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Novel, Biocatalytically Produced Hydroxytyrosol Dimer Protects against Ultraviolet-Induced Cell Death in Human Immortalized Keratinocytes

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ABSTRACT: Compounds derived from botanicals, such as olive trees, have been shown to possess various qualities that make them function as ideal antioxidants and, in doing so, protect them against the damaging effect of ultraviolet (UV)-derived oxidative stress. The aim of this study was to biocatalytically synthesize a dimeric product (compound **II**) from a known botanical, 3-hydroxytyrosol, and test it for its antioxidant ability using a human immortalized keratinocyte cell line (HaCaT). 2,2-Diphenyl-picryhydrazyl (DPPH) antioxidant assays showed 33 and 86.7% radical scavenging activity for 3-hydroxytyrosol and its dimer, respectively. The ferric-reducing antioxidant power (FRAP) assay corroborated this by showing a 3-fold higher antioxidant activity for the dimer than 3-hydroxytyrosol. Western blot analyses, showing cells exposed to 500 μM of the dimeric product when ultraviolet A (UVA)-irradiated, increased the anti-apoptotic protein Bcl-2 expression by 16% and reduced the pro-apoptotic protein Bax by 87.5%. Collectively, the data show that the dimeric product of 3-hydroxytyrosol is a more effective antioxidant and could be considered for use in skin-care products, health, and nutraceuticals.

KEYWORDS: *Biocatalysis, reactive oxygen species, antioxidants, UV, keratinocytes*

INTRODUCTION

The medicinal properties of *Olea europaea* (olive tree) have been known for centuries. Increasingly, the constituents of this tree, particularly hydroxytyrosol and its metabolites, have gained recognition for their antioxidant, antiatherogenic, and antitumorigenic properties[1] in both the pharmaceutical and cosmetic industries.[2] Despite human skin possessing efficient antioxidant systems, such as superoxide dismutase (SOD), catalases (CAT), and glutathione peroxidase (GSH),[3] exposure to environmental factors, such as ultraviolet light radiation (UVR), specifically ultraviolet A (UVA, 320−400 nm), results in increased intracellular free radicals, which overwhelm these endogenous systems.[4] It is therefore imperative in situations such as these to enhance the antioxidant capacity of the skin cells through the addition of exogenous antioxidants. Despite the polyphenolic hydroxytyrosol being touted as an effective antioxidant,[5] biocatalytically produced derivatives may prove to be even more efficacious in their endogenous antioxidant ability to protect against long-wavelength UVR. To this end, the use of plant-derived polyphenols has been widely used.[6] The effect of polyphenols to protect the skin against the damaging effects of ultraviolet (UV) has been highlighted in a number of recent papers. Yu et al. examined the antioxidant activity of bran extracts growing in three locations in Colorado and found that wheat was able to significantly reduce lipid peroxidation *in vitro*. This suggested that wheat-based products show great potential in suppressing oxidation of biological substrates *in vivo*.[7] In addition, a study on green tea polyphenolic antioxidants, using both chronic oral feeding and topical application of green tea polyphenols, resulted in significant protection against UVR-induced cutaneous edema and erythema, lipid peroxidation, and depletion of the epidermal antioxidant defense enzyme system.[8−10]

The purpose of this study was 2-fold. First, to biocatalytically synthesize oxidized oligomeric products using isolated enzymes, tyrosinase from *Agaricus biosporus* and laccase from *Trametes pubescens*. Oxidation of tyrosol by tyrosinase yielded 3-hydroxytyrosol (compound **I**), and further oxidation by laccase yielded a dimer (compound **II**) with a molecular weight (MW) of 306. Second, this study shows that this novel, dimeric derivative exhibits higher antioxidant activity than its parent compound (3-hydroxytyrosol) and mechanistically protects human skin keratinocyte cells (HaCaT, a gift from Professor Fusenig, Germany)[11] against damaging effects of UVA-induced stress. Overall, we propose a mechanism for the protection of these cells against UV-induced stress and believe that the data contributes in a novel way toward inclusion of these compounds in formulations to enhance future antioxidant therapies.

MATERIALS AND METHODS

Safety. All tissue culture was conducted in an appropriate biohazard facility, and all of the necessary precautions were strictly adhered.

Chemicals. All chemicals, unless stated otherwise, were obtained from Sigma-Aldrich (Pty), Ltd. (Johannesburg, South Africa).

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Extraction of Tyrosinase from *A. biosporus*. Tyrosinase was partially purified according to the standard method.[12] The activity of tyrosinase was determined by monitoring the production of dopachrome at 475 nm in 3 mL of 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA) in 50 mM potassium phosphate buffer (pH 7).[13] One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of dopachrome from L-DOPA at a rate of 1 μmol min^{-1}, where the extinction coefficient is 3600 M^{-1} min^{-1}. A Unicam Merck UV/vis spectrophotometer (Merck, Hitachi, Darmstad, Germany) was used for all spectrophotometric assays. The protein concentration in the enzyme extract was determined using the Bradford method.[14]

Maintenance and Growth of *T. pubescens*. A culture of *T. pubescens* (CBS 696.94) obtained from the Boku Institute in Austria, was grown on agar containing 50 g/L malt extract agar, supplemented with a laccase inducer [1% phenol mixture (phenol, 82.8 mM; *p*-cresol, 25.8 mM; and *o*-cresol, 77.03 mM)].[15] *T. pubescens* mycelial blocks were aseptically inoculated onto the plates and incubated at 28 °C for 6 days.

Mycelial plugs from agar plates were homogenized in 200 mL *Trametes*-defined medium (TDM) and inoculated aseptically into flasks. Cultures were incubated at 28 °C with agitation at 180 rpm.

Large-Scale Production of Laccase in Airlift Bioreactors. Large-scale production of laccase was performed according to the method by Ryan et al.[15] Briefly, 200 mL of a 9-day-old culture was used to inoculate 3.6 L of TDM (supplemented with 0.03% antifoam) in the airlift bioreactor. The culture medium was monitored daily for laccase activity. At 5 days post-inoculation, the medium was supplemented with 30 mL of phenol-inducing mixture (phenol, 82.8 mM; *p*-cresol, 25.8 mM; and *o*-cresol, 77.03 mM) and 1 g of glucose was added daily to the medium. After 13 days, the medium containing laccase with activity of 2−3 units/mL, was harvested by centrifugation at 10 000 rpm for 10 min.

Isolation of the Laccase Enzyme. The laccase in the culture medium was precipitated using acetone. For acetone precipitation, 300 mL of cold acetone was added to 300 mL of culture medium. The mixture was then kept at −20 °C for 30 min. The precipitated protein was recovered by centrifugation at 10 000 rpm for 15 min at 4 °C. The pellet was resuspended in 0.1 M sodium acetate buffer at pH 5 and stored at −20 or 4 °C. The protein solution was then dialyzed against the 0.1 M sodium acetate buffer (pH 5) for 2 days. Laccase activity was determined with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Roche, Johannesburg, South Africa) as the substrate.[16] The assay mixture contained 0.33 mL of 5 mM ABTS, 2.5 mL of 0.1 M sodium acetate buffer (pH 5), and 0.17 mL aliquots of sample. Oxidation of ABTS was monitored by following the increase in absorbance at 420 nm. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS/min at 25 °C.

Oxidation of Tyrosol by Tyrosinase To Yield 3-Hydroxytyrosol. Tyrosol (0.30 g) and ascorbic acid (2.04 g) were added to 240 mL of 500 mM potassium phosphate buffer (pH 7), followed by the addition of 80 units of tyrosinase.[17] The reaction was shaken at 180 rpm, at 30 °C, and samples were taken periodically and analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Oxidation of 3-Hydroxytyrosol by Laccase To Yield Dimer. 3-Hydroxytyrosol (1 g) was added to 200 mL of 0.1 M sodium acetate buffer (pH 5) and 200 mL of methanol. To initiate the reaction, 80 units of laccase was added, covered in foil, and shaken at 180 rpm at 30 °C. Samples were taken periodically and analyzed by TLC and HPLC.

The reactions were monitored by TLC analysis with eluent chloroform/ethyl acetate/formic acid solution (5:4:1).

HPLC−MS Analysis of Oxidation Products. Liquid chromatography−mass spectrometry (LC−MS) was performed on a Dionex HPLC system (Dionex Softron, Germering, Germany) equipped with a binary solvent manager and autosampler coupled to a Bruker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The products were separated using a linear gradient of acetonitrile (solvent B) and 0.1% formic acid (solvent A; BDH Chemicals, Heidelberg, Germany) at a flow rate of 0.001 L/min, using an injection volume of 10 μL and an oven temperature of 30 °C. The gradient was set as follows: 95% solvent A and 5% solvent B (0−2 min), 20% A and 80% B (2−25 min), 100% B (25−30 min), and 95% A and 5% B (30−40 min). The mass spectrometer was operated in negative ionization mode with an electrospray capillary voltage of +3500 V. The dry gas flow was set to 9 L/min with a temperature of 350 °C. The nebulizer pressure was set at 35 psi. The masses were scanned from 100 to 1000 atomic mass units (amu).

Nuclear Magnetic Resonance (NMR) Analysis. NMR spectra were recorded using a Varian 600 MHz NMR spectrometer (^{1}H, 600 MHz; ^{13}C, 125 MHz) (Varian, Palo Alto, CA). The spectra were determined at ambient temperature in deuterated chloroform ($CDCl_3$) and methanol solutions, with $CHCl_3$ at δ 7.26 for ^{1}H NMR spectra and chloroform at δ 77.00 for ^{13}C NMR spectra as internal standards. In the NMR spectra, assignments of signals with the same superscripts are interchangeable. Splitting patterns are designated as "s", "d", "t", "q", "m", and "br s". These symbols indicate "singlet", "doublet", "triplet", "quartet", "multiplet", and "broad singlet".

Antioxidant Assays. All of the antioxidant assays were conducted in triplicate and biologically repeated 3 times (n = 3).

2,2-Diphenyl-picryhydrazyl (DPPH) Radical Assay of the Antioxidant Activity. A total of 0.003 mmol of the respective antioxidant was added to 3.9 mL of DPPH solution (0.025 g/L in methanol). The decrease in absorbance at 515 nm was monitored until the reaction reached a steady state.[18]

Percentage of Radical Scavenging Activity of the Product Calculated as Follows.[19]

$$\frac{\text{initial absorbance} - \text{final absorbance}}{\text{initial absorbance}} \times 100$$

Ferric-Reducing Antioxidant Power (FRAP) Assay of the Antioxidant Activity. A total of 0.003 mmol of the respective sample was added to 2.5 mL of 500 mM potassium phosphate buffer (pH 7). A total of 2.5 mL of potassium ferricyanide was added and incubated at 50 °C for 20 min. Thereafter, 10% (v/v) trichloroacetic acid was added to stop the reaction, followed by 2.5 mL of water and 0.5 mL of iron chloride. The reaction was allowed to stand for 30 min, after which the absorbance was read at 700 nm.

Low-Density Lipoprotein (LDL) Assay of the Antioxidant Activity. Oxidation of LDL was performed according to the method developed by Nardini et al.[20] LDL was dialyzed in a 200-fold volume of phosphate-buffered saline (PBS) at pH 7 in the dark for 18 h. LDL (100 μg/mL) was oxidized with 5 μM $CuCl_2$ for 4 h at 37 °C, in the presence and absence of 50 μM test antioxidant. Conjugated diene formation was measured spectroscopically at 234 nm.

Maintenance of Keratinocytes. An immortal human keratinocyte cell line (HaCaTs)[11] was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at pH 7.4 in humidified air and 5% CO_2 at 37 °C.

Cell Viability Assay. The Cell Titer XTT proliferation assay (Roche, Johannesburg, South Africa) was used to investigate the cell viability of HaCaTs following treatment with the putative antioxidants. Briefly, HaCaTs were seeded into a 96-well plate at a density of 5 × 10^3 cells/well and incubated overnight. The cells were treated with different concentrations (0−3000 μM) of the putative antioxidants (dissolved in 1% ethanol) and incubated for 24 h to investigate cytotoxic levels of the putative antioxidants if any. The XTT solution was then added, and cells were incubated for 4 h at 37 °C. The plate was then read at an absorbance of 450 nm using a multiwell reader (VERSAmax tunable microplate reader, Labotec Molecular, Midrand, South Africa) with associated SOFTmaxPRO 4.3.1 software. In addition, cells were exposed to concentrations of the compounds that showed nontoxicity for 3 days to investigate the long-term effects. Absorbance readings were converted into percentage of the untreated control values. Results are reported as the mean ± standard deviation (SD) of three separate biological experiments (n = 3).

UVA Irradiation of HaCaT Cells. Human immortalized keratinocyte (HaCaT) cells were grown to 80% confluency and then incubated for 18 h with the different concentrations of the putative

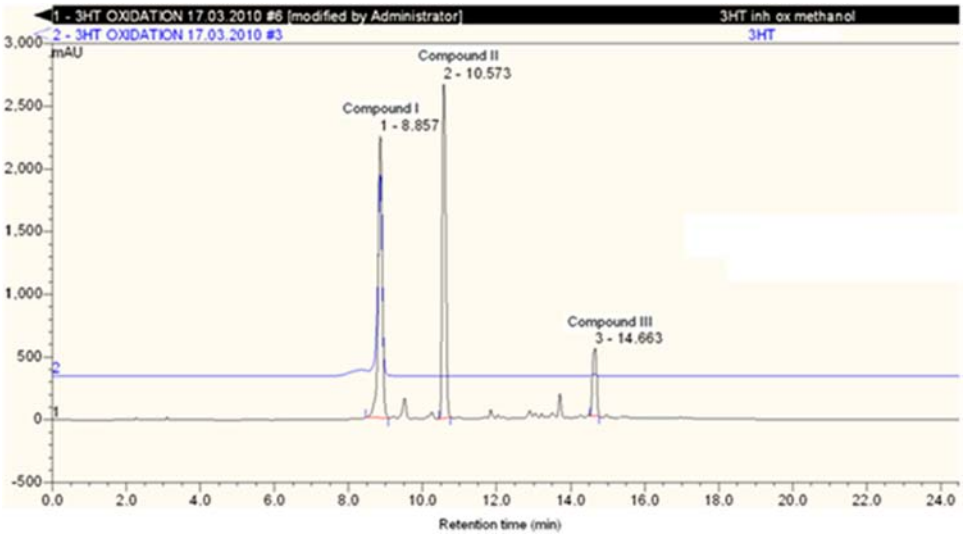

Figure 1. HPLC chromatogram and LC−MS analysis of the oxidation of 3-hydroxytyrosol (compound **I**) by *T. pubecsens* laccase to produce a dimer (compound **II**, 5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) and a trimer (compound **III**). 3HT, 3-hydroxytyrosol; ox, laccase-catalyzed oxidation.

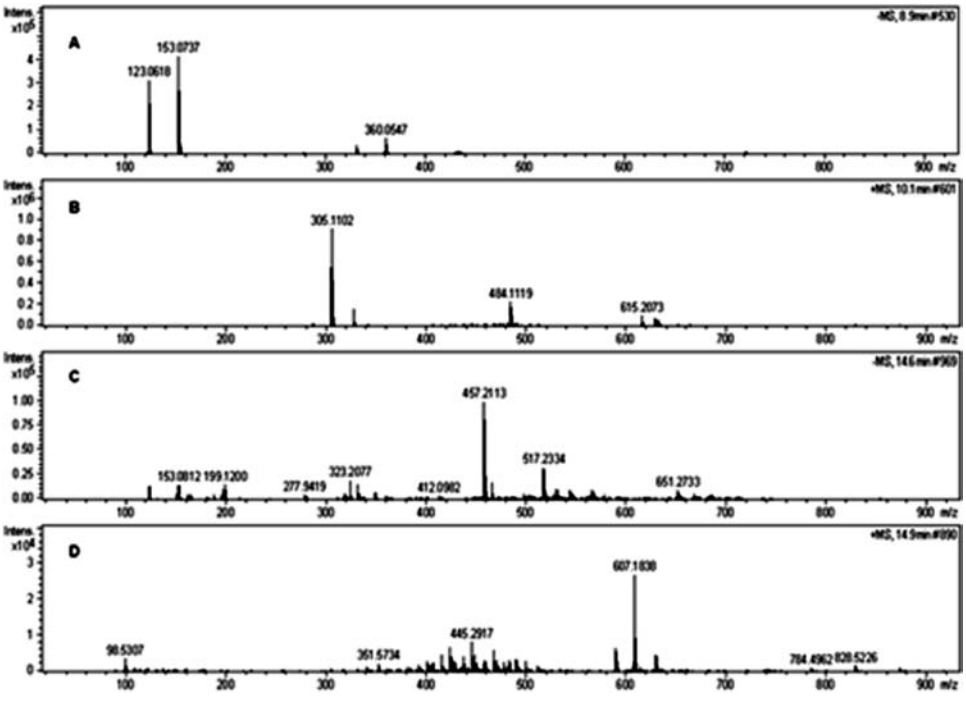

Figure 2. Mass spectra of (A) 3-hydroxytyrosol and its oxidation products (B) dimer (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol), (C) trimer, and (D) tetramer.

antioxidant dissolved in 1% ethanol and diluted in DMEM. At the end of the incubation, the medium was removed and cells were washed and replaced with 3 mL of PBS. The cells were then exposed to UVA, performed using a calibrated UVA light source (315−400 nm) (Waldmann, Dortmund, Germany) at a dose of 22.3 J/cm^2. Subsequent to irradiation, the cells were allowed to recover for 2 h at 37 °C in medium.

Protein Extraction Following UVA Exposure. Following the recovery period, the media was removed and the cells were placed on ice, washed with PBS, and prepared for western blot analyses. Proteins were electrotransferred onto a nitrocellulose membrane and probed with the primary antibodies of interest (concentrations of primary and secondary antibodies are listed in the figure captions). Protein expression was quantified from bands using densitometric analyses. Western blot analyses were conducted on two biological repeats, and the quantification was presented as mean densities.

Flow Cytometric Analysis: Reactive Oxygen Species (ROS) Assay. To quantify the amount of ROS, dihydrorhodamine 123 (DHR 123, Invitrogen, Carlsbad, CA) was added and cells were incubated at 37 °C for 30 min. The cells were then washed once with PBS and trypsinized. The pellet was washed twice with PBS and centrifuged at 3500 rpm for 5 min. A total of 10 000 events were then analyzed at 488 nm on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with the associated software CELLQuest software (Becton Dickinson, Franklin Lakes, NJ). Results are reported from three separate biological experiments (n = 3).

RESULTS AND DISCUSSION

Oxidation of Tyrosol by Tyrosinase To Yield 3-Hydroxytyrosol. The method used here for the oxidation of tyrosol to 3-hydroxytyrosol using a biocatalyst is advantageous in that the biocatalysis eliminates the use of toxic reagents required for chemical synthesis, the substrate tyrosol is cheaper than the precursors needed for chemical synthesis of 3-hydroxytyrosol, and the process is considered natural.[21] In addition, the product formed during chemical synthesis is usually rapid, but the compound obtained has to be purified further using chromatography.[22,23] Therefore, the enzymatic synthesis of 3-hydroxytyrosol can be used as an alternative procedure to obtain 3-hydroxytyrosol.

Mushroom tyrosinase catalyzed the hydroxylation of tyrosol to give 3-hydroxytyrosol (t_R, 8.857; m/z, 153.0737; and exact mass, 154.1) (Figure 2A). The results obtained were in agreement with previous reports.[17,21,23]

Oxidation of 3-Hydroxytyrosol by Laccase. Hydroxytyrosol was oxidized using the laccase from *T. pubescens* in sodium acetate buffer containing 50% methanol. This ratio of methanol (50%) to buffer was chosen on the basis of the work by Ncanana.[17]

HPLC analysis of the oxidation of 3-hydroxytyrosol (compound **I**) showed one major product, 5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol; yield, 33.9 ± 3.06%; t_R, 10.573 (hereafter designated as compound **II**; Figure 1). A minor product (compound **III**; yield, 5.5 ± 1.54%) was observed at retention time t_R of 14.663. The rest of the products were polymeric compounds. LC−MS analysis showed that this main product (compound **II**) was a dimer of 3-hydroxytyrosol (observed $[M - H]^-$ ion signals at m/z, 305.1102; calculated [M], 306.1; Figure 2B). The $[M - H]^-$ ion signals of the minor product (compound **III**; m/z, 457.2113; Figure 2C) suggest the formation of a trimer of 3-hydroxytyrosol ([M] 458.2). An additional $[M - H]^-$ ion signal was observed at m/z 607.1838 (Figure 2D), which could indicate the formation of a tetramer. This product was only observed with the more sensitive MS detector (not observed with HPLC analysis).

NMR analysis (Table 1) showed that the dimer (compound **II**) was formed through 5−5 (C−C) coupling to form 5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol (Figure 3). The methylene protons of the ethyl alcohol side chain appeared as a triplet at δ 2.75 with coupling of 7.2 Hz to the adjacent aryl methylene protons, which appeared at δ 3.80 with similar coupling. The aliphatic hydroxyl groups appeared as a broad single peak at δ 4.80. Two *meta*-coupled doublets (J = 2.2 Hz) appeared at δ 7.42 and 7.67 for H-4 and H-6, respectively. The four phenolic protons were not observed because of the very low concentration of product in $CDCl_3$, which exchanged with residual $CHCl_3$. The symmetry in ^{13}C NMR was quite dramatic, with signals appearing at δ 38.0 because of the aryl methylene C and at 67.1 for the C of the ethyl alcohol side chain. As expected, six signals were observed in the aromatic region. The two strongest signals as a result of the C−H groups were observed at δ 127.6 and 129.4 assigned to C6 and C4, respectively. The weaker signals at 121.1, 131.3, 137.9, and 146.5 were assigned to C3, C2, C1, and C5, respectively, on the basis of calculations from a predictor program.

Table 1. NMR Spectra Data for the Dimer (5,5′-Bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) Formed through Laccase-Catalysed Oxidation of 3-Hydroxytyrosol[a]

^1H and ^{13}C NMR spectra data	
^1H δ	^{13}C δ
2.75 (4H, t, J = 7.2 Hz, $-CH_2OH$)	38.0 ($PhCH_2-$)
3.80 (4H, t, J = 7.2 Hz, $PhCH_2$)	67.1 (CH_2OH)
4.80 (2H, br s, $2\times CH_2OH$)	121.1 (C3)
7.42 (2H, d, J = 2.2 Hz, H-4)	127.6 (C6)
7.65 (2H, d, J = 2.2 Hz, H-6)	129.9 (C4)
	131.3 (C2)
	137.9 (C1)
	146.5 (C5)

[a]Structure numbering only refers to NMR assignments and not the conventional numbering of phenolic compounds.

The hydroxyl group on the benzene ring is *ortho*- or *para*-directing,[24−26] and molecules with a free C-5 position usually dimerize through 5−5 linkages because of the stability of C−C bonds[27] and low heat of formation of 5−5 linkages when compared to ether linkages.[28] In addition, the 3-hydroxytyrosol structure resembles lignin monomers that are known to form oligomers through 5−5 and 4−O−5 linkages.[29] In our previous work, we have reported the formation of 5−5 linkages during laccase-catalyzed coupling of functional molecules to lignin model compounds.[30,31] It was therefore not surprising that the oxidized 3-hydroxytyrosol coupled through 5−5 (C−C) linkages (Figure 3).

Antioxidant Activity of Phenolic Products as Assessed by *in Vitro* Assays. In this study, three different antioxidant assays (DPPH, FRAP, and LDL) were used to assess the putative antioxidant ability of both 3-hydroxytyrosol and 5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol (compound **II**).

Both compounds (3-hydroxytyrosol) and compound **II** were found to be effective radical scavengers against the DPPH radical, with radical scavenging activities of 33.2 and 86.7%, respectively, relative to the standard, ascorbic acid (Table 2). In addition, compound **II** showed higher antioxidant activity compared to the starting material, compound **I**. This increased radical scavenging activity suggest that the laccase-catalyzed dimerization resulted in a product with increased antioxidant activity. Dimerization or polymerization also results in an increase in the number of hydroxyl groups per molecule, which is thought to contribute to the increased antioxidant activity.[32]

The kinetic behavior of the putative antioxidants were classified as follows: <5 min (rapid), 5−30 min (intermediate), and >30 min (slow).[33] Compounds **II** and **I** (3-hydroxytyrosol) showed intermediate behavior at 7 and 5 min, respectively.

Results obtained from the FRAP assay indicated that compound **II** showed the highest antioxidant activity, with an antioxidant activity equivalent to 363 mg/L of ascorbic acid compared to 3-hydroxytyrosol, with an antioxidant activity equivalent to 124 mg/L (Table 3). Thus, the ferric-reducing power increased with an increase in the molecular weight of the putative antioxidants, and the dimer (compound **II**) showed a higher antioxidant activity than its substrate precursor, 3-hydroxytyrosol. Compound **II** may therefore be a strong electron donor, which terminates oxidation chain reactions by reducing the Fe complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the ferrous form.[34]

In the LDL oxidation assay, the compounds showed an absorbance-decreasing effect (Figure 4). Both compounds

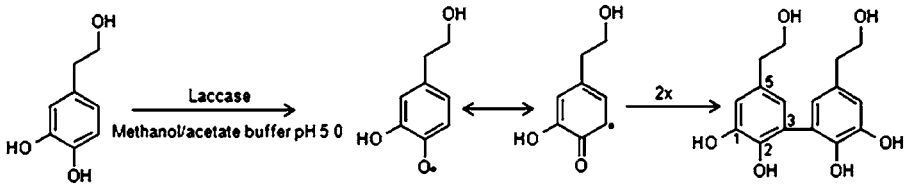

Figure 3. Proposed mechanism for the laccase-catalyzed oxidation of 3-hydroxytyrosol to form 5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol.

Table 2. Hydrogen-Donating Ability of Compound I (3-Hydroxytyrosol) and Compound II (5,5′-Bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) Using the DPPH Assay

compound name	radical scavenging activity (%)	final reaction time (min)
ascorbic acid (standard)	100	4
compound **I**	33.2	5
compound **II**	86.7	7

Table 3. Ferric-Reducing Ability of Compound I (3-Hydroxytyrosol) and Compound II (5,5′-Bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) Obtained Using the FRAP Assay, Represented Relative to the Ascorbic Acid Standard

sample name	concentration (mmol)	ascorbic acid equivalence (mg/L)
compound **I**	0.003	124
compound **II**	0.003	363

showed high inhibition of LDL oxidation compared to the standard, ascorbic acid. Thus, compounds **I** and **II** were able to prevent the chain reaction needed for diene conjugation during lipid peroxidation, rendering them as good chain-breaking antioxidants.[35]

Protective Effects of the Putative Antioxidants on Human Skin Cells. The efficacy of both the putative antioxidants (3-hydroxytyrosol and its dimeric product) were tested on UVA-treated immortalized human keratinocyte cells (HaCaTs). Their protective effects against UVA irradiation (320−400 nm) were measured by investigating cell viability, levels of ROS, and levels of proteins involved in UV-induced apoptosis.

Cell Viability. Over a 24 h period, 3-hydroxytyrosol displayed no significant cytotoxic effect on cell viability at concentrations of 200−1000 μM. In contrast, concentrations higher than 1000 μM caused a marked decrease in cell viability (Figure 5). Compound **II**, however, had no deleterious effect on cell viability at concentrations of 200−500 μM but started displaying significant cytotoxicity at concentrations exceeding 500 μM (Figure 5). It should be noted that compound cytotoxity is often dependent upon cell type. Hydroxytyrosol, for example, has been found to completely inhibit HL60 (human promyelocytic leukemia) cell proliferation at 100 μM, leading to more than 50% loss in cell viability in both human prostate cancer cells and in immortalized renal proximal tubule cells.[36] In our study, the presence of 3-hydroxytyrosol only led to a loss of keratinocyte cell viability at concentrations higher than 1000 μM. This suggests that keratinocytes contain an inherent ability to absorb much higher concentrations of potentially cytotoxic compounds.

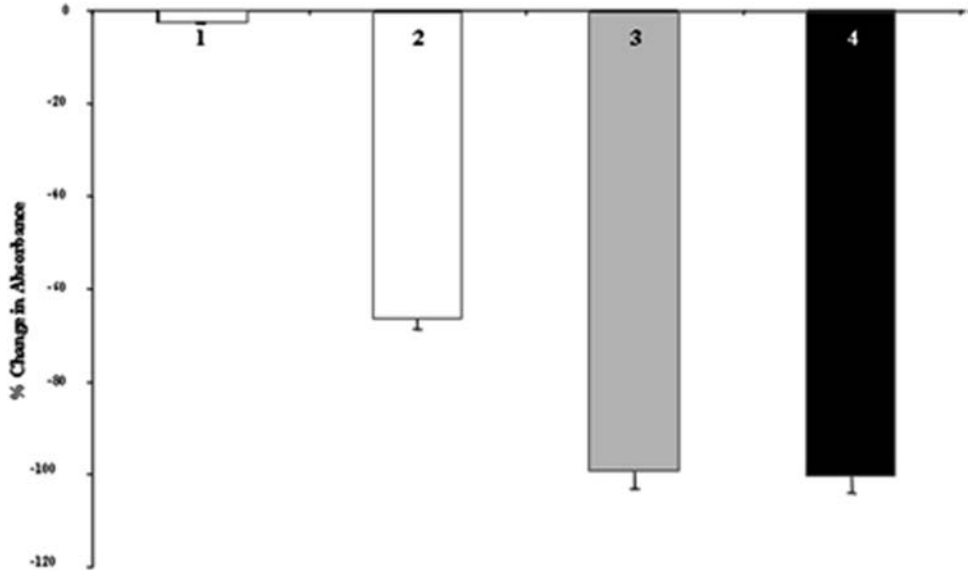

Figure 4. Inhibition of LDL peroxidation by compound **I** (3-hydroxytyrosol) and compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol). LDL was oxidized with $CuCl_2$ for 4 h at 37 °C, in the presence or absence of the antioxidant. All experiments were performed in triplicate. (1) Control, (2) ascorbic acid (standard), (3) compound **I** (3-hydroxytyrosol), and (4) compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol).

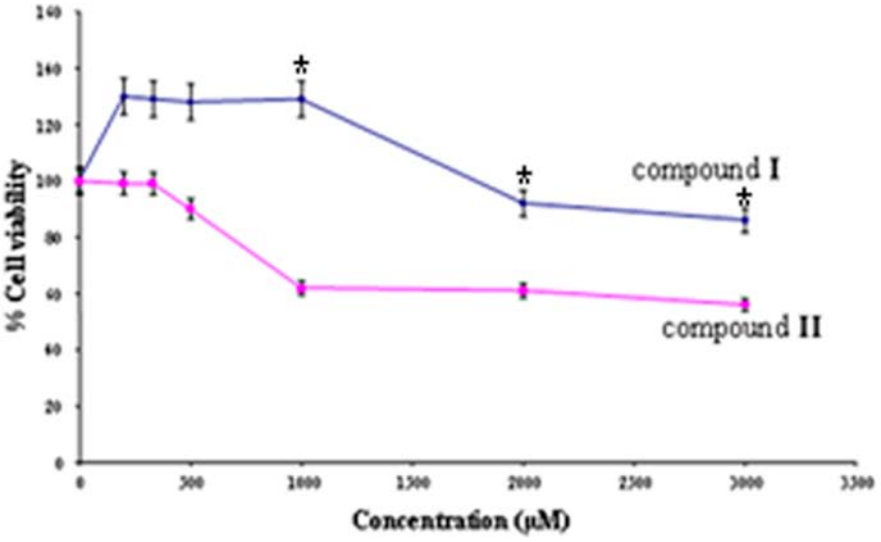

Figure 5. Effect of the various concentrations of compound **I** (3-hydroxytyrosol) and compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) on HaCaT cell viability. Cells were incubated with various concentrations of the test compound for 24 h. The XTT reagent was added; cells were incubated for 4 h at 37 °C; and the absorbance was read. Cell viability is expressed as a percentage of the control. The blue line represents compound **I** (3-hydroxytyrosol), and the pink line represents compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) (∗, $p < 0.05$).

To further explore the long-term exposure effect of the putative antioxidants on these cells, a dose−response study was conducted over a period of 72 h. The full concentration ranges for both 3-hydroxytyrosol and compound **II** proved to be nontoxic over the growth period (data not shown), with the cells displaying a typical sigmoidal growth curve indicative of minimal cytotoxicity. Concentrations extrapolated from these curves were chosen for all subsequent experiments.

Protective Effects of the Putative Antioxidants on the UV-Induced Intracellular ROS Production. UVA irradiation of cultured human skin cells results in elevated levels of H_2O_2 and other ROS, with H_2O_2 being the predominant species.[37] To test the efficacy of our antioxidants on the UV-irradiated keratinocytes, we quantified the fluorescence using the intracellular ROS probe, DHR 123. The quantitative analysis of the data showed that both compounds inhibited UVA-induced ROS production in a dose-dependent manner (Figure 6). At 200 μM, both compounds reduced ROS levels to normal physiological levels relative to the no UV control (100%). At 500 μM, 3-hydroxytyrosol resulted in the same relative ROS reduction effect as 333 μM for compound **II**, both displaying a 1.6-fold reduction in ROS compared to the control. The greatest effects were observed with the highest concentrations of 3-hydroxytyrosol and its dimer with a 1.79- and 1.97-fold reduction in the production of ROS, respectively. Thus, the dimer (compound **II**) is considered a more effective inhibitor of ROS production in HaCaTs than its parent compound.

These results directly demonstrate that pre-incubation with both compounds results in potent radical scavenging activity, which can prevent the overproduction of intracellular ROS through a free radical scavenging pathway. Although the exact mechanisms of this pathway have not been fully uncovered, we can speculate two possibilities. First, the hydroxytyrosol compounds may prolong the half-lives of other radical scavengers, such as ascorbate, as shown by other electron spin resonance studies,[38] and/or protect and, thus, enhance the basal endogenous levels of α-tocopherol in much the same way that has been proposed for the effect of maritime pine bark

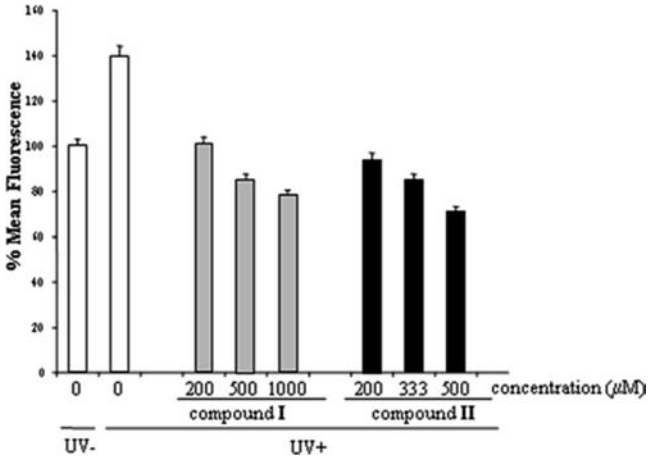

Figure 6. Effect of various concentrations of compound **I** (3-hydroxytyrosol) and compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) on UVA-induced ROS levels. HaCaTs were treated with various concentrations of the test compounds for 24 h. The cells were UVA-irradiated (320−400 nm; 22.3 J/cm^2) and incubated with DHR 123 for 30 min. The cells were harvested and analyzed on a FACSCalibur flow cytometer. ROS levels are presented as a mean percentage ± standard error of the mean (SEM) of the sham-irradiated control (100%) ($n = 3$; $p < 0.05$).

extract (PBE) on endothelial cells.[39] Second, the antioxidant efficiency may be related to the molecular weights of the compounds because the degree of ROS reduction seemed to correlate with an increase in the molecular weights (respective molecular weights of 306 and 153 for 3-hydroxytyrosol and compound **II**, respectively). It further suggests that the effect of dimerization may lead to an increase in the antioxidant activity. Because UV exposure has been shown to produce both an increase in ROS as well as DNA damage, the effect of pre-incubation of these compounds may have a profound effect on downstream cellular signaling, such as NF-κB activation in keratinocytes, in much the same way as was speculated for

PBE.[40] Although not addressed in this paper, it deserves to be studied further.

Overall, this study provides the first evidence that a novel, dimeric derivative of 3-hydroxytyrosol acts as a potent protective agent for keratinocytes after UVA irradiation and further evidence of the protective effect of 3-hydroxytyrosol in scavenging hydrogen peroxide.[41]

Evaluating the Effect of Putative Antioxidants on UVA-Induced Apoptosis in HaCaTs. Because UVA-induced oxidative stress in human skin leads to ROS-associated cellular damage with a consequent induction of apoptosis,[42−44] the addition of effective antioxidants to provide further protection is warranted. To evaluate whether the putative compounds used in this study could function as effective antioxidants and abrogate the induction of apoptotic cell death mechanisms, we studied the protein expression of known pro- and anti-apoptotic proteins, B-cell lymphoma protein-2 (Bcl-2), Bcl-2-associated X protein (Bax), and apoptosis-inducing factor (AIF). These proteins represent both the caspase-dependent (Bcl-2 and Bax) and -independent (AIF) mediated pathways in apoptosis.[45,46]

The pretreatment of cells with 3-hydroxytyrosol at concentrations of 200 and 500 μM resulted in an increase in Bcl-2 protein expression in a concentration-dependent manner (Figure 7). At 1000 μM, both compounds resulted in an increase in Bcl-2 levels relative to the control (Figure 6). Interestingly, 500 μM of compound **II** increased Bcl-2 levels by 16% relative to the control (Figure 6).

Whereas 1000 μM 3-hydroxytyrosol resulted in the greatest decrease in the pro-apoptotic Bax protein expression (Figure 8), 200 μM of its dimeric product reduced Bax levels by 75%.

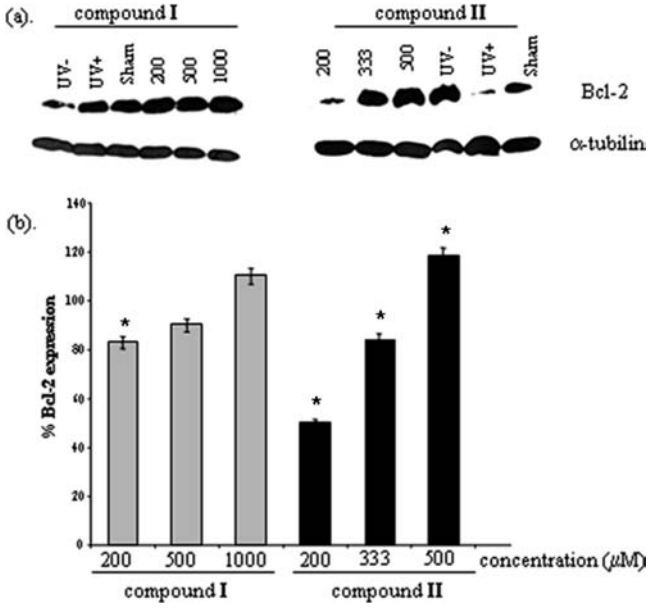

Figure 7. Effect of the various concentrations of compound **I** (3-hydroxytyrosol) and compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) on Bcl-2 expression in response to UVA exposure. HaCaTs were treated with varying concentrations of the test compounds for 18 h and then irradiated at 22.3 J/cm^2 with UVA. Protein lysates were analyzed by western blotting for (a) Bcl-2 expression and (b) quantitation by densitometry. Bcl-2 levels are presented as a percentage of the sham-irradiated untreated control (100%). Data are presented as the mean percentage ± SEM (n = 3) (∗, p < 0.05).

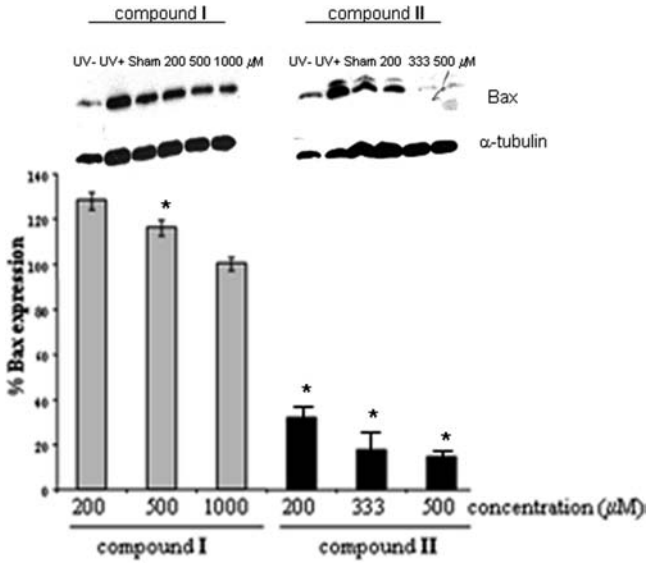

Figure 8. Effect of the various concentrations of compound **I** (3-hydroxytyrosol) and compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) on Bax expression in response to UVA exposure. HaCaTs were treated with varying concentrations of the test compounds for 18 h and then irradiated at 22.3 J/cm^2. Protein lysates were analyzed by western blotting for Bax expression, and levels were quantified by densitometry. Bax levels are presented as a percentage of the sham-irradiated control (100%). Data are presented as the mean percentage ± SEM (n = 3) (∗, p < 0.05).

In addition, the other two concentrations tested (333 and 500 μM) reduced Bax levels up to 87.5% relative to the control (Figure 8). This further confirmed that the dimeric product produced a greater anti-apoptotic effect than the parent compound.

Because apoptosis can occur in a caspase-dependent or -independent manner, we additionally investigated the expression of AIF, a marker of caspase-independent apoptosis. Noteworthy was that only the highest concentration (1000 μM) of 3-hydroxytyrosol caused any change to the AIF expression levels (reduction by 25%), whereas 200, 333, and 500 μM concentrations of compound **II** significantly reduced AIF levels to 20, 28, and 32% expression levels lower than the control, respectively (Figure 9). These results suggest that, despite the compounds having an anti-apoptotic effect, the apoptosis that does indeed occur is caspase-dependent.[47]

In conclusion, the aim of this study was to develop methodologies to produce biocatalytically active products and measure their antioxidant effect in living cells, thus allowing for an assessment of the protective role of the compounds as antioxidants at a metabolic level. Biotransformation of 3-hydroxytyrosol lead to oligomeric products with added antioxidant activity compared to the parent compound *in vitro* and *ex vivo*. The applicability of this study is that these compounds may thus be considered for use in health, nutraceutical, or cosmetic markets. Although the exact mechanism is still unknown, 3-hydroxytyrosol and its dimeric product, compound **II**, were both shown to protect HaCaT cells against the cytotoxic effects induced by a potent, oxidizing dose (22.3 J/cm^2) of UVA, rendering them good potential candidates for the protection of skin cells against the damaging effects of UVA irradiation. Of course, the skin comprises other cell types, such as the epidermal melanocytes and dermal

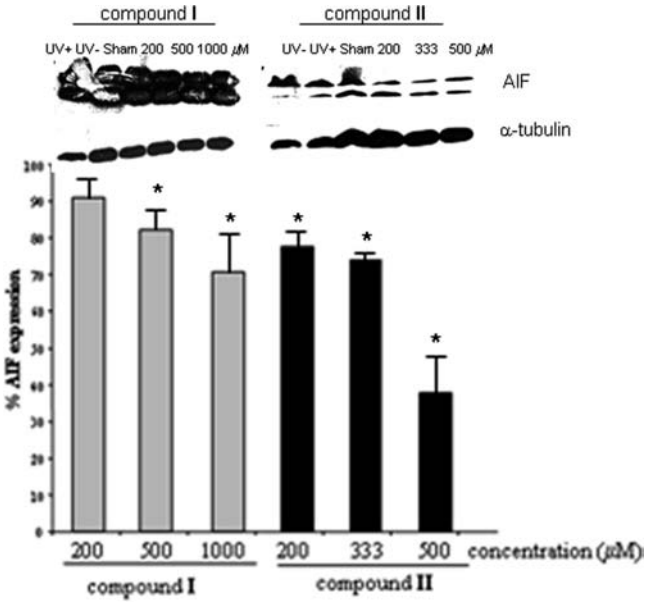

Figure 9. Effect of the various concentrations of compound **I** (3-hydroxytyrosol) and compound **II** (5,5′-bis(2-hydroxyethyl)-1,2-dihydro-[1,1′-biphenyl]-2,2′,3,3′-tetraol) on AIF expression in response to UVA exposure. Cells were cultured and treated with varying concentrations of the test compounds for 18 h and then irradiated at 22.3 J/cm^2. Protein lysates were analyzed by western blotting for AIF expression, and levels were quantified by densitometry. AIF levels are presented as a percentage of the sham-irradiated control (100%). Data are presented as the mean percentage ± SEM (n = 3) (∗, p < 0.05).

fibroblasts, and future studies will address the interaction of these compounds with the other resident skin cells in a three-dimensional co-culture assay. Moreover, despite these compounds being worthy candidates, future studies with these compounds will have to include elucidation of their protective mechanism, whether any synergistic action exists between the parent compound and the products formed, and their transdermal penetrative ability.

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ABBREVIATIONS USED

TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; LC−MS, liquid chromatography−mass spectrophotometry; TDM, *Trametes*-defined medium; DPPH, 2,2-diphenyl-picryhydrazyl; FRAP, ferric-reducing antioxidant power; LDL, low-density lipoprotein; Bcl-2, B-cell lymphoma protein-2; Bax, Bcl-2-associated X protein; AIF, apoptosis-inducing factor

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