

Octadecyl ferulate behavior in 1,2-Dioleoylphosphocholine liposomes[☆]Kervin O. Evans^{a,*}, David L. Compton^a, Nathan A. Whitman^{a,1}, Joseph A. Laszlo^a, Michael Appell^b, Karl E. Vermillion^c, Sanghoon Kim^d^a Renewable Products Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University Street, Peoria, IL 61604, USA^b Bacterial Foodborne Pathogens and Mycology, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University Street, Peoria, IL 61604, USA^c Functional Foods Research, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University Street, Peoria, IL 61604, USA^d Plant Polymer Research, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University Street, Peoria, IL 61604, USA

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ABSTRACT

Octadecyl ferulate was prepared using solid acid catalyst, monitored using Supercritical Fluid Chromatography and purified to a 42% yield. Differential scanning calorimetry measurements determined octadecyl ferulate to have melting/solidification phase transitions at 67 and 39 °C, respectively. AFM imaging shows that 5-mol% present in a lipid bilayer induced domains to form. Phase behavior measurements confirmed that octadecyl ferulate increased transition temperature of phospholipids. Fluorescence measurements demonstrated that octadecyl ferulate stabilized liposomes against leakage, maintained antioxidant capacity within liposomes, and oriented such that the feruloyl moiety remained in the hydrophilic region of the bilayer. Molecular modeling calculation indicated that antioxidant activity was mostly influenced by interactions within the bilayer.

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1. Introduction

Plants and fungi containing bioactive compounds have been used for centuries for their medicinal benefits. In recent years, identification and exploration of these bioactive compounds have been of particular interest to researchers. One such compound is ferulic acid (FA), which itself and its derivatives, are readily found in foods such as tomatoes, grain foods, citrus fruits, and coffee [1]. FA has been shown to inhibit oxidative stress in human lymphocytes [2] and its derivatives have been shown to have anti-cancer activity [3].

Octadecyl ferulate (ODF) is one of many FA derivatives that are found throughout the plant and fungi kingdoms. For instance, ODF can be found naturally in fungi such as *Annulohypoxylon squamulosum* [4], in plants such as potato tubers [5], and in herbs [6]. Despite the natural sources, ODF is not particularly abundant in nature and thus the need for de novo synthesis of ODF and other phenolic derivatives in order

to assess their potential as cosmeceutical, nutraceutical, and pharmaceutical ingredients [3,7–15]. One of the features sought in these cosmeceutical, nutraceutical and pharmaceutical ingredients is their ability to retard lipid oxidation.

Lipid peroxidation is damaging to cells because the radical products can propagate to polyunsaturated lipids creating a chain reaction within a cell. Nature counters lipid peroxidation through use of both enzymatic and non-enzymatic antioxidants [16]. Non-enzymatic antioxidants include a large variety of antioxidants that are either aqueous-based or lipophilic like ODF, which is readily produced by plants. We recently demonstrated the antioxidant capacity of the lipophilic feruloyl glyceride derivative, feruloyl dioleoylglycerol [17]. We further explore feruloyl derivatives for antioxidant capacity by demonstrating the antioxidant capacity of ODF. Specifically, we used a Trolox-based assay because Trolox can readily incorporate into liposomes [18]. Peroxyl radicals play a substantial role in peroxidation of polyunsaturated fatty acids of cells which leads to membrane structural loss [19]. The use of liposomes is important because they can readily serve as model cellular systems and can be used as delivery systems of active agents like antioxidants in formulations [20,21]. Thus, it is important to characterize the behavior of ODF within liposomes.

In this work, we report the chemical synthesis of ODF from ferulic acid and octadecanol using a solid acid catalyst as monitored by Supercritical Fluid Chromatography (SFC), the phase transition temperature

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of ODF and its behavior in a lipid matrix of phospholipid liposomes. We compare it to that of slightly less lipophilic ethyl ferulate (EF) in liposomes.

2. Materials and methods

2.1. Reagents and materials

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid), 1-octadecanol, *p*-toluenesulfonic acid monohydrate (*p*-TSA), anhydrous toluene, magnesium sulfate, cobalt (II) chloride hexahydrate, ethylenediaminetetraacetic acid disodium dehydrate (EDTA), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and all common solvents were purchased from Sigma-Aldrich. Ferulic acid ethyl ester (ethyl ferulate) was obtained from Sinova Corporation (Ningbo, China). Ethanol (200 proof) was purchased from Decon Laboratories, Inc. (King of Prussia, PA). Supercritical Fluid Chromatography (SFC)/supercritical fluid extraction (SFE) grade CO₂ was purchased from Airgas Products Co. (Radnor Township, PA). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was bought from Avanti Polar Lipids (Alabaster, AL). High purity calcein, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C₁₁-Bodipy® 581/591), 1,6-diphenylhexatriene (DPH), and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were purchased from Invitrogen (Carlsbad, CA). Tris(hydroxymethyl)aminomethane hydrogen chloride (Tris-HCl), potassium phosphate monobasic, potassium phosphate dibasic, Sephadex G-75 column beads, columns and sodium chloride were obtained from Fisher Scientific. Octaethylene glycol monododecyl ether (C₁₂E₈) was purchased from Sigma-Aldrich.

2.2. Octadecyl ferulate (ODF) synthesis

The procedure was adapted from Taniguchi et al. [22]. Ferulic acid (10.07 g, 520 mmol), octadecanol (21.10 g, 780 mmol), and *p*-TSA (1.05 g, 5.5 mmol) were combined in a 500-mL Schlenk flask under a dry, nitrogen atmosphere using standard Schlenk line techniques. Anhydrous toluene (250 mL) was added via syringe, and the Schlenk flask was fitted with a reflux condenser. The slurry was heated to 85 °C with stirring. Octadecanol and *p*-TSA dissolved after 24 h, but not the ferulic acid. The entire slurry became a clear, yellow solution after stirring and heating for an additional 30 h (54 h total). The solution was then cooled to ambient temperature for 18 h, resulting in the formation of an off-white precipitate (confirmed by SFC analysis to be unreacted ferulic acid). MgSO₄ (1.00 g) was added and the solution stirred for 15 min. The solid was removed by filtration through a fine frit, and the solvent removed from the filtrate resulting in ~30.0 g of off-white solid.

The crude product was purified in 10-g batches by flash chromatography. A 10-g fraction of the crude product was placed in a 1-L round bottom flask and dissolved in 500 mL of acetone. Silica gel (20 g, Silica Gel 60, 70–230 mesh ASTM) was added and the slurry thoroughly mixed. The acetone was then removed by rotor-evaporation. The product-loaded silica was slurried in hexane and poured into a Pyrex, 600-mL, glass frit (10–15 ASTM) fitted on top of a 1-L Erlenmeyer filtration flask. A vacuum was applied and the resultant 5-cm bed of product-loaded silica was developed with 3.5 L of 2% acetone/hexane, 2.5 L of 5% acetone/hexane, 0.5 L of 10% acetone/hexane, and finally 0.5 L of 100% acetone, respectively. Fractions containing ODF (as monitored by SFC) were placed on ice and a white precipitate formed. The precipitates were collected by filtration (Whatman #2, 4.25 cm filter paper) and combined. The supernatants were combined and placed on ice, and a second precipitant formed. The second precipitant was collected by filtration and combined with the first, and the white precipitate was dried at 35 °C in vacuo.

Yield: 9.77 g, 42.2% (based on ferulic acid). Analytical calculated for C₂₈H₄₆O₄: C, 75.29 and H, 10.38; found: C, 73.36 and H, 10.86. ESI-MS (*m/z*) calculated for C₂₈H₄₇O₄ [M + H]⁺: 447.34; found: 447.34749. ¹H NMR (500 MHz, d₆-acetone): δ 8.12 (bs, 0.60 H, —OH), 7.60 (d, *J* = 16.0 Hz, 1.00 H, α), 7.35 (d, *J* = 1.90, 1.00 H, A2), 7.15 (dd, *J* = 8.12, 2.03, 1.99 Hz, 1.00 H, A6), 6.88 (d, *J* = 8.16 Hz, 0.98 H, A5), 6.40 (d, *J* = 15.90 Hz, 1.01 H, β), 4.16 (t, 2.04 H, 1), 3.93 (s, 2.99 H, A7), 1.63 (p, 2.07 H, 2), 1.37 (bm, 30.97 H, 3–17), and 0.89 (t, 3.15 H, 18) ppm. ¹³C NMR (125.77 MHz, d₆-acetone): δ 166.6 (γ), 149.2 (A3), 147.9 (A4), 144.6 (α), 126.6 (A1), 123.0 (A6), 115.2 (A5), 115.1 (β), 110.4 (A2), 63.8 (1), 55.4 (A7), 31.7 (16), 29.4 (11 C, 5–15), 29.2 (4), 28.7 (2), 25.8 (3), 22.4 (17), and 13.4 (18) ppm (Fig. 1).

2.3. Supercritical fluid chromatography (SFC)

The synthesis of ODF was monitored by SFC using a Selerity Technologies Series 4000 chromatograph. Samples (100 μL) taken from the reduction reactions were diluted in 1.0 mL of ethanol and 10-μL aliquots were used for injection. The samples were developed with CO₂ on a Selerity Technologies SB-methyl-100 SFC column (PN AE002, 50 μm × 10 m × .25 μm film) starting at 100 atm and 100 °C. The starting pressure was held for 5 min and ramped at 15 atm/min to a final pressure of 310 atm, which was held for 11 min. The column temperature was isothermal for the duration of the analysis. The FID was set at 350 °C. Under these analytical SFC conditions octadecanol, ferulic acid, and ODF resulted in *R*_t of 11.96, 12.52, and 15.00 min, respectively.

2.4. NMR

¹H and ¹³C NMR spectra were obtained on a Bruker Avance 500 spectrometer (500 MHz ¹H/125.77 MHz ¹³C) using a 5 mm BBI probe. All samples were dissolved in d₆-acetone, and all spectra were acquired at 27 °C. Chemical shifts are reported as ppm from tetramethylsilane calculated from the lock signal (Ξ_D = 15.350609%). Noteworthy: The reaction as monitored by SFC showed nearly 85% conversion of ferulic acid to ODF, which belies the reported yield of 40%. Much of the product was lost in optimizing the flash chromatography and in the precipitations. The ODF is >95% pure by SFC and ¹H NMR.

2.5. Differential scanning calorimetry (DSC)

The thermal properties of ODF were characterized using a Q2000 MDSC™ (TA Instruments; New Castle, DE). ODF (2–3 mg) was weighed into aluminum pans and hermetically sealed. The pans were heated from 10 °C to 100 °C at a heating rate of 1.0 °C/min, followed by cooling from 100° to 10 °C at the same rate (1.0 °C/min). The heating/cooling cycle was repeated twice per sample to determine if ODF exhibited any property changes. All measurements were conducted under a nitrogen atmosphere and run in quadruplicate. Universal Analysis 2000 software (TA Instruments) was used to conduct analysis of the thermographs.

2.6. Fluorescence measurements

Fluorescence emission measurements were conducted using a Jobin-Yvon Horiba Fluorolog 3-21 spectrofluorometer (Edison, NJ) equipped with a 450-W xenon lamp and four-position cuvette holder. Constant temperature was maintained using a Thermo Neslab RTE-7 refrigerated water circulator. Fluorescence measurements were conducted in a 10 mm × 10 mm quartz cuvette (Starna Cells, Inc., Atascadero, CA) with constant stirring. All spectra were corrected and background subtracted using a solution containing only buffer or buffer/liposomes with the corresponding amount of ODF only. Excitation and emission slits were 5 and 10 nm, respectively (unless stated otherwise).

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