Chemoenzymatic Synthesis and Radical Scavenging of Sulfated Hydroxytyrosol, Tyrosol and Acetylated Derivatives

Paloma Begines[†], David Biedermann[‡], Kateřina Valentová[‡], Lucie Petrásková[‡], Helena Pelantová[‡], Inés Maya[†], José G. Fernández-Bolaños[†*], Vladimír Křen[‡*]

[†]Department of Organic Chemistry, Faculty of Chemistry, University of Seville, E-41012, Spain

[‡]Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, CZ 142 20 Prague, Czech Republic

* Corresponding author. (Tel.: +34-954550996. E-mail: bolanos@us.es)

* Corresponding author. (Tel.: +420-296442510. E-mail: kren@biomed.cas.cz)

Abstract

Potential metabolites of bioactive compounds are important for their biological activities and as authentic standards for metabolic studies. The phenolic compounds contained in olive oil are an important part of the human diet, and therefore their potential metabolites are of utmost interest. We developed a convenient, scalable, one-pot chemoenzymatic method using the arylsulfotransferase from *Desulfitobacterium hafniense* for the sulfation of the natural olive oil phenols tyrosol, hydroxytyrosol and of their monoacetylated derivatives. Respective monosulfated (tentative) metabolites were fully structurally characterized using LC-MS, NMR and HRMS. In addition, Folin-Ciocalteu reduction, 1,1-diphenyl-2-picrylhydrazyl radical scavenging and anti-lipoperoxidant activity in rat liver microsomes damaged by *tert*-butylhydroperoxide were measured and compared with the parent compounds. As expected, the sulfation diminished the radical scavenging properties of the prepared compounds. These compounds will serve as authentic standards of phase II metabolites.

Keywords: olive phenols; arylsulfotransferase; hydroxytyrosol, tyrosol; chemoenzymatic; metabolites.

Introduction

Sulfation is one of the major pathways of the phase II of biotransformation and detoxification of xenobiotics as well as eubiotics (e.g. steroid hormones). Sulfation converts the compounds into more hydrophilic metabolites, facilitating their excretion. In humans, sulfate conjugation is catalyzed by a superfamily (at least ten functional genes)[1] of membrane-associated and cytosolic sulfotransferases, which transfer a sulfate moiety (SO_3^-) from the donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) onto a wide variety of substrates. Sulfotransferases are present in tissues such as the brain, kidney, liver, adrenal glands, gastrointestinal tissue and gut. Sulfotransferases are also able to reverse the process of sulfation in cells (desulfation).[2]

Recently, much attention has been focused on the phenolic components of olive fruit and especially of olive oil for their beneficial effects on health. Extra virgin olive oil (EVOO) is considered to be one of the main components of the Mediterranean diet; its consumers have a reduced incidence of neurodegenerative diseases, coronary heart disease, atherosclerosis and certain cancers.[3] The phenolic compounds found in olive oil with important bioactive properties include: simple phenols (phenolic acids and phenolic alcohols such as tyrosol (**1**) and hydroxytyrosol (**2**), and their esters: hydroxytyrosol-elenolic acid ester Hy-EA, tyrosol-elenolic acid ester Tyr-EA, and the dialdehyde derivatives, oleocanthal and oleacein (Figure 1). Oleacein (2-(3,4-dihydroxyphenyl)ethyl (3*S*,4*E*)-4-formyl-3-(2-oxoethyl)hex-4-enoate) and oleocanthal (2-(4-hydroxyphenyl)ethyl (3*S*,4*E*)-4-formyl-3-(2-oxoethyl)hex-4-enoate) are the most abundant dialdehydes in olive oil and have important biological properties.[4-7]

Hydroxytyrosol has been reported to promote apoptosis in several tumor cell lines,[8] it inhibits their proliferation and, in addition, contributes to protecting humans in terms of bone health, platelet function, oxidative damage, cellular aging and plasma lipoproteins due to its anti-inflammatory, antimicrobial, anticancer, neuroprotective and antioxidant activities.[9] Hydroxytyrosol acetate was shown to be useful for cancer,[10] systemic lupus erythematous,[11] and arthritis[12] prevention. Tyrosol proved to be less active; however, it was able to decrease LPS-stimulated cytokine production and increase mouse survival in endotoxemia induced by LPS;[13] tyrosol and its acetylated derivative inhibited the synthesis of PAF (platelet-activating factor), a potent mediator of platelet aggregation and inflammation.[14]

The bioactivity of these phenolic compounds *in vivo* depends on their absorption and metabolism. The study of the metabolic fate of olive oil polyphenols is an area of active research.[15-19] Due to low aqueous solubility of most polyphenols[20], these compounds are biotransformed in humans into more polar derivatives, typically sulfates and glucuronides.[21,22] Moreover, the process of conjugation reduces the amount of polyphenols in the blood, increasing metabolite excretion, and also producing some active metabolites;[23] in this sense, sulfated polyphenols have been proven to be biologically active.[24] The sulfation process is considered to be reversible, involving sulfotransferases, which catalyze the sulfation reaction and sulfatases, which catalyze the hydrolysis of sulfate esters.[25] Therefore, such conjugated metabolites are required as reference compounds and standards for investigating their bioavailability in humans.

The health benefits attributed to extra virgin olive oil (EVOO) such as antioxidant, antimicrobial, anti-inflammatory, neuroprotective, cardioprotective and anti-cancer properties can be mostly associated with its phenolic content.[26-29] The bioactivity of the phenolic compounds from EVOO has been widely studied. It is known than olive

phenols undergo an extensive conjugation during their metabolism forming sulfated, methylated and glucuronidated derivatives, and often before reaching the target tissues; these metabolites are finally excreted in the urine. In previous literature, hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate have been detected as the main metabolites in human plasma when consuming VOO.[21] Moreover, these phase II metabolites has been identified as the most suitable biomarkers for monitoring compliance with olive oil intake.[22] Therefore, sulfated metabolites are of great interest as standards to study their biological properties.[30,31]

Although there are number of chemical methods for the sulfation of phenols, they generally suffer from the lack of regioselectivity and the products are often hard to purify. The use of enzymes offers the way to improve the regioselectivity under mild reaction conditions. The chemical methods for sulfation of small molecules was reviewed recently.[32]

The arylsulfate sulfotransferase[33] (AST) from *Desulfitobacterium hafniense* catalyzes the transfer of the sulfate group from various sulfate donors (typically *p*-nitrophenyl sulfate) onto various acceptors with free OH groups. This commercially unavailable enzyme has virtually no hydrolytic activity, *i.e.* it does not transfer the sulfate group to water. This enzyme also exhibits some regioselectivity, as demonstrated e.g. on the sulfation of quercetin and its derivatives[34,35] or the flavonolignans from silymarin.[36] This sulfotransferase using cheap donor *p*-nitrophenyl sulfate has a great advantage over "classical sulfotransferases", which employ very expensive and unstable PAPS.

Sulfated derivatives of tyrosol and hydroxytyrosol were prepared previously using chemical procedures, as regioisomeric mixtures of monosulfates in the case of hydroxytyrosol.[37, 38] As enzymatic procedures showed to be good methods of choice for

the preparation of standards of sulfated metabolites of various xenobiotics, the aim of the present study was to prepare pure isomers of sulfated natural olive oil phenolics tyrosol (**1**), hydroxytyrosol (**2**) and their monoacetylated derivatives: tyrosol-2′-acetate (**3**) and hydroxytyrosol-2′-acetate (**4**)[39,40] using AST from *D. hafniense*. Although these compounds are not the main phenols in olive oil, they are the metabolic precursors and also the degradation products[19] of the main phenolics in that oil, i.e., Tyr-EA, Hy-EA, oleocanthal, and oleacein. The reducing, radical-scavenging and anti-lipoperoxidant properties of the sulfated derivatives was compared with that of parent compounds.

Experimental Section

Materials. Tyrosol (**1**), hydroxytyrosol (**2**) and *p*-nitrophenyl sulfate potassium salt were purchased from Sigma-Aldrich. Hydroxytyrosol-2′-acetate (**4**) was prepared by refluxing **2** in ethyl acetate (EtOAc) in the presence of the strong acidic resin Amberlite IR-120 H^+ (The Dow Chemical Company, USA), *i.e.* by a chemoselective acid-catalyzed acetylation reaction.[41] Tyrosol-2′-acetate[42] (**3**) was synthesized using the above procedure.

Methods. NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) in DMSO-d_6 at 30 ºC, using the residual solvent signal (δ_H 2.499 ppm, δ_C 39.46 ppm) as a reference. NMR experiments ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, and gHMBC were performed using the manufacturer's software.

Mass spectra in negative-ion mode were measured in an Orbitrap Elite (Thermo Fisher) equipped with an electrospray ion source (HESI), using a spray voltage of 3,500 V (+) and a resolution of 60,000. The acquisition range was from 60 to 900. The samples were dissolved in methanol/water 50% (v/v) with 0.1% formic acid.

The enzymatic sulfation was monitored by thin-layer chromatography (TLC) [silica gel 60 F_{254} plates (Merck, DE); mobile phase EtOAc/MeOH/HCO_2H, 4:1:0.2, v/v and EtOAc/MeOH 9:2.5, v/v].

All analytical HPLC analyses were performed in a Shimadzu Prominence LC analytical system consisting of a Shimadzu LC-20AD binary HPLC pump, Shimadzu SIL-20ACHT cooling auto sampler, Shimadzu CTO-10AS column oven, Shimadzu CBM-20A system controller and Shimadzu SPD-20MA diode array detector (Shimadzu, JP); there was no coupling to a MS detector. The sample (0.5 mg) was dissolved in the mobile phase A (50 µL) and analyzed in a Kinetex PFP (150 × 4.6 mm, 5 µm) column (Phenomenex, USA) coupled with a PFP security guard cartridge kit (4 × 3 mm). Binary gradient elution was used: mobile phase A = 0.1% trifluoroacetic acid in water; mobile phase B = 100% methanol; gradient: 0 min 10% B, 20 min 40% B, 21 min 10% B. The flow rate was 0.6 mL/min at 45 °C and the injection volume was 1 µL; the peaks were detected at 275 nm (compounds **7**, **8**, **9**, **10**) or at 254 nm (compounds **5**, **6**).

Preparative HPLC separations were performed using an ASAHIPAK GS-310 20F column (Shodex, Munich, Germany), with the mobile phase specified for each experiment, flow rate 5 mL/min and detection at 254 and 369 nm. The preparative HPLC (Shimadzu, Kyoto, Japan) system consisted of an LC-8A high-pressure pump with an SPD-20A dual-wavelength detector (with semi-preparative cell), and fraction collector FRC-10A. The system was connected to a PC using a CBM-20A command module and controlled *via* the LabSolution 1.24 SPI software suite supplied with the machine.

Preparation of the Enzyme. Frozen cells transformed with the plasmid containing the AST gene[33,34] (100 µL, the plasmid was kindly provided by Dr. van der Horst,

University of Amsterdam, The Netherlands), were incubated in LB (Luria-Bertani) medium (100 mL) with kanamycin (KNM, 25 μM, 100 μL) at 37 ºC and 200 rpm, to an optical density (OD) of 600. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM, 160 μL) was then added. The mixture was incubated at 25 ºC overnight, at 200 rpm, and was centrifuged (5000 g, 20 min, 8 ºC). The cells were resuspended in Tris-Gly buffer (100 mM, pH 8.9, 2 mL), then they were sonicated for 4 × 4 min in an ice bath. The cell debris were then centrifuged (5000 g, 20 min, 8 ºC), thus obtaining the enzyme as a crude cell lysate.[34]

Preparation of 4-Hydroxyphenethyl Acetate (Tyrosol-2′-Acetate) (3). Tyrosol (**1**) (1.0 g, 7.24 mmol) in EtOAc (25 mL) containing Amberlite IR-120 H^+ (2 g) was refluxed and stirred thoroughly for 11 h under argon atmosphere (Ar from cylinder), followed by filtration with a Buchner funnel in vacuum, the solvent was eliminated in a rotary evaporator under reduced pressure, and then the residue was purified by column chromatography (cyclohexane/EtOAc 5:1 to cyclohexane/EtOAc 2:1) yielding **3** as a white solid (1.14 g, 87 %). R_F 0.65 (cyclohexane/EtOAc 1:1). ^{1}H NMR (399.87 MHz, DMSO-d_6, 30 ºC) δ: 7.02 (m, 2H, *o*-H), 6.68 (m, 2H, *m*-H), 4.12 (t, 2H, $J_{2',1'}$ = 7.06 Hz, OCH_2), 2.75 (t, 2H, $J_{1',2'}$ = 7.08 Hz, $ArCH_2$), 1.97 (s, 3H, CH_3); ^{13}C NMR (100.55 MHz, DMSO-d_6, 30 ºC) δ: 170.28 (CO), 155.79 (*p*-C), 129.74 (*o*-C), 127.86 (*i*-C), 115.09 (*m*-C), 64.67 (C-2′), 33.54 (C-1′), 20.62 (CH_3); HRESIMS *m/z* calcd for $C_{10}H_{12}O_3Na$ $[M+H]^+$ 203.0679, found 203.0676.

General Method for the Preparation of Sulfated Phenolic Derivatives and their Purification. A solution of potassium *p*-nitrophenyl sulfate (*p*-NPS, 1.2 eq) in Tris-Gly buffer (100 mM, pH 8.9, 15 mL) and the enzyme (AST, 2.5 mL) were added to a solution of the phenolic compound (100 or 150 mg, as indicated in each case) in acetone

(2 mL). The reaction mixture was purged with argon, incubated in the dark under a positive pressure of argon (balloon) at 30 ºC, with stirring on an orbital shaker (Labnet) at 220 rpm for 5 h. The organic solvent (acetone) was evaporated on a rotary evaporator at room temperature under reduced pressure, and pH was adjusted to 7.5 – 7.7 (formic acid). *p*-Nitrophenol and residual starting materials were extracted with ethyl acetate (3 × 20 mL) and the aqueous phase (15 mL) with the sulfated compounds was evaporated. The residue was dissolved in the mobile phase, filtered on a 0.45-mm PTFE, injected into preparative HPLC, and eluted with an isocratic flow (MeOH/H_2O 1:4; 3:2; 0:1; 1:19, for the different experiments). The combined fractions containing the products were evaporated and lyophilized from water.

Antioxidant Activity Evaluation

Folin-Ciocalteu Reduction Assay. Folin-Ciocalteu reduction (FCR) capacity was measured according to a previously reported protocol,[43,44] where 5 µL of the native compounds **1**–**4** and the sulfated samples **5**–**10** (1 mM) or standards (gallic acid, 0–4 mM) in phosphate-buffered saline (PBS, pH 7.4) were mixed with 100 µL of Folin-Ciocalteu reagent diluted tenfold with distilled water. It was incubated for 5 min, then 100 µL Na_2CO_3 (75 g/L) was added and the mixture was further incubated for 90 min at room temperature. The absorbance was measured at 700 nm using a Tecan Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland) and the reducing capacity was expressed as gallic acid equivalents (GAE).

DPPH Assay. The antiradical activity of the compounds was tested as the capacity to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich), thus decreasing the violet color of DPPH as previously described[44,45] with minor modifications. A 15 µL solution of the tested substance (final concentration 0 – 5 mM in MeOH) was mixed

with 285 µL of a freshly prepared methanolic DPPH solution (final concentration 20 µM) in a microtiter plate well (total volume 300 µL). After 30 min at 25 °C, the absorbance at 517 nm was measured. The concentration of the antioxidant required for reducing the DPPH concentration to 50% (IC_{50}) of its initial value was calculated.

Inhibition of Microsomal Lipid Peroxidation. This assay was performed according to the reported method.[44] Pooled microsomes from male rat liver (M9066, Sigma-Aldrich) were washed 5× using centrifugation (13,500 rpm, 5 min, 4 ºC) and PBS to remove sucrose, and diluted to 0.625 mg protein/mL with PBS before use. The protein concentration was determined using the Bradford method.[46] A 0.4 mL solution of the diluted microsomal suspension was then mixed with the compounds **1**–**10** (final concentration 5 µM – 2 mM in 50 µL PBS), *tert*-butyl hydroperoxide (*t*-BH, a pro-oxidant, 50 µL in PBS; final concentration 1 mM) was then added and the mixture was incubated at 37 ºC for 60 min. The products of lipid peroxidation were determined as thiobarbituric acid reactive substances (TBARS): 0.7 mL of trichloroacetic acid (26 mM) with thiobarbituric acid (918 mM) were added, the mixture was heated (90 ºC, 15 min), cooled, centrifuged (13,500 rpm, 10 min, 4 ºC) and the absorbance of the supernatant at 535 nm was measured. The activity was calculated as the concentration of the analyzed compounds that inhibited the color reaction with the thiobarbiturate (without the analyzed compounds) by 50% (IC_{50}).

Determination of log P Values. The hydrophobicity of the compounds (miLogP) was calculated using the Molinspiration property engine v2016.10 (http://www.molinspiration.com, Molinspiration Cheminformatics, Slovensky Grob, Slovakia, accessed on 26th September 2018).[47]

Statistical Analysis. Data were expressed as means ± standard deviation (SD). Assays were done in triplicate. The differences in mean values were analyzed by Student's *t*-tests. A p value of less than 0.05 was considered to be statistically significant.

Results and Discussion

Preparation and Purification of the Synthesized Compounds

Various methods based on chemical procedures have been used to synthesize sulfate phenols, most of them consist of using sulfur trioxide-pyridine complex as the sulfating reagent.[38,48] Sulfation of hydroquinone derivatives with SO_3·Py at 60 ºC gave the disulfated compounds, whereas at room temperature led to monosulfated compounds; no regioselectivity was described.[48] Paiva Martins *et al*. described the sulfation of hydroxytyrosol acetate with SO_3·Py (2 equiv., -20 ºC) to afford a mixture of monosulfated regioisomers on the phenolic hydroxyls with preference for 4-hydroxyl group; whereas with 8 equiv. of the sulfating agent the disulfated product was obtained.[38] Furthermore, sulfation of unprotected hydroxytyrosol led to occurred preferentially in the aliphatic hydroxyl group. The microwave-assisted *O*-sulfation reaction was performed using SO_3·NMe_3 to prepare sulfate derivatives of olive polyphenols,[49] however this method involves various protection-deprotection steps. Moreover, these chemical syntheses are not chemoselective, generating a mixture of monosulfates and disulfates, which is hard to separate.

We employed a single-step, efficient and inexpensive chemoenzymatic methodology to the transfer of a sulfate group from *p*-nitrophenyl sulfate (*p*-NPS) to the natural unprotected and monoacetylated phenolic compounds.

First, tyrosol-2′-acetate (**3**) and hydroxytyrosol-2′-acetate (**4**) were synthesized by refluxing tyrosol **1** and hydroxytyrosol **2**, respectively, in EtOAc, in strong acid resin.[41] The resin can be easily removed from the reaction medium by filtration and reused, thus constituting a green, economical, and effective method for the chemoselective monoacetylation of phenolic alcohols.

In the sulfation of tyrosol (**1**), a preference for the aromatic hydroxyl was observed, only tyrosol-4-*O*-sulfate (**5**) was obtained as a product in a good yield (81%) and a chemoselective fashion (Figure 2); in the same way, tyrosol-2′-acetate-4-*O*-sulfate (**6**) was obtained in a 59 % yield. Both products were purified by preparative HPLC and characterized by spectral techniques (see analytical HPLC chromatograms of **5** (supporting S7), and of **6** (supporting S12), after purification).

With hydroxytyrosol (**2**), the formation of two monosulfated compounds (3-*O*-sulfate/4-*O*-sulfate ca 2:1, calculated from ^{1}H NMR integration) was observed in a chemoselective manner, as no sulfation was detected in the side chain (Figure 3). The enzymatic sulfation of hydroxytyrosol-2′-acetate (**4**) also yielded both regioisomers (3-*O*-sulfate/4-*O*-sulfate ca 1:6), although the preferred reaction site changed from C3-OH to C4-OH, with acetylation in the side chain. Again, the selectivity of the enzyme (AST) for the aromatic hydroxyls was confirmed. Therefore, the regioselective outcome of the enzymatic sulfation of the catechol fragment of **2** and **4** depends on the features of the side chain. No disulfated derivatives were formed, presumably because the charge and the size of the sulfate group preclude subsequent sulfation.

From the ^{1}H NMR spectra of the reaction mixture we could deduce that hydroxytyrosol-3-*O*-sulfate (**7**) and hydroxytyrosol-4-*O*-sulfate (**8**) were formed in a 1.3:1 ratio;

whereas, hydroxytyrosol-2′-acetate-3-*O*-sulfate (**9**) and hydroxytyrosol-2′-acetate-4-*O*-sulfate (**10**) were in a 1:1.4 ratio. Due to their physicochemical properties, the regioisomers **7** and **8** derived from hydroxytyrosol, and **9** and **10**, derived from hydroxytyrosol acetate were difficult to separate by preparative HPLC (ASAHIPAK GS-310 20F column). After chromatography, partial separation was achived (**7**/**8** in a 2:1 ratio, and **9**/**10** in a 1:6 ratio). At analytical scale (Kinetex PFP column), the best conditions to separate both pairs of regioisomers involved the use of a gradient of 0.1% trifluoroacetic acid in water (phase A) and methanol (phase B) using an analytical Kinetex PFP HPLC column (Figure 4).

Only monosulfate and glucuronide conjugates of olive phenols were found to be the main metabolites in human plasma and excreted in urine after olive oil intake.[50,51] In accordance with this, no disulfated or trisulfated compounds were detected in any case, even using up to four equivalents of *p*-NPS as the sulfate donor using the chemoenzymatic method in this work. This is in contrast to the situation with flavonols and flavonolignans.[35,36] This might be due to the high polarity (low log P) of the monosulfates of these small phenolics (see Table 1), which are therefore probably not accepted as substrates for AST.

4-(2-Hydroxyethyl)phenyl sulfate (tyrosol-4-*O*-sulfate; 5): Tyrosol (**1**, 150 mg, 1.08 mmol) was sulfated according to the general procedure and purified by preparative HPLC in MeOH/H_2O 1:4 to obtain **5** as a white solid (192 mg, (69%). R_F 0.45 (EtOAc/MeOH/HCO_2H 4:1:0.2). ^{1}H NMR (399.87 MHz, DMSO-d_6, 30 ºC) δ: 7.090 (2H, m, *o*-H), 7.050 (2H, m, *m*-H), 4.579 (1H, br t, $J_{2',2'\text{-}OH}$ = 4.9 Hz, 2′-OH), 3.562 (2H, dt, $J_{2',2'\text{-}OH}$ = 4.9 Hz, $J_{2',1'}$ = 7.2 Hz, H-2′), 2.660 (2H, t, $J_{1',2'}$ = 7.2 Hz, H-1′); ^{13}C NMR (100.55 MHz, DMSO-d_6, 30 ºC) δ: 151.64 (*p*-C), 133.90 (*i*-C), 128.91 (*o*-C),

120.29 (*m*-C), 62.28 (C-2′), 38.30 (C-1′); HRESIMS *m/z* calcd for $C_8H_9O_5S$ $[M-H]^-$ 217.0165, found 217.0168.

4-(2-Acetoxyethyl)phenyl sulfate (tyrosol-2′-acetate-4-*O*-sulfate; 6): Tyrosol-2′-acetate (**3,** 100 mg, 0.55 mmol) was sulfated according to the general procedure and purified by preparative HPLC in MeOH/H_2O 3:2 to obtain **6** as a white solid (85 mg, 46%). R_F 0.65 (EtOAc/MeOH 9:2.5). ^{1}H NMR (399.87 MHz, DMSO-d_6, 30 ºC) δ: 7.131 (2H, m, *o*-H), 7.082 (2H, m, *m*-H), 4.174 (2H, t, $J_{1',2'}$ = 6.9 Hz, H-2′), 2.822 (2H, t, $J_{1',2'}$ = 6.9 Hz, H-1′), 1.973 (3H, s, CH_3); *tris*(hydroxymethyl)methylammonium: 7.25 (br s, NH_3), 5.05 (s, OH), 3.46 (s, CH_2);^{13}C NMR (100.55 MHz, DMSO-d_6, 30 ºC) δ: 170.19 (CO), 152.03 (*p*-C), 132.28 (*i*-C), 128.92 (*o*-C), 120.36 (*m*-C), 64.40 (C-2′), 33.55 (C-1′), 20.62 (CH_3); *tris*(hydroxymethyl)methylammonium: 60.4 (C-N), 59.6 (C-O); HRESIMS *m/z* calcd for $C_{10}H_{11}O_6S$ $[M-H]^-$ 259.0271, found 259.0270.

2-Hydroxy-5-(2-hydroxyethyl)phenyl sulfate (hydroxytyrosol-3-*O*-sulfate; 7) and 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate (hydroxytyrosol-4-*O*-sulfate; 8): Hydroxytyrosol (2, 100 mg, 0.64 mmol) was sulfated according to the general procedure and purified by preparative HPLC in H_2O to obtain a mixture of 7 and 8 as a colorless viscous substance (ratio 3-*O*-sulfate/4-*O*-sulfate 2:1, total yield: 86 mg, 49%). R_F 0.48 (EtOAc/MeOH/HCO_2H 4:1:0.2). The position of sulfates was unambiguously assigned in NMR using typical changes in carbon chemical shifts compared to the parent compound.[34] ^{1}H NMR (399.87 MHz, DMSO-d_6, 30 ºC) δ: 3-*O*-sulfate (7) 8.669 (1H, br s, 4-OH), 6.933 (1H, d, $J_{2,6}$ = 2.1 Hz, H-2), 6.793 (1H, dd, $J_{6,2}$ = 2.1 Hz, $J_{6,5}$ = 8.0 Hz, H-6), 6.710 (1H, d, $J_{5,6}$ = 8.0 Hz, H-5), 4.553 (1H, br s, 2′-OH), 3.530 (2H, t, $J_{2',1'}$ = 7.2 Hz, H-2′), 2.592 (2H, t, $J_{1',2'}$ = 7.2 Hz, H-1′); 4-*O*-sulfate (8) 8.669 (1H, br s, 3-OH), 6.961 (1H, d, $J_{5,6}$ = 8.0 Hz, H-5), 6.670 (1H, d, $J_{2,6}$ = 2.1 Hz, H-2), 6.577 (1H,

306 $J_{6,2}$ = 2.1 Hz, $J_{6,5}$ = 8.0 Hz, H-6), 4.553 (1H, br s, 2′-OH), 3.549 (2H, t, $J_{2',1'}$ = 7.2 Hz,
307 H-2′), 2.601 (2H, t, $J_{1',2'}$ = 7.2 Hz, H-1′); ^{13}C NMR (100.55 MHz, DMSO-d_6, 30 ºC) δ:
308 3-*O*-sulfate (7) 147.25 (C-4), 140.41 (C-3), 130.40 (C-1), 125.17 (C-6), 123.45 (C-2),
309 116.83 (C-5), 62.26 (C-2′), 38.07 (C-1′); 4-*O*-sulfate (8) 148.78 (C-3), 138.95 (C-4),
310 136.34 (C-1), 122.84 (C-5), 119.74 (C-6), 117.65 (C-2), 62.14 (C-2′), 38.46 (C-1′);
311 HRESIMS *m/z* calcd for $C_8H_9O_6S$ $[M-H]^-$ 233.0114, found 233.0114.

312 **5-(2-Acetoxyethyl)-2-hydroxyphenyl sulfate (hydroxytyrosol-2′-acetate-3-*O*-**
313 **sulfate; 9) and 4-(2-acetoxyethyl)-2-hydroxyphenyl sulfate (hydroxytyrosol-2′-**
314 **acetate-4-*O*-sulfate; 10):** Hydroxytyrosol-2’-acetate (4, 150 mg, 0.76 mmol) was
315 sulfated according to the general procedure and purified by preparative HPLC in
316 MeOH/H_2O 1:19 to obtain a mixture of 9 and 10 as a colorless viscous substance (ratio
317 3-*O*-sulfate/4-*O*-sulfate 1:6; 96 mg, total yield 34%) R_F 0.63 (EtOAc/MeOH/HCO_2H
318 4:1:0.2). ^{1}H NMR (399.87 MHz, DMSO-d_6, 30 ºC) δ: 4-*O*-sulfate (10) 7.450 (1H, br s,
319 3-OH), 7.009 (1H, d, $J_{5,6}$ = 8.0 Hz, H-5), 6.708 (1H, d, $J_{2,6}$ = 2.1 Hz, H-2), 6.614 (1H,
320 dd, $J_{6,2}$ = 2.1 Hz, $J_{6,5}$ = 8.0 Hz, H-6), 4.161 (2H, t, $J_{2',1'}$ = 6.9 Hz, H-2′), 2.763 (2H,
321 $J_{1',2'}$ = 6.9 Hz, H-1′), 1.986 (3H, s, CH_3); 3-*O*-sulfate (9): 8.630 (1H, br s, 4-OH),
322 6.989 (1H, d, $J_{2,6}$ = 2.1 Hz, H-2), 6.824 (1H, dd, $J_{6,2}$ = 2.1 Hz, $J_{6,5}$ = 8.0 Hz, H-6), 6.739
323 (1H, d, $J_{5,6}$ = 8.0 Hz, H-5), 4.126 (2H, t, $J_{2',1'}$ = 6.9 Hz, H-2′), 2.751 (2H, $J_{1',2'}$ = 6.9
324 Hz, H-1′), 1.982 (3H, s, CH_3); *tris*(hydroxymethyl)methylammonium: 7.45 (br s, NH_3),
325 5.08 (s, OH), 3.46 (s, CH_2); ^{13}C NMR (100.55 MHz, DMSO-d_6, 30 ºC) δ: 4-*O*-sulfate
326 (10) 170.23 (CO), 148.95 (C-3), 139.39 (C-4), 134.73 (C-1), 122.98 (C-5), 119.67 (C-
327 6), 117.65 (C-2), 64.32 (C-2′), 33.72 (C-1′), 20.67 (CH_3); 3-*O*-sulfate (9) 170.24 (CO),
328 147.64 (C-4), 140.60 (C-3), 128.81 (C-1), 125.09 (C-6), 123.47 (C-2), 117.05 (C-5),
329 64.43 (C-2′), 33.37 (C-1′), 20.63 (CH_3); *tris*(hydroxymethyl)methylammonium: 60.4

330 (C-N), 59.6 (C-O); signals for HRESIMS *m/z* calcd for $C_{10}H_{11}O_7S$ $[M-H]^-$ 275.0220,
331 found 275.0218.

332 **Antioxidant activity.**

333 In order to better characterize the obtained compounds, their ability to reduce Folin-
334 Ciocalteu reagent, to scavenge DPPH radicals and to inhibit lipid peroxidation (ILP)
335 were determined and evaluated with respect of the calculated miLogP values (Table 1).

336 FCR assay is known as a total phenol determination, however it is based on reducing
337 capacity measurement, and thus it indicates the overall antioxidant status of the tested
338 compound.[52] As expected, replacing hydroxyl groups with sulfate groups in the
339 aromatic ring decreased the reducing capacity (Table 1).

340 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay is one of the most
341 widely used methods to compare antioxidant activity of natural and (semi)synthetic
342 biologically active compounds. Although this assay has no direct physiological
343 relevance, it allows quick comparison of free radical scavenging potential as this
344 activity has been described for many compounds in the literature.[53] In our experimental
345 setup, only hydroxytyrosol (**2**) and hydroxytyrosol-2′-acetate (**4**) displayed measurable
346 activity with IC_{50} values of 11 and 9 μM, respectively. This is in contrast with
347 previously published activity of these two compounds,[54] probably due to slightly
348 different experimental conditions. Tyrosol (**1**), tyrosol-2′-acetate (**3**) and all sulfate
349 conjugates **5**–**10** exhibited no detectable activity in this assay (IC_{50} > 225 μM, Table 1).

350 Using a more biologically relevant system, we determined the ability of all compounds
351 to inhibit the lipid peroxidation of rat liver microsomes induced by the pro-oxidant *tert*-
352 butyl hydroperoxide (*t*-BH) in the ILP assay. The results are expressed as the

concentration of the tested compound needed to inhibit lipid peroxidation by 50 % (IC_{50}). The results for hydroxytyrosol (**2**) and hydroxytyrosol-2′-acetate (**4**) correlated with their DPPH scavenging activity with IC_{50} values of 42 and 7 µM, respectively. This is in good agreement with previously reported effect of both compounds on microsomes from vitamin E deficient rats.[55] Among the sulfated compounds, the best inhibitors of the lipid peroxidation were the acetylated derivatives with more aromatic hydroxyls; in this way, hydroxytyrosol-2′-acetate monosulfates (**9**, **10**) were the most active compounds (0.43 ± 0.04 mM). In addition, the hydroxytyrosol monosulfates (**7**, **8)** (1.3 ± 0.3 mM) exhibited better activity than tyrosol (**1**) (2 ± 1 mM). Lipid peroxidation can be considered as a process under which free radicals transform lipids containing C=C bonds, especially polyunsaturated fatty acids.[56] It has been shown that the radical scavenging activity of lipophilic phenols is not linearly correlated with hydrophobicity, and efforts to correlate lipophilicity (calculated log P) with antioxidant capacity failed due to the influence of the antioxidant location in biphasic environments on their properties.[57] In agreement with this, no good correlation between ILP and lipophilicity/hydrophilicity (miLogP) of the tested compounds was found in the present work. However for hydroxytyrosol (**2**) and its derivatives (**4, 9**/**10**, **7**/**8**), the more positive the values of miLogP (1.22, 0.52, -2.27, and -2.98), the stronger the lipid peroxidation inhibition (IC_{50} 7.0, 42.1, 433, and 1300 µM, respectively).

In general, the sulfated derivatives have a lower antioxidant activity than their respective parent phenols as expected, as sulfation involves reduction in the number of phenolic hydroxyl groups. The results of ILP and DPPH highlight the structural importance of the catechol moiety in phenols for potent antioxidant activity.

In conclusion, chemoenzymatic sulfation using AST from *D. hafniense* is a relatively rapid one-step method that is efficient and widely applicable to a number of catechol

scaffolds. We demonstrate that AST is chemoselective towards aromatic hydroxyls, so it is not necessary to protect aliphatic hydroxyls allowing thus preparation of respective metabolites in a single step. When comparing aromatic hydroxyls, the enzyme slightly prefers the 3-position in hydroxytyrosol, and the 4-position in hydroxytyrosol-2′-acetate. The sulfation decreased the anti-lipoperoxidant, radical scavenging and reducing properties of the tested phenolics and increased their hydrophilicity. The monosulfate metabolites synthesized here will be used as reference compounds and standards to determine their bioavailability in humans and thus clarify their metabolism.

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Supporting Information

Supporting information contains: ^{1}H NMR, ^{13}C NMR, HMBC, MS-ESI and HPLC chromatograms of the compounds.

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Figure captions

Figure 1. Structures of phenolic compounds in olive oil

Figure 2. Sulfation of tyrosol and tyrosol-2′-acetate.

Figure 3. Sulfation of hydroxytyrosol and hydroxytyrosol-2′-acetate.

Figure 4. HPLC chromatograms of (A) a mixture of hydroxytyrosol sulfate regioisomers **7** and **8**, and (B) a mixture of hydroxytyrosol-2′-acetate sulfate regioisomers **9** and **10**. Small signals to the right are not identified.

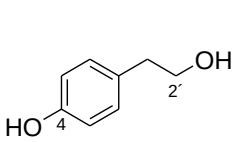
Tables

Table 1: Lipoperoxidation Inhibition, Reducing Capacity and Lipophilicity of Olive Oil Phenolic Compounds and their Sulfated Derivatives[a]

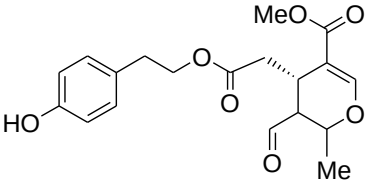
Compound	FCR (GAE)[b]	DPPH (IC_{50} [µM])[bc]	ILP (IC_{50} [µM])[b]	miLogP[e]
Tyrosol (**1**)	0.82 ± 0.02	> 225	> 2000	1.00
Hydroxytyrosol (**2**)	0.87 ± 0.01	11 ± 1	42 ± 26	0.52
Tyrosol-2′-acetate (**3**)	0.38 ± 0.02	> 225	> 3000	1.71
Hydroxytyrosol-2′-acetate (**4**)	0.86 ± 0.03	9 ± 1	7 ± 5	1.22
Tyrosol-4-*O*-sulfate (**5**)	0.04 ± 0.01**	> 225	> 3000	-2.46
Tyrosol-2′-acetate-4-*O*-sulfate (**6**)	0.04 ± 0.02**	> 225	> 3000	-1.75
Hydroxytyrosol-sulfate (**7**, **8**)	0.42 ± 0.03**	> 225	1300 ± 300*	-2.98, -2.98
Hydroxytyrosol-2′-acetate-sulfate (**9**, **10**)	0.35 ± 0.03**	> 225	433 ± 41**	-2.27, -2.27

[a] Values are given as means ± standard deviation calculated from three independent experiments. [b] Folin-Ciocalteu reagent reduction (gallic acid equivalents), [c] 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, [d] Inhibition of lipoperoxidation of rat liver microsomal membranes induced by *tert*-butylhydroperoxide, [e] hydrophobicity of compounds. ** p <0.001 statistically significant difference from value obtained with corresponding non-sulfated phenol. * p <0.01 statistically significant difference from value obtained with corresponding non-sulfated phenol.

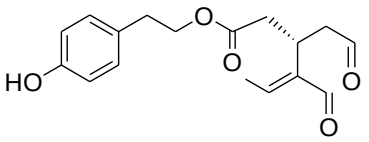
Figure graphics



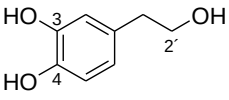
Tyrosol (**1**)



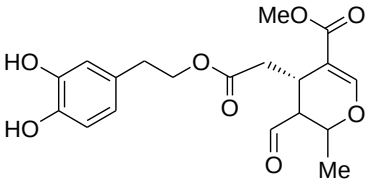
Tyrosol-elenolic acid ester (Tyr-EA)



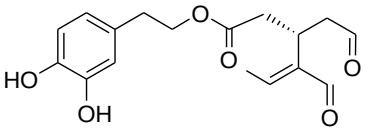
Oleocanthal



Hydroxytyrosol (**2**)



Hydroxytyrosol-elenolic acid ester (Hy-EA)



Oleacein

Figure 1.

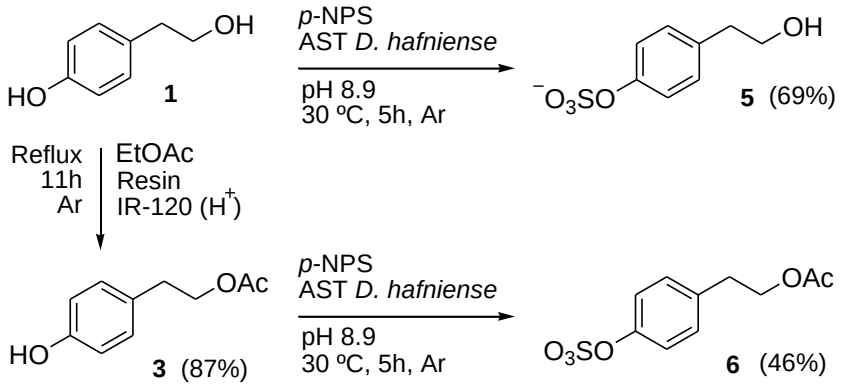

OH
HO
1
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
OH
⁻O3SO
5 (69%)
Reflux
11h
Ar
EtOAc
Resin
IR-120 (H+)
OAc
HO
3 (87%)
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
OAc
O3SO
6 (46%)

Figure 2.

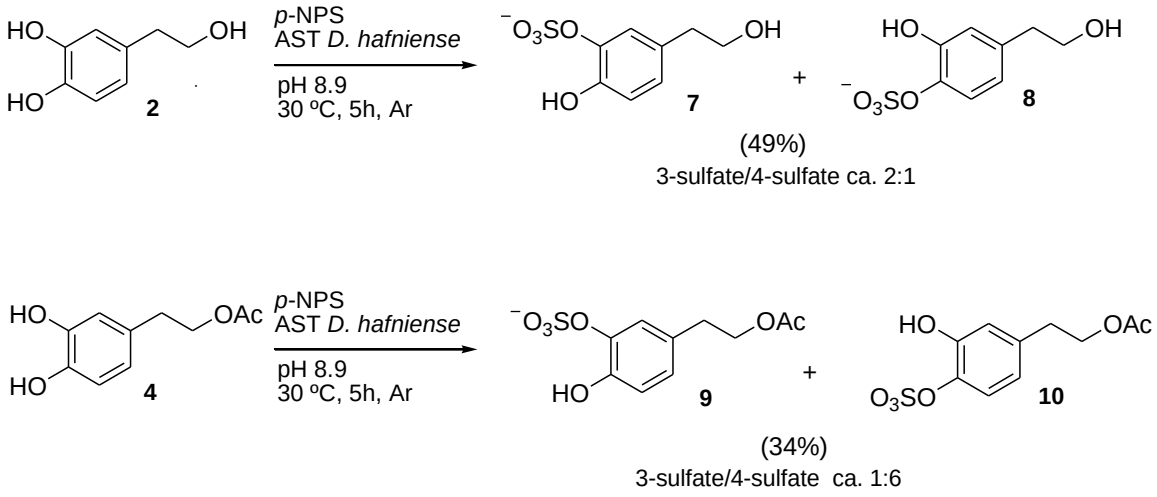

HO
OH
HO
2
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
⁻O3SO
OH
HO
7
+
HO
OH
⁻O3SO
8
(49%)
3-sulfate/4-sulfate ca. 2:1
HO
OAc
HO
4
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
⁻O3SO
OAc
HO
9
+
HO
OAc
O3SO
10
(34%)
3-sulfate/4-sulfate ca. 1:6

Figure 3.

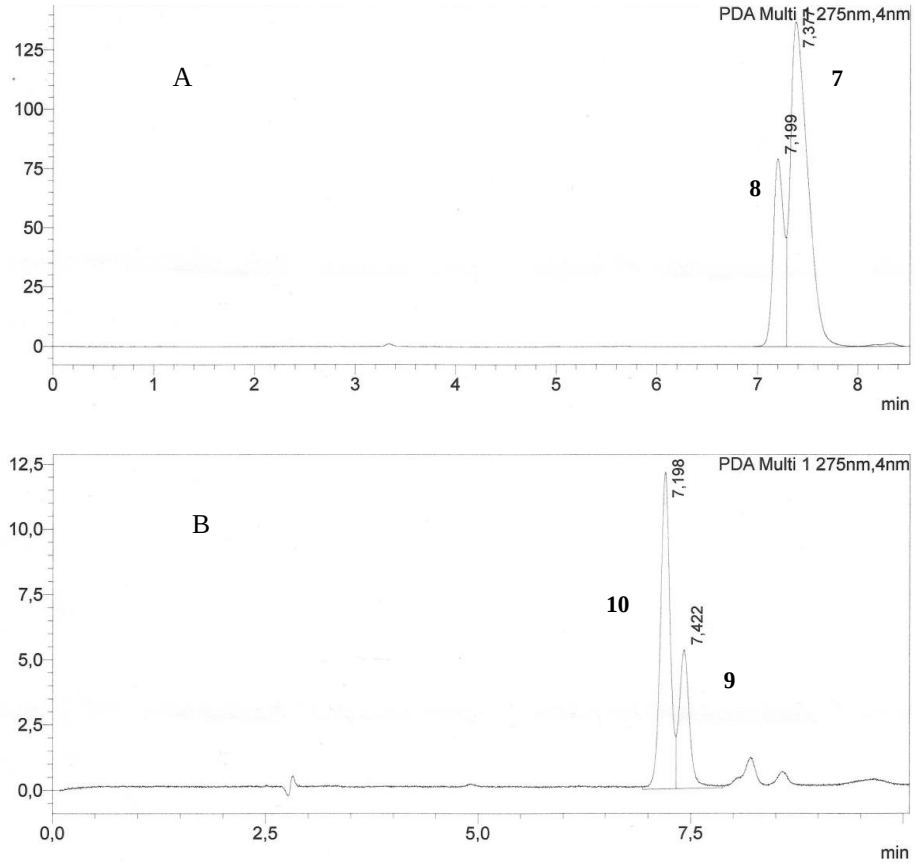

A
PDA Multi 1 275nm,4nm
7,377
7,199
8
7
125
100
75
50
25
0
0
1
2
3
4
5
6
7
8
min
B
PDA Multi 1 275nm,4nm
7,198
7,422
10
9
12,5
10,0
7,5
5,0
2,5
0,0
0,0
2,5
5,0
7,5
min

Figure 4.

Graphic for table of contents