Bioorganic & Medicinal Chemistry 21 (2013) 684–692

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Biocatalytic synthesis, structural elucidation, antioxidant capacity and tyrosinase inhibition activity of long chain fatty acid acylated derivatives of phloridzin and isoquercitrin

Ziaullah, Khushwant S. Bhullar, Sumudu N. Warnakulasuriya, H. P. Vasantha Rupasinghe *

*Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia, Canada B2N 5E3*

ARTICLE INFO

*Article history:*
Received 25 September 2012
Revised 19 November 2012
Accepted 26 November 2012
Available online 5 December 2012

*Keywords:*
Flavonoid
Phloridzin
Isoquercitrin
Acylation
*Candida antarctica* lipase B
$^{1}$H NMR
$^{13}$C NMR spectroscopy
Antioxidant capacity
Tyrosinase

ABSTRACT

Our present investigation describes the regioselective enzymatic acylation of two series of acylated derivatives of phloridzin and isoquercitrin with six different long chain saturated, mono- and poly-unsaturated fatty acids. The biocatalytic synthesis was optimized to achieve 81–98% yields, using immobilized lipase B, from *Candida antarctica* (Novozym 435®), in acetone at 45 °C. The synthesized esters have been analyzed by $^{1}$H NMR, $^{13}$C NMR spectroscopy and evaluated for their antioxidant capacity and tyrosinase inhibition, using in vitro assays. Among all the phloridzin and isoquercitrin derivatives, the greatest potential for inhibition of tyrosinase activity ($p \leqslant 0.05$) was exhibited by the α-linolenic acid ester of isoquercitrin.



## 1. Introduction

Flavonoids have been receiving a great deal of attention recently, due to their valuable biological, pharmacological and medicinal properties. These polyhydroxylated phenolic compounds have antioxidant properties that include free radical termination and metal chelation.[1] Flavonoids are also inhibitors of many enzymes, such as cyclooxygenase and lipoxygenase, which can lead to the formation of reactive oxygen species (ROS).[2] In addition, flavonoids have been discovered to have a variety of biological activities, which include anti-inflammatory, anti-viral, antibiotic, anti-spasmolytic, anti-allergenic, anti-diarrheic, anti-tumor, anti-ulcer, oestrogenic and vasodilatory properties.[2–7] The multiple functionalities make the polyphenols distinguished chelators, through their hydroxyl groups, to the metallic part of the enzymes and thus, act as competitive inhibitors of many enzymes.

So far, a number of studies have been carried out to identify natural inhibitors of tyrosinase, as well as their synthetic analogs. Flavonoids, which are one of the most investigated groups of plant secondary metabolites, show tyrosinase inhibition. For example, quercetin, kaempferol and morin act as competitive inhibitors of tyrosinase and rhamnetin acts as a cofactor or substrate of tyrosinase.[8] This copper containing enzyme is involved in enzymatic browning of plant tissues, formation of cuticle in insects and most importantly, melanogenesis in humans.[9] Tyrosinase triggers melanogenesis by hydroxylation of L-tyrosinase to L-3,4-dihydroxyphenylalanine (L-DOPA) and its oxidation to dopaquinone.[10] Tyrosinase is involved in the treatment of dermatological disorders such as hyperpigmentation, melanoma, along with other skin disorders.[11] Numerous chemicals of natural origin, such as alkaloids,[11] phenolics,[12–14] chalcones,[15] flavonoids,[16] and tetraketones[17] are promising tyrosinase inhibitors. In this study we investigated the tyrosinase inhibition of novel quercetin and phloridzin esters using L-3,4-dihydroxyphenylalanine (L-DOPA) as the binding enzyme substrate.

These recognized health benefits have sparked the potential for many industrial applications in food ingredients, cosmetics and pharmaceuticals. However, these applications are significantly limited, due to the poor solubility of flavonoids in both hydrophobic and hydrophilic media.[18]

Previous studies have shown that enzyme-catalyzed esterification and transesterification of flavonoid glycosides have been successful in increasing lipophilicity. With ideal reaction conditions, relatively high conversion yields of the flavonoid esters have been obtained.[18–24] Herein, using long chain fatty acids as acyl donors would have a wide range of health benefits. As described in earlier

* Corresponding author. Tel.: +1 902 893 6623; fax: +1 902 893 1404.
*E-mail address:* vrupasinghe@dal.ca (H.P.V. Rupasinghe).

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http://dx.doi.org/10.1016/j.bmc.2012.11.034

reports, they provide an important fuel source to humans, specifically to the heart and skeletal muscles.[18] Besides, polyunsaturated fatty acids are also responsible for the regulation of important biological functions, as they are the precursors of many metabolites.[18] The ω-3 and ω-6 polyunsaturated acids, which are essential fatty acids, have unique beneficial biological activities. Recently, antitumour activities of ω-3 and ω-6 polyunsaturated acids have been discovered.[25,26] The use of these fatty acids, in natural product forms, is limited as they are prone to autooxidation, due to free radicals. These free radicals can have deleterious effects on the human body, such as the formation of DNA lesions, loss of enzymatic activities and potential for cell death.[27] It would be advantageous to combine the fatty acids with certain antioxidants that would prevent the harmful effects of oxidation and preserve the health benefits of the fatty acid. Recently, Salem et al.[19] have found that isoquercitrin esters increased antioxidant properties, including a higher inhibition of xanthine oxidase, an enzyme that catalyzes the production of superoxide radicals.

Chemical, enzymatic and chemo-enzymatic acylation of phenolic compounds, with various acyl donors (C2–C18 chain length), has been already reported.[19,21,28–34] However, the enzymatic approach is preferred over the chemical acylation,[24] due to its regioselectivity and simple one-step reaction. Regioselective reactions ensure that some hydroxyl groups remain on the flavonoid, preserving its antioxidant properties.[19,21,28–42]

The aim of this study was to perform lipase-catalyzed acylation of flavonoid glycosides, using very long chain unsaturated and saturated fatty acids (C18–C22), with Lipase B (Novozym 435®) from *Candida antarctica* as the catalytic enzyme. The focus of interest was to investigate the amphipathic/amphiphilic properties, by changing the types of acyl donor fatty acids. Specifically, modifications in the chain lengths and degrees of unsaturation, to synthesize a variety of flavonoid esters, with the long-term aim of improving the bioavailability and biological activity. This current study provides, for the first time, the detailed NMR structural assignments, along with tyrosinase enzyme inhibition of the synthesized esters of phloridzin and isoquercitrin.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Phloridzin, isoquercitrin (quercetin-3-*O*-glucoside), oleic, stearic, linolenic, linoleic, eicosapentanenoic, docosahexanoic acids and Novozyme 435®, an immobilized lipase B from *Candida antarctica* (with an activity of 10,000 propyl laurate units), 3 Å molecular sieves, *p*-anisaldehyde, mushroom tyrosinase, phosphate buffer and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma–Aldrich (Mississauga, ON, Canada) while sodium acetate trihydrate, ferric chloride, 2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ), Trolox, fluorescein, phosphate buffer, 2,2-diphenyl-1-picrylhydrazyl (DPPH), spectrophotometric grade dimethyl sulfoxide, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma–Aldrich (St. Louise, MO, USA). Phosphorus (V) oxide ($P_2O_5$) was purchased from *A*lfa *A*esar (Ward Hill, MA, USA). Acetone (extra dry), Acetic acid, hydrochloric acid, HPLC grade chloroform and HPLC grade methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). Ethanol anhydrous was obtained from Commercial Alcohols (Montreal, QC, Canada). Round bottom 96-well microplates were obtained from Corning Incorporated (Edison, NY, USA).

### 2.2. TLC analysis

The progress of reactions was monitored by thin-layer chromatography (TLC) on silica gel 60 $F_{254}$ plates from *Merck* (Darmstadt, Germany), using a solvent mixture system of acetone/toluene (60:40, few drops of acetic acid). The products were detected under UV light, as well as by using anisaldehyde (4-methoxybenzaldehyde) as spray reagent, followed by heating at 110 °C. The acylated products were purified by column chromatography, using silica gel 60 (0.040–0.063 mm; 230–400 mesh, *Merck*, Darmstadt, Germany) and eluted with acetone toluene. The purity of the samples was tested, using silver-ion thin-layer chromatography (Ag-TLC) as well as NMR spectroscopy. TLC-plates were impregnated with $AgNO_3$ by immersion in a 10% (w/v) solution of $AgNO_3$ in water/methanol (95:5), under dark. After the plates were dried, compounds were loaded onto them and developed with acetone–toluene (6:4, v/v).The plates were then sprayed with 10% $H_2SO_4$ in methanol and heated at 110 °C. The purified compounds appeared as dark black spots on the TLC plates.[43]

### 2.3. NMR and IR spectroscopic analysis

The chemical structures of the acylated products were determined by $^1$H NMR and $^{13}$C NMR spectroscopic analysis in solvent DMSO-$d_6$ or MeOD, on Bruker AVANCE 300 MHz spectrometer (Bruker Corp., Billerica, MA, USA). Chemical shifts were expressed in $\delta$ ppm, relative to an internal standard of tetramethylsilane (TMS). The IR data was collected on Nicolet 6700 FT-IR spectrometer, from Thermo Scientific (Ottawa, ON, Canada).

### 2.4. The ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to the method described by Rupasinghe et al.[44] The working assay reagent (FRAP solution) was prepared daily by mixing 300 mmol $l^{-1}$ acetate buffer of pH 3.6, 10 mmol $l^{-1}$ TPTZ solution and 20 mmol $l^{-1}$ ferric chloride in the ratio of 10:1:1. As the standard material, Trolox solution was prepared by dissolving 0.025 g of Trolox in 100 ml of 95% ethanol. The appropriate dilutions of Trolox solution was carried out using 95% ethanol to obtain 50, 100, 200, 300, 400, 500, 1000 μmol $l^{-1}$ concentrations and calibration curve was developed. All the sample compounds were dissolved in 95% ethanol to prepare desired concentrations. To perform the assay, 20 μl of blank, standard or sample was reacted with 180 μl of FRAP solution in 96-well polystyrene plates after warming up to 37 °C. The absorbance was measured at 595 nm after 10 min reaction time, including 3 s shaking time using the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). Antioxidant capacity was calculated as μmol $l^{-1}$ Trolox equivalents of 1 mmol $l^{-1}$ of solution.

### 2.5. The oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out using the method explained by Rupasinghe et al.[44] with slight modifications. The Trolox solution and AAPH [2,2′-azobis(2-amidino-propane) dihydrochloride] were prepared daily immediate before the assay. The Trolox standard solution was made using phosphate buffer and suitable dilutions were carried out to develop the calibration curve. The buffer, standard, or sample (35 μl) and 0.957 μmol $l^{-1}$ fluorescein (130 μl) solutions were placed in the 96-well plates and pre-incubated at 37 °C for 10 min. The fluorescence was measured using FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA) after injection of 35 μl pre-warmed (37 °C) AAPH to each well in every 60 s up to 3201 s. Antioxidant capacity was calculated as μmol $l^{-1}$ Trolox equivalents of 0.01 or 0.001 mmol $l^{-1}$ of solution.

### 2.6. DPPH radical scavenging assay

The DPPH radical scavenging assay was carried out as described by Brand-Williams et al.[45] with modifications. In solution, DPPH

has a stable free radical and is a deep purple colour, with a characteristic absorbance around 520 nm, but with the addition of an antioxidant, the free radical is scavenged and DPPH becomes 2,2-diphenyl-1-picryl hydrazine, a colourless compound. Thus, the change in absorbance of a solution with DPPH with an added compound, compared to solution of DPPH alone, is indicative of the antioxidant activity of the compound. The reaction mixture containing 40 μl of 0.2 mM DPPH solution and 40 μl from different concentrations of test compounds or control in the solvent system of dimethyl sulfoxide/chloroform/methanol in a ratio of 1:8:4 was placed in 96-well plates following incubation for 30 min at 25 °C. The absorbance was measured using FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA) at 520 nm, at 0 s, and every 300 s until the reaction reached a plateau. The antiradical activities of the compounds were expressed by their $IC_{50}$ values, the concentration required to scavenge 50% of the DPPH radical.

### 2.7. Tyrosinase inhibition assay

The tyrosinase inhibition assay was performed using L-DOPA, as the substrate as recently described by Sirat et al.[46] Tyrosinase inhibition is depended upon the amount of dopachrome, produced in the reaction mixture. Absorption was measured at 490 nm to calculate the amount of dopachrome produced using 96-well clear polystyrene plates and FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). The tyrosinase inhibitory effect of the compounds was measured by calculating the percent inhibition using the following equation:

$$\%\ \text{Inhibition} = \frac{\text{absorbance (blank)} - \text{absorbance (sample)}}{\text{absorbance (blank)}} \times 100$$

### 2.8. Statistical analysis

One way ANOVA was performed to analyse all data from antioxidant assays using MINITAB 16 and when there were significant differences at $P \leqslant 0.05$, the means were compared using Tukey's multiple means comparison test. All the analyses were performed in triplicate.

### 2.9. Biocatalytic synthesis

#### 2.9.1. Procedure for the synthesis of saturated/unsaturated fatty acid esters of phloridzin and isoquercitrin (2–7 and 9–14)

To flame dried 3 Å molecular sieves, in round bottom flask, was added phloridzin (0.500 g; 1.15 mmol), stearic acid (1.62 g, 5.72 mmol), Novozyme 435® (1.30 g). It was followed by the addition of dry acetone (5 ml) and the mixture was stirred and heated at 45 °C for 12–24 h. The progress of reaction was monitored by thin layer chromatography (TLC), followed by staining with anisaldehyde spray reagent and then heating at 110 °C. After completion of reaction, it was filtered, evaporated and passed through column chromatography (acetone/toluene; 35:75 to 50:50) to get the pure stearic acid ester (**2**) of phloridzin. All other reactions followed the same procedure and produced esters **3–7** and **9–14**, with yields in the range of 81–98%. The pure compounds were then analyzed by IR, $^1$H NMR and $^{13}$C NMR spectroscopy. All other compounds were prepared by the same method.

#### 2.9.2. (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl] phenoxy}-3,4,5-trihydroxytetra-hydro-2*H*-pyran-2-yl)methyl stearate (2)

Yield: 98%; light yellow solid; $R_f$: 0.53 (acetone/toluene; 4:6: few drops of AcOH); IR (KBr) cm$^{-1}$: 3383, 2944, 2832, 2523, 2247, 2043, 1719, 1631, 1591, 1512, 1450, 1269, 1113, 1028, 913, 737, 652; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.60 (s, 1H, ArOH), 9.18 (br s, 1H, ArOH), 7.06 (d, 2H, $J$ = 8.4 Hz, H-2, H-6), 6.67 (d, 2H, $J$ = 8.4 Hz, H-3, H-5), 6.13 (d, 1H, 1.8 Hz, H-3′), 5.98 (d, 1H, $J$ = 1.8 Hz, H-5′), 5.46 (br s, 1H, OH), 5.37 (br s, 2H, 2OH), 5.04 (d, 1H, H-1″), 4.35 (br d, 1H, $J$ = 11.6 Hz, H-6a″), 4.14 (dd, 1H, $J$ = 11.6 Hz, 6.9 Hz, H-6b″), 3.65 (br t, $J$ = 7.2 Hz, H-4″), 3.46–3.20 (m, 6H, 2 × H$_\alpha$, H-2″, H-3″, H-5″, OH), 2.81 (br t, 2H, $J$ = 7.5 Hz, 2 × H$_\beta$), 2.30 (t, 2H, $J$ = 7.2 Hz, 2 × H-2‴), 1.49 (m, 2H, 2 × H-3‴), 1.25–1.19 (m, 28H, 2(CH$_2$)), 0.87 (br t, 3H, $J$ = 6.9 Hz, CH$_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 205.34 (CO), 173.44 (OCO), 166.05 (C-4′), 165.13 (C-6′), 161.30 (C-2′), 156.03 (C-4), 132.25 (C-1), 129.72 (C-2, C-6), 115.74 (C-3, C-5), 106.18 (C-1′), 101.55 (C-1″), 97.79 (C-3′), 95.44 (C-5′), 77.30 (C-3″), 74.77 (C-5″), 73.94 (C-2″), 70.68 (C-4″), 63.81 (C-6″), 45.61 (C$_\alpha$), 34.17 (C-2‴), 31.96 (C$_\beta$), 29.84, 29.34, 29.13 (C-4‴, C-5‴, C-6‴, C-7‴, C-8‴, C-9‴, C-10‴, C-11‴, C-12‴, C-13‴, C-14‴, C-15‴, C-16‴), 25.08 (C-3‴), 22.73 (C-17‴), 14.53 (C-18‴).

#### 2.9.3. (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl] phenoxy}-3,4,5-trihydroxytetra-hydro-2*H*-pyran-2-yl)methyl (*Z*)-9-octadecenoate (3)

Yield: 96%; light brownish yellow spongy solid; $R_f$: 0.56 (acetone/toluene; 4:6: few drops of AcOH); IR (KBr) cm$^{-1}$: 3383, 2927, 2855, 2253, 1714, 1627, 1599, 1514, 1455, 1264, 1203, 1078, 908, 734, 650; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 11.82 (br s, 1H, ArOH), 10.63 (br s, 1H, ArOH), 9.18 (br s, 1H, OH), 7.09 (d, 2H, $J$ = 8.7 Hz, H-2, H-6), 6.72 (d, 2H, $J$ = 8.7 Hz, H-3, H-5), 6.17 (d, 1H, 1.8 Hz, H-3′), 6.01 (s, 1H, H-5′), 5.48–5.29 (m, 4H, H-9‴, H-10‴), 5.04 (d, 1H, $J$ = 6.9 Hz, H-1″), 4.38 (br d, 1H, $J$ = 11.4 Hz, H-6a″), 4.18 (dd, 1H, $J$ = 11.4 Hz, 6.6 Hz, H-6b″), 3.67 (br t, 1H, $J$ = 7.8 Hz, H-4″), 3.55–3.23 (m, 6H, 2 × H$_\alpha$, H-2″, H-3″, H-5″, OH), 2.84 (br t, 2H, $J$ = 6.9 Hz, 2 × H$_\beta$), 2.32 (br t, 2H, $J$ = 6.9 Hz, 2 × H-2‴), 2.01–1.98 (m, 4H, 2 × H-8‴, 2 × H-11‴), 1.54–1.50 (m, 2H, 2 × H-3‴), 1.27–1.20 (m, 20H, 10(CH$_2$)), 0.87 (br t, 3H, $J$ = 6.9 Hz, CH$_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 205.37 (CO), 173.41 (OCO), 166.13 (C-4′), 165.15 (C-6′), 161.34 (C-2′), 156.06 (C-4), 132.30 (C-1), 130.30 (C-9‴, C-10‴), 129.73 (C-2, C-6), 115.78 (C-3, C-5), 106.25 (C-1′), 101.63 (C-1″), 97.86 (C-3′), 95.50 (C-5′), 77.37 (C-3″), 74.83 (C-5″), 73.98 (C-2″), 70.74 (C-4″), 63.85 (C-6″), 45.63 (C$_\alpha$), 34.19 (C-2‴), 31.97 (C$_\beta$), 29.83 (C-7‴, C-12‴), 29.54 (C-15‴), 29.33 (C-4‴, C-5‴, C-14‴), 29.20 (C-6‴, C-13‴, C-16‴), 27.33 (C-8‴, C-11‴), 25.10 (C-3‴), 22.74 (C-17‴), 14.50 (C-18‴).

#### 2.9.4. (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl] phenoxy}-3,4,5-trihydroxytetra-hydro-2*H*-pyran-2-yl)methyl (9*Z*,12*Z*)-9,12-octadecadienoate (4)

Yield: 93%; light brownish yellow spongy solid; $R_f$: 0.57 (acetone/toluene; 4:6: few drops of AcOH); IR (KBr) cm$^{-1}$: 3396, 2928, 2856, 2253, 1711, 1628, 1599, 1515, 1454, 1378, 1263, 1204, 1173, 1080, 907, 733, 650; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.62 (br s, 1H, ArOH), 9.16 (br s, 1H, ArOH), 7.08 (d, 2H, $J$ = 8.1 Hz, H-2, H-6), 6.70 (d, 2H, $J$ = 8.1 Hz, H-3, H-5), 6.16 (d, 1H, $J$ = 1.2 Hz, H-3′), 6.01(d, 1H, $J$ = 1.2 Hz, H-5′), 5.47 (br s, 1H, OH), 5.40–5.27 (m, 5H, H-9‴, H-10‴, H-12‴, H-13‴, OH), 5.04 (d, 1H, $J$ = 6.9 Hz, H-1″), 4.37 (br d, 1H, $J$ = 11.4 Hz, H-6a″), 4.18 (dd, 1H, $J$ = 11.4 Hz, 6.6 Hz, H-6b″), 3.66 (br t, 1H, $J$ = 7.8 Hz, H-4″), 3.57 (br s, 2 × OH), 3.48–3.22 (m, 5H, 2 × H$_\alpha$, H-2″, H-3″, H-5″), 2.83 (br t, 2H, $J$ = 6.9 Hz, 2 × H$_\beta$), 2.74 (br t, 2H, $J$ = 5.4 Hz, 2 × H-11‴), 2.30 (br t, 2H, $J$ = 7.5 Hz, 2 × H-2‴), 2.15–1.99 (m, 4H, 2 × H-8‴, 2 × H-14‴), 1.53–1.48 (m, 2H, 2 × H-3‴), 1.37–1.18 (m, 14H, 7(CH$_2$)), 0.86 (br t, 3H, $J$ = 6.6 Hz, CH$_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 205.42 (CO), 173.45 (OCO), 166.20 (C-4′), 165.16 (C-6′), 161.38 (C-2′), 156.09 (C-4), 132.36 (C-1), 130.50 (C-9‴, C-13‴), 129.75 (C-2, C-6), 128.48 (C-10‴, C-12‴), 115.84 (C-3, C-5), 106.33 (C-1′), 101.68 (C-1″), 97.94 (C-3′), 95.56 (C-5′), 77.43 (C-3″), 74.89 (C-5″), 74.03 (C-2″), 70.80 (C-4″), 63.88 (C-6″), 45.66

($C_\alpha$), 34.23 (C-2‴), 31.64 ($C_\beta$), 29.95 (C-7‴), 29.73 (C-15‴), 29.44 (C-4‴), 29.23 (C-5‴, C-6‴, C-11‴), 27.39 (C-8‴, C-14‴), 25.99 (C-16‴), 25.13 (C-3‴), 22.65 (C-17‴), 14.47 (C-18‴).

### 2.9.5. (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl] phenoxy}-3,4,5-trihydroxytetra-hydro-2*H*-pyran-2-yl)methyl (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoate (5)

Yield: 94%; light brownish yellow spongy solid; $R_f$: 0.52 (acetone/toluene; 4:6: few drops of AcOH); IR (KBr) cm $^{-1}$: 3373, 2944, 2831, 2520, 2247, 2043, 1708, 1449, 1365, 1270, 1228, 1112, 1028, 915, 736, 653; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.62 (br s, 1H, OH), 9.15 (br s, 1H, OH), 7.07 (d, 2H, $J$ = 8.4 Hz, H-2, H-6), 6.70 (d, 2H, $J$ = 8.4 Hz, H-3, H-5), 6.15 (d, 1H, $J$ = 1.2 Hz, H-3′), 6.00(d, 1H, $J$ = 1.8 Hz, H-5′), 5.49–5.25 (m, 8H, H-9‴, H-10‴, H-12‴, H-13‴, H-15‴, H-16‴, 2 × OH), 5.03 (d, 1H, $J$ = 6.6 Hz, H-1″), 4.36 (br d, 1H, $J$ = 11.4 Hz, H-6a″), 4.16 (dd, 1H, $J$ = 11.4 Hz, 7.2 Hz, H-6b″), 3.65 (br t, 1H, $J$ = 8.4 Hz, H-4″), 3.49–3.21 (m, 7H, 2 × $H_\alpha$, H-2″, H-3″, H-5″, 2 × OH), 2.84–2.77 (m, 6H, 2 × $H_\beta$, 2 × H-11‴, 2 × H-14‴), 2.30 (br t, 2H, $J$ = 7.2 Hz, 2 × H-2‴), 2.15–1.98 (m, 4H, 2 × H-8‴, 2 × H-17‴), 1.52–1.48 (m, 2H, 2 × H-3‴), 1.30–1.18 (m, 8H, 4($CH_2$)), 0.98 (br t, 3H, $J$ = 7.5 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 205.37 (CO), 173.43 (OCO), 166.08 (C-4′), 165.12 (C-6′), 161.31 (C-2′), 156.04 (C-4), 132.25 (C-1, C-16‴), 130.66 (C-9‴), 129.73 (C-2, C-6), 128.68 (C-10‴, C-13‴), 127.70 (C-12‴), 115.78 (C-3, C-5), 106.27 (C-1′), 101.60 (C-1″), 97.85 (C-3′), 95.49 (C-5′), 77.35 (C-3″), 74.82 (C-5″), 73.97 (C-2″), 70.73 (C-4″), 63.83 (C-6″), 45.62 ($C_\alpha$), 34.18 (C-2‴), 31.22 ($C_\beta$), 29.89 (C-7‴), 29.67 (C-5‴), 29.17 (C-4‴, C-6‴), 27.36 (C-8‴), 25.93 (C-11‴), 25.85 (C-14‴), 25.08 (C-3‴), 20.72 (C-17‴), 14.67 (C-18‴).

### 2.9.6. (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl] phenoxy}-3,4,5-trihydroxytetra-hydro-2*H*-pyran-2-yl)methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-icosapentaenoate (6)[47–49]

Yield: 85%; light brownish yellow spongy solid; $R_f$: 0.57 (acetone/toluene; 4:6: few drops of AcOH); IR (KBr) cm $^{-1}$: 3386, 3013, 2964, 2931, 2836, 2253, 1710, 1628, 1599, 1514, 1451, 1366, 1261, 1204, 1079, 908, 733, 650; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.62 (br s, 1H, OH), 9.15 (br s, 1H, OH), 7.06 (d, 2H, $J$ = 8.4 Hz, H-2, H-6), 6.69 (d, 2H, $J$ = 8.4 Hz, H-3, H-5), 6.15 (d, 1H, $J$ = 1.5 Hz, H-3′), 5.99 (d, 1H, $J$ = 1.5 Hz, H-5′), 5.45–5.32 (m, 12H, H-5‴, H-6‴, H-8‴, H-9‴, H-11‴, H-12‴, H-14‴, H-15‴, H-17‴, H-8‴, 2 × OH), 5.03 (d, 1H, $J$ = 6.6 Hz, H-1″), 4.36 (br d, 1H, $J$ = 11.4 Hz, H-6a″), 4.15 (dd, 1H, $J$ = 11.4 Hz, 6.6 Hz, H-6b″), 3.65 (br t, 1H, $J$ = 8.4 Hz, H-4″), 3.49–3.24 (m, 7H, 2 × $H_\alpha$, H-2″, H-3″, H-5″, 2 × OH), 2.83–2.76 (m, 10H, 2 × $H_\beta$, 2 × H-7‴, 2 × H-10‴, 2 × H-13‴, 2 × H-16‴), 2.32 (br t, 2H, $J$ = 7.5 Hz, 2 × H-2‴), 2.15–2.01 (m, 4H, 2 × H-4‴, 2 × H-19‴), 1.63–1.53 (m, 2H, 2 × H-3‴), 0.94 (br t, 3H, $J$ = 7.5 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 205.02 (C=O), 174.69 (OC=O), 166.28 (C-4′), 162.77 (C-6′), 160.31 (C-2′), 153.85 (C-4), 133.73 (C-1), 131.99 (C-17‴), 129.28 (C-2, C-6), 129.03 (C-6‴), 128.62 (C-5‴, C-14‴), 128.36 (C-12‴), 128.31 (C-8‴), 128.12 (C-11‴ or C-9‴), 128.06 (C-11‴ or C-9‴), 127.86 (C-15‴), 127.05 (C-18‴), 115.52 (C-3, C-5), 106.77 (C-1′), 100.63 (C-1″), 98.72 (C-3′), 95.06 (C-5′), 76.90 (C-3″), 74.17 (C-5″), 73.36 (C-2″), 70.32 (C-4″), 63.21 (C-6″), 45.31 ($C_\alpha$), 33.55 (C-2‴), 29.45 ($C_\beta$), 29.23 (C10‴ or C-13‴), 26.46 (C-4‴), 25.61 (C-16‴, C-7‴, C-13‴ or C-10‴), 24.66 (C-3‴), 20.43 (C-19‴), 13.97 (C-20‴).

### 2.9.7. (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetra-hydro-2*H*-pyran-2-yl)methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-4,7,10,13,16,19-docosahexaenoate (7)[47–49]

Yield: 82%; light brownish yellow spongy solid; $R_f$: 0.58 (acetone/toluene; 4:6: few drops of AcOH); IR (KBr) cm$^{-1}$: 3386, 3014, 2964, 2927, 2832, 2253, 1718, 1627, 1599, 1514, 1450, 1390, 1262, 1204, 1173, 1078, 907, 733, 650; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.62 (br s, 1H, OH), 9.15 (br s, 1H, OH), 7.06 (d, 2H, $J$ = 8.4 Hz, H-2, H-6), 6.68 (d, 2H, $J$ = 8.4 Hz, H-3, H-5), 6.14 (d, 1H, $J$ = 1.5 Hz, H-3′), 5.98 (d, 1H, $J$ = 1.5 Hz, H-5′), 5.46–5.25 (m, 14H, H-4‴, H-5‴, H-7‴, H-8‴, H-10‴, H-11‴, H-13‴, H-14‴, H-16‴, H-17‴, H-19‴, H-20‴, 2 × OH), 5.02 (d, 1H, $J$ = 6.3 Hz, H-1″), 4.38 (br d, 1H, $J$ = 12.0 Hz, H-6a″), 4.17 (dd, 1H, $J$ = 12.0 Hz, 6.9 Hz, H-6b″), 3.65 (br t, 1H, $J$ = 8.4 Hz, H-4″), 3.44–3.22 (m, 7H, 2 × $H_\alpha$, H-2″, H-3″, H-5″, 2 × OH), 2.85–2.78 (m, 12H, 2 × $H_\beta$, 2 × H-6‴, 2 × H-9‴, 2 × H-12‴, 2 × H-15‴, 2 × H-18‴), 2.40–2.28 (m, 4H, 2 × H-2‴, 2 × H-3‴), 2.15–2.00 (m, 2H, 2 × H-21‴), 0.93 (br t, 3H, $J$ = 7.5 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 205.27 (C=O), 174.86 (OC=O), 166.65 (C-4′), 163.61 (C-6′), 161.18 (C-2′), 154.49 (C-4), 134.27 (C-1), 132.51 (C-20‴), 130.22 (C-5‴), 129.81 (C-2, C-6), 129.15 (C-17‴), 129,03 (C-14‴), 128.89 (C-11‴, C-16‴), 128.59 (C-8‴, C-10‴), 128.40 (C-13‴, C-7‴), 128.00 (C-4‴), 127.60 (C-19‴), 115.52 (C-3, C-5), 107.53 (C-1′), 101.32 (C-1″), 99.51 (C-3′), 95.97 (C-5′), 76.96 (C-3″), 74.87 (C-5″), 73.94 (C-2″), 70.88 (C-4″), 63.81 (C-6″), 45.89 ($C_\alpha$), 34.57 (C-2‴), 31.05 (C-18‴), 30.21 (C-12‴), 29.82 ($C_\beta$), 26.17 (C-21‴, C-15‴), 26.07 (C-9‴), 23.08 (C-6‴), 20.94 (C-3‴), 14.47 (C-22‴).

### 2.9.8. (6-{[2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-4,5-dihy-droxytetrahydro-2*H*-pyran-2-yl)methyl stearate (9)

Yield: 97%; greenish solid; $R_f$: 0.42 (acetone/toluene; 1:1: few drops of AcOH); IR (KBr) cm$^{-1}$: 3449, 3271, 3070, 3007, 2926, 2854, 2559, 2250, 2124, 1997, 1830, 1767, 1655, 1454, 1366, 1308, 1203, 1172, 1057, 931, 822, 759, 731, 623; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 10.63–8.98 (br s, 2H, ArOH), 7.56 (br s, 2H, H-2′, H-6′), 6.86 (br s, 1H, H-5′), 6.41 (s, 1H, H-8), 6.22 (s, 1H, H-6), 5.47–5.26 (m, 3H, H-1″, 2OH), 4.20 (br d, 1H, $J$ = 10.5 Hz, H-6a″), 4.00 (br s, 1H, H-6b″), 3.55–3.20 (m, 5H, H-2″, H-3″, H-4″, H-5″, OH), 2.00 (br s, 2H, H-2‴, OH), 1.24–1.09 (m, 31H, 15x$CH_2$, OH), 0.83 (br. s, 3H, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 178.14 (CO), 173.09 (OCO), 165.09 (C-7), 162.09 (C-5), 157.16 (C-2, C-9), 149.25 (C-4′), 145.59 (C-3′), 133.97 (C-3), 122.19 (C-1′), 121.95 (C-6′), 116.98 (C-5′), 115.90 (C-2′), 104.66 (C-10), 101.73 (C-1″), 99.47 (C-6), 94.21 (C-8), 77.26 (C-3″), 75.01 (C-5″), 74.79 (C-2″), 71.01 (C-4″), 63.78 (C-6″), 34.00 (C-2‴), 31.99 (C-16‴), 29.73 29.37, 29.23, 29.06 (C-4‴, C-5‴, C-6‴, C-7‴, C-8‴, C-9‴, C-10‴, C-11‴, C-12‴, C-13‴, C-14‴, C-15‴), 24.95 (C-3‴), 22.74 (C-17‴), 14.51 (C-18‴).

### 2.9.9. (6-{[2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-3,4,5-trihy-droxytetrahydro-2*H*-pyran-2-yl)methyl (*Z*)-9-octadecenoate (10)

Yield: 92%; greenish yellow spongy solid; $R_f$: 0.58 (acetone/toluene; 1:1: few drops of AcOH); IR (KBr) cm$^{-1}$: 3373, 3065, 2947, 2836, 2497, 2251, 2121, 2042, 1898, 1763, 1640, 1596, 1560, 1459, 1448, 1229, 1165, 1026, 942, 739; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 11.45–9.01 (br s, 2H, ArOH), 7.53 (d, 2H, $J$ = 9.0 Hz, H-2′, H-6′), 6.85 (d, 1H, $J$ = 9.0 Hz, H-5′), 6.41 (d, 1H, $J$ = 1.8 Hz, H-8), 6.22 (d, 1H, $J$ = 1.8 Hz, H-6), 5.48–5.25 (m, 5H, H-9‴, H-10‴, H-1″, 2OH), 4.20 (br.d, 1H, $J$ = 11.7 Hz, H-6a″), 3.99 (dd, 1H, $J$ = 11.7 Hz, $J$ = 7.2 Hz, H-6b″), 3.45–3.13 (m, 6H, H-2″, H-3″, H-4″, H-5″, 2 × OH), 2.06–1.97 (m, 6H, 2 × H-2‴, 2 × H-8‴, 2 × H-11‴), 1.27–1.09 (m, 23H, 11x$CH_2$), 0.85 (br t, 3H, $J$ = 6.6 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 178.12 (CO), 173.05 (OCO), 164.99 (C-7), 162.06 (C-5), 157.12 (C-2, C-9), 149.21 (C-4′), 145.56 (C-3′), 133.92 (C-3), 130.29 (C-9‴, C-10‴), 122.16 (C-1′), 121.93 (C-6′), 116.95 (C-5′), 115.88 (C-2′), 104.65 (C-10), 101.65 (C-1″), 99.42 (C-6), 94.18 (C-8), 77.23 (C-3″), 74.98 (C-5″), 74.76 (C-2″), 70.98 (C-4″), 63.75 (C-6″), 33.96 (C-2‴), 31.92 (C-16‴), 29.79 (2C), 29.49, 29.29 (3C), 29.13, 29.03 (C-4‴, C-5‴, C-6‴, C-7‴, C-12‴, C-

13‴, C-14‴, C-15‴), 27.31 (C-8‴, C-11‴), 27.90 (C-3‴), 22.69 (C-17‴), 14.49 (C-18‴).

#### 2.9.10. (6-{[2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-3,4,5-tri-hydroxytetrahydro-2*H*-pyran-2-yl)methyl (9*Z*,12*Z*)-9,12-octadecadienoate (11)

Yield: 94%; greenish yellow spongy solid; $R_f$: 0.59 (acetone/toluene; 1:1: few drops of AcOH); IR (KBr) $cm^{-1}$: 3346, 2946, 2835, 2492, 2181, 2043, 1897, 1762, 1643, 1591, 1466, 1447, 1230, 1027, 941, 737, 700; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 11.46–8.97 (br s, 2H, ArOH), 7.58 (br s, 2H, H-2′, H-6′), 6.88 (br d, 1H, $J$ = 6.9 Hz, H-5′), 6.43 (s, 1H, H-8), 6.24 (s, 1H, H-6), 5.50–5.37 (m, 7H, H-9‴, H-10‴, H-12‴, H-13‴, H-1″, 2OH), 4.22 (br.d, 1H, $J$ = 10.5 Hz, H-6a″), 4.02 (dd, 1H, $J$ = 10.5 Hz, $J$ = 7.2 Hz, H-6b″), 3.55–3.20 (m, 6H, H-2″, H-3″, H-4″, H-5″, 2 × OH), 2.78 (br s, 2H, 2 × H-11‴), 2.16–2.04 (m, 6H, 2 × H-2‴, 2 × H-8‴, 2 × H-14‴), 1.29–1.11 (m, 17H, 8x$CH_2$, OH), 0.88 (br s, 3H, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 178.12 (CO), 173.05 (OCO), 165.01 (C-7), 162.06 (C-5), 157.13 (C-2, C-9), 149.22 (C-4′), 145.58 (C-3′), 133.91 (C-3), 130.49 (C-9‴, C-10‴), 128.45 (C-12‴, C-13‴),122.17 (C-1′), 121.93 (C-6′), 116.96 (C-5′), 115.89 (C-2′), 104.65 (C-10), 101.64 (C-1″), 99.43 (C-6), 94.19 (C-8), 77.22 (C-3″), 74.99 (C-5″), 74.77 (C-2″), 70.98 (C-4″), 63.75 (C-6″), 33.96 (C-2‴), 31.57 (C-16‴), 29.66 (C-15‴), 29.38 (C-7‴), 29.16 (C-5‴), 29.03 C-4‴, C-6‴), 27.34 (C-8‴, C-14‴), 25.94 (C-11‴), 24.91 (C-3‴), 22.59 (C-17‴), 14.48 (C-18‴).

#### 2.9.11. (6-{[2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-3,4,5-tri-hydroxytetrahydro-2*H*-pyran-2-yl)methyl (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienoate (12)

Yield: 91%; greenish yellow spongy solid; $R_f$: 0.56 (acetone/toluene; 1:1: few drops of AcOH); IR (KBr) $cm^{-1}$: 3346, 2946, 2835, 2492, 2180, 2049, 1895, 1765, 1645, 1594, 1464, 1447, 1230, 1027, 941, 736, 701; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 11.48–8.99 (br s, 2H, ArOH), 7.52 (br s, 2H, H-2′, H-6′), 6.82 (br d, 1H, $J$ = 8.7 Hz, H-5′), 6.38 (s, 1H, H-8), 6.18 (s, 1H, H-6), 5.43–5.31 (m, 9H, H-9‴, H-10‴, H-12‴, H-13‴, H-15‴, H-16‴, H-1″, 2OH), 4.16 (br.d, 1H, $J$ = 11.4 Hz, H-6a″), 3.96 (dd, 1H, $J$ = 11.4 Hz, $J$ = 7.1 Hz, H-6b″), 3.43–3.15 (m, 5H, H-2″, H-3″, H-4″, H-5″, OH), 2.84–2.71 (m, 4H, 2 × H-11‴, 2 × H-14‴), 2.11–1.98 (m, 6H, 2 × H-2‴, 2 × H-8‴, 2 × H-17‴), 1.26–1.05 (m, 12H, 5x$CH_2$, 2OH), 0.90 (br t, 3H, $J$ = 7.2 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 178.11 (CO), 173.03 (OCO), 164.95 (C-7), 162.04 (C-5), 157.10 (C-2, C-9), 149.19 (C-4′), 145.55 (C-3′), 133.91 (C-3), 132.22 (C-16‴), 130.67 (C-9‴), 128.65 (C-10‴, C-12‴), 128.18 (C-13‴), 127.68 (C-15‴), 122.15 (C-1′), 121.91 (C-6′), 116.94 (C-5′), 115.87 (C-2′), 104.64 (C-10), 101.63 (C-1″), 99.40 (C-6), 94.16 (C-8), 77.21 (C-3″), 74.96 (C-5″), 74.74 (C-2″), 70.96 (C-4″), 63.73 (C-6″), 33.93 (C-2‴), 29.62 (C-7‴), 29.12 (C-5‴), 29.00 (C-4‴, C-6‴), 27.33 (C-8‴), 25.90 (C-11‴), 25.81 (C-14‴), 24.87 (C-3‴), 20.68 (C-17‴), 14.65 (C-18‴).

#### 2.9.12. (6-{[2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-3,4,5-tri-hydroxytetrahydro-2*H*-pyran-2-yl)methyl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-5,8,11,14,17-icosapentaeno-ate (13)[47–49]

Yield: 81%; greenish yellow spongy solid; $R_f$: 0.57 (acetone/toluene; 1:1: few drops of AcOH); IR (KBr) $cm^{-1}$: 3346, 2946, 2835, 2490, 2180, 2044, 1893, 1768, 1647, 1591, 1466, 1446, 1231, 1027, 941, 737, 706; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 12.23–8.82 (br s, 2H, ArOH), 7.55 (br d, 2H, $J$ = 8.5 Hz, H-2′, H-6′), 6.87 (d, 1H, $J$ = 8.5 Hz, H-5′), 6.42 (s, 1H, H-8), 6.23 (s, 1H, H-6), 5.49–5.17 (m, 13H, H-5″, H-6″, H-8″, H-9‴, H-11‴, H-12‴, H-14‴, H-15‴, H-17‴, H-18‴, H-1″, 2OH), 4.20 (br.d, 1H, $J$ = 11.4 Hz, H-6a″), 3.99 (dd, 1H, $J$ = 11.4 Hz, $J$ = 7.2 Hz, H-6b″), 3.49–3.14 (m, 6H, H-2″, H-3″, H-4″, 2 × OH), 2.82–2.71 (m, 8H, 2 × H-7‴, 2 × H-10‴, 2 × H-13‴, 2 × H-16‴), 2.14–1.85 (m, 6H, 2 × H-2‴, 2 × H-4‴, 2 × H-19‴), 1.39–1.24 (m, 3H, 2 × H-3‴, OH), 0.92 (br t, 3H, $J$ = 7.8 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 178.16 (CO), 172.95 (OCO), 165.05 (C-7), 162.09 (C-5), 157.16 (C-2, C-9), 149.26 (C-4′), 145.60 (C-3′), 133.96 (C-3), 132.31 (C-18‴), 129.61 (C-5‴), 129.01, 128.89 128.79, 128.64, 128.47 (C-6‴, C-8‴, C-9‴, C-11‴, C-12‴, C-14‴, C-15‴, C-17‴), 122.21 (C-1′), 121.96 (C-6′), 116.99 (C-5′), 115.93 (C-2′), 104.69 (C-10), 101.71 (C-1″), 99.47 (C-6), 94.24 (C-8), 77.24 (C-3″), 75.01 (C-5″), 74.79 (C-2″), 70.98 (C-4″), 63.85 (C-6″), 33.46 (C-2‴), 26.63 (C-4‴), 25.95 (C-7‴, C-10‴), 25.88 (C-16‴, C-13‴), 24.92 (C-3‴), 20.74 (C-19‴), 14.71 (C-18‴).

#### 2.9.13. (6-{[2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-3,4,5-tri-hydroxytetrahydro-2*H*-pyran-2-yl)methyl (4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)-4,7,10,13,16,19-docosa-hexaenoate (14)[47–49]

Yield: 81%; greenish yellow spongy solid; $R_f$: 0.57 (acetone/toluene; 1:1: few drops of AcOH); IR (KBr) $cm^{-1}$: 3373, 2944, 2913, 2835, 2678, 2499, 2251, 2182, 2045, 1890, 1767, 1651, 1592, 1466, 1447, 1383, 1229, 1027, 909, 732, 649; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 11.61–8.96 (br s, 2H, ArOH), 7.52 (br d, 2H, $J$ = 8.1 Hz, H-2′, H-6′), 6.83 (d, 1H, $J$ = 8.1 Hz, H-5′), 6.38 (s, 1H, H-8), 6.19 (s, 1H, H-6), 5.44–5.15 (m, 15H, H-4″, H-5″, H-7″, H-8″, H-10‴, H-11‴, H-13‴, H-14‴, H-16‴, H-17‴, H-19‴, H-20‴, H-1″, 2OH), 4.17 (br d, 1H, $J$ = 11.4 Hz, H-6a″), 3.96 (dd, 1H, $J$ = 11.4 Hz, $J$ = 6.6 Hz, H-6b″), 3.49 (br s, OH), 3.34–3.17 (m, 5H, H-2″, H-3″, H-4″, H-5″, OH), 2.75–2.66 (m, 10H, 2 × H-6‴, 2 × H-9‴, 2 × H-12‴, 2 × H-15‴, 2 × H-18‴), 2.11–1.95 (m, 6H, 2 × H-2‴, 2 × H-3‴, 2 × H-21‴), 1.20 (br s, 1H, OH), 0.88 (t, 3H, $J$ = 7.5 Hz, $CH_3$); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta$ 178.14 (CO), 172.53 (OCO), 165.07 (C-7), 162.07 (C-5), 157.16 (C-2, C-9), 149.26 (C-4′), 145.60 (C-3′), 134.00 (C-3), 132.30 (C-20‴), 129.17 (C-4‴), 128.88, 128.64, 128.44, 127.66 (C-5‴, C-7‴, C-8‴, C-10‴, C-11‴, C-13‴,C-14‴, C-16‴, C-17‴, C-19‴), 122.20 (C-1′), 121.95 (C-6′), 117.01 (C-5′), 115.92 (C-2′), 104.68 (C-10), 101.81 (C-1″), 99.49 (C-6), 94.27 (C-8), 77.24 (C-3″), 74.96 (C-5″), 74.79 (C-2″), 70.94 (C-4″), 63.94 (C-6″), 33.94 (C-2‴), 25.95 (C-6‴, C-9‴, C-12‴, C-15‴, C-18‴), 22.86 (C-3‴), 20.73 (C-21‴), 14.69 (C-18‴).

## 3. Results and discussion

### 3.1. Chemistry

Acylation of phloridzin and isoquercitrin with long chain fatty acids were carried out at 45–50 °C in extra dry acetone along with constant stirring. Briefly, defined quantities of flavonoids and fatty acids were dissolved in acetone. Enzymatic reactions were initiated by the addition of lipase (Novozyme 435®; with an activity of 10,000 propyl laurate units). As the water content (<200 ppm) is very important parameter in enzymatic catalysis in organic media. It was assumed that the activity of the enzyme could be improved by reducing the hydration level of the different components in the reaction medium. In the present investigation, the highest efficiency was reached by drying the enzyme for 18–20 h over $P_2O_5$ before use; besides, using flame dried molecular sieves (3 Å) to remove any in situ generated water in the reaction mixture (Schemes 1 and 2).

#### 3.1.1. Effect on regioselectivity

The synthesized esters have been evaluated qualitatively by using NMR spectroscopy. The $^1$H and $^{13}$C NMR assignment have confirmed their structures. The location of the acylated site has been well documented in previous studies for a range of acyl donors and is in agreement with our studies.[19,20,23] In our case it was confirmed by comparing the downfield and upfield shifts between the newly synthesized products and the reported phloridzin

and isoquercetin.[19] $^{13}C$ NMR spectra showed that the acylation on the 6″-OH position led to an downfield shift of the C-6″ signal by approximately 3 ppm (~60.97 to ~63.83 ppm) due to the resonance effect towards carbonyl of the newly generated ester as well as an upfield shift of the C-1‴ (OCO) signal by approximately 3–4 ppm from their corresponding fatty acids[47] (Fig. 1).

### 3.1.2. Effect of acyl chain length

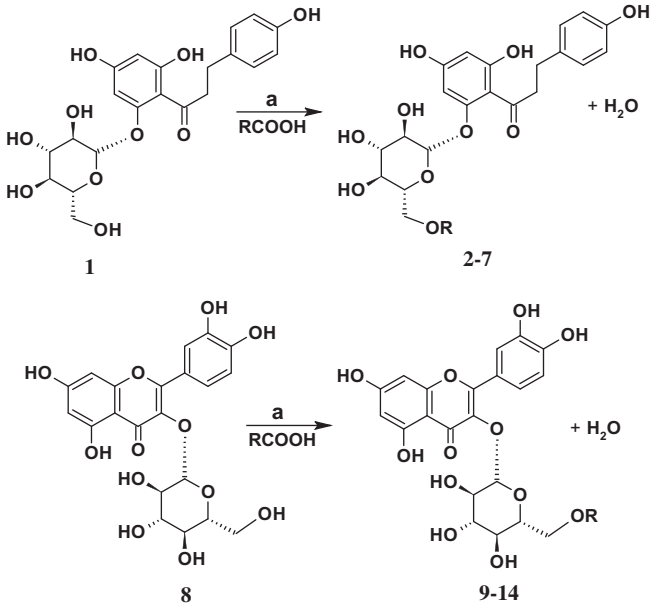
A slight decrease in yield (compounds **6**, **7** and **13**, **14**) was observed with the increase in chain length which was possibly because the longer chain length of fatty acids might have impact on the active sites of enzyme.

### 3.1.3. Effect of molar ratios of substrate and acyl donor

The time course of enzymatic acylation of phloridzin and isoquercitrin was significantly affected by using different molar ratios of substrate and acyl donor. The acyl donor concentration was adjusted to have a flavonoid/acyl donor molar ratio of 1:5 for the complete conversion of phloridzin and isoquercitrin into their corresponding esters in shorter time.

## 3.2. Antioxidant capacity of flavonoid esters

For the two series of compounds the FRAP assay has resulted in significantly higher ($P \leqslant 0.05$) antioxidant capacity for the parent compounds phloridzin (**1**) than their respective esters (**2–7**) (Table 1). The phloridzin showed significantly higher ($P \leqslant 0.05$) antioxidant capacity than all of its esters in ORAC assay. Of the isoquercitrin series, the isoquercitrin (**8**) showed greater antioxidant capacity in both FRAP and DPPH assays than their esters. Phloridzin was found to have an $IC_{50}$ value of 1.489 mmol $l^{-1}$, a value similar to that was found by Yang et al.[38] Isoquercitrin was found to have an $IC_{50}$ value of 18 μmol $l^{-1}$, which was very comparable to that found by Shibano et al.[39] The esters of isoquercitrin showed to have poorer antioxidant capacity, and higher $IC_{50}$ values than their parent flavonoid, which was a consistent trend of acylated flavonoids found in literature.[19,41,42] Lue et al.[42] reported the same for rutin and its esters and it is explained that accessibility of hydroxyl groups to the free radicals is obstructed by the steric hindrance created by long acyl chains of fatty acids. In this study, no significance in such correlation was observed between the saturation number of the fatty acids attached to the esters and their antioxidant capacity. Although the radical scavenging ability of flavonoids were not completely diminished by esterification with fatty acids but rather the high antioxidant capacity of these esters was significantly retained. The isoquercitrin esters had a much greater antioxidant activity, according to FRAP assay and DPPH assay which shows lower $IC_{50}$ values than the phloridzin esters. This can be due to the high potential of radical scavenging ability possessed by isoquercitrin than phloridzin. To our assumption, the incorporation of amphipathic/amphiphilic properties in the flavonoid skeleton through esterification would facilitate their interaction with membrane bound proteins or receptors as well as enhance their absorption through cell membrane thus increasing their bioavailability.




**Scheme 1.** Acylation of phloridzin and isoquercetrin using lipases. (a) Acetone, 3° A molecular sieves, novozyme 435®, 45 °C, stirring, 24 h; R = oleic, stearic, linoleic, linolenic, eicosapentaenoic (EPA), and docosahexaenoic acids or their esters.

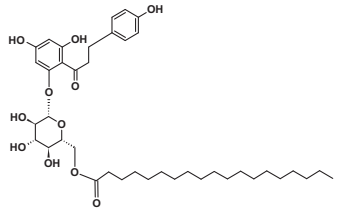
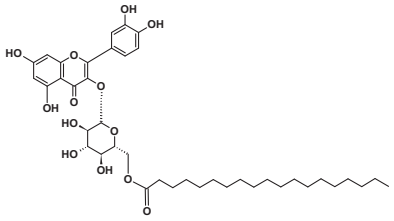
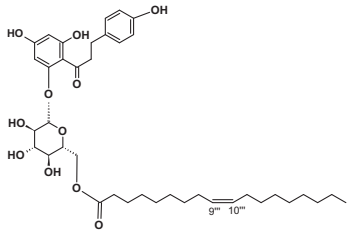
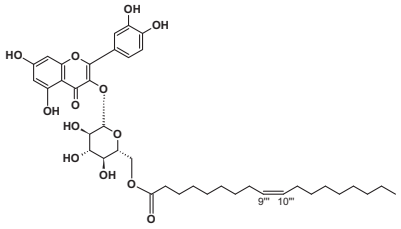
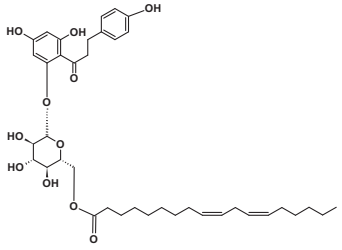
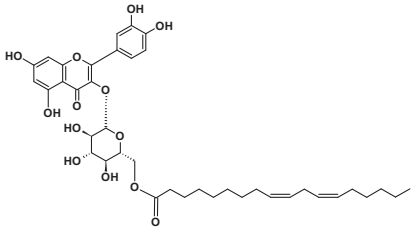
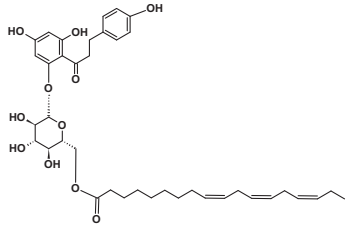
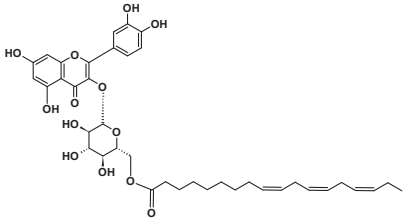
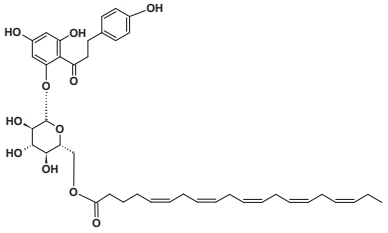
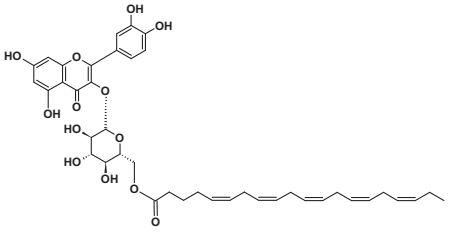
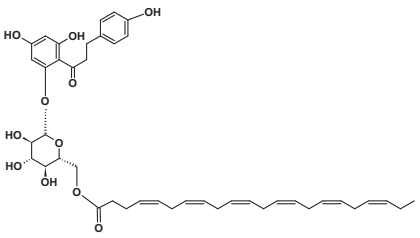
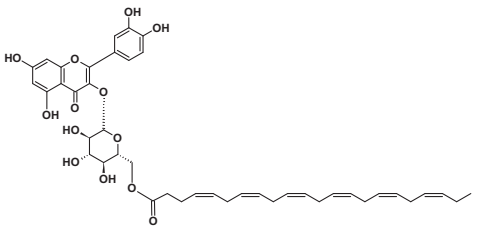
## 3.3. Tyrosinase inhibition activity

The percent tyrosinase inhibition by the two novel series of flavonoid esters were concentration dependent (Table 2). Of the two precursors, the phloridzin (**1**) exhibited weak tyrosinase inhibition, as compared to isoquercitrin (**8**). Among the series of phloridzin and its esters **(1–7)**, the α-linolenic and stearic acid esters (**2** and **5**) also exhibited weak tyrosinase inhibition. Similar to our present results, Shoji et al.[50], using B 16 mouse melanoma cells, also showed that phloridzin was a weak tyrosinase inhibitor. However, docosahexaenoic acid ester (**7**) of phloridzin exhibited the highest tyrosinase inhibition ($P \leqslant 0.05$) among all the tested esters of phloridzin **(2–7)**.

In this study, it was discovered that **3** and **4** of phloridzin esters showed stronger tyrosinase inhibition, with the increase in number of double bonds of fatty acids *via* structure-activity relationships (SAR). The same trend of the increase in inhibitory potential, with the increasing number of double bonds, has been almost followed by compounds **6** and **7**. However, as opposed to isoquercitrin eicosapentaenoic acid and docosahexaenoic acid esters (**12** and **13**, respectively), the eicosapentaenoic acid and docosahexaenoic acid esters of phloridzin (**6** and **7**, respectively) exhibited moderate to significant inhibition potency, which can be explained based on the rationale of the structure of phloridzin. The phloridzin backbone is more flexible (more conformational isomers) in 3-dimensions, as compared to isoquercitrin, due to the free rotation at C-α and C-β (Fig. 1). Once the bulky unsaturated fatty acids (C20–C22) are attached to their skeleton, they result in the orientation (through rotation at C-α and C-β) of these flexible molecules (**2–7**) in a way that some of their conformations may help in better interaction with tyrosinase, in terms of adjusting the positions of phenolic functionalities, as well as of the fatty acid side chains. On the other hand, an increase in double bonds in the side chains makes the fatty acid parts of the molecules conformationally less flexible and hence, the π-electrons are frequently available for some lipophilic interactions with the tyrosinase, in addition to interaction of the polar polyphenolic functionalities of these molecules with the binuclear copper metal of the enzyme.

Among the isoquercitrin series (**8–14**), the α-linolenic acid ester (**12**) of isoquercitrin was the strongest tyrosinase inhibitor, at all tested concentrations ($P \leqslant 0.05$). It was followed by linoleic acid (**11**), stearic acid (**9**), and oleic acid (**10**) esters of isoquercitrin, respectively. The parent flavonoid, isoquercitrin (**8**), exhibited reasonable tyrosinase activity at the tested concentration levels.

Considering the structure-activity relationship, it can be found that the inhibition potential of the isoquercitrin series (**8**–**14**) increases first, rises to its maximum (**12**) and then decreases (**13** and **14**) with the addition of double bonds and/or the chain length. From the activity profile, it can be concluded that the presence of double bonds is crucial for inhibition but the increase in chain

| Compd. | Structure | Yield | Compd. | Structure | Yield |
|---|---|---|---|---|---|
| **2** |  | 98 % | **9** |  | 97 % |
| **3** |  | 96 % | **10** |  | 92 % |
| **4** |  | 93 % | **11** |  | 94 % |
| **5** |  | 94 % | **12** |  | 91 % |
| **6** |  | 85 % | **13** |  | 81 % |
| **7** |  | 82 % | **14** |  | 81 % |

**Scheme 2.** Structures and percent yield of acylated fatty acid derivatives of phloridzin and isoquercetrin.

length, after a certain limit, prohibits the molecule from entering the enzyme core, which can be seen in case of esters **13** and **14**, where the addition of double bonds is counterfeited by the size of the chain lengths (C-20 and C-22, respectively).

Based on these results, it can be concluded that these fatty acid esters of isoquercitrin and phloridzin are potential tyrosinase inhibitors. The docosahexaenoic acid ester of phloridzin (**7**), followed by α-linoleic acid ester of isoquercitrin, (**12**), emerged as

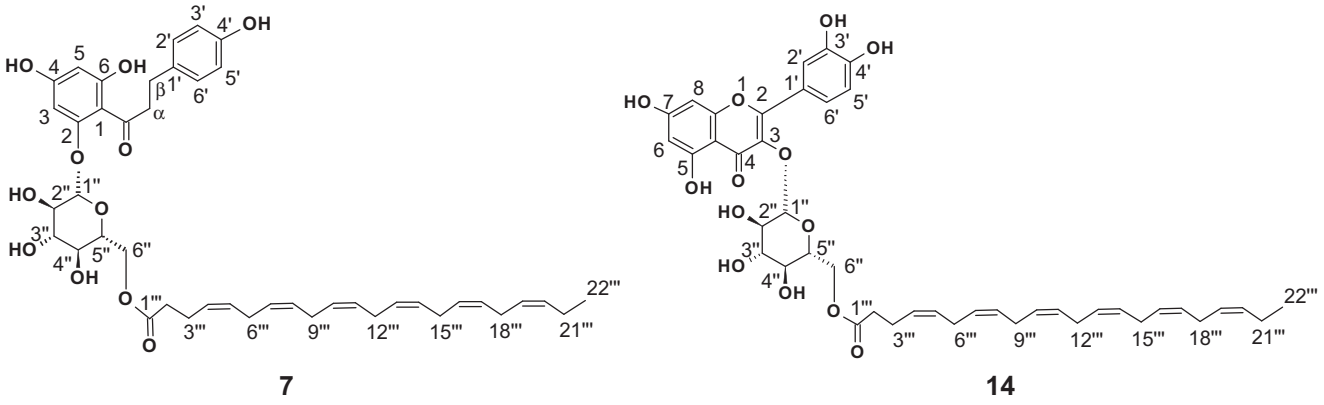



**Figure 1.** Numbering of the esters of phloridzin (**7**) and isoquercitrin (**14**).

**Table 1**
Antioxidant capacity measured by FRAP, ORAC and DPPH radical assays for the two parent flavonoids and their modified esters

| Compd no. | Compound | FRAP[Z] (μmol TE $l^{-1}$) | ORAC[Y] (μmol TE $l^{-1}$) | DPPH $IC_{50}$ (μmol $l^{-1}$) |
|---|---|---|---|---|
| 1 | Phloridzin (Pz) | 166.3 ± 14.6 f | 120.2 ± 2.6 e | 1488.9 ± 68.4 e |
| 2 | Stearic acid ester of Pz | 88.5 ± 6.1 g | 26.0 ± 2.3 h | 5265.3 ± 228.4 i |
| 3 | Oleic acid ester of Pz | 90.2 ± 6.6 g | 76.1 ± 6.9 f | 9720.0 ± 149.4 j |
| 4 | Linoleic acid ester of Pz | 96.0 ± 8.1 g | 61.2 ± 2.6 g | 901.8 ± 44.7 d |
| 5 | α-Linolenic acid ester of Pz | 87.6 ± 7.2 g | 57.8 ± 3.0 g | 2873.6 ± 144.8 h |
| 6 | EPA ester of Pz | 94.3 ± 5.6 g | 34.9 ± 4.3 h | 1688.8 ± 35.9 f |
| 7 | DHA ester of Pz | 112.3 ± 10.1 fg | 64.7 ± 7.7 fg | 1907.7 ± 96.4 g |
| 8 | Isoquercitrin (Q3G) | 1500.0 ± 16.7 a | 473.1 ± 1.4 b | 17.9 ± 1.7 a |
| 9 | Stearic acid ester of Q3G | 622.3 ± 27.9 b | 310.0 ± 5.4 c | 42.0 ± 3.6a |
| 10 | Oleic acid ester of Q3G | 536.0 ± 22.9 cd | 265.4 ± 5.1 d | 38.2 ± 0.6 a |
| 11 | Linoleic acid ester of Q3G | 340.4 ± 18.9 e | 491.2 ± 0.9 a | 60.4 ± 2.8 ab |
| 12 | α-Linolenic acid ester of Q3G | 613.8 ± 62.0 b | 490.3 ± 1.8 a | 97.9 ± 4.7 b |
| 13 | EPA ester of Q3G | 569.4 ± 18.4 bc | 491.8 ± 3.1a | 36.95 ± 2.1 a |
| 14 | DHA ester of Q3G | 485.0 ± 28.1 d | 488.9 ± 4.6 a | 146.5 ± 1.8 c |

(Means ± standard deviation followed by the same letter within column are not significantly different, Tukey's multiple means comparison test, $P \leqslant 0.05$); [Z]μmol Trolox equivalents (TE) $l^{-1}$ of 1 mmol $l^{-1}$ of solutions of all the test compounds; [Y]For phloridzin and its esters: μmol $l^{-1}$ Trolox equivalents of 0.01 mmol $l^{-1}$ of solution; For Isoquercitrin and its esters: μmol $l^{-1}$ Trolox equivalents of 0.001 mmol $l^{-1}$ of solution; Pz: Phloridzin; Q3G: Isoquercitrin; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

**Table 2**
Percentage inhibition of tyrosinase activity in vitro by flavonoid esters

| Compd no. | Compound | Concentration (μM) | | |
|---|---|---|---|---|
| | | 10 | 100 | 1000 |
| 1 | Phloridzin (Pz) | 0 | 0 | 16.56 j |
| 2 | Stearic acid ester of Pz | 0 | 16.59 i | 17.17 j |
| 3 | Oleic acid ester of Pz | 0 | 20.53 h | 31.85 i |
| 4 | Linoleic acid ester of Pz | 0 | 28.16 g | 66.96 e |
| 5 | α-Linolenic acid ester of Pz | 0 | 0 | 12.53 l |
| 6 | EPA ester of Pz | 0 | 19.59 h | 46.25 h |
| 7 | DHA ester of Pz | 25.71 d | 62.09 c | 91.21 a |
| 8 | Isoquercitrin (Q3G) | 0 | 40.59 f | 54.55 g |
| 9 | Stearic acid ester of Q3G | 0 | 56.44 d | 69.20 d |
| 10 | Oleic acid ester of Q3G | 38.89 b | 47.54 e | 62.44 f |
| 11 | Linoleic acid ester of Q3G | 32.07 c | 73.31 b | 80.58 c |
| 12 | α-Linolenic acid ester of Q3G | 55.78 a | 78.77 a | 85.55 b |
| 13 | EPA ester of Q3G | 0 | 0 | 7.06 m |
| 14 | DHA ester Q3G | 0 | 19.28 h | 32.53 i |

PZ: Phloridzin; Q3G: Isoquercitrin; μM: Micromole; a–j: The same letter within each column indicates that corresponding means are not significantly different. Means separation by Tukey's multiple means comparison test ($P \leqslant 0.05$).

promising tyrosinase inhibitors, in vitro. These compounds exhibited significantly higher tyrosinase inhibition ($P \leqslant 0.05$) compared to parent flavonoids, at tested concentrations (Table 2). The results suggest that these flavonoid esters can serve as candidate molecules for the design and synthesis of tyrosinase inhibitory drugs and/or skin care products, which can be useful in treatment of various dermatological disorders.

## 4. Conclusions

Two series of long chain fatty acid esters of phloridzin and isoquercetin were synthesized and their structures were confirmed by NMR analysis. The study explains how to increase the efficiency of the acylation reactions by using the excess of acyl donors, as well as by maintaining the extra dry conditions through the use of extra dry acetone, flame dried molecular sieve, dried enzyme over $P_2O_5$ and keeping the reaction under inert atmosphere, which limits the possibility of hydrolysis and drags the reaction forward. From the bioactivity point of view, all the flavonoid esters retained substantial antioxidant capacity after the esterification. Although the parent compounds have greater free radical scavenging ability than their esters, the amphipathic/amphiphilic properties could make them unique in terms of enhanced bioavailability and biological activity. The chain lengths and the number of double bonds of acyl donors, influenced the antioxidant capacity and ability to inhibit tyrosinase in vitro, by the acylated derivatives of phloridzin and isoquercetin. The α-linolenic acid ester of isoquercitrin (**12**) and docosahexaenoic acid ester of phloridzin (**7**) showed the greatest tyrosinase inhibition ($P \leqslant 0.05$) potential. Further investigations are warranted to confirm their biological activities in vivo and to investigate their possible candidacy for pharmaceutical and cosmetic applications.

## Acknowledgments

We are thankful to the financial support of the Discovery Grant and USRA Programs of the Natural Sciences and Engineering Council (NSERC) of Canada and the Atlantic Innovation Funds (AIF) program of the Atlantic Canada Opportunities Agency. We are grateful to Dr. Amitabh Jha at Acadia University for providing us the NMR facility and Dr. Bobby Ellis for facilitating us in the NMR operation. Thanks to Emily Fraser for the assistance given in conducting antioxidant capacity assays.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.034.

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