

Synthesis, Characterization and Free Radical Scavenging Properties of Rosmarinic Acid Fatty Esters

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Abstract The hydrophobation of rosmarinic acid with saturated aliphatic primary alcohols of various chain lengths (methanol to eicosanol) was achieved via an acid-catalyzed esterification in the presence of a highly acidic sulfonic resin. The resulting alkyl rosmarinates were isolated, characterized and their global free radical scavenging activity was determined by the 2,2-diphenyl-1-picrylhydrazyl method in the stationary state. Only the dodecyl ester showed a stronger activity than rosmarinic acid.

Keywords Rosmarinic acid fatty esters · Syntheses · NMR and mass spectrometry characterization · Radical scavenging properties · DPPH · Stationary state

Introduction

Rosmarinic acid (RO) [(*R*)-a-[[3-(3,4-dihydroxyphenyl)-1-oxo-2*E*-propenyl]oxy]-3,4-dihydroxy-benzenepropanoic acid] is a polyphenolic compound present in high amount ($\geq 3\%$ w/w dry matter) in many herbal plants [1] of the

Lamiaceae family such as rosemary (*Rosmarinus officinalis*), lemon balm (*Melissa officinalis*), sage (genus *Salvia*), oregano (*Origanum vulgare*) or savory (genus *Satureja*). Several biological activities have been described for rosmarinic acid, the most known being anti-inflammatory [2] antiviral [3] and antioxidative [4, 5]. Concerning the later activity, rosmarinic acid as well as its naturally occurring methyl, ethyl and butyl esters have shown strong free radical scavenging properties, especially towards superoxide anion [6]. These properties make them good candidates to replace some current synthetic antioxidants (i.e. butylated hydroxytoluene and butylated hydroxyanisole) in foods and cosmetics, which are increasingly controversial [7]. However, the implementation of such polar molecules in lipid-based systems (emulsion or other) is difficult and can lead to a decrease of their ability to counteract oxidation of unsaturated lipids at the oil–water interface [8, 9]. To solve this issue, one strategy consists of adjusting their polarity by the grafting of aliphatic chains of increasing length [10, 11]. Herein, we reported for the first time the synthesis and characterization (^1H - ^{13}C -NMR and ESI-MS) of alkyl esters of rosmarinic acid with carbon chain lengths ranging from 1 to 20 carbon atoms (compounds **R1**–**R20**), and the evaluation of their global free radical scavenging activity by the well known 2,2-diphenyl-1-picrylhydrazyl (DPPH) method.

Materials and Methods

Materials

Sulfonic resin Amberlite® IR-120H, molecular sieve (3 Å), rosmarinic acid (96.5%), 2,2-diphenyl-1-picrylhydrazyl, *n*-butanol (99.8%), *n*-octanol (99%), *n*-dodecanol (99.5%),

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n-hexadecanol (99%), *n*-octadecanol (99%), *n*-eicosanol (98%) and solvents (analytical or HPLC grade) were purchased from Sigma-Aldrich (Saint Quentin, France). Silica (60–200 μm, pore size 60 Å) was from Acros organics (Geel, Belgium). Available immobilized lipase from *C. Antarctica* lipase B (Novozym® 435) was purchased from Novozymes A/S (Bagsvaerd, Denmark).

Methods

Chemical Synthesis and Purification of Alkyl Rosmarinates

The chemical esterification of rosmarinic acid (56 μmol) was carried out in sealed flasks each containing 5 mL of alcohol (methanol, 123.44 mmol; *n*-butanol, 54.64 mmol; *n*-octanol, 31.905 mmol; *n*-dodecanol, 22.46 mmol; *n*-hexadecanol, 16.95 mmol; *n*-octadecanol, 15.09 mmol and *n*-eicosanol, 13.6 mmol). The reaction mixtures were stirred (orbital shaker, 250 rpm, 55–70 °C) prior to the addition of the catalyst, the strongly acidic sulfonic resin Amberlite® IR-120H (5% w/w—total weight of both substrates) previously dried at 110 °C for 48 h. The water generated during the reaction was removed by absorption on molecular sieves (40 mg/mL) added to the medium. Samples (20 μL) were regularly withdrawn from the reaction medium then mixed with 980 μL of methanol, filtered (0.45 μm syringe filter Millex®-FH, Millipore Corporation Bedford, MA, USA) and finally analyzed by reverse phase HPLC with UV detection at 328 nm. After complete (4–21 days) conversion of rosmarinic acid into the corresponding ester the latter was purified in a two-step procedure. Firstly, a liquid–liquid extraction using hexane and acetonitrile was achieved to remove the excess of alcohol. Then, the remaining traces of the alcohol and rosmarinic acid were eliminated by flash chromatography on a CombiFlash® Companion® system (Teledyne Isco Inc., Lincoln, NE, USA). Separation was achieved on a silica column using an elution gradient of hexane and ether (20–100% in 35 min). The purity of the esters, obtained as pale yellow to yellow amorphous powders, was checked by HPLC and the absence of residual alcohol was confirmed by TLC.

HPLC Monitoring of Rosmarinic Acid Conversion

Monitoring of ester formation was carried out with a ACE® 5 C18 reversed phase column (5 μm, 250 × 4.6 mm, 100 Å) (ACT, Aberdeen, Scotland) using a Dionex Ultimate 3000 HPLC system (Dionex, Jouy En Josas, France). Peak integration was performed using Chromeleon software (Version 6.8). Briefly, gradient elution was performed using methanol and phosphoric acid 3 mM at 1 mL/min and 25 °C, in linear gradients from 0/100 (v/v) to 100/0 (v/v) for 30 min, then 100/0 (v/v) for 10 min and

back to 0/100 (v/v) in 5 min. Rosmarinic acid (R0) and its esters (R1–R20) were detected under UV light at 328 nm and their retention time, in minutes, were (R0): 19.6, (R1): 21.4, (R4): 25.4, (R8): 29.6, (R12): 32.5, (R16): 34.4, (R18): 35.5 and (R20): 36.7. As the alkyl rosmarinate were the only product of the reaction between rosmarinic acid and the alcohol, the molar yield of the ester formed was defined as follows:

$$\text{Molar yield (\% of ester)} = 100(\text{mol of ester}) / (\text{mol of ester} + \text{mol of remaining R0}).$$

It was calculated from the calibration curves established from purified esters. The reaction was stopped when the molar yield of the ester was at a maximum.

ESI-MS, ¹H-NMR and ¹³C-NMR Characterization

Pure esters were first characterized by electrospray ionisation-mass spectrometry (ESI-MS) in negative mode using a mass spectrometer composed of a quadrupole ion trap mass analyzer, an external atmospheric pressure ion source and an integrated syringe pump (Finnigan LCQ Serie MS detector, San Jose, CA, USA). Methanolic solutions of esters (100 mg/L) were directly injected into the ESI-source (infusion mode) at a 5-μL/min flow rate. The zoom scan mode was used for an accurate determination of the parent ion *m/z*, while fragment ions were obtained in MS² mode. Basic operating conditions in the negative mode were the following: collision energy of 30–35%, ionization voltage of 4.5 kV, capillary temperature of 300 °C and a sheath gas (nitrogen) flow of 20 arbitrary units.

¹H- and ¹³C-NMR spectra were recorded in DMSO with a Bruker DRX 400 spectrometer at 400 and 100 MHz respectively. In the case of alkyl rosmarinates represented as RCO–O–^αCH₂–^βCH₂–(CH₂)_{*n*-3}–CH₃, three types of methylene were distinguished in the alkyl chain for the attribution of chemical shifts: –^αCH₂– and –^βCH₂– correspond to methylene in alpha- and beta-position related to oxygen respectively, and –(CH₂)_{*n*-3}– correspond to the other methylene groups, where *n* is the total number of carbon of the alkyl chain.

Radical Scavenging Activity Assessment by the DPPH Method and Determination of Stationary Parameters

Basically, the DPPH method consists in measuring the ability of a molecule to reduce the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) in methanol and its subsequent bleaching at 515 nm. The radical scavenging activity of rosmarinic acid and its esters against DPPH[•] was performed spectrometrically at 515 nm and 20 °C, as described by Brand-Williams et al. [12] and recently applied to

chlorogenate fatty esters by López Giraldo et al. [13]. Briefly, the absorbance decay was monitored on solutions consisting in 1,950 μL methanolic DPPH \cdot solution (60 μM) and 50 μL methanolic solution of phenolic compound (14.6–585 μM). The absorbance decay was monitored each minute until it reached a steady state (14 h for rosmarinic acid and 6 h for its esters) that corresponds to the complete consumption of the antioxidant. In absence of antioxidant (blank), the spontaneous DPPH \cdot bleaching during the experiment appeared to be low and did not exceed 3–4% and 6–7% after 6 and 14 h respectively. However, a correction of this intrinsic DPPH \cdot bleaching was systematically made in order to avoid any bias. The global radical scavenging activity was defined as the total number of reduced DPPH \cdot at stationary state (n^{ss}) and expressed as a mol DPPH \cdot radical reduced by a mol of tested compound. It was calculated as $1/(2 \times \text{EC}_{50})$ where EC_{50} corresponds to the amount of antioxidant necessary to reduce the initial DPPH \cdot concentration by 50%. All determinations were done in triplicate.

Results and Discussion

Synthesis of Alkyl Ester of Rosmarinic Acid

In a first attempt, rosmarinic acid or its methyl ester were enzymatically esterified following a procedure already used in a previous work [10] for the esterification of chlorogenic acid (5-caffeoyl quinic acid, 5-CQA). However, none of the corresponding esters was obtained whatever the alcohol used and the reaction conditions. The reason for such a failure was attributed to the putative inhibition of the enzyme due to steric hindrance at the catalytic site. Then, alkyl rosmarinates were obtained by acid catalyzed esterification of rosmarinic acid with the corresponding saturated aliphatic primary alcohols (Fig. 1) in the presence of a highly acidic sulfonic resin. The reaction was achieved at low temperature (55–70 $^{\circ}\text{C}$) to avoid rosmarinic degradation, in an excess of the alcohol that played, when melted, both the role of substrate and solvent. The reaction was stopped after 4–21 days, when the molar yield of the ester has reached its maximum:

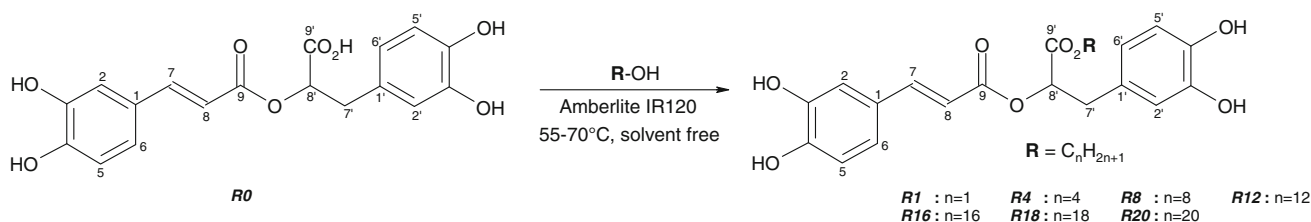


Fig. 1 Heterogeneous acid-catalyzed esterification of rosmarinic acid with aliphatic alcohols of various chain length

(**R1**): 98.5%, (**R4**): 99.3%, (**R8**): 99.5%, (**R12**): 94.4%, (**R16**): 81.6%, (**R18**): 99.0% and (**R20**): 99.0%. It corresponded to the quasi complete conversion of rosmarinic acid, except for hexadecyl rosmarinate where the maximum acid conversion was 82%. After the purification steps, the purity of esters was at least 96% and only traces of rosmarinic acid were detected by HPLC. The remaining 4% were attributed to the impurities already present in the starting material (rosmarinic acid, 96.5% purity) or to their derivatives, that could not be eliminated by flash chromatography.

NMR and MS Characterization of Alkyl Rosmarinates

Rosmarinic acid and its esters were first characterized by ^1H NMR and ^{13}C NMR (Table 1). Data obtained for rosmarinic acid, and its methyl and butyl esters were highly consistent with those reported in the literature [14–16]. For alkyl chains of more than four carbon atoms, ^1H NMR of $-\alpha\text{CH}_2-$, $-\beta\text{CH}_2-$ and other methylene group give multiplets with chemical shifts of 4.07–4.03, 1.54–1.51 and 1.37–1.23 ppm respectively. On the other hand, ^{13}C -NMR chemical shifts of the same methylene were respectively 64.6–64.3, 31.3–31.1 and 29.3–22.1 ppm.

ESI-MS spectra (full scan mode) of both rosmarinic acid and its esters (Table 2) showed two peaks corresponding to the molecular ion of the deprotonated compound [MM–H] and its adduct [2MM–H]. The MS 2 fragmentation of rosmarinic acid molecular ion (358.97) lead to three peaks at 197, 179 and 161 (m/z) corresponding to the deprotonated form of 3-(3,4-dihydroxyphenyl)lactic and caffeic acids and their dehydrated forms, respectively (Fig. 2; Table 2). These results were in agreement with the fragmentation scheme proposed by Møller et al. [17].

In case of alkyl rosmarinates, fragmentation of molecular ion always leads to only two peaks at 179 and 135 (m/z) whose correspond to the deprotonated form of caffeic acid and its residue after decarboxylation, respectively (Fig. 3; Table 2). The differences in fragmentation between rosmarinic acid and its esters may be due to the higher weakness of the ester bond at carbon 9 compared to carbon 9'.

Table 1 ^{13}C -NMR (100 MHz, DMSO) and ^1H -NMR (400 MHz, DMSO) data for rosmarinic acid (**R0**) and its esters (**R1–R20**)

C	$(\delta/\text{ppm})^{13}\text{C}$		$(\delta/\text{ppm})^1\text{H}$			
	R0	R1–R20	R0		R1–R20	
1	125.3	125.3	–	–	–	–
2	113.2	112.8	1H	7.08	s	7.07 s ^a
3	144.9	145.0	–	–	–	–
4	148.6	148.7	–	–	–	–
5	115.7	115.7	1H	6.80–6.78	d, $J = 9.2$ Hz	6.79–6.77 d ^a , $J = 8.1$ Hz
6	121.6	121.6	1H	7.04–7.02	d, $J = 8.5$ Hz	7.02–7.00 d, $J = 8.3$ Hz
7	145.9	146.2	1H	7.51–7.47	d, $J_{\text{trans}} = 16.9$ Hz	7.52–7.48 d, $J_{\text{trans}} = 15.8$ Hz
8	114.8	114.9	1H	6.28–6.24	d, $J_{\text{trans}} = 16.2$ Hz	6.28–6.24 d, $J_{\text{trans}} = 15.9$ Hz
9	165.9	165.9	–	–	–	–
1'	127.3	126.6	–	–	–	–
2'	116.7	116.6	1H	6.70–6.69	d, $J = 2.0$ Hz	6.68–6.67 d, $J = 2.0$ Hz
3'	145.6	145.6	–	–	–	–
4'	144.0	144.1	–	–	–	–
5'	115.4	115.3	1H	6.67–6.65	d, $J = 9.3$	6.66–6.64 d, $J = 8.0$ Hz
6'	120.0	120.0	1H	6.56–6.54	dd, $J = 8.1, 2.1$ Hz	6.52–6.50 dd, $J = 8.1, 1.9$ Hz
7'	36.1	36.2	2H	3.04–2.99, 2.96–2.90	dd, $J = 14.9, 4.5$ Hz	dd, $J = 14.5, 8.7$ Hz
8'	72.8	72.9	1H	5.07–5.04	dd, $J = 8.3, 4.6$ Hz	5.12–5.08 dd, $J = 7.2, 5.7$ Hz
9'	170.9	169.4	–	–	–	–
–$^{\alpha}\text{CH}_2$–	64.6–64.3	2H	–	–	–	4.07–4.03 m
–$^{\beta}\text{CH}_2$–	31.3–31.1	2H	–	–	–	1.54–1.51 m
–CH₂–	29.3–22.1	$x\text{H}^{\text{c}}$	–	–	–	1.37–1.23 m
–CH₃	13.91 (51.96 ^b)	3H	–	–	–	0.90–0.82 (3.69 ^b) m (s ^b)

^a s singlet, d doublet, m multiplet, dd doublet of doublet

^b Data for methyl rosmarinate (–OCH₃)

^c $x = 2(n-3)$ with n total number of carbon in the alkyl chain

Table 2 Negative ion and MS² data for rosmarinic acid (**R0**) and some of its esters

Compound (Id. no.)	MM (g mol ⁻¹)	Full scan (m/z) [MM–H] ^a	MS ²
Rosmarinic acid (R0)	360.31	358.97 (100) 718.90 (72)	161.04 (100) 179.02 (26) 197.01 (22)
Methyl rosmarinate (R1)	374.34	373.04 (100) 786.84 (58)	135.12 (26) 178.96 (100)
Butyl rosmarinate (R4)	416.42	415.11 (26) 831.21 (100)	135.14 (28) 178.97 (100)
Dodecyl rosmarinate (R12)	528.63	527.25 (52) 1,055.21 (100)	135.11 (23) 178.99 (100)
Octadecyl rosmarinate (R18)	612.79	611.36 (62) 1,223.36 (100)	135.09 (27) 178.95 (100)
Eicosyl rosmarinate (R20)	640.85	639.41 (58) 1,279.42 (100)	ND

MM molar mass, ND not determined

^a Value into brackets corresponds to percentage relative intensity

As a consequence, the fragmentation of molecular ion of each ester theoretically led to the breaking of this weaker ester bond into the deprotonated form of caffeic acid and

3-(3,4-dihydroxyphenyl) lactates. However, the fragment containing the alkyl chain was not detected under our working conditions.

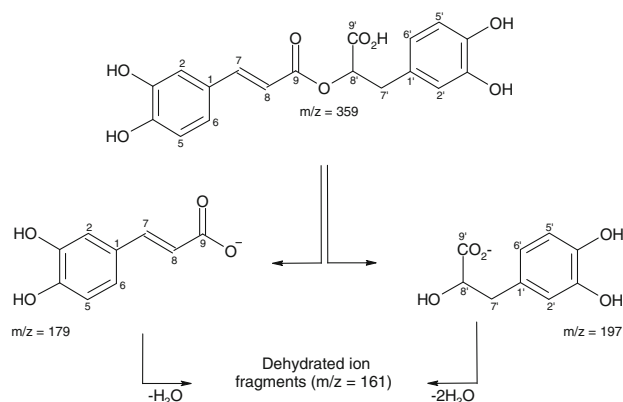


Fig. 2 MS² fragmentation scheme of rosmarinic acid as proposed by Møller et al. [17]

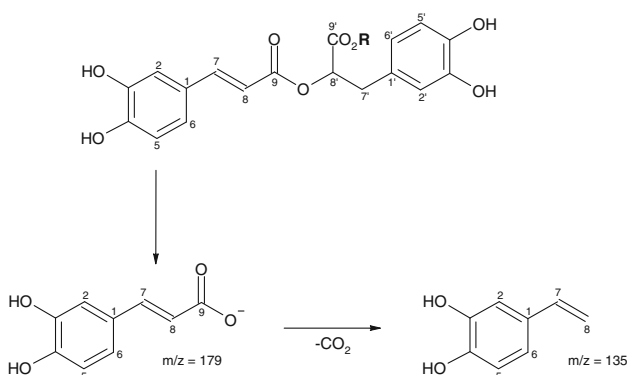


Fig. 3 MS² fragmentation scheme of alkyl rosmarinates

Table 3 Global radical scavenging capacity (n^{SS}) of rosmarinic acid (R0) and its esters (R1–R20) determined at the stationary state

Compound	Alkyl chain (–OC ₉ '–OR)	n^{SS}
R0	R=H	9.78 ± 0.06
R1	R=CH ₃ (methyl)	9.22 ± 0.07
R4	R=(CH ₂) ₃ CH ₃ (butyl)	8.61 ± 0.12
R8	R=(CH ₂) ₇ CH ₃ (octyl)	6.24 ± 0.02
R12	R=(CH ₂) ₁₁ CH ₃ (dodecyl)	12.36 ± 0.19
R16	R=(CH ₂) ₁₅ CH ₃ (hexadecyl)	9.22 ± 0.07
R18	R=(CH ₂) ₁₇ CH ₃ (octadecyl)	6.82 ± 0.28
R20	R=(CH ₂) ₁₉ CH ₃ (eicosyl)	6.35 ± 0.08

n^{SS} : mol DPPH radical reduced by mol of compound, at the stationary state

Data are means of triplicate values

Radical Scavenging Properties of Alkyl Rosmarinates

In terms of antioxidant properties, we reported in Table 3 the global free radical scavenging activity (n^{SS}) of rosmarinic acid and its ester determined by the DPPH method. The global free radical scavenging activity was defined as the total mole of DPPH reduced by mole of

antioxidant at stationary state. Surprisingly, it appeared that only dodecyl rosmarinate (R12) with a n^{SS} value of 12.36 ± 0.19 could reduce more DPPH[•] than rosmarinic acid ($n^{SS} = 9.78 \pm 0.06$).

Indeed, in a similar work on chlorogenic acid (5-CQA) and its alkyl esters [13] it was found that all chlorogenates exhibit higher n^{SS} values (6.2–8.5) than 5-CQA (4.9). However, it is worth mentioning that rosmarinic acid reached a steady state in 14 h, whereas all esters reached it in 6 h. Interestingly, such a strong influence of the esterification on the DPPH[•] bleaching was already observed with chlorogenic acid and its alkyl ester [13] and protocatechuic acid and its methyl ester [18]. In addition, it can be observed that all n^{SS} values were greater than 4 which is the number of available phenolic hydroxyl groups present in each tested molecule and known as being responsible for the antiradical activity. These results suggest, as proposed by other authors [19] the possible nucleophilic methanol regeneration of oxidized forms (quinone) of antioxidant into new reducing phenolic compounds and its combination with dimerization phenomena. In the future, further investigations on structure of reaction products will be required to determine the part of each mechanism involved according to alkyl chain length.

In conclusion, this work relates the first heterogeneous synthesis of a homologous series of alkyl rosmarinates of chain lengths ranging from 1 (methyl) to 20 (eicosyl) carbon atoms and their full characterization. In terms of global radical scavenging properties according to the DPPH method at the stationary state, it shows that the grafting of an alkyl chain to rosmarinic acid does not necessarily lead to an increase of its stoichiometry against DPPH[•] (except for dodecyl), despite the drastic acceleration of the free radical scavenging process.

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