

Synthesis and Bioactivity Profile of 5-S-Lipoylhydroxytyrosol-Based Multidefense Antioxidants with a Sizeable (Poly)sulfide Chain

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S Supporting Information

ABSTRACT: Novel polyfunctionalized antioxidants, 5-S-lipoylhydroxytyrosol (**1**) and its disulfide **2**, trisulfide **3**, and tetrasulfide **4**, were prepared from tyrosol and dihydrolipoic acid in the presence, when appropriate, of sulfur. Compound **1** exhibited significant activity in the ferric reducing/antioxidant power (FRAP) assay (1.60 Trolox equiv), whereas polysulfides **2–4** were more efficient in the DPPH reduction assay (88–93% reduction vs 68% by Trolox). At 10 μ M concentration, all compounds **1–4** proved to be efficient hydroxyl radical scavengers (56–69% inhibition) in a Fenton reaction assay. When administered to human HepG2 cells, **1–4** proved to be nontoxic and exhibited marked protective effects against reactive oxygen species (ROS) generation (60–84% inhibition at 1 μ M concentration) and cell damage induced by 400 μ M *tert*-butylhydroperoxide. All compounds **1–4** exhibited overall greater antioxidant activity than hydroxytyrosol.

KEYWORDS: hydroxytyrosol, lipoic acid, antioxidant, HepG2 cells, bioactive compounds

INTRODUCTION

Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol, HTy), the most representative phenolic constituent of extra virgin olive oil, has been the focus of increasing interest over the past decades because of a range of biological properties that have been implicated to account for the lower risk of cardiovascular diseases and malignant neoplasms observed in the populations of Mediterranean countries.^{1,2} The beneficial role of HTy has been ascribed to its potent antioxidant and scavenging properties against reactive oxygen (ROS) and nitrogen (RNS) species^{3–7} generated in settings of oxidative stress, and its reactivity with oxidizing systems of physiological relevance has been elucidated.^{8–10} In vitro, HTy has been shown to exert protective effect against free radical-induced oxidative stress in different cell lines, such as Caco-2, melanoma, and human hepatoma HepG2 cells.^{11–13}

Several efforts have been directed toward the preparation of HTy derivatives and analogues with improved solubility properties, particularly enhanced lipophilicity,^{14–16} and antioxidant and pharmacological activities. The latter include alkyl HTy ethers, which showed protective effects against oxidative stress in HepG2 cells,¹⁷ and a novel ester of HTy with α -lipoic acid (LA) exhibiting a significant antiproliferative effect on human colon cancer HT-29 cells.¹⁸

In pursuing a research program on the design and synthesis of novel bioinspired antioxidant agents, we recently became interested in exploring the potential of chalcogen-containing conjugates of HTy as prototypes of novel multifunctional polyphenolic derivatives combining diverse scavenging and inhibitory activities against oxidative stress inducing species and systems. Organochalcogen-substituted phenols are efficient scavengers of hydroperoxides,¹⁹ which is attributed to low

O–H bond dissociation enthalpy induced by chalcogens.²⁰ Furthermore, S-glutathionyl conjugates of catechols were identified as most potent antinitrosating agents^{21,22} exceeding the parent catechols, a finding that supported the important role of chalcogen atoms, especially sulfur and selenium, in enhancing the H-atom donating, metal chelating, and reducing power of polyphenolic systems.

On the basis of the above rationale, the aim of the present study was the synthesis and characterization of a novel class of HTy derivatives comprising 5-S-lipoylhydroxytyrosol (**1**), an unprecedented S-conjugate of HTy with dihydrolipoic acid (DHLA), the reduced form of LA or vitamin N, and a set of dimers (**2–4**) linked by di-, tri-, and tetrasulfide chains, in that order (Figure 1). DHLA features two SH groups onto a short carbon chain ending with a carboxyl group, thus providing not only the necessary SH functionality for conjugation with HTy but also a second SH group as reactive site for secondary functionalization as well as a flexible chain expected to favor interaction with membranes, lipid bilayers, and other hydrophobic environments. Polysulfides exhibit diverse beneficial properties for human health, including inhibition of proliferation and induction of apoptosis in cancer cells, suggesting a chemopreventive action of these compounds for cancer therapy.^{23–25} Natural polysulfides, such as diallyl disulfide, diallyl trisulfide, and diallyl tetrasulfide from garlic, are also

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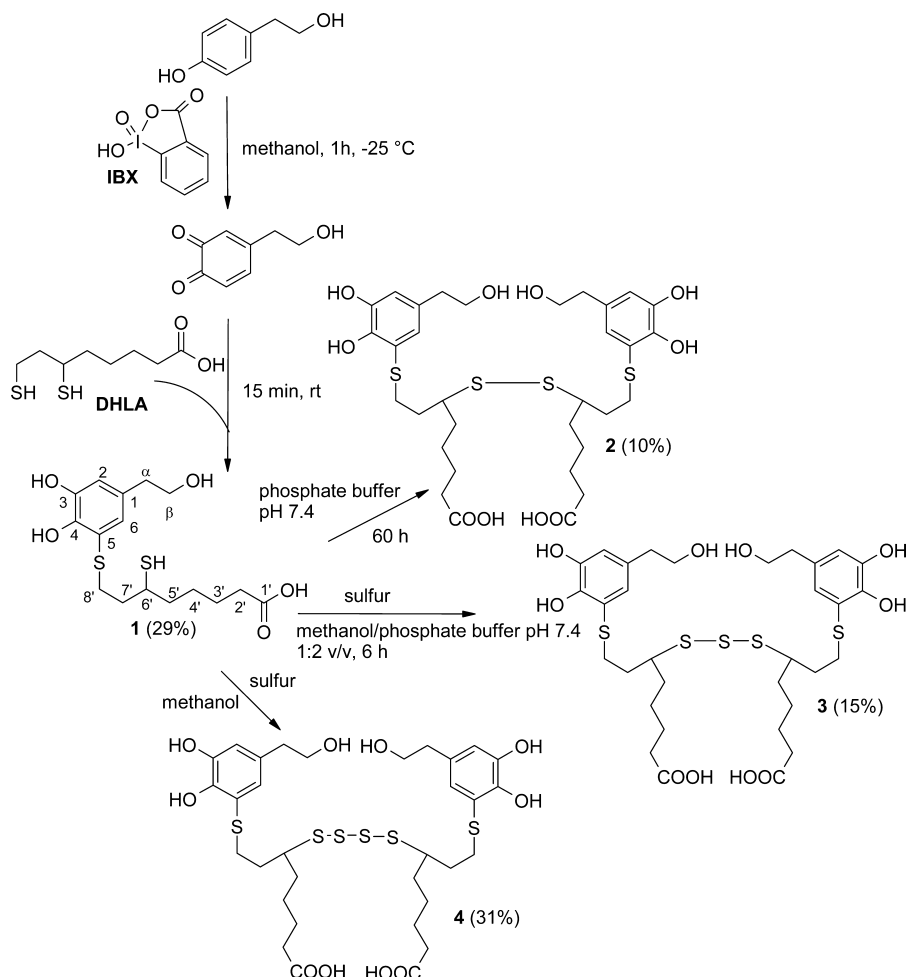


Figure 1. Synthetic route to 5-S-lipoylhydroxytyrosol (1) and polysulfides 2–4.

known for their potent antioxidant activities, which increase with the increasing number of S atoms.^{26,27}

Specific goals of the study were therefore (a) to develop an expeditious and versatile entry to the new HTy derivatives through one-pot oxygenation/oxidation of commercially available tyrosol in the presence of DHLA followed by controlled exposure to elemental sulfur to provide polysulfide bridges when required; (b) to compare the effects of the different sulfur functionalities (SH vs polysulfides) in a battery of chemical tests for antioxidant properties as well as in vitro assays on cultured HepG2 cells; and (c) to draw simple and preliminary structure–property relationships that may guide the rational design of new members of this class of antioxidants with improved activity for diverse applications.

MATERIALS AND METHODS

Caution. IBX is explosive under impact or heating >200 °C.³⁰

Chemicals. 2-Iodobenzoic acid, oxone, (±)-lipoic acid (LA), tyrosol, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), neocuproine, ethylenediaminetetraacetic disodium salt (Na₂EDTA) dihydrate, hydrogen peroxide (30% w/w solution in water), 2-hydroxybenzoic acid (salicylic acid), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and catalase from bovine liver (EC 1.11.1.6) were purchased from Sigma-Aldrich (Milan, Italy). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from CalbioChem (Darmstadt, Germany). Ascorbic acid was purchased from AppliChem (Darmstadt, Germany). DMEM F-12 medium and fetal bovine serum (FBS) were purchased from Biowhitaker (Lonza, Madrid, Spain); *tert*-butylhy-

droperoxide (*t*-BOOH), reduced nicotinic adenine dinucleotide (NADH), 2',7'-dichlorofluorescein diacetate, gentamycin, penicillin G, and streptomycin were purchased from Sigma-Aldrich (Madrid, Spain). Other chemicals used were of analytical grade. (±)-Dihydrolipoic acid (DHLA)²⁸ and 2-iodoxybenzoic acid (IBX)²⁹ were prepared as described.

General Experimental Methods. UV–vis spectra were recorded on a Beckman (Milan, Italy) DU 640 spectrophotometer. ¹H and ¹³C NMR spectra were recorded in CD₃OD at 400 and 100 MHz, respectively, on a Bruker Avance NMR spectrometer. H–H COSY, HSQC, and HMBC data were collected using standard pulse programs. Chemical shifts are reported in δ values downfield from TMS. HPLC analysis was carried out on an Agilent (Santa Clara, CA, USA) instrument equipped with a UV detector set at 280 nm. Chromatographic separation was achieved on a Phenomenex (Castel Maggiore, Italy) Spherclone ODS column (250 mm \times 4.6 mm, 5 μ m) using binary gradient elution conditions as follows: 0.1% trifluoroacetic acid (solvent A), acetonitrile (solvent B), from 5 to 90% B, 0–45 min, flow rate 0.7 mL/min (eluent system I); 10 mM phosphoric acid (pH 2.5)/acetonitrile 80:20 v/v, flow rate 0.7 mL/min (eluent system II). For preparative purposes an Alltech (Passirana di Rho, Milan, Italy) Econosil C18 column (250 mm \times 10 mm, 10 μ m) was used, with 0.1% trifluoroacetic acid/acetonitrile 60:40 v/v as eluent, at a flow rate of 3 mL/min. LC-MS analysis was carried out with an LC-MSD 1100 VL system (Agilent, Santa Clara, CA, USA) equipped with a UV–vis detector (G1314A) and a quadrupole mass spectrometer with electrospray ionization source (G1956A) operating in positive ionization mode (ESI+): nebulizer pressure, 50 psi; drying gas (nitrogen), 10 L/min, 350 °C; capillary voltage, 4000 V; fragmentor voltage, 50 V. Chromatographic separation was performed

on an Eclipse XDB-C18 (150 × 4.60 mm, 5 μm, Agilent) column, at a flow rate of 0.4 mL/min, with eluent system I. HRMS analysis were performed on an FT-ICR Bruker Daltonics mass spectrometer using an ESI source.

Preparation of 5-S-Lipoylhydroxytyrosol (1). To a reaction flask charged with tyrosol (1.0 g, 7.2 mmol), methanol (100 mL), and a stir bar at −25 °C was added IBX (2.9 g, 10.4 mmol, 1.4 equiv), and the resulting reaction mixture was stirred for 1 h. Then, DHLA (6.0 g, 28.8 mmol, 4.0 equiv) in methanol solution (100 mL) was added. Stirring was continued at room temperature for 15 min, and then the mixture was diluted 1:5 with water, acidified to pH 3 with 6 M HCl, and extracted with toluene (6 × 500 mL) to remove 2-iodobenzoic acid, with chloroform (3 × 500 mL) to obtain 1 and finally with ethyl acetate (3 × 500 mL) to recover HTy. Residual IBX in the water phase was destroyed by adding sodium dithionite. The combined chloroform extracts were dried over Na₂SO₄ and taken to dryness to give 1 as a light yellow oil in pure form (752 mg, 29% yield): HRESIMS (negative), *m/z* 359.0985 ([M − H][−]), calcd for C₁₆H₂₃O₅S₂, *m/z* 359.0992; UV, λ_{max} (CH₃OH) 256, 291 nm; ¹H NMR (400 MHz, CD₃OD), δ 1.44 (m, 3, H-4', H-5'), 1.59 (m, 4, H-3', H-5', H-7'), 1.85 (m, 1, H-7'), 2.27 (t, 2, *J* = 7.2 Hz, H-2'), 2.65 (t, 2, *J* = 7.2 Hz, H-α), 2.92 (m, 2, H-6', H-8'), 3.02 (m, 1, H-8'), 3.68 (t, 2, *J* = 7.2 Hz, H-β), 6.63 (d, 1, *J* = 2.0 Hz, H-2), 6.72 (d, 1, *J* = 2.0 Hz, H-6); ¹³C NMR (100 MHz, CD₃OD), δ 25.9 (C-3'), 27.8 (C-4'), 32.6 (C-8'), 35.1 (C-2'), 39.6 (C-α), 39.7 (C-5'), 39.8 (C-7'), 40.3 (C-6'), 64.5 (C-β), 116.8 (C-2), 121.7 (C-5), 125.5 (C-6), 132.0 (C-1), 144.9 (C-4), 146.4 (C-3), 178.0 (C-1').

Preparation of Polysulfides 2–4. Disulfide 2. To a reaction flask charged with 1 (250 mg, 0.69 mmol) in methanol solution (15 mL) was added 0.1 M phosphate buffer (pH 7.4) (255 mL), and the resulting reaction mixture was taken under vigorous stirring at room temperature and periodically analyzed by HPLC and LC-MS (eluent system I). After ca. 60 h, when consumption of 1 was complete, the mixture was acidified to pH 3 and extracted with ethyl acetate (3 × 150 mL). The combined organic layers were dried over Na₂SO₄ and taken to dryness. The residue obtained was dissolved in methanol and fractionated by semipreparative HPLC to give 2 as a light yellow oil in pure form (25 mg, 10% yield): HRESIMS (positive), *m/z* 741.1892 ([M + Na]⁺), calcd for C₃₂H₄₆O₁₀S₄Na, *m/z* 741.1871; UV, λ_{max} (CH₃OH) 256, 291 nm; ¹H NMR (400 MHz, CD₃OD), δ 1.39 (m, 2 × 2, H-4'), 1.57 (m, 4 × 2, H-3', H-5'), 1.80 (m, 2 × 2, H-7'), 2.27 (t, 2 × 2, *J* = 7.6 Hz, H-2'), 2.65 (t, 2 × 2, *J* = 7.2 Hz, H-α), 2.80 (m, 1 × 2, H-6'), 2.89 (m, 1 × 2, H-8'), 2.96 (m, 1 × 2, H-8'), 3.68 (t, 2 × 2, *J* = 7.2 Hz, H-β), 6.63 (d, 1 × 2, *J* = 1.6 Hz, H-2), 6.72 (d, 1 × 2, *J* = 1.6 Hz, H-6); ¹³C NMR (100 MHz, CD₃OD), δ 26.0 (C-3'), 27.4 (C-4'), 32.1 (C-8'), 34.9 (C-2'), 35.0 (C-5', C-7'), 39.6 (C-α), 51.9 (C-6'), 64.5 (C-β), 116.8 (C-2), 121.5 (C-5), 125.6 (C-6), 131.9 (C-1), 144.9 (C-4), 146.3 (C-3), 177.7 (C-1').

Trisulfide 3. To a reaction flask charged with 1 (250 mg, 0.69 mmol) in methanol solution (15 mL) and 0.1 M phosphate buffer (pH 7.4) (180 mL) was added S (80 mg, 2.5 mmol) in methanol solution (90 mL), and the resulting reaction mixture was taken under vigorous stirring at room temperature and periodically analyzed by HPLC and LC-MS (eluent system I). After ca. 6 h, when consumption of the starting material was complete, the reaction mixture was diluted 1:4 with water, acidified to pH 3, washed with *n*-hexane (3 × 700 mL) to remove excess sulfur, and extracted with ethyl acetate (3 × 700 mL). The combined organic layers were dried over Na₂SO₄ and taken to dryness. The residue obtained was dissolved in methanol and fractionated by semipreparative HPLC to give 3 as a light yellow oil in pure form (40 mg, 15% yield): HRESIMS (positive), *m/z* 773.1611 ([M + Na]⁺), calcd for C₃₂H₄₆O₁₀S₃Na, *m/z* 773.1592; UV, λ_{max} (CH₃OH) 260, 293 nm; ¹H NMR (400 MHz, CD₃OD), δ 1.43 (m, 2 × 2, H-4'), 1.59 (m, 4 × 2, H-3', H-5'), 1.84 (m, 1 × 2, H-7'), 1.94 (m, 1 × 2, H-7'), 2.27 (t, 2 × 2, *J* = 7.2 Hz, H-2'), 2.66 (t, 2 × 2, *J* = 7.2 Hz, H-α), 2.93 (m, 1 × 2, H-8'), 3.01 (m, 2 × 2, H-6', H-8'), 3.68 (t, 2 × 2, *J* = 7.2 Hz, H-β), 6.63 (d, 1 × 2, *J* = 1.6 Hz, H-2), 6.72 (d, 1 × 2, *J* = 1.6 Hz, H-6); ¹³C NMR (100 MHz, CD₃OD), δ 25.7 (C-3'), 27.5 (C-4'), 32.6 (C-8'), 34.6 (C-5'), 34.7 (C-7'), 35.0 (C-2'), 39.4

(C-α), 52.9 (C-6'), 65.1 (C-β), 117.0 (C-2), 121.5 (C-5), 126.0 (C-6), 132.1 (C-1), 144.9 (C-4), 146.2 (C-3), 178.2 (C-1').

Tetrasulfide 4. To a reaction flask charged with 1 (250 mg, 0.69 mmol) in methanol solution (270 mL) was added S (80 mg, 2.5 mmol), and the resulting reaction mixture was taken under vigorous stirring at room temperature and periodically analyzed by HPLC and LC-MS (eluent system I). After ca. 48 h, when consumption of the starting material was complete, the mixture was washed with *n*-hexane (3 × 100 mL) to remove excess sulfur and taken to dryness, and the residue obtained was dissolved in methanol and fractionated by semipreparative HPLC to give 4 as a light yellow oil in pure form (85 mg, 31% yield): HRESIMS (negative), *m/z* 781.1362 ([M − H][−]), calcd for C₃₂H₄₅O₁₀S₄, *m/z* 781.1342; UV, λ_{max} (CH₃OH) 256, 294 nm; ¹H NMR (400 MHz, CD₃OD), δ 1.38 (m, 1 × 2, H-4'), 1.52 (m, 1 × 2, H-5'), 1.60 (m, 4 × 2, H-3', H-4', H-5'), 1.87 (m, 1 × 2, H-7'), 1.93 (m, 1 × 2, H-7'), 2.28 (t, 2 × 2, *J* = 7.2 Hz, H-2'), 2.65 (t, 2 × 2, *J* = 7.2 Hz, H-α), 2.94 (m, 1 × 2, H-8'), 3.03 (m, 1 × 2, H-6'), 3.13 (m, 1 × 2, H-8'), 3.69 (t, 2 × 2, *J* = 7.2 Hz, H-β), 6.62 (d, 1 × 2, *J* = 1.6 Hz, H-2), 6.72 (d, 1 × 2, *J* = 1.6 Hz, H-6); ¹³C NMR (100 MHz, CD₃OD), δ 25.8 (C-3'), 27.5 (C-4'), 32.3 (C-8'), 34.8 (C-2'), 35.1 (C-5', C-7'), 39.6 (C-α), 52.9 (C-6'), 64.5 (C-β), 116.9 (C-2), 121.3 (C-5), 125.7 (C-6), 131.9 (C-1), 145.0 (C-4), 146.3 (C-3), 178.0 (C-1').

DPPH Assay. The assay was performed as described.³¹ Briefly, to 1.98 mL of 200 μM DPPH in methanol was added 20 μL of a 5 mM solution of compounds 1–4, HTy, or LA. The reaction was followed by spectrophotometric analysis measuring the absorbance at 515 nm every 30 s for 10 min. Trolox was used as standard.

Ferric Reducing/Antioxidant Power (FRAP) Assay. The assay was performed as described.³² To 3.6 mL of a solution of FRAP reagent was added 5–150 μL of 5 mM solutions of compounds 1–4, HTy, or LA (7–200 μM final concentration). After 10 min, the absorbance at 593 nm was measured. Trolox was used as standard. The FRAP reagent was prepared freshly by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride in water, in the ratio 10:1:1, in that order.

Copper Reducing Antioxidant Capacity (CUPRAC) Assay. The assay was performed as described.³³ To a test tube containing 1 mL of 10 mM CuCl₂ × 2H₂O in water were added 1 mL of 7.5 mM neocuproine in 96% ethanol, 1 mL of 1 M ammonium acetate buffer (pH 7.0), 1 mL of water, and 2–25 μL of 5 mM solutions of compounds 1–4, HTy, or LA (2.5–30 μM final concentration). After 1 h, the absorbance at 450 nm was measured. Trolox was used as standard.

Hydroxyl Radical Scavenging Assay. The assay was performed as described.³⁴ The following solutions were prepared: 20 mM ferrous chloride in 40 mM HCl; 20 mM Na₂EDTA in water; 50 mM H₂O₂ in water; 10 mM salicylic acid in 0.2 M phosphate buffer (pH 7.4). To 1.5 mL of 0.2 M phosphate buffer (pH 7.4) was added 500 μL of salicylic acid solution, followed by 250 μL of EDTA solution, 250 μL of Fe²⁺ solution, 2 mL of water or 25 μM solutions of compounds 1–4, HTy, or LA in water, and 500 μL of H₂O₂ solution. After 10 min, 500 μL of 268 U/mL catalase solution was added, and the mixtures were analyzed by HPLC (eluent system II).

Cell Culture and Treatment. Human hepatic HepG2 cells were maintained in a humidified incubator containing 5% CO₂ and 95% air at 37 °C. They were grown in DMEM F-12 supplemented with 2.5% FBS and 50 mg/L each of gentamicin, penicillin, and streptomycin. Compound 1–4, HTy, or LA was dissolved at different concentrations (1, 5, 10, and 20 μM) in serum-free culture medium and added to the cell plates for 24 h (crystal violet assay) and 2 h (dichlorofluorescein assay). In the experiments to evaluate the protective role of the compounds against an oxidative insult, cells were pretreated with the same concentrations of the compounds for 20 h, then the medium was discarded and fresh medium containing 400 μM *t*-BOOH was added for 3 h (lactate dehydrogenase (LDH) assay) or 2 h (dichlorofluorescein assay).

Evaluation of Cell Viability, Cell Damage, and ROS Generation. Cell viability was determined using the crystal violet assay.³⁵ HepG2 cells were seeded at low density (10⁴ cells/well) in 96-

well plates, grown for 24 h, treated with the different compounds for 24 h, and then incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with water and allowed to dry, and 1% sodium dodecyl sulfate was added. The absorbance of each well was measured using a microplate reader at 570 nm.

Cellular damage was evaluated by LDH leakage.¹³ Cells were seeded (2×10^5 cells/plate) in 60 mm plates and grown for 20 h with the different treatments, and then the cell culture medium was collected, and the cells were scraped off in phosphate buffer saline. LDH activity was determined by the disappearance of NADH at 340 nm. LDH leakage was estimated from the ratio between the LDH activity in the culture medium and the total LDH activity in the culture plus intracellular medium.

Cellular ROS generation was quantified by the dichlorofluorescein assay using a microplate reader.¹³ Cells were seeded in 24-well plates (2×10^5 cells/well), treated with the noted conditions, and measured in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Statistical Analysis. One-way analysis of variance was used for cellular assays followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. Differences were considered to be statistically significant if $P < 0.05$ (statistical package SPSS v. 19.0). Excel Student's t test was used for chemical antioxidant assays.

RESULTS AND DISCUSSION

Preparation of 5-S-Lipoylhydroxytyrosol (1). The reaction of DHLA with quinones is known to lead to the S-conjugation products.^{36,37} Access to the desired compound **1** was thus obtained by an expedient protocol involving regioselective oxidation of tyrosol with IBX to HTy *o*-quinone,^{10,22} followed by addition of DHLA (Figure 1). Tyrosol (75 mM in methanol) was treated with 1.5 mol equiv of IBX, at -25°C , giving an instable *o*-quinone. After 1 h, DHLA (300 mM) was added, and the mixture was taken at room temperature for 15 min, diluted 1:5 with water, and then acidified to pH 3 with 6 M HCl. HPLC analysis (eluent system I) of the reaction mixture showed the presence of **1**, 2-iodobenzoic acid, and HTy, most likely deriving from a redox exchange *o*-quinone/DHLA competing with the nucleophilic addition. To isolate **1** in pure form, a sequential extraction with solvents of increasing polarity was performed: removal of 2-iodobenzoic acid, DHLA, and LA was achieved by extraction of the mixture with toluene; subsequent extraction with chloroform allowed isolation of **1** in pure form in the organic phase, whereas the more polar HTy was recovered by extraction with ethyl acetate. This procedure led to isolation of **1** in 29% yield.

Preparation of Polysulfides 2–4. **Disulfide 2.** It is well-known that thiols give corresponding disulfides by oxidation. On this basis, **2** was prepared by aerial oxidation of **1** (2.5 mM) under vigorous stirring in 0.1 M phosphate buffer (pH 7.4). HPLC analysis (eluent system I) showed a complete consumption of **1** after about 60 h with concomitant formation of a main product eluted at 25 min. LC-MS/ESI+ analysis showed for this product pseudomolecular ion peaks $[M + \text{Na}]^+$ and $[M + \text{K}]^+$ at m/z 741 and 757, respectively, suggestive of a disulfide derivative. Consistently, an intense $M/2$ fragmentation peak at m/z 359 was also observed. The reaction mixture was taken to pH 3 and extracted with ethyl acetate, and the residue obtained from the combined organic layers was fractionated by semipreparative HPLC to give **2** in pure form (10% yield).

Trisulfide 3. For the preparation of the trisulfide derivative, **1** was reacted at pH 7.4 in the presence of elemental sulfur. In fact, it is known that polysulfides can be obtained in good yields from the reaction of thiols with sulfur at room temperature, and

trisulfides, in particular, are the main products in the case of secondary thiols such as **1**.³⁸ Sulfur dissolved in methanol was added to compound **1** dissolved in phosphate buffer at pH 7.4, with a final methanol/phosphate buffer ratio of 1:2 v/v. Under these conditions a fast consumption of **1** was observed (90% after 6 h), with concomitant formation of a main product eluted at 26 min (HPLC analysis, eluent system I). LC-MS/ESI+ analysis indicated for this product pseudomolecular ion peaks $[M + \text{Na}]^+$ and $[M + \text{K}]^+$ at m/z 773 and 789, respectively, as expected for the trisulfide derivative of **1**; moreover, significant fragmentation peaks at m/z 391 and 359 most likely related to S–S bond cleavage were observed. The reaction mixture was diluted 1:4 with water, taken to pH 3, washed with *n*-hexane to remove any excess sulfur, and extracted with ethyl acetate. The residue obtained from the combined organic layers was fractionated by semipreparative HPLC to give **3** in pure form (15% yield).

Tetrasulfide 4. Tetrasulfide **4** was prepared by reacting **1** under the conditions adopted for the preparation of **3** but in pure methanol, to optimize solubilization of sulfur. A rather slow consumption of **1** (90% after 48 h) was obtained, with formation of a main product eluted at 27 min (HPLC analysis, eluent system I). LC-MS analysis in ESI+ mode indicated for this product pseudomolecular ion peaks $[M + \text{H}]^+$ and $[M + \text{Na}]^+$ at m/z 783 and 805, respectively, with significant fragmentation peaks at m/z 391 and 423. The reaction mixture was washed with *n*-hexane to remove excess sulfur and fractionated by semipreparative HPLC as previously described to give **4** in pure form (31% yield).

Chemical Properties of 1–4. Compounds **1–4** were fairly stable when stored dry or in organic solvent in the cold and were fairly soluble in methanol, acetonitrile, and water. However, they were rapidly degraded in alkaline media in the presence of air or oxygen, consistent with the notorious instability of catechol moieties. Interestingly, the observed mild conversion of **1** to **2** in aerated phosphate buffer at pH 7.4 suggests that the SH functional group is more susceptible to aerial oxidation than the catechol moiety, making an equilibrium of the two compounds likely to occur in a biological environment.

Evaluation of the Antioxidant Properties of Compounds 1–4 by Chemical Assays. **DPPH Assay.** The hydrogen donor capacity of compounds **1–4** was estimated through the DPPH assay,³¹ which determines the ability of molecules to reduce the colored and stable DPPH radical against a suitable reference donor (typically Trolox). Figure 2

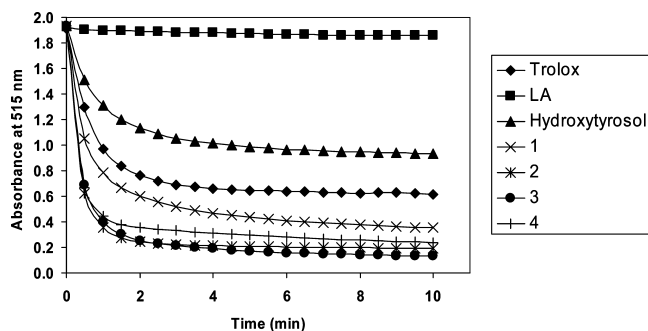


Figure 2. Decrease in the absorbance of 200 μM DPPH (515 nm) in the presence of 50 μM compounds **1–4**, HTy, LA, or Trolox. Reported are the mean \pm SD values for two separate experiments.

Table 1. H-Atom Transfer Reaction from Compounds 1–4 (50 μM Each) to DPPH (200 μM) in Comparison to Trolox, HTy, and LA^a

antioxidant	DPPH reduced ^b (%)	k_1^c ($\text{M}^{-1} \text{s}^{-1}$)	n^d	n_{total}^e
1	82 \pm 4a	515 \pm 17a	2.04 \pm 0.05a	2.81 \pm 0.14ab
2	90 \pm 1ab	1412 \pm 81bc	2.81 \pm 0.07b	3.10 \pm 0.03a
3	93 \pm 1b	1067 \pm 35b	2.73 \pm 0.10b	3.20 \pm 0.03a
4	88 \pm 1a	1531 \pm 22c	2.65 \pm 0.02b	3.01 \pm 0.02a
HTy	52 \pm 1c	175 \pm 39de	1.11 \pm 0.16cd	1.79 \pm 0.04c
LA	4 \pm 1d	4 \pm 1d	0.12 \pm 0.01d	0.12 \pm 0.02d
Trolox	68 \pm 2e	312 \pm 12e	1.67 \pm 0.07c	2.31 \pm 0.01b

^aValues are the mean \pm SD ($n = 2$). Values in a column without a common letter differ, $P < 0.05$. ^bCalculated after 10 min of reaction. ^cRate constant for the fast step. ^dNumber of H atoms transferred in the fast step. ^eNumber of H atoms transferred after 10 min.

shows the decrease in absorbance of DPPH at 515 nm in the presence of compounds 1–4. HTy and LA were also tested for comparison. DHLA was not tested because of its well-known instability under oxidative conditions. Data obtained by kinetic analysis are reported in Table 1. Polysulfides 2–4 were found to have the strongest hydrogen donor ability. No significant differences were observed related to the number of sulfur atoms. Compound 1 displayed high DPPH reducing activity, whereas HTy was less active than Trolox. Almost no reduction was observed with LA. Higher rates for the fast hydrogen atom transfer step were exhibited by the polysulfides 2–4 with respect to 1.

FRAP and CUPRAC Assays. Whereas the reducing capacity of thiol-containing compounds cannot be determined accurately by the FRAP assay,^{32,39,40} the CUPRAC assay³³ has been proven to be suitable. Here, the reducing capacity of compounds 1–4 was measured by the above two assays for comparison. Table 2 reports the results for the two assays,

Table 2. Fe^{3+} and Cu^{2+} Reducing Activity of Compounds 1–4 in Comparison to HTy and LA

antioxidant	Trolox equiv (FRAP) ^a	Trolox equiv (CUPRAC) ^a
1	1.60 \pm 0.15a	3.32 \pm 0.21a
2	1.00 \pm 0.05ab	2.35 \pm 0.08b
3	0.77 \pm 0.08bc	1.85 \pm 0.07c
4	0.71 \pm 0.04c	1.75 \pm 0.04c
HTy	0.92 \pm 0.04ab	2.42 \pm 0.11b
LA	0.03 \pm 0.01d	0.02 \pm 0.01d

^aValues are the mean \pm SD ($n = 2$). Results are expressed as Trolox equivalents. Values in a column without a common letter differ, $P < 0.05$.

expressed as Trolox equivalents. It is shown that 1 is endowed with the best reducing capacity in both tests. The activity of polysulfides 2–4 decreases apparently with increasing number of sulfur atoms, whereas LA is, as expected, a very poor reducing agent.

***OH Scavenging Assay.** The $\cdot\text{OH}$ scavenging activity of compounds 1–4 was evaluated by a Fenton inhibition assay based on HPLC determination of salicylic acid hydroxylation products, which are catechol, 2,3-dihydroxybenzoic acid, and 2,5-dihydroxybenzoic acid.³⁴ Briefly, salicylic acid at 1 mM concentration was reacted with Fe^{2+} (1 mM), EDTA (1 mM), and H_2O_2 (5 mM) in 0.2 M phosphate buffer (pH 7.4), in the presence of compounds 1–4, HTy, or LA. After 10 min, catalase (25 U/mL) was added to stop the reaction, and the reaction mixture was analyzed by HPLC (eluent system II); inhibition (%) of the formation of catechol and 2,3- and 2,5-

dihydroxybenzoic acids was calculated with respect to a control reaction mixture run in the absence of antioxidant. As reported in Table 3, all compounds 1–4 acted as efficient hydroxyl radical scavengers at concentration as low as 10 μM .

Table 3. Inhibition of Salicylic Acid Hydroxylation by Compounds 1–4, HTy, and LA (10 μM Each)

antioxidant	inhibition ^a (%)
1	69 \pm 9
2	56 \pm 2
3	57 \pm 2
4	59 \pm 2
HTy	64 \pm 4
LA	61 \pm 6

^aValues are the mean \pm SD ($n = 2$).

Evaluation of the Antioxidant Properties of Compounds 1–4 in Cell Culture. Following demonstration of the marked antioxidant properties of compounds 1–4 in chemical assays, further experiments were aimed at a screening of their activities in validated cellular systems.⁴¹ The concentrations of compounds 1–4 used in this study (1–20 μM) were in the range of plasma concentrations of HTy in individuals who consume extra virgin olive oil in Mediterranean countries. Daily consumption of 25–50 mL of virgin olive oil, which contains between 180 and 300 mg/kg of olive oil phenols, results in an estimated daily intake of about 9 mg of olive oil phenols, which is equivalent to 58 μmol of HTy equiv per day, of which 30–90% could be absorbed,⁴² leading to plasma concentrations of 5–10 μM .

Preliminarily, the effects of 1–4 (concentration range of 1–20 μM) on liver cell viability were evaluated, and the results showed that none of the compounds tested exerted significant toxicity (data not shown). Figure 3 reports the effects of 1–4 on ROS production in HepG2 cells. The data show that all compounds tested can efficiently abate ROS levels, with 2–4 being more effective than the parent HTy. These results were in agreement with previous studies showing a direct effect on ROS quenching in the same cell line by other natural olive oil phenolics^{13,43} and also synthetic derivatives of HTy.¹⁷

Figures 4 and 5 show the effects of pretreating HepG2 cells with compounds 1–4 at 1–20 μM concentrations before exposure to 400 μM *t*-BOOH.^{41,44} Again, marked inhibitory properties could be demonstrated against cell damage and ROS generation under the stressing conditions of the assay. These results demonstrate that the new compounds are efficient protecting agents in *in vitro* models of oxidative stress, preventing cell damage caused by potent oxidative insults.

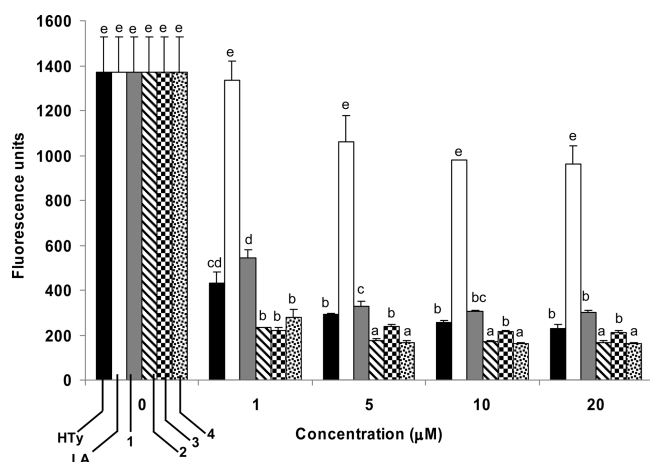


Figure 3. Direct effect of HTy, LA, and compounds 1–4 on ROS generation. HepG2 cells were treated with the noted concentrations of the mentioned compounds during 2 h. Results are expressed as fluorescence units, and values are the mean \pm SD of four to six different samples per condition. Means without a common letter differ, $P < 0.05$.

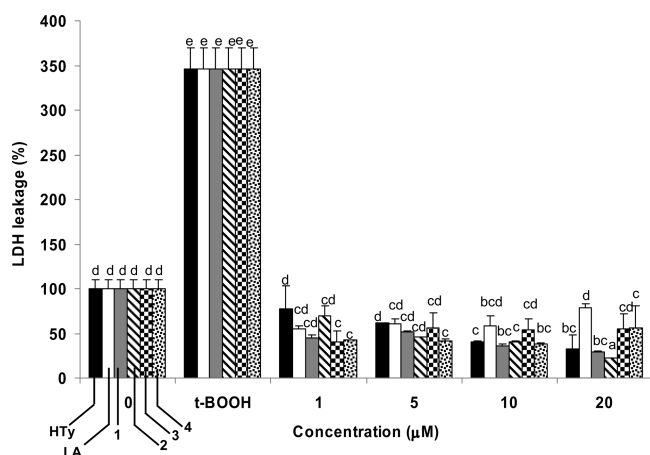


Figure 4. Protective effect of HTy, LA, and compounds 1–4 on cell damage. HepG2 cells were treated with the noted concentrations of mentioned compounds during 20 h, and then the cultures were washed and 400 μ M *t*-BOOH was added for 3 h to all cultures except controls. Results of LDH leakage are expressed as the ratio of LDH activity in the culture medium to that in culture plus intracellular medium. A value of 100% was given to controls, and the other values are referred to them. Values are the mean \pm SD of four to six data. Means without a common letter differ, $P < 0.05$.

Although antioxidant activity data in chemical assays and in cell culture were similar for all compounds, it is worth noting that in cells compounds 2, 3, and 4 were relatively more efficient than compound 1, perhaps due to their higher hydrophobicity, enabling them to penetrate the membranes and reach the radicals formed within the cell. In line with this view, Lima et al.⁴⁴ evaluated the protective effects of phenolic acids and their esters against oxidative damage in HepG2 cells and emphasized the important role of hydrophobicity in the hepatoprotective potential of the phenolic compounds. Likewise, Spencer et al.⁴⁵ suggested that the antioxidant bioactivity of polyphenols strongly depends on the extent to which they interact with, and permeate, cell membranes.

In conclusion, we have reported herein the synthesis and characterization of a new set of HTy derivatives as promising

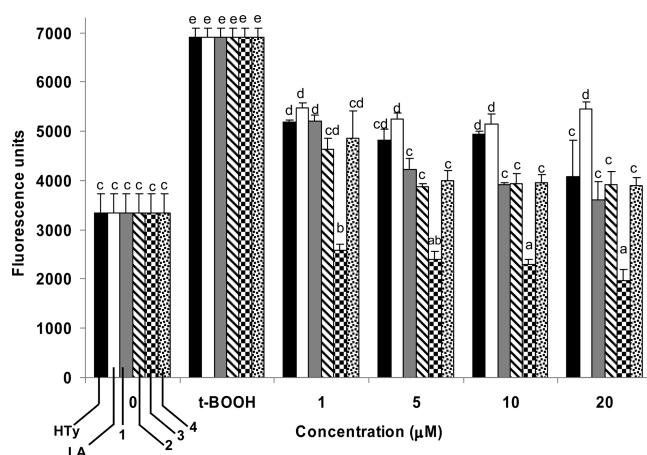


Figure 5. Protective effect of HTy, LA, and compounds 1–4 on ROS generation. HepG2 cells were treated with the noted concentrations of mentioned compounds during 20 h, and then the cultures were washed and 400 μ M *t*-BOOH was added for 2 h to all cultures except controls. Data are expressed as fluorescence units, and values are the mean \pm SD of four to six different samples per condition. Means without a common letter differ, $P < 0.05$.

lead structures for the development of innovative antioxidants inspired by natural products. The compounds were synthesized by a versatile and expedient synthetic protocol, which capitalized on the IBX-mediated conjugation of tyrosol with DHLA to afford the unprecedented adduct 1, from which the di- to tetrasulfide derivatives 2–4 were easily obtained without complex purification stages. Determination of the antioxidant activities of 1–4 in the DPPH, FRAP, CUPRAC, and Fenton reaction inhibition assays revealed significantly more potent effects compared to the parent HTy, LA, or the reference compound Trolox. Compounds 1–4 also exerted protective effects against ROS generation and oxidative cell damage in human liver HepG2 cells. On the basis of these results, it is concluded that (1) insertion of the sulfur-containing chain potentiates the overall antioxidant activity of HTy, conferring to the core system a more pronounced lipophilic character and a novel putative metal-chelating site;⁴⁶ (2) compounds 1–4 behave as broad-spectrum, multidimensional antioxidants acting through different mechanisms, including H-atom release, free radical quenching, ferric and cupric ion reduction, and OH radical scavenging; (3) the antioxidant effects of 1–4 vary within the product series with the nature of the assay, for example, 1 being more efficient as a ferric reducing agent and OH radical scavenger and polysulfides 2–4 being more active as H-atom donors in DPPH reduction exhibiting a higher protecting capacity in vitro against ROS generation; (4) the overall properties of compounds 1–4 reflect both electronic effects caused by the sulfur substituent on the catechol ring and solubility effects induced both by the *S*-lipoyl side chain and the bridging polysulfide chain, which may play a crucial role in cell membrane permeation. On this basis, DHLA–HTy conjugation can be proposed as a convenient access route to versatile multidimensional antioxidant systems that can be finely tuned through proper selection of molecular size (monomer 1 versus dimers 2–4), functional groups (SH versus S–(S)_{*n*}–S, with 0 $\leq n \leq 2$ and possibly more) and lipophilicity. Pursuit of this approach may benefit also from future work aimed at testing new members of the series, for example, as antinitrosating and hypochlorous acid scavengers as most natural polysulfides and

the rational formulation of suitable cocktail mixtures for a broader activity spectrum.

■ ASSOCIATED CONTENT

■ Supporting Information

NMR and MS spectra of compounds 1–4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS USED

HTy, hydroxytyrosol; ROS, reactive oxygen species; RNS, reactive nitrogen species; LA, lipoic acid; DHLA, dihydrolipoic acid; TPTZ, 2,4,6-tris(2-pyridyl)-s-triazine; Na₂EDTA, ethylenediaminetetraacetic disodium salt; DPPH, 2,2'-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; t-BOOH, *tert*-butylhydroperoxide; NADH, reduced nicotinic adenine dinucleotide; IBX, 2-iodoxybenzoic acid; FRAP, ferric reducing/antioxidant power; CUPRAC, copper reducing antioxidant capacity; LDH, lactate dehydrogenase

■ REFERENCES

- (1) Frankel, E. N. Nutritional and biological properties of extra virgin olive oil. *J. Agric. Food Chem.* **2011**, *59*, 785–792.
- (2) Visioli, F.; Galli, C. Olive oil phenols and their potential effects on human health. *J. Agric. Food Chem.* **1998**, *46*, 4292–4296.
- (3) Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60–64.
- (4) Cornwell, D. G.; Ma, J. Y. Nutritional benefit of olive oil: the biological effects of hydroxytyrosol and its arylating quinone adducts. *J. Agric. Food Chem.* **2008**, *56*, 8774–8786.
- (5) Deiana, M.; Aruoma, O. I.; Bianchi, M. P.; Spencer, J. P. E.; Kaur, H.; Halliwell, B.; Aeschbach, R.; Banni, S.; Dessi, M. A.; Corongiu, F. P. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radical Biol. Med.* **1999**, *26*, 762–769.
- (6) Napolitano, A.; Panzella, L.; Savarese, M.; Sacchi, R.; Giudicianni, I.; Paolillo, L.; d'Ischia, M. Acid-induced structural modifications of unsaturated fatty acids and phenolic olive oil constituents by nitrite ions: a chemical assessment. *Chem. Res. Toxicol.* **2004**, *17*, 1329–1337.
- (7) d'Ischia, M.; Napolitano, A.; Manini, P.; Panzella, L. Secondary targets of nitrite-derived reactive nitrogen species: nitrosation/nitration pathways, antioxidant defense mechanisms and toxicological implications. *Chem. Res. Toxicol.* **2011**, *24*, 2071–2092.

- (8) Vogna, D.; Pezzella, A.; Panzella, L.; Napolitano, A.; d'Ischia, M. Oxidative chemistry of hydroxytyrosol: isolation and characterisation of novel methanooxocinobenzodioxinone derivatives. *Tetrahedron Lett.* **2003**, *44*, 8289–8292.

- (9) De Lucia, M.; Panzella, L.; Pezzella, A.; Napolitano, A.; d'Ischia, M. Oxidative chemistry of the natural antioxidant hydroxytyrosol: hydrogen peroxide-dependent hydroxylation and hydroxyquinone/o-quinone coupling pathways. *Tetrahedron* **2006**, *62*, 1273–1278.

- (10) Napolitano, A.; De Lucia, M.; Panzella, L.; d'Ischia, M. The chemistry of tyrosol and hydroxytyrosol: implications for oxidative stress. In *Olive and Olive Oil in Health and Disease Prevention*; Preedy, V. R., Watson, R. R., Eds.; Academic Press: Oxford, UK, 2010; pp 1225–1232.

- (11) Deiana, M.; Corona, G.; Incani, A.; Loru, D.; Rosa, A.; Atzeri, A.; Melis, M. P.; Dessi, M. A. Protective effect of simple phenols from extra virgin olive oil against lipid peroxidation in intestinal Caco-2 cells. *Food Chem. Toxicol.* **2010**, *48*, 3008–3016.

- (12) D'Angelo, S.; Ingrosso, D.; Migliardi, V.; Sorrentino, A.; Donnarumma, G.; Baroni, A.; Masella, L.; Tufano, M. A.; Zappia, M.; Galletti, P. Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. *Free Radical Biol. Med.* **2005**, *38*, 908–919.

- (13) Goya, L.; Mateos, R.; Bravo, L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells: protection against oxidative stress induced by *tert*-butylhydroperoxide. *Eur. J. Nutr.* **2007**, *46*, 70–78.

- (14) 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.

- (15) Grasso, S.; Siracusa, L.; Spatafora, C.; Renis, M.; Tringali, C. Hydroxytyrosol lipophilic analogues: enzymatic synthesis, radical scavenging activity and DNA oxidative damage protection. *Bioorg. Chem.* **2007**, *35*, 137–152.

- (16) Bernini, R.; Crisante, F.; Barontini, M.; Tofani, D.; Balducci, V. Synthesis and structure/antioxidant activity relationship of novel catecholic antioxidants structurally analogues to hydroxytyrosol and its lipophilic esters. *J. Agric. Food Chem.* **2012**, *60*, 7408–7416.

- (17) Pereira-Caro, G.; Sarriá, B.; Madrona, A.; Espartero, J. L.; Goya, L.; Bravo, L.; Mateos, R. Alkyl hydroxytyrosol esters show protective effects against oxidative stress in HepG2 cells. *J. Agric. Food Chem.* **2011**, *59*, 5964–5976.

- (18) Bernini, R.; Crisante, F.; Merendino, N.; Molinari, R.; Soldatelli, M. C.; Velotti, F. Synthesis of a novel ester of hydroxytyrosol and α -lipoic acid exhibiting an antiproliferative effect on human colon cancer HT-29 cells. *Eur. J. Med. Chem.* **2011**, *46*, 439–446.

- (19) Kumar, S.; Johansson, H.; Engman, L.; Valgimigli, L.; Amorati, R.; Fumo, M. G.; Pedulli, G. F. Regenerable chain-breaking 2,3-dihydrobenzo[*b*]selenophene-5-ol antioxidants. *J. Org. Chem.* **2007**, *72*, 2583–2595.

- (20) Amorati, R.; Pedulli, G. F.; Valgimigli, L.; Johansson, H.; Engman, L. Organochalcogen substituents in phenolic antioxidants. *Org. Lett.* **2010**, *12*, 2326–2329.

- (21) Panzella, L.; Napolitano, A.; d'Ischia, M. Oxidative conjugation of chlorogenic acid with glutathione: structural characterization of addition products and a new-nitrite promoted pathway. *Bioorg. Med. Chem.* **2003**, *11*, 4797–4805.

- (22) De Lucia, M.; Panzella, L.; Pezzella, A.; Napolitano, A.; d'Ischia, M. Plant catechols and their S-glutathionyl conjugates as antinitrosating agents: expedient synthesis and remarkable potency of S-S-glutathionylpiceatannol. *Chem. Res. Toxicol.* **2008**, *21*, 2407–2413.

- (23) Busch, C.; Jacob, C.; Anwar, A.; Burkholz, T.; Ba, L. A.; Cerella, C.; Diederich, M.; Brandt, W.; Wessjohann, L.; Montenarh, M. Diallylpolysulfides induce growth arrest and apoptosis. *Int. J. Oncol.* **2010**, *36*, 743–749.

- (24) Saidu, N. E. B.; Valente, S.; Bana, E.; Kirsch, G.; Bagrel, D.; Montenarh, M. Coumarin polysulfides inhibit cell growth and induce apoptosis in HCT116 colon cancer cells. *Bioorg. Med. Chem.* **2012**, *20*, 1584–1593.

- (25) Munday, R. Harmful and beneficial effects of organic monosulfides, disulfides and polysulfides in animals and humans. *Chem. Res. Toxicol.* **2012**, *25*, 47–60.
- (26) Jacob, C. A scent of therapy: pharmacological implications of natural products containing redox-active sulfur atoms. *Nat. Prod. Rep.* **2006**, *23*, 851–863.
- (27) Argüello-García, R.; Medina-Campos, O. N.; Pérez-Hernández, N.; Pedraza-Chaverrí, J.; Ortega-Pierres, G. Hypochlorous acid scavenging activities of thioallyl compounds from garlic. *J. Agric. Food Chem.* **2010**, *58*, 11226–11233.
- (28) Gunsalus, I. C.; Barton, L. S.; Gruber, W. Biosynthesis and structure of lipoic acid derivatives. *J. Am. Chem. Soc.* **1956**, *78*, 1763–1766.
- (29) Frigerio, M.; Santagostino, M.; Sputore, S. A user-friendly entry to 2-iodoxybenzoic acid (IBX). *J. Org. Chem.* **1999**, *64*, 4537–4538.
- (30) Plumb, J. B.; Harper, D. J. 2-Iodoxybenzoic acid. *Chem. Eng. News* **1990**, *68* (29), 3.
- (31) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615–622.
- (32) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.
- (33) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *J. Agric. Food Chem.* **2004**, *52*, 7970–7981.
- (34) Özyürek, M.; Bektasoglu, B.; Güçlü, K.; Apak, R. Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. *Anal. Chim. Acta* **2008**, *616*, 196–206.
- (35) Granado-Serrano, A. B.; Angeles Martín, M.; Bravo, L.; Goya, L.; Ramos, S. Time-course regulation of quercetin on cell survival/proliferation pathways in human hepatoma cells. *Mol. Nutr. Food Res.* **2008**, *52*, 457–464.
- (36) Tsuji-Naito, K.; Hatani, T.; Okada, T.; Tehara, T. R. Evidence for covalent lipoyl adduction with dopaquinone following tyrosinase-catalyzed oxidation. *Biochem. Biophys. Res. Commun.* **2006**, *343*, 15–20.
- (37) Greco, G.; Panzella, L.; Pezzella, A.; Napolitano, A.; d’Ischia, M. Reaction of dihydrolipoic acid with juglone and related naphthoquinones: unmasking of a spirocyclic 1,3-dithiane intermediate en route to naphtho[1,4]dithiepinones. *Tetrahedron* **2010**, *66*, 3912–3916.
- (38) Vineyard, B. D. Mercaptan-sulfur reaction. Alkyl trisulfides. *J. Org. Chem.* **1966**, *31*, 601–602.
- (39) Prior, R. L.; Wu, X. L.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.
- (40) Ou, B.; Huang, D.; Hampsch-Woodhill, M.; Flanagan, J. A.; Deemer, E. K. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *J. Agric. Food Chem.* **2002**, *50*, 3122–3128.
- (41) Goya, L.; Martín, M. A.; Ramos, S.; Mateos, R.; Bravo, L. A cell culture model for the assessment of the chemopreventive potential of antioxidant compounds. *Curr. Nutr. Food Sci.* **2009**, *5*, 56–64.
- (42) Vissers, M. N.; Zock, P. L.; Roodenburg, A. J.; Leenen, R.; Katan, M. B. Olive oil phenols are absorbed in humans. *J. Nutr.* **2002**, *132*, 409–417.
- (43) Pereira-Caro, G.; Mateos, R.; Sarriá, B.; Cert, R.; Goya, L.; Bravo, L. Hydroxytyrosyl acetate contributes to the protective effects against oxidative stress of virgin olive oil. *Food Chem.* **2012**, *131*, 869–878.
- (44) Lima, C. F.; Fernandes-Ferreira, M.; Pereira-Wilson, C. Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels. *Life Sci.* **2006**, *79*, 2056–2068.
- (45) Spencer, J. P.; Abd-el-Mohsen, M. M.; Rice-Evans, C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch. Biochem. Biophys.* **2004**, *423*, 148–161.
- (46) Napolitano, A.; Memoli, S.; Nappi, A. J.; d’Ischia, M.; Protta, G. S-S-Cysteinyl-dopa, a diffusible product of melanocyte activity, is an efficient inhibitor of hydroxylation/oxidation reactions induced by the Fenton system. *Biochim. Biophys. Acta* **1996**, *1291*, 75–82.