Enzymatic Synthesis and Spectroscopic Characterization of 1,3-Divernoloyl Glycerol from *Vernonia galamensis* Seed Oil

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***cis*-12,13-Epoxy-*cis*-octadecenoic (vernolic) acid occurs in triglycerides of the seed oil of *Vernonia galamensis*. The seeds also contain a lipase capable of hydrolyzing the triglycerides. Previous investigators incubated the seed of *Vernonia anthelmintica* and isolated 5.6% yield of 1,3-divernoloyl glycerol. We used crude lipase extract from *V. galamensis* seed to synthesize 1,3-divernoloyl glycerol from vernonia oil in pentane at 40% yield. A 94% conversion of the 1,3-divernoloyl glycerol to pure vernolic acid (5.34% oxirane = 98.9% purity) was achieved by a low-energy saponification process. The carbon-13 nuclear magnetic resonance (NMR) spectrum of the 1,3-divernoloyl glyceride indicates a potential for using carbon-13 NMR spectroscopy in the identification of isomeric diglycerides. Thus the paper describes the synthesis, spectroscopic and chemical characterization of 1,3-divernoloyl glycerol, in addition to providing quantitative carbon-13 NMR studies of *V. galamensis* oil.**

KEY WORDS: Carbon-13 NMR, 1,3-divernoloyl glycerol, hydrogenation, lipase, lipolysis, mass spectrometry, saponification, transesterification, vernolic acid, *Vernonia galamensis*.

The potential commercial applications of *Vernonia galamensis* seed oil are well documented in recent literature (1-5). The seeds contain about 40% of a triglyceride oil, of which about 76% is comprised of an epoxy acid commonly known as vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) acid. One of the most promising commercial applications of the oil is in coatings formulation, where there is urgent need to reduce volatile organic compounds (VOC). Another area of potential application is the use of vernonia lipase to catalyze hydrolytic and esterification reactions. Carlson and co-workers demonstrated the presence of lipase in ground *V. galamensis* seed (5), and Olney and co-workers (6) showed that the lipase extracted from *V. anthelmintica* seeds was nonspecific in lipolytic cleavages of olive oil. Krewson and co-workers (7) incubated ground *V. anthelmintica* seed for 26 d and subsequently isolated *ca.* 6% of 1,3-divernoloyl glycerol. That they isolated only the 1,3-isomer was a surprising finding, because when pancreatic lipase was used on vernonia oil, 2-vernoloyl glycerol was isolated. Thus, Krewson and his group concluded that *V. anthelmintica* lipase was specific for cleaving the acid moiety in the 2-position of vernonia oil glycerides, a finding that contradicts Olney and co-workers (6).

The present paper describes the synthesis, isolation, spectroscopic and chemical characterization of 1,3-divernoloyl glycerol from *V. galamensis* seed oil. A low-energy saponification process was used to convert the 1,3-divernoloyl glycerol to chromatographically pure vernolic acid. In addition, quantitative carbon-13 nuclear magnetic resonance (NMR) studies of vernonia oil and 1,3-divernoloyl glycerol were undertaken to demonstrate its potential use in the identification of isomeric diglycerides.

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EXPERIMENTAL PROCEDURES

Refined vernonia oil was prepared from crude oil as previously described (8). The lipolysis reactions were monitored with a Finnigan (San Jose, CA) MAT 4500 gas chromatograph/mass spectrometer (GC/MS), operated at 70 eV in the electron impact (EI) mode. The interface oven and transfer line were maintained at 300°C, the ionizer temperature was set at 140°C and the injector temperature at 240°C. High-resolution gas chromatography was obtained with a Supelco (Bellefonte, PA) fused-silica SPB-1 capillary column (30 m, 0.32 mm i.d. 0.25 μm film), temperature was programmed from 50 to 300°C, with helium as the carrier gas. GC/MS analysis of transesterified 1,3-diglyceride was performed by the previously described procedure of Ayorinde *et al.* (9). Proton- and carbon-13 NMR spectra were obtained with a GE QE 300 MHz FT-NMR (General Electric, Fremont, CA). Quantitative carbon-13 spectra were obtained at 75.6 MHz with a sample concentration of 10 wt% in deuterated chloroform and the NOE-suppressed, inverse-gated proton decoupled technique. Infrared spectra were obtained with a Perkin-Elmer (Norwalk, CT) 983G spectrophotometer with 3.5 cm^{-1} resolution in nujol mull (5 wt%), or in dilute CCl_4 solution (1 g sample to 50 mL solution). Melting points were determined on a 9100 model Electrothermal instrument (Electrothermal Engineering Ltd., Southend-On-Sea, Essex, England). Percent oxirane was determined by a previously published method (10).

Extraction of vernonia lipase (acetone powder). A 1.2-L glass blender was charged with 100 g *V. galamensis* seed and 200 mL acetone. The seed was ground for 2 min, the resulting mixture was stirred for 1 min, and then the suspension, containing low-density material, was vacuum filtered. The remaining ground seed was washed with two 200-mL portions of acetone that were then filtered through the original filter cake. The grayish filter cake was washed with two 150-mL portions of acetone, then by a 100-mL portion. The combined acetone washes were vacuum filtered to give 13.27 g of light grayish powder after evaporation of the acetone.

Preparation of 1,3-divernoloyl glycerol from vernonia oil. A 100-mL round-bottom flask equipped with an air condenser and magnetic stir bar was charged with 5.01 g vernonia oil, 20 mL pentane and 1.02 g vernonia lipase extract (acetone powder). The mixture was stirred continuously for 14 d in a cold room (4°C). The resulting mixture was transferred to a 500-mL Erlenmeyer flask to which 200 mL pentane was added. The mixture was stirred for 15 min then vacuum filtered. The filtrate was evaporated to obtain 2.33 g (46.6%) oil. The filter cake was transferred to a 500-mL Erlenmeyer flask, stirred for 30 min in 100 mL methanol and then vacuum filtered. The methanol extraction was repeated, the filtrates combined, cooled for 1 h and vacuum filtered to give 2.01 g of 1,3-divernoloyl glycerol (40.12% yield, 4.85% oxirane, m.p. 55.6-56.6°C, lit. m.p. 55-57°C).

Isolation of vernolic acid from 1,3-divernoloyl glycerol.

A 250-mL ground-joint Erlenmeyer flask, equipped with a magnetic stir bar and a water-bath (70°C), was charged with 30 mL methanol and 1.49 g (37.3 mmol) sodium hydroxide. The mixture was refluxed with continuous stirring until complete dissolution of the sodium hydroxide, after which 4.98 g (7.7 mmol) 1,3-divernoloyl glycerol was added in one portion. After refluxing for five minutes, the water-bath was removed. The resulting brownish solution was stirred continuously for an additional 20 min. After mixing with *ca.* 50 g ice and 50 mL water, the mixture was vacuum filtered to give an off-white soap cake, which was transferred into a beaker containing *ca.* 50 g ice and 50 mL water. The mixture was acidified with 7 mL of acetic acid, then vacuum filtered to give a whitish, semi-solid that was dissolved in 50 mL hexane and transferred to a separatory funnel. The hexane layer was washed with 50 mL water and then stripped to give 4.32 g (93.9% yield) pure vernolic acid (5.34% oxirane = 98.9% purity). Mass-spectral data of the methylated product was consistent with the previously reported spectrum of methyl vernolate (11).

RESULTS AND DISCUSSION

Characterization of the 1,3-divernoloyl glycerol. Based on the percent oxirane of 4.85 g, the product was 98.9% pure (m.p. 55.6–56.6°C, lit. m.p. 55–57°C). Mass-spectral data indicated fragment ions that were similar to those previously reported for vernolic acid ester (11), in addition to some diagnostic ions (Table 1). A weak molecular ion [$M^{+\cdot}$] was observed at *m/z* 648. Infrared (IR) data (cm^{-1}) in nujol: 3506, 3450, 1726, 1702, 846, 822; in dilute CCl_4: 3642, 3604 and 3483; Proton-NMR (δ = ppm): 5.49 (multiplet, 4H), 4.15 (multiplet, 5H), 2.94 (broad singlet, 4H, epoxy protons), 2.4 (triplet, 4H), 2.2 (multiplet, 4H), 2.1 (multiplet, 4H), 1.6 (multiplet, 4H), 1.3–1.5 (complex, 32H), 0.9 (triplet, 6H). Figure 1 shows the proton-decoupled carbon-13 NMR spectrum of the diglcyeride: 173.651 (2 carbonyl carbons), 132.396 (2 C-10 carbons), 123.806 (2 C-9 carbons).

In earlier studies of the *V. anthelmintica* lipase by Krewson and co-workers, vernonia seed was incubated for several days, after which about 6% 1,3-diglyceride was obtained. However, in the present investigation, lipolysis was achieved by the interaction of the lipase extract directly with the oil, thus the higher product yield could be due, in part, to the greater interaction between the enzyme and the triglycerides. Further evidence to support this conclusion was derived from an experiment that used crushed vernonia seed in place of the lipase extract, in which case little reaction was observed. Initial characterization of the diglyceride was obtained from the mass spectrometric and proton-NMR analyses. The 1,3-divernoloyl glycerol was introduced directly into the mass spectrometer. A weak molecular ion ($M^{+\cdot}$) was observed at *m/z* 648, which apparently lost two water molecules, probably from the epoxy groups, to give a daughter ion at *m/z* 612. The ion at *m/z* 517 was attributed to cleavage alpha to the epoxy group (C11-C12), followed by loss of water. Fragments at *m/z* 279 and 295 could represent ions attributable to the acyl group of vernolic acid and the alkoxy group from vernolic acid, respectively (Table 1). To further support these conclusions,the 1,3-divernoloyl glycerol was hydrogenated. The mass-spectral data of the saturated diglyceride confirmed all the diagnostic ions observed in the mass-spectral data of the 1,3-divernoloyl glycerol. Transesterification followed by GC/MS analysis gave methyl vernolate with trace amounts of methyl linoleate, methyl oleate, methyl stearate and methyl palmitate. The IR of the 1,3-divernoloyl glycerol were consistent with those of earlier investigators (7).

The proton-NMR data were in agreement with those reported by Krewson's group. The primary and secondary protons of the glycerol moiety apparently exhibit close chemical shifts, hence the complex signals at 4.15 ppm. This is in contrast to the chemical shift of the secondary proton of a triglyceride glycerol moiety, in which the secondary proton has a chemical shift almost identical with those of the olefinic protons at about 5.41 ppm. To lend additional support, the proton-NMR data of the hydrogenated product indicated the presence of the epoxy protons at 2.91 ppm, protons alpha to the carbonyl group at 2.38 ppm, and the glycerol protons at 4.16 ppm. The chemical shifts due to the olefinic and allylic protons in

TABLE 1

Mass Spectrometric Diagnostic Ions for 1,3-Divernoloyl Glycerol

Fragment ions (%)	Structural species
648 (0.2)	Molecular ion [$M^{+\cdot}$]
630 (0.2)	$M-H_2O$
612 (0.2)	$M-2H_2O$
517 (0.4)	$M-[H_2O + CH_3(CH_2)_4CH(O)CH-]$
295 (0.2)	$CH_3(CH_2)_4CH(O)CH-CH_2-CH=CH-(CH_2)_7-CO_2$
279 (35.4)	$CH_3(CH_2)_4CH(O)CH-CH_2-CH=CH-(CH_2)_7-CO$

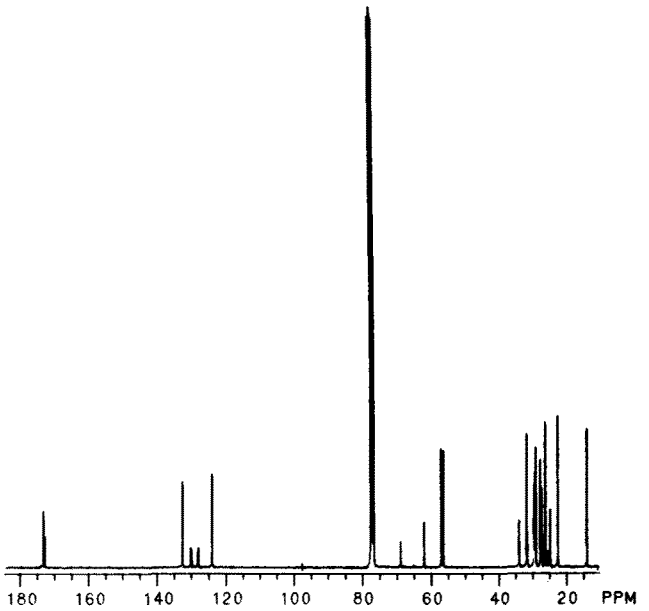

FIG. 1. Proton-decoupled carbon-13 nuclear magnetic resonance spectrum of *Vernonia galamensis* oil. Peaks are referenced to $CDCl_3$ at 76.998 ppm. Samples were made to 10 wt% in $CDCl_3$. Operating frequency was at 75.6 MHz. Chemical shifts (δ = ppm): vernoloyl carbonyl carbons, 173.104 (1,3-) and 172.695 (2-); vernoloyl olefinic carbons, C-10, 132.413, C-9, 123.889. Glycerol carbons, 62.010 (1,3-), 68.822 (2-), epoxy carbons, 56.437 and 57.101.

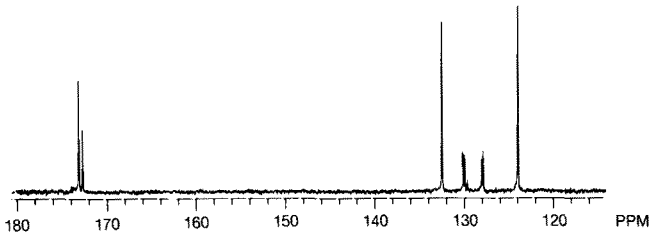

FIG. 2. Proton-decoupled carbon-13 nuclear magnetic resonance spectrum carbonyl and olefinic region of *Vernonia galamensis* oil. Peaks are referenced to $CDCl_3$ at 76.998 ppm. Samples were made to 10 wt% in $CDCl_3$. Operating frequency was at 75.6 MHz. Chemical shifts (δ = ppm): vernoloyl carbonyl carbons, 173.104 (1,3-) and 172.695 (2-); vernoloyl olefinic carbons, C-10, 132.413, C-9, 123.889. Olefinic carbons from non-epoxy acids, 127.731, 127.891, 129.787, 129.985.

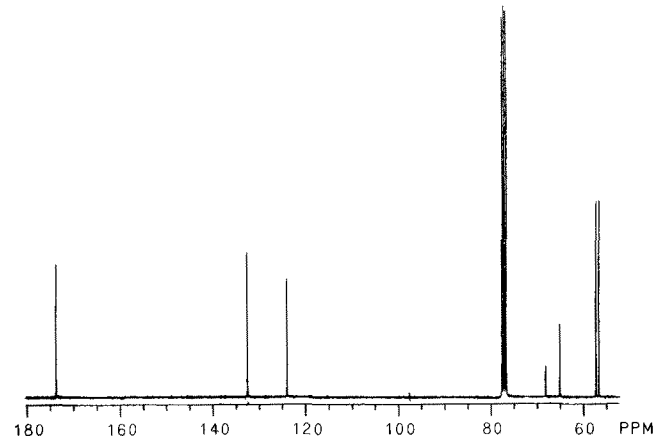

FIG. 3. Proton-decoupled carbon-13 nuclear magnetic resonance spectrum of 1,3-divernoloyl glycerol. Peaks are referenced to $CDCl_3$ at 76.998 ppm. Samples were made to 10 wt% in $CDCl_3$. Operating frequency was at 75.6 MHz. Chemical shifts (δ = ppm): vernoloyl carbonyl carbons, 173.651 (1,3-); vernoloyl olefinic carbons, C-10, 132.413, C-9, 123.889. Glyceryl carbons, 64.910 (1,3-), 68.022 (2-), epoxy carbons, 56.440 and 57.105.

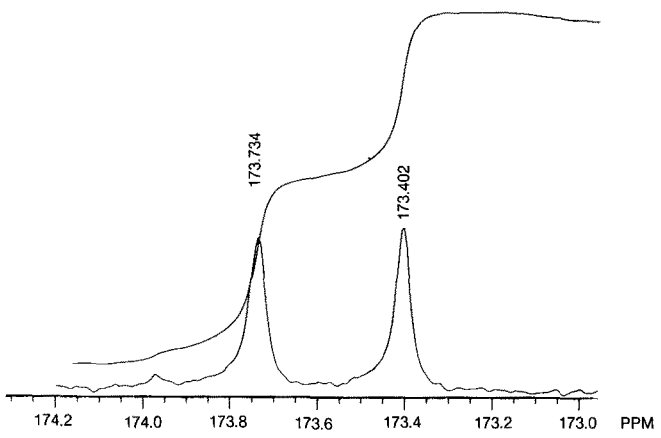

FIG. 4. Proton-decoupled carbon-13 nuclear magnetic resonance spectrum carbonyl region of 1,2-dipalmitin. Peaks are referenced to $CDCl_3$ at 76.998 ppm. Samples were made to 10 wt% in $CDCl_3$. Operating frequency was at 75.6 MHz. Chemical shifts (δ = ppm): carbonyl carbons, 173.734 (1- or 3-), 173.402 (2-).

the 1,3-divernoloyl glycerol were absent in the spectrum of the hydrogenated diglyceride.

Because there have been no published carbon-13 NMR spectra of 1,3-divernoloyl glycerol and vernonia oil, we investigated their quantitative carbonyl spectra. Figures 1 and 2 show the carbon-13 carbonyl NMR spectrum and partial spectrum of vernonia oil. The carbon-13 carbonyl NMR spectrum (partial) of the synthetic 1,3-diglyceride is shown in Figure 3. Recent studies by Wollenberg (12) and Ng (13) on quantitative carbon-13 of vegetable oils have shown that the acyl carbonyl groups on the primary and secondary glycerol carbons exhibit different chemical shifts, as well as primary acyl carbonyl carbons being shifted downfield relative to the secondary acyl carbonyl carbons. A comparison of the carbon-13 spectra of the 1,3-divernoloyl glycerol (Fig. 3) and vernonia oil (Fig. 2) shows the absence of linoleic and oleic acid moieties in the diglyceride. Furthermore, a single carbonyl signal (attributed to two carbonyl carbons) in the spectrum of the diglyceride indicates the absence of a secondary acyl group.

To lend support for these assignments and because there were no available reference standards for 1,3- or 1,2-divernoloyl glycerol, we undertook a study of the carbon-13 NMR carbonyl spectra of 1,3- and 1,2-dipalmitins (purchased from Sigma Chemical Company, St. Louis, MO). Figure 4 shows the quantitative carbonyl spectrum of 1,2-dipalmitin. As expected, 1,2-dipalmitin showed two peaks at 173.734 ppm (primary acyl carbon) and 173.402 ppm (secondary acyl carbon). On the other hand, the carbonyl spectrum of 1,3-dipalmitin showed only one peak, at 173.839 ppm. We found that the chemical shift of the 1,3-dipalmitin carbonyl carbon was consistently shifted downfield relative to the α-acyl carbon of the 1,2-dipalmitin, hence the spectrum of a mixed sample containing equimolar concentration of 1,3- and 1,2-dipalmitin was run to confirm this finding. The resulting spectrum gave three peaks in the carbonyl region with relative intensity ratios of 2:1:1, the peak attributed to 1,3-dipalmitin being shifted downfield relative to the 1,2-dipalmitin peaks, thus establishing that proton-decoupled carbon-13 NMR spectroscopy may constitute a valuable tool for the identification of isomeric diglycerides. We continue to pursue further investigations in this direction.

To our knowledge the present work represents the first reported data on the carbon-13 NMR spectra of vernonia oil and 1,3-divernoloyl glycerol. We found that the latter compound constitutes a quantitative source of vernolic acid, which has potential as a chemical feedstock.

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