New Lipophilic Tyrosyl Esters. Comparative Antioxidant Evaluation with Hydroxytyrosyl Esters

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New lipophilic esters of tyrosol, a naturally occurring phenol with interesting biological properties, have been synthesized in good yields by a chemoselective procedure, using lipase from *Candida antarctica* or *p*-toluenesulfonic acid as catalysts. Their antioxidant activities have been evaluated by the Rancimat test in lipophilic food matrices, as well as by FRAP and ABTS assays in methanolic solutions, and compared with those of previously synthesized hydroxytyrosyl esters. Free tyrosol, hydroxytyrosol, butylhydroxytoluene, and α-tocopherol were used as standards. All methods used for the antioxidant activity evaluation emphasized the high influence of the ortho-diphenolic structure on the antioxidant capacity, tyrosol and its derivatives being less active than hydroxytyrosol and its analogues and even less than BHT and α-tocopherol. In addition, the Rancimat test revealed a lower activity for ester derivatives than for their respective reference compounds (HTy or Ty), in agreement with the polar paradox. On the other hand, FRAP and ABTS methods reported an opposite behavior between the synthetic esters and their respective references. Thus, hydroxytyrosyl esters were more active than HTy, whereas tyrosyl esters were less active than Ty. The length and nature of the acyl side chain did not seem to play an important role in the antioxidant activity of either the hydroxytyrosyl or tyrosyl ester series, since no significant differences were observed among them.

KEYWORDS: Tyrosol; hydroxytyrosol; synthesis; selective esterification; lipases; Rancimat; ABTS; FRAP; lipophilic antioxidants

INTRODUCTION

Phenolic compounds, the biggest group of natural antioxidants, have attracted much attention due to their known and wide-ranging biological activities, as well as to their health effects (*1, 2*). Of them, particular attention has been focused on 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HTy, **1**), an ortho-diphenolic compound, essential component of oleuropein, and present in virgin olive oil, mainly either as secoiridoid derivatives or as the acetate ester (*3*). The presence of these biophenols in virgin olive oil contributes to its beneficial properties (*2, 4*).

In order to obtain a better knowledge of its mechanism of action of free HTy, it has been the subject of various in vitro and in vivo studies. Thus, it has been reported that this biophenol reduces the risk of coronary heart disease and atherosclerosis (*5*). Other biological properties of the phenolic components of olive oil include antimicrobial (*6*), antiinflammatory (*7*) activity

as well as inhibition of several lipoxygenases (*8*) and apoptosis induction in HL-60 cells (*9*).

In olive oil, besides HTy and its derivatives, there are appreciable amounts of 2-(4-hydroxyphenyl)ethanol (tyrosol, Ty, **2**) and its secoiridoid derivatives. It has been well established (*10*) that Ty has a significantly lower antioxidant activity than HTy, due to the absence of the ortho-diphenolic group in its chemical structure. However, and despite its weak antioxidative efficacy, recent studies suggest that Ty exerts a powerful protective effect against oxidative injuries in cell systems (*11*) and improves the intracellular antioxidant defense systems (*12*). An ibuprofen-like antiinflammatory activity of oleocanthal, one of the tyrosyl secoiridoid derivatives present in olive oil, was also recently reported (*13*). Other tyrosyl derivatives have been found in diverse natural sources: thus, the presence of its acetate (**10**) was reported in virgin olive oil (*14*), its lipophilic palmitate, stearate, and oleate esters were isolated from the flowers of *Ligustrum ovalifolium* (*15*), from the stem bark of *Buddleja cordata* (*16*), and from olive fruits (*17*), respectively, and, in the past few years, the use of some tyrosyl esters for antiaging and/or pharmacological applications has been attracting interest (*18*).

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Last but not least, the food industry is demanding powerful, insipid, and cheap antioxidants with nutritional properties to improve the nutritional value and the quality of foods (*19*). HTy and Ty may be effectively recovered from olive oil wastewaters (*20*). However, because of the limited solubility of these biophenols in lipid media, the search for new lipophilic derivatives with enhanced properties is of great interest. A selective procedure for the preparation of lipophilic hydroxy-tyrosyl esters (*3–9*) was developed by our group (*21*), and their antioxidant activity was further evaluated (*22*). Results obtained in lipid matrices by the Rancimat test, as well as in stressed-brain homogenates, indicated that esterification of HTy did not significantly affect its antioxidant activity but improved its liposolubility (*22*). Combining the new biological activity described for Ty and the ease of obtaining it from wastewaters, the synthesis of its ester derivatives could be an interesting alternative to satisfy the needs of the food industry. Recently, the synthesis of tyrosyl acetate, catalyzed by lipase from *Staphylococcus xylosus*, has been studied and optimized by Aissa et al. (*23*). For these reasons, and as a continuation of our work, the synthesis of new lipophilic tyrosyl esters (*10–16*), as well as the evaluation of their antioxidant activity, is presented herein. Different reaction conditions have been tested and optimized. Data obtained on their radical-scavenging capacity, using the ABTS method, and of their reducing capacity, by the FRAP method, are compared with the results on the evaluation of their antioxidant capacity in lipid matrices using the Rancimat method. α-Tocopherol and BHT have been used as reference compounds. Finally, data obtained from ABTS and FRAP experiments for the known hydroxytyrosyl esters are also included for comparative purposes.

MATERIALS AND METHODS

Materials. All solvents and reagents were of analytical grade unless otherwise stated. α-Tocopherol, 2,6-di-*tert*-butyl-4-methylphenol (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and tyrosol were from Aldrich (Madrid, Spain). 2,2′-Azino bis(3-ethylben-zothiazoline-6-sulfonic acid) diammonium salt (98%), 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ), and lipase from *Candida antarctica* (Novozime 435) were from Sigma (Madrid, Spain). Hydroxytyrosol was recovered with 95% purity from olive oil wastewaters (*20*), and further purified by column chromatography. Hydroxytyrosyl lipophilic esters (*3–9*) were prepared as previously reported (*22*) NMR spectra were recorded on a Bruker AMX-500 spectrophotometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Chemical shifts are given in ppm, with the residual solvent signals (2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C) as references. Samples were dissolved (10–20 mg/mL) in hexadeuterated methyl sulfoxide (DMSO-d_6), and spectra were recorded at 303 K. Elemental analyses were made on a Leco CHNS-932 apparatus. High-resolution EI and CI mass spectra were obtained on a Micromass AUTOSPECQ spectrometer.

Synthetic Method. General procedures: (a) by enzymatic catalysis: tyrosol (1 mmol), the acylating agent (10 mL), and lipase from *Candida antarctica* (50 mg) were stirred at 55 °C until completion of the reaction (by thin layer chromatography, TLC, using hexane/*tert*-butyl methyl ether (2:1) as eluant). The mixture was filtered and concentrated in vacuo. Pure tyrosyl esters were obtained after column chromatography (see **Table 1**); (b) by acid catalysis: tyrosol (1 mmol) and the acylating agent (10 mL) were heated at 70 °C in presence of *p*-toluenesulfonic acid (10 mg) until completion of the reaction (TLC). The resulting mixture was concentrated, and the products were purified in each case by column chromatography (see **Table 1**).

Data for Tyrosyl Acetate (10). The pure compound was obtained, using ethyl acetate as acylating agent, after column chromatography, eluting with hexane/diethyl ether (1:1). NMR data (see **Tables 2** and **3**) were in good accordance with those previously reported (*14*).

Table 1. Results in the Synthesis of Tyrosyl Esters

compd	catalyst	T (°C)	time (h)	yield (%)[a]
10	LCA	55	1.5	92
	TsOH	70	2	95
11	LCA	55	5	90
	TsOH	70	7	98
12	LCA	55	22	66
	TsOH	70	24	92
13	LCA	55	40	60
	TsOH	70	20	74
14	LCA	55	42	85
	TsOH	70	32	65
15	LCA	55	6	64
	TsOH	70	3	90
16	LCA	55	42	80
	TsOH	70	15	76

[a] Calculated yield for pure isolated compounds.

Data for Tyrosyl Butyrate (11). Pure **11** was obtained, using methyl butyrate as acylating agent, after column chromatography, eluting with hexane/*tert*-butyl methyl ether (2:1). NMR data (see **Tables 2** and **3**). HRMS (EI) *m/z* calcd for $C_{12}H_{16}O_3$ [M]$^{+\bullet}$ 208.1099, found 208.1101 (0.7 ppm). Anal. Calcd for $C_{12}H_{16}O_3$: C, 69.21; H: 7.74. Found: C, 68.65; H, 7.75.

Data for Tyrosyl Laurate (12). THF (3 mL) was used as solvent and methyl laurate as acylating agent. Pure **12** was obtained after column chromatography, eluting with hexane/*tert*-butyl methyl ether (6:1). Mp: 43 °C. NMR data (see **Tables 2** and **3**). HRMS (EI) *m/z* calcd for $C_{20}H_{32}O_3$ [M]$^{+\bullet}$ 320.2351, found 320.2368 (5.2 ppm). Anal. Calcd for $C_{20}H_{32}O_3$: C, 74.96; H, 10.06. Found: C, 74.98; H, 9.89.

Data for Tyrosyl Palmitate (13). THF (3 mL) was used as solvent and methyl palmitate as acylating agent. Pure **13** was obtained after column chromatography, eluting with hexane/dichloromethane (1:1). Mp: 63 °C. NMR data (see **Tables 2** and **3**). HRMS (EI) *m/z* calcd for $C_{24}H_{40}O_3$ [M]$^{+\bullet}$ 376.2977, found 376.2973 (1.2 ppm). Anal. Calcd for $C_{24}H_{40}O_3$: C, 76.59; H, 10.64. Found: C, 76.32; H, 10.91.

Data for Tyrosyl Stearate (14). THF (3 mL) was used as solvent and methyl stearate as acylating agent. Pure **14** was obtained after column chromatography, eluting with hexane/*tert*-butyl methyl ether (8:1). Mp: 69 °C. NMR data (see **Tables 2** and **3**). HRMS (EI) *m/z* calcd for $C_{26}H_{44}O_3$ [M]$^{+\bullet}$ 404.3290, found 404.3274 (4.1 ppm). Anal. Calcd for $C_{26}H_{44}O_3$: C, 77.18; H, 10.96. Found: C, 76.88; H, 10.67.

Data for Tyrosyl Oleate (15). Pure **15** was obtained after column chromatography solvent, using methyl oleate as acylating agent, eluting with hexane/*tert*-butyl methyl ether (10:1). NMR data (see **Tables 2** and **3**). HRMS (EI) *m/z* calcd for $C_{26}H_{42}O_3$ [M]$^{+\bullet}$ 402.3134, found 402.3143 (2.2 ppm). Anal. Calcd for $C_{26}H_{42}O_3$: C, 77.56; H, 10.51. Found: C, 78.71; H, 10.52.

Data for Tyrosyl Linoleate (16). THF (3 mL) was used as cosolvent and methyl linoleate as acylating agent. Pure **16** was obtained after column chromatography, eluting with hexane/*tert*-butyl methyl ether (10:1). NMR data (see **Tables 2** and **3**). HRMS (CI) *m/z* calcd for $C_{26}H_{41}O_3$ [M + H]$^+$ 401.305571, found 401.304694 (2.2 ppm). Anal. Calcd for $C_{26}H_{40}O_3$: C, 77.95; H, 10.06. Found: C, 77.29; H, 9.77.

Lipid Matrices. A lipid matrix was obtained from virgin olive oil (VOO) of 'Arbequina' variety by purification through alumina, according to the 'free solvent' procedure (*24*). The purified matrix, free of antioxidants, was stored at −18 °C under nitrogen atmosphere. The fatty acid composition of the matrix was C16:0 (15.5%), C16:1 (1.7%), C17:0 (0.1%), C17:1 (0.2%), C18:0 (2.0%), C18:1 (67.5%), C18:2 (11.8%), C18:3 (0.5%), C20:0 (0.4%), C20:1 (0.2%), and C22:0 (0.1%).

Evaluation of Oxidative Stability by the Rancimat Method. Aliquots of this purified glyceridic matrix were spiked with increasing amounts of antioxidants (ranging from 0.2 mM to 3 mM) and then subjected to accelerated oxidation in a Rancimat apparatus, model 743 (Metrohm Co., Basel, Switzerland). A flow of air (15 L/h) was bubbled successively through the oil heated at 80 °C and cold water. In this process, the volatile oxidation products are stripped from the oil and dissolved in the water, increasing the water conductivity. The time taken

Table 2. ^{1}H NMR Data (500.13 MHz, DMSO-d_6, 303 K) for Compounds **2** and **10−16**[a]

	2	10	11	12	13	14	15	16
phenethyl unit								
1	3.51 (dt) ($J_{1',OH} = 5.2$)	4.11 (t) ($J_{1',2'} = 7.1$)	4.13 (t) ($J_{1',2'} = 7.0$)	4.13 (t) ($J_{1',2'} = 7.0$)	4.12 (t) ($J_{1',2'} = 7.0$)	4.12 (t) ($J_{1',2'} = 7.0$)	4.12 (t) ($J_{1',2'} = 7.0$)	4.12 (t) ($J_{1',2'} = 7.0$)
2	2.59 (t) ($J_{1',2'} = 7.0$)	2.74 (t) ($J_{1',2'} = 7.0$)	2.74 (t) ($J_{1',2'} = 7.0$)	2.74 (t) ($J_{1',2'} = 7.0$)	2.74 (t) ($J_{1',2'} = 7.0$)	2.74 (t) ($J_{1',2'} = 7.0$)	2.73 (t) ($J_{1',2'} = 7.0$)	2.73 (t) ($J_{1',2'} = 7.0$)
4	6.97 (d) ($J_{4',5'} = 8.4$)	7.02 (d) ($J_{4',5'} = 8.4$)	7.01 (d) ($J_{4',5'} = 8.5$)	7.00 (d) ($J_{4',5'} = 8.5$)	7.00 (d) ($J_{4',5'} = 8.5$)	7.00 (d) ($J_{4',5'} = 8.5$)	7.00 (d) ($J_{4',5'} = 8.5$)	7.00 (d) ($J_{4',5'} = 8.5$)
5	6.64 (d) ($J_{4',5'} = 8.4$)	6.67 (d) ($J_{4',5'} = 8.4$)	6.67 (d) ($J_{4',5'} = 8.5$)	6.66 (d) ($J_{4',5'} = 8.5$)	6.66 (d) ($J_{4',5'} = 8.5$)	6.66 (d) ($J_{4',5'} = 8.5$)	6.66 (d) ($J_{4',5'} = 8.5$)	6.66 (d) ($J_{4',5'} = 8.5$)
acyl chain								
2'		1.96 (s)	2.22 (t) ($^3J = 7.3$)	2.22 (t) $^3J = 7.3$	2.22 (t) $^3J = 7.3$	2.22 (t) $^3J = 7.3$	2.22 (t) $^3J = 7.3$	2.22 (t) $^3J = 7.3$
3'			1.50 (m)	1.46 (m)	1.46 (m)	1.46 (m)	1.46 (m)	1.46 (m)
4'			0.83 (t) ($^3J = 7.3$)	1.23 (m)	1.24 (s.a.)	1.23 (s.a.)	1.23 (s.a)	1.26 (m)
(CH$_2$)$_3$				1.23 (m)	1.24 (s.a.)	1.23 (s.a.)	1.23 (s.a)	1.26 (m)
8'				1.23 (m)	1.24 (s.a.)	1.23 (s.a.)	1.97 (q) ($^3J = 6.4$)	2.00 (q) $^3J = 6.9$
9'				1.23 (m)	1.24 (s.a.)	1.23 (s.a.)	5.31 (m)	5.31 (m)
10'				1.23 (m)	1.24 (s.a.)	1.23 (s.a.)	5.31 (m)	5.31 (m)
11'				1.23 (m)	1.24 (s.a.)	1.23 (s.a.)	1.97 (q) ($^3J = 6.4$)	2.72 (t) $^3J = 6.9$
12'				0.84 (t) ($^3J = 7.0$)	1.24 (s.a.)	1.23 (s.a.)	1.23 (s.a.)	5.31 (m)
13'					1.24 (s.a.)	1.23 (s.a.)	1.23 (s.a.)	5.31 (m)
14'					1.24 (s.a.)	1.23 (s.a.)	1.23 (s.a.)	2.00 (q) ($^3J = 6.9$)
(CH$_2$)$_n$					1.24 (s.a.)	1.23 (s.a.)	1.23 (s.a.)	1.26 (m)
Me					0.84 (t) ($^3J = 7.1$)	0.84 (t) $^3J = 7.0$	0.84 (t) $^3J = 7.1$	0.84 (t) $^3J = 7.1$

[a] Chemical shifts (δ, ppm) and coupling constants (J, Hz).

Table 3. ^{13}C NMR Chemical Shifts (ppm) (125.76 MHz, DMSO-d_6, 303 K) for Compounds **2** and **10−16**
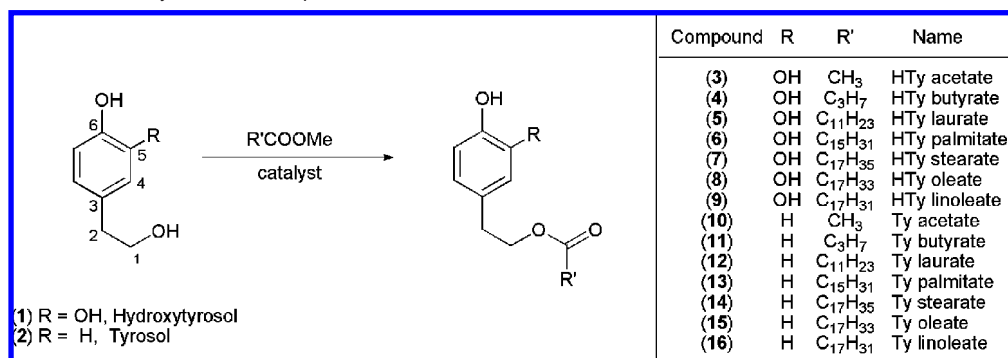
	2	10	11	12	13	14	15	16
phenethyl unit								
1	62.5	64.6	64.5	64.5	64.4	64.5	64.4	64.4
2	38.2	33.5	33.6	33.5	33.5	33.5	33.5	33.5
3	129.4	127.8	127.8	127.8	127.8	127.8	127.8	127.8
4	129.6	129.6	129.7	129.6	129.6	129.6	129.5	129.6
5	114.9	115.1	115.1	115.0	115.0	115.0	115.0	115.0
6	155.4	155.8	155.8	155.8	155.8	155.8	155.8	155.8
acyl chain								
1'		172.2	172.6	172.7	172.7	172.7	172.7	172.7
2'		20.6	35.3	33.4	33.4	33.4	33.4	33.5
3'			17.9	24.4	24.3	24.3	24.3	24.3
4'			13.3	28.9−28.3	28.9−28.3	28.9−28.3	29.0−28.2	28.6−28.3
(CH$_2$)$_3$				28.9−28.3	28.9−28.3	28.9−28.3	29.0−28.2	28.6−28.3
8'				28.9−28.3	28.9−28.3	28.9−28.3	26.5	26.5
9'				28.9−28.3	28.9−28.3	28.9−28.3	129.6	129.6
10'				31.2	28.9−28.3	28.9−28.3	129.6	127.7
11'				22.0	28.9−28.3	28.9−28.3	26.5	25.1
12'				13.8	28.9−28.3	28.9−28.3	29.0−28.2	127.7
13'					28.9−28.3	28.9−28.3	29.0−28.2	129.6
14'					31.2	28.9−28.3	29.0−28.2	26.5
15'					22.0	28.9−28.3	29.0−28.2	28.6−28.3
16'					13.8	31.2	31.2	30.8
17'						22.0	22.0	21.8
18'						13.8	13.8	13.8

until there is a sharp increase of conductivity is termed the induction time (IT) and is expressed in hours. All determinations were carried out in duplicate.

Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was carried out according to the procedure described by Benzie and Strain (25), with some modifications (26). The antioxidant potential of the synthesized compounds was estimated from their ability to reduce TPTZ-Fe[III] complex to TPTZ-Fe[II] complex. Briefly, the FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, plus 2.5 mL of 20 mM FeCl$_3$·6H$_2$O and 25 mL of 0.3 M acetate buffer to a

final pH of 3.6. This reagent was freshly prepared and warmed to 37 °C prior to its use. A 900 μL amount of FRAP was mixed with 90 μL of distilled water and 30 μL of either test sample (ranging from 50 μM to 500 μM for HTy and its derivatives and from 250 μM to 4000 μM for Ty and its derivatives), standard, or methanol (as appropriate reagent blank), and the mixture was shaken. Readings at the absorption maximum at 595 nm were taken every 20 s, and the reaction was monitored up to 30 min at 37 °C, using a UV−visible Varian (Cary 50 BIO) spectrophotometer, equipped with a thermostatted autocell-holder. The reading at 30 min was selected in each case for the calculation of

Scheme 1. General Structure of Synthesized Compounds

Compound	R	R'	Name
(3)	OH	CH_3	HTy acetate
(4)	OH	C_3H_7	HTy butyrate
(5)	OH	$C_{11}H_{23}$	HTy laurate
(6)	OH	$C_{15}H_{31}$	HTy palmitate
(7)	OH	$C_{17}H_{35}$	HTy stearate
(8)	OH	$C_{17}H_{33}$	HTy oleate
(9)	OH	$C_{17}H_{31}$	HTy linoleate
(10)	H	CH_3	Ty acetate
(11)	H	C_3H_7	Ty butyrate
(12)	H	$C_{11}H_{23}$	Ty laurate
(13)	H	$C_{15}H_{31}$	Ty palmitate
(14)	H	$C_{17}H_{35}$	Ty stearate
(15)	H	$C_{17}H_{33}$	Ty oleate
(16)	H	$C_{17}H_{31}$	Ty linoleate

(1) R = OH, Hydroxytyrosol
(2) R = H, Tyrosol

FRAP values. Methanolic solutions of Trolox were used for calibration. The FRAP values are expressed as Trolox equivalent antioxidant capacity (TEAC, mM). All analyses were run in triplicate.

ABTS Assay. The free-radical scavenging capacity was measured using the ABTS decoloration method (27) with some modifications. Briefly, ABTS was dissolved in water to get a 7 mM concentration. ABTS radical cation ($ABTS^{•+}$) was produced by reacting this stock solution with a 2.45 mM $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The $ABTS^{•+}$ solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 730 nm. After the addition of 0.1 mL of sample (ranging from 50 μM to 500 μM for HTy and its derivatives and from 100 μM to 550 μM for Ty and its derivatives), methanol as a blank, or Trolox standard to 2.9 mL of diluted $ABTS^{•+}$ solution, absorbance readings were taken every 20 s at 30 °C over 6 min, using a UV−visible spectrophotometer. The percentage inhibition of absorbance versus time was plotted, and the area below the curve (0−360 s) was calculated. Methanolic solutions of known concentrations of Trolox were used for calibration. This standard curve was linear between 0.2 mM and 0.8 mM of Trolox. Results are expressed in TEAC (mM). Each value is the average of three determinations.

Statistical Analysis. Results are expressed as means ± standard deviation (SD) of three measurements for the analytical determination. The data were subjected to a one-way analysis of variance (ANOVA) using Statistix 8.0. The level of significance was $p < 0.05$.

RESULTS

Preparation and Characterization of Tyrosyl Esters. New lipophilic tyrosyl esters (**10−16**) have been prepared by a simple, chemoselective procedure (**Scheme 1**), previously applied to the synthesis of hydroxytyrosyl esters (**3−9**) (*21, 22*). Enzymatic (lipase from *Candida antarctica*, LCA) or acidic (*p*-toluenesulfonic acid, TsOH) catalysts have been used for the transesterification reactions, and the obtained results are shown
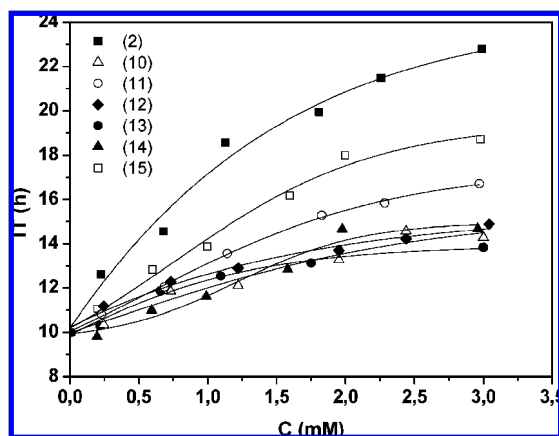

Figure 1. Induction times (ITs) of lipid matrices spiked with tyrosol (**2**), tyrosyl acetate (**10**), tyrosyl butyrate (**11**), tyrosyl laurate (**12**), tyrosyl palmitate (**13**), tyrosyl stearate (**14**), and tyrosyl oleate (**15**).

in **Table 1**. As can be seen, with esters of short alkyl chain (**10** and **11**), good yields were obtained by both methods. In these cases, the enzymatic reaction workup was easier, making the chromatographic purification unnecessary. Moreover, when TsOH was used as catalyst, temperature and time of reaction were higher. With increasing alkyl chain length, the enzymatic reaction is not so favorable, perhaps because of the need to use THF as cosolvent (*23*), while in the acid-catalyzed reactions, high yields were still obtained. Synthesized tyrosyl esters were characterized by NMR and MS spectroscopy, as well as by their elemental analyses. NMR chemical shifts (**Tables 2** and 3) were unequivocally assigned by comparison with data from starting materials. As can be seen in **Table 2**, there are differences of 0.60 and 0.15 ppm, respectively, for the chemical shifts (δ) of H_1 and H_2 with respect to the same values in free Ty (**2**). For the aromatic protons, this difference is very weak but still appreciable (0.03 and 0.05 ppm). Similar deshielding effects can be observed from ^{13}C data (**Table 3**), although the expected β-shielding effect is evident for C_2 in each ester.

Antioxidant Activity in Lipid Matrices. The efficacy of the new synthesized compounds as antioxidants in food was evaluated using the accelerated Rancimat method. Taking into account the low activity previously reported for free Ty (*10*), and in order to find greater differences in the activity of the new synthesized compounds, an experimental temperature of 80 °C was selected for this determination. The induction time (IT) values corresponding to purified matrices of olive oil spiked with different antioxidant concentrations are plotted in **Figure 1**. All the tyrosyl esters showed an IT slightly, but significantly, lower than that of free Ty.

Ferric-Reducing Antioxidant Power. The reducing capacities of the new tyrosyl esters (**10−15**), as well as of the known hydroxytyrosyl esters (**3-8**), were determined by the FRAP assay and are summarized in **Table 4**. Results are expressed as Trolox equivalent antioxidant capacity (TEAC, mM). HTy (**1**), Ty (**2**), α-tocopherol, and BHT were used in this assay as reference compounds.

These results indicated that free Ty and its esters had significantly less ferric reducing power than did free HTy and its derivatives. On the other hand, the reducing capacity of HTy and its esters was even higher than that shown by BHT and α-tocopherol, which significantly exceeded the low reducing activity associated to Ty and its new synthesized derivatives. Comparison of the results obtained for the two series of synthetic esters (**Table 4**) with their respective references (free HTy and Ty) revealed a contradictory trend: while hydroxytyrosyl esters (**3−8**) showed a significantly higher reducing activity than their natural precursor, HTy (**1**), all the tyrosyl esters (**10−15**) showed a lower antioxidant activity than that of Ty (**2**).

Table 4. Reducing Antioxidant Power of Hydroxytyrosyl (HTy) and Tyrosyl (Ty) Ester Derivatives (**3**–**8** and **10**–**15**) Evaluated by the FRAP Assay[a,b]

compd	TEAC (mM)[c]	compd	TEAC (mM)[c]
HTy (**1**)	1.39 ± 0.05 [b]	Ty (**2**)	0.31 ± 0.03 [b]
HTy acetate (**3**)	1.62 ± 0.06 [d]	Ty acetate (**10**)	0.15 ± 0.03 [d]
HTy butyrate (**4**)	2.05 ± 0.06 [e]	Ty butyrate (**11**)	0.16 ± 0.03 [a]
HTy laurate (**5**)	1.67 ± 0.06 [d]	Ty laurate (**12**)	0.14 ± 0.03 [a]
HTy palmitate (**6**)	1.58 ± 0.06 [c,d]	Ty palmitate (**13**)	0.12 ± 0.02 [a]
HTy stearate (**7**)	1.67 ± 0.06 [d]	Ty stearate (**14**)	0.15 ± 0.03 [a]
HTy oleate (**8**)	1.46 ± 0.06 [b,c]	Ty oleate (**15**)	0.16 ± 0.02 [a]
α-Tocopherol	0.80 ± 0.04 [a]	α-Tocopherol	0.80 ± 0.04 [c]
BHT	1.32 ± 0.05 [b]	BHT	1.32 ± 0.05 [d]

[a] Each value is the mean of triplicate measurements ± standard deviations. [b] All values within a column with different superscript letter are significantly different, $p < 0.05$. [c] Results are expressed as Trolox equivalent antioxidant capacity (TEAC) in units of mmol Trolox/L.

Table 5. Radical-Scavenging Capacity of Hydroxytyrosyl (HTy) and Tyrosyl (Ty) Ester Derivatives (**3**–**8** and **10**–**15**) Evaluated by the ABTS Assay[a,b]

compd	TEAC (mM)[c]	compd	TEAC (mM)[c]
HTy (**1**)	0.84 ± 0.02 [b]	Ty (**2**)	0.37 ± 0.01 [d]
HTy acetate (**3**)	1.09 ± 0.02 [d]	Ty acetate (**10**)	0.29 ± 0.01 [b]
HTy butyrate (**4**)	0.90 ± 0.02 [c]	Ty butyrate (**11**)	0.29 ± 0.01 [b]
HTy laurate (**5**)	1.13 ± 0.02 [d,e]	Ty laurate (**12**)	0.25 ± 0.01 [a]
HTy palmitate (**6**)	1.10 ± 0.02 [d,e]	Ty palmitate (**13**)	0.26 ± 0.01 [a]
HTy stearate (**7**)	1.10 ± 0.02 [d,e]	Ty stearate (**14**)	0.32 ± 0.01 [c]
HTy oleate (**8**)	1.16 ± 0.02 [e]	Ty oleate (**15**)	0.25 ± 0.01 [a]
α-tocopherol	1.01 ± 0.02 [c]	α-tocopherol	1.01 ± 0.02 [e]
BHT	0.27 ± 0.01 [a]	BHT	0.27 ± 0.01 [a,b]

[a] Each value is the mean of triplicate measurements ± standard deviations. [b] All values within a column with different superscript letter are significantly different, $p < 0.05$. [c] Results are expressed as Trolox equivalent antioxidant capacity (TEAC) in units of mmol Trolox/L.

The ABTS Assay. A widely used method for measuring the radical-scavenging activity of antioxidants is the ABTS assay, where the activity toward a stable free radical, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{•+}$), is evaluated.

The radical-scavenging activities of the evaluated antioxidants hydroxytyrosyl (**3**–**8**) and tyrosyl (**10**–**15**) esters, HTy (**1**), Ty (**2**), BHT, and α-tocopherol are summarized in **Table 5**. Results are again expressed as Trolox equivalent antioxidant capacity (TEAC, mM). The order of the scavenging activities toward the ABTS radical, derived from kinetic studies, was hydroxytyrosyl esters ≥ α-tocopherol > hydroxytyrosol > tyrosol > tyrosyl esters ∼ BHT. Again, the highest activity was provided by the ortho-diphenolic unit present in (**1**, **3**–**8**) when compared with monophenolic compounds (**2**, **10**–**15**). In addition, as in the previous method, an inverse behavior was observed among the synthesized esters and their references.

DISCUSSION

Phenolic antioxidants (PhOH) are free-radical terminators and can play their protective role by donating an H atom or acting as electron donors. This activity depends mainly on different structural features, such as O−H bound dissociation energy, resonance delocalization of the phenol radical (PhO$^{•}$), and steric hindrance derived from bulky groups substituting hydrogen in the aromatic ring (*28*). Starting from HTy and Ty, two characteristic phenolic compounds of virgin olive oil and with inherent and widely demonstrated biological activities, two series of potentially biologically active compounds (**Scheme 1**) have been synthesized by a chemoselective transesterification procedure. Their potential antioxidant activities have been evaluated by different methods and compared with those free HTy, Ty,

and another two controls traditionally used as food antioxidants: butylhydroxytoluene (BHT) and α-tocopherol. The Rancimat test is a method commonly used to evaluate the potency of antioxidants in lipophilic food matrices, such as oils and fats, while the FRAP and ABTS assays are two usual methods used for the evaluation of antioxidant activity in hydrophilic medium, the FRAP method determining the reducing activity and the ABTS assay evaluating the radical-scavenging capacity. An overall evaluation of the Rancimat values for tyrosyl derivatives is shown in **Figure 1**. Comparison of these values with those previously reported for hydroxytyrosyl esters (*10*–*22*) emphasized the high influence of the ortho-diphenolic structure on the antioxidant capacity. These results are in accord with the widely demonstrated contribution of the number of phenolic hydroxyl groups to the antioxidant activity of the evaluated compounds (*29*). On the other hand, in the Rancimat experiments all the ester derivatives were less active than their respective reference compounds (HTy or Ty). In this sense, the different activity in bulk oil autooxidation within a particular family of phenolic compounds is usually related to their polarity (*30*). According to the polar paradox concept, in apolar medium the most potent antioxidant should be the most polar one. Our results are in agreement with this paradox, the most-polar compounds in each series (HTy and Ty) being the most-effective antioxidants. However, for each compared series (hydroxytyrosyl and tyrosyl esters), matrices spiked with each specific concentration showed very similar induction times, independent of the length and nature of the acyl side chain.

The radical-scavenging and reducing capacities of the newly synthesized compounds were evaluated and compared with those of hydroxytyrosyl esters, which were not previously reported. The results, summarized in **Tables 4** and 5, confirm the influence of the ortho-diphenolic structure in the antioxidant activity, according to the higher TEAC values obtained for the hydroxytyrosyl esters in comparison with the tyrosyl ones. This is in agreement with the widely demonstrated lower efficacy shown by monophenols in comparison with polyphenols (*31*). With regard to HTy and its derivatives, the higher TEAC values obtained for the lipophilic hydroxytyrosyl derivatives in comparison with free HTy are in accordance with the polar paradox. However, an inverse behavior was observed for tyrosyl esters in comparison with free Ty, a priori contradictory to the above-mentioned results: according to the polar paradox, the apolar nature of tyrosyl esters should provide higher antioxidant activity in comparison with Ty in these assays. For this reason, the solubility of these compounds in the methanol used in the two procedures, FRAP and ABTS, was questioned. Different solvents, compatible with the aqueous buffers used in the solution of the different reagents involved, were tested to ensure the complete solution of the analyte. With this aim, DMSO, THF, ethanol, and methanol were tested as single solvents for the FRAP assay. Additionally, a combination of water−methanol (2:3, v/v) containing 1 mol/L of formic acid, reported as a good solvent for both hydrophilic and hydrophobic compounds (*32*), was evaluated. The reaction involved in the FRAP analysis was effective only using methanolic solution. In a similar way, the ABTS assay was investigated using ethanol as solvent for the solution of Ty and its derivatives (*33*). In this case, the results obtained with ethanol as solvent were identical to those obtained using methanol.

Recently, the radical-scavenging activity of HTy, Ty, and homovanillic alcohol, as well as some hydroxytyrosyl and homovanillyl (HMV) esters, determined by the DPPH method has been reported (*34*). Lipophilic hydroxytyrosyl analogues

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showed the best antioxidant capacity in comparison with the homovanillyl series or free Ty. Substitution of a hydroxyl function in the aromatic ring by a methoxy group provoked a drastic decrease in the antioxidant activity. HMV alcohol and its esters were even less active than free Ty. The nature and length of the acyl side chain in HTy or HMV derivatives seem to be negligible with regard to the antioxidant activity, and a slight rise in activity for the lipophilic esters as compared with the free compounds was observed. These results are in good agreement with ours and with those recently reported by Aissa et al. (*23*) for tyrosyl acetate. However, contrary results were recently reported in a similar study (*35*). For those authors, the nature of the acyl side chain surprisingly seems to play an important role in radical-scavenging capacity.

In conclusion, these results represent a pool of interesting new derivatives of HTy and Ty in which the lipophilic nature improved the activity of HTy esters as reducing agents and radical scavengers, while this activity decreased in Ty esters. The results obtained for their evaluation in lipid matrices using the accelerated Rancimat method were in accord with the polar paradox. Finally, the length and nature of the fatty acyl side chain seemed not to influence the antioxidant activity of the tested compounds, since no significant differences were observed between them in any of the three selected methods (Rancimat, ABTS, and FRAP).

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