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Bioactive Constituents, Metabolites, and Functions

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

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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.

116 The enzymatic sulfation was monitored by thin-layer chromatography (TLC) [silica gel
117 60 F_{254} plates (Merck, DE); mobile phase EtOAc/MeOH/HCO_2H, 4:1:0.2, v/v and
118 EtOAc/MeOH 9:2.5, v/v].

119 All analytical HPLC analyses were performed in a Shimadzu Prominence LC analytical
120 system consisting of a Shimadzu LC-20AD binary HPLC pump, Shimadzu SIL-
121 20ACHT cooling auto sampler, Shimadzu CTO-10AS column oven, Shimadzu CBM-
122 20A system controller and Shimadzu SPD-20MA diode array detector (Shimadzu, JP);
123 there was no coupling to a MS detector. The sample (0.5 mg) was dissolved in the
124 mobile phase A (50 μL) and analyzed in a Kinetex PFP (150 × 4.6 mm, 5 μm) column
125 (Phenomenex, USA) coupled with a PFP security guard cartridge kit (4 × 3 mm).
126 Binary gradient elution was used: mobile phase A = 0.1% trifluoroacetic acid in water;
127 mobile phase B = 100% methanol; gradient: 0 min 10% B, 20 min 40% B, 21 min 10%
128 B. The flow rate was 0.6 mL/min at 45 °C and the injection volume was 1 μL; the peaks
129 were detected at 275 nm (compounds **7**, **8**, **9**, **10**) or at 254 nm (compounds **5**, **6**).

130 Preparative HPLC separations were performed using an ASAHIPAK GS-310 20F
131 column (Shodex, Munich, Germany), with the mobile phase specified for each
132 experiment, flow rate 5 mL/min and detection at 254 and 369 nm. The
133 preparative HPLC (Shimadzu, Kyoto, Japan) system consisted of an LC-8A
134 high-pressure pump with an SPD-20A dual-wavelength detector (with semi-
135 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

182 reader (Tecan Group Ltd., Männedorf, Switzerland) and the reducing capacity was
183 expressed as gallic acid equivalents (GAE).

184 **DPPH Assay.** The antiradical activity of the compounds was tested as the capacity to
185 reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich), thus decreasing the
186 violet color of DPPH as previously described[44,45] with minor modifications. A 15 µL
187 solution of the tested substance (final concentration 0 – 5 mM in MeOH) was mixed
188 with 285 µL of a freshly prepared methanolic DPPH solution (final concentration 20
189 µM) in a microtiter plate well (total volume 300 µL). After 30 min at 25 °C, the
190 absorbance at 517 nm was measured. The concentration of the antioxidant required for
191 reducing the DPPH concentration to 50% (IC_{50}) of its initial value was calculated.

192 **Inhibition of Microsomal Lipid Peroxidation.** This assay was performed according to
193 the reported method.[44] Pooled microsomes from male rat liver (M9066, Sigma-Aldrich)
194 were washed 5× using centrifugation (13,500 rpm, 5 min, 4 ºC) and PBS to remove
195 sucrose, and diluted to 0.625 mg protein/mL with PBS before use. The protein
196 concentration was determined using the Bradford method.[46] A 0.4 mL solution of the
197 diluted microsomal suspension was then mixed with the compounds **1**–**10** (final
198 concentration 5 µM – 2 mM in 50 µL PBS), *tert*-butyl hydroperoxide (*t*-BH, a pro-
199 oxidant, 50 µL in PBS; final concentration 1 mM) was then added and the mixture was
200 incubated at 37 ºC for 60 min. The products of lipid peroxidation were determined as
201 thiobarbituric acid reactive substances (TBARS): 0.7 mL of trichloroacetic acid (26
202 mM) with thiobarbituric acid (918 mM) were added, the mixture was heated (90 ºC, 15
203 min), cooled, centrifuged (13,500 rpm, 10 min, 4 ºC) and the absorbance of the
204 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

252 enzymatic sulfation of the catechol fragment of **2** and **4** depends on the features of the
253 side chain. No disulfated derivatives were formed, presumably because the charge and
254 the size of the sulfate group preclude subsequent sulfation.

255

256 From the ^{1}H NMR spectra of the reaction mixture we could deduce that hydroxytyrosol-
257 3-*O*-sulfate (**7**) and hydroxytyrosol-4-*O*-sulfate (**8**) were formed in a 1.3:1 ratio;
258 whereas, hydroxytyrosol-2′-acetate-3-*O*-sulfate (**9**) and hydroxytyrosol-2′-acetate-4-*O*-
259 sulfate (**10**) were in a 1:1.4 ratio. Due to their physicochemical properties, the
260 regioisomers **7** and **8** derived from hydroxytyrosol, and **9** and **10**, derived from
261 hydroxytyrosol acetate were difficult to separate by preparative HPLC (ASAHIPAK
262 GS-310 20F column). After chromatography, partial separation was achived (**7**/**8** in a
263 2:1 ratio, and **9**/**10** in a 1:6 ratio). At analytical scale (Kinetex PFP column), the best
264 conditions to separate both pairs of regioisomers involved the use of a gradient of 0.1%
265 trifluoroacetic acid in water (phase A) and methanol (phase B) using an analytical
266 Kinetex PFP HPLC column (Figure 4).

267 Only monosulfate and glucuronide conjugates of olive phenols were found to be the
268 main metabolites in human plasma and excreted in urine after olive oil intake.[50,51] In
269 accordance with this, no disulfated or trisulfated compounds were detected in any case,
270 even using up to four equivalents of *p*-NPS as the sulfate donor using the
271 chemoenzymatic method in this work. This is in contrast to the situation with flavonols
272 and flavonolignans.[35,36] This might be due to the high polarity (low log P) of the
273 monosulfates of these small phenolics (see Table 1), which are therefore probably not
274 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 $C_8H_9O_5S$ $[M\text{-}H]^-$ 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 $C_{10}H_{11}O_6S$ $[M\text{-}H]^-$ 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, $J_{6,2}$ = 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, 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: 3-*O*-sulfate (7) 147.25 (C-4), 140.41 (C-3), 130.40 (C-1), 125.17 (C-6), 123.45 (C-2), 116.83 (C-5), 62.26 (C-2′), 38.07 (C-1′); 4-*O*-sulfate (8) 148.78 (C-3), 138.95 (C-4), 136.34 (C-1), 122.84 (C-5), 119.74 (C-6), 117.65 (C-2), 62.14 (C-2′), 38.46 (C-1′); HRESIMS *m/z* calcd for $C_8H_9O_6S$ $[M-H]^-$ 233.0114, found 233.0114.

5-(2-Acetoxyethyl)-2-hydroxyphenyl sulfate (hydroxytyrosol-2′-acetate-3-*O*-sulfate; 9) and 4-(2-acetoxyethyl)-2-hydroxyphenyl sulfate (hydroxytyrosol-2′-acetate-4-*O*-sulfate; 10): Hydroxytyrosol-2'-acetate (4, 150 mg, 0.76 mmol) was sulfated according to the general procedure and purified by preparative HPLC in MeOH/H_2O 1:19 to obtain a mixture of 9 and 10 as a colorless viscous substance (ratio 3-*O*-sulfate/4-*O*-sulfate 1:6; 96 mg, total yield 34%) R_F 0.63 (EtOAc/MeOH/HCO_2H 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, 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, 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, $J_{1',2'}$ = 6.9 Hz, H-1′), 1.986 (3H, s, CH_3); 3-*O*-sulfate (9): 8.630 (1H, br s, 4-OH),

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 (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 Hz, H-1′), 1.982 (3H, s, CH_3); *tris*(hydroxymethyl)methylammonium: 7.45 (br s, NH_3), 5.08 (s, OH), 3.46 (s, CH_2); ^{13}C NMR (100.55 MHz, DMSO-d_6, 30 ºC) δ: 4-*O*-sulfate (10) 170.23 (CO), 148.95 (C-3), 139.39 (C-4), 134.73 (C-1), 122.98 (C-5), 119.67 (C-6), 117.65 (C-2), 64.32 (C-2′), 33.72 (C-1′), 20.67 (CH_3); 3-*O*-sulfate (9) 170.24 (CO), 147.64 (C-4), 140.60 (C-3), 128.81 (C-1), 125.09 (C-6), 123.47 (C-2), 117.05 (C-5), 64.43 (C-2′), 33.37 (C-1′), 20.63 (CH_3); *tris*(hydroxymethyl)methylammonium: 60.4 (C-N), 59.6 (C-O); signals for HRESIMS *m/z* calcd for $C_{10}H_{11}O_7S$ $[M-H]^-$ 275.0220, found 275.0218.

Antioxidant activity.

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

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

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay is one of the most widely used methods to compare antioxidant activity of natural and (semi)synthetic biologically active compounds. Although this assay has no direct physiological relevance, it allows quick comparison of free radical scavenging potential as this activity has been described for many compounds in the literature.[53] In our experimental setup, only hydroxytyrosol (**2**) and hydroxytyrosol-2′-acetate (**4**) displayed measurable

activity with IC_{50} values of 11 and 9 μM, respectively. This is in contrast with previously published activity of these two compounds,[54] probably due to slightly different experimental conditions. Tyrosol (**1**), tyrosol-2′-acetate (**3**) and all sulfate conjugates **5**–**10** exhibited no detectable activity in this assay (IC_{50} > 225 μM, Table 1).

Using a more biologically relevant system, we determined the ability of all compounds to inhibit the lipid peroxidation of rat liver microsomes induced by the pro-oxidant *tert*-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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393

394 **Supporting Information**

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

397

398 **References**

1 Strott, C. A. Sulfonation and molecular action. *Endocr. Rev*. **2002**, *23*, 703–732.

2 Tan, E.; Pang, S. K. Sulfation in rate limiting in the futile cycling between estrone and estrone sulfate in enriched periportal and perivenous rat hepatocytes. *Drug Metab. Dispos.* **2001**, *29*, 335–346.

3 Cicerale, S.; Lucas, L.; Keast, R. S. Biological activities of phenolic compounds present in virgin olive oil. *Int. J. Mol. Sci.* **2010**, *11*, 458–479.

4 Polini, B.; Digiacomo, M.; Carpi, S.; Bertini, S.; Gado, F.; Saccomanni, G.; Macchia, M.; Nieri, P.; Manera, C.; Fogli, S. Oleocanthal and oleacein contribute to the *in vitro* therapeutic potential of extra virgin oil-derived extracts in non-melanoma skin cancer. *Toxicol. in Vitro* **2018**, *52*, 243–250.

5 Batarseh, Y. S.; Kaddoumi, A. Oleocanthal-rich extra-virgin olive oil enhances donepezil effect by reducing amyloid-β load and related toxicity in a mouse model of Alzheimer's disease. *J. Nutr. Biochem.* **2018**, *55*, 113–123.

6 Fogli, S.; Arena, C.; Carpi, S.; Polini, B.; Bertini, S.; Digiacomo, M.; Gado Saba, A.; Saccomanni, G.; Breschi, M. C.; Nieri, P.; Manera, C.; Macchia, M.

Cytotoxic activity of oleocanthal isolated from virgin olive oil on human melanoma cells. *Nutr. Cancer* **2016**, *68*, 873–877.

7 LeGendre, O.; Breslin, P. A.; Foster, D. A. (-)-Oleocanthal rapidly and selectively induces cancer cell death *via* lysosomal membrane permeabilization. *Mol. Cell Oncol.* **2015**, *2*, e1006077.

8 Granados-Principal, S.; Quiles, J. L.; Ramírez-Tortosa, C. L.; Sánchez-Rovira, P.; Rámirez- Tortosa, M. C. Hydroxytyrosol: from laboratory investigations to future clinical trials. *Nutr. Rev.* **2010**, *68*, 191–206.

9 Fernández-Bolaños, J. G.; López, O.; Fernández-Bolaños, J.; Rodríguez-Gutiérrez, G. Hydroxytyrosol and derivatives: Isolation, synhtesis, and biological properties. *Curr. Org. Chem*. **2008**, *12*, 442–463.

10 Bernini, R.; Gilardini Montani, M. S.; Merendino, N.; Romani, A.; Velotti, F. Hydroxytyrosol-derived compounds: A basis for the creation of new pharmacological agents for cancer prevention and therapy. *J. Med. Chem*. **2015**, *58*, 9089–9107.

11 Aparicio-Soto, M.; Sanchez-Hidalgo, M.; *Cardeno*, A.; Gonzalez-Benjumea, A.; Fernandez-Bolanos, J.G. Alarcón-de-la-Lastra, C. Dietary hydroxytyrosol and hydroxytyrosyl acetate supplementation prevent pristane-induced systemic lupus erythematous in mice. *J. Funct. Food* **2017**, *29*, 84–92.

12 Rosillo, M. A.; Sánchez-Hidalgo, M.; González-Benjumea, A.; Fernández-Bolaños, J. G.; Lubberts, E.; Preventive effects of dietary hydroxytyrosol acetate, an extra virgin olive oil polyphenol in murine collagen-induced arthritis. *Mol. Nutr. Food Res.* **2015**, *59,* 2537–2546.

13 Lu, J.; Huang, G.; Wang, Z.; Zhuang, S.; Xu, L.; Song, B.; Xiong, Y.; Guan, S. Tyrosol exhibits negative regulatory effects on LPS response and endotoxemia. *Food Chem. Toxicol*. **2013**, *62*, 172–178.

14 Vlachogianni, I. C.; Fragopoulou, E.; Stamatakis, G. M.; Kostakis, I. K.; Antonopoulou, S. Platelet activating factor (PAF) biosynthesis is inhibited by phenolic compounds in U-937 cells under inflammatory conditions. *Prostaglandins Other Lipid Mediat*. **2015**, *121*, 176–183.

15 Serreli, G.; Deiana, M. Biological relevance of extra virgin olive oil polyphenols metabolites. Antioxidants **2018**, *7*, 1–17

16 Lopez de las Hazas, M. C.; Motilva, M. J.; Pinol, C.; Macia, A. Application of dried blood spot cards to determine olive oil phenols (hydroxytyrosol metabolites) in human blood. *Talanta* **2016**, *159*, 189–193.

17 Rubió, L.; Valls, R. M.; Macià, A.; Pedret, A.; Giralt, M.; Romero, M. P.; de la Torre, R.; Covas, M. I.; Solà, R.; Motilva, M. J. Impact of olive oil concentration on human plasmatic phenolic metabolites. *Food Chem.* **2012**, *135*, 2922−2929.

18 Pinto, J.; Paiva-Martins, F.; Corona, G.; Debnam, E. S.; OrunaConcha, M. J.; Vauzour, D. Absorption and metabolism of olive oil secoiridoids in the small intestine. *Br. J. Nutr*. **2011**, *105*, 1607−1618.

19 Corona, G.; Tzounis, X.; Dessi, M. A.; Deiana, M.; Debnam, E. S.; Visioli, F.; Spencer, J. P. E. The fate of olive oil polyphenols in the gastrointestinal tract: Implications of gastric and colonic microflora-dependent biotransformation. *Free Radical Res*. **2006**, *40*, 647−658.

20 De Souza, J.; Casanova, L.; Costa, S. Bioavailability of phenolic compounds: a major challenge for drug development? *Rev. Fitos* **2015**, *9*, 1–72.

21 López de las Hazas, M. C.; Rubió, L.; Kotronoulas, A.; De la Torre, R.; Solà. R.; Motilva, M. J. Dose effect on the uptake and accumulation of hydroxytyrosol and its metabolites in target tissues in rats. *Mol. Nutr. Food Res*. **2015**, *59*, 1395–1399.

22 Rubió, L.; Farràs, M.; de La Torre, R.; Macià, A.; Romero, M. -P.; Valls, R. M.; Solà, R.; Farré, M.; Fitó, M.; Motilva, M. Metabolite profiling of olive oil and thyme phenols after a sustained intake of two phenol-enriched olive oils by humans: Identification of compliance markers. *Food Res. Int.* **2014**, *65*, 59−68.

23 D'Archivio, M.; Filesi, C.; Varì, R.; Scazzocchio, B.; Masella, R. Bioavailability of the polyphenols: Status and controversies. Int. J. Mol. Sci. **2010**, *11*, 1321–1342.

24 Walker, J.; Schueller, K.; Schaefer, L. M.; Pignitter, M.; Esefelder. L.; Somoza V. Resveratrol and its metabolites inhibit pro-inflammatory effects of lipopolysaccharides in U-937 macrophages in plasma-representative concentrations. *Food Funct*. **2014**, *5*, 74–84.

25 Coughtrie, M. W. H.; Sharp, S.; Maxwell, K.; Innes, N.P. Biology and function of the reversible sulfation pathway catalysed by human sulfotransferases and sulfatases. *Chem. Biol. Interact.* **1998**, *109*, 3–27.

26 Cicerale, S.; Lucas, L. J.; Keast, R. S. J. Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. *Curr. Opin. Biotechnol.* **2012**, *23*, 129–135.

27 Corona, G.; Spencer, J. P. E.; Dessi, M. A. Extra virgin olive oil phenolics: absorption, metabolism, and biological activities in the GI tract. (2009). *Toxicol. Ind. Health* **2009**, *25*, 285–293.

28 Angeloni, C.; Malaguti, M.; Barbalace, M. M.; Hrelia, S. Bioactivity of olive oil phenols in neuroprotection. *Int. J. Mol. Sci.* **2017**, *18*, 2230.

29 Fortes, C.; García-Vilas, J. A.; Quesada, A. R.; Medina, M. A. Evaluation of the anti-angiogenic potential of hydroxytyrosol and tyrosol, two bio-active phenolic compounds of extra virgin olive oil, in endothelial cell cultures. *Food Chem.* **2012**, *134*, 134–140.

30 Lopez, S.; Montserrat-de la Paz, S.; Lucas, R.; Bermudez, B.; Abia, R.; Morales, J. C.; Muriana, F. J. G. Effect of metabolites of hydroxytyrosol on protection against oxidative stress and inflammation in human endothelial cells. *J. Funct. Food* **2017**, *29*, 238–247.

31 Belmonte-Reche, E.; Martínez-García, M.; Peñalver, P.; Gómez-Pérez, V.; Lucas, R.; Gamarro, F.; Pérez-Victoria, J. M.; Morales, J. C. Tyrosol and hydroxytyrosol derivatives as antitrypanosomal and antileishmanial agents. *Eur. J. Med. Chem.* **2016**, *119*, 132–140.

32 Al-Horani, R. A.; Desai, U. R. Chemical sulfation of small molecules. Advances and challenges. *Tetrahedron* **2010**, *66*, 2907–2918.

33 Van der Horst, M. A.; van Lieshout, J. F. T.; Bury, A.; Hartog, A. F.; Wever, R. Sulfation of various alcoholic groups by an arylsulfate sulfotransferase from *Desulfitobacterium hafniense* and synthesis of estradiol sulfate. *Adv. Synth. Catal.* **2012**, *354*, 3501–3508.

34 Purchartová, K.; Valentová, K.; Pelantová, H.; Marhol, P.; Cvačka, J.; Havlíček, L.; Křenková, A.; Vavříková, E.; Biedermann, D.; Chambers, C. S.; Křen, V. Prokaryotic and eukaryotic aryl sulfotransferases: Sulfation of quercetin and its derivatives. *Chem Cat Chem* **2015,** *7*, 3152–3162.

35 Valentová, K.; Káňová, K.; Di Meo, F.; Pelantová, H.; Chambers, C.; Rydlová, L.; Petrásková, L.; Křenková, A.; Cvačka, J.; Trouillas, P.; Křen, V. Chemoenzymatic preparation and biophysical properties of sulfated quercetin metabolites. *Int. J. Mol. Sci.* **2017**, *18*, 2231.

36 Valentová, K.; Purchartová, K.; Rydlová, L.; Roubalová, L.; Biedermann, D.; Petrásková, L.; Křenková, A.; Pelantová, H.; Holečková-Moravcová, V.; Tesařová, E.; Cvačka, J.; Vrba, J.; Ulrichová, J.; Křen, V. Sulfated metabolites of flavonolignans and 2,3-dehydroflavonolignans: Preparation and properties. *Int. J. Mol. Sci.* **2018**, *19*, 2349.

37 Atzeri, A.; Lucas, R.; Incani, A.; Peñalver, P.; Zafra-Gómez, A.; Melis, M. P.; Pizzala, R.; Morales, J. C.; Deiana, M. Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells. *Food Funct.* **2016**, *7*, 337–346.

38 Martins Gomes, V. P.; Torres, C.; Rodríguez-Borges, J. E.; Paiva-Martins, F. A Convenient Synthesis of Hydroxytyrosol Monosulfate Metabolites. *J. Agric. Food Chem.* **2015**, 63, 9565–9571.

39 Christophoridou, S.; Dais, P.; Tseng, L. H.; Spraul, M. Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with postcolumn solid-phase extraction to nuclear

magnetic resonance spectroscopy (LC-SPE-NMR). *J. Agric. Food Chem*. **2005**, *53*, 4667–4679.

40 Olmo-García, L.; Polari, J. J.; Li, X.; Bajoub, A.; Fernández-Gutiérrez, A.; Wang, S. C.; Carrasco-Pancorbo, A. Deep insight into the minor fraction of virgin olive oil by using LC-MS and GC-MS multi-class methodologies. *Food Chem*. **2018**, *261*, 184–193.

41 Sánchez-Fidalgo, S.; Villegas, I.; Aparicio-Soto, M.; Cárdeno, A.; Rosillo, M. A.; González-Benjumea, A.; Marset, A.; López, O.; Maya, I.; Fernández-Bolaños, J. G.; Alarcón de la Lastra, C. Effects of dietary virgin olive oil polyphenols: hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol on DSS-induced acute colitis in mice. *J. Nutr. Biochem.* **2015**, *26*, 513–520.

42 Bernini, R.; Mincione, E.; Barontini, M.; Crisante, F. Convenient synthesis of hydroxytyrosol and its lipophilic derivatives from tyrosol or homovanillyl alcohol. *J. Agric. Food Chem.* **2008**, *56*, 8897–8904.

43 Velioglu, Y. S.; Mazza, G.; Gao, L.; Oomah, B. D. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food Chem.* **1998**, *46*, 4113–4117.

44 Pyszková, M.; Biler, M.; Biedermann, D.; Valentová, K.; Kuzma, M.; Vrba, J.; Ulrichová, J.; Sokolová, R.; Mojovic, M.; Popovic-Bijelic, A.; Kubala, M.; Trouillas, P.; Křen, V.; Vacek, J. Flavonolignan 2,3-dehydroderivatives: Preparation, antiradical and cytoprotective activity. *Free Radic. Biol. Med.* **2016**, *90*, 114–125.

45 Joyeux, M.; Mortier, F.; Fleurentin, J. Screening of antiradical, antilipoperoxidant and hepatoprotective effect of nine plant extracts used in Caribbean folk medicine. *Phythother. Res.* **1995**, *9*, 228–230.

46 Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

47 Pyka, A.; Babuska, M.; Zachariasz, M. A comparison of theoretical methods of calculation of partition coefficients for selected drugs. *Acta Pol. Pharm.* **2006**, *63*, 159–167.

48 Kawai, N.; Fujibayashi, Y.; Kuwabara, S.; Takao, K.; Ijuin, Y.; Kobayashi, S. Synthesis of a potential key intermediate of akaterpin, specific inhibitor of PI-PLC. *Tetrahedron* **2000**, *56*, 6467–6478.

49 Atzeri, A.; Lucas, R.; Incani, A.; Peñalver, P.; Zafra-Gómez, A.; Melis, M. P.; Pizzala, R.; Morales, J. C.; Deiana, M. Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells. *Food Funct.* **2016**, *7*, 337–346.

50 López de las Hazas, M. C.; Godinho-Pereira, J.; Macià, A.; Almeida, A. F.; Ventura, M. R.; Motilva, M. J.; Santos, C. N. Brain uptake of hydroxytyrosol and its main circulating metabolites: Protective potential in neuronal cells. *J. Funct. Food* **2018**, *46*, 110–117.

51 Tuck, K. L.; Hayball, P. J.; Stupans, I. Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. *J. Agric. Food Chem.* **2002**, *50*, 2404–2409.

52 Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *23*, 1841–1856.

53 Sharma, O. P.; Bhat, T. K. The chemistry behind antioxidant capacity assays. *Food Chem.* **2009**, *113*, 1202–1205.

54 Gordon, M. H.; Paiva-Martins, F.; Almeida, M. Antioxidant Activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. **2001**, *49*, 2480–2485.

55 Rubio-Senent, F.; De Roos, B.; Duthie, G.; Fernández-Bolaños, J.; Rodríguez-Gutiérrez, G. Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E-deficient rats. *Eur. J. Nutr.* **2015**, *54*, 1287–1295.

56 Ayala, A.; Muñoz, M. F.; Argüelles, S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid. Med. Cell. Longev*. **2014**, *2014*, 360–438.

57 Bernini, R.; Crisante, F.; Barontini, M.; Tofani, D.; Balducci, V.; Gambacorta, A. Synthesis and structure/antioxidant activity relationship of novel catecholic antioxidant structural analogues to hydroxytyrosol and its lipophilic esters. *J. Agric. Food Chem*. **2012**, *60*, 7408–7416.

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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

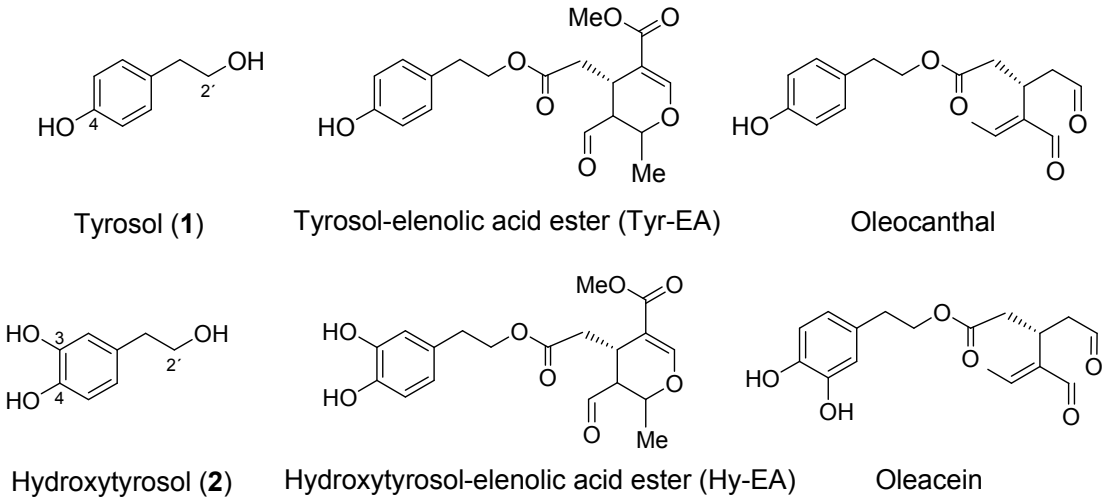

Figure 1.

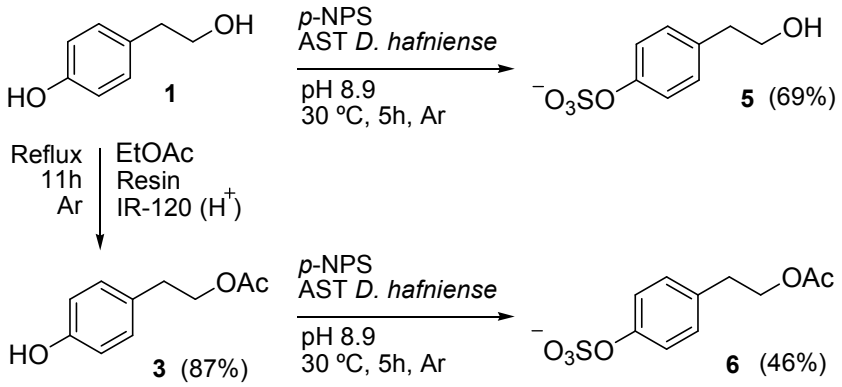
OH
HO
1
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
OH
⁻O3SO
5 (69%)
Reflux
11h
Ar
EtOAc
Resin
IR-120 (H+)
OAc
HO
3 (87%)
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
OAc
⁻O3SO
6 (46%)

Figure 2.

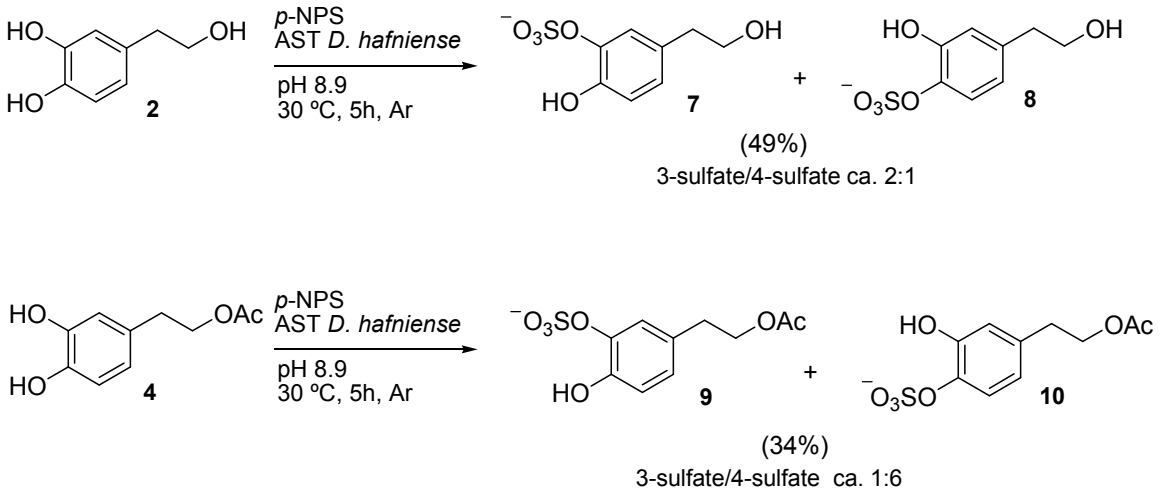
HO
OH
HO
2
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
⁻O3SO
OH
HO
7
+
HO
OH
⁻O3SO
8
(49%)
3-sulfate/4-sulfate ca. 2:1
HO
OAc
HO
4
p-NPS
AST D. hafniense
pH 8.9
30 ºC, 5h, Ar
⁻O3SO
OAc
HO
9
+
HO
OAc
⁻O3SO
10
(34%)
3-sulfate/4-sulfate ca. 1:6

Figure 3.

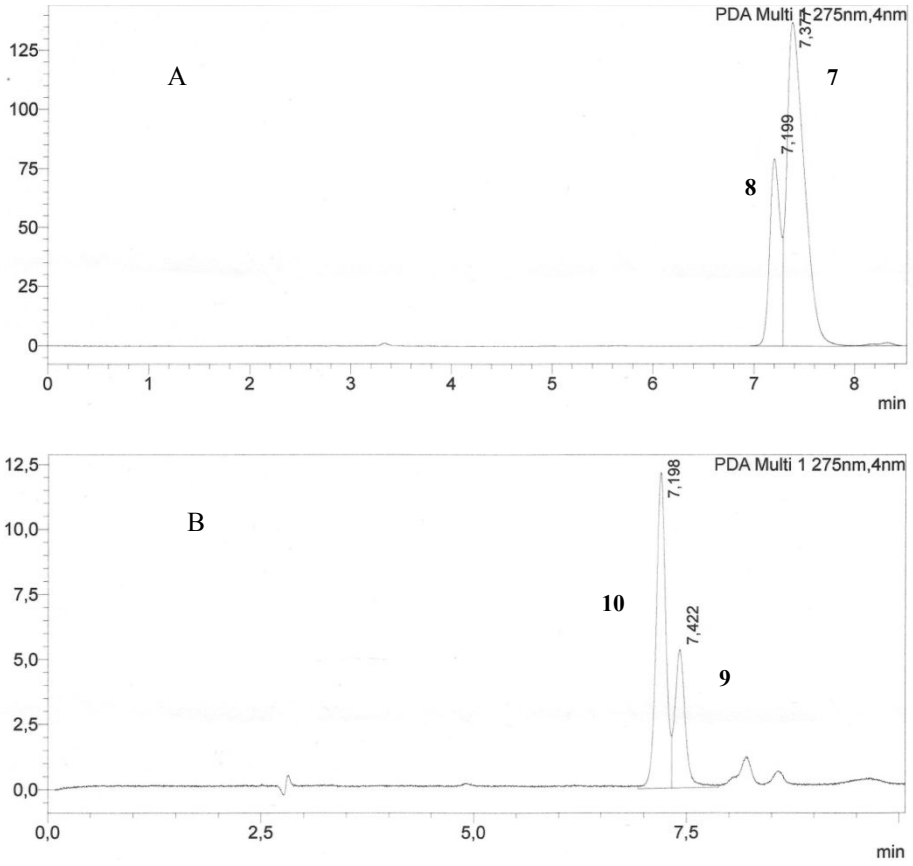
A
PDA Multi 1 275nm,4nm
7,377
7,199
7
8
125
100
75
50
25
0
0
1
2
3
4
5
6
7
8
min
B
PDA Multi 1 275nm,4nm
7,198
7,422
10
9
12,5
10,0
7,5
5,0
2,5
0,0
0,0
2,5
5,0
7,5
min

Figure 4.

Graphic for table of contents