t-Butyl 6-Methyl-1-cyclohexenecarboxylate (XIb).—The ester was obtained pure by preparative gas chromatography. The infrared spectrum of ester 4 (neat) contained absorption bands at 1700 (C=C–C=O), 1640 w-m (C=C), 1160 (C–O), and 750 (H–C=C–COOR) cm^{-1}; nmr spectrum: one olefinic proton (δ 6.73), a doublet equivalent to the three protons of the ring methyl group (δ 1.04), and a singlet equivalent to the nine protons of the *t*-butyl group (δ 1.46); the remaining seven cyclohexyl protons formed a complex multiplet (δ 1.20–2.80). The refractive index of XIb was $n^{25}D$ 1.4629.

6-Methyl-1-cyclohexenecarboxylic Acid (XIa) and Its Periodate Oxidation.—The acid was obtained in pure form both by saponification of XIb and by crystallization from a mixture of the acids from the dehydrohalogenation reaction. In the latter case the liquid acids were decanted from the solid which was washed with cold pentane. Recrystallization from aqueous ethanol yielded a white crystalline product that melted at 104–105° (lit.[26] 105.5°). The infrared spectrum (potassium bromide pellet) contained absorption bands at 1680 (C=C–C=O), 1640 (C=C), 1220 (C–O), and 930 broad (OH deformation) cm^{-1}; ultraviolet absorption data: λ_{max}^{EtOH} 214 mμ (ϵ 6600);[16] nmr spectrum: one olefinic proton (δ 7.04), a multiplet equivalent to one proton (δ 2.70), a multiplet equivalent to two protons (δ 2.23), a multiplet equivalent to four protons (δ 1.65), and a doublet equivalent to the three protons of the ring methyl group (δ 1.12).

Anal. Calcd for $C_8H_{12}O_2$: C, 68.54; H, 8.57. Found: C, 68.78; H, 8.46.

XIa was oxidized by the method of von Rudloff[23] to α-methyladipic acid (52% yield). Its methyl ester was identical with that of a known sample by infrared spectrum and gas chromatographic retention time.

t-Butyl 2-Methylbicyclo[3.1.0]hexane-1-carboxylate (XIIb).—Ester 1 (XIb) was obtained pure by preparative gas chromatography. Its infrared spectrum (neat) contained absorption bands at 3080, 3040, 3000, 1025 (cyclopropyl group),[18d,19] 1709 (C=O), and 1140 (C–O) cm^{-1}. There was no C=C absorption band; nmr spectrum: a multiplet equivalent to one proton (δ 2.55), a multiplet equivalent to four protons (δ 1.72), a singlet equivalent to nine protons of the *t*-butyl group (δ 1.43), a doublet equivalent to three protons of the ring methyl group superimposed on a multiplet for one proton (δ 1.03), and a multiplet equivalent to two protons (δ 0.70). The spectrum did not contain absorption associated with olefinic protons. The refractive index of XIIb was $n^{25}D$ 1.4498.

Anal. Calcd for $C_{12}H_{20}O_2$: C, 73.42; H, 10.72. Found: C, 73.67; H, 10.11.

(25) W. Kuster, *Ber.*, **35**, 2949 (1902).
(26) W. S. Rapson and R. G. Shutteworth, *J. Chem. Soc.*, 636 (1940).

Hydroxymonoenoic Acids of *Lesquerella densipila* Seed Oil

R. G. BINDER AND A. LEE

Western Regional Research Laboratory,[1] *Albany, California 94710*

Received December 3, 1965

Two hydroxy fatty acids of *Lesquerella densipila* seed oil are characterized as (+)-12D-hydroxy-*cis*-9-octadecenoic (ricinoleic) acid and as the previously unknown (+)-12-hydroxy-*cis*-9-hexadecenoic acid. The fatty acid composition of the oil is given.

Most seed oils of examined[2] *Lesquerella* species contain large percentages of hydroxyeicosenoic acid, but *L. densipila* and *L. lescurii* seed oils instead contain C-18 hydroxy acids. Smith and co-workers characterized the hydroxyeicosenoic acid as (+)-14-hydroxy-*cis*-11-eicosenoic acid[3] and the major hydroxy acid of *L. densipila* as 12-hydroxy-*cis*-9-*cis*-15-octadecadienoic acid.[4] Recent work[5] shows that these acids have the D configuration. Smaller amounts of a C-18 hydroxy acid, presumed[4] to be ricinoleic acid, and of an apparently monoenoic C-16 hydroxy acid were also found[2,4] in *L. densipila* oil. The present paper describes the characterization of these minor hydroxy acids.

After methyl esters were prepared by sodium methoxide-catalyzed methanolysis of *L. densipila* seed oil, distillation provided a C-18 hydroxy ester fraction. The monoenoic ester was separated from methyl densipolate (12-hydroxy-*cis*-9-*cis*-15-octadecadienoate) on a silver ion macroreticular ion-exchange resin.[6] Chromatography on silicic acid then gave a liquid ester that showed no impurity by gas–liquid partition chromatography (glpc) or thin layer chromatography (tlc).

The nuclear magnetic resonance (nmr) spectrum of this ester indicated a methyl ester of a straight chain monounsaturated fatty acid with a secondary hydroxyl group and the double bond not β to the terminal methyl group (*cf.* methyl densipolate). Mass spectral analysis of the hydrogenated derivative located the hydroxyl group at C-12.[7] To locate the double bond, the parent ester was cleaved by a modification of von Rudloff's[8] permanganate–periodate oxidation procedure. Methyl esters of the fragments were identified as dimethyl azelate and methyl 3-hydroxynonanoate by mass spectrometry, so the double bond is in the 9,10 position. The infrared, nmr, and mass spectra of the parent ester are indistinguishable from the corresponding spectra of methyl ricinoleate [(+)-12D-hydroxy-*cis*-9-octadecenoate]. That the compound is in fact methyl ricinoleate is shown by the coincidence of the optical rotatory dispersion (ORD) curves of the ester and its hydrogenated derivative with the curves of methyl ricinoleate and hydrogenated methyl ricinoleate.

The compound tentatively identified as a monoenoic C16 hydroxy ester was concentrated from *L. densipila* methyl esters and finally purified by repeated chromatography on silicic acid.

Features of the pure C-16 methyl ester shown by the nmr spectrum included an unbranched chain containing one ethylenic bond not β to the terminal methyl group and a secondary hydroxyl group. As there is no absorption in the infrared spectrum at 10.3 μ, the

(1) A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.
(2) K. L. Mikolajczak, F. R. Earle, and I. A. Wolff, *J. Am. Oil Chemists' Soc.*, **39**, 78 (1962).
(3) C. R. Smith, Jr., T. L. Wilson, T. K. Miwa, H. Zobel, R. L. Lohmar, and I. A. Wolff, *J. Org. Chem.*, **26**, 2903 (1961).
(4) C. R. Smith, Jr., T. L. Wilson, R. B. Bates, and C. R. Scholfield, *ibid.*, **27**, 3112 (1962).
(5) T. H. Applewhite, R. G. Binder, and W. Gaffield, *Chem. Commun.*, **12**, 255 (1965); T. H. Applewhite, *Tetrahedron Letters*, **No. 38**, 3391 (1965).
(6) E. A. Emken, C. R. Scholfield, and H. J. Dutton, *J. Am. Oil Chemists' Soc.*, **41**, 388 (1964).
(7) R. Ryhage and E. Stenhagen, *Arkiv Kemi*, **15**, 545 (1960).
(8) E. von Rudloff, *J. Am. Oil Chemists' Soc.*, **33**, 126 (1956).

double bond is *cis*. The hydroxyl group was found at C-12 in the unsaturated and hydrogenated esters by mass spectral analyses. Oxidative cleavage of the double bond yielded acidic fragments that were esterified and then chromatographed on silicic acid. As shown by mass spectral analysis and glc, dimethyl azelate predominated in the nonpolar fraction. Analysis of the polar fraction by glpc disclosed a component having the retention characteristics expected for a methyl hydroxyheptanoate. Although the mass spectrum did not clearly indicate a parent ion for this, it did indicate the fragment CH_3OOCCH_2CHOH. Thus, the double bond is in the 9,10 position and the ester is methyl 12-hydroxy-*cis*-9-hexadecenoate.

At 589 mμ, methyl 12-hydroxy-*cis*-9-hexadecenoate is slightly more dextrorotatory than methyl ricinoleate though the ORD curves of the two compounds are virtually identical between 600 and 250 mμ. That these compounds have the same sign of rotation suggests they have the same absolute configuration, but the sign of rotation of the unsaturated compound can be misleading. Both methyl ricinoleate (dextrorotatory) and methyl densipolate (levorotatory) have the D configuration.[5] Applewhite, *et al.*,[5] have recommended reduction to the saturated alcohol before attempting to assign absolute configuration. Because reduced methyl 12-hydroxy-*cis*-9-hexadecenoate displays a plain negative ord curve, as does methyl 12D-hydroxyoctadecanoate, it is highly likely to have the D configuration. But without comparison to methyl 12-hydroxyhexadecanoate of known configuration, the assignment is not unequivocal.

The three hydroxy acids of *L. densipila* oil thus are very similar: they appear to have the same absolute configuration, and each has a *cis* double bond in the 9,10 position and a hydroxyl group at C-12.

The fatty acid composition of the oil was determined by combined column chromatography and glpc. Free fatty acids were obtained from saponified oil after extraction of the nonsaponifiables and converted to methyl esters with methanolic hydrochloric acid. Chromatography on silicic acid gave 40% nonhydroxy esters and 60% hydroxy esters. Both fractions were then analyzed by glpc. Our best estimate of the fatty acid composition of *L. densipila* seed oil is given in Table I. The analysis (glpc) by Mikolajczak, *et al.*,[2] differs somewhat, particularly in indicating only 50% of C-18 unsaturated hydroxy esters. Their lower result may be due to the lower relative response of the hydroxy esters in the thermal conductivity detector.

TABLE I

ESTIMATED FATTY ACID COMPOSITION OF *L. densipila* SEED OIL

Acid	% by wt
12-Hydroxy-*cis*-9-*cis*-15-octadecadienoic (densipolic)	48.2
Octadecenoic (oleic)[a]	19.7
12-Hydroxy-*cis*-9-octadecenoic (ricinoleic)	9.9
Octadecatrienoic (linolenic)[a]	8.2
Hexadecanoic (palmitic)	4.2
Octadecanoic (stearic)	3.4
Octadecadienoic (linoleic)[a]	1.9
12-Hydroxy-*cis*-9-hexadecenoic	1.9
Hexadecenoic (palmitoleic)[a]	1.3
Eicosanoic	0.5
Hexadecadienoic	0.4
Eicosenoic	0.3
Sum of C-14, C-15, C-17, C-19 acids	0.1

[a] Probable identity.

Experimental Section

Infrared spectra of thin films were determined with an Infracord Model 137 spectrophotometer.[9] Ultraviolet spectra of cyclohexane solutions were obtained with an extended-range Beckman Model DK-2 spectrophotometer. Optical rotations at 589 mμ were measured with a Rudolph Model 200 photoelectric polarimeter, and the ord curves for methanol solutions with a Cary Model 60 recording spectropolarimeter. A Varian A-60 spectrometer was used to obtain nmr spectra on 10% carbon tetrachloride solutions containing 1% tetramethylsilane. Mass spectra were obtained with a Consolidated Electrodynamics Corporation Model 21-110 mass spectrometer operated with a source temperature of 225° and an ionization voltage of 70 ev. For the analysis of nonhydroxy esters, a 6-ft, 0.25-in. column of 15% diethylene glycol succinate on 60–80 mesh Gas Chrom P was used at 192° with a helium flow of 50 ml/min in an Aerograph Model A-90-AC gas chromatograph. For the analysis of hydroxy esters, a 4-ft column with the same packing was used at 210° with helium flow of 60 ml/min. Programmed temperature operation of an F + M Model 720 gas chromatograph fitted with 3-ft columns of 10% ECNSS-S on 100–120 mesh Gas Chrom P was also used for qualitative glc.

Isolation of the C-18:1 Hydroxy Ester.—Methyl esters of *L. densipila* seed oil were prepared by sodium methoxide-catalyzed methanolysis of the oil.[10] Distillation of the mixed esters gave a fraction that was almost exclusively C-18 hydroxy esters. Two portions, totaling 1.120 g, were passed through ion-exchange resin converted to the silver ion form[6] to give a 244-mg sample that, analyzed by glc, contained no methyl densipolate but did contain a small amount of several other impurities. Chromatography on silicic acid by the method of Frankel, *et al.*,[11] followed by treatment with decolorizing carbon gave a 180-mg sample of pure (glpc, tlc) C-18:1 hydroxy ester. This ester had the same infrared, mass, and nmr spectra as methyl ricinoleate and their optical rotatory dispersion curves were superimposible. The corresponding hydrogenated derivatives similarly showed like mass spectra and ord curves.

Permanganate–Periodate Oxidation of the C-18:1 Hydroxy Ester.—A 60-mg portion of the ester was mixed with 550 mg of sodium iodate, 11 mg of potassium permanganate, 240 mg of potassium carbonate, 45 ml of water, and 10 ml of methanol, and stirred at room temperature for 18 hr.[12] The mixture was reduced with sodium bisulfite, acidified with hydrochloric acid, and extracted with ether. Acids from the ether extracts were esterified with methanol–hydrochloric acid. The methyl esters obtained (64 mg) were chromatographed on a silicic acid column and yielded a nonpolar fraction (35 mg) and a polar fraction (31 mg). Gas chromatographic analysis of the nonpolar fraction revealed a compound (>97% purity) that showed the retention characteristics of a dimethyl nonanedioate when compared with dimethyl sebacate and dimethyl dodecandioate. Infrared and mass spectra established its identity as dimethyl azelate. Methyl 3-hydroxynonanoate was a major component of the polar fraction, as indicated by its mass spectrum.

Isolation of the C-16:1 Hydroxy Ester.—A fraction distilled from mixed *L. densipila* methyl esters contained about 4% of the C-16 hydroxy ester (glpc). This ester mixture was chromatographed on a column prepared from a slurry of dried (120°) Mallinckrodt 100-mesh silicic acid in benzene. Benzene and benzene–ether mixtures were eluting solvents. Nonhydroxy esters readily separated from hydroxy esters, and the C-18 hydroxy esters tended to elute before the C-16 hydroxy ester. Serial chromatographies of C-16 hydroxy ester concentrates eventually gave a sample of the pure (glpc, tlc) C-16 ester. *Anal.* Calcd for $C_{17}H_{32}O_3$: C, 71.78; H, 11.34. Found: C, 71.6; H, 11.2.

(9) Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

(10) M. J. Diamond, R. E. Knowles, R. G. Binder, and L. A. Goldblatt, *J. Am. Oil Chemists' Soc.*, **41**, 430 (1964).

(11) E. N. Frankel, C. D. Evans, H. A. Moser, D. G. McConnell, and J. C. Cowan, *ibid.*, **38**, 130 (1961).

(12) These reaction conditions differ from those of von Rudloff;[8] the solution is more basic and methanol is present in the mixture to promote mutual solution of the reactants.

This C-16 hydroxy ester and methyl ricinoleate have nmr and infrared spectra which are identical except for differences attributable to unequal chain lengths. The mass spectra of the unsaturated ester and its hydrogenated derivative indicate[7] that the hydroxyl group is at the C-12 position and that the double bond is between C-1 and C-12.

Permanganate–Periodate Oxidation of the C-16:1 Hydroxy Ester.—A 53-mg portion of the ester was mixed with 480 mg of sodium iodate, 10 mg of potassium permanganate, 210 mg of potassium carbonate, 40 ml of water, and 10 ml of methanol[12] and stirred for 15 hr at room temperature. Products were isolated, esterified, and chromatographed as described in the preceding section. Pooling of consecutive eluate portions yielded four fractions totaling 47 mg. By gas chromatographic analysis, fraction 2 (33.2 mg) was 85% dimethyl azelate and 15% unknowns, fraction 3 (5.7 mg) was 70% dimethyl azelate, 20% unknowns and 10% methyl hydroxyheptanoate, and fraction 4 (6.7 mg) was 90% methyl hydroxyheptanoate. Mass spectral analysis confirmed the presence of dimethyl azelate in fraction 2 and indicated a CH_3OOCCH_2CHOH fragment from methyl 3-hydroxyheptanoate in fraction 4.

Optical Rotation of the C-16:1 Hydroxy Ester.—Methyl 12-hydroxy-*cis*-9-hexadecenoate, $[\alpha]^{26}D$ +6.2° (*c* 0.036 g/ml, methanol), shows a positive background curve which becomes less positive at low wavelengths: $[\alpha]^{27}_{500}$ +8.3°, $[\alpha]^{27}_{400}$ +13.9°, $[\alpha]^{27}_{300}$ +19.6°, $[\alpha]^{27}_{275}$ +20.8°, $[\alpha]^{27}_{250}$ +4.5° (*c* 0.775). Hydrogenation with platinum oxide catalyst in acetic acid gave methyl 12-hydroxyhexadecanoate, mp 45.6–46.0° after crystallization from petroleum ether, which had a plain negative ORD curve. *Anal.* Calcd for $C_{17}H_{34}O_3$: C, 71.28; H, 11.96. Found: C, 71.3; H, 11.9.

Optical Rotation of Methyl Densipolate.—Methyl densipolate (preparation described elsewhere)[13] was found to be levorotatory, $[\alpha]^{26}D$ −0.58° (*c* 0.131 g/ml, methanol). Smith, *et al.*,[3] reported $[\alpha]^{27}D$ 0 ± 1° (*c* 3.7, methanol).

Fatty Acid Composition of *L. densipila* Seed Oil.—*L. densipila* seeds were minced in a Brabender sample chopper, then steeped in petroleum ether at room temperature. From 64.4 g of seed, 16.9 g of oil was obtained. The oil had no selective ultraviolet adsorption between 220 and 350 mμ. An 11.2-g sample was saponified at room temperature by 4.0 g of potassium hydroxide in 20 ml of 50% ethanol. Eleven portions of ether extracted the nonsaponifiables (450 mg). After acidification of the alkaline solution, ether extracted 10.47 g of fatty acids. Methyl esters were prepared with methanolic hydrochloric acid at 60°, and a 650-mg sample was separated into nonhydroxy esters (40%) and hydroxy esters (60%) by chromatography on silicic acid.[11] Peaks in gas chromatograms were identified by comparison to standards and by use of equivalent chain length[14] data. Integrated peak areas were corrected by relative detector response factors determined for nonhydroxy esters on a purchased standard mixture and for hydroxy esters on a synthetic mixture of the pure esters. Chromatograms of hydrogenated and nonhydrogenated esters were compared to determine the relative amounts of C-18:3, C-20:0, and C-20:1 esters.

Acknowledgment.—We thank William Gaffield for ORD data, R. E. Lundin for analyses of nmr spectra, G. E. Secor for carbon–hydrogen determinations, and Quentin Jones, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, for providing *L. densipila* seed.

(13) R. G. Binder, L. A. Goldblatt, and T. H. Applewhite, *J. Org. Chem.*, **30**, 2371 (1965).

(14) T. K. Miwa, K. L. Mikolajczak, F. R. Earle, and I. A. Wolff, *Anal. Chem.*, **32**, 1739 (1960).

Studies on the Fine Structure of Clam Glycogen[1]

OM P. BAHL AND F. SMITH[2]

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota

Received November 3, 1965

The fine structure of glycogen isolated from six species of fresh-water clam has been determined by periodate oxidation, methylation, and β-amylolysis. Periodate oxidation of the glycogen, followed by sodium borohydride reduction, yielded a polyalcohol which on methylation and degradation yielded methoxyacetaldehyde dimethylacetal, 1,3-di-*O*-methylglyceritol, 1,4-di-*O*-methylerythritol and 1-*O*-methyl-D-erythritol. The results obtained indicate that the specimens of glycogen from the different clam species possess a similar structure composed of linear chains of 1,4-α-linked glucose units with 1,6-α-linkages at the branch points. The average size of the repeating unit varies from 11 to 13 glucose units. All the clam glycogens show an identical behavior toward concanavalin-A and, therefore, can not be differentiated on the basis of this reaction.

Since the isolation of glycogen from dog liver by Claud Bernard in 1857,[3] its extraction from other animals[4] has been reported. Certain plants and microorganisms have been found to contain glycogen-like materials.[4] The structural investigations of glycogen from these sources have been carried out by the use of techniques based on methylation,[5] periodate oxidation,[6] and enzymic[7–9] and chemical degradation.[10] Although the periodate oxidation has been widely employed in the structural studies, limited work has been reported on glycogen polyaldehyde particularly, from the point of view of its application to the study of the fine structure of glycogen. This paper deals with the isolation and characterization of glycogen from the fresh-water clam and a detailed chemical account of glycogen polyaldehyde.

The glycogen was isolated from six species of fresh-water clams by extraction with alkali.[11] All the samples of glycogen showed similar physical and chemical behavior. Attempts to differentiate them by concanavalin-A[12] were unsuccessful as indicated by their glycogen values given in Table I.

Oxidation with periodate[6] was carried out on all six samples of glycogen. The consumption of the oxidant[13]

(1) Paper No. 5797 of the Scientific Journal Series, Minnesota Agricultural Experiment Station.

(2) Deceased, Feb 1, 1965.

(3) C. Bernard, *Compt. Rend.*, **44**, 578 (1857).

(4) R. L. Whistler and C. L. Smart, "Polysaccharide Chemistry," Academic Press Inc., New York, N. Y., 1953, p 436.

(5) W. N. Haworth and E. G. V. Percival, *J. Chem. Soc.*, 2277 (1932).

(6) M. Abdel-Akher and F. Smith, *J. Am. Chem. Soc.*, **73**, 994 (1951).

(7) K. H. Meyer, *Advan. Enzymol.*, **3**, 109 (1943).

(8) B. Illingworth, J. Larner, and G. T. Cori, *J. Biol. Chem.*, **199**, 631 (1952); J. Larner, B. Illingworth, G. T. Cori, and C. F. Cori, *ibid.*, **199**, 641 (1952).

(9) P. J. P. Roberts and W. J. Whelan, *Biochem. J.*, **76**, 246 (1960).

(10) M. L. Wolfrom, E. N. Lassettre, and A. N. O'Neill, *J. Am. Chem. Soc.*, **73**, 595 (1951).

(11) E. F. W. Pfluger, "Das Glykogen," Bonn, Germany, 1905, p 53.

(12) J. A. Cifonelli, R. Montgomery, and F. Smith, *J. Am. Chem. Soc.*, **78**, 2485 (1956).

(13) P. F. Fleury and J. Lange, *J. Pharm. Chim.* [8], **17**, 107 (1933).