Enzymatic continuous-flow preparation of nature-inspired phenolic esters as antiradical and antimicrobial agents

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Abstract: A collection of nature-inspired lipophilic phenolic esters have been prepared by an enzymatic synthesis under flow conditions, using the immobilized lipase B from *Candida antarctica* (Novozyme 435®) as a catalyst in cyclopentyl methyl ether (CPME), a non-conventional and green solvent. Their antimicrobial activity against four selected bacterial strains together with their efficiency as radical scavengers were evaluated. The obtained compounds were characterized by enhanced lipophilicity in comparison with the parent non-esterified compounds, which increased the possibility of their use as additives in the food industry.

Keywords: phenolic esters, antimicrobial agents, radical scavenger, food preservatives, flow chemistry, lipase

1. Introduction

A large number of phenols and phenolic derivatives occurs in nature, particularly in the plant kingdom, and possess a variety of health-promoting effects such as antioxidant, metal chelator, free radical scavenger, antimicrobial and anti-inflammatory properties (Bucciantini et al., 2021; Prabhu et al., 2021). Therefore, plant-derived phenols are of considerable interest from the viewpoint of dietary antioxidant supplementation and food preservation. In fact, food additives are widely used to limit the detrimental effects of food processing and storage. In this context, plant-derived phenolic compounds have great potential as food preservatives (Zieniuk et al., 2021). However, due to their hydrophilic character, their applicability as active ingredients in lipophilic food matrices and cosmetic products requiring solubility in non-aqueous media is limited. With the aim to overcome this limitation, common for almost all phenolic compounds, a growing interest has been devoted to synthesizing lipophilic derivatives (Farooq, Abdullah, Zhang, & Weiss, 2021). In fact, the esterification of phenolic compounds for the obtainment of molecules with medium or long aliphatic chains

can be used as a strategy for the modification of physical properties, such as solubility and miscibility in emulsion-based systems. Other strategies include the incorporation of one or more halogen atoms (Bernini et al., 2018) or, more commonly, the introduction of different aliphatic chain into the molecular skeleton (Sun, Zhou, & Shahidi, 2018), avoiding the derivatization of the phenolic moiety, to which the biological effects are attributed. Lipophilization of phenolic acids or alcohols can be performed either following traditional chemical procedures (Wang et al., 2018; Bernini et al., 2019), enzymatically (Yang, Guo, & Xu, 2012), or chemo-enzymatically (Almeida et al., 2016; Contente et al., 2021). Chemical lipophilization is commonly achieved under drastic conditions of pH and temperature using strong acidic heterogeneous or homogeneous catalysts resulting in low selectivity with consequent unwanted side products and the need for purification steps that generate extra waste to dispose of. With the evolving demand for green and sustainable processes, the use of biocatalysis has dramatically increased in the last two decades (Truppo, 2017; Bell et al., 2021; Heath, Ruscoe, & Turner, N. J., 2021; Wu et al., 2021). In fact, enzymatic synthesis offers different advantages such as the ability to promote highly chemo-, regio- and stereo-selective transformations under mild reaction conditions, minimization of side reactions and formation of by-products, and more environmentally friendly processes with lower energy consumption and waste production. Enzymatic lipophilization of phenolic acids or alcohols with hydrophobic chains can be performed in free or added-solvent systems in some hours or several days at temperatures ranging from 30 °C to 80 °C (Compton, Laszlo, & Berhow, 2000; Figueroa-Espinoza, & Villeneuve, 2005). Further research should be dedicated to the development of productive and sustainable biocatalytic processes, capable of producing the desired products in sufficient amounts to evaluate their functional, toxicological and sensory profile.

In this context, the possibility of merging the advantages of enzymatic catalysis with continuous flow processes can lead to green, versatile, scalable, and highly productive synthetic tools (De Santis, Meyer, & Kara, 2020; Guajardo, & Domínguez de María, 2019; Santi, et al., 2021; Xu, Wang, Huang, & Zheng, 2022). Exploiting our previous experiences in this field (Annunziata, et al., 2021, Semproli, et al., 2020, Contente, Tamborini, Molinari, & Paradisi, 2020; Annunziata, et al. 2022), a simple biocatalyzed flow procedure to prepare lipophilic tyrosyl, vanillyl and homo-hydroxytyrosyl alkyl esters was planned to extend their use in pharma, food and cosmetic sectors (Figure 1). In fact, a selective biocatalyzed esterification of the primary alcohol of the phenol derivatives with fatty acids (*i.e.,* propionic acid, sorbic acid, caproic acid and lauric acid) was performed to obtain a multifunctional amphiphilic molecule readily available, with a reproducible quality. The scaffolds were selected among natural ones, endowed with interesting antioxidant or antimicrobial properties to achieve a certain variability in the series both on the phenolic and fatty acid moieties (Matsue, et al., 2019; Borrelli et al., 2021; Fischer, 2020; Guzman, 2014). Sorbic acid was selected also to evaluate if the presence of the unsaturations conjugated with the carboxylic group could affect the reactivity in comparison with caproic acid and the biological activity of the obtained compounds. Finally, the activity of

the obtained compounds as radical scavengers and antimicrobial agents was tested and compared with the parent non-esterified compounds.

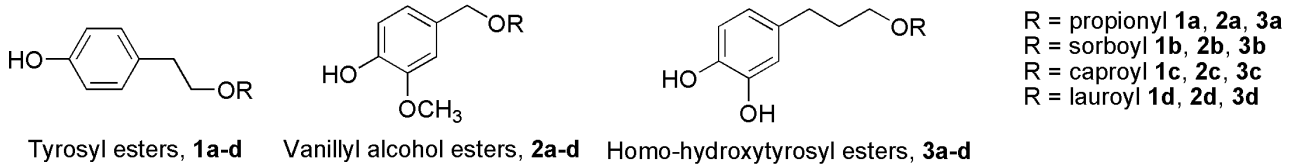


Figure 1. Structure of the synthesized phenolic esters.

2. Materials and Methods

Reagents and solvents were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^{1}H chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) in hertz (Hz). Continuous flow biotransformations were performed using a R2$^+$/R4 Vapourtec flow reactor or using Asia Flow Chemistry Syringe pumps (Syrris) equipped with an Omnifit® glass column (6.6 mm i.d. × 100 mm length). The temperature sensor sits on the wall of the reactors. Pressure was controlled by using back-pressure regulators. HPLC analyses were carried out on a Jasco PU-980 pump equipped with a Jasco UV-975 UV/Vis Detector. Column Luna 5u Silica (2) 100 Å, 250x4.6 mm; flow rate 1 mL/min; λ= 254 nm; mobile phase hexane / iPrOH 8:2. Retention time: **1d:** 3.6 min, Ty: 5.8 min. Immobilized lipase B from *Candida antarctica* (Novozyme 435®) was purchased from Merck. 4-(3-Hydroxypropyl)benzene-1,2-diol (homo-HTy) was prepared from caffeic acid following a literature procedure (Wang, et al., 2020).

2.1 General procedure for the flow esterification and in-line work up

A glass column (i.d.: 6.6 mm) was packed with a previously prepared mixture of Novozyme 435® (692 mg) and powder molecular sieves 4 Å (692 mg) to obtain a packed bed reactor (PBR) with a final volume of 3.0 mL. A 0.1 M solution of the phenolic alcohol in CPME (total volume: 20 mL) and a 0.3 M solution of the fatty acid in CPME were mixed in a T-piece and the resulting flow stream was directed into the reactor column kept at 60 °C. The total flow rate was 0.2 mL/min (residence time: 15 minutes). The exiting flow stream was flowed through a column (i.d.: 10 mm) packed with Amberlite IRA67 (2.8 g, total exchange capacity ≥ 1.60 eq/L FB form, PBR volume: 5.5 mL) at a total flow rate of 0.4 mL/min thanks to an inlet of CPME delivered through a third peristaltic pump. The organic solvent was collected and evaporated under pressure and, if necessary, the crude was purified by flash chromatography.

2.2 General procedure for the flow esterification with sorbic acid

A glass column (i.d.: 6.6 mm) was packed with a previously prepared mixture of Novozyme 435® (692 mg) and powder molecular sieves 4 Å (692 mg) (PBR volume: 3.0 mL). A 0.1 M solution of the phenolic alcohol in

CPME (20 mL) and a 0.3 M solution of the sorbic acid in CPME were mixed in a T-piece and the resulting flow stream was directed into the reactor column kept at 60 °C. The total flow rate was 0.04 mL/min (residence time: 75 minutes). The organic solvent was collected and evaporated under pressure and the crude was purified by flash chromatography.

2.3 Compound characterization

*4-Hydroxyphenethyl propionate (**1a**)* (Botta, et al, 2015): amber oil; yield: 80%; Rf = 0.56 (cyclohexane/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.7 Hz, 2H), 6.77 (d, *J* = 8.7 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.31 (q, *J* = 7.6 Hz, 2H), 1.12 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.10, 154.51, 130.02, 129.60, 115.37, 65.29, 34.21, 27.67, 9.09.

*4-Hydroxyphenethylexa-2,4-dienoate (**1b**):* white solid; yield: 24%; Rf = 0.84 (cyclohexane/EtOAc 1:1); m.p.: 73.3 – 74.4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 – 7.16 (m, 1H), 7.09 (d, *J* = 8.1 Hz, 2H), 6.77 (d, *J* = 8.1 Hz, 2H), 6.27 – 6.04 (m, 2H), 5.76 (d, *J* = 15.4 Hz, 1H), 4.30 (t, *J* = 7.1 Hz, 2H), 2.89 (t, *J* = 7.1 Hz, 2H), 1.85 (d, *J* = 5.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.78, 154.55, 145.58, 139.85, 130.01, 129.72, 129.62, 118.61, 115.41, 65.26, 34.29, 18.66.

*4-Hydroxyphenethyl hexanoate (**1c**)* (Lucas, et al, 2010): colourless oil; yield: 68%; Rf = 0.72 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.6 Hz, 2H), 6.76 (d, *J* = 8.6 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.28 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.52 (m, 2H), 1.37 – 1.21 (m, 4H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.15, 154.33, 130.02, 129.84, 115.32, 65.03, 34.33, 34.24, 31.25, 24.62, 22.29, 13.88.

*4-Hydroxyphenethyl dodecanoate (**1d**)* (Mateos, et al, 2008): white solid; yield: 90%; Rf = 0.66 (cyclohexane/EtOAc 7:3); m.p.: 40.5 – 42.0 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.08 (d, *J* = 8.1 Hz, 2H), 6.76 (d, *J* = 8.1 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.28 (t, *J* = 7.0 Hz, 2H), 1.57 (m, 2H), 1.37 – 1.21 (m, 16H), 0.88 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.31, 154.42, 130.00, 129.71, 115.34, 65.12, 34.39, 34.24, 31.90, 29.60, 29.44, 29.33, 29.24, 29.11, 24.94, 22.68, 14.11.

*4-Hydroxy-3-methoxybenzyl propionate (**2a**)* (Antoniotti et al, 2008): pale yellow oil; yield: 75%; Rf = 0.61 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95 – 6.83 (m, 3H), 5.03 (s, 2H), 3.90 (s, 3H), 2.36 (q, *J* = 7.6 Hz, 2H), 1.15 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.45, 146.49, 145.76, 127.96, 121.99, 114.36, 111.28, 66.37, 55.92, 27.64, 9.09.

*4-Hydroxy-3-methoxybenzylhexa-2,4-dienoate (**2b**):* white solid; yield: 30%; Rf = 0.58 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 – 7.20 (m, 1H), 6.91 - 6.88 (m, 3H), 6.24 – 6.06 (m, 2H), 5.80 (d, *J* = 15.4 Hz, 1H), 5.64 (s, OH), 5.10 (s, 2H), 3.90 (s, 3H), 1.85 (d, *J* = 5.3 Hz, 3H).

*4-Hydroxy-3-methoxybenzyl hexanoate (**2c**)* (Kobata, et al, 2002)*:* colourless oil; yield: 67%; Rf = 0.74 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.95 – 6.83 (m, 3H), 5.64 (s, OH), 5.03 (s, 2H), 3.90 (s, 3H), 2.39 – 2.27 (m, 2H), 1.72 – 1.54 (m, 2H), 1.36 – 1.21 (m, 4H), 0.88 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.82, 146.48, 145.75, 128.00, 121.97, 114.35, 111.26, 66.28, 55.91, 34.34, 31.28, 24.64, 22.29, 13.88.

*4-Hydroxy-3-methoxybenzyl dodecanoate (**2d**)* (He, et al, 2009)*:* yellow solid; yield: 61%; Rf = 0.74 (cyclohexane/EtOAc 6:4); m.p.: 35.5 – 36.9 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.94 – 6.83 (m, 3H), 5.64 (s, OH), 5.03 (s, 2H), 3.90 (s, 3H), 2.33 (t, *J* = 7.5 Hz, 2H), 1.61 (m, 2H), 1.30 - 1.25 (m, 16H), 0.93 – 0.82 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$**)** δ 173.80, 146.44, 145.73, 128.02, 121.98, 114.32, 111.22, 66.26, 55.91, 34.39, 31.89, 29.58, 29.45, 29.31, 29.25, 29.12, 24.96, 22.67, 14.10.

*3-(3,4-Dihydroxyphenyl)propyl propionate (**3a**):* light brown solid; yield: 76%, Rf = 0.55 (cyclohexane/EtOAc 1:1); m.p.: 65.6 – 67.9 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.77 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 1.5 Hz, 1H), 6.60 (dd, *J* = 8.1, 1.5 Hz, 1H), 4.08 (t, *J* = 6.5 Hz, 2H), 2.57 (t, *J* = 6.5, 2H), 2.35 (q, *J* = 7.6 Hz, 2H), 1.90 (p, *J* = 6.5 Hz, 2H), 1.15 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.31, 143.64, 141.80, 134.08, 120.66, 115.44, 115.30, 63.86, 31.34, 30.19, 27.68, 9.14.

*3-(3,4-Dihydroxyphenyl)propyl-hexa-2,4-dienoate (**3b**):* pale yellow solid; yield: 30%; Rf = 0.48 (cyclohexane/EtOAc 6:4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.17 (m, 1H), 6.77 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 2.0 Hz, 1H), 6.59 (dd, *J* = 8.1, 2.0 Hz, 1H), 6.27 – 6.05 (m, 2H), 5.92 (s, OH), 5.78 (d, *J* = 15.4 Hz, 1H), 5.77 (s, OH), 4.14 (t, *J* = 7.0 Hz, 2H), 2.58 (t, *J* = 7.0 Hz, 2H), 1.93 (p, *J* = 7.0 Hz, 2H), 1.86 (d, *J* = 5.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$**)** δ 167.90, 145.46, 143.61, 141.81, 139.82, 134.12, 129.71, 120.69, 118.65, 115.47, 115.29, 63.74, 31.39, 30.28, 18.67.

*3-(3,4-Dihydroxyphenyl)propyl hexanoate (**3c**)* (Bernini, et al, 2012)*:* white solid; yield: 68%; Rf = 0.64 (cyclohexane/EtOAc 1:1); m.p.: 62.4 – 64.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.77 (d, *J* = 8.1 Hz, 1H), 6.70 (d, *J* = 1.4 Hz, 1H), 6.60 (dd, *J* = 8.1, 1.4 Hz, 1H), 5.44 (s, OH), 5.31 (s, OH), 4.07 (t, *J* = 6.6 Hz, 1H), 2.57 (t, *J* = 7.6 Hz, 3H), 2.31 (t, *J* = 7.6 Hz, 2H), 1.90 (p, *J* = 6.6 Hz, 2H), 1.63 (p, *J* = 7.6 Hz, 2H), 1.46 – 1.21 (m, 4H), 0.90 (t, *J* = 6.5 Hz, 3H). ^{13}C NMR (75 MHz**,** $CDCl_3$**)** δ 174.82, 143.67, 141.82, 134.03, 120.64, 115.43, 115.30, 63.86, 34.41, 31.36, 31.29, 30.21, 24.68, 22.28, 13.89.

*3-(3,4-Dihydroxyphenyl)propyl dodecanoate (**3d**)* (Bernini, et al, 2012)*:* white solid; yield: 59%; Rf = 0.69 (cyclohexane/EtOAc 1:1); m.p.: 65 – 66.9 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.77 (d, *J* = 8.0 Hz, 1H), 6.70 (d, *J* = 2.1 Hz, 1H), 6.60 (dd, *J* = 8.0, 2.1 Hz, 1H), 5.27 (s, OH), 5.12 (s, OH), 4.07 (t, *J* = 6.6 Hz, 2H), 2.57 (t, *J* = 7.5 Hz, 2H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.90 (p, *J* = 6.6 Hz, 2H), 1.62 (p, *J* = 7.5 Hz, 2H), 1.37 – 1.21 (m, 16H), 0.88 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.69, 143.64, 141.78, 134.09, 120.66, 115.43, 115.29, 63.78, 34.45, 31.90, 31.37, 30.23, 29.59, 29.46, 29.32, 29.25, 29.16, 25.01, 22.67, 14.10.

2.4 DPPH radical-scavenging assay

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical-scavenging activity was performed using a commercial kit (Bioquochem, Asturie, Spain) following manufacturer's instructions. Briefly, samples were appropriately diluted in dimethyl sulfoxide (DMSO) and mixed with the DPPH solution provided by the kit. Trolox at different concentration was used to build the standard curve. Antioxidant Activity was determined by measuring absorbance at 517 nm by spectrophotometer (Eppendorf, Milan, Italy) and calculating the corresponding percentage of inhibition as reported in the kit instructions. All the assays were performed in triplicate and standard deviations varied between 1 and 5%.

2.5 Bacterial Strains and Culture Conditions.

The evaluation was performed using *Escherichia coli* ATCC 25922 (*Ec*), *Salmonella enterica enterica* subsp. *enterica* ser. Enteritidis ISM 8324 (*Se*), *Pseudomonas aeruginosa* IMV 1 (*Pa*) and *Staphylococcus aureus* ATCC 6538 (*Sa*). Bacteria were plated by streaking on blood agar plates (Tryptic Soy Agar + 5% sheep blood [Microbiol, Italy]) and incubated at 37 °C for 24 h under aerobic atmosphere.

2.6 Determination of the Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) was determined using the microdilution assay, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute (CLSI) (2018) Performance Standards for Antimicrobial Susceptibility Testing. CLSI Approved Standard M100-S15. Clinical and Laboratory Standards Institute, Wayne). Briefly, all the strains were grown on Tryptic Soy Broth (TSB, Oxoid, Milan, Italy) and 3 or 4 isolated colonies were suspended in fresh sterile saline solution (9 gr/L NaCl) to reach an initial concentration of 1.5 x 10^8 CFU/mL (equivalent to 0.5 MacFarland standard). One hundred microliters of the 1:100 diluted cell suspensions were dispensed into each well of a 96-well microtiter plate. The strains were exposed to 2-fold dilution series of each derivative (dissolved in DMSO).

After incubation for 24 h at 37 °C under aerobic condition, the MICs were determined as the lowest dilution of molecules able to inhibit visible bacterial growth. All the tests were performed in triplicate.

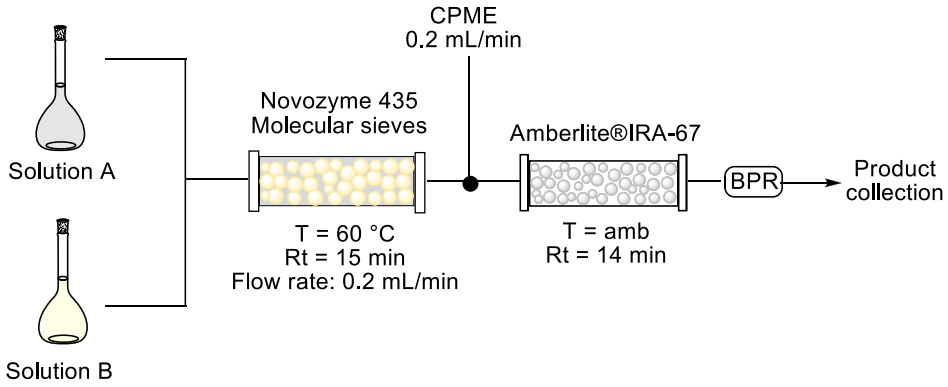
2.7 Calculation of Selected Properties of Tested Compounds

Selected properties of the compounds (i.e., molecular weights, cLogP, cLogS) were calculated with OSIRIS DataWarrior. Calculated properties are summarized in Table 3.

3. Results and discussion

Since the quest for green solvents derived from renewable sources in biocatalytic reactions is currently matter of intense research (Clarke et al., 2018; Alcántara, &. Domínguez de María, 2018; Mourelle-Insua, Lavandera, & Gotor-Fernández, 2019; Pätzold, 2019), cyclopentyl methyl ether (CPME) was chosen as the solvent for the biotransformation (de Gonzalo, Alcántara, & Domínguez de María, 2019; Azzena, 2019). (CPME) is currently synthesized by petrochemical-based routes with high atom economy, but some bio-based alternatives are becoming available, paving the way for a future biogenic source of the solvent, potentially minimizing waste production and energy consumption. The reaction using tyrosol (Ty) and lauric acid as the substrates was studied to optimize the reaction parameters, including the molar ratio of the substrates (1:1, 1:2, 1:3), concentration (range: 0.02-0.1 M), temperature (range: 40-80 °C), residence time (range: 7-30 min), and stability under continuous work. The conversion was monitored by HPLC. A packed bed reactor (PBR) was prepared mixing the immobilized *Candida antarctica* lipase B (CalB) with molecular sieves (4 Å, 1:1 w/w). After a first set of experiments, a 1:2 ratio between Ty (0.1 M in CPME) and lauric acid (0.2 M in CPME) was selected. Then, the effect of temperature was investigated. Different temperatures between 40 °C and 80 °C were tested. At 80 °C, the conversion remained constant as at 60 °C, reaching 84% conversion in 15 min of residence time. Thus, the optimal temperature of 60 °C was identified. By increasing the equivalents of lauric acid to 3 eq., full conversion was achieved (residence time: 15 min, T = 60 °C, SP_f = 29 μmol/min*g) (Bolivar & López-Gallego, 2020; Zambelli, et al., 2016), thus, these were identified as the best conditions. A time on stream study was performed to evaluate the stability of the catalyst in an unconventional medium over a prolonged time. In particular, the production of 4-hydroxyphenethyl dodecanoate **1d** was monitored under continuous work carrying out the biotransformation for 24 h under optimized conditions (residence time: 15 min, T = 60 °C, back pressure regulator: 75 psi, 3 eq. of lauric acid, flow bioreactor productivity = 4.1 $g_{pruduct}$/day) and monitoring the exiting flow stream by HPLC. The bioreactor showed excellent stability, obtaining constant conversion (> 99%) during the monitoring. Then, the catalytic bed was rinsed with CPME at 0.5 mL/min for 15 minutes. The bioreactor was then used for the second cycle of reaction under the same conditions. Similarly, ten set of cycles were repeated. The results show again almost full conversion (98%) at each cycle, proving the

reusability of the enzyme. Moreover, with the aim of developing a fully automated procedure for the synthesis of the desired esters, an in-line purification procedure was developed by adding a column packed with Amberlite®IRA-67 to scavenge the excess of the carboxylic acid (Scheme 1), that can be released and recovered by flowing through the scavenger column a 2% v/v solution of acetic acid in CPME. Exploiting the final set up, all the substrates were submitted to the esterification reaction. However, the reactions using sorbic acid appeared more challenging, giving very low conversions (5-10%), probably due to the presence of double bonds conjugated to the carboxylic group that negatively affects the lipase activity. Therefore, in this case, the residence time was increased to 75 min to isolate the desired esters **1b**, **2b** and **3b** in acceptable yields (24-30%). A further increase of the residence time (i.e., 90 min and 120 min), of the temperature (i.e., 70 °C and 80 °C) or of the equivalents of sorbic acid was not beneficial, leading to similar conversions and productivities.



Scheme 1. Schematic representation of the flow reactor configuration. Solution A: 0.1 M solution of the phenolic alcohol in CPME. Solution B: 0.3 M solution of the fatty acid in CPME. Flow stream: CPME. Backpressure regulator (BPR): 75 psi.

Since phenolic compounds can act as free radical scavengers due to their ability to donate a hydrogen radical forming aryloxy radicals, their efficiency as radical scavengers was evaluated performing a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Kedare & Singh, 2011). The results are summarized in Table 1.

Table 1. Determination of the free radical scavenging capacity. Ty: tyrosol; VA: vanillic alcohol; homo-HTy: homo-hydroxytyrosol.

Cmp	% Inhibition
Ty (0.1 mM)	45±1

1a (0.1 mM)	32±2
1b (0.1 mM)	38±0.5
1c (0.1 mM)	22±2
1d (0.1 mM)	35±4
VA (0.1 mM)	51±3
2a (0.1 mM)	26±2
2b (0.1 mM)	40±2
2c (0.1 mM)	3±1
2d (0.1 mM)	33±2
Homo-HTy (0.01 mM)	35±1
3a (0.01 mM)	50±2
3b (0.01 mM)	62±4
3c (0.01 mM)	40±1
3d (0.01 mM)	52±2

The structures of the aromatic alcohol as well as of the carboxylic acid used for the ester formation significantly affect the radical scavenger properties of the obtained compounds. As expected, two hydroxyl group in the aromatic ring, *i.e.,* **homo-HTy**, leads to more efficient radical scavengers. The presence of a methoxy group in the meta- position relative to the carbon chain in the aromatic ring, *i.e.,* vanillyl alcohol and its derivatives, does not significantly affect the radical scavenger properties in comparison to **Ty** and its esters. In the case of tyrosyl (**1a-d**) and vanillyl esters (**2a-d**), the newly synthetized lipophilic esters are less reactive than the corresponding alcohol. On the other hand, the series of homo-tyrosyl esters (**3a-d**) are more effective than the starting alcohol. Notably, the **homo-HTy** series has been used in the assay in a 10-fold lower concentration than the other derivatives demonstrating its higher antioxidant power with a significant correlation with molecular weight and number of non-hydrogen atoms (Table 3).

Finally, the obtained compounds have been submitted to antibacterial screening against a panel of Gram-negative and Gram-positive bacteria (Table 2). The mechanism of antimicrobial action of phenolic compounds and their esters is not well understood yet; they could affect membrane permeability or inhibit key enzymes that are involved in the growth and multiplication of microorganism cells (Rempe, Burris, Lenaghan, & Stewart, 2017).

Table 2. Minimum inhibitory concentrations (MIC) (μg/mL) of tested compounds *Ec* = *E. coli*, *Se* = *S. enterica* subsp. *enterica* ser. Enteritidis, *Pa* = *P. aeruginosa*, *Sa* = *S. aureus*. All the tests were performed in triplicate.

Cmp	*Ec* MIC (μg/mL)	*Se* MIC (μg/mL)	*Pa* MIC (μg/mL)	*Sa* MIC (μg/mL)
Ty	256	256	128	256
1a	128	128	128	128
1b	128	128	128	128
1c	256	128	128	64
1d	128	128	128	128
VA	256	256	256	256
2a	128	128	64	256
2b	128	256	128	256
2c	128	128	64	128
2d	128	128	128	256
homo-HTy	128	128	64	64
3a	128	128	256	128
3b	256	256	128	128
3c	128	128	128	32
3d	128	128	128	128
Propionic acid	128	128	128	256
Sorbic acid	128	128	128	256
Caproic acid	128	256	128	256
Lauric acid	256	256	128	128

In general, all the esters show a similar o slightly better antimicrobial activity against the selected bacteria in comparison with the parent alcohol and fatty acid. Interestingly, compound **3c** showed a MIC of 32 μM against the Gram-positive *Staphylococcus aureus*, 3-fold lower than against the Gram-negative bacteria selected for this study, being also more active than **homo-HTy** (MIC = 64 μM) and sorbic acid (MIC = 256 μM). Compound **1c** showed a MIC of 64 μM against *Staphylococcus aureus*, 3-fold lower than the corresponding starting alcohol **Ty** (MIC = 256 μM) and sorbic acid (MIC = 256 μM). Vanillyl ester **2a** and **2c** resulted more active on the Gram-negative *Pseudomonas aeruginosa* (MIC = 64 μM), being again 2- or 3-fold more active than the starting acids and **VA**. On the contrary, *E. coli and S. S. enterica* subsp. *enterica* ser. Enteritidis showed high MIC values *versus* all the tested molecules.

Both cLogP and cLogS values (calculated using OSIRIS DataWarrior, see Matherials and Methods) reported in Table 3 indicate that the obtained esters should dissolve better in the lipid phase than in the aqueous phase, making possible an application in lipid-rich food matrices.

Table 3. Calculated properties of tested compounds.

Cmp	MW	cLogP	cLogS	Non-H Atoms	Electronegative Atoms
Ty	138.17	1.147	-1.316	10	2
VA	154.16	0.647	-1.222	11	3
homo-HTy	168.19	1.255	-1.290	12	3
1a	194.23	2.086	-1.996	14	3
2a	210.23	1.586	-1.902	15	4
3a	224.26	2.194	-1.970	16	4
1b	232.28	2.945	-2.350	17	3
1c	236.31	3.449	-2.806	17	3
2b	248.28	2.444	-2.256	18	4
2c	252.31	2.949	-2.712	18	4
3b	262.30	3.053	-2.324	19	4
3c	266.34	3.558	-2.780	19	4
1d	320.47	6.175	-4.426	23	3
2d	336.47	5.675	-4.332	24	4
3d	350.50	6.284	-4.400	25	4

4. Conclusions

The functional properties of phenolic compounds are not only influenced by their intrinsic structural features (e.g., number, position, nature of hydrogen- or electron-donating groups), but also by their physico-chemical properties (e.g., lipophilicity, steric hindrance, polarizability…). Hence, the same molecule can be efficient in one system (e.g. emulsion) but not in another one (e.g. bulk oil). Lipophilization of phenolic derivatives with fatty acids represents an efficient strategy to obtain amphiphilic compounds that can be used as multifunctional additives in the food, cosmetic, and pharmaceutical industries, as they should conserve their other functional properties (UV A and UV B filters, antimicrobial, antiviral, bacteriostatic, etc.). The lipophilization should preserve the phenolic groups that are responsible for the antioxidant activity. In this context, a biocatalyzed continuous flow approach for the synthesis of a series of phenolic derivatives selectively coupling the primary alcohol of a phenolic derivative with selected fatty acids was developed. The biotransformations were carried out in CPME as the solvent and the biocatalyst (Novozyme 435®) showed good stability in this unconventional medium. The compounds were isolated in moderate to good yields and were tested as antimicrobials and radical scavengers. Esterification increases the lipophilicity of the starting

phenolic compounds without losing their ability to neutralize free radicals and their antimicrobial properties, increasing their utility as preservative agents in lipid-rich media and their possible use in the food industry.

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