended in PBS for FACS analysis. As a positive control, the results were compared with the effect of ionophore carbonyl cyanide 3-chlorophenylhydrazone CCCP (5 μM), and with ciprofloxacin (15 μM) as a negative control. After the incubation of this bacterial strain with each antibiotic, the cell viability was determined by conventional dilution plating. FACS analysis was performed in a BD Sciences LSRII flow cytometer and data analyzed with the BD FACSDiva Software. Stained bacteria can be assayed in a flow cytometer equipped with a laser emitting at 488 nm and a total of 20,000 events were recorded. Fluorescence intensity was monitored in the green and red channels; filters were used for detecting fluorescein and the Texas Red dye. The forward scatter, side scatter, and fluorescence were collected with logarithmic signal amplification.

ATP Measurement

Evaluation of the levels of ATP after the incubation of *B. cereus* and the resveratrol-*trans*-dihydrodimer were determined using the ATP

Bioluminescence Assay Kit HS II (Roche, Indianapolis, IN) in black-clear-bottom 96-well plates. Briefly, *B. cereus* cells (10^8 cells/mL) were incubated at respective MIC levels for 30 min with the resveratrol-trans-dihydrodimer, CCCP, and ciprofloxacin at 30°C. After centrifugation (8000*g*) the supernatant was removed, and cells were re-suspended with dilution buffer (50 μL) and an equal volume of cell lysis buffer was added for 5 min at room temperature. Finally, 50 μL of luciferase reagent was added and luminescence was measured at 452 nm using an integration time of 1.5 s in a Spectra Max MS Plate Reader (Molecular Devices, Sunnyvale, CA). The blank luminescence was subtracted from the raw data and ATP concentrations were calculated from a log–log plot standard curve. All experiments were performed in triplicate.

Quantification of Bacterial DNA by Flow Cytometry

Quantification of bacterial DNA was performed by FACS using DRAQ5 (Cell Signaling Technology, Danvers, MA) (Silva et al., 2010), a membrane-permeable anthraquinone dye with high affinity for double-stranded DNA to label live cells. *B. cereus* cells and *E. coli BL21** (1×10^8 cells/mL) were incubated with resveratrol-*trans*-dihydrodimer (30 μM) for 1 h at 37°C and 30°C, respectively. Cells were centrifuged (9000*g*) to remove the resveratrol-*trans*-dihydrodimer. Cells were re-suspended in PBS and incubated with DRAQ5 (5 μM) diluted 1:50 for 30 min at 30°C (for *B. cereus*), and 37°C (for *E. coli BL21**) supplemented with 4 mM EDTA (pH 7.4). Cells were centrifuged twice to remove the excess dye and re-suspended in PBS for FACS analysis (a total of 20,000 events were recorded). After the washing step, a sample of each suspension was collected for viability assessment by conventional dilution plating. The results were compared with the DNA gyrase inhibitor ciprofloxacin (15 μM) as a positive control, and CCCP (5 μM) as a negative control. FACS analysis was performed in a BD Sciences LSRII flow cytometer. Acquisition was performed with BD FACS Diva Software based on light-scatter and fluorescence signals resulting from illumination at 488 nm and 635 nm. Light-scatter and fluorescence measurements were acquired logarithmically.

In Vitro Assay of DNA Topoisomerase II (Gyrase) Activity

The activity of DNA gyrase was evaluated in vitro using the DNA Topoisomerase II (Gyrase) Assay Kit Plus (ProFoldin, Worcester, MA) following the procedure provided by the vendor. All the reactions were performed in a black-clear-bottom 96-well plate and the total volume was 40 μL /reaction. Briefly, relaxed plasmid DNA (25 μg/mL), resveratrol-*trans*-dihydrodimer (at MIC), DNA gyrase (20 nM), and ATP (1 mM) were dissolved in 1 × buffer for 1 h at room temperature with 5% DMSO. The final composition of the 1 × buffer (provided by ProFoldin) was 20 mM Tris-HCl, 35 mM NH_4OAc, 4.6% glycerol, 1 mM DTT, 0.005% Brij35, 8 mM $MgCl_2$. After 1 h, 250 μL of freshly prepared H19 dye solution (provided by ProFoldin) were added to each well for 5 min and fluorescence was measured at 535 nm using the excitation wavelength at 485 nm in a Spectra Max MS Plate Reader (Molecular Devices). The activity of DNA gyrase was normalized against the reaction in the absence of antibiotic. Positive controls using ciprofloxacin (at MIC), a well-known inhibitor of DNA gyrase, and another without ATP were used in the assay. CCCP (at MIC) was used as a negative control. All the experiments were performed in triplicate.

Reactive Oxygen Species (ROS) Detection by Flow Cytometry

Intracellular ROS detection was measured by flow cytometry using the fluorescent probe dihydrorhodamine 123 (DHR123, Sigma–Aldrich). *B. cereus* 10987 and *E. coli* BL21* (1×10^8 cells/mL) were incubated with the resveratrol-*trans*-dihydrodimer (IC_{50} and MIC) for 1 h. The cells were washed three times in PBS buffer, and DHR123 (5 μg/mL) was added to the cells for 1 h. The cells were then washed three times in PBS buffer, and transferred to flow cytometry tubes. As a positive control, both strains were incubated with ampicillin (100 μg/mL), which has previously shown to generate ROS in bacteria (10). FACS analysis was performed in a BD Sciences LSRII flow cytometer and data analyzed with the BD FACS Diva Software. Fluorescence intensity was monitored in the green channel. The forward scatter, side scatter, and fluorescence were collected with logarithmic signal amplification; a total of 20,000 events were recorded.

Results

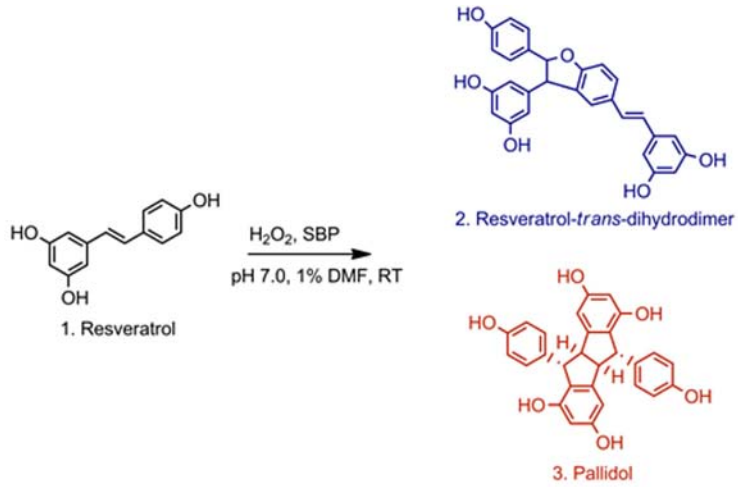
Synthesis and Antimicrobial Activity of Resveratrol-*trans*-Dihydrodimer and Pallidol

The enzymatic oligomerization of resveratrol catalyzed by SBP (Fig. 1A) results in a mixture of at least two dimers of identical molecular weights (454 *m/z*) (Supplementary Figure S1). NMR characterization of the two dimers revealed the structures of pallidol and resveratrol-*trans*-dihydrodimer (Supplementary Figures S2 and S3). The enzymatic conversion of resveratrol is >99%. LC-MS analysis did not reveal the presence of residual resveratrol. Resveratrol-*trans*-dihydrodimer was the main product (>60% of total product) while pallidol represents the second main product (~20% of total product). Other minor products were not further characterized.

The antimicrobial activities of resveratrol-*trans*-dihydrodimer and pallidol were evaluated against four bacterial strains (Gram-negative *E. coli*, and Gram-positive *B. cereus*, *L. monocytogenes*, and *S. aureus*) in the presence or absence of efflux pump inhibitors specific to each strain. Efflux pump inhibitors decrease elimination of antibiotics from bacteria enhancing their ability to eradicate pathogens (Schindler et al., 2013; Tanaka et al., 2013). Unlike pallidol, resveratrol-*trans*-dihydrodimer was active against the bacterial strains tested (Fig. 1B). In addition, the antimicrobial activity of resveratrol-*trans*-dihydrodimer was at least two orders of magnitude than that of its precursor, resveratrol (data not shown). The effect of efflux pump inhibitors was only significant in Gram-negative *E. coli* (Fig. 1B).

Dose response studies on *E. coli tolC* (a key component of the RND efflux systems driven by membrane potential in *E. coli*) deletion strain indicated increased susceptibility to the active dimer (MIC and IC_{50} values were 2.5 and 7.0 μM, respectively) (Fig. 1B).

(A)



(B)

Strain (Gram +/-)	MIC (μM)	IC_{50} (μM)	MIC +Inhibitor (μM)	IC_{50} +Inhibitor (μM)
B. cereus (+)	30.0 (13.6)	12.1 (5.5)	15.0 (6.8)	5.1 (2.3)
Listeria (+)	250.0 (113.5)	72.0 (32.7)	250.0 (113.5)	30.2 (13.6)
S. aureus (+)	62.0 (28.1)	27.1 (12.3)	62.0 (28.1)	27.2 (12.3)
*E. coli BL21** (-)	>250.0 (>113.5)	>250.0 (113.5)	250.0 (113.5)	58.9 (26.7)
E. Coli BL21 ΔTolC (-)*	7.0 (3.2)	2.5 (1.1)	3.0 (1.4)	1.2 (0.5)

Figure 1. (**A**) Scheme of the enzymatic oligomerization of resveratrol mediated by soybean peroxidase (SBP) in aqueous buffer. The major products are resveratrol-*trans*-dihydrodimer, and pallidol; (**B**) MIC and IC_{50} values of the resveratrol-*trans*-dihydrodimer against Gram-positive strains *B. cereus*, *L. monocytogenes*, *S. aureus*, and the Gram-negative *E. coli* (parenthetical values are indicated in μg/mL). Efflux pump inhibitors: Phe-Arg-β-naphthylamide (PABN, 48.1 μM) for *E. coli,* 1(1-naphthylmethyl)-piperazine (NMP, 441.9 μM) for *B. cereus*, reserpine (16 μM) for *L. monocytogenes*, and piperine (350 μM) for *S. aureus*.

This confirms that the RND efflux pumps are important in defense of Gram-negative bacteria against the active resveratrol derivative.

The effect of efflux pump inhibitors was considerably less significant against Gram-positive bacteria. Resveratrol-trans-dihydrodimer was also effective against *L. monocytogenes* and *S. aureus* (Fig. 1B). Images taken with a Nikon Eclipse TE200 microscope showed that the resveratrol-*trans*-dihydrodimer did not cause aggregation of bacteria (Supplementary Figure S4) (which could lead to an observed decrease in bacterial colonies). Morphological changes of *B. cereus* and *L. monocytogenes* were observed; conventional rod-shape bacteria were drastically altered into small structures at lethal and sub-lethal concentrations (Figure S4), which is a response of bacteria after exposure to antimicrobials for surviving. These results are consistent with previous studies with polyphenols (Figure S4) (Garcia-Ruiz et al., 2011; Paulo et al., 2010).

Effect of the Resveratrol-*trans*-Dihydrodimer on Membrane Potential

Based on the aforementioned results, the resveratrol-*trans*-dihydrodimer appears to be a unique derivative capable of acting against both Gram-positive and Gram-negative bacteria. A significant difference between the active resveratrol-*trans*-dihydrodimer and the inactive pallidol prompted us to explore the specific mechanism of action of the active molecule. To that end, we evaluated the effect of the resveratrol-*trans*-dihydrodimer at a gene regulation level in *B. cereus 10987*. RNA isolated from 8 h cell culture (Supplementary Figure S5) treated with resveratrol-*trans*-dihydrodimer (at a concentration half

of the calculated IC_{50}) was analyzed using microarray and compared against untreated cells. Genes showing a ~twofold or higher change (up or down) were of interest and validated via qRT-PCR. Only genes with clearly established biological function were examined (Supplementary Table S1). In particular, two genes (BCE2421 and BCE2420), responsible for encoding the ATP-binding cassette transporters (ABC transporters), were significantly downregulated (~3.0 and >7.0-fold from microarray and qRT-PCR, respectively).

In line with the gene regulation study, we evaluated the effect of resveratrol-*trans*-dihydrodimer on bacterial membrane potential by flow cytometry using the fluorescent dye 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$) (Lunde et al., 2009; Silverman et al., 2003), which displays green fluorescence in all bacterial cells. A fluorescent red shift occurs as the dye self-associates in the cytosol due to larger membrane potentials. A decrease in the membrane potential, leads to an increase in the ratio of green/red fluorescence.

B. cereus and *E. coli* were incubated separately with resveratrol-trans-dihydrodimer (at the MIC = 30 and 250 μM respectively) and PABN (only for *E. coli*), the known proton ionophore CCCP (as a positive control), ciprofloxacin (as a negative control) and analyzed by flow cytometry using $DiOC_2$ (cell viability was ~95% for both strains after 1 h incubation, Supplementary Figure S6). The incubation with the resveratrol-*trans*-dihydrodimer led to a decrease in red fluorescence indicating the disruption of the membrane potential and preventing the internalization of $DiOC_2$; similar results were observed with CCCP (Fig. 2). Upon quantification of the ratio of green fluorescence/red fluorescence (GF/RF), the active dimer treated cells showed an ~12-fold and ~10-fold increase in the GF/RF ratio for *B. cereus* (Fig. 2A) and *E. coli* (Fig. 2B), respectively; CCCP treated cells showed an ~16-fold and 10-fold increase in the GF/RF ratio for *B. cereus* and *E. coli* respectively, while ciprofloxacin did not show a shift in red to green fluorescence.

Membrane potential is implicated in modulating the distribution of several cell division (e.g., MinD, FtsA) and cytoskeletal (i.e., MreB) proteins; such proteins are localized at specific positions near the membrane and require energy for their maintenance (Strahl and Hamoen, 2010). Other natural phenolic compounds (i.e., carvacrol) exhibit antimicrobial activity against bacteria by disrupting the membrane potential; a previous study suggested that hydroxyl groups play a role in destabilizing the membrane potential, as well as affecting the pH gradient across the cytoplasmic membrane (Ultee et al., 2002). We may speculate that polyphenolic hydroxyl groups alter the interaction of lipids (and potentially proteins) resulting in conformational changes on the phospholipid layer leading to destabilized membrane potential as a result of more mobile membrane structure.

Effect of Resveratrol-*trans*-Dihydrodimer on DNA Replication

Inhibition of DNA replication has been reported to be a mechanism of action of several plant polyphenols (Cushnie and Lamb, 2011; Gradišar et al., 2006; Paulo et al., 2010). In particular, flow cytometry studies have revealed a slight decrease in the DNA content of *B. cereus* after incubation with resveratrol. Furthermore, our gene regulation study showed a decrease in the gene BCE0294 responsible of encoding a DNA-binding response regulator (Table I) and ABC transporters (involved in DNA repair and elongation). Therefore, we proceeded to study the effect of the active dimer on the total DNA content of the treated cells. The DNA content of live cells was quantified by flow cytometry using the red fluorescent dye DRAQ5, which permeates live cells and intercalates with double stranded DNA. Independent incubations of *B. cereus* and *E. coli* in mid-log growth phase with resveratrol-trans-dihydrodimer were performed to evaluate its impact on overall DNA amount. Ciprofloxacin, a known DNA gyrase inhibitor, was used as a positive control (Supplementary Figure S7) and CCCP as a negative control (Supplementary Figure S8). Three distributions for the cell population (regions I, II, and III) of low, medium and high fluorescence, respectively were defined (Fig. 3). A side scattered versus red fluorescence (APC) plot of untreated *B. cereus* showed a cell population of ~4% within the region of high fluorescence and 57% within the region of medium fluorescence (Fig. 3C). After treatment with resveratrol-trans-dihydrodimer, <1% of cells were highly stained; cells within

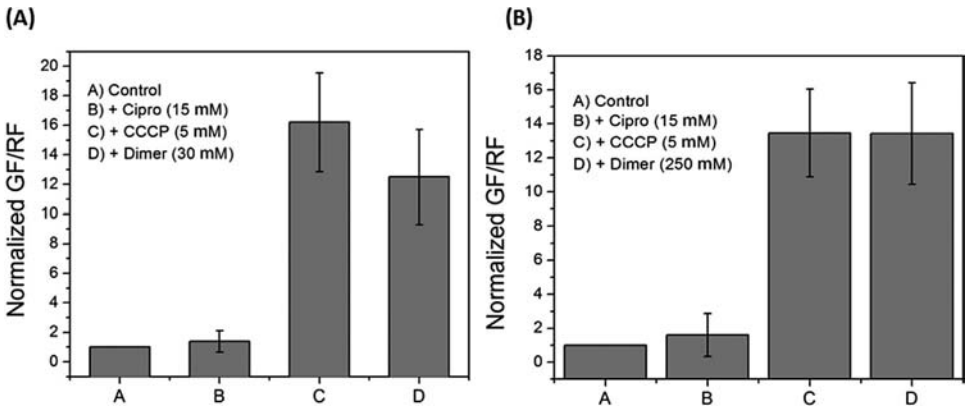


Figure 2. Effect of the resveratrol-*trans*-dihydrodimer on membrane potential. Normalized green fluorescence/red fluorescence ratio (GF/RF) of stained (**A**) *B. cereus* and (**B**) *E. coli* in presence of ciprofloxacin, CCCP, and resveratrol-*trans*-dihydrodimer. Fluorescence was measured by flow cytometry and the GF/RF ratio considers the overall fluorescence of 20,000 cells. The results were based on triplicate experiments.

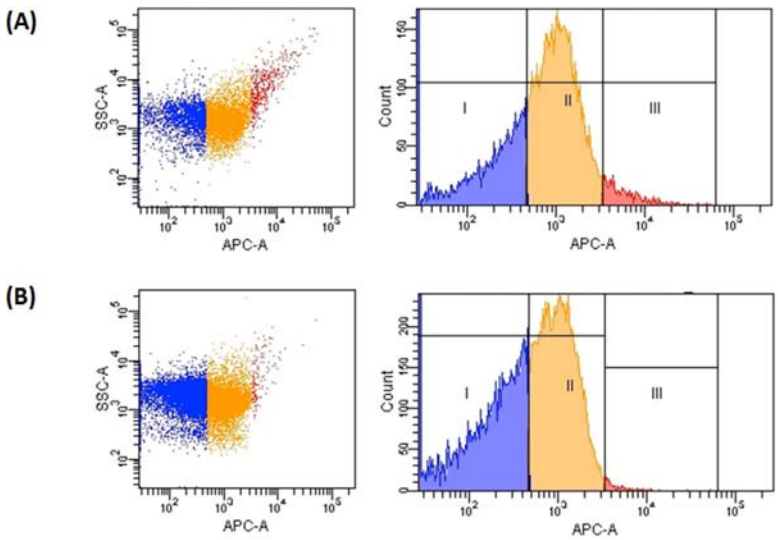
Table I. Gene regulation of ABC transporters and DNA-binding response regulator in *B. cereus*, after incubation with the resveratrol-*trans*-dihydrodimer (0.5 IC_{50}, 8 h). Data were obtained from a microarray analysis, and validated by qRT-PCR.

Gene name	Gene ID	Function	Microarray	qRT-PCR
BCE2421	2749121	ABC transporter, permease protein, putative	2.8↓	9.9↓
BCE0294	2751936	DNA-binding response regulator	2.1↓	2.0↓
BCE2420	2748761	ABC transporter, ATP-binding protein	2.7↓	8.5↓

the region of medium fluorescence decreased by ∼14% while cells in the region of low fluorescence increased by ∼18% (Fig. 3A and B). A decrease in highly stained cell population was observed in treated *E. coli* and *E. coli ΔTolC* strains (Supplementary Figure S9).

Several reports have shown that plant polyphenols (e.g., EGCG and quercetin) affect DNA replication by inhibiting DNA gyrase (topoisomerase II and IV), possibly through competitive binding to the enzyme's ATP binding site (Gradišar et al., 2006; Plaper et al., 2003). DNA gyrase is composed of two subunits, GyrA and GyrB, which relieve strain and causes negative supercoiling of DNA by binding to it as an A2B2 tetramer and hydrolyzes ATP (Brino et al., 2000). Consistent with the literature of other polyphenols, we hypothesize that resveratrol-trans-dihydrodimer could act as an inhibitor of DNA gyrase, by potentially targeting the ATP binding site. Based on sequence alignment (data not shown), the ATP binding site of all four bacterial strains tested in this work have 100% homology. Specifically, we evaluated the inhibitory effect of the resveratrol derivative on DNA gyrase activity in vitro using an isolated topoisomerase II from *E. coli* (Fig. 4A). The results were compared to ciprofloxacin (a known DNA gyrase inhibitor, and used as a positive control), CCCP (as a negative control), and a reaction carried out in the absence of ATP. Over 90% inhibition of DNA gyrase was induced by resveratrol-*trans*-dihydrodimer (Fig. 4B), which is comparable to the inhibitory effect of ciprofloxacin. In contrast, CCCP did not show any significant inhibition on the enzymatic activity and no enzymatic activity was detected in the absence of ATP.

To further investigate our hypothesis, we performed molecular modeling using the 24 kDa fragment of DNA gyrase subunit B (PDB ID: 1AJ6) with the grid centered at the biocin (the saccharide portion of novobiocin) binding site (See Supplementary Information for detailed description of docking analysis). Based on docking studies, resveratrol-trans-dihydrodimer can form hydrogen bonds with Asp73 and Gly117 residues (Fig. 5). The docking analysis



(C)

	B. cereus	*B. cereus* + resveratrol-*trans*-dihydrodimer
I	7752 (38.8%)	11269 (56.3%)
II	11338 (56.7%)	8598 (43%)
III	900 (4.5%)	133 (0.7%)

Figure 3. Flow cytometry genomic DNA quantification for *B. cereus*. (**A**) Untreated *B. cereus* + DRAQ5; (**B**) *B. cereus* incubated with resveratrol-*trans*-dihydrodimer (at MIC) + DRAQ5; (**C**) Cell quantification in different regions (I, II, and III) of red-fluorescence level.

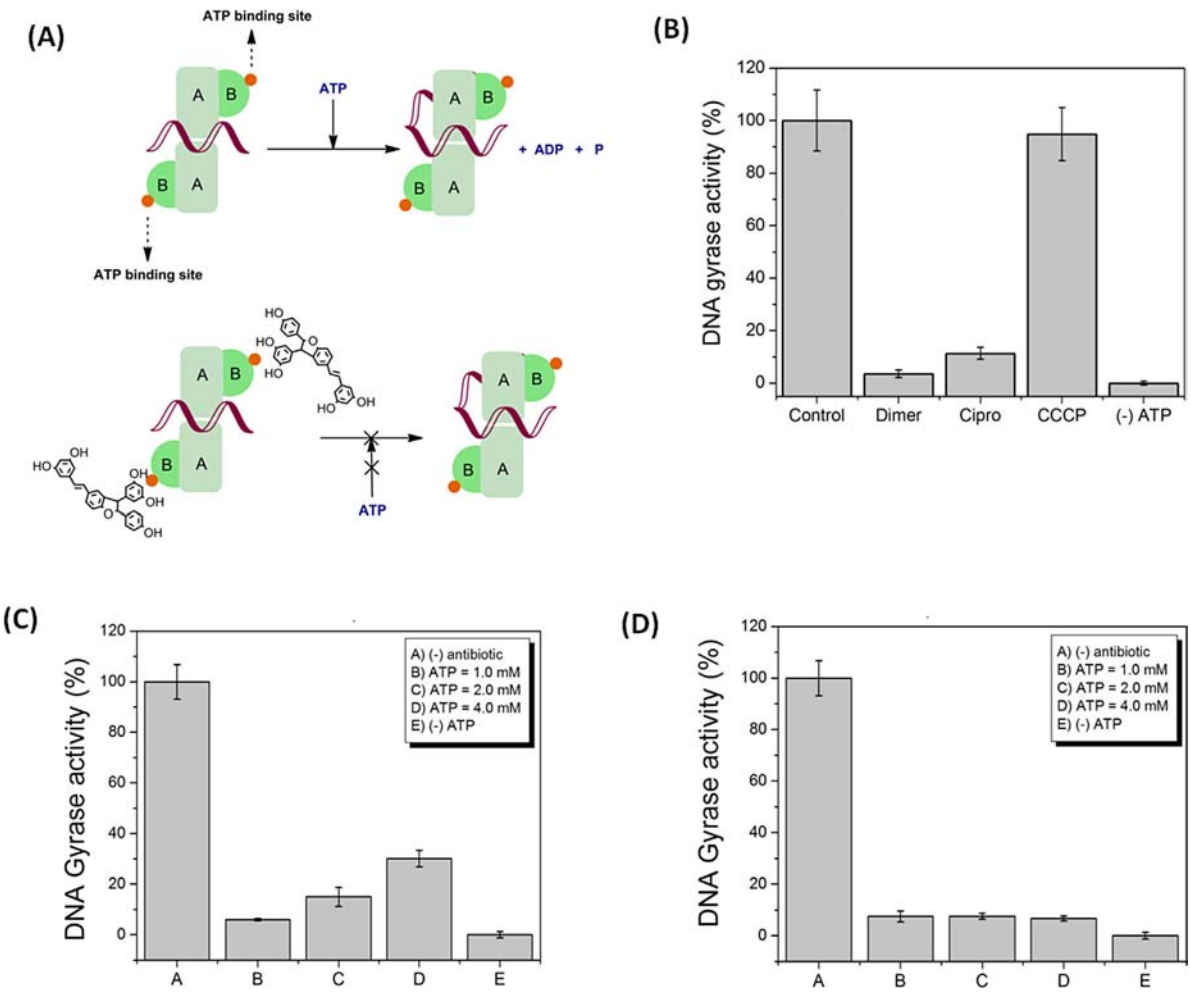


Figure 4. (**A**) Scheme representing negatively supercoiling of DNA by DNA gyrase and the enzymatic inhibition by resveratrol-*trans*-dihydrodimer using the ATP binding site as a potential target; (**B**) in vitro enzymatic activity of DNA gyrase from *E. coli* (1 mM ATP) after incubation with resveratrol-*trans*-dihydrodimer (MIC), CCCP (5 μM), ciprofloxacin (15 μM) and a control in the absence of ATP; (**C**) DNA gyrase activity using different ATP concentrations (1.0, 2.0, and 4.0 mM) incubated with resveratrol-*trans*-dihydrodimer (MIC) and (**D**) ciprofloxacin (15 μM).

suggests that resveratrol-*trans*-dihydrodimer fills the pocket for ATP binding (Supplementary Figure S10A). Resveratrol also docks within the ATP binding site but does not block ATP (Figure S10B) from accessing this pocket.

ATP and ROS Measurement

Many membrane potential disrupting antibiotics have been reported to cause a decrease in ATP levels in bacteria (Strahl and Hamoen, 2010; Ultee et al., 2002), likely as a result of since ATP synthesis is a membrane potential driven process (Kaim and Dimroth, 1998). However, it should be noted that not all membrane potential disrupting antibiotics exhibit the same effects, as this depends on the chemical structure and specific interactions with the membrane components. In addition, induction of oxidative stress in bacteria has been shown to result in variation of total ATP content of bacterial cells causing a rapid initial increase in ATP concentrations (∼twofold), followed by ATP dissipation (Dahan-Grobgeld et al., 1998; Dwyer et al., 2007).

Thus, to gain more insight into the biochemical effects of resveratrol-*trans*-dihydrodimer, ATP and ROS levels in treated cells were studied by luminescence and flow cytometry, respectively. Resveratrol-*trans*-dihydrodimer caused a ∼2.5-fold increase in ATP levels in *B. cereus* (Fig. 6A). In contrast, the active dimer caused a decrease in ATP levels in *E. coli*, comparable to the ATP depletion observed upon treatment with CCCP (positive control) (Fig. 6A). Ciprofloxacin (negative control) did not affect ATP levels in either *B. cereus* or *E. coli*.

ATP levels can also be increased within bacteria as a consequence of ROS generation, which act as inhibitors of ATPases. Flow cytometry studies using the fluorescent dye dihydrorhodamine 123 (DHR123) were performed to identify ROS in *B. cereus* and *E. coli* after incubation with resveratrol-trans-dihydrodimer at IC_{50} and MIC (Fig. 1B). FACS analysis revealed a shift of green fluorescence

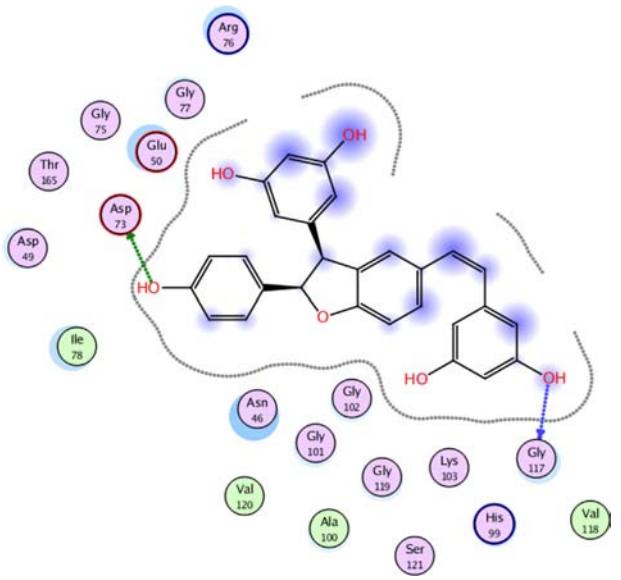


Figure 5. Two-dimensional display of ATP binding site of DNA gyrase from *E. coli* (PDB ID 1AJ6) interacting with resveratrol-*trans*-dihydrodimer obtained by docking; hydrogen bonds are shown explicitly.

signal in *B. cereus* (Fig. 6B) after incubation with resveratrol-trans-dihydrodimer at the MIC indicating the presence of ROS (Fig. 6D). ROS generation was not detected in *E. coli* upon treatment with the dimer at MIC or IC_{50} (Figs. 6C and D).

Bactericidal drugs kill bacteria partly by induction of ROS formation (Foti et al., 2012; Kohanski et al., 2007). The detection of ROS in treated *B. cereus* is a common signal in cellular death pathways (Kohanski et al., 2007). Production of oxygen radicals is known to attack polyunsaturated fatty acids in membranes and cause lipid peroxidation leading to changes in, for example, membrane fluidity and ultimately membrane degradation (Cui et al., 2012). In contrast, *E. coli* cells showed a decrease in ATP levels (Fig. 6A) and no increase in bacterial fluorescence (previously incubated with DHR 123) indicating the absence of ROS (Figs. 6C and D).

Cytotoxicity of the Resveratrol-*trans*-Dihydrodimer

Given that resveratrol-*trans*-dihydrodimer impacts gene expression and/or interacts with essential proteins involved in cellular metabolism, we evaluated the cytotoxicity of the resveratrol derivative against mammalian cells. For this purpose, we incubated the resveratrol-*trans*-dihydrodimer (0 to 1.0 mM) with HepG2 cells and used a lactate dehydrogenase (LDH) assay to quantify cell viability (See Supplementary Information for detailed description of methodology). The viability of HepG2 cells in the presence of resveratrol-*trans*-dihydrodimer (Supplementary Figure S11) was compared to that after incubation with resveratrol. Cell viability was ~80% after 72 h of incubation with both resveratrol and the active dimer (at the highest concentration; 1.0 mM). This value is higher than the obtained MIC values indicating a potentially wide therapeutic window.

Discussion

We show that the in vitro diversification of resveratrol mediated by SBP can be used as a tenable strategy to generate oligomeric polyphenolic molecules with enhanced bioactivity. In particular, the resveratrol-*trans*-dihydrodimer generated in this study was identified as having antimicrobial activity against Gram-positive bacteria and low cytotoxicity against mammalian cells. We suggest that this oligomerization approach can be expanded to other plant polyphenols with poor antimicrobial properties and potentiate their activity.

Transcriptomic analysis revealed the down regulation of couple essential genes. The BCE2420 gene encodes an ABC transporter responsible for the export of macrolides, lipoprotein and cell division protein across the membrane, and also possesses ATPase activity. BCE2421 encodes for FstX, the transmembrane domain of the putative *B. cereus* ABC-transporter. FstX is known to play an important role in bacterial cell division and differentiation by localizing the septal ring (Davidson et al., 2008; De Leeuw et al., 1999). ABC systems also couple the energy of ATP hydrolysis to perform several non-transport related processes, such as translation, DNA elongation and repair (Davidson et al., 2008). The downregulation of the putative ATP binding cassette transporters suggests increased uptake of the active compound.

It has been suggested that hydroxyl groups play a role in destabilizing the membrane potential, as well as affect the pH gradient across the cytoplasmic membrane (Ultee et al., 2002), and some natural phenolic compounds (e.g., carvacrol) have exhibited antimicrobial activity against bacteria by disrupting the membrane potential. Disrupting membrane potential has critical implications on spatial organization of cell division and cytoskeletal proteins within bacteria (Strahl and Hamoen, 2010).

In line with the microarray data, we observed an overall decrease in the total DNA content of the treated cells and the active dimer showed high inhibitory effect (~90% inhibition) against bacterial DNA gyrase in vitro (Fig. 4B).

Antibacterials that target DNA gyrase act by: (1) inhibiting the binding of the enzyme to DNA or preventing hydrolysis of ATP, resulting in impairment of the enzyme activity (e.g., simocyclinone and novobiocin); or (2) binding to the enzyme-DNA complex converting the enzyme into a lesion-inducing agent (e.g., ciprofloxacin), which is also known as gyrase poisoning (Collin et al., 2011). An increase in ATP concentration in the presence of the resveratrol-*trans*-dihydrodimer resulted in a recovery of up to 30% of DNA gyrase activity (Fig. 4C). As a control, the same assays were performed using ciprofloxacin (a DNA gyrase inhibitor), and as expected no increase in DNA gyrase activity was observed as a function of increased ATP concentrations (Fig. 4D). Since we were able to recover ~30% of the DNA gyrase activity upon increasing ATP concentrations we suggest that the active dimer inhibits DNA gyrase by preferentially replacing ATP from its ATP binding site and not by DNA gyrase poisoning (Fig. 4). Moreover, molecular modeling carried out for understanding the interaction between resveratrol-*trans*-dihydrodimer and the 24 kDa fragment of DNA gyrase subunit B from *E. coli* indicated that the resveratrol-*trans*-dihydrodimer can form hydrogen bonds with Asp73 and Gly117 residues (Fig. 5). The active dimer can fill the pocket for ATP

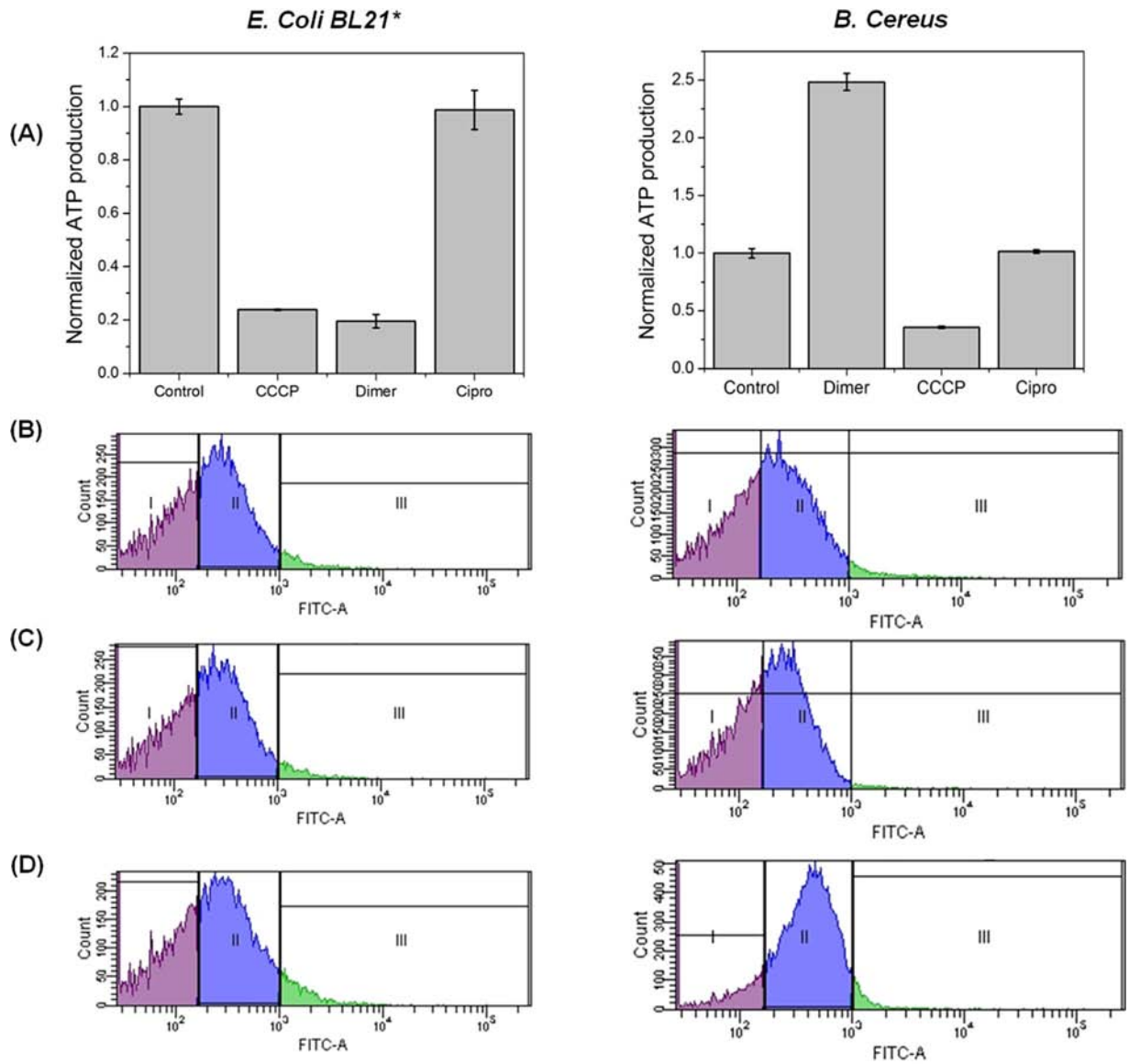


Figure 6. (**A**) Total ATP measurement after incubating *E. coli* and *B. cereus* with resveratrol-*trans*-dihydrodimer (MIC), CCCP (5 μM), and ciprofloxacin (15 μM). Determination of ROS in bacteria by flow cytometry. (**B**) Untreated *E. coli* BL21* and *B. cereus* 10987; (**C**) Treated *E. coli* BL21* and *B. cereus* 10987 with resveratrol-*trans*-dihydrodimer (IC_{50}); (**D**) Treated *E. coli* BL21* and *B. cereus* 10987 with resveratrol-*trans*-dihydrodimer.

binding site (Figure S10A). Resveratrol also docks within the ATP binding site but does not block ATP from accessing this pocket (Figure S9B). These results support our hypothesis that the resveratrol-*trans*-dihydrodimer inhibits DNA gyrase activity by targeting the ATP-binding site of the enzyme.

In conclusion, we show that the peroxidase-catalyzed oxidation of resveratrol leads to a dimeric polyphenol with antimicrobial activity. This methodology may be used in a broader strategy to generate new oligomeric polyphenols, including with plant polyphenols with poor antimicrobial properties to potentiate their activity. Resveratrol-*trans*-dihydrodimer was identified as an active antimicrobial against Gram-positive bacteria with minimal mammalian cytotoxicity (against the HepG2 cell line). Mechanistically, resveratrol-*trans*-dihydrodimer may have at least two modes of action: (1) disruption of the membrane potential; and/or (2) inhibition of DNA replication through the inhibition of DNA gyrase. Our results suggest that the active dimer down-regulates two ABC transporters that play critical roles in cell division and transport of molecules across the membrane. However, independent measurements of ROS and ATP in both *B. cereus* and *E. coli* showed opposite results. While measurements in *E. coli* showed a decrease in ATP levels and ROS were not detected, ATP levels increased in *B. cereus* and ROS were generated and contributed to the lethality of the antimicrobial agent (Dwyer et al., 2014), and potentially inducing a programed cell death pathway mechanism (Brynildsen et al., 2013). Furthermore, resveratrol-trans-dihydrodimer acts as a strong inhibitor of DNA gyrase and to preventing DNA replication. Experimental and molecular modeling data

suggest that resveratrol-trans-dihydrodimer the resveratrol dimer acts as a competitive inhibitor of the ATP binding site of DNA gyrase. The multiple targets of action could help the active dimer to fight effectively against bacteria.

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