

Hydroxytyrosol Acyl Esters: Biosynthesis and Activities

Zouhaier Bouallagui · Mohamed Bouaziz ·
Salwa Lassoued · Jean Marc Engasser ·
Mohamed Ghoul · Sami Sayadi

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Abstract We previously reported the production of high yields of hydroxytyrosol through the bioconversion of tyrosol. In the present work, hydroxytyrosol was subjected to the lipase catalyzed acylation aiming for the recovery of more lipophilic esters that might be easily incorporated in cosmetic and food preparations. Hydroxytyrosyl acetate and hydroxytyrosyl oleate were produced with respective molar esterification yields of 98% and 78%. DPPH free radical quenching potency demonstrated that the acylation of hydroxytyrosol did not alter its antioxidant activity. The acylated esters were shown to be more effective than the natural antioxidant: caffeic acid and two synthetic ones as BHA and BHT. Antiproliferative activity on human cervical cells (HeLa) resulted in IC_{50} values of 0.46, 0.42 and 0.33 mM for hydroxytyrosol and its acetyl and oleyl esters, respectively. Additionally, when used at a non-cytotoxic concentration (100 μ M), these compounds showed significant effectiveness in preventing iron-induced oxidative stress, resulting in a reduction of 30%, 36% and 38% in thiobarbituric acid-reactive substance production, respectively.

Keywords Hydroxytyrosol · Esterification · Novozym 435 · Kinetics · Cytotoxicity · Oxidative stress

Abbreviations

BHA Butylated hydroxyanisole
BHT Butylated hydroxytoluene

Z. Bouallagui · M. Bouaziz · S. Sayadi (✉)
Environnemental Bioprocesses Laboratory, Sfax Biotechnology Centre,
P.O. Box 1177, 3038 Sfax, Tunisia
e-mail: sami.sayadi@cbs.mrt.tn

S. Lassoued
Unité de Biotechnologie et Pathologie, Higher Institute of Biotechnology of Sfax, University of Sfax,
P.O. Box “261”, 3038 Sfax, Tunisia

J. M. Engasser · M. Ghoul
Laboratoire d'ingénierie des biomolécules, ENSAIA-National Polytechnic Institute of Lorraine,
2, Av de la Forêt de Haye, 54500 Vandoeuvre, France

DPPH	2,2-Diphenyl-1-picrylhydrazyl
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
TBARS	Thiobarbituric acid-reactive substances

Introduction

Natural antioxidants are being, recently, largely investigated owing to their safety and their usefulness in foods, cosmetics and pharmaceuticals [1]. Then, biolabelled products made through enzymatic and cellular bioconversion reactions will be a promising alternative for their recovery. During the last two decades, many studies have been dedicated to understanding polyphenols bioactivities and developing processes for their production [2]. Particular interest was focused on hydroxytyrosol, the major phenolic antioxidant found in olive oil. This molecule has shown not only a powerful antioxidant activity but also anti-carcinogenic, anti-thrombotic and many other bioactivities [3, 4].

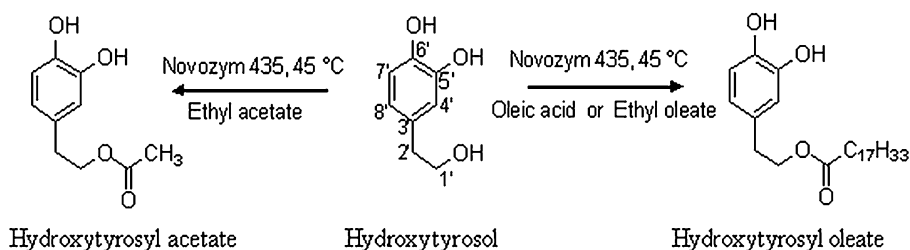
In recent times, different hydroxytyrosol preparations are being sold as aqueous solutions or as tablets. But the incorporation of hydroxytyrosol as an additive in foods and cosmetics, as oil matrices, is still a challenge. The hydrophilic character of such antioxidant stands as a serious disadvantage, reducing then its effectiveness in stabilizing fats and oils. Therefore, more lipophilic hydroxytyrosol derivatives will promote its properties with respect to the dispersion medium, to passage across cell membranes and also in regard to protecting the substrate during delivery. These considerations trigger off the development of a suitable esterification approach to overcome this dilemma.

Concomitantly with the natural origin of the being esterified molecule, lipase-catalyzed reactions are believed as economically and ecologically more advantageous [5]. Indeed, lipases are explored for racemic mixture resolution, fat transformation and also for the synthesis of molecules with moisturizing or antioxidant activities allowing the control of human skin integrity [6, 7]. In the present work, the synthesis of lipophilic hydroxytyrosol derivatives through lipase-catalyzed acylation is reported (Scheme 1). In addition, chemico-biological activities of the newly synthesized esters are investigated.

Materials and Methods

Materials

Oleic acid ($\geq 93\%$ purity), ethyl oleate (98% purity), acetonitrile ($\geq 99.8\%$), dimethyl sulfoxide (99.9% purity), MTT (thiazolyl blue tetrazolium bromide, 98% purity) and molecular sieves (5 Å) were from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate and



Scheme 1 Regioselective acylation of hydroxytyrosol catalyzed by lipase B from *C. antarctica*

2-methyl 2-butanol were from Merck (Darmstadt, Germany). *Candida antarctica* lipase SP 435 (7,000 PLU/g: propyl laurate units synthesized per gram of catalyst) was purchased from Novozymes (Dittingen, Switzerland). DPPH was from Fluka Chemicals (Steinheim, Germany). RPMI 1640 culture medium and foetal bovine serum (FBS) were from Invitrogen (Paisley, UK). Hydroxytyrosol was produced in our laboratory as previously reported by Bouallagui and Sayadi [8].

Biosynthesis of Hydroxytyrosol Esters

For the synthesis of hydroxytyrosyl acetate, the polyphenolic substrate was dissolved in ethyl acetate at a final concentration of 5 g/l. The reaction was initiated by the addition of 10 g/l immobilized *C. antarctica* lipase B (Novozym 435). All reactions were performed in a 50-ml double-jacket batch reactor with 25 ml working volume. The operating conditions were 200-rpm stirring rate and 45 °C.

The enzymatic synthesis of hydroxytyrosyl oleate was carried out in 2-methyl 2-butanol using oleic acid as acyl donor or in solvent-free medium where ethyl oleate is the acylating reactant.

Analytical Methods

HPLC analyses were conducted with a Merck system (LaChrom, Merck) equipped with a reverse phase C18 column (Purospher® RP-18, 250×4.0 mm, Merck). Phenolic compounds were detected using a diode array detector at 280 nm. In the case of the oleate ester, the reaction kinetic was followed by a light-scattering detector (LSD 31, Eurosep). The mobile phase was composed of 0.1% acetic acid in water (solvent A)–methanol (solvent B). During the elution time, solvent B was varied as follows: 0–5 min, 30–100%; 5–10 min, 100% and 10–15 min, 100–30%.

Mass spectrometry was carried out with an Agilent Series 1100 liquid chromatography system (Agilent, Waldbronn, Germany) coupled with an Agilent series MSD trap XCT instrument.

Structures were established by ¹H NMR (300 MHz) and ¹³C NMR (75 MHz) on a BRUCKER 300 Ultrashield spectrometer using deuterated chloroform (CDCl₃) as solvent.

Spectroscopic Characterization of Hydroxytyrosol and Acylated Derivatives

Hydroxytyrosol: MS *m/z* 155 (calcd for C₈H₁₀O₃ [M+H]⁺+155.16). ¹H NMR (300 MHz, CDCl₃): δ 3.67 (2H, t, H-1'), 2.67 (2H, t, H-2'), 6.67 (1H, d, H-4'), 6.69 (1H, d, H-7') and 6.52 (1H, dd, H-8'). ¹³C NMR (75 MHz, CDCl₃): δ 62.8 (CH₂, C-1'), 37.8 (CH₂, C-2'), 115.4, 115.3 and 119.46 (CH, C-4', C-7' and C-8') and 130.0, 144.3 and 142.8 (C, C-3', C-5' and C-6').

Hydroxytyrosyl acetate: MS *m/z* 197 (calcd for C₁₀H₁₃O₄ [M+H]⁺+197.081). ¹H NMR (300 MHz, CDCl₃): δ 4.12 (2H, t, H-1'), 2.73 (2H, t, H-2'), 6.67 (1H, d, H-4'), 6.71 (1H, d, H-7'), 6.54 (1H, dd, H-8') and 2.00 (3H, s, CH₃-2). ¹³C NMR (75 MHz, CDCl₃): δ 21.4 (CH₃, C-2), 65.8 (CH₂, C-1'), 34.5 (CH₂, C-2'), 116.4, 115.8 and 121.1 (CH, C-4', C-7' and C-8') and 129.1, 144.2, 142.8 and 171.6 (C, C-3', C-5', C-6' and C-1).

Hydroxytyrosyl oleate: MS *m/z* 441 (calcd for C₂₆H₄₂O₄Na [M+Na]⁺+441.298). ¹H NMR (300 MHz, CDCl₃): δ 4.15 (2H, t, H-1'), 2.74 (2H, t, H-2'), 6.67 (1H, d, H-4'), 6.72 (1H, d, H-7'), 6.54 (1H, dd, H-8'), 2.25 (2H, t, H-2), 1.54 (2H, m, H-3), 1.21 (14H, m, H-4_H-6 and H-13_H-16), 1.25 (4H, m, H-7 and H-12), 1.96 (4H, m, H-8 and H-11), 5.28

(2H, m, H-9 and H-10), 1.22 (2H, m, H-17) and 0.84 (3H, t, H-18). ^{13}C NMR (75 MHz, CDCl_3): δ 65.4 (CH_2 , C-1'), 34.7 (CH_2 , C-2'), 116.4, 115.4 and 121.1 (CH, C-4', C-7' and C-8'), 130.0, 144.7, 142.7 and 174.2 (C, C-3', C-5', C-6' and C-1), 34.3 (CH_2 , C-2), 25.0 (CH_2 , C-3), 29.4–29.6 (CH_2 , C-4_6/C-13_15), 29.8 (CH_2 , C-7/C-12), 27.5 (CH_2 , C-8/C-11), 129.8 (CH, C-9/C-10), 32.2 (CH_2 , C-16), 23.0 (CH_2 , C-17) and 14.4 (CH_3 , C-18).

Antioxidant Assay

The free radical scavenging effect of the studied compounds was evaluated using the DPPH radical quenching method derived from that described by Sabally et al. [9]. The reaction consists of 2 ml of a methanolic DPPH solution (40 μM) and 50 μl sample solution. A series of varying concentrations was used for each compound. After standing the reaction mixture under dark for 15 min, the reduction of DPPH free radicals was optically monitored at 517 nm. Results are expressed as IC_{50} corresponding to the antioxidant concentration necessary for reducing 50% of the initially present DPPH radicals.

Antiproliferative Activity

Antiproliferative effects were evaluated via the MTT assay. Human cervical cells (ATCC) were grown as monolayer in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS and maintained at 37 °C in a humidified atmosphere in the presence of 5% CO_2 . Cells were harvested over 80% confluence and seeded in 96-well plates at 5×10^4 cells per well. After overnight incubation, cells were treated with increasing concentrations of test compounds. Forty-eight hours later, the medium was changed and 10 μl MTT (5 mg/ml) was added to each well, and the plates were further incubated for 4 h. The formazan crystals were solubilized with 10% SDS, and the absorbance of each well was read at 570 nm using a multiwell plate reader.

Prevention of Oxidative Stress in Human Cells

HeLa cells were seeded at 4×10^5 cells/ml and allowed to grow for 48 h then treated with hydroxytyrosol, hydroxytyrosyl acetate or hydroxytyrosyl oleate for 4 h. Subsequently, oxidative stress was induced by incubation of cells in the presence of 100 μM FeSO_4 for 1 h. Finally, cells were harvested and lysed by sonication and their protein content quantified using the Bradford reagent and BSA as standard.

Oxidative stress was evaluated through the thiobarbituric acid-reactive substances (TBARS) protocol [10]. Results are presented as TBARS quantity (μg) per milligram of protein. Data given are the means of three independent replicates wherein the statistical significance was evaluated at $p < 0.05$.

Results and Discussion

Enzymatic Synthesis of Hydroxytyrosol Esters

Hydroxytyrosyl acetate was synthesized following the enzymatic transesterification between hydroxytyrosol and ethyl acetate. This reaction resulted in 98% conversion of hydroxytyrosol initially present in the medium, within 7 h. Indeed, the conversion was favoured in the presence of molecular sieves as they prevented the establishment of a

thermodynamic equilibrium by absorbing the released ethanol. NMR-based spectroscopy showed that the enzyme preferably catalyzed the acylation of the primary hydroxyl in the hydroxytyrosol ethylic chain (Scheme 1). Once again, the enzymatic esterification has allowed the recovery of hydroxytyrosyl acetate without any secondary product formation, proving the regioselectivity earlier reported for *C. antarctica* lipase B [11]. In addition, the esterified compound could be used without further purification.

Hydroxytyrosyl oleate was obtained as a light yellow syrup after esterification between hydroxytyrosol and oleic acid in 2-methyl 2-butanol. In this case also, the lipase showed the same regioselectivity towards the primary hydroxyl group. The esterification yield was found to be affected by the molar ratio of both substrates: hydroxytyrosol and oleic acid (Fig. 1a). The highest conversion yield was obtained at a molar ratio of hydroxytyrosol/oleic acid of 1:2, resulting in 69%. Under these optimal conditions, oleic acid disappeared exponentially during the first 5 h. Subsequently, the kinetic reached a steady state where the released water coupled with the esterification reaction could explain the establishment of such equilibrium (Fig. 1b).

Similarly, the synthesis of hydroxytyrosyl oleate was also performed in ethyl oleate as a solvent-free medium. The transesterification yield in the absence of molecular sieves was 41%. The released ethanol was considered to be the most competing agent with the primary hydroxy group and the likely cause of this low yield [12]. The addition of molecular sieve at 200 g/l was responsible for the equilibrium shifting in favour of the accumulation of hydroxytyrosyl oleate, resulting in 78% molar transesterification yield.

Antioxidant Activity

Hydroxytyrosol as well as hydroxytyrosyl acetate and hydroxytyrosyl oleate exhibited lower IC_{50} values when compared to BHT and BHA, used as chemical food-preserving agents, during the DPPH radical quenching assay (Table 1). Thus, on the molar basis, hydroxytyrosol and the corresponding acyl esters are considered as more powerful antioxidants. More interestingly, similar observations can be concluded for the natural antioxidant caffeic acid, being less potent. Analogous data for hydroxytyrosol were previously reported by Fki et al. [13]. The DPPH radical quenching properties are mainly

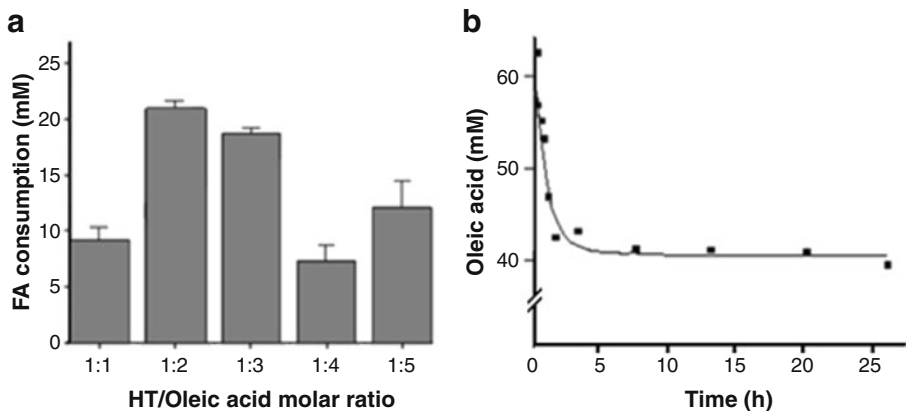


Fig. 1 Kinetic of hydroxytyrosyl oleate synthesis in organic medium (2-methyl 2-butanol). The initial hydroxytyrosol concentration is 32.4 mM (5 g/l). **a** Effect of molar ratio of hydroxytyrosol/oleic acid (HT/oleic acid) on the final oleic acid consumption. **b** Fatty acid (FA) consumption kinetic at a molar ratio of 1:2

Table 1 DPPH free radical scavenging activities of hydroxytyrosol and its corresponding esters

Compound	IC ₅₀ (μM) ^a	Compound	IC ₅₀ (μM)
Hydroxytyrosol	12.3	BHA	16.4
Hydroxytyrosyl acetate	13.3	BHT	13.7
Hydroxytyrosyl oleate	13.1	Caffeic acid	15.7

Conditions: 40 μM DPPH in methanol (2 ml), sample solutions with varying concentrations (50 μl), incubation of 15 min under dark, optical density at 517 nm

^aIC₅₀ value corresponds to the compound concentration necessary for reducing 50% of the initially present DPPH radicals. Data are the means of three independent experiments

attributed to the *ortho*-diphenolic structure of the tested compounds. An argument has been recently offered by our team showing that chemically synthesized tri-acetylated hydroxytyrosol was unable to reduce DPPH free radicals [10].

Bioactivities of Hydroxytyrosol and Synthesized Esters

Hydroxytyrosol and its enzymatically synthesized esters exhibited a dose-dependent cytotoxicity against HeLa cells. The IC₅₀ values were 0.46, 0.42 and 0.33 mM for hydroxytyrosol, hydroxytyrosyl acetate and hydroxytyrosyl oleate, respectively (Fig. 2a).

Hydroxytyrosol was previously shown to exert cytotoxic effects through the induction of apoptosis in human leukaemia cells HL-60 [14]. This apoptosis was subsequent to a G0/G1 cell cycle arrest. The same behaviour is being proposed for hydroxytyrosol esters. Furthermore, acylating groups are thought to contribute to the enhancement of the functionality and bioavailability of hydroxytyrosol [15–17].

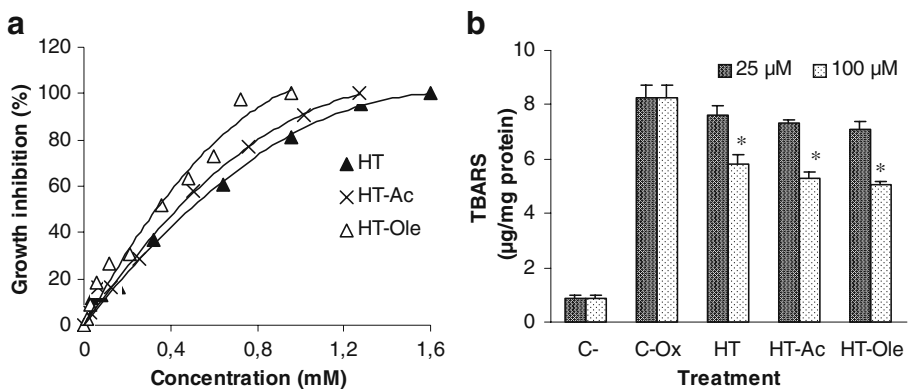


Fig. 2 **a** Cytotoxic effects on human cervical (HeLa) cells. Filled triangle hydroxytyrosol (HT); Multiplication sign hydroxytyrosyl acetate; Empty triangle hydroxytyrosyl oleate (HT-Ole). IC₅₀ values were calculated using the equations obtained from a nonlinear regression for a relation between the compound concentrations and the percentage of growth inhibition. Data are the means of at least three independent experiments. **b** Ability of hydroxytyrosol and acylated derivatives to reduce iron-induced oxidative stress within HeLa cells. C- untreated control cells, C-Ox iron-stressed or positive control cells, HT HT-Ac and HT-Ole refer to cells treated with both FeSO₄ and the corresponding compound. Statistical significance at $p < 0.05$ was calculated relatively to the positive control. Data with asterisks are statistically different from the positive control

Concerning the ability of hydroxytyrosol and derivatives to prevent iron-induced oxidative stress, data are shown in Fig 2b. Pre-incubation of HeLa cells in the presence of 100 μ M phenolic compounds led to a significant improvement of the oxidative status. In fact, TBARS production was decreased by 30%, 36% and 38% with hydroxytyrosol, hydroxytyrosyl acetate and hydroxytyrosyl oleate, respectively.

As far as the antioxidant activity of hydroxytyrosol in bioassays is considered, both in vitro and in vivo studies were undertaken [17, 18]. Nevertheless, few data are available regarding the activities of hydroxytyrosol esters. Recent data have shown that hydroxytyrosyl acetate inhibited platelet aggregation through a decreased thromboxane synthesis and an increased nitric oxide production in rats and in human whole blood [19, 20]. Moreover, hydroxytyrosyl acetate was previously shown to be metabolized in human hepatoma cells HepG2, yielding hydroxytyrosol which is further converted to methylated, glucuronidated and methylglucuronidated metabolites [21].

Hence, both hydroxytyrosyl acetate and hydroxytyrosyl oleate are suggested to be enzymatically metabolized in vivo, contributing then to the improvement of hydroxytyrosol effectiveness. Consequently, acylation through enzymatic esterification reactions is considered as a promising method for the preparation of lipophilic and more stable derivatives of hydroxytyrosol and probably many other valuable molecules.

To conclude, the exploitation of lipases in biocatalytic reactions represents a successive method for the preparation of lipophilic hydroxytyrosol esters. Along with its economic and ecological benefits, this procedure contributed not only to the decrease of hydroxytyrosol polarity but also seems responsible for the augmentation of its bioavailability.

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