Characterization of Estolides Produced from the Acid-Catalyzed Condensation of Oleic Acid

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Estolides produced from an acid-catalyzed condensation of oleic acid were characterized by high-performance liquid chromatography (HPLC), gas chromatography (GC), GC–mass spectrometry (MS) and nuclear magnetic resonance (NMR). C-8 reverse-phase HPLC provided a clean resolution of the estolide oligomers present in the reaction mixtures, allowing an average oligomer distribution to be calculated. Corroboration of HPLC results were obtained either through hydrolysis of the estolide mixture and quantitation of the hydroxy fatty acid content by GC, through the integration of the α-methylene protons adjacent to the carbonyl of the acids vs. the esters in the ^{1}H NMR spectrum, or by titration of the carboxylic acid with standardized base. GC and GC–MS analysis of the hydrolyzed estolide mixture indicated that the ester positions were centered around the original double-bond position, with linkages ranging from positions 5–13. Likewise, the unsaturation was distributed along the fatty acid backbone.

KEY WORDS: Estolide, GC–MS, HPLC, NMR, oleic acid, oleic homopolymer, oleic oligoesters, polyestolide.

An estolide, **1**, is a unique oligomeric fatty acid that contains secondary ester linkages on the alkyl backbone of the molecule. Estolides have typically been synthesized by the homopolymerization of castor oil fatty acids (1,2) or 12-hydroxystearic acid (3,4) under thermal or acid-catalyzed conditions. The homopolymerization of hydroxy fatty acids can also be catalyzed by lipases (5). In addition to the estolides derived from hydroxy fatty acids, hydroxy oils will also provide estolides (6–9) when reacted with fatty acids. These estolide-containing triglycerides are used mainly as drying oils (1,6–9) after the ester moiety has been decomposed to yield conjugated dienes. A new technique for the production of estolides was developed by Erhan *et al.* (10), in which estolides were formed from a high-temperature and pressure condensation of unsaturated fatty acids over clay catalysts.

Our laboratory has developed a new process for the acid-catalyzed oligomerization of unsaturated fatty acids into estolide (11). Conversion of the fatty acid double bond into an ester functionality is a strikingly different method than the hydroxy esterification process and had only been observed in limited yields (12–15) prior to our work. Unfortunately, a detailed characterization of the polymeric estolides had yet to be made, although some work with infrared (IR) spectroscopy (14), thin-layer chromatography (TLC) (2), supercritical fluid chromatography (SFC) (3) and gel permeation chromatography (GPC) (5) has been done. In an effort to address this problem, we have developed four analysis techniques that utilize high-pressure liquid chromatography (HPLC), gas chromatography (GC), nuclear magnetic resonance (NMR) and titration as general methods for the determination of polyestolides. In addition, we have fully characterized the structure of polyestolide through NMR and GC–mass spectrometry (MS).

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EXPERIMENTAL PROCEDURES

Materials. Estolide samples were obtained by a previous technique developed in our laboratory (11). Oleic acid (90%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Boron trifluoride/methanol complex (14% wt/vol) and fatty acid methyl esters (FAMEs) standard mixtures were obtained from Alltech Associates, Inc. (Deerfield, IL). Solvents for chromatography or extraction were of HPLC or an equivalent grade and used without further purification. *Bis*(trimethylsilyl)trifluoroacetamide silylating reagent was obtained from Sigma (St. Louis, MO).

Instrumentation. GC was performed on a Hewlett-Packard 5890 Series II gas chromatography (Palo Alto, CA) equipped with a flame-ionization detector and an autosampler/injector. Analyses were conducted on two columns, a CP SIL-84 25 m × 0.22 mm i.d. (Chrompack, Bridgewater, NJ) and an SGE-BP1 25 m × 0.22 mm i.d. (Scientific Glass Engineering Pty. Ltd., Austin, TX). CP SIL-84 analysis conditions: column flow, 0.95 mL/min; split ratio, 35:1; programmed ramp, 120 to 250°C at 3°C/min with a 2-min hold at 250°C. SGE-BP1 analysis conditions: column flow, 0.87 mL/min; split ratio, 37:1; programmed ramp, 170 to 250°C at 2°C/min with a 5-min hold at 250°C. Injector and detector temperatures were set at 250°C for all analyses. Saturated C8-C30 FAMEs provided standards for calculating equivalent chainlength values. Ozonolysis fragments were analyzed on the CP SIL-84 column with a programmed ramp of 50 to 250°C at 5°C/min and a 5-min hold at 250°C. Injector and detector temperatures were maintained at 250°C.

HPLC analyses were performed on a Spectra-Physics 8100 extended LC system (San Jose, CA) with autosampler/injector coupled to a Varex evaporative light scattering detector (Burtonsville, MD). C-8 reverse-phase analysis was carried out on the methyl ester derivative with an IBM octyl end-capped column (250 mm × 4.5 mm, 5 μ particle size) and the following gradient elution: time 0 to 2 min, 60% acetonitrile 40% acetone; 20–25 min, 100% acetone; 30—35 min, 60% acetonitrile 40% acetone. Retention times for eluted peaks: monomer, 5.0 min; monoestolide, 7.1 min; diestolide, 10.3 min; triestolide, 13.4 min; tetraestolide, 16.5 min; pentaestolide, 18.6 min; hexaestolide, 20.1 min; heptaestolide, 21.9; and polyestolides, 22–26 min. A chromatogram of a polyestolide sample is shown in Figure 1.

GC–MS was performed on a Hewlett-Packard 5890A GC with a 25 m DB-1 column and a Hewlett-Packard 5970 mass selective detector. GC conditions for trimethylsiloxy FAMEs: Programmed ramp 150 to 250°C at 5°C/min with a 10-min hold at 250°C. Injector temperature was set at 250°C and transfer line at 280°C.

NMR was performed on a Bucker WM300 (Billericka, MA) with a dual 5-mm probe. $CDCl_3$ served as the solvent in all analyses. ^{1}H NMR (300 MHz) of polyestolide sample: δ 5.34 (*m* 1H), 4.84 (*m*, 2H), 2.30 (*t*, $J = 7.4$, 2H), 2.25 (*t*, $J = 7.5$, 2H), 2.00–1.87 (*m*, 2H), 1.65–1.55 (*m*, 6H), 1.55–1.43 (*m*, 8H), 1.43–1.00 (*m*, 60H), and 0.85 ppm (*t*, 9H). ^{13}C NMR (75.5 MHz) δ 179.7 (*s*), 173.7 (*s*), 130.5 (*d*),

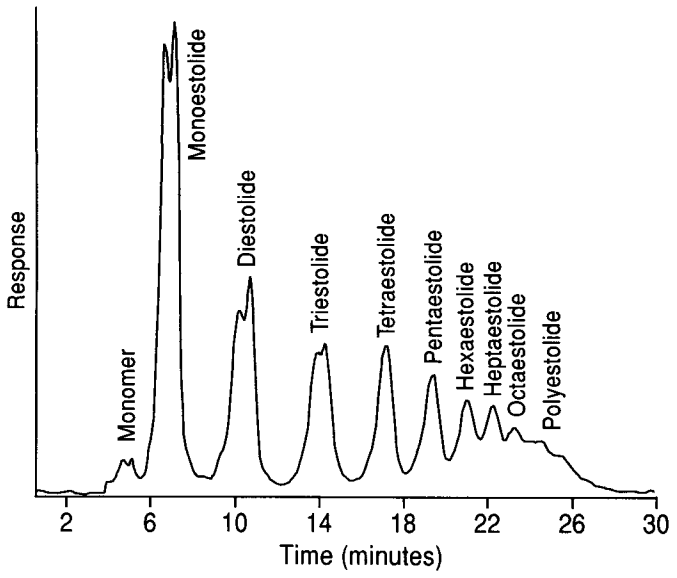


FIG. 1. Reverse-phase high-performance liquid chromatography separation of polyestolide oligomers.

74.0 (*d*), 34.5 (*t*), 33.8 (*t*), 32.5 (*t*), 31.8 (*t*), 31.7 (*t*), 31.5 (*t*), 28.9 (*t*), 27.4 (*t*), 27.1 (*t*) 25.0 (*t*), 24.9 (*t*), 24.6 (*t*), 24.5 (*t*), 22.5 (*t*) and 13.9 ppm (*q*).

Methods. Methyl esters of the estolides were made by dissolving ≈100 mg of the estolide in 2 mL tetrahydrofuran (THF) and 2 mL BF_3 • MeOH (14% wt/vol) and held at reflux over a steam bath for 15 min. The crude estolide methyl ester was poured into 10 mL hexane and washed with 3 × 100 mL H_2O, dried over Na_2SO_4, filtered and concentrated *in vacuo.*

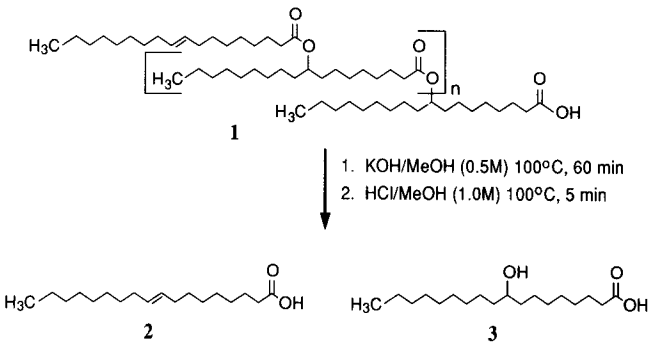
Isolation of individual polyestolide oligomers by HPLC. Polyestolide (10-mg portions) was injected onto a C-8 reverse-phase column, and the individual estolide oligomers were collected as they eluted from the column. This procedure was repeated seven times to obtain a sufficient mass for subsequent derivatization and analysis. The estolide fractions were then diluted in 1 mL hexane and split into two fractions. One 200-μL portion was injected onto the HPLC to confirm oligomer purity. The remaining 800 μL was concentrated under a stream of nitrogen and then subjected to base hydrolysis.

Base hydrolysis of polyestolide. Polyestolide (10 mg) was placed in a sealed vial with 0.5 mL of 0.5 M KOH/CH_3OH and heated at 100°C for 60 min. The reaction was acidified with 0.5 mL of 1.0 M HCl/CH_3OH and heated 5 min at 100°C. Solid $NaHCO_3$ was added to the reaction mixture, and the methanol was removed under a stream of nitrogen at 50°C. The residual esters were dissolved in ethyl acetate and injected onto a CP SIL-84 GC column. Hydroxy esters were separated from unsaturated fatty esters by crystallization from hexane. The isolated hydroxy fatty acids were then treated with an excess of *bis*(trimethylsilyl)trifluoroacetamide to give silyloxy fatty esters which were then analyzed by GC–MS.

Olefin position. Ozonolysis was carried out, as previously described (16), on the unsaturated fatty acid fraction obtained from the mother liquor during crystallization of the hydroxy fatty acids from hexane.

RESULTS AND DISCUSSION

HPLC peak assignment of estolide oligomers. Estolide **1**, produced from the acid-catalyzed condensation of oleic acid (11), was separated into its individual components by C-8 reverse-phase HPLC as shown by the trace in Figure 1. Peak assignments for the reverse-phase HPLC chromatogram were made by first isolating the individual estolide oligomers as they eluted from the column. The fractions were then divided into two portions, one portion was injected onto the C-8 column to confirm oligomer purity, and the second portion was subjected to base hydrolysis (Scheme 1). The resulting hydroxy fatty acids, **3**, and monoenoic acids, **2**, were then analyzed by GC. A comparison of the HPLC hydroxy hydrolysis equivalent (HHE) values to the observed percentage of hydroxy fatty acids determined by GC are shown in Table 1. The GC analysis of the hydrolyzed estolide oligomers fractions confirmed our original HPLC peak assignments.



SCHEME 1

TABLE 1

HPLC Isolation of Polyestolide Oligomers

Fraction[a]	HHE[b] (HPLC)	Observed percent hydroxy (GC)[c]	Percent deviation
1	51.2	51.8	0.6
2	66.7	65.1	1.6
3	75.0	74.5	0.5
4	80.0	78.2	1.8
5	83.2	83.3	0.1
6	85.6	85.9	0.3
7	87.4	87.0	0.4
8	88.7	88.6	0.1
9	89.0	92.2	3.2
10	90.5	89.6	0.9
11	90.1	90.1	0.0

[a]Fractions were obtained by collecting the individual oligomers as they eluted from the C-8 column.
[b]HHE (hydroxy hydrolysis equivalent); HPLC, high-performance liquid chromatography.
[c]Gas chromatography (GC) analysis performed on a CP SIL-84 25 m × 0.22 mm i.d. column with a programmed ramp from 120 to 250°C @ 3°C/min.

TABLE 2

Calculation of Estolide Number (EN) and Hydroxy Hydrolysis Equivalent (HHE)

Peak	HPLC[a] (%)	Formula weight (g/mol)	Mole fraction	Number of ester linkages	Fractional EN
Monomer	2.4	282.47	0.086	0	0.00
Monostolide	17.2	564.93	0.314	1	0.31
Diestolide	15.4	847.40	0.187	2	0.37
Triestolide	15.3	1129.87	0.140	3	0.42
Tetraestolide	13.0	1412.33	0.095	4	0.38
Pentaestolide	9.7	1694.79	0.059	5	0.29
Hexaestolide	7.9	1977.25	0.041	6	0.25
Heptaestolide	6.0	2259.71	0.027	7	0.19
Octaestolide	5.1	2542.17	0.021	8	0.16
Polyestolides	8.1	2824.63	0.030	≥9	0.27
				EN	2.65
				HHE	72.63

[a]Integration data shown as area percentage obtained from the HPLC chromatogram shown in Figure 1, along with abbreviation.

HHE (Equation 1) is a calculated hydroxy value (based on the area percentage from HPLC) that an estolide would yield if base-hydrolyzed under the conditions outlined in Scheme 1. Estolide number (EN), the average oligomeric distribution of estolides in a mixture, is calculated by multiplying the mole fraction for each individual oligomer by the number of ester linkages present in the oligomer to provide the fractional estolide number. Summation of the fractional EN values gives the total EN. Calculation of the representative polyestolide sample, in Figure 1, is detailed in Table 2.

$$\% \text{ hydroxy} = \text{HHE} = (\text{EN}/\text{EN} + 1) \times 100 \quad [1]$$

NMR characterization of polyestolide. A proton spectrum for a polyestolide sample is shown in Figure 2. A key feature of the proton NMR is the estolide methine signal (H_m) at 4.84 ppm. In addition to the estolide

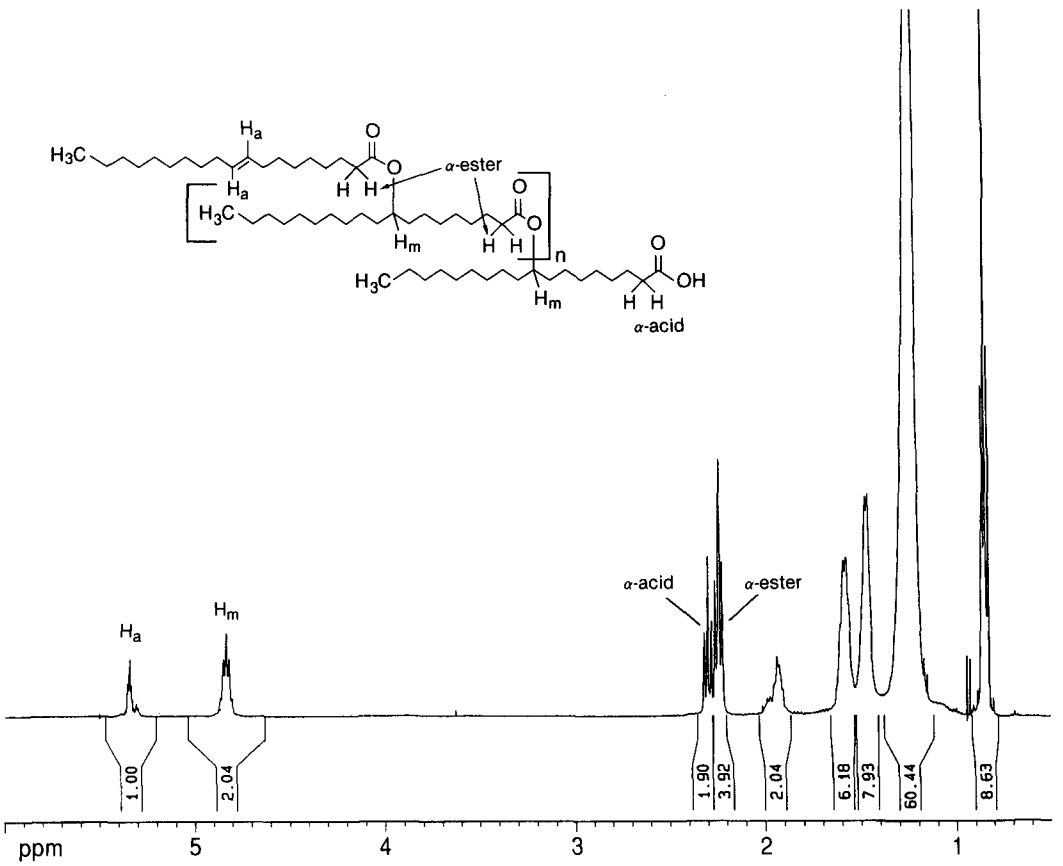


FIG. 2. ^{1}H nuclear magentic resonance of polyestolide (300 MHz).

TABLE 3

Mass Spectral Data for Silylated Hydroxy Fraction from Polyestolide Hydrolysis

Position	Mass carbonyl fragment (*m/e*)	Normalized abundance	Mass alkyl terminus fragment (*m/e*)	Normalized abundance
5	203	0.79	285	0.03
6	217	3.64	271	1.69
7	231	8.59	257	4.48
8	245	13.63	243	8.14
9	259	18.64	229	13.94
10	273	17.08	215	16.67
11	287	9.74	201	11.61
12	301	6.30	187	8.40
13	315	1.80	173	4.59

methine signal, the other distinctive feature for the estolide moiety is the α-carboxylic protons. These α-methylene protons (2.25 ppm), adjacent to the ester, are upfield of the α-methylene protons (2.30 ppm) that are adjacent to the acid. Both have triplet coupling patterns as expected for a proton adjacent to a methylene. Integration of the α-methylene signals provided a ratio for the number of ester bonds with respect to acid functionalities. This ratio (α-ester/α-acid) is the EN. Thus, HHE can be calculated directly from the ^{1}H-NMR spectrum by Equation 1.

The main feature of the ^{13}C NMR is the estolide methine carbon at 74.0 ppm, which is confirmed in the DEPT experiment at a 135°C pulse. A second distinctive feature for the estolide in the ^{13}C NMR is the two different carbonyl carbons at 179.7 ppm (acid) and 173.7 ppm (estolide).

GC–MS of silylated hydroxy esters obtained from hydrolysis of polyestolide. Polyestolide was hydrolyzed in 0.5 M KOH/CH_3OH to give the corresponding hydroxy and normal-chain fatty acids (Scheme 1). The isolated hydroxy fatty esters (silylated) were then analyzed by GC–MS (17). The main mass spectral features are *m/e* 371 ($M^+ - 15$, 2%), 73 (TMS+, 100%) and two sets of gaussian fragments representing cleavage at the silyloxy positions (masses 173 to 315). This fragments and their abundances are summarized in Table 3 relative to their respective positions on the fatty ester backbone. The gaussian envelope that contains each set of fragments indicates that the original estolide position is distributed from positions 5–13 with the original Δ9 and Δ10 positions having the largest abundances in the mass spectrum. One might expect a high concentration of estolide with ester linkages at the 9 and 10 positions because the estolide is derived by addition across the double bond of oleic acid (11). However, the intensity of a fragment in MS is not solely dependent on concentration of a species in a mixture but more so on ionization energies and charge stabilization. Thus, the apparent gaussian distribution of hydroxy positions derived from the estolide, though likely due to structural similarities, does not necessarily represent the amount of each position in the mixture other than to indicate their presence.

Alkene position within the estolide. The structure of the estolide, shown in Figure 2, indicates that the estolide has two components, hydroxy fatty acid and unsaturated fatty acid. Hydrolysis of the estolide, as described in the preceding section, provided both hydroxy fatty ester and unsaturated fatty esters. The MS of the silylated hydroxy esters indicated that a distribution of the ester position down the backbone of the fatty acid had occurred. Similarly, the unsaturation has migrated along the backbone of the fatty acid molecule to positions 6 through 16, as seen by the ozonolysis (16) data summarized in Table 4. It is not surprising that positional isomerization is occurring under these acidic reaction conditions and provides further support to the mechanism proposed for estolided formation (11).

Acid value (AV) as a means to determine HHE. AVs were determined by the AOCS Method Te la-64 (18), but substituting ethanol for methanol to increase the solubility of the estolide during the titration. Titration not only provided the AV but also yielded the neutralization equivalent (NE), which is the average formula weight of the estolide mixture. NE was calculated by Equation 2 and can be converted to HHE by Equations 3 and 1.

$$NE = (1000 \times 56.11)/AV \quad [2]$$

$$EN = (NE - 282.47)/282.47 \quad [3]$$

Table 5 provides a summary of all four analysis techniques and indicates that there is a good correlation between these four independent methods for determing the extent of polyestolide formation.

TABLE 4

Ozonolysis Data[a]

Alkene position	Mole percentage
6	2.40
7	15.83
8	15.90
9	16.20
10	16.09
11	13.48
12	8.40
13	5.60
14	3.34
15	1.80
16	0.96

[a]Ozonolysis and analysis performed by the technique outlined by Isbell *et al.* (16).

TABLE 5

Comparison of Estolide Analysis Techniques

	HPLC	GC (hydrolysis)[a]	Titration (AV)[b]	NMR
EN	2.26	2.29	2.34	2.10
HHE	69.31	69.60	70.06	67.74
Neutralization equivalent	920.47	929.16	943.50	875.64
AV	60.96	60.39	59.47	64.08

[a]Observed percent hydroxy fatty acid from hydrolysis of the estolide mixture. NMR, nuclear magnetic resonance. See Tables 1 and 2 for other abbreviations.
[b]Titration with standardized base to give the acid value (AV).

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