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Lignin Nanoparticles Support Lipase-Tyrosinase Enzymatic Cascade in the Synthesis of Lipophilic Hydroxytyrosol Ester Derivatives

Elisabetta Tomaino,^[a] Eliana Capecchi,^[a] Davide Piccinino,^[a] and Raffaele Saladino^{*[a]}

Lipophilic esters of hydroxytyrosol have been synthesized in a one-pot procedure from tyrosol and long side-chain carboxylic acids by a lignin nanoparticles supported enzymatic cascade of lipase M and tyrosinase. The reaction involved the initial esterification of the aliphatic carboxylic group with the hydroxyl group of tyrosol followed by *ortho*-hydroxylation of the aromatic moiety. Lipase M and tyrosinase were immobilized on lignin nanoparticles by layer-by-layer procedure using chitosan

as positive charged polyelectrolyte and Concanavalin A as molecular spacer to control the optimal distance between the active sites of the two enzymes. The occurrence of substrate channeling effect was hypothesized on the basis of FRET analysis. Lipophilic hydroxytyrosol esters were synthesized in high yield in 2-methyltetrahydrofuran and in the presence of a very low amount of buffer to generate the hydration sphere of the enzyme.

Introduction

In the last years, lignin nanoparticles received a great attention due to the optimal chemo-physical properties they showed in the immobilization of different enzymes.^[1–6] In this context, organosolv lignin nanoparticles (OLNPs) have been applied as functional nanoplatfroms for the immobilization of laccase,^[2,6] horseradish peroxidase,^[4] and hydrolase,^[5] showing the beneficial effect to improve the enzymatic activity by pseudo direct electron transfer (pseudo-DET) and mediated electron transfer (MET) processes.^[2,7] Tyrosol, [2-(4-hydroxyphenyl) ethanol] and hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol] are secondary metabolites produced in the cell by the shikimic acid pathway.^[8] These compounds are well-recognized components in olive oil wastes, where they are recovered in high amount (about 1.0 g/L),^[9] representing a valuable resource for sustainable green processes.^[10] Tyrosol is resistant toward microbial degradation,^[11] and it is characterized by a lower biological activity than hydroxytyrosol.^[12] Pharmaceutical, nutraceutical and cosmeceutical properties of hydroxytyrosol are reported, including antioxidant activity,^[13] and protective effect against cardiovascular diseases,^[14] inflammation and neurodegenerative disorders.^[14–16] Unfortunately, the high polarity of hydroxytyrosol limited its absorption, distribution, and excretion properties.^[17] Chemical procedures for the oxidation of tyrosol

to hydroxytyrosol are reported, encompassing the use of toxic halogenated reagents and solvents,^[18] and tedious multi steps-procedures.^[19,20] Enzymes are potent and selective catalysts in the transformation of phenol derivatives^[18,21] under mild experimental conditions.^[22] Examples of the enzymatic mediated oxidation of tyrosol to hydroxytyrosol are reported^[18,23,24] under both homogeneous and heterogeneous conditions.^[25] In addition, the activity and biological availability of hydroxytyrosol was improved through the synthesis of lipophilic esters^[26] with high affinity for the cellular membrane,^[27,28] by using multi-steps chemical^[29] and enzymatic procedures.^[30,31] These compounds showed antioxidant and antiviral activity dependent from the number of carbon atoms in the side-chain.^[32] Enzymatic cascade is an advanced tool for multiple synthetic transformations in one-pot condition,^[33,34] promoting the fast exchange of substrate between adjacent enzymes.^[35,36] Supported multienzyme cascades are reported, and their efficiency, selectivity, stability and reusability deeply discussed.^[1,37] Here we describe the one-pot synthesis of lipophilic hydroxytyrosol esters from tyrosol and long side-chain carboxylic acids by lignin nanoparticles supported enzymatic cascade involving lipase M from *Mucor javanicus* (MJL) and tyrosinase (Ty) from *Agaricus bisporus*. Previous examples of immobilization of Ty and MJL alone on lignin nanoparticles were reported by us in connection to the synthesis of bioactive catechols^[3] and the esterification of butyric acid,^[5] respectively. The novel lignin nanoparticles supported enzymatic cascade worked in mild experimental conditions using bio-renewable 2-methyltetrahydrofuran^[38] and sodium phosphate buffer as reaction medium. MJL performed as a lyase between tyrosol and carboxylic acids,^[39] while Ty catalyzed the selective *ortho*-hydroxylation of newly produced tyrosol esters to corresponding hydroxytyrosol counterpart. MJL and Ty were co-immobilized on lignin nanoparticles by a layer-by-layer (LBL) procedure using chitosan (CH) as positive charged polyelectrolyte to balance negative charged lignin and enzymes. Concanavalin A

[a] Dr. E. Tomaino, Dr. E. Capecchi, Dr. D. Piccinino, Prof. R. Saladino
Department of Biological and Ecological Sciences
University of Tuscia
Via S.C. De Lellis s.n.c., 01100 Viterbo (Italy)
E-mail: saladino@unitus.it

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cctc.202200380>

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(Con A) was also used as a molecular spacer to confer an ordered co-orientation at the two enzymes, thus favoring the optimal distance between their active sites.^[40] The molecular recognition properties of Con A in the immobilization of enzymatic cascade was previously reported in the preparation of glucose oxidase and horseradish peroxidase biosensors.^[1,40]

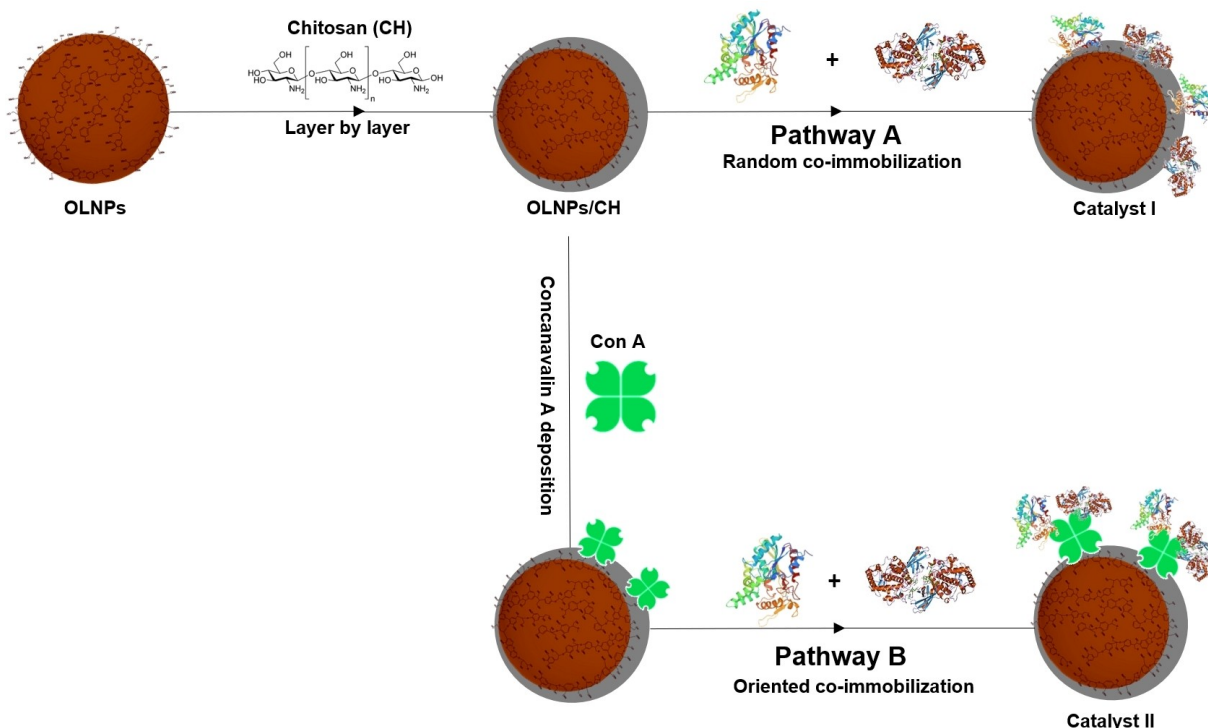
Results and Discussion

OLNPs were prepared by nanoprecipitation of organosolv lignin (OL) in the presence of tetrahydrofuran (THF) as primary solvent and deionized water as anti-solvent.^[41] In order to immobilize MJL and Ty, the positive charged layer of chitosan (CH) was initially coated on the negative charged surface of OLNPs to yield the OLNPs/CH intermediate (Scheme 1).^[42] Briefly, a solution of CH in diluted acetic acid was added dropwise to a suspension of OLNPs in Milli-Q water under gentle magnetic stirring for 2 h at room temperature. The final suspension was centrifuged and lyophilized to afford OLNPs/CH intermediate in 96% yield. **Catalyst I** was prepared by random immobilization of similar enzymatic units of MJL and Ty on OLNPs/CH intermediate. MJL (25 U) and Ty (25 U) in sodium phosphate buffer were added to OLNPs/CH intermediate (1.5 mg mL^{-1}) in PBS. The mixture was stirred at 4°C in orbital shaking for 24 h. The suspension was centrifuged and washed two times with PBS to afford **Catalyst I** in 85% yield (Scheme 1, pathway A). **Catalyst II** was prepared by co-oriented immobilization of MJL and Ty on OLNPs/CH intermediate by using Con A as a molecular spacer. Negative charged (ζ -potential -14 mV) Con A

(0.5 mg)^[43] in PBS was deposited on OLNPs/CH intermediate (1.5 mg mL^{-1}) in PBS under gentle sonication conditions for 5 min at room temperature to afford OLNPs/CH/Con A intermediate (Scheme 1, pathway B). Next, MJL (25 U) and Ty (25 U) in PBS were added to OLNPs/CH/Con A intermediate (1.5 mg mL^{-1}) in PBS, and the mixture was stirred at 4°C for 24 h. The suspension was centrifuged and washed two times with PBS to afford **Catalyst II** in 88% yield (Scheme 1, pathway B).

Field emission scanning electron microscopy (FE-SEM) analysis of **Catalyst I** and **Catalyst II** (Figure 1) showed nanoparticles with regular spherical shape characterized, in the case of **Catalyst II**, by the typical corona-like structural motif associated to the presence of Con A (Figure 1, panel B) (FE-SEM analysis of OLNPs are reported in Supporting Information #1).^[44] This different morphology was appreciable from that observed for **Catalyst I**, in which case roughly aggregates of small clumps, typical of CH,^[45] were present on the surface of the nanoparticles (Figure 1, panel A).

Dynamic light scattering (DLS) and ζ -potential values of **Catalyst I** and **Catalyst II** are reported in Table 1 and compared with OLNPs, and OLNPs/CH and OLNPs/CH/Con A intermediates, respectively (entries 1–5). **Catalyst I** and **Catalyst II** showed an average diameter of 378.8 nm and 526.5 nm , respectively, higher than OLNPs (Table 1, entries 4–5 versus entry 1). Note that, the diameter of **Catalyst I** increased of c.a. 50 nm with respect to OLNPs/CH intermediate after the deposition of the enzymes (Table 1, entry 4 versus entry 2, respectively). In a similar way, the diameter of **Catalyst II** increased of c.a. 74 nm with respect to OLNPs/CH/Con A intermediate (Table 1, entry 5



Scheme 1. Schematic representation of **Catalyst I** and **Catalyst II**. Random immobilization (pathway A); Oriented co-immobilization (pathway B)

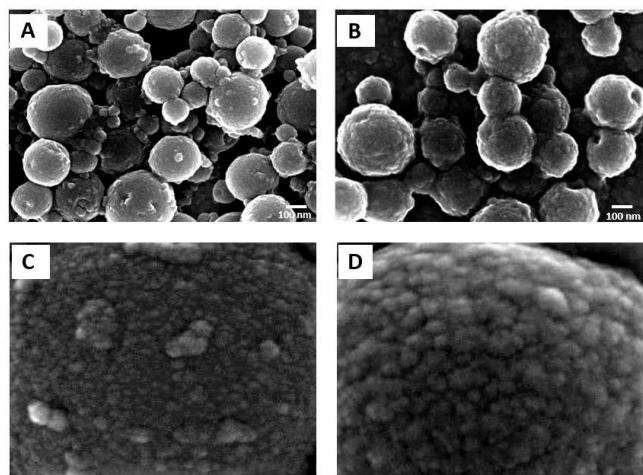


Figure 1. Field emission scanning electron microscopy (FE-SEM) images of Catalyst I (panel A) and Catalyst II (panel B). Panel C–D: magnification (x800k) of Catalyst I and Catalyst II, respectively.

Entry	Sample	Size [nm]	ζ -potential [mV]
1	OLNPs	290.8	−43
2	OLNP/CH	328.6	+26
3	OLNP/CH/Con A	452.2	−30
4	Catalyst I	378.8	−36
5	Catalyst II	526.5	−48

versus entry 3). In addition, an increase of about 100 nm was measured after deposition of Con A on OLNP/CH intermediate. This latter trend was in accordance with previous reported data on the immobilization of enzymes on organic supports when mediated by Con A.^[44] The value of the ζ -potential confirmed the occurrence of the expected coating sequence in the preparation of Catalyst I and Catalyst II. Catalyst I inverted the ζ -potential value with respect to OLNP/CH intermediate which was characterized by a positive ζ -potential value (+26 mV), as a consequence of the balance with the negative charged enzymes (Table 1, entry 4 versus entry 2). Conversely, the ζ -potential of Catalyst II was similar to that of OLNP/CH/Con A intermediate, having this latter compound a negative ζ -potential (Table 1, entry 5 versus entry 3). Probably, the efficacy of the supramolecular recognition between OLNP/CH/Con A intermediate and MJL and Ty was controlled by the formation of H-bonding network than electrostatic interactions, in accordance with data previously reported.^[46]

The immobilization of MJL and Ty on OLNP/CH intermediate was further confirmed by attenuated total reflectance FT-IR analysis (FT-IR analysis of OLNP, OLNP/CH and OLNP/CH/Con A intermediates are reported in SI#2; Figure S2). The FT-IR spectrum of Catalyst I showed the typical bands of OLNP/CH intermediate (Figure 2). In addition, the signal about 1500 cm^{-1} and 1600–1700 cm^{-1} were attributed to the stretching vibration of the amide II and amide I bands, respectively, which are characteristic of the polypeptide structure. FT-IR analysis was

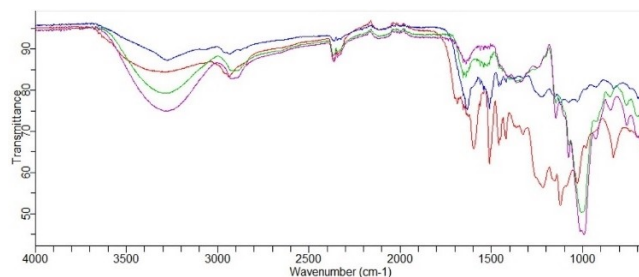


Figure 2. FT-IR spectra of Catalyst I (red line) and Catalyst II (blue line). The FT-IR spectra of free MJL (green line) and Ty (purple line) are reported as reference.

not diagnostic in the case of Catalyst II, due to the presence of bands associated to Con A.

The activity parameters of Catalyst I and Catalyst II were evaluated as reported in Equations (1)–(3), respectively:

$$\text{Activity yield (\%)} = [U_x / (U_a - U_r)] \times 100 \quad (1)$$

$$\text{Immobilization yield (\%)} = [(U_a - U_r) / U_a] \times 100 \quad (2)$$

$$\text{Activity (units/mg)} = U_x / W_{\text{support}} \quad (3)$$

where U_x are the immobilized enzyme units, U_a are the total units of the enzyme added to the solution, and U_r are the residual units of the enzyme in the washing solution. The activity parameters of MJL were evaluated following the increase in absorbance (λ_{max} 405 nm) produced during the release of *para*-nitrophenol in the hydrolysis of *para*-nitrophenyl laurate (*p*-NPL) in Tris-HCl (0.1 M, pH 7.4) at 25 °C for 15 min.^[47,48] In a typical experiment, Catalyst I and Catalyst II (1.5 mg mL^{-1} in PBS) were added to *p*-NPL solution (20 mM) in Tris-HCl at 25 °C. The international unit of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1.0 μmol of *p*-NPL in 1 min at 25 °C and optimal pH. The activity of Ty was measured by the dopachrome assay (λ_{max} 475 nm) in PBS at 25 °C for 10 min.^[49] In a typical experiment, Catalyst I and Catalyst II (1.5 mg mL^{-1} in PBS) were added to L-tyrosine solution (2.5 mM) in PBS at 25 °C. The activity of Ty was determined following the increase in absorbance (λ_{max} 475 nm) during the oxidation of L-tyrosine in PBS at 25 °C for 10 min. The international unit of tyrosinase activity was defined as the amount of enzyme necessary to oxidize 1.0 μmol of L-tyrosine in 1 min at 25 °C and optimal pH. As reported in Table 2, the immobilization yield of MJL and Ty in Catalyst II was higher than that of Catalyst I, confirming the beneficial role of Con A in the immobilization procedure (entry 4 versus entry 3). In a similar way, the activity yield and the activity of MJL and Ty in Catalyst II were higher than that of Catalyst I. This effect was most probably due to the optimal co-orientation of the two enzymes, as successively confirmed by FRET analysis (see next paragraph).

Kinetic parameters (K_m and V_{max}) of MJL and Ty in Catalyst I and Catalyst II (Table 3, entries 3 and 4) were determined by measuring the activity of the appropriate enzyme at different

Table 2. Immobilization and activity parameters of Catalyst I and Catalyst II. ^[a]					
Entry	Catalyst	IY ^[b] of MJL [%]	IY of Ty [%]	AY ^[c] [%] and A ^[d] of MJL	AY [%] and A of Ty
1	MJL	–	–	10.6	–
2	Ty	–	–	–	11.8
3	I	80	50	20 (8.4) ^[d]	14 (12.2) ^[d]
4	II	87	69	60 (15.9) ^[d]	45 (15.7) ^[d]

[a] All experiments were conducted in triplicates. [b] Immobilization yield (%). [c] Activity yield (%). [d] Activity (U/W_{support}).

Table 3. Kinetic parameters of Catalyst I and Catalyst II. ^[a]					
Entry	Catalyst	K _m [mM] ^[b] of MJL	K _m [mM] of Ty	V _{max} [μmol L ⁻¹ s ⁻¹] ^[c] of MJL	V _{max} [μmol L ⁻¹ s ⁻¹] of Ty
1	MJL	5.3	–	0.24	–
2	Ty	–	0.18	–	0.62
3	I	8.30	0.45	0.22	0.54
4	II	3.17	0.16	0.45	0.75

[a] All experiments were conducted in triplicates. [b] Average error is 2–4%. [c] Average error is 1–3%.

concentration of *p*-NPL (0–10 mM) and L-tyrosine (0.1–0.33 mM) by using the *p*-NPL^[47] and dopachrome assays,^[49] respectively. All measurements were performed by using the same amount of catalyst (1.5 mg). The data were analyzed by Lineweaver-Burk plots and the Michaelis-Menten constant and V_{max} calculated in triplicate with the GraphPad software. Kinetic parameters of MJL and Ty were also calculated as a reference (Table 3, entries 1 and 2). **Catalyst II** showed a value of V_{max} for both MJL and Ty of the same order of magnitude than the native counterpart (Table 3, entries 3–4 versus 1–2), and higher than **Catalyst I** (Table 3, entry 4 versus entry 3), confirming the beneficial effect played by Con A in the immobilization process. K_m of MJL and Ty followed a similar trend (Table 3). This result is in accordance with data previously reported on the role played by the correct spatial distribution of the enzymes during the immobilization process in the overall catalytic activity of the enzymatic cascade.^[1,50]

In order to unambiguously confirm the proximity of MJL and Ty on the surface of **Catalyst II**, we measured the Förster resonance energy transfer (FRET) spectra by Zeiss LSM-710 laser confocal microscopy (CLSM) analysis,^[51] comparing the result with **Catalyst I** as a reference (Figure 3, panel a–h). The FRET effect occurs between two fluorophores by non-radiative dipole-dipole coupling as a consequence of the energy transfer between a donor and acceptor dyes in close proximity (typically 1–10 nm).^[52] **Catalyst I** and **Catalyst II** were labelled with fluorescein isothiocyanate (FITC) as donor dye, and with rhodamine B (Rho B) as the acceptor counterpart, respectively (labelling of MJL and Ty and confocal FRET microscopy procedure are reported in SI#3).^[53] The effective presence of FITC-MJL on **Catalyst I–II** was detected in the donor channel mode at 490 nm (Figure 3, panel a and e; green fluorescence signal), while Rho B–Ty was detected in the acceptor channel mode at 560 nm (Figure 3, panel b and f; red fluorescence

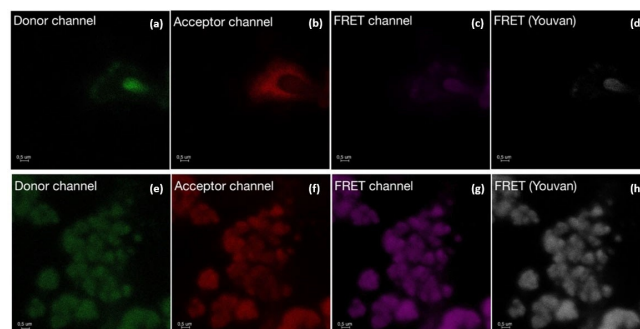
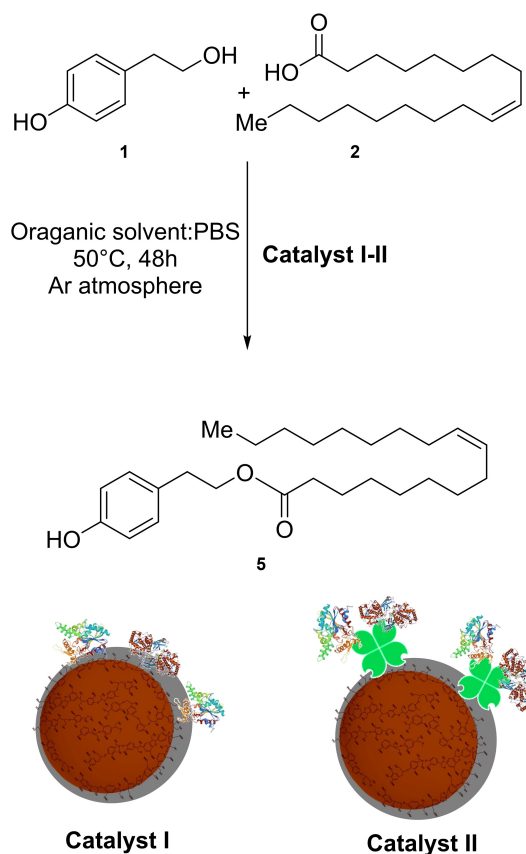


Figure 3. Laser confocal scanning microscopy of Catalyst I–II. Panel a: MJL labelled with FITC in the donor channel at 490 nm (green pixels) of Catalyst I. Panel b: Ty labelled with Rho B in the acceptor channel at 560 nm (red pixels) of Catalyst I. Panel c: FRET channel of merged fluorescence effect of Catalyst I. Panel d: original FRET signal of Catalyst I. PANEL e–h: donor, acceptor, FRET and original FRET channels respectively of Catalyst II.

signal). The proximal localization of fluorophores, highlighted by the presence of a merged fluorescent signal, was observed only in the case **Catalyst II**, (Figure 3, panel g versus panel c). The elaboration of the FRET signal of **Catalyst II** (Figure 3, panel h)^[54] indicated the presence of a distance of 6.7 nm between the two enzymes, a value that is comprised in the range of values for which the substrate channeling effect was operative.^[55] The absence of the FRET signal in the case of **Catalyst I** (Figure 3 panel d) suggested the random co-immobilization of the enzymes.

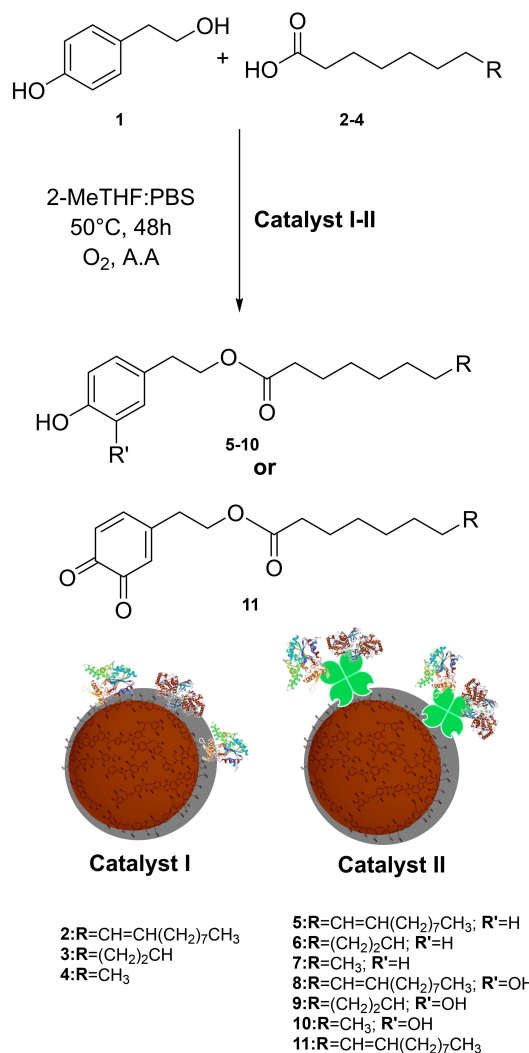
Lipophilic esters of hydroxytyrosol were synthesized by reaction of tyrosol 1 with long-chain carboxylic acids including oleic acid (C_{18:1}) 2, lauric acid (C₁₂) 3 and octanoic acid (C₈) 4. We first evaluated the effect played by the ratio between the two reagents and the polarity of the reaction solvent in the esterification step, analyzing the reaction of 1 and 2 as a selected case under argon atmosphere in order to selectively inhibit the activity of Ty (Scheme 2).

The reaction was performed using three different molar ratio of the reagents (1:1, 1:1.78 and 3:1 ratio, respectively). Compounds 1 and 2 were solubilized in organic solvents (5.0 mL) with different polarity (n-hexane, 2-methyltetrahydrofuran, and n-hexane/ethanol 9:1 v/v) in the presence of a low amount of PBS (0.1 mL) containing the appropriate amount of catalyst (165 μg) under argon atmosphere at 50 °C and gentle magnetic stirring (200 rpm). The organic solvents were selected among that previously reported in the lipase transformations,^[38,56,57] while the low amount of PBS was required to hydrate the enzyme.^[58] Higher amount of aqueous buffer are known to inhibit the esterase activity of lipase.^[57] The range of temperature between 40 °C and 50 °C is a well-recognized temperature for the enzymatic synthesis of ester derivatives.^[59] Similar reactions were performed with free MJL as a reference. Table 4 summarizes the conversion of 1 and the yield of tyrosol oleate 5 depending on the ratio value of the reagents, the nature of the reaction solvent and the catalyst applied in the transformation. As expected, hydroxytyrosol was not detected in the reaction mixture due to the presence of the



Scheme 2. Synthesis of tyrosol oleate **5** by reaction of tyrosol **1** and oleic acid **2** in the presence of **Catalyst I** and **Catalyst II**. The tyrosinase activity of **Catalyst I** and **Catalyst II** was inhibited by performing the reaction under argon atmosphere.

argon atmosphere inhibiting Ty activity. As a general trend, 2-methyltetrahydrofuran (2-MeTHF) performed as the best organic solvent in the synthesis of **5** under both homogeneous and heterogeneous conditions (Table 4, entries 10–18). A lower yield of **5** was obtained in the presence of n-hexane (n-Hex) and n-Hex/Ethanol (EtOH; 9:1 v/v). Irrespective from the experimental conditions, the highest yield of **5** was generally obtained using the 1:1 ratio of the two reagents. 2-MeTHF is a bio-renewable reaction solvent^[60] widely applied in bio-catalysis and esterification processes.^[36,61] Among the different experimental conditions tested with 2-MeTHF, **Catalyst II** afforded **5** in yield higher than **Catalyst I** (Table 4, entry 16 versus entry 13). This result is in accordance with the higher activity and kinetic parameters of **Catalyst II** than **Catalyst I**. In addition, the reaction performed with **Catalyst II** in 2-MeTHF afforded **5** in a yield comparable to (Table 4, entry 16 versus entry 10), or higher than (Table 4, entries 17 and 18 versus entries 11 and 12), free MJL. On the basis of these data, we selected 2-MeTHF and the molar ratio value of 1:1 between **1** and long-chain carboxylic acids **2–4** to generalize the procedure for the synthesis of hydroxytyrosol ester derivatives **8–10**. In this latter case, the reaction was performed under dioxygen atmosphere (O_2) to activate the catecholase activity of Ty in the enzymatic cascade (Scheme 3).



Scheme 3. Synthesis of hydroxytyrosol esters **8–10** from tyrosol **1** and carboxylic acids **2–4** in the presence of **Catalyst I** and **Catalyst II**. The reaction was performed under dioxygen atmosphere to activate also tyrosinase.

Briefly, tyrosol **1** (35 mg, 0.25 mmol) and carboxylic acids **2–4** (0.25 mmol) were added with **Catalyst I–II** (165 µg in PBS, 0.1 mL) and ascorbic acid (0.5 mmol) in 2-MeTHF (5.0 mL) under dioxygen atmosphere at 50 °C for 48 h. Ascorbic acid (A.A) was used to avoid the formation of quinone side chain derivatives.^[62] Under these experimental conditions, hydroxytyrosol esters **8–10** were synthesized as the main reaction products from acceptable to high yield (from 49% to 80%), besides low amount of tyrosol esters **5** and **6–7**, and unreacted substrate (Table 5). As a general trend, the highest conversion of **1** and yield of compounds **8–10** were obtained in the presence of **Catalyst II**, further confirming the beneficial effect of Con A as molecular spacer (Table 5, entries 4–7 versus entries 1–3). Irrespective from the experimental conditions, a low amount of tyrosol esters **5–7** was also obtained. In order to evaluate the possible occurrence of substrate-tunneling effect between the active sites of the two enzymes, the performance

Table 4. Synthesis of tyrosol oleate **5** from tyrosol **1** and oleic acid **2** in the presence of Catalyst I and Catalyst II. [a]

Entry	Organic solvent	MJL/Catalyst	1 [mmol]	2 [mmol]	Ratio 1/2	Conversion ^[b] [%]	Yield ^[c] [%]
1	n-Hex	MJL	0.25	0.25	1:1	38	35
2	n-Hex	MJL	0.14	0.25	1:1.78	5	3.2
3	n-Hex	MJL	0.36	0.12	3:1	16	15
4	n-Hex	Catalyst I	0.25	0.25	1:1	3.8	2.7
5	n-Hex	Catalyst I	0.14	0.25	1:1.78	1.6	1.4
6	n-Hex	Catalyst I	0.36	0.12	3:1	3.0	3.0
7	n-Hex	Catalyst II	0.25	0.25	1:1	12.5	11
8	n-Hex	Catalyst II	0.14	0.25	1:1.78	2.3	2.1
9	n-Hex	Catalyst II	0.36	0.12	3:1	5.6	5.6
10	2-MeTHF	MJL	0.25	0.25	1:1	57	57
11	2-MeTHF	MJL	0.14	0.25	1:1.78	23	22
12	2-MeTHF	MJL	0.36	0.12	3:1	29.5	29
13	2-MeTHF	Catalyst I	0.25	0.25	1:1	27	27
14	2-MeTHF	Catalyst I	0.14	0.25	1:1.78	20	19
15	2-MeTHF	Catalyst I	0.36	0.12	3:1	13	12
16	2-MeTHF	Catalyst II	0.25	0.25	1:1	51	50
17	2-MeTHF	Catalyst II	0.14	0.25	1:1.78	30	30
18	2-MeTHF	Catalyst II	0.36	0.12	3:1	40	40
19	n-Hex/EtOH	MJL	0.25	0.25	1:1	19	18
20	n-Hex/EtOH	MJL	0.14	0.25	1:1.78	7	5.6
21	n-Hex/EtOH	MJL	0.36	0.12	3:1	8.5	6.0
22	n-Hex/EtOH	Catalyst I	0.25	0.25	1:1	2.3	1.1
23	n-Hex/EtOH	Catalyst I	0.14	0.25	1:1.78	0.4	0.4
24	n-Hex/EtOH	Catalyst I	0.36	0.12	3:1	1.6	1.6
25	n-Hex/EtOH	Catalyst II	0.25	0.25	1:1	6	5.9
26	n-Hex/EtOH	Catalyst II	0.14	0.25	1:1.78	4.2	4.0
27	n-Hex/EtOH	Catalyst II	0.36	0.12	3:1	4.7	4.7

[a] All experiments were conducted in triplicates at 50 °C for 48 h. MJL: Lipase M. [b] Conversion of tyrosol **1** was evaluated on the basis of the recovered mmol of substrate. [c] The yield of tyrosol oleate **5** was evaluated on the basis of the recovered mmol of the reaction product.

Table 5. Synthesis of lipophilic hydroxytyrosol esters **8–10** from **1** by **Catalyst I** and **Catalyst II**^[a]

Entry	Catalyst	Carboxylic acid	Product(s)	Conversion ^[b] [%]	Yield ^[c] [%]
1	I	2	5 (8)	24	6.1 (18)
2	I	3	6 (9)	32	8.0 (24)
3	I	4	7 (10)	38	5.1 (33)
4	II	2	5 (8)	53	4.2 (49)
5	II	2 ^[d]	5 (8) [11]	53	5.3 (25) [25]
6	II	3	6 (9)	70	7 (63)
7	II	4	7 (10)	86	6 (80)
8	III–IV	2	5 (8) [12]	70	32.4 (9) [27]
9	II	2	8	51 (Run n.1) ^[e]	49
10	II	2	8	49 (Run n.2)	47
11	II	2	8	43 (Run n.3)	39
12	II	2	8	38 (Run n.4)	35
13	II	2	8	35 (Run n.5)	29

[a] All experiments were conducted in triplicate. [b] Conversion of tyrosol **1**. [c] The yield was evaluated on the basis of the recovered mmol of reaction product. [d] Reaction performed in the absence of ascorbic acid. [e] **Catalyst II** was recovered by centrifugation and re-used in next runs.

of **Catalyst II** was compared with that of **Catalyst III** and **Catalyst IV**, prepared by the individual immobilization of MJL and Ty on OLNP/CH/Con A intermediate (the preparation and structural characterization of **Catalyst III** and **Catalyst IV** are reported in SI#4). The treatment of **1** and **2** under previously reported experimental conditions in the presence of a mixture of **Catalyst III** and **Catalyst IV**, bearing the same total amount of the two enzymes as in **Catalyst II**, afforded hydroxytyrosol ester **8** in low yield, besides to appreciable amount of tyrosol ester **5** and hydroxytyrosol **12** (Table 5, entry 8 versus entry 4). The presence of relevant amount of **5** and **12** in the reaction mixture suggested that the occurrence of the substrate-tunneling effect cannot be completely ruled out. As a general trend, the yield of esters **8–10** increased by decreasing the number of carbon atoms in the side-chain of the molecule (Table 5, entry 4 versus entries 6–7). The role of carboxylic acids in the efficacy of esterification of tyrosol and hydroxytyrosol under biocatalytic conditions is reported, focusing on the deleterious effect exerted by the increase of the number of carbon atoms in the efficacy of the transformation.^[63] Note that, the treatment of **1** with **Catalyst II** in the absence of ascorbic acid afforded a mixture of hydroxytyrosol ester **8** and quinone **11** in a similar yield (Table 5, entry 5), opening the possibility to apply this procedure for the synthesis of hydroxytyrosol quinone esters.

The reusability of **Catalyst II** was evaluated after its recovering at the end of the reaction (centrifugation, followed by washing with PBS) and its re-using in the next runs. As a general trend, **Catalyst II** retained a relevant reactivity after 5 runs in the synthesis of hydroxytyrosol ester **8** as a selected example (Table 5, entries 9–13). The decrease in conversion of the substrate observed during the recycling experiments (c.a. 20% with respect to the first run) may be probably due to the partial thermic denaturation of Con A,^[64,65] responsible for the

occurrence of unfavorable conformational changes of immobilized enzymes.

Conclusion

Lignin nanoparticles supported lipase M and tyrosinase enzymatic cascade efficiently catalyzed the synthesis of lipophilic hydroxytyrosol esters **8–10** by one-pot esterification and ortho-hydroxylation of tyrosol **1** with carboxylic acids **2–4**. The highest yield was obtained in the presence of Concanavalin A (**Catalyst II**), which controlled the optimal orientation and distance between the two enzymes, favoring the occurrence of substrate-tunneling effect. As confirmed by previous studies, the presence of Con A on the surface of lignin nanoparticles shielded the conformational changes caused by the direct enzyme-nanoparticles interaction.^[44] Noteworthy, Con A selectively binds the carbohydrate region of glycoproteins such as lipase M and tyrosinase in peptide sequences which are not directly involved in the catalytic activity of the enzymes.^[40]

Probably, the synthetic pathway started from the esterification step, in accordance with the isolation of low amount of tyrosol esters **5–7** as side-products, as well as by the absence of hydroxytyrosol in the reaction mixture. In addition, the same reaction performed with MJL and Ty supported alone on different batch of lignin nanoparticles afforded, in a selected case, hydroxytyrosol ester **8** in yield lower than **Catalyst II**, confirming the efficacy of the enzymatic cascade in the overall transformation. It is interesting to note that both MJL and Ty showed, once supported on lignin nanoparticles, kinetic parameters (K_m and V_{max}) higher than the native counterpart, in accordance with the beneficial effect we previously observed in the stabilization of peroxidase and glucose oxidase by lignin nanoparticles.^[1,4] The yield of the reaction products was found to be dependent from the chemical complexity of the carboxylic acid, the highest yield being obtained in the presence of medium length chain derivatives. **Catalyst II** was stable enough to be recovered and used for at list five successive runs. This procedure opens a new entry for a green and low-cost valorization procedure of olive oil wastes containing tyrosol in order to improve the overall amount of bioactive lipophilic hydroxytyrosol ester derivatives.

Experimental Section

Materials

Organosolv lignin (OL) was purchased from Chemical Point (Oberhaching, Germany). Chitosan (medium molecular weight: 100,000–300,000), 2-(4-Hydroxyphenyl)ethanol (Tyrosol), octanoic acid, lauric acid, oleic acid, Amano lipase M from *Mucor javanicus* (300 U/mg), Concanavalin A (Con A) type IV from *Canavalia ensiformis* (Jack bean), mushroom tyrosinase from *Agaricus bisporus* (1000 U/mg), 4-nitrophenyl laurate, L-tyrosine, trizma base (primary standard and buffer) and sodium sulfate anhydrous (Na_2SO_4), were purchased from Sigma Chemical Co. (St Louis, MO, USA). Reagents including *n*-hexane, 2-methyltetrahydrofuran, and ethanol were purchased from Sigma Chemical Co. (St Louis, MO, USA) and were

used without further purification. Sodium phosphate buffer (PBS 0.1 M, pH 7) and Tris-HCl (0.1 M, pH 7.4) were prepared using Milli-Q water ($\rho = 18.2 \text{ M}\Omega \text{ cm}$ at 25°C ; $\text{TOC} < 10 \mu\text{g L}^{-1}$, Millipore, Molsheim, France). Spectrophotometric analysis was performed with Cary 60 UV-Vis spectrophotometer equipped with a single cell peltier thermostated cell holder, Agilent, Santa Clara, USA. Data were elaborated with Cary Win UV software. Chemical reactions were monitored using thin layer chromatography on precoated aluminium silica gel Merck 60 F_{254} plates and a UV lamp ($\lambda_{\text{max}} = 254 \text{ nm}$) was used for visualization.

Preparation of Catalyst I

Organosolv lignin nanoparticles (OLNPs) were prepared by previously reported nanoprecipitation procedure.^[2] Briefly, CH solution (5 mg, 1.0 mg mL^{-1} in diluted acetic acid 1.0% v/v) that was added dropwise to OLNPs (5 mg, 1.0 mg mL^{-1}) in Milli-Q water under slow magnetic stirring (200 rpm) for 2 h at room temperature. The final suspension was centrifuged (2616 g, 20 min) and rinsed with deionized water, followed by centrifugation (2616 g, 20 min) and freeze-dry to yield OLNPs/CH intermediate. The yield of OLNPs/CH intermediate is defined as total amount in weight of nanoparticles recovered with respect to the total amount of the two components (lignin and CH) (yield 96%). In order to prepare **Catalyst I**, the OLNPs/CH intermediate (1.5 mg mL^{-1}) were dispersed in sodium phosphate buffer (PBS 1.0 mL; 0.1 M, pH 7) and added of the appropriate amount of MJL (25 U; 3.0 mg mL^{-1}) and Ty (25 U; 1.0 mg mL^{-1}) dissolved in PBS (0.1 M; pH 7) under gentle stirring (200 rpm). The mixture was stirred at 4°C in orbital shaking for 24 h and the suspension was centrifuged and washed two times with PBS (pH 7, 0.1 M) to afford **Catalyst I** (yield 85%). Note that the enzymes were not released from **Catalyst I** in the reaction conditions (that is 2-MeTHF and PBS), as evaluated by Bradford analysis of the supernatant after recovery of the catalyst by centrifugation.

Preparation of Catalyst II

For the preparation of **Catalyst II**, 0.5 mL of Con A solution (1.0 mg mL^{-1}) in PBS (0.1 M, pH 7.3) was added to OLNPs/CH intermediate (1.5 mg mL^{-1}) in PBS (1.0 mL; 0.1 M, pH 7) under gentle sonication conditions at 25°C , followed by the addition of MJL (25 U; 3.0 mg mL^{-1}) and Ty (25 U; 1.0 mg mL^{-1}) dissolved in PBS (0.1 M; pH 7). The mixture was stirred at 4°C in orbital shaking for 24 h. The solution was centrifuged and washed two times with PBS (pH 7; 0.1 M) to afford **Catalyst II** (yield 88%).

Field emission scanning electron microscopy (FE-SEM) characterization.

The field emission scanning electron microscopy (FE-SEM) of **Catalyst I** and **Catalyst II** was acquired on FESEM ZEISS Gemini-SEM500, operated at 5 kV after drop $20 \mu\text{L}$ (with deionized water) of sample dispersion on specimen stubs, air dried and coated with gold by sputtering with AGAR (Auto Sputter Coater). Before the observations, the sample received a deposition of chromium thin film (5 nm) by sputter coating using a QUORUM Q 150T ES plus coater.

Hydrodynamic diameter and ζ -potential analyses

The hydrodynamic diameter and ζ -potential values were evaluated in H_2O by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) apparatus, equipped with an

He-Ne laser (633 nm, fixed scattering angle of 173° , 25°C). Measurements were performed in triplicate at 25°C .

Fourier-transform infrared spectroscopy (FT-IR)

Catalyst I and **II** were analyzed by attenuated total reflection Fourier transform infrared (ATR-IR) spectroscopic using an Agilent Cary 630 FT-IR spectrometer (UATR unit cell) averaging 32 scans (resolution of 4 cm^{-1}).

Kinetic and activity parameters

The enzymatic activity and kinetic parameters of MJL and Ty were determined by using the *p*-NPL^[47,48] and dopachrome assay,^[49] respectively. In particular, the activity of MJL was evaluated by measuring the increase in absorbance ($\lambda_{\text{max}} 405 \text{ nm}$) produced during the release of *para*-nitrophenol in the hydrolysis of *para*-nitrophenyl laurate (*p*-NPL) in Tris-HCl (0.1 M, pH 7.4) at 25°C for 15 min. In a typical experiment, free MJL (25 U), or in alternative, **Catalyst I** and **Catalyst II** (1.5 mg mL^{-1} in PBS, 0.1 M, pH 7) were added to *p*-NPL solution (0.1 mL; 20 mM; CNCH_3 ; water 1:1) in Tris-HCl. The international unit of lipase activity was defined as the amount of enzyme necessary to hydrolyse $1.0 \mu\text{mol}$ of *p*-NPL in 1.0 min at 25°C and optimal pH. The activity of Ty was measured by the dopachrome assay ($\lambda_{\text{max}} 475 \text{ nm}$) in PBS (pH 7; 0.1 M) at 25°C for 10 min. In a typical experiment, free Ty (25 U), or in alternative **Catalyst I** and **Catalyst II** (1.5 mg mL^{-1} in PBS, 0.1 M, pH 7), were added to L-tyrosine solution (1.0 mL; 2.5 mM) in PBS (pH 7; 0.1 M). The international unit of tyrosinase activity was defined as the amount of enzyme necessary to oxidize $1.0 \mu\text{mol}$ of L-tyrosine in 1.0 min at 25°C and optimal pH. Kinetic parameters (K_m and V_{max}) of MJL, Ty and **Catalyst I** and **Catalyst II** were determined by measuring the activity of the appropriate enzyme (25 U) kept constant using different concentration of *p*-NPL (from 0 to 10 mM) in Tris-HCl (pH 7.4) and L-tyrosine (from 0.1 to 0.33 mM) in PBS (pH 7), respectively.

Förster resonance energy transfer (FRET)

The detailed procedure for the labelling of MJL and Ty and for the calculation of the FRET effect are reported in supporting (SI#3). The confocal images of **Catalyst I** and **Catalyst II** were obtained by ZEISS LSM-710 NLO confocal system equipped with a Plan ApoChromat $100\times/1.40$ oil DIC M 27 objectives.

Synthesis of tyrosol oleate 5 by esterification of tyrosol 1 with Catalyst I and II

The reactions were performed starting from different amount of tyrosol 1 (0.25, 0.14 and 0.36 mmol) and oleic acid 2 (0.25, 0.25 and 0.12 mmol) in the appropriate organic solvent (5.0 mL) and PBS (0.1 mL) containing **Catalyst I-II** ($165 \mu\text{g}$) at 50°C for 48 h under argon atmosphere. The reactions were monitored at regular intervals of time by thin layer chromatography (CH_2Cl_2 : MeOH 9.8:0.2). At the end of the reaction the catalyst was recovered by centrifugation (6000 rpm) for 20 min, and the organic phase was evaporated under reduce pressure. The crude was dissolved in EtOAc (5.0 mL) and washed with NaCl saturated solution, dried over anhydrous Na_2SO_4 and evaporated. Reaction products were recovered after chromatographic purification using CH_2Cl_2 : MeOH as eluent. The reaction with free MJL was also performed as a reference. Briefly, different amount of tyrosol 1 (0.25, 0.14 and 0.36 mmol) and oleic acid 2 (0.25, 0.25 and 0.12 mmol) were reacted in the appropriate organic solvent (5.0 mL) and PBS

(0.1 mL) with MJL (25 U) at 50 °C for 48 h. The reactions were monitored at regular intervals by thin layer chromatography (CH₂Cl₂: MeOH 9.8:0.2). At the end of the reaction the organic phase was recovered and evaporated under reduce pressure. The crude was dissolved in EtOAc (5.0 mL) and washed with NaCl saturated solution, dried over anhydrous Na₂SO₄ and evaporated.

4-Hydroxyphenethyl oleate (tyrosol oleate) **5**. Yield 50% (47 mg); yellow oil. ¹H NMR (400 MHz, [D]CDCl₃): δ = 0.90 (3H, m, CH₃), 1.29–1.30 (20H, m, CH₂), 1.59 (2H, m, CH₂), 2.02 (4H, m, CH₂), 2.30 (2H, m, O=C-CH₂), 2.88 (2H, m, CH₂), 4.26 (2H, m, OCH₂), 5.37 (2H, m, CH=CH), 6.79 (2H, d, J 9.0 Hz, Ph-H), 7.09 (2H, d, J 8.4 Hz, Ph-H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 14.8 (CH₃), 23.2 (CH₂), 25.4 (CH₂), 28.2 (CH₂x2), 28.7–30.8 (CH₂, x8), 32.4 (CH₂), 34.2 (CH₂), 36.3 (CH₂), 66.4 (CH₂), 117.2 (CH x2), 128 (CH x2), 129.9 (C), 130.8 (CH, x2), 156.3 (C), 173.2 (C) ppm. MS (EI) m/z 402.62

Synthesis of hydroxytyrosol esters 8–11 by cascade enzymatic transformation of tyrosol **1** and carboxylic acids 2–4

The reactions were performed starting from tyrosol **1** (0.25 mmol) and the appropriate carboxylic acid **2–4** (0.25 mmol) in 2-MeTHF (5.0 mL) and PBS (0.1 mL) containing **Catalyst I–II** (165 µg) and ascorbic acid (0.5 mmol) under dioxygen atmosphere (O₂) at 50 °C for 48 h. The reaction was monitored at regular times by thin layer chromatography (CH₂Cl₂: MeOH 9.8:0.2). At the end of the reaction the catalyst was recovered by centrifugation (2616 g) for 20 min, followed by evaporation of the solvent under reduced pressure. The crude was dissolved in EtOAc (5.0 mL) and washed with NaCl saturated solution, dried over anhydrous Na₂SO₄ and evaporated. Reaction products were recovered after chromatographic purification using CH₂Cl₂: MeOH as eluent. In one selected case, tyrosol **1** (0.25 mmol) was reacted with **2** (0.25 mmol) and **Catalyst II** (165 µg) in absence of ascorbic acid under previously reported experimental conditions. The work-up of the reaction was performed in the same conditions previously described to yield hydroxytyrosol ester **8** and hydroxytyrosol quinone ester **11**. As an alternative, tyrosol **1** (0.25 mmol) and oleic acid **2** (0.25 mmol) in 2-MeTHF (5.0 mL) and PBS (0.1 mL) were reacted in the presence of **Catalyst III** (165 µg) and **Catalyst IV** (165 µg) and ascorbic acid (0.5 mmol) under dioxygen atmosphere (O₂) at 50 °C for 48 h. The reaction was monitored at regular times by thin layer chromatography (CH₂Cl₂: MeOH 9.8:0.2). The work-up of the reaction was performed as reported in the previous procedures. The reusability of **Catalyst II** was evaluated after its recovering at the end of the reaction by centrifugation at 15000 g for 10 mins, followed by washing with PBS (pH 7.0, 0.1 M) and re-using it in next runs.

4-Hydroxyphenethyl dodecanoate (tyrosol laurate) **6**. Yield 7% (6 mg); yellow oil. ¹H NMR (400 MHz, [D] CDCl₃): δ = 0.90 (3H, m, CH₃), 1.29 (16H, m, CH₂), 1.62 (2H, m, CH₂), 2.31 (2H, m, O=C CH₂), 2.88 (2H, m, CH₂), 4.27 (2H, m, OCH₂), 6.80 (2H, d, J 9.0 Hz, Ph-H), 7.07 (2H, d, J 8.4 Hz, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 12.8 (CH₃), 22.5 (CH₂), 25.4 (CH₂), 28.2–30.5 (CH₂, x6), 32.4 (CH₂), 34.3 (CH₂, x2), 66.4 (CH₂), 116.2 (CH, x2), 130.9 (C), 132.8 (CH, x2), 156.3 (C), 173.9 (C) ppm. MS (EI) m/z 320.47.

4-Hydroxyphenethyl octanoate (tyrosol octanoate) **7**. Yield 6% (4.26 mg); yellow oil. ¹H NMR (400 MHz, [D]CDCl₃): δ = 0.94 (3H, m, CH₃), 1.26–1.30 (8H, m, CH₂), 1.64 (2H, m, CH₂), 2.31 (2H, m, O=C CH₂), 2.89 (2H, m, CH₂), 4.26 (2H, m, OCH₂), 6.79 (2H, d, J 9.0 Hz, Ph-H), 7.09 (2H, d, J 8.4 Hz, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 14.8 (CH₃), 22.7 (CH₂), 25.4 (CH₂), 28.2–30.5 (CH₂, 3x), 34.3 (CH₂), 35.2 (CH₂), 66.4 (CH₂), 115.9 (CH, x2), 130.9 (C), 132.8 (CH, x2), 156.3 (C), 174.2 (C) ppm. MS (EI) m/z 264.37.

3,4-Dihydroxyphenethyl oleate (Hydroxytyrosol oleate) **8**. Yield 49% (48 mg); colourless oil. ¹H NMR (400 MHz, [D]CDCl₃): δ = 0.88 (3H, m, CH₃), 1.27–1.33 (20H, m, CH₂), 1.66 (2H, m, CH₂), 2.03 (4H, m, CH₂), 2.37 (2H, m, O=C-CH₂), 2.83 (2H, m, CH₂), 4.24 (2H, m, OCH₂), 5.35 (2H, m, CH=CH), 6.67 (1H, dd, J 7.9 Hz, Ph-H), 6.76 (1H, d, J 2.1 Hz, Ph-H), 6.81 (1H, d, J 8.0 Hz, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 14.5 (CH₃), 23.2 (CH₂), 25.4 (CH₂), 27.2 (CH₂ x2), 28.5–30.1 (CH₂, x8), 30.2 (CH₂ x2), 31.5 (CH₂), 34.3 (CH₂), 35.2 (CH₂), 66.7 (CH₂), 116.2 (CH), 117.9 (CH), 122.5 (CH), 130.9 (CH x2), 131.7 (CH), 145.8 (C), 146.3 (C), 173.2 (C) ppm. MS (EI) m/z 418.62.

3,4-Dihydroxyphenethyl dodecanoate (Hydroxytyrosol laurate) **9**. Yield 63% (46 mg); yellow oil. ¹H NMR (400 MHz, [D]CDCl₃): δ = 0.90 (3H, m, CH₃), 1.28–1.32 (16H, m, CH₂), 1.63 (2H, m, CH₂), 2.37 (2H, m, O=C-CH₂), 2.83 (2H, m, CH₂), 4.25 (2H, m, OCH₂), 6.65 (1H, dd, J 7.9 Hz, Ph-H), 6.76 (1H, d, J 2.1 Hz, Ph-H), 6.79 (1H, d, J 8.0 Hz, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 13.9 (CH₃), 22.5 (CH₂), 24.8 (CH₂), 28.2–29.8 (CH₂ x6), 32.8 (CH₂), 34.3 (CH₂), 35.8 (CH₂), 65.4 (CH₂), 116.2 (CH), 117.2 (CH), 120.2 (CH), 131.9 (C), 142.3 (C), 145.1 (C), 173.2 (C) ppm. MS (EI) m/z 336.47.

3,4-Dihydroxyphenethyl octanoate (Hydroxytyrosol octanoate) **10**. Yield 80% (56.8 mg); colourless oil. ¹H NMR (400 MHz, [D]CDCl₃): δ = 0.90 (3H, m, CH₃), 1.27–1.29 (8H, m, CH₂), 1.66 (2H, m, CH₂), 2.35 (2H, m, O=C-CH₂), 2.83 (2H, m, CH₂), 4.23 (2H, m, OCH₂), 6.76 (1H, dd, J 7.9 Hz, Ph-H), 6.77 (1H, d, J 2.1 Hz, Ph-H), 6.81 (1H, d, J 8.0 Hz, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 13.8 (CH₃), 23.2 (CH₂), 24.8 (CH₂), 29.3–29.5 (CH₂ x2), 31.8 (CH₂), 33.9 (CH₂), 34.5 (CH₂), 64.8 (CH₂), 115.7 (CH), 116.3 (CH), 121.2 (CH), 130.9 (C), 145.3 (C), 146.3 (C), 173.4 (C) ppm. MS (EI) m/z 280.36.

2-(3,4-Dioxocyclohexa-1,5dien-1-yl) ethyl oleate (hydroxyl tyrosol quinone oleate) **11**. Yield 25% (26.5 mg); yellow oil. ¹H NMR (400 MHz, [D]CDCl₃): δ = 0.88 (3H, m, CH₃), 1.28 (21H, m, CH₂), 1.62 (2H, m, CH₂), 2.10 (4H, m, CH₂), 2.29 (2H, m, CH₂), 2.80 (2H, m, CH₂), 4.13 (2H, m, CH₂), 5.30 (2H, m, CH=CH), 5.89 (1H, s, Ph-H), 6.80 (1H, s, Ph-H), 7.25 (1H, s, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 14.3 (CH₃), 23.2 (CH₂), 25.2 (CH₂), 27.2 (CH₂ x2), 28.5–29.9 (CH₂ x8), 32.3 (CH₂), 34.2 (CH₂), 39.1 (CH₂), 64.7 (CH₂), 113.5 (CH), 127.5 (CH), 130.7 (CH x2), 153.8 (CH), 156.3 (C), 173.8 (C), 180.4 (C), 183.1 (C) ppm. MS (EI) m/z 416.60.

3,4-dihydroxyphenethyl alcohol (hydroxytyrosol) **12**. Yield 10% (10.6 mg); white solid. ¹H NMR (400 MHz, [D]CDCl₃): δ = 2.77 (2H, m, CH₂), 3.85 (2H, m, CH₂), 4.58 (OH, m), 6.67 (1H, s, Ph-H), 6.77 (1 h, m, Ph-H), 6.84 (1H, s, Ph-H) ppm. ¹³C NMR (100 MHz, [D]CDCl₃): δ = 39.2 (CH₂), 61.1 (OCH₂), 115.9 (CH), 116.4 (CH), 122.2 (CH), 131.5 (C), 144.5 (C), 145.6 (C) ppm. MS (EI) m/z 154.06.

Acknowledgements

The Large Equipment Center (CGA) of Tuscia University are acknowledged and dr. Elena Paccosi is acknowledged for confocal analysis and FRET measurements. Lorenzo Arrizza and Microscopies center of L'Aquila University are acknowledged for SEM analyses. Open Access funding provided by Università degli Studi della Tuscia within the CRUI-CARE Agreement.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: biocatalysis · enzymatic cascade · esterification · hydroxytyrosol · lignin nanoparticles

- [1] E. Capecchi, D. Piccinino, E. Tomaino, B. M. Bizzarri, F. Polli, R. Antiochia, F. Mazzei, R. Saladino, *RSC Adv.* **2020**, *10*, 29031–29042.
- [2] D. Piccinino, E. Capecchi, L. Botta, P. Bollella, R. Antiochia, M. Crucianelli, R. Saladino *Catal. Sci. Technol.* **2019**, *9*, 4125–4134.
- [3] E. Capecchi, D. Piccinino, I. Delfino, P. Bollella, R. Antiochia, R. Saladino, *Nanomaterials* **2018**, *8*, 438.
- [4] E. Capecchi, D. Piccinino, B. M. Bizzarri, L. Botta, M. Crucianelli, R. Saladino, *Catalysts* **2020**, *10*, 313.
- [5] M. H. Sipponen, M. Farooq, J. Koivisto, A. Pellis, J. Seitsonen, M. Österberg, *Nat. Commun.* **2018**, *9*, 2300.
- [6] E. Capecchi, D. Piccinino, B. M. Bizzarri, D. Avitabile, C. Pelosi, C. Colantonio, G. Calabrò, R. Saladino, *Biomacromolecules* **2019**, *20*, 1975–1988.
- [7] E. Kim, W. T. Leverage, Y. Liu, I. M. White, W. E. Bentley, G. F. Payne, *Analyst* **2014**, *139*, 32–43.
- [8] J. Rodríguez-Morató, A. Boronat, A. Kotronoulas, M. Pujadas, A. Pastor, E. Olesti, C. Pérez-Mañá, O. Khymenets, M. Fitó, M. Farré, R. de la Torre, *Drug Metab. Rev.* **2016**, *48*, 218–236.
- [9] R. Delisi, F. Saiano, M. Pagliaro, R. Ciriminna, *Chem. Cent. J.* **2016**, *10*, 63.
- [10] F. S. Markhali, *Processes* **2021**, *9*, 953.
- [11] N. Allouche, M. Damak, R. Ellouz, S. Sayadi, *Appl. Environ. Microbiol.* **2004**, *70*, 2105–2109.
- [12] A. K. Marković, J. Torić, M. Barbarić, C. J. Brala, *Molecules* **2019**, *24*, 2001.
- [13] C. Manna, P. Galletti, V. Cucciolla, G. Montedoro, V. Zappia, *J. Nutr. Biochem.* **1999**, *10*, 159–165.
- [14] Lopez-Huertas Leon E., Geerlings A., Morales Sanchez J. C., J. Boza Puerta, *WO-200308798 A1*, **2003**.
- [15] R. Fuccelli, R. Fabiani, P. Rosignoli, *Molecules* **2018**, *23*, 3212.
- [16] R. M. de Pablos, A. M. Espinosa-Oliva, R. Hornedo-Ortega, M. Cano, S. Arguelles, *Pharmacol. Res.* **2019**, *143*, 58–72.
- [17] M. Robles-Almazan, M. Pulido-Moran, J. Moreno-Fernandez, C. Ramirez-Tortosa, C. Rodriguez-Garcia, J. L. Quiles, M. Ramirez-Tortosa, *Food Res. Int.* **2018**, *105*, 654–667.
- [18] P. Ziosi, C. Paolucci, F. Santarelli, T. Tabanelli, S. Passeri, F. Cavani, P. Righi, *ChemSusChem* **2018**, *11*, 2202–2210.
- [19] R. Bernini, E. Mincione, M. Barontini, F. Crisante, *J. Agric. Food Chem.* **2008**, *56*, 8897–8904.
- [20] A. D. Kalampaliki, V. Giannouli, A. L. Skaltsounis, I. K. Kostakis, *Molecules* **2019**, *24*, 3239.
- [21] Z. Li, H. Chen, Y. Fang, Y. Ma, H. Chen, B. Yang, Y. Wang, *J. Agric. Food Chem.* **2021**, *69*, 5481–5490.
- [22] B. Zieniuk, K. Groborz, M. Wołoszynowska, K. Ratusz, E. Bialecka-florjańczyk, A. Fabiszewska, *Biomol. Eng.* **2021**, *11*, 1–12.
- [23] P. P. Liebgott, M. Labat, L. Casalot, A. Amouric, J. Lorquin, *FEMS Microbiol. Lett.* **2007**, *276*, 26–33.
- [24] F. Annunziata, M. L. Contente, C. Pinna, L. Tamborini, A. Pinto, *Antioxidants* **2021**, *10*, 1142.
- [25] T. Bozzini, G. Botta, M. Delfino, S. Onofri, R. Saladino, D. Amatore, R. Sgarbanti, L. Nencioni, A. T. Palamara, *Bioorg. Med. Chem.* **2013**, *21*, 7699–7708.
- [26] D. Y. Zhou, Y. X. Sun, F. Shahidi, *J. Funct. Foods* **2017**, *37*, 66–73.
- [27] T. Ginex, J. Vazquez, E. Gilbert, E. Herrero, F. J. Luque, *Future Med. Chem.* **2019**, *11*, 1177–1193.
- [28] E. Rutkowska, K. Pajk, K. J'èwiak, *Acta Pol. Pharm.* **2013**, *70*, 3–18.
- [29] R. Bernini, I. Carastro, F. Santoni, M. Clemente, *Antioxidants* **2019**, *8*, 174–174.
- [30] R. Fotiadou, A. v. Chatzikonstantinou, M. A. Hammami, N. Chalmpes, D. Moschovas, K. Spyrou, A. C. Polydera, A. Avgeropoulos, D. Gournis, H. Stamatis, *Nanomaterials* **2021**, *11*, 1–22.
- [31] G. Botta, B. M. Bizzarri, A. Garozzo, R. Timpanaro, B. Bisignano, D. Amatore, A. T. Palamara, L. Nencioni, R. Saladino, *Bioorg. Med. Chem.* **2015**, *23*, 5345–5351.
- [32] D. Tofani, V. Balducci, T. Gasperi, S. Incerpi, A. Gambacorta, *J. Agric. Food Chem.* **2010**, *58*, 5292–5299.
- [33] J. H. Schrittwieser, S. Velikogne, M. Hall, W. Kroutil, *Chem. Rev.* **2018**, *118*, 270–348.
- [34] K. Chauhan, A. Zárate-Romero, P. Sengar, C. Medrano, R. Vazquez-Duhalt, *ChemCatChem* **2021**, *13*, 3732–3748.
- [35] A. Giannakopoulou, A. V. Chatzikonstantinou, N. Chalmpes, G. Tsapara, D. Gournis, A. C. Polydera, H. Stamatis, *Catalysts* **2021**, *11*, 749.
- [36] E. T. Hwang, S. Lee, *ACS Catal.* **2019**, *9*, 4402–4425.
- [37] J. Rocha-Martin, S. Velasco-Lozano, J. M. Guisán, F. López-Gallego, *Green Chem.* **2014**, *16*, 303–311.
- [38] Y. Simeó, J. V. Sinisterra, A. R. Alcántara, *Green Chem.* **2009**, *11*, 855–86.
- [39] P. Adlercreutz, *Chem. Soc. Rev.* **2013**, *42*, 6406–6436.
- [40] E. Ortiz, P. Gallay, L. Galicia, M. Eguilaz, G. Rivas, *Sens. Actuators B* **2019**, *292*, 254–262.
- [41] T. Leskinen, M. Smyth, Y. Xiao, K. Lintinen, M.-L. Mattinen, M. A. Kostiaainen, P. Oinas, M. Österberg, *Nord. Pulp Pap. Res. J.* **2017**, *32*, 586–596.
- [42] G. Decher, *Science* **1997**, *277*, 1232–1237.
- [43] A. Makky, J. P. Michel, P. Maillard, V. Rosilio, *Biochim. Biophys. Acta Biomembr.* **2011**, *1808*, 656–666.
- [44] Y. Yong, R. Su, X. Liu, W. Xu, Y. Zhang, R. Wang, P. Ouyang, J. Wu, J. Ge, Z. Liu, *Biochem. Eng. J.* **2018**, *129*, 26–32.
- [45] V. T. Perchyonok, J. Souza, M. F. Küll, T. Y. U. Suzuki, A. T. Maluly-Proni, P. H. dos Santos, *Braz. Oral Res.* **2019**, *33*, 0024.
- [46] Y. Zhang, Y. Yong, J. Ge, Z. Liu, *ACS Catal.* **2016**, *6*, 3789–3795.
- [47] S. Hernández-García, M. I. García-García, F. García-Carmona, *Anal. Biochem.* **2017**, *530*, 104–106.
- [48] S. K. Verma, K. K. Ghosh, R. Verma, S. Verma, *Int. J. Chem. Kinet.* **2021**, *53*, 308–316.
- [49] Y. Masamoto, S. Lida, M. Kubo, *J. Med. Plants Res.* **1980**, *40*, 361–365.
- [50] O. Idan, H. Hess, *Curr. Opin. Biotechnol.* **2013**, *24*, 606–611.
- [51] F. Wu, S. D. Minter, *Biomacromolecules* **2013**, *14*, 2739–2749.
- [52] D. Shrestha, A. Jenei, P. Nagy, G. Vereb, J. Szöllösi, *Int. J. Mol. Sci.* **2015**, *16*, 6718–6756.
- [53] Y. Zhang, F. Lyu, J. Ge, Z. Liu, *Chem. Commun.* **2014**, *50*, 12919–12922.
- [54] Z. Xia, Y. Liu, *Biophys. J.* **2001**, *81*, 2395–2402.
- [55] J. Fu, Y. R. Yang, A. Johnson-Buck, M. Liu, Y. Liu, N. G. Walter, N. W. Woodbury, H. Yan, *Nat. Nanotechnol.* **2014**, *9*, 531–536.
- [56] A. Zaidi, J. L. Gainer, G. Carta, A. Mrani, T. Kadir, Y. Belarbi, A. Mir, *J. Biotechnol.* **2002**, *93*, 209–216.
- [57] J. Sun, S. Q. Liu, *J. Food Biochem.* **2015**, *39*, 11–18.
- [58] G. v. Chowdary, S. G. Prapulla, *Pocess Biochem.* **2002**, *38*, 393–397.
- [59] S. Wang, X. Meng, H. Zhou, Y. Liu, F. Secundo, Y. Liu, *Catalysts* **2016**, *6*, 32.
- [60] G. Englezou, K. Kortsens, A. A. C. Pacheco, R. Cavanagh, J. C. Lentz, E. Krumins, C. Sanders-Velez, S. M. Howdle, A. J. Nedoma, V. Taresco, *J. Polym. Sci.* **2020**, *58*, 1571–1581.
- [61] C. Zippilli, B. M. Bizzarri, S. Gabellone, L. Botta, R. Saladino, *ChemCatChem* **2021**, *13*, 4151–4158.
- [62] M. P. Bradshaw, C. Barril, A. C. Clark, P. D. Prenzler, G. R. Scollary, *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 479–498.
- [63] Y. Liu, E. Lotero, J. G. Goodwin, *J. Catal.* **2006**, *243*, 221–228.
- [64] V. Vetri, C. Canale, A. Relini, F. Librizzi, V. Militello, A. Gliozzi, M. Leone, *Biophys. Chem.* **2007**, *125*, 184–190.
- [65] A. K. Locke, B. M. Cummins, A. A. Abraham, G. L. Cotei, *Anal. Chem.* **2014**, *86*, 9091–9097.

Manuscript received: March 18, 2022

Revised manuscript received: June 10, 2022

Accepted manuscript online: June 13, 2022

Version of record online: July 18, 2022