J Am Oil Chem Soc (2013) 90:1751–1759
DOI 10.1007/s11746-013-2314-0

ORIGINAL PAPER

Synthesis, Molecular Characterization and Preliminary Antioxidant Activity Evaluation of Quercetin Fatty Esters

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Received: 18 January 2013 / Revised: 20 June 2013 / Accepted: 12 July 2013 / Published online: 6 September 2013

Abstract Quercetin shows interesting pharmacological effects, but its use in topical applications is limited by its low skin permeability and solubility. In this work, the synthesis of highly lipophilic quercetin esters with oleic, linoleic and linolenic acid useful as topical quercetin prodrugs is reported. Partial OH esterification is advisable to maintain the antioxidant activity of these compounds; tetraesters and triesters can be achieved by modulating the reaction conditions utilized for the total esterification of quercetin. The chemical structures of the esters were proven by spectroscopic techniques; quantum chemical NMR calculation were mandatory to unequivocally assign the free position in triesters. Finally, the antioxidant activity of all the synthesized compounds was determined by the 2,2-diphenyl-1-picryl-hydrazyl method and by 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) assay.

Keywords Quercetin pentaesters · Quercetin tetraesters · Quercetin trimesters · Fatty acids · Bi-dimensional NMR techniques · Quantum chemical NMR calculation · DPPH · ABTS

Electronic supplementary material The online version of this article (doi:10.1007/s11746-013-2314-0) contains supplementary material, which is available to authorized users.

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Introduction

In recent years the interest in natural substances useful to counteract aging has risen sharply. In fact, there is a significant increase in skin cosmetics, nutraceuticals and even pharmaceutical products based on the use of natural compounds or on their semi-synthetic derivatives.

Besides, a strong interest in natural substances with high antioxidant activity has been particularly observed. Indeed oxidative stress induced by multiple factors is the main cause of many pathological conditions such as inflammation, cancer, coronary heart disease and even skin aging.

Quercetin, 3,3′,4′,5,7-pentahydroxyflavone, is a natural polyphenol abundant in plant food (apples, onions, grapes, red wine and green tea) which exerts many pharmacological effects, such as antioxidant activity [1–3], anti-inflammatory effects [4–6], antitumor [7], pro-apoptotic [8, 9] and cardiovascular activity [10] inhibiting platelet aggregation and thrombus formation. Moreover quercetin shows in-vitro antiviral [11] and antibacterial activity [12–14]. Recently it has been reported that the topical application of quercetin inhibits oxidative skin damage [15] and the inflammatory processes induced by solar UV radiation [16]. However quercetin topical use is limited by its low skin permeability and solubility making the development of dermocosmetic and nutraceutical applications difficult.

In this work, the synthesis of new quercetin pentaesters with ω-3, ω-6 and ω-9 fatty acids is presented with the aim of obtaining topical prodrugs with two moieties both of them pharmacologically active. These molecules could lead to an increase in quercetin lipophilicity which could positively affect its bioavailability, skin permeability and overall pharmacological activity. Since in the literature [17–19] is reported that the different hydroxyl groups of quercetin are responsible for the various pharmacological

activities, we also report herewith on the synthesis and analytical characterization of tetra and triesters of quercetin with oleic, linoleic and linolenic acids. Furthermore, a preliminary evaluation of the antioxidant activity by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method and by 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) assay (ABTS) is reported.

Experimental

Materials

Quercetin, oleic, linolenic and linoleic acid, solvents, reagents and deuterated solvents were purchased from Sigma Aldrich (Milan, Italy). Reaction were monitored by thin layer chromatography on silica gel plates F254, visualized with UV light or iodine vapor. Silica gel 230–400 mesh/60A was employed for column chromatography. Antioxidant Assay Kits were from Sigma-Aldrich, St. Louis, USA.

Characterization Methods

FT-IR spectra were collected using a Perkin Elmer (Waltham, MA, USA) FT-IR Spectrometer "Spectrum One" in a spectral region between 4,000 and 600 or 450 cm^{-1} for solid or liquid compounds respectively and analysed by transmittance technique through 32 scanning and 4 cm^{-1} resolution. Solid samples were mixed in a mortar with KBr (1:100) and pressed in a hydraulic press (14 tons) to small tablets, while for liquid samples one drop was placed between two widows of sodium chloride. The ^{1}H- and ^{13}C-NMR and Bi-dimensional analyses, COSY, heteronuclear multiple-bond correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) were carried out on a Bruker Avance 500 (Billerica, MA, USA) operating at 500 MHz for ^{1}H and 125.75 MHz for ^{13}C or with a Varian Mercury Plus 200, operating at 200 MHz for ^{1}H and 50.3 MHz for ^{13}C. Chemical shifts were expressed as ppm (δ) using the central peak of chloroform as the internal reference ($\delta_H = 7.23$ ppm; $\delta_C = 77.3$ ppm). The APT sequence was used to distinguish methine and methyl carbon signals from those due to methylene and quaternary carbons.

HPLC/DAD analyses were performed using an HPLC system equipped with a quaternary pump Merck Hitachi L-7100, an injector Rheodyne 7125 with a 20 µl loop, a thermostat Column Block Heater (Mod. 7940 Hichrom Ltd.), a detector DAD Hewlett Packard HP 1050. The acquired data were processed by software HP CHEM. The detector was a Diode Array (Waters mod. 2996, Milford, MA, USA) operating at 254 nm. The column employed was silica Ascentis 15 × 4.6 mm (3 µm particle size); the mobile phase was a mixture of hexane/isopropanol 99.9:0.2 (v/v) eluted at 1.5 ml min^{-1} at room temperature.

Low resolution mass analyses were recorded with a Thermo-Finnigan LCQ advantage AP electrospray/ion trap equipped instrument by using a syringe pump device to directly inject sample solutions.

The UV–Vis spectra were collected using a Jasco V530 instrument.

*General Procedure for the Preparation of Acyl Chloride (**1b–d**)*

Under nitrogen atmosphere, oxalyl chloride (16 mmol, 1.31 ml) was added dropwise to an ice cold stirred solution of the unsaturated fatty acid (2.47 mmol, 0.77 ml) in anhydrous toluene (10 ml). The reaction mixture was then allowed to reach room temperature and magnetically stirred for 3 h. The reaction was monitored by FT-IR. At the end of the reaction (3 h) the solution was evaporated under a vacuum (77 mbar, 40 °C) in an inert atmosphere and the acyl chloride was employed without further purification for the following reaction.

Oleoyl chloride. IR (NaCl) cm^{-1} 3005, 2926, 2855, 1801, 1464, 723.

Linoleoyl chloride. IR (NaCl) cm^{-1} 3010, 2928, 2857, 1801, 1464, 729.

Linolenoyl chloride. IR (NaCl) cm^{-1} 3011, 2961, 2930, 2857, 1801, 1463, 727.

*General Procedure for the Preparation of Quercetin Esters (**2, 3, 4 a–d**)*

Under nitrogen flow, the opportune acyl chloride (for molar ratio see Table 1) was added dropwise to a magnetically stirred ice cold solution of quercetin (0.3 mmol, 0.10 g) and TEA (2.47 mmol, 0.35 ml) in anhydrous dioxane

Table 1 Polyesters obtained by the modulation of quercetin and fatty acid molar ratio

Quercetin/fatty acid molar ratio	Stearoyl ester	Unsaturated fatty ester
1/10	Pentaester	Pentaester
1/7.5	Pentaester	Pentaester
1/6	Pentaester	Penta and tetraester
1/5	Pentaester	Tetraester
1/4	Pentaester[a]	Tetra and triester
1/3	Pentaester[a]	Tetra and triester[a]
1/2	Pentaester[a]	Tetra and triester[a]
1/1	Pentaester[a]	Tetra and triester[a]

[a] And unreacted quercetin

(10 ml). The reaction mixture obtained was then magnetically stirred while being protected from daylight at room temperature for 20 h. After solvent evaporation under a vacuum (107 mbar, 40 °C), the residue was dissolved in chloroform (20 ml), washed with a saturated aqueous $NaHCO_3$ solution (20 ml) and then with water (3 × 20 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

2-(3,4-Distearoylossiphenyl)-4-oxo-4H-chromene-3,5,7-tristearate (2a). Recrystallization from ethanol gave a white solid with 57 % yield. IR (KBr) cm^{-1} 2957, 2918, 2850, 1769, 1641, 1618, 1468, 1438, 1266, 1233, 1208, 1178, 1112, 990, 721. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.70 (dd, 1 H, J = 8.3, 1.8 Hz), 7.66 (d, 1 H, J = 1.8 Hz), 7.32 (d, 1 H, J = 8.3 Hz), 7.30 (d, 1 H, J = 2.2 Hz), 6.83 (d, 1 H, J = 2.2 Hz), 2.73 (t, 2 H, J = 7.6 Hz), 2.62–2.52 (m, 8 H), 1.78–1.71 (m, 10 H), 1.55–1.26 (m, 100 H), 0.88 (t, 15 H, J = 5.6 Hz). ^{13}C NMR ($CDCl_3$) δ 172.2, 171.0, 170.9, 170.8, 170.2, 157.1, 154.5, 153.8, 150.8, 144.7, 142.5, 134.3, 128.0, 126.6, 124.0, 115.0, 114.1, 109.1, 34.6, 34.3, 34.3, 34.0, 32.2, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.4, 29.3, 25.1, 24.9, 24.8, 24.7, 22.9, 14.3. ESI–MS: $C_{105}H_{180}O_{12}$, calc 1634.5, found m/z (%) = 1635 $[M + H]^+$. UV/Vis ($CHCl_3$) 261, 298 nm.

2-(3,4-Dioleoylossiphenyl)-4-oxo-4H-chromene-3,5,7-trioleate (2b). A light yellow liquid, obtained in 60 % yield. IR (NaCl) cm^{-1} 3005, 2925, 2854, 1770, 1653, 1632, 1466, 1434, 1266, 1234, 1205, 1178, 1114, 723. ^{1}H NMR(500 MHz, $CDCl_3$) δ 7.73 (dd, 1 H, J = 8.5, 2.0 Hz), 7.69 (d, 1 H, J = 2.0 Hz), 7.34 (d, 1 H J = 8.5 Hz), 7.33 (d, 1 H J = 2.2 Hz), 6.86 (d, 1 H, J = 2.2 Hz), 5.4–5.3 (m, 10 H), 2.75 (t, 2 H, J = 7.6), 2.64–2.58 (m, 8 H), 2.05 (m, 20 H), 1.82–1.70 (m, 10 H), 1.48–1.25 (m, 100 H), 0.91 (t, J = 6.6 Hz, 15 H). ^{13}C NMR δ 171.3, 170.1, 170.0, 169.9, 169.8, 169.3, 156.2, 153.6, 152.9, 149.9, 143.8, 141.6, 133.4, 129.4, 129.3, 129.1 (2 × C), 129.0, 128.9, 127.1, 125.7, 123.1 (2 × C), 114.1, 113.2, 108.1, 33.7, 33.4 (2 × C), 33.3, 33.1, 31.3, 29.1 (2 × C), 29.0(3 × C), 28.8, 28.7, 28.6 (3 × C), 28.5 (4 × C), 28.4 (4 × C), 28.3, 26.5 (2 × C), 26.4 (2 × C), 24.2, 24.1, 24.0, 23.9, 23.7, 22.0 (3 × C), 13.4. ESI–MS: $C_{105}H_{170}O_{12}$, calc 1624.5, found m/z = 1647 $[M + Na]^+$. Rt = 2.93 min. UV/Vis ($CHCl_3$) 250, 296 nm.

2-(3,4-Dioleoylossiphenyl)-4-oxo-4H-chromene-5-hydroxy-3,7-dioleate (3b). Separation from compound **4b** by column chromatography was performed using a mixture hexane/acetone 9:1 giving a light yellow liquid in 65 % yield. IR (NaCl) cm^{-1} 3008, 2922, 2853, 1767, 1651, 1605, 1488, 1468, 1297, 1224, 1203, 1187, 1114, 723. ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.15 (s, 1 H, exch. D_2O), 7.75 (dd, 1 H, J = 2.1, 8.5 Hz), 7.73 (d, 1 H, J = 2.1 Hz), 7.37 (d, 1 H, J = 8.5 Hz), 6.87 (d, 1 H, J = 2.0 Hz), 6.61 (d, 1 H, J = 2.0 Hz), 5.41-5.35 (m, 8 H,), 2.65 (t, 2 H, J = 7.6), 2.61–2.57 (m, 6 H), 2.05 (m, 16 H), 1.81–1.74 (m, 8 H), 1.44–1.29 (m, 80 H), 0.90 (t, 12 H, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$) δ 175.6, 170.4, 169.9, 169.9, 169.8, 161.0, 155.8, 155.3, 154.8, 144.1, 141.7, 131.6, 129.4, 129.3, 129.2, 129.0, 128.9 (2 × C), 126.8, 125.8, 123.3, 123.2, 108.1, 104.8, 100.4, 33.7, 33.4, 33.3, 33.0, 31.2, 29.1, 29.0 (2 × C), 28.9, 28.8, 28.6 (2 × C), 28.5 (2 × C), 28.4 (2 × C), 28.3 (3 × C), 26.6, 26.5, 26.4, 24.2, 24.1 24.0, 23.9, 21.9, 13.4. ESI–MS: $C_{87}H_{138}O_{11}$, calc 1360.0, found m/z = 1383 $[M + Na]^+$. Rt = 2.49 min. UV/Vis ($CHCl_3$) 269, 337 nm.

2-(3,4-Dioleoylossiphenyl)-4-oxo-4H-chromene-3,5-dihydroxy-7-oleate (4b). Separation from compound **3b** by column chromatography was performed using a mixture hexane/acetone 9:1 giving a light yellow liquid in 15 % yield. IR (NaCl) cm^{-1} 3322, 3099, 3005, 2959, 2854, 1765, 1647, 1603, 1563, 1489, 1466, 1308, 1263, 1179, 1111, 722. ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.65 (s, 1 H, exch. D_2O), 8.14 (dd, 1 H, J = 8.6, 2.1), 8.08 (d,1 H, J = 2.1 Hz), 7.38 (d, 1 H, J = 8.6 Hz), 6.90 (d, 1 H, J = 2.0 Hz), 6.8 (s, 1 H, exch. D_2O), 6.59 (d, 1H, J = 2.0 Hz), 5.40–5.31 (m, 8 H), 2.06–2.57 (m, 6 H,), 2.06–2.02 (m, 12 H,), 1.82–1.78 (m, 6 H), 1.48–1.28 (m, 60 H,), 0.92 (t, 9 H, J = 5.2 Hz).^{13}C NMR ($CDCl_3$) δ 175.1, 170.5, 170.2, 169.9, 159.9, 155.8, 154.9, 143.8, 143.2, 141.7, 136.4, 129.4, 129.0, 128.9, 128.1, 125.3, 123.1, 122.4, 106.3, 103.9, 100.6, 33.7, 33.4, 33.3, 31.2, 29.1, 29.0, 28.8, 28.6, 28.5, 28.4, 28.3 (2 × C), 26.5, 26.4, 24.2, 24.1, 21.9, 13.4. ESI–MS: $C_{69}H_{106}O_{10}$, calc 1095.6, found m/z = 1094 $[M - H]^+$. Rt = 3.58 min. UV/Vis ($CHCl_3$) 250, 271, 316, 366 nm.

2-(3,4-Dilinoleoylossiphenyl)-4-oxo-4H-chromene-3,5,7-trilinoleate (2c). A light yellow liquid obtained in 85 % yield. IR (NaCl) cm^{-1} 3009, 2926, 2855, 1771, 1653, 1622, 1464, 1433, 1177, 1115, 724. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.71 (dd, 1 H, J = 8.2, 1.8 Hz), 7.57 (d, 1 H, J = 1.8 Hz), 7.33 (d, 1 H, J = 8.2 Hz,), 7.32 (d,1 H, J = 2.4 Hz), 6.84 (d, 1 H, J = 2.4 Hz), 5.46–5.26 (m, 20 H), 2.77 (t, 10 H, J = 5.5 Hz), 2.69–2.41 (m, 10 H), 2.10 (m, 20 H), 1.78–1.71 (m, 10 H), 1.36–1.07 (m, 70 H), 0.89 (t, 15 H, J = 6.1 Hz). ^{13}C NMR δ 172.2, 171.0, 170.8, 170.7, 170.2, 169.8, 157.1, 154.5, 153.8, 150.81, 144.7, 142.5, 134.3, 130.5-130.4, 130.4, 130.3, 130.3, 130.2, 130.2, 130.1, 128.4, 128.3, 128.3, 128.2, 128.2, 128.1, 128.1, 128.0, 128.0, 126.6, 124.0, 115.0, 114.1, 109.0, 35.5, 34.6, 34.2, 34.1, 34.0, 31.7, 29.9, 29.8,29.6, 29.5, 29.4, 29.2, 29.1, 27.4, 25.9, 25.11, 24.9, 24.8, 24.7, 24.4, 22.8, 13.3. ESI–MS: $C_{105}H_{160}O_{12}$, calc 1614.2, found m/z (%) = 1615 $[M + H]^+$. Rt = 3.92 min. UV/Vis ($CHCl_3$) 281 nm.

2-(3,4-Dilinoleoylossiphenyl)-4-oxo-4H-chromene-5-hydroxy-3,7-dilinoleate (3c). Separation from compound **4c** by column chromatography, eluent hexane/

acetone 9:1 gave a light yellow liquid in 56 % yield. IR (NaCl) cm^{-1} 3010, 2927, 2856, 1772, 1655, 1615, 1465, 1434, 1266, 1236, 1196, 1179, 1128, 724. ^1H NMR (200 MHz, $CDCl_3$) δ 12.12 (s, 1 H, exch. D_2O), 7.72 (dd, 2 H, $J = 2.1$, 8.2 Hz), 7.34 (d, 1 H, $J = 8.2$ Hz), 6.84 (d, 1 H, $J = 2.1$ Hz), 6.58 (d, 1 H, $J = 1.8$ Hz), 5.45–5.29 (m, 16 H), 2.77 (t, 8 H, $J = 5.5$ Hz), 2.66–2.53 (m, 8 H), 2.00 (m, 16 H), 1.75–1.56 (m, 8 H), 1.29–1.25 (m, 56H), 0.88 (t, 12 H, $J = 6.8$ Hz). ^{13}C NMR ($CDCl_3$) δ 176.5, 171.3,170.8, 170.8, 170.7, 161.9, 156.7, 156.2, 155.7, 145.0, 142.6, 132.5, 130.5, 130.4, 130.3, 130.2, 130.1, 128.4, 128.3, 128.2, 128.1, 128.1, 127.7, 126.7, 124.2, 108.9, 105.7, 101.4, 34.6, 34.3, 34.3, 33.9, 31.7, 29.8, 29.6, 29.5, 29.4, 29.3, 29.2, 27.4, 25.9, 25.1, 24.9, 24.8, 22.8, 14.3. ESI–MS: $C_{87}H_{130}O_{11}$, calc 1351.9, found $m/z = 1374$ $[M + Na]^+$. Rt = 2.57 min. UV/Vis ($CHCl_3$) 273 nm.

2-(3,4-Dilinoleoylossiphenyl)-4-oxo-4H-chromene-3,5-dihydroxy-7-linoleate (4c). Separation from compound **3c** by column chromatography, eluent hexane/acetone 9:1 gave a light yellow liquid in 20 % yield. IR (NaCl) cm^{-1} 3351, 3009, 3005, 2927, 2856, 1771, 1652, 1606, 1492, 1466, 1307, 1268, 1194, 1133, 724. ^1H NMR (200 MHz, $CDCl_3$) δ 11.64 (s, 1 H, exch. D_2O), 8.12 (dd, 1 H, $J = 8.5$, 2.1 Hz,), 8.05 (d, 1 H, $J = 2.1$ Hz), 7.36 (d, 1 H, $J = 8.5$ Hz), 6.88 (d, 1 H, $J = 2.1$ Hz), 6.70 (s, 1 H, exch. D_2O), 6.57 (d, 1 H, $J = 2.1$ Hz), 5.45–5.26 (m, 12 H), 2.77 (t, 6 H, $J = 5.5$ Hz), 2.66–2.53 (m, 6 H), 2.07–2.00 (m, 12 H), 1.75–1.59 (m, 6 H,), 1.30–1.21 (m, 42 H), 0.88 (t, 9 H, $J = 6.7$ Hz). ^{13}C NMR ($CDCl_3$) δ 176.5, 171.4, 171.1, 170.9, 160.9, 156.7, 155.9, 144.7, 144.1, 142.6, 137.4, 130.5, 130.3, 130.2, 130.1, 129.0, 128.4, 128.3, 128.1, 126.8, 124.0, 123.3, 107.2, 104.8, 101.5, 34.6, 34.3, 34.2, 31.7, 29.8, 29.6, 29.5, 29.4, 29.3, 29.2, 27.4, 25.9, 25.2, 25.0, 22.8, 14.3. ESI–MS: $C_{69}H_{100}O_{10}$, calc 1088.7, found $m/z = 1090$ $[M + H]^+$. Rt = 4.13 min. UV/Vis ($CHCl_3$) = 269, 311, 359 nm.

2-(3,4-Dilinolenylossiphenyl)-4-oxo-4H-chromene-3,5,7-trilinolenate (2d). A light yellow liquid obtained in 74 % yield. IR (NaCl) cm^{-1} 3011, 2960, 2929, 2855, 1772, 1655, 1621, 1463, 1434, 1177.68, 1123, 758, 723. ^1H NMR (200 MHz, $CDCl_3$) δ 7.70 (dd, 1 H, $J = 8.5$, 1.8 Hz), 7.65 (d, 1 H, $J = 1.8$ Hz), 7.32 (d, 1 H, $J = 8.5$ Hz), 7.31 (d, 1 H, $J = 2.1$ Hz), 6.84 (d, 1 H, J = 2.1 Hz), 5.58–5.24 (m, 30 H), 2.84 (t, 20 H, $J = 5.8$ Hz), 2.65–2.55 (m, 10 H), 2.26–2.00 (m, 20 H), 1.77–1.59 (m, 10 H), 1.36–1.21 (m, 40 H), 0.94 (t, 15 H, $J = 7.6$ Hz). ^{13}C NMR ($CDCl_3$) δ 172.2, 171.0, 170.8, 170.7, 170.1, 157.1, 154.5, 153.9, 150.8, 144.7, 142.5, 134.3, 130.5, 130.4, 130.3, 130.2, 130.1, 128.5, 128.4, 128.4, 128.2, 128.1, 127.9, 127.3, 126.6,124.1, 115.0, 114.1, 109.1, 35.5, 34.6, 34.3, 34.3, 33.9, 29.8, 29.7, 29.6, 29.5, 29.3, 29.4, 29.1, 27.4, 25.1, 24.9, 24.8, 24.7, 24.4, 22.8, 20.8, 14.5, 14.5. ESI–MS: $C_{105}H_{150}O_{12}$, calc 1604.3, found $m/z = 1605$ $[M + H]^+$. Rt = 3.54 min. UV/Vis ($CHCl_3$) 260, 284 nm.

2-(3,4-Dilinolenylossiphenyl)-4-oxo-4H-chromene-5-hydroxy-3,7-dilinolenate (3d). Separation from compound **4d** by column chromatography, eluent hexane/acetone 9:1 gave a light yellow liquid in 48 % yield. IR (NaCl) cm^{-1} 3011, 2960, 2929, 2855, 1772, 1655, 1615, 1488, 1455, 1266, 1236, 1197, 1127, 722. ^1H NMR (200 MHz, $CDCl_3$) δ 12.12 (s, 1 H, exch. D_2O), 7.73 (dd, 2 H, $J = 2.1$, 8.2 Hz), 7.34 (d, 1 H, $J = 8.2$ Hz), 6.84 (d, 1 H, $J = 2.1$ Hz), 6.58 (d, 1 H, $J = 2.1$ Hz), 5.46–5.24 (m, 24 H), 2.81 (t, 16 H, $J = 5.5$ Hz), 2.66–2.52 (m, 8 H), 2.15–2.00 (m, 16 H), 1.78–1.56 (m, 8 H), 1.27–1.21 (m, 32 H), 0.94 (t, 12 H, $J = 7.6$ Hz). ^{13}C NMR ($CDCl_3$) δ 176.5, 171.3, 170.9, 170.8, 170.7, 161.9, 156.7, 156.2, 155.7, 145.0, 142.6, 132.5, 132.2, 130,5, 130.4, 130.3, 130.2, 130.1, 128.6, 128.5, 128.5, 128.4, 128.23, 128.1, 128.0, 127.9, 127.7, 127.3, 126.7, 124.2, 108.9, 105.7, 101.4, 34.6, 34.3, 34.3, 33.9, 29.8, 29.6, 29.5, 29.4, 29.3, 29.2, 27.4, 25.9, 25.8, 25.1, 24.9, 24.8, 22.8, 14.5, 14.5. ESI–MS: $C_{87}H_{122}O_{11}$, calc 1343.9, found: m/z 1346 $[M + H]^+$. Rt = 2.46 min. UV/Vis ($CHCl_3$) 269, 301, 340 nm.

2-(3,4-Dilinolenylossiphenyl)-4-oxo-4H-chromene-3,5-dihydroxy-7-linolenate (4d). Separation from compound **3d** by column chromatography, eluent hexane/acetone 9:1 gave a yellow liquid in 15 % yield. IR (NaCl) cm^{-1} 3369, 3011, 2960, 2928, 2855, 1771, 1652, 1606, 1491, 1464, 1194, 1128, 723. NMR (200 MHz, $CDCl_3$) δ 11.64 (s, 1 H, exch. D_2O), 8.12 (dd, 1 H, $J = 8.5$, 1.8 Hz,), 8.05 (d, 1 H, $J = 1.8$ Hz), 7.36 (d, 1 H, $J = 8.5$ Hz), 6.89 (d, 1 H, $J = 1.8$ Hz), 6.84–6.69 (s, 1 H, exch. D_2O), 6.57 (d, 1H, $J = 1.8$ Hz), 5.46–5.31 (m, 18 H), 2.81 (t, 12 H, $J = 5.5$ Hz), 2.62–2.53 (m, 6 H), 2.15–2.01 (m, 12 H), 1.76–1.56 (m, 6 H), 1.26–1.17 (m, 24 H), 0.98 (t, 9 H, $J = 7.6$ Hz).^{13}C NMR ($CDCl_3$) δ 176.1, 171.4, 171.1, 170.9, 160.9, 156.7, 155.9, 144.7, 144.1, 142.6, 137.3, 132.2, 130,4, 129.0, 128.5, 128.4, 128.1, 127.3, 126.2, 124.0, 123.3, 107.2, 104.8, 101.5, 34.6, 34.3, 34.2, 29.8, 29.6, 29.4, 29.3, 27.4, 25.9, 25.8, 25.1, 25.0, 20.9, 14.5. ESI–MS: $C_{69}H_{94}O_{10}$, calc 1083.5, found $m/z = 1085$ $[M + H]^+$. Rt = 3.92 min. UV/Vis ($CHCl_3$) 270, 313, 362 nm.

*Synthesis of 2-(3,4-Distearolylossiphenyl)-4-oxo-4H-chromene-5-hydroxy-3,7-distearate (**3a**)*

Under nitrogen flow, stearoyl chloride (1.32×10^{-3} mol, 0.45 ml) was added dropwise to a magnetically stirred ice cold solution of quercetin (0.3 mmol, 0.10 g) and TEA (2.47 mmol, 0.35 ml) in anhydrous dioxane (10 ml). The solution was magnetically stirred at 5 °C for 3 h and then evaporated to dryness under vacuum (107 mbar, 40 °C). The obtained residue was dissolved in chloroform (20 ml)

and washed with a saturated aqueous $NaHCO_3$ solution (20 ml) and then with water (3 × 20 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure, leaving a light yellow solid in 53 % yield. IR (KBr) cm^{-1} 3436, 2957, 2918, 2850, 1766, 1655, 1616, 1468, 1438, 1266, 1234, 1195, 1178, 1145. ^1H NMR (200 MHz, $CDCl_3$) δ 12.12 (s, 1 H, exch. D_2O), 7.72 (dd, 2 H, $J = 2.1$, 8.4 Hz), 7.35 (d, 1 H, $J = 8.4$ Hz), 6.84 (d, 1 H, $J = 2.1$ Hz), 6.58 (d, 1 H, $J = 2.1$ Hz), 2.67–2.52 (m, 8 H), 1.75–1.71 (m, 8 H), 1.26–1.05 (m, 112 H), 0.88 (t, 12 H, $J = 6.1$ Hz). ^{13}C NMR ($CDCl_3$) δ 176.5, 171.3, 170.9, 170.8, 170.2, 161.9, 156.7, 156.2, 155.7, 145.0, 142.6, 132.5, 127.7, 126.6, 124.2, 108.9, 105.7, 101.3, 34.6, 34.3, 34.2, 33.9, 32.1, 29.9, 29.74 29.6, 29.5, 29.4, 29.4, 29.3, 25.1, 24.9, 24.7, 22.9, 14.3. ESI–MS: $C_{87}H_{146}O_{11}$, calc 1368.1, found m/z = 1369 $[M + H]^+$. UV/Vis ($CHCl_3$) 260, 300 nm.

Theoretical Calculations

DFT calculations were performed using the Gaussian 09 package [20]. Starting geometries were initially obtained through a conformational search conducted in vacuo using the Low Mode molecular dynamics method and the MMFF94× force field implemented in MOE [21]. The eight most stable conformers, included in an energy range of 2 kcal mol^{-1}, were completely optimized in vacuo at the mPW1B95/6-31+G (d,p) level [22], a level of theory that provided excellent results in previous works from our group [23–25]. Moreover the frequencies were calculated at the same level to confirm the minimized structures as a minimum (no imaginary frequencies). NMR computations were performed on the most stable conformations at the mPW1B95/TZVP level. As in other works this basis set provided the best results in terms of accuracy/computation time [26, 27]. The absolute shieldings of penta, tetra and triesters were calculated using the GIAO method [28, 29]. All calculations were performed in the gas phase. As in previous works the inclusion of the solvent provided no improvements over the general quality of results, but resulted in severely more expensive computation time [26].

Antioxidant Activity

The Trolox equivalent antioxidant capacity (TEAC) measures the ability of a compound to scavenge the ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as radical cation, $ABTS^{\bullet+}$, a soluble chromogen that can be determined spectrophotometrically at 734 nm.

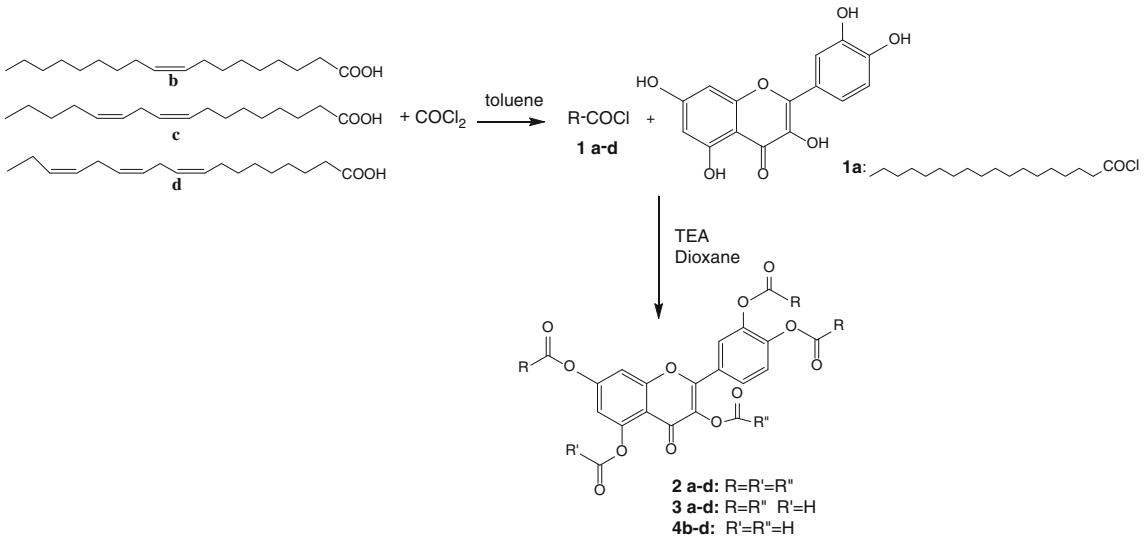
In the presence of antioxidants the radical cation is suppressed to an extent dependent on the activity of the antioxidant and the color intensity is decreased proportionally.

The assay is performed with the Antioxidant Assay Kit, as suggested by the producer. Trolox, a water-soluble vitamin E analogue, serves as a standard.

Results are expressed as TEAC g^{-1} of dry powder (TEAC, μmol g^{-1}) and are the means ± SD of three different experiments performed in triple.

The DPPH assay utilizes the stable DPPH free radical that produces a purple color with strong absorbance at 517 nm [31]. When DPPH is placed in an assay system containing free radical scavengers, the color vanishes: therefore the change in absorbance is a measurement of antioxidant scavenging capacity of test samples.

Results are expressed as the sample concentration that quenches the 50 % of DPPH radical (IC_{50}, μg ml^{-1}), calculated by linear regression, plotting together data obtained



Scheme 1 Synthesis of total and partial quercetin esters with stearoyl chloride (**a**), oleic (**b**), linoleic (**c**) and linolenic (**d**) acids

by at least two different experiments performed in duplicate

Statistical analysis was performed between the TEAC activity of the different derivatives by unpaired Student's *t* test.

Results and Discussion

Synthesis of Quercetin Fatty Esters

As reported in Scheme 1 the quercetin pentaesters were easily synthesized following the well-established esterification procedure by the reaction between quercetin and the proper acyl chloride, obtained from the corresponding carboxylic acid with an excess of oxalyl chloride in anhydrous toluene. Furthermore, stearoyl pentaester was synthetized from stearoyl chloride commercially available.

As summarized in Table 1, quercetin and unsaturated acyl chloride molar ratio modulation allowed us to obtain a mixture of tetra and triesters which were then purified by column chromatography. As reported in the literature [31] for quercetin pivaloyl esters, the tetrastearoyl derivative was obtained modulating the reaction temperature at 0 °C instead of room temperature and not in the reagents' molar ratio.

Molecular Characterization

In FT-IR spectra, the C=O stretching of the carboxylic acids, acyl chlorides and quercetin fatty esters allowed us to verify the esterification; in fact, this band shifted from 1,710 to 1,810 to 1,770 cm^{-1} respectively. In addiction in all the synthetized compounds at about 1,654 cm^{-1} is shown the quercetin C=O stretching. Quercetin shows intense absorption broad bands between 3,445 and 3,000 cm^{-1} due to O–H stretching, which disappear in the pentaesters; while in the tetraester and mainly in the triester, a broad band between 3,445 and 3,000 cm^{-1} is evident.

The full mass spectra of compounds **3a–d** and **4a–d** showed a molecular ion peak in agreement with the tetra and the triester respectively.

In order to identify the unesterified –OH groups, quercetin pentaoleyl ester **2b**, tetraoleyl ester **3b** and trioleyl ester **4b** were analyzed by bi-dimensional NMR techniques, such as COSY, HMBC and HSQC. The ^{1}H-NMR shifts of the tetraoleyl ester **3b** showed for H_6 (6.61 ppm) and H_8 (6.87 ppm) a downfield shift compared with that of the corresponding pentaester (6.86 and 7.31 ppm respectively); furthermore, the ^{13}C-NMR analysis showed that 4-C (174.60 ppm) had a higher frequency in comparison with that in the pentaester (169.25 ppm) suggesting that the hydrolyzed –OCO group is in the five position. This hypothesis, in agreement with the literature data [31] for quercetin tetracetyl esters, was confirmed by HMCQ and HSQC analyses.

The identification of free hydroxyl groups in the trioleoyl ester **4b** led to ambiguous results. The H_6, H_8 and 4-C shifts confirmed that the OH on 5-C was free, while the comparison between the $H_{6'}$, $H_{2'}$, and $H_{5'}$ shifts in penta and tetraester (7.73, 7.69 and 7.32 ppm) with that in **4b** ^{1}H spectrum (8.14, 8.08 and 7.38 ppm) allow us to hypothesize that the second free –OH group could be in either the 3′ or 4′ position; not even ^{13}C- and 2D-NMR techniques were able to distinguish between them.

Quantum chemical NMR calculation were then performed on 3,5,7,3′,4′-pentaester, 3,7,3′,4′-tetraester, 3,7,3′ triester, 3,7,4′-triester and 7,3,4′-triester to assist the experimental structural assignment. Indeed, this technique was successfully used by our group to solve similar questions [26, 27]. Calculations on long and highly flexible fatty acid quercetin esters could not be performed, as the huge conformational flexibility would have made both of the calculations too expensive, in terms of CPU time, as well as the interpretation of results more difficult, due to the large noise introduced by the side-chain signals. Concerned that discriminating signals for the mentioned esters are principally those of the quercetin nucleus, for the above reported reason, we adopted the acetyl esters as a model for NMR calculations. Beside the triesters whose structure was equivocal, the penta and tetraacetyl esters were also included in the study to test the general reliability of the method and to eventually rule out systematical errors. A conformational analysis (see "Experimental" for details) was performed and the eight most stable conformation were selected for further geometry optimization by density functional theory (DFT) at the mPW1B95/6-31+G(d,p) level of theory [22]. Preliminary NMR calculations were conducted on tetra and pentaesters by computing ^{13}C absolute shieldings for the most stable conformation, as resulted from DFT calculation, or for selected conformations (from 2 to 8), where average ^{13}C absolute shieldings were obtained. Theoretical results were then compared to the experimental results, although no significant variation in the computed NMR values was observed within the "multi conformation" or the "single conformation" methods, so the less computationally expensive latter one was chosen for our analysis.

Both ^{13}C absolute shieldings sets calculated for 3,5,7,3′,4′-pentaacetyl quercetin and 3,7,3′,4′-tetraacetyl quercetin were compared with the corresponding experimental chemical shifts through a linear regression analysis. As shown in Fig. 1, the calculated regression coefficient (R^2) is quite good (0.9925), proving that the chosen computational method performs well in predicting NMR values for the systems reported herein.

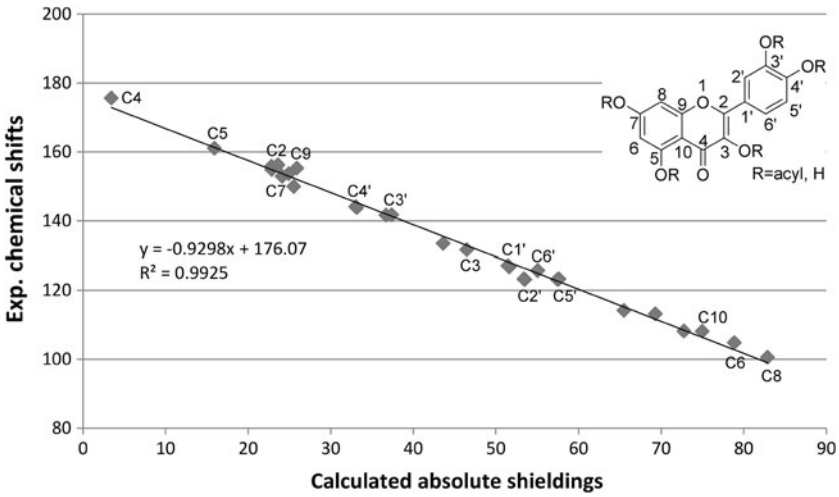


Fig. 1 Correlation between experimental ^{13}C chemical shifts (ppm) and absolute shieldings calculated at the mPW1B95/TZVP//mPW1B95/6-31+G(d,p) level for 3,5,7,3′,4′-pentaacetyl quercetin and 3,7,3′,4′-tetraacetyl quercetin

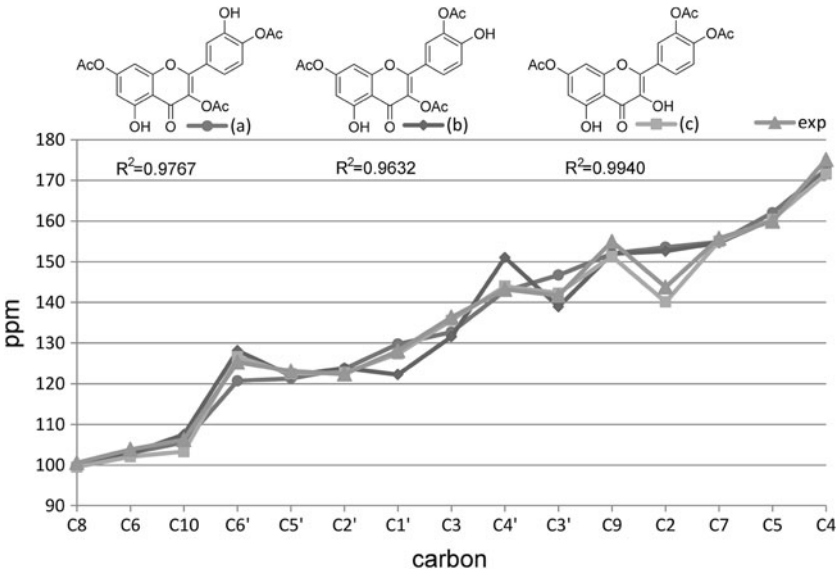


Fig. 2 Comparison between experimental ^{13}C chemical shifts (ppm) of **4b** and theoretical ^{13}C chemical shifts computed for the hypothetical quercetin triesters (*a*), (*b*) and (*c*). Correlation coefficients (R^2) between experimental and theoretical chemical shifts are also reported

The linear equation obtained was then used to convert the absolute shieldings, computed for the hypothesized triesters 3,7,4′-triacetyl quercetin (a), 3,7,3′-triacetyl quercetin (b) and 7,3′,4′-triacetyl quercetin (c), into chemical shifts directly comparable with those experimentally obtained. As can be clearly observed in Fig. 2, the only theoretical set that fitted perfectly with experimental ^{13}C-NMR chemical shifts is the one obtained from the triester (c) ($R^2 = 0.9940$). Indeed, compounds (a) and (b) ($R^2 = 0.9767$ and 0.9632, respectively) could be safely ruled out due to the severely discordant values computed for 6′-C, 1′-C, 3′-C, 4′-C and most of all 2-C. Indeed, the chemical shift for this last carbon atom is particularly low if compared to the corresponding values computed for (a) and (b), suggesting that 2-C is more severely shielded by the –OCO-hydrolysis at 3-C than that at either 3′-C or 4′-C.

In Table 2 the ^{1}H and ^{13}C chemical shifts assignment for quercetin oleic esters are summarized.

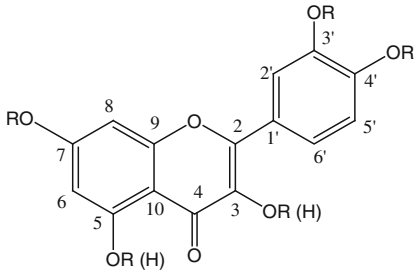
The purity of all the synthetized compounds was evaluated by HPLC/DAD analysis.

Antioxidant Activity

In order to evaluate the antioxidant power of different quercetin fatty polyesters, we performed two common and reliable in-vitro assays, i.e. ABTS, expressed as TEAC, and the stable DPPH radical, expressed as IC_{50}. Results are reported in Table 3; with both assays the quercetin esters display a lower antioxidant activity respect to free quercetin; in fact they display a high IC_{50} and a low TEAC both indicating a lower antioxidant capacity.

On the other hand, quercetin triesters with oleic, linoleic and linolenic fatty acids displayed a higher antioxidant activity with respect to their corresponding pentaester compounds. The trilinolenoyl quercetin esters display the highest antioxidant power even if still 20 times lower than

Table 2 Chemical shifts (δ) of the aromatic protons and carbons of pentaoleoyl quercetin (**2b**), 3,3′,4′,7-tetraoleoyl quercetin (**3b**) and 3′,4′,7 trioleoyl quercetin (**4b**) in $CDCl_3$



Compound	δ (H-6)	δ (H-8)	δ (H-2′)	δ (H-5′)	δ (H-6′)
2b	6.86	7.31	7.69	7.32	7.73
3b	6.61	6.87	7.73	7.37	7.75
4b	6.59	6.90	8.08	7.38	8.14

Compound	δ (4-C)	δ (3-C)	δ (2-C)	δ (1′-C)	δ (3′-C)	δ (4′-C)
2b	169.25	133.42	152.9	127.09	141.61	143.79
3b	175.60	131.57	154.83	126.78	141.67	144.09
4b	175.15	136.39	143.79	128.09	141.69	143.22

Table 3 Antioxidant capacity of the different fatty esters

Compounds	IC_{50} (μg ml^{-1})	TEAC mean ± SD (mg g^{-1})
Quercetin	4.72	1930.6 ± 127.5
2a	1101.90	4.64 ± 0.84
3a	990.20	1.82 ± 0.08*
2b	2693.00	1.26 ± 0.30
3b	2101.6	0.7 ± 0.28
4b	808.00	1.37 ± 0.10
2c	1853.70	0.55 ± 0.17
3c	1071.70	1.56 ± 0.15[b]
4c	224.42	1.26 ± 0.40[a]
2d	607.26	1.33 ± 0.21
3d	480.27	1.67 ± 0.60
4d	107.26	2.92 ± 0.16[c]

$n = 3$

* $p < 0.05$ vs 2A

[a] $p < 0.05$

[b] $p < 0.01$ vs 2C

[c] $p < 0.01$ vs 2D

quercetin. As evidenced by the results, the difference in antioxidant activity between penta and tetraesters with the same fatty acid depends upon the acyl chain. While with stearic acid the tetraester displays a lower TEAC compared to the pentaester, no differences were measured with oleic acid and an increase in antioxidant activity was measured for linoleic and linolenic tetraesters compared to the respective pentaester. These data are probably related to the free OH on 5-C of the tetraesters which is the least acidic and reactive due to intramolecular H-bonding with 4-C carbonyl; whereas the triesters, in which also OH at 3-C is free, showed a better antioxidant activity.

Conclusion

In conclusion, following a well-known procedure, we successfully prepared a series of new quercetin fatty pentaesters and a series of esters bearing a free OH group on 5-C or on 5-C and 3-C. Quantum chemical calculations allowed the unequivocal assignment of the free position in triesters through the comparison of theoretically predicted and experimental chemical shifts. The antioxidant activity of the new compounds was also evaluated, highlighting the relevance of the free OH on 3-C for biological activity.

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