



# Enzymatic alkylsuccinylation of tyrosol: Synthesis, characterization and property evaluation as a dual-functional antioxidant



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## ABSTRACT

This work reports a novel approach to generate a new group of tyrosol-based amphiphilic lipid alkylsuccinylated tyrosol by lipase-catalyzed succinylation of tyrosol with alkylsuccinic anhydrides of varying alkyl chain lengths, in high yields (80–95%). The structures of the compounds were confirmed by MS, FTIR & <sup>1</sup>H NMR; and their properties were characterized by Temperature-Ramp FTIR, DSC & CMC measurements. The synthesized compounds integrate water-soluble phenylethanoid and hydrophobic alkyl into one molecule thus are endowed with dual functions: retaining the antioxidant property of tyrosol and entailing tyrosol with new surface-active property. The DPPH activity of tyrosol (13.77%) was significantly enhanced by 2-dodecen-1-ylsuccinylated tyrosol (16.01%). Compared to tyrosol-based emulsions (76.63%), the lipid oxidation is reduced to 21.57% and 42.32% in 2-octen-1-ylsuccinylated/2-dodecen-1-ylsuccinylated tyrosol emulsions, respectively. This work brings new members to the library of functional lipid excipients and open a novel and effective synthetic pathway for derivation of phenyl alcohols.

## 1. Introduction

Food products containing natural ingredients are perceived by consumers as ‘green and healthy’, and therefore considerable research is devoted to enriching food formulations with natural bioactive components. Of particular interest is the use of natural bioactive extracts to prevent the oxidation and degradation of fish oil based foods (Mrabet et al., 2017). This is because of the well proven beneficial health effects of the oxidatively labile omega-3 (n-3) polyunsaturated fatty acids (PUFAs) (Ruxton, Reed, Simpson, & Millington, 2004; Shahidi & Miraliakbari, 2004) in fish oil. Therefore the search for natural and novel ingredients to stabilize omega-3 oil-in-water delivery emulsions has progressed steadily over the last years.

Biophenols (e.g. hydroxytyrosol and tyrosol) are important antioxidants from olive oil and are responsible for the antioxidant activity of olive plant components and extracts (Bernini, Gilardini Montani, Merendino, Romani, & Velotti, 2015; Bernini, Merendino, Romani, & Velotti, 2013; Ricciutelli et al., 2017; Shahidi & Zhong, 2010). Recently, tyrosol has attracted the attention of organic chemists and pharmacologists as a versatile and cheap substrate for the synthesis of a variety of esters exhibiting diverse and improved biological effects (Barontini, Bernini, Carastro, Gentili, & Romani, 2014). This is partly due to tyrosol being the most abundant biophenol in extra virgin olive oil and its

ability to exert protective effects against oxidative injuries in cell systems (Giovannini et al., 1999) and improve intracellular antioxidant defense systems (Di Benedetto et al., 2007). Furthermore, the peculiar ability of tyrosol esters to partition at the interfacial regions of oil-and-water structures (Laszlo et al., 2013) implies that they could find applications in stabilizing omega-3 oil-in-water emulsions.

In this work we report the synthesis and characterization of an array of novel dual functional amphiphilic lipids based on tyrosol with both surface active and antioxidant properties. It is hypothesized that the free carboxylic acid group in the molecules will promote interactions with biopolymers such as proteins in the aqueous phase of oil-in-water emulsions similar to the interactions reported for Diacetyltartaric Esters of Mono- and Di-glycerides (DATEM) with gluten proteins in dough (Köhler, 2001). Therefore, this new group of compounds via interactions with biopolymers will have improved ability to stabilize oil droplets and hence can support high loads of omega-3 oil in delivery emulsions. Because anhydrides are excellent electrophiles, which readily react with nucleophiles (e.g. alcohols), anhydrides were preferred for alkyl succinylation of tyrosol. In addition, previous work in our laboratory showed that the ring opening mechanisms of anhydrides with various alcohols yield compounds with multi-functional properties including surface activity (Anankanbil, Pérez, Yang, Banerjee, & Guo, 2017; Falkeborg & Guo, 2015).

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To find optimal structure of phenol alkanyl succinyl derivatives, an array of products with alteration of alkanyl chain lengths were synthesized. The new amphiphilic lipids were characterized by Differential Scanning Calorimetry (DSC) and FT-IR. Moreover, fish oil-in-water emulsions stabilized by the synthesized compounds were characterized by dynamic light scattering studies and zeta potential determination. The antioxidant efficacy of the compounds were assessed by means of thiobarbituric acid reactive substances (TBARS), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging activity assays.

## 2. Materials and methods

### 2.1. Materials

Lipozyme RM IM, Lipozyme 435 and Novozym 435 were obtained from Novozymes A/S (Bagsvaerd, Denmark). 2-(4-Hydroxyphenyl) ethanol (tyrosol) (98%), succinic anhydride ( $\geq 99\%$ ), butylsuccinic anhydride, 2-octen-1-ylsuccinic anhydride, mixture of *cis* and *trans* ( $\geq 97\%$ ), 2-dodecen-1-yl succinic anhydride ( $\geq 95\%$ ), other chemicals and all solvents used (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, USA). All water used was de-ionized water obtained from a Milli-Q (Millipore, MA) system.

### 2.2. General procedure for lipase-catalyzed synthesis

The reaction mixture consisting of 1 mmol of tyrosol, 6 mmol of the respective anhydride and 18 mg molecular sieves (3 Å, activated at 180 °C for 8 h) in 10 mL hexane:2-MTHF (7:3) was incubated at selected temperatures in a 50 mL jacketed glass reactor with temperature controlled by a circulating water bath. After heating for 30 min to ensure the complete solubility of the reagents, the reaction was initiated by the addition of 15% Novozyme 435 (based on weight of 2-(4-hydroxyphenyl) ethanol). Agitation was continued for 20 h at 350 rpm. Control reactions with immobilized but thermally inactivated enzymes were carried to rule out the possibility that succinylation of tyrosol was not being catalyzed by the enzyme support.

Thin layer chromatography (TLC) analysis was used to monitor the reaction progress. Briefly, aliquots from reaction mixtures were taken at set time intervals, diluted with 300  $\mu$ L CHCl<sub>3</sub>:methanol 3:1 (v/v) and analyzed on TLC plates (TLC silica gel 60, 5 cm  $\times$  10 cm, Merck, Germany). The plates were developed with diethyl ether:petroleum ether:acetic acid 85:15:1 (v/v/v) until the solvent front moved 8 cm, dried and checked under UV lamp at 254 nm. At the end of the reaction, the lipase and molecular sieves were removed by filtration and the solvent evaporated under vacuum. The conversion of 2-(4-hydroxyphenyl)ethanol was estimated with HPLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a UV-visible detector (Thermo Fisher Scientific, Waltham, MA, USA) at an emission wavelength of 280 nm. A Supelco (250 mm  $\times$  4.6 mm i.d., 5  $\mu$ m particle size) column at a temperature of 35 °C was used for HPLC quantification of reaction products. Two solvents mixtures were used with solvent A being methanol containing 0.1% of H<sub>3</sub>PO<sub>4</sub>. Solvent B was water:methanol (90:10) with 1.2% of H<sub>3</sub>PO<sub>4</sub>. Flow rate and injection volume were 1 mL/min and 10  $\mu$ L respectively. Compound peaks were identified using retention times of tyrosol standards and its acid esters, purified in this work. Area percentage was used as weight for the calculation of the yields of the products. The determinations were performed in duplicates. Optimization of the reaction conditions was done using 2-dodecen-1-yl succinic anhydride as a representation. The parameters for the optimization of were substrate molar ratios, type of solvents and enzymes, reaction temperature, and enzyme loading. Six solvents (hexane, *t*-BuOH, toluene, 2-methyltetrahydrofuran, MTBE and a mixture of hexane:2-MTHF 7:3), three types of enzymes (Lipozyme RM IM, Lipozyme 435 and Novozym 435), four substrate molar ratios (alcohol:anhydride, 1:1, 1:2, 1:4 and 1:6), five temperature levels (40 °C, 50 °C, 60 °C, 65 °C and 70 °C) and four levels of enzyme loading (2%, 5%, 10%

and 15%) were considered.

### 2.3. Purification of synthetic compounds

Synthesized compounds were purified on a glass column packed with silica gel using diethyl ether:petroleum:ether:acetic acid 85:15:1 (v/v/v) as elution solvent. Identification of the compounds was carried out by <sup>1</sup>H NMR spectroscopic analysis (Bruker Avance III spectrometer) at 400 MHz. MS spectra were obtained on a Bruker Maxis Impact electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-QTOF-MS) (Bruker Daltonics GmbH, Bremen, Germany). FT-IR Spectra were recorded using an ATR-FTIR (PIKE, Madison, WI; Bruker, Ettlingen, Germany).

**Synthesis of 4-(3-(4-hydroxyphenyl)propoxy)-4-oxobutanoic acid (1a).** 1 mmol of tyrosol and 6 mmol of succinic anhydride were catalyzed by Novozym 435 (15% of total weight of equivalent amount of tyrosol) in 10 mL hexane:2-MTHF 7:3 for 20 h.

White liquid; isolated yield after purification: 40.61%, *R*<sub>f</sub>: 0.48; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, 25 °C, TMS):  $\delta$  = 2.15–2.50 (m), 3.5–4.25 (m), 6.30–6.40 (m), 6.58–6.67 (m). MS, *m/z* calcd for C<sub>13</sub>H<sub>16</sub>O<sub>5</sub>: 238.08; found: 261.08 (M + Na<sup>+</sup>).

### 2.4. Differential scanning calorimetry

The thermal properties of compound **4a** were analyzed using differential scanning calorimetry on a Pyris 6 DSC system (Perkin-Elmer Cetus, Norwalk, USA). Approximately 8 mg of **4a** was put into an aluminum pan and placed in the DSC under a constant flow of nitrogen (20 mL/min). An empty pan was used as a reference. The heating and cooling profile was: (1) initial temperature 20 °C; (2) ramp 10 °C/min to 90 °C; (3) isothermal for 5 min; (4) ramp –10 °C/min to –60 °C; (5) isothermal for 5 min; (5) ramp 10 °C/min to 90 °C. The DSC scans were evaluated using MicroCal Origin 8.6 Software.

### 2.5. Temperature-ramp fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) was used to determine the packing behavior of the compounds. FT-IR Spectra were recorded using an ATR-FTIR (PIKE, Madison, WI; Bruker, Ettlingen, Germany). The synthesized compounds were dried under vacuum over night, and then pressed onto a ZnSe ATR crystal mounted in a trough plate. The ATR crystal was coupled with an Auto Pro Temperature Controller (Pike Technologies, Madison, WI) for gradual heating of the crystal from 25 °C to 60 °C. Spectra were collected with a spectral resolution of 4 cm<sup>–1</sup> with 8 scans over the range of 3500–650 cm<sup>–1</sup>. The FTIR spectra were analyzed by using MicroCal Origin 8.6 software.

### 2.6. Critical micelle concentration

The critical micelle concentrations (CMCs) of the synthesized compounds were determined by pyrene fluorescence using a fluorescence spectrometer (Varian Cary Eclipse, Agilent Technology, California, USA). Sample solutions of different concentrations (1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 mM) were prepared using water previously saturated with pyrene (final concentration of 1  $\mu$ M). Emission spectra were obtained by exciting the samples at 343 nm. The fluorescence intensity ratio of *I*<sub>1</sub>/*I*<sub>3</sub> (*I*<sub>1</sub> = 373 nm, *I*<sub>3</sub> = 383 nm) was plotted against sample concentration. The CMC was determined as the sample concentration at which the *I*<sub>1</sub>/*I*<sub>3</sub> ratio remained constant after the initial abrupt decrease. Analysis was conducted in triplicate.

### 2.7. Free radical scavenging activity

The free radical scavenging activity of different products (**1a**, **2a**, **3a**, **4a**) was determined based on DPPH method (Chen, Bertin, & Foldi, 2013) with slight modifications. Briefly, 1 mL of 0.01 mM DPPH in

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