Synthesis and biological evaluation of new natural phenolic 2*E*, 4*E*, 6*E* octatrienoic esters

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In the present study the esterification of the hydroxyl groups of resveratrol, caffeic acid, ferulic acid and ß-sitosterol with an antioxidant polyconjugated fatty acid, 2E,4E,6E-octatrienoic acid, was achieved. As the selective esterification of hydroxyl groups of natural compounds can affect their biological activity, a selective esterification of resveratrol and caffeic acid was performed by an enzymatic approach. The new resulting compounds were characterized spectroscopically (FT-IR, NMR mono and bidimensional techniques); when necessary the experimental data were integrated by quantum chemical calculations. The antioxidant, anti-inflammatory and proliferative activity was evaluated. The good results encourage the use of these molecules as antioxidant and/or anti-inflammatory agents. in dermocosmetic application.

**Keywords**: natural products, biocatalysis, antioxidant, anti-inflammatory.

Introduction

In recent years the interest for natural substances with beneficial activity in humans, and in particular for the ones that are useful to counteract aging, has sharply risen. In fact, there is a significant increase in skin cosmetic, nutraceuticals and even pharmaceutical products based on natural compounds or on their semi-synthetic derivatives. The main interest has been observed for natural substances such as polyphenols and phytosterols with strong anti-oxidant activity, as 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.[1] As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

The most common phytochemicals include resveratrol (trans 3,5,4'-trihydroxystilbene, RESV), which is found in grapes and leguminous products,[2] caffeic (3,4-dihydroxycinnamic acid, CA) and ferulic acid (3-methoxy-4-hydroxycinnamic acid, FA) which are in grain, blueberries, apples, grapes, other fruits and vegetables,[3] and ß-sitosterol which is also found in plant oils and leguminous products.[4]

Although these compounds are present in significant amounts in foods, their dietary intake is generally characterized by a low bioavailability, either due to their low absorption or to the high rate with which they are metabolized, mainly by methylation, sulfation and glucuronidation of -OH groups[5] which are responsible for their antioxidant activity. These factors reduce the potential preventive and therapeutic activities of these classes of molecules, hampering their use as antioxidants in pharmaceutical and nutraceutical areas. Their dermocosmetic application is reduced by their low skin permeability and poor solubility in water media, making the development of pharmaceutical formulations difficult. In drugs containing carboxylic and hydroxyl groups esterification[6] represents one of the main reactions in organic synthesis used to improve the above mentioned properties. The selective esterification of hydroxyl groups of resveratrol[7] [8] can affect its biological activity, stability, availability, solubility and metabolism,[9] [10] and that resveratrol partial acetyl esters increase its affinity for the constituents of the lipophilic membranes and may be useful for structure-activity relationship studies. In fact, the antioxidant properties of resveratrol are found to be directly proportional to the number of free hydroxyl groups and even the position of the esterified hydroxyl group can influence the antioxidant activity of the molecule.[11] On the contrary, the literature does not contain any evidence concerning the esterification of the hydroxyl group of ferulic acid nor of the total or partial esterification of caffeic acid at the OH in C3 and C4.

The aim of this study was to modify the structure of resveratrol, caffeic acid, ferulic acid and ß-sitosterol leading to the development of new semi-synthetic esters[12] at the hydroxyl groups with the polyconjugated C8 chain of all *trans* 2,4,6-octatrienoic acid (OCTA, Fig. 1) in order to enhance the antioxidant activity of these compounds. OCTA[13] is a fatty acid known as a promising antioxidant and anti-inflammatory substance[14] which share some structural features with carotenoids and retinoids. It belongs to the class of parrodienes,[15] synthetic analogues of psittacofulvines,[16] which are a class of pigments found in the red plumage of *Ara macao*. Parrodienes are antioxidant compounds which inhibit membrane lipoperoxidation and possess anti-inflammatory activity.[17.]

Figure 1. 2E,4E,6E-octatrienoic acid (1), OCTA

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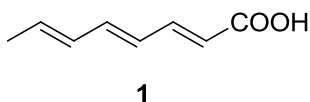
In order to obtain a regioselective esterification of resveratrol and caffeic acid hydroxyl groups, the enzymes selectivity was exploited for direct substrate esterification or for hydrolysis of full esters synthetized by conventional chemical reactions. After esterification, the potential antioxidant, proliferative and anti-inflammatory activities of all the newly synthetized compounds were evaluated comparing with that of the starting compounds.

Results and Discussion

Synthesis

The synthesis of sitosterol, resveratrol, caffeic, ferulic esters can easily be performed by means of esterification with an appropriate molar amount of OCTA (Figure 1), activated as acyl chloride or anhydride, using respectively trimethylamine or dimethylaminopyridine (DMPA) as catalyst. Following this procedure in the cases of resveratrol (Scheme 1) and caffeic acid (Scheme 2) all the hydroxyl groups were esterified. With the purpose to obtain the regioselective esterification of the hydroxyl groups, the molar amount of OCTA moiety was decreased, but only reacting an equimolar amount of RESV, octatrienoic anhydride and trimethylamine was isolated a partial ester, the monoester **5a**. In light of these results a chemo-enzymatic approach, starting from the synthetized compounds **5** and **6**, was evaluated to obtain the mono and dioctatrienoic derivatives. In the first phase of this work, a screening of the most suitable enzymes and of the proper reaction conditions were performed using commercially available Lipases and Acylase. The progress of the hydrolysis reactions was monitored by HPLC/DAD.

For resveratrol derivatives, the biotransformations carried out using a Lipase from *Candida rugosa* in TRIS HCl buffer (pH 8) did not produce any chemoselectivity whereas using a mixture of TMBE/H_2O (1%), the HPLC analyses showed after 24h the presence of compound **5b** (46% yield), a small amount (1%) of monoester **5c** and resveratrol (Scheme 1).

**Scheme 1.** Synthesis of resveratrol derivatives.
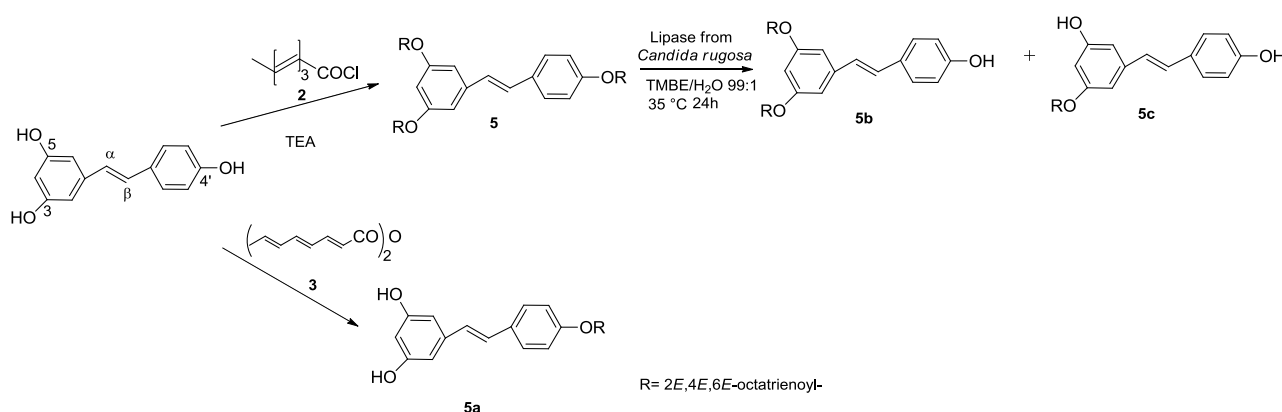
In order to isolate and characterize the main products by mass spectrometry and NMR spectroscopy the biotransformation was scaled-up. The NMR mono and bidimensional experiments allowed the complete attribution of all signals. Comparing the ^{1}H and ^{13}C spectra of compounds **5** and **5b**, the para aromatic and the =CH of RESV moiety showed the greatest differences in chemical shifts. In fact the H2'6' system shifted upfield from 7.51 to 7.35, the α =CH from 7.10 to 7.00 and the ß =CH from 7.01 to 6.89 ppm suggesting the hydrolysis of the OCO bond in 4'. Furthermore, the integration ratio between the aromatic and polyconjugated systems confirmed the presence of two unsaturated chains. In the ^{13}C spectrum of the triester **5** two carbonyl peaks respectively at 165.58 (CO in C3, C5) and 165.26 (CO in C4') are evident. Since in the diester the C3, C5 carbonyl peak have a negligible difference in chemical shift (165.47 ppm), and the peak corresponding to C4' shifted downfield from 150.90 to 155.93, the chemoselective hydrolysis in 4' is suggested. As RESV is a symmetrical molecule in which positions 3 and 5 are chemically and spectroscopically equivalent, all the NMR data evidenced that in compound **5b** the symmetry is maintained allowing to ascertain that Lipase from *Candida rugosa* showed selectivity for the hydrolysis at the 4' position.

Likewise, was confirmed the structure of compound **5a** which is also in agreement with literature[9] data for 4' monoacetyl resveratrol ester. In fact the proton in C4 exhibit a resonance that fall at 6.17 ppm, while in tri and diesters at 6.95 and 6.85 respectively.

Considering caffeic acid derivatives (Scheme 2), the reaction between octatrienoic anhydride (1:0.5) and NaH in DMSO gave, in very low yield, a mixture of the monoesters (60:40 ratio). The HPLC/DAD chromatogram evidenced two peaks at 23.9 min and 25.8 min with a UV-vis absorption spectrum with a maximum at 310 nm; moreover the LC/MS analysis evidenced for these two peaks the same molecular weight (301 Dalton). Contrarily, the biocatalytic hydrolysis of diester **6** using the Lipase from *Rhizopus arrhizus* in a biphasic system (dioxane/H$_2$O 1:1) at room temperature for 17h, allowed to obtain a smaller amount of a single monoester. In fact, the HPLC/DAD chromatogram evidenced one peak at 23.84 min with a UV-vis maximum at 310 nm and the LC/MS one peak at 301 m/z corresponding to the major compound in the mixture chemically obtained.

Scheme 2. Synthesis of caffeic acid derivatives.

R = 2*E*,4*E*,6*E*-octatrienoyl-
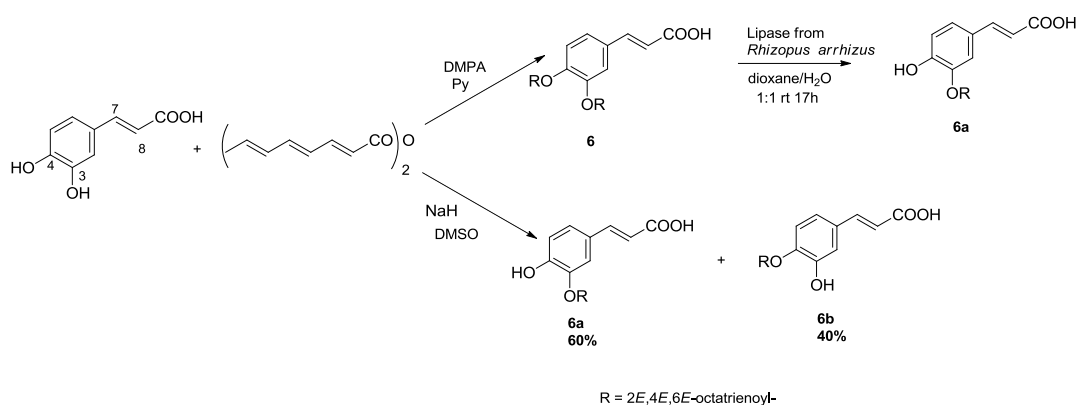
The mono and bidimensional NMR of the chemically obtained mixture allow to confirm the formation of the monoesters, but not the unambiguous position of esterification.

Since the unequivocal assignment of the structure of **6a** and **6b** was not possible, experiments were integrated by quantum chemical (QM) calculations. Indeed, NMR parameters of organic molecules can be accurately computed by QM calculation,[18] and the comparison of theoretical and experimental chemical shifts can allow a complete structural attribution to non-trivial structures like tautomers or regioisomers.[19-21] In our case, the comparison of these data with the experimental NMR shifts (Table 1) suggests that the major compound is that in which the hydrolysis undergone at the hydroxyl in 4, thus giving caffeic monoester at position 3.

Table 1. Theoretical and experimental ^{13}C shifts (ppm) for monoesters **6a** and **6b**

Carbon	Exp. (δ)	Pred. 6a	Pred. 6b	abs err 6a	abs err 6b
COOH	168.25	165.72	165.66	2.53	2.59
CO	164.80	163.07	163.58	1.73	1.22
C3	139.14	139.93	147.97	0.79	8.83
C4	151.73	151.10	143.23	0.63	8.5
C7	143.80	148.12	148.27	4.32	4.47
C1	126.30	128.32	133.44	2.02	7.14
C6	127.78	132.75	127.67	4.97	0.11
C5	117.40	120.12	122.91	2.72	5.51
C2	123.45	119.88	117.65	3.57	5.80
C8	117.03	113.29	115.18	3.74	1.85
aMAE=				2.70	5.29
bR2=				0.971	0.908

^{a}.Mean Absolute Error. ^{b} Coefficient of determination between experimental and theoretical chemical shifts.

Another approach to obtain partial esters was the direct biocatalytical esterification in organic solvents (TBME, ter butanol or hexane) of RESV and CA with OCTA as such, or activated as vinyl or trifluoroethyl ester. For this purpose Acylase I from *Aspergillus melleus*, PPL, Lipase from

Candida rugosa, *Candida antarctica B* and *Rhizopus arrhizus* were employed. The HPLC/DAD analyses evidenced traces of di- and monoester only in the biotransformation carried out with Lipase from *Candida rugosa* in hexane containing pyridine (0.4%).

With the purpose to employ these compounds in dermocosmetic formulations the antioxidant, anti-inflammatory and proliferative activity were studied. In the biological assays the concentration of the newly synthetized molecules were established taking into account the molar ratio between octatrienoic acid and natural alcohol.

Antioxidant activity

The antioxidant activity of the compounds was first evaluated as radical scavenging activity (RSA%) on stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Table 2). When in the presence of an antioxidant or a hydrogen donor the colored stable DPPH radical is reduced to DPPH-H (non-radical form). The DPPH radical without antioxidants was stable over time. Under the assay conditions, 100% of activity corresponds to the complete scavenging of DPPH radical (50µM final concentration) after 10 min of incubation with the antioxidant compounds

Table 2. Radical scavenging activity towards DPPH• of OCTA (5mM), β-sitostero l, resveratrol, caffeic acid, ferulic acid, octatrienoylsitosterol (**4**), trioctatrienoylresveratrol (**5**), 3,5-dioctatrienoylresveratrol (**5b**), 4'-octatrienoylresveratrol (**5a**), 3,4-dioctatrienoil caffeic acid (**6**), monooctatrienoyl caffeic acid (**6a+6b**) and 4-octatrienoylferulic acid (**7**). OCTA and OCTA-conjugates were tested at 5mM; free compounds were tested in equimolar ratio with OCTA, 1:2, 1:4, 1:3, 1:2, 1:3, 1:2 and 1:4 for compounds 4, 5, 5b, 5a, 6, 6a+6b, and 7 respectively.

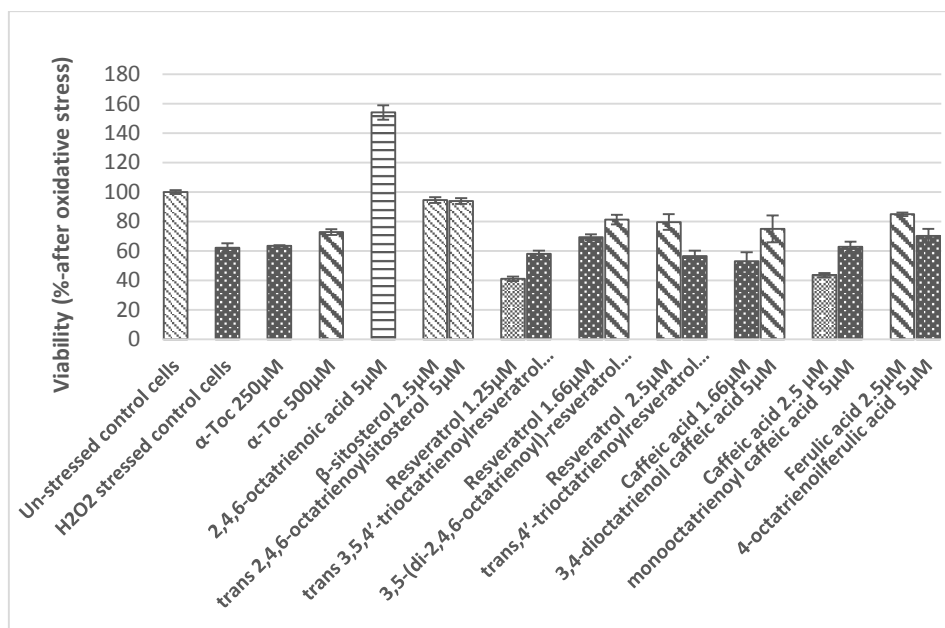| Compounds	% RSA
2,4,6-octatrienoic acid 5mM	23.69 ± 1.21[a]
β-sitosterol 2.5mM	7.00 ± 0.64[b]
4 5mM	49.72 ± 1.88[c]
RESV 1.25mM	84.225 ± 0.02[d]
5 5mM	92.50 ± 1.36[e]
RESV 1.66mM	82.41± 1.28[d]
5b 5mM	93.82 ± 2.53[f]
RESV 2.5mM	41.75 ± 2.19[c]
5a 5mM	32.14 ± 3.70[g]
Caffeic acid 1.66mM	99.03 ± 7.21[h]
6 5mM	100.00 ± 0.02[h]
Caffeic acid 2.5mM	94.03± 0.15[e]
6a+6b 5mM	96.25± 0.34[e]
Ferulic acid 1.25mM	40.58± 0.54[c]
7 5mM	5.43± 3.70[i]

Data are the mean of three independent analyses.

[a-i] Values with different superscript letters, differ significantly ($P<0.05$)

According to previous studies, resveratrol[22] [23] and caffeic acid[24] [25] showed a strong radical scavenging activity for the DPPH radical. β-sitosterol also showed an appreciable radical scavenging activity, as reported by Baskar et al.[26] and Ruijie et al.[27] OCTA had a radical scavenging activity towards the stable radical DPPH of 24 ± 1%, esterification significantly ($P<0.05$) increased the radical scavenging activity (RSA), depending on the starting compound used. In general, esterification with caffeic acid led to the higher increase, although the derivative (3,4-dioctatrienoil caffeic acid) % RSA wasn't higher than caffeic acid alone. Therefore, the resveratrol diester showed a RSA ca. 6% higher than resveratrol at 5 mM. β-sitosterol was the less effective towards the stable radical DPPH (RSA < 10%) but, most interesting, its derivative showed a RSA 43% higher than β-sitosterol.

The capacity of all synthetized compounds to protect cells from induced-oxidative stress was further assayed. NCTC2544 cells were treated for 16h (Figure 2a) with (0.5-5 µM) starting compounds in equimolar ratio and corresponding esters and then treated with 1 mM hydrogen peroxide. Compared to H_2O_2-stressed control cells (62.3 ± 3.0% of cell viability compared with un-stressed control cells), α-tocopherol (500 µM) significantly ($P < 0.05$) increased cell survival. The highest viability was found for cells treated with OCTA (103.9 ± 5.2%). β-sitosterol and its derivative **4** also significantly ($P < 0.05$) protected cells from induced-oxidative stress with % of cell viability similar to the un-stressed cells (94.5 ± 10.9 and

93.9 ± 14.9%, respectively). Similar results were also obtained for resveratrol, ferulic acid and corresponding 4-O-octatrienoyl ester **7**. Most interesting, unlike caffeic acid, compound **6** (3,4-dioctatrienoyl caffeic acid) allowed a recovery of NCTC 2544 cell viability (75.9 ± 9.1 vs 55.9 ± 6.1%). These results were confirmed by determining the concentration of intracellular reactive oxygen species (ROS; Fig. 2b). The assay uses DCFH-DA as a probe that emits a fluorescence proportionate to the formation of intracellular hydrogen peroxide, once deacetylated. Higher emitted fluorescence is related to a higher ROS intracellular production; therefore, a decrease of fluorescence indicate an antioxidant activity of the samples. Data are reported as ROS percentage with respect to the control (H_2O_2 stressed cells). Pretreatment with α-tocopherol (250-500 μM) significantly ($P < 0.05$) decreased ROS production (14.8 ±1.7 and 18.7 ±3.7%, respectively). Similar results were found when cells were pre-treated with starting compounds and their derivatives, with the exception of caffeic acid and 4'-octatrienoylresveratrol **5a**. Both compounds showed the lower radical scavenging activity (49.7 ±1.4 and 50.9 ±1.0%, respectively) between tested compounds. The higher significantly ($P < 0.05$) antioxidant effect was found for OCTA (5.2 ±1.4%).

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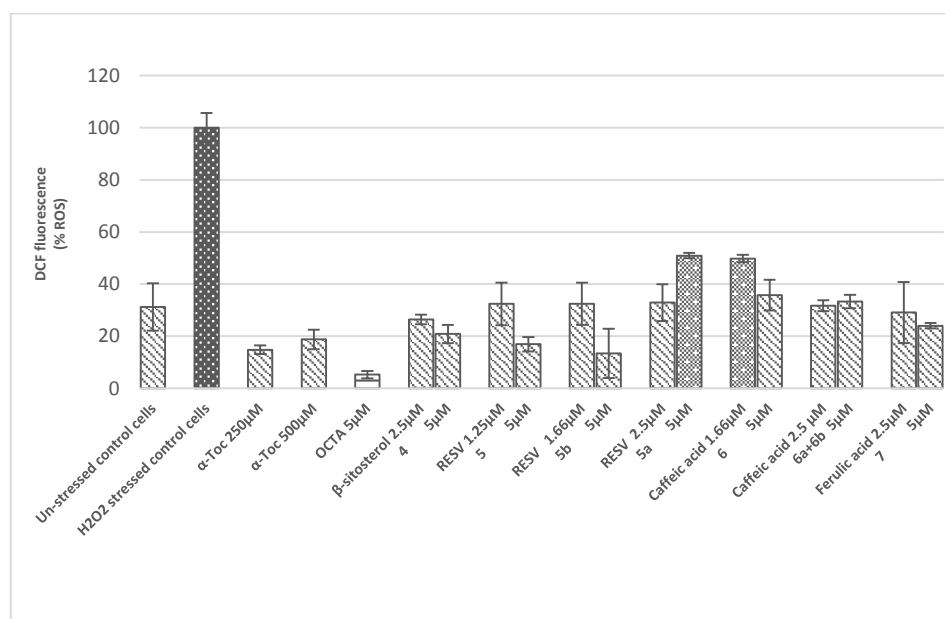
b

Figure 2. Effect of compounds on cell viability (%) (a) and intracellular ROS generation (b) in human NCTC 2544 keratinocytes during oxidative stress. Data are the means of three independent experiments twice analyzed. Columns with different background, in each graph, differ significantly ($P < 0.05$).

Proliferative activity on human keratinocyte NCTC 2544 cells

As the new derivatives of the octatrienoic acid shares some structural features with retinoids and carotenoids, we hypothesized that they might also influence cell proliferation[28-32] as well as possess antioxidant properties.

We used the MTT assay to assess proliferation of NCTC2544 cells in response to new synthesized compounds and compared to OCTA. The concentrations ranged from 0.5 to 5μM. Starting compounds were also tested at equimolar ratio. Cytotoxicity was measured as the effect causing a decrease of the cell viability below 70% (table S1 Supporting Information). No cytotoxic effect was found at tested concentrations, independently from the time of incubation and tested compound. Compared to basal medium (negative control) no significant (P > 0.05) increase of cell proliferation was found during incubation with 2,4,6-octatrienoic acid, caffeic acid and its derivative, 3,4-dioctatrienoil caffeic acid **6**. Compared to negative control and OCTA, β-sitosterol and its ester **4** significantly (P < 0.05) increased the cell proliferation after 72 h of treatment. Also exposure to 0.125–2.5μM of resveratrol caused a significant (P > 0.05) increase in cell proliferation after 72h of incubation and the same effect was observed for the triester **5**. On the contrary, no significant (P < 0.05) induction of cell proliferation was produced by monoester 5a. Ferulic acid also caused a significant (P < 0.05) induction of proliferation when cells were treated for 24 and 72 h at 0.25 μM and for 72 h at 1.25 μM. Most interesting 4-octatrienoylferulic acid significantly (P < 0.05) increased NCTC2544 proliferation after 72 h of treatment at 0.5 to 5μM.

Transcriptional regulation of tumor necrosis factor- α (TNF-α) genes

Lipopolysaccharide (LPS), the major constituent of the outer membrane of bacterial pathogens, is a well-known initiator of inflammation.[33] Its binding to cell surface receptors, i.e., Toll-like receptors (TLRs), lead to several signaling pathways initiation and consequently to the activation of cellular responses, including the production of pro-inflammatory cytokines, such as TNF-α.[34] [35]

The expression of TNF-α was significantly (P < 0.05) affected by treatments with compounds tested (Fig. 3). Compared to positive control (Control + LPS), the expression of TNF-α decreased starting from 16 h of treatment. In general, OCTA was the most effective in decreasing TNF-α, especially after 24 h of treatment with values similar to not-inflamed cells (control). Also the derivatives significantly (P < 0.05) reduced TNF-α expression and the effect was more evident after 24 h of treatments. Among tested compounds, treatment with caffeic acid and the corresponding diester 6 were the less effective. Most interesting, after 24 h of incubation, treatment with octatrienoylsitosterol **4** produced a reduction of TNF-α gene expression significantly (P < 0.05) higher than β-sitosterol, and this effect was comparable to OCTA.

Obtained data confirmed the previous studies on anti-inflammatory activity of parrodienes[36] and OCTA modulation of Peroxisome proliferator-activated receptor γ (PPARγ),[14] a transcription factor strictly involved in immunoregulatory balance together with cytokine TNF-α.
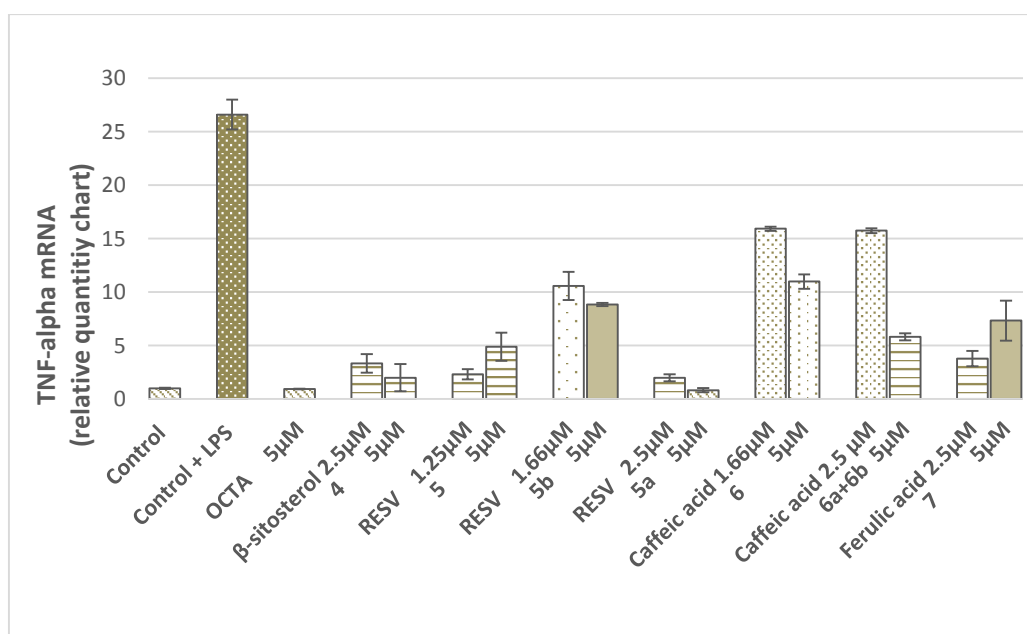

Figure 3. Expression of the Th1 cytokine tumor necrosis factor-a (TNF-α) gene in human NCTC 2544 keratinocytes, determined using RT-PCR. Data are the means ± SD of three separate experiments, performed in triplicate. Statistical differences between mean values were determined using Student's t-test. Columns with different background, in each graph, differ significantly (P < 0.05).

Conclusions

In this paper new all trans octatrienoic esters with natural polyphenolic compounds and sitosterol have been synthetized and biologically evaluated. This work confirmed the usefulness of biocatalysed reactions for the regioselective modification of polyphenols.

The study of antioxidant potential of new compounds highlighted the synergic action between OCTA and resveratrol and caffeic acid. In particular, diester **5b** shows the strongest antioxidant activity. Synergism was found also for compounds **5**, triester of resveratrol, and for di- and monoester of caffeic acid **6a** + **6b**.

Mono-esterification of resveratrol with OCTA (**5a**) lead to the highest anti-inflammatory activity. A strong synergic action into inflammation inhibition was also found when OCTA was esterified with caffeic acid (**6** and **6a+b**).

Most interesting, esterification reflects also into an improvement of cell proliferation; this is true for compounds **4**, **5** and **7**. The obtained data suggest a possible role of such esters in promoting wound healing, but further investigations are needed.

Taking together, these results encourage the use of new synthesized compounds for dermatological purposes as antioxidant and/or anti-inflammatory agents.

Experimental Section

General

All commercial available solvents and reagents were used after distillation or treatment with drying agents. 2E,4E,6E-octatrienoic acid **1** was a gift of Giuliani S.p.A.(Milan, Italy). *Candida rugosa* (4.9 U/mg) and *Candida antarctica B* were purchased from Sigma Aldrich, Acylase I from *Aspergillus melleus* (0.72U/mg) and *PPL* (23.9 U/mg) from Fluka BioChemika.

All of the reactions that involved the use of reagents sensitive to oxygen or hydrolysis were carried out under an inert atmosphere and the glassware was previously dry in oven at 110 °C.

The reactions were monitored by thin layer chromatography (TLC) on Merck precoated silica GF254 plates or Alumina using when necessary iodine vapor for spot visualization. Mps: Opto-lab (Italy) instrument are uncorrected. FTIR spectra: Spectrum One (Perkin Elmer) (MA, USA) in a spectral region between 4000 and 450 cm^{-1} and analyzed by transmittance technique with 32 scansions 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 plates of sodium chloride. The ^1H and ^{13}C NMR and bidimensional analyses were taken on a Bruker Avance 500 (Billerica, MA, USA) operating at 500 MHz for ^1H and 125.75 MHz for ^{13}C or with a Varian Mercury Plus 200, operating at 200 MHz for ^1H and 50.3 MHz for ^{13}C. Chemical shifts were expressed as ppm; J in Hertz. The APT sequence was used to distinguish methyl and methine signals from those due to methylene and quaternary carbons.

MS analyses: Thermo Finnigan (MA, USA) LCQ Advantage system equipped with a quaternary pump, Diode Array Detector (working wavelength 254 nm) and MS spectrometer with an Electrospray ionization source and an Ion Trap mass analyzer; ionization: ESI positive or ESI negative; capillary temperature: 250°C; source voltage: 5.50 kV; source current: 4.00 µA; multipole 1 and 2 offset, -5.50 V and -7.50 V respectively; intermultipole lens voltage: -16.00 V; trap DC offset voltage: -10.00 V.

The enzymatic reactions were incubated at 35 °C under stirring; control reactions without enzyme were carried out under the same conditions. The progress of the chemo-enzymatic reactions was monitored, at regular time intervals, by thin-layer chromatography (TLC) and HPLC/DAD performed using a Waters Delta Prep 600E DAD equipped with a manual injector Rheodyne 7125 (loop 20 µl), an online degasser and a column block heater. The column was a Purosphere STAR RP 18-e (Merck, MA, USA), 150 x 4.6 mm i.d. (5 µm particle size). The mobile phase employed for resveratrol esters was methanol 100% eluted at a flow rate of 0.6 ml/min, while for caffeic esters was used the following gradient steps of solvents A (MeOH 95%, H_2O 5%, trifluoroacetic acid 0.1%) and B (H_2O 95%, MeOH 5%, trifluoroacetic acid 0.1%): 60:40 (A:B) for 5 min, then from 5 to 25 min gradually to 100 % A at a flow rate of 0.8 mL/min. All analyses were carried out under isothermal conditions at 30 °C.

Synthesis of 2*E,4E,6E*-octatrienoyl chloride (2).

To a suspension of 2*E,4E,6*E-octatrienoic acid (2.0 g, 14.5 mmol) in anhydrous toluene (20 mL), oxalyl chloride (4.6 mL, 58 mmol) was added dropwise at 0°C under nitrogen flow. The mixture was than stirred at room temperature for 3 h monitoring by FTIR the disappearance of the CO stretching at 1682 cm^{-1} and the appearance of the acylchloride CO band at 1749 cm^{-1}. When the reaction was complete, the solvent was evaporated under reduced pressure and the obtained acyl chloride was directly used without further purifications. Yield 94%. FTIR (NaCl): v 3025 (Csp^2-H), 2968 (Csp^3-H), 2849 (Csp^3-H), 1749 (C=O), 1601 (C=C), 1574(C=C), 1444, 927.

Synthesis of 2*E*,4*E*,6*E*-octatrienoyl anhydride (3)

TEA (2 mL, 14.5 mmol) was added at 0 °C to a suspension of OCTA in anhydrous toluene (30 mL) under nitrogen atmosphere and to the mixture was added dropwise the chloride **2** diluted in a small amount of anhydrous toluene. Upon cooling to room temperature the mixture was stirred under nitrogen atmosphere at for 12 h while protected from daylight. The precipitate was filtered off the solution evaporated under reduced pressure. The residue was diluted in CH_2Cl_2 (6 mL) and extracted with a saturated aqueous solution of $NaHCO_3$ (1 x 6 mL) and then with water (2 X 10 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Yield 55%. Rf = 0.8 (hexane/acetone 1:1). FTIR (NaCl): 3019, 2992, 2918, 1755, 1716, 1617, 1577, 1447. ^{1}H NMR (200 MHz, $CDCl_3$): 7.40 (1H, dd, J = 11.4, 15.03, H(3)); 6.61 (1H, dd, J = 9.9, 14.9, H(5)); 6.1-6.3 (2H, m, H(4), H(6)) 6.00 (1H. dq, J = 6.2, 15.4, H(7)); 5.88 (1H, d, J = 15.0, H(2)), 1.85 (3H, d, J = 6.2, CH_3). ^{13}C NMR (50.3 MHz, $CDCl_3$): 163.0 (CO); 148.9 (C(3)); 143.7 (C(5)); 137.2 (C(7)); 131.4 (C(6)); 127.4 (C(4)); 118.6 (C(2)); 18.9 (CH_3). ESI-MS: $C_{16}H_{18}O_3$, calc 259, found m/z 281.1 [M+Na]$^+$.

Synthesis of 2*E*,4*E*,6*E*-octatrienoylsitosterol (4)

A suspension of **2** obtained from OCTA (500 mg, 0.0036 mmol) in anhydrous toluene (10 mL), was added dropwise at 0°C to a stirred mixture of sitosterol (500 mg, 0.0012 mmol) and DMPA (629 mg, 0.0051 mmol) in 10 mL of anhydrous toluene. After few minutes, the ice-bath was removed and the mixture was allowed to warm to room temperature and stirred for 48 h. The DMPA chloride was filtered and the filtrate was washed with water (3 x 25 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Rf = 0.9 (hexane/AcOEt/diethylether 5:2:1 developing in iodine vapor). Yield 95%. FTIR (KBr): 3015, 2958, 2939, 2868, 2851, 1715, 1646, 1622, 1466, 1446, 992. ^{1}H NMR (200MHz, $CDCl_3$): 7.28 (1 H, dd, J = 11.0, 16.2, H(3octa)); 6.50 (1H, dd, J = 10.5, 15.0, H(5octa)); 6.22-6.06 (2H, m, H(4 octa), H(6 octa)); 5.95 (1H, dq, J = 6.6, 15.7, H(7octa)); 5.80 (1H, d, J = 15.0, H(2octa)); 5.38 (1H, d, J = 4.4, H(6)); 4.8 (1H, m, H(1)); 2.0-0.8 (48H, m, CH_2, CH3); 1.8 (3H, d, J = 6.6, CH_3-CH=). ^{13}C NMR (50.3 MHz, $CDCl_3$): 166.8 (CO); 144.8 (C(3octa)); 141.0 (C(5octa)); 139.9 (C(6)); 135.0 (C(7octa)); 131.50 (C(6octa)); 127.8 (C(4octa)); 122.8 (C(7)); 120.8 (C(2octa)); 73.9 (C(2)); 56.9 (C(14)); 56.3 (C(17)); 50.3 (C(10)); 46.1 (C(21)); 42.5-12.1 (24C). ESI-MS: $C_{37}H_{58}O_2$, calc 534; found m/z 1091 m/z [2M+Na]$^+$.

Synthesis of 3,5,4'-tri-O-(2*E*,4*E*,6*E*-octatrienoyl) resveratrol (5).

A suspension of **2**, obtained from OCTA (500 mg, 0.0036 mmol), in anhydrous chloroform (10 mL) not stabilized with ethanol was added dropwise at 0°C to a stirred mixture of resveratrol (250 mg, 1.095 mmol), TEA (435 µl) and anhydrous chloroform (20 mL) not stabilized with ethanol. The ice-bath was removed after few minutes and the resulting mixture was allowed to warm to room temperature and stirred for 12 h after which TEA salts were filtered, the filtrate washed with a saturated $NaHCO_3$ solution 1 x 15 mL) and with water (2 x 15 mL), anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Rf = 0.67 (Esano/AcOEt 1:1). t_R: 7,56 min UV (MeOH): 315, 297. Yield 55%. FTIR (KBr): 3019, 2967, 2932, 2908, 2845, 1725, 1615, 1585, 1435, 1218, 1198, 1164, 1119, 962, 733. ^{1}H NMR (500 MHz, $CDCl_3$): 7.53-748 (5H, m, H(2'), H(6'), H(3octa)); 7.18 (2H, d, J = 2.0, H(2), H(6)); 7.14 (d, 2H, J=8.0, H(3'), H(5')); 7.10 (1H, d, J =16.0, H(α)); 7.01 (1H, d, J = 16.0, H(β)); 6.95 (1H, t, J = 2.0, H(4)); 6.64 (3H, dd, J = 15.0, 11.0, H(4octa)); 6.30 (3H, dd, J=11.0, 15.0 H(5octa)); 6.23 (3H, dd, J = 15.0, 0.9, H(6octa)); 6.07-6.00 (3H, m, H(7octa)); 6.01 (3H, d, J= 15.0, H(2octa)); 1.86 (9H, d, J=6.6, CH_3). ^{13}C- NMR (125.75 MHz, $CDCl_3$): δ = 165.6 (C(4')OCO); 165.3 (C(3,5)OCO); 151.8 (C(4')); 150.9 (C(3,5)); 147.3 (C(3octa)); 142.4 (C(5octa)); 139.6 (C(1)); 136.1 (C(7octa)); 134.6 (C(1')); 131.4 (C(6octa)); 129.8 (C(α)); 127.8 (C(2',6')); 127.6 (C(ß, C4octa)); 122.1 (C(3',5')); 118.9 (C(2octa)); 116.9 (C(2,6)); 114.2 (C(4)); 18.7 (CH_3) ppm. ESI-MS: $C_{70}H_{104}O_6$, calc 588; found 611 m/z [M+Na]$^+$.

Synthesis of 4'-O-(2*E*,4*E*,6*E*-octatrienoyl)resveratrol (5a)

Under nitrogen atmosphere in a Schlenk tube, to a stirred solution of resveratrol (0,11 mmol, 25 mg) in anhydrous DMSO (3 mL), anhydrous TEA (0,11 mmol, 15 µL) was added. After 20 min a solution of octatrienoic anhydride (0,11 mmol, 29 mg) in anhydrous DMSO (2 mL) was added dropwise and the solution stirred in the dark for 16 h. The mixture was then quenched with brine (5 mL) and extracted twice with diethyl ether (2 x 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography with hexane/acetone 7:3 to afford the monoester. Rf = 0.1 (hexane/acetone 7:3). t_R: 4,35 min UV: 315. Yield 20%. FTIR (KBr): 3382, 3327, 3291, 3023, 2969, 2926. 2906, 2844, 1710, 1636, 1609, 1505, 1445, 1347, 1299, 1268, 1219, 1197, 1165, 1148, 1130, 1005, 966, 839. ^{1}H-NMR (500 MHz, $CDCl_3$): 9.20 (2H, broad s exch D_2O, OH); 7.62 (2H, d, J = 8.7, H(2'), H(6')); 7.47 (1H, dd, J =11.2, 14.9, H(3octa)); 7.16 (2H, d, J = 8.7, H(3'),H(5')); 7.08 (1H, d, J = 16.1, H(α)); 7.03 (1H, d, J=16.1, H(β)); 6.81 (1H, dd, J=10.8, 15.1, H(5octa)); 6.45 (d, 2H, J = 2.2, H(2), H(6)); 6.43 (dd, 1H, J=10.8, 14.6, H(4octa)); 6.28 (dd, 1H, J = 11, 14.8, H(6octa)); 6.17 (t, 1H, J = 2, H(4)); 6.15 (d, 1H, J = 14.9, H(2octa)); 6.06 (1H, dq, J= 6.2, 14.8, H(7octa)); 1.82 (3H, d, J = 6.2, CH_3). ^{13}C- NMR (50.3 MHz,$CDCl_3$): 165.6 (C(4')OCO); 159.2 (C(3,5); 150.5 (C(4')); 147.7 (C(3octa)); (C(5octa)); 139.4 (C(1)); 136.8 (C(7octa)); 135.4 (C(1')); 132.0 (C(6octa)); 129.8 (C(α)); 128.1 (C(4octa)); 128.0 (C(2',6')); 127.6 (C(ß)); 122.7 (C(3',5')); 119.2 (C(2octa)); 116.2 (C(4)); 105.1 (C(2,6)); 19.1 (CH_3) ppm. ESI-MS: $C_{22}H_{20}O_4$, calc 348; found 349 m/z [M+H]$^+$; 371 m/z [M+Na]$^+$; 719 m/z [2M+Na]$^+$.

General procedure for synthetizing compounds (6, 7).

To a stirred solution of caffeic or ferulic acid (349 mg, 1.936 mmol) in dry pyridine (30 mL) under nitrogen atmosphere and at 0°C a catalytic amount of DMPA and octatrienoic anhydride (2 eq for caffeic acid and 1 eq for ferulic acid) were added. After stirring at room temperature for 12h, the mixture was diluted in $CHCl_3$ (20 mL) and poured in HCl 1N (20 mL). The organic layer was then washed with water (20 mL), with a saturated aqueous solution of $NaHCO_3$ 2 x 20 mL) and with water again (20 mL). The organic layer was exsiccated over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was recrystallized from methanol.

Synthesis of 3,4-di-O-(2*E*,4*E*,6*E*-octatrienoyl) caffeic acid (6).

Rf = 0.2 (AcOEt 100%). t_R: 30.2 min UV (MeOH): 320. Yield 55%. FTIR (KBr): 3412, 3019, 2961, 2923, 1732, 1687, 1615, 1430, 1111, 1004. ^1H NMR (500 MHz, DMSO): 12.5 (1H,broad s exch D_2O , COOH); 7.72 (1H, d, J = 2, H(2)); 7.65 (1H, dd, J = 8.5, 2, H(6)); 7.60 (1H, d, J = 16.0, H(7)); 7.46–7.40 (2H, m, H(3octa)); 7.38 (1H, d, J = 8.5, H(5)); 6.81-6.73 (2H, m, H(5octa)); 6.57 (1H, d, J = 16, H(8)); 6.42–6.35 (2H, m, H(4octa)); 6.23(1H, dd, J = 14.8, 11.2, H(6octa)); 6.08 (2H, d, J = 14.6, H(2octa)); 6.06-6.00 (2H, m, H(7octa); 1.8 (3H, d, J = 6.8, CH₃). ^{13}C-NMR (125.75 MHz, DMSO): 167.8 (COOH); 164.3 (CO); 164.1 (CO); 148.3 (C(3octa)); 143.9 (C(3)); 143.7 (C(5octa)); 143.0 (C(4)); 142.5 (=CH); 137.1 (C(7octa)); 133.5 (C(1)); 131.7 (C(6octa)); 127.8(C(4octa)); 127.1 (C(6)); 124.4 (C(5)); 123.4 (C(2)); 120.7 (=CHCOOH); 117.8, (C(2octa)); 18.6 (CH₃) ppm. ESI-MS: $C_{25}H_{24}O_6$ calc 420, found 443 m/z [M+Na]⁺.

Synthesis of 4-O-(2*E*,4*E*,6*E* –octatrienoyl) ferulic acid (7).

Rf = 0.3 (AcOEt/hexane 7:3). Yield 30%. FTIR (KBr): 3436, 3017, 2961, 2924, 1727, 1683, 1615, 1508, 1467, 1111, 1004. ^1H NMR (200 MHz, DMSO): 12.5 (1H, broad s exch D_2O, COOH); 7.72 (1H, d, J = 2.0, H(2)); 7.65 (1H, dd, J = 8.5, 2, H(6)); 7.60 (d, 1H, J = 16.0, H(7)); 7.46 – 7.40 (2H, m, H(3octa)); 7.38 (1H, d, J = 8.5, H(5)); 6.81-6.73 (2H, m, H(5octa)); 6.57 (1H, d, J = 16, H(8)); 6.42–6.35 (2H, m, H(4octa)); 6.23(1H, dd, J = 14.8, 11.2, H(6octa)); 6.08 (2H, d, J = 14.6, H(2octa)); 6.06-6.00 (2H, m, H(7octa)); 3.8(3H, s, CH₃); 1.8 (3H, d, J = 6.8, CH₃). ^{13}C- NMR (50.3 MHz, DMSO): 168.2 (COOH); 164.8 (CO); 151.9 (C(3)); 147.8 (C(3octa)); 143.9 (=CH); 143.4 (C(5octa)); 141.6 (C(4)); 136.9 (C(7octa)); 133.9 (C(1)); 132.0 (C(6octa)); 128.1 (C(4octa)); 123.9 (C(5)); 122.0 (C(6)); 120.2 (=CHCOOH); 118.8 (C(2octa)); 112.5 (C(2)); 56.7 (OCH₃); 19.1 (CH₃). ESI-MS: $C_{18}H_{18}O_5$ calc 314, found 337 m/z [M+Na]⁺.

Synthesis of monooctatrienoyl caffeic acid (6a + 6b).

Under nitrogen flow to a stirred suspansion of NaH (3,84 mmol, 153 mg) in anhydrous DMSO (2 mL) a solution of caffeic acid (0,96 mmol, 173 mg) in DMSO (5 mL) was added dropwise. After stirring for 20 min, a solution octatrienoic anhydride (0,96 mmol, 250 mg) in DMSO (8 mL) was added dropwise and the mixture was stirred in the dark for 16 h. The reaction was quenched with HCl 10% (5 mL) and extracted with diethyl ether (3x10 mL). The combined organic phases were washed with H_2O (30 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The ^1H-NMR allowed to verify the achievement of the two monoesters mixture in the ratio 60:40. Rf = 0.3 (AcOEt/hexane 1:1). t_R: A: 22.2, B: 24.4 min UV (MeOH): 310. Yield 20%. FTIR (KBr): 3380, 3020, 2974, 1705, 1698, 1615, 1585, 1512, 1436, 1271, 1219, 1200, 1150, 1131, 1005, 979, 930, 859, 825. ^1H NMR (500 MHz, CD₃OD): 7.60 (1H, d, J = 15.9, H(7a)); 7.58 (1H, d, J = 15.5, H(7b)); 7.25 (2H, dd, J = 11.3, 15.0, H(10)) 7.37 (1H, dd, J = 8.4, 2.1, H(6a)); 7.31 (1H, d, J = 2.0, H(2a)); 7.15 (1H, d, J = 1.8, H(2b)); 7.09 (1H, dd, J = 8.3, 1.8, H(6b)); 7.06 (1H, d, J = 8.3, H(5b)); 7.06 (1H, d, J = 8.4, H(5a)); 6.95 (1H, d, J = 8.4, H(5a)); 6.71 (2H, dd, J = 10.7, 14.9, H(3octa)); 6.41 (1H, d, J = 15.5, H(8b)); 6.39 (2H, dd, J = 10.7, 14.9, H(5octa)); 6.33 (1H, d, J = 15.9, H(8a)); 6.26 (2H, dd, J = 10.0, 14.9, H(6octa)) 6.12 (1H, d, J = 14.3, H(9aocta)); 6.11 (1H, d, J = 14.8, H(9bocta)); 6.06 (2H, dq, J = 6.8, 14.9, H(7octa)); 1.86 (6H, d, J = 6.8, CH₃). ^{13}C-NMR (125.75 MHz, DMSO): 168.2 (COOHa); 167.9 (COOHb); 164.8 (COa); 164.7 (COb), 151.7 (C(4a)); 149.8 (C(4b)); 147.2 (C(3octa)); 143.8 (C(7)); 142.8 (C(5octa)); 140.6 (C(3b)); 139.1 (C(3a)); 136.5 (C(7aocta)); 135.2 (C(7bocta)); 133.2 (C(1b)); 131.8 (C(6aocta)); 128.2 (C(6bocta)); 127.9 (C(4aocta)); 127.7 (C(6a)); 126.30 (C(1a)); 124.1 (C(5b)); 123.4 (C(2)); 119.9 (C(2bocta)); 119.4 (C(8b)); 119.1 (C(2aocta)); 117.4 (C(5a)); 117.0 (C(8a)); 116.4 (C(6b)); 18.9 (CH₃). ESI-MS: $C_{17}H_{16}O_5$ calc 300, found 623 m/z [2M+Na]⁺; 299 m/z [M-H]⁺.

General procedure for the enzymatic synthesis

5 mg/ml of substrate **4** and 20 mg/mL of enzyme (Lipase from *Candida rugosa*, *Candida antarctica B*, *PPL, Rhizopus arrhizus* and Acylase I from *Aspergillus melleus*).were added in 2 ml of TRIS HCl buffer (pH 8) or TBME/0.1% H_2O; in the case of substrate **7** as solvent was also used a biphasic system dioxane/H_2O (1:1). The biotransformations were carried out in screw-cap test tubes at 35 °C under magnetic stirring. Control reactions without enzyme were carried out under the same conditions. The progress of the chemo-enzymatic reactions was monitored, at regular time intervals, by thin-layer chromatography (TLC) and HPLC/DAD.

Synthesis of 3,5-di-O-(2E,4E,6E-octatrienoyl) resveratrol (5b).

The reaction mixture contained substrate **5** (500 mg), 100 mL of TMBE/H_2O (99:1) and 2 g of Lipase from *Candida rugosa* was incubated at 35 °C under magnetically stirring. After 24h the mixture was filtered and extracted with EtOAc (3x30 mL). The organic layers were washed with

a saturated solution of NaHCO$_3$, dried over Na$_2$SO$_4$, filtered and evaporated under reduced pressure. The crude residue was purified by column chromatography with hexane/acetone 7:3 to afford the diester. Rf = 0.5 (hexane/ACOEt 1:1). t$_R$: 5,4 min UV: 320. Yield 46%. FTIR (KBr): 3019, 2967, 2932, 2908, 2845, 1725, 1615, 1585, 1435, 1218, 1198, 1164, 1119, 962, 733. ^{1}H NMR (500 MHz, CDCl$_3$): 7.48 (1H, dd, J =11.4, 15.4, H(3octa)); 7.35 (2H, d, J = 8.8, H(2'), H(6')); 7.11 (1H, d, J = 1.9, H(2),H(6)); 7.00 (d, 1H, J = 16.1, H(α)); 6.89 (1H, d, J = 16.1 H(β)); 6.85 (1H, t, J = 2, H(4)) 6.80 (2H, d, J = 8.5, H(3'),H(5')); 6.62 (1H, dd, J = 10.2, 14.5, H(5octa)); 6.27 (1H, dd, J =11.3, 14.6, H(4octa)); 6.14 (1H, dd, J = 11, 14, H(6octa)); 6.10-5.80 (1H, m, H(7octa)); 6.01 (d, 1H, J =1 5, H(2octa)); 1.82 (6H, d, J = 6.6, CH3). ^{13}C- NMR (125.75 MHz, CDCl$_3$): = 165.4 (CO); 155.9 (C(4')); 151.7 (C(3,5)); 147.4, (C(3octa)); 142.6 ((5octa)); 140.1 C(1)); 136.4(C(7octa)); 131.4 (C(6octa)); 130.2 (C(α); 129.9 (C(1')); 128.4 (C(2',6')); 127.6 (C(4octa)); 125.3(C(ß)); 118.9 (C(2octa)); 116.8 (C(2,6)); 115.9 (C(3',5')); 114.2, (C(4); 18.8 CH$_3$. ESI-MS: C$_{30}$H$_{28}$O$_5$ calc 468, found 467 m/z [M-H]$^+$.

Theoretical calculations.

The unsaturated acyl groups in diesters **6** and monoesters **6a** (meta) and **6b** (para) were approximated by a crotonate (2-butenoate), thus obtaining models M6, M6a and M6b. These models, in addition to caffeic acid, were subjected to a "low-mode" conformational search in gas phase, using the MMFF94x forcefield as implemented in the software MOE.[34] All the conformations within a interval of 3 kcal/mol were then optimized at the M06-2X/6-31+G(d,p) level, using the IEFPCM formalism and the UA0 set of atomic radii to model the effect of DMSO solvation.[38] [39] Three unique conformations were obtained for caffeic acid, eight conformations for diester M6, two conformation for monoester M6a and six for M6b. All conformations were confirmed as minima on the potential energy surface by performing a vibrational analysis, at the same theoretical level used for geometry optimization, and verifying the absence of imaginary frequencies. NMR absolute shieldings were then computed for all conformations using the GIAO method, at the B3LYP/6-311+G(2d,p), accordingly to a protocol reported in the literature.[40]

Mean absolute shielding were then obtained by computing weighted shielding accordingly to the Boltzmann equation. The electronic energy corrected by thermal free energy correction (298.15 K, 1 atm) was used to compute the Boltzmann factor for each conformation.

The conversion of absolute shieldings to chemical shift was done by performing a regression analysis between the experimental chemical shifts obtained for caffeic acid and diester M6 (see Figure) and the weighted absolute shieldings of the corresponding computational models.

The following linear equation was obtained for ^{13}C chemical shifts:

$$\delta(H, ppm) = -0.9256 \cdot Abs.\ Shielding + 30.5580\ (r2 = 0.87) \qquad [eq.\ 1]$$

and ^{13}C chemical shifts for M6a and M6b were computed accordingly.

Biological assays

DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of starting and esterified compounds was determined as reported by Shimada et al.[41] with some modification.[42] OCTA and esterified compounds were dissolved in 95% ethanol at 5mM. Starting compounds were dissolved taken into account final equimolar ratio in equimolar ratio with OCTA, 1:2, 1:4, 1:3, 1:2, 1:3, 1:2 and 1:4 for compounds 4, 5, 5b, 5a, 6, 6a+6b and 7 respectively. 2 mL of dissolved compounds were added to 2 mL of 0.1 mM DPPH, which was dissolved in 95% ethanol. The mixture was shaken and left at room temperature for 30 min. The reaction was monitored by reading the absorbance at 517 nm every 2 min for 30 min. A blank reagent was used to verify the stability of DPPH over the test time. The absorbance value measured after 10 min was used for the calculation of the μmoles DPPH scavenged by extracts. Lower absorbance values indicate higher free-radical-scavenging activity. The scavenging activity was expressed as follows: DPPH scavenging activity (%) = [(blank absorbance – sample absorbance) / blank absorbance] x 100.

MTT assay

Normal human keratinocyte NCTC 2544 cells were supplied by Istituto Nazionale di Ricerca sul Cancro (Italy). NCTC 2544 cells were cultured under humidified atmosphere (5% CO$_2$, 37°C), using RPMI 1640 medium supplemented with 10% (w/v) fetal bovine serum (FBS), 2mM l-glutamine, and 1% penicillin (10,000 U/ml)/streptomycin (10,000 U/mL). RPMI medium and others reagents were purchased from Sigma (Sigma Aldrich CO., St. Louis, MO). The culture medium was renewed every two days and after four passages the cultures were used for viability assays. Aiming at determining the cytotoxicity of esterified compounds, the cell viability of human keratinocytes NCTC2544 was assayed by MTT (3- (4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) assay according to the method of Mosmann[43] with minor modifications. Twenty-four hours after seeding on 96-well plates, 80% confluent NCTC 2544 cells were exposed to OCTA, starting compounds and the esterified ones. The concentrations ranged from 0.125 to 10μM. A control in basal medium, without addition of compounds, was used. Incubation was carried out for 24, 48 and 72 h. After incubation, the medium was removed and replaced by 100 μL/well of MTT solution. MTT was dissolved (5 mg/mL) in PBS and diluted 1:10 in basal medium without phenol red. Then, plates were incubated in the dark for 3 h (37 °C, 5 % CO$_2$). Then the basal serum free medium was aspirated and 100 μl per well of dimethyl sulphoxide (DMSO) were added to dissolve purple formazan product. The solution was shacked in dark for 15 min at room temperature. The absorbance of the solutions was read at 550nm in a microplate reader (BioTek

Instruments Inc., Bad Friedrichshall, Germany) and elaborated with the ELX808 software (BioTek Instruments Inc., Bad Friedrichshall, Germany). Data were expressed as the mean percentage of viable cells compared to control culture, grown in basal media without addition of compounds .Each experiment was carried out in triplicate.

Viability of oxidation-induced cells

NCTC2544 cells viability after induced oxidative stress was assessed by means of MTT assay as described by Coda et al.[44] with some minor modification. NCTC 2544 cells (~80% confluent) were harvested with trypsin/EDTA and seeded, at a density of 5x 10^4 cells per well, in 96-well plates.

Subsequently, cells were treated with OCTA, starting compounds and the esterified ones for 16 h in RPMI medium (2.5% FBS). The following concentrations of OCTA and esterified compounds were assayed: 0.5, 1 and 5µM. Starting compounds were assayed at concentrations ranging from 0.166 to 2.5µM, according to final equimolar ratio with OCTA. α-Tocopherol (250 and 500µM) was used as the positive control. Cells cultivated on basal serum free medium was used as the control. A negative control, only basal 2.5% FBS medium, was used. After treatment, medium was removed from each well and, after washing, cells were exposed to 1mM hydrogen peroxide (100 µl/well) for 2 h. A control without addition of hydrogen peroxide treatment, was used. After the incubation, MTT assay was performed as described above. Data were expressed as the mean percentage of viable cells compared to the control culture, without oxidative stress. Each experiment was carried out in triplicate.

Intracellular reactive oxygen species (ROS) generation

Production of ROS was monitored spectrofluorometrically using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method, as described by Tobi et al.[45] After seeding at 5x 10^4 cells per well, in 96-well plates, NCTC 2544 were treated with OCTA, starting and esterified compounds at the same concentrations described above. α-tocopherol (250 and 500µM) and H_2O_2-stressed cells were also used. Plates were incubated at 37°C, in 5% CO_2, for 16 h. Medium (RPMI 2.5% FBS) was then removed, cells were rinsed twice with PBS 1X and loaded with 100 µM DCFH-DA (dissolved in DMSO) for 30 min at 37°C in the dark. Following washing of cells with PBS, cells were then exposed to treatment with 100 µL pre-warmed RPMI medium (serum free) containing 1 mM H_2O_2 at 37°C for 2 h in the dark in 5% CO_2. At the end of the treatment, cells were washed twice, lysed with Cell Lytic M lysis buffer (Sigma Aldrich S.r.l., Gallarate, MI, Italy) and transferred into a black 96-well plate. Fluorescent 2',7'-dichlorofluorescein (DCF) was read spectrofluorometrically using a Fluoroskan Ascent FL Microplate Fluorescence Reader (Thermo Fisher Scientific Inc., Waltham, MA) at excitation and emission wavelengths of 485 and 538 nm, respectively. Each experiment was carried out in triplicate.

Effect of OCTA derivatives on the expression of TNF-α gene

Sub confluent monolayers of NCTC 2544 cells were subjected to treatment in basal RPMI 1640 medium with OCTA and esterified compounds at 5µM. Equimolar concentration of starting compound were also tested. Plates were incubated at 37°C for 24h, in 5% CO_2. For quantitative real-time PCR (RT-PCR), total RNA from NCTC2544 cells was extracted using Tri Reagent (Sigma Aldrich S.r.l.) as described by Chomczynski and Mackey.[46] cDNA was synthesized from 2µg RNA template in a 20 µL reaction volume, using PrimeScriptTM RT Reagent Kit (Takara Bio, Tokyo, Japan) in a thermal cycler (Stratagene Mx3000P Real Time PCR System, Agilent Technologies Italia S.p.A., Milan, Italy). The conditions were the following: 37 °C for 15min, 85°C for 5 sec, 4°C hold. Following analysis of mRNA levels was performed using 20X TaqMan® assay (Applied Biosystems): Hs00174128-m1 (Tumor necrosis factor alpha, TNF–α) and Hs999999-m1 (human glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Human GAPDH was used as the housekeeping gene. PCR amplifications were carried out by 2X Premix Ex Taq (Takara Bio, Tokyo, Japan) in a 20 µL of total volume. 4 and 1µL of 40ng cDNA were used for target and hose keeping gene, respectively under the following PCR conditions: 95°C for 30 s followed by 40 amplification cycles (95∘C for 5 s; 60°C for 20 s). Analyses were carried out in triplicate. GAPDH was used as an endogenous control to normalize the amount of total RNA in each and the relative quantification of the levels of gene expression was determined by comparing Δ cycle threshold (Δ Ct) value.[47]

Statistical analysis

All data were obtained at least in three replicates and are reported as the sample mean ± the standard deviation (SD). Pair-comparison between groups was achieved by Student's t-test at P < 0.05, using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The difference between two subsets of data is considered statistically significant if the Student t-test gives a significance level P (P value) less than 0.05. In some cases, the P value can be well below than 0.05, as reported in the text where necessary.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

Author Contribution Statement

Conception and design of the study: Elena Pini

Synthesis of compounds: Elena Pini

Enzymatic synthesis: Raffaella Gandolfi

Quantum chemical calculations: Alessandro Contini

Analysis: Donatella Nava, Stefano Pandini

Interpretation data: Elena Pini, Donatella Nava

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