Synthesis and characterization of 3-ketohexadecanoic acid-1-^{14}C, DL-3-hydroxyhexadecanoic acid-1-^{14}C, and *trans*-2-hexadecenoic acid-1-^{14}C

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ABSTRACT The chemical synthesis and characterization of three intermediates in the β oxidation of palmitic acid-1-^{14}C by rat liver mitochondria, namely, 3-ketohexadecanoic acid-1-^{14}C, DL-3-hydroxyhexadecanoic acid-1-^{14}C, and *trans*-2-hexadecenoic acid-1-^{14}C, are described.

KEY WORDS 3-ketohexadecanoic acid-1-^{14}C · DL-3-hydroxyhexadecanoic acid-1-^{14}C · *trans*-2-hexadecenoic acid-1-^{14}C · synthesis · thin-layer chromatography · IR spectra · intermediates · β oxidation · palmitic acid

In the course of our studies on the endocrinological control of fatty acid metabolism (1, 2), we required supplies of radioactive compounds suspected of being intermediates in mitochondrial β oxidation of palmitic acid. These labeled compounds were not available, nor had they been previously prepared or employed as substrates in studies of long-chain fatty acid oxidation in mammalian tissues. We report here details of the chemical synthesis and characterization of three of the compounds: *trans*-2-hexadecenoic acid-1-^{14}C, DL-3-hydroxyhexadecanoic acid-1-^{14}C, and 3-ketohexadecanoic acid-1-^{14}C.

METHODS

Optical rotations of solutions in absolute ethanol were determined in a Rudolph polarimeter equipped with a cell of 0.75 ml capacity.

Infrared spectra of fatty acids and esters were obtained in a Perkin-Elmer 212 infrared spectrophotometer; the pellet contained 1 mg of compound to 300 mg of KBr.

For TLC, 250-μ layers were prepared from suspensions of 30 g of Silica Gel G (E. G. Merck, A. G., Darmstadt, W. Germany) in 60 ml of water or 70 ml of 12.5% aqueous silver nitrate solution. Adsorbents were activated at 110°C for 1 hr before use. Fatty acids were separated with *n*-hexane–diethyl ether–glacial acetic acid–methanol 90:20:3:4, and fatty esters with *n*-hexane–diethyl ether in 85:15, to a predetermined solvent front of 15 cm. Spots were made visible either by brief exposure to iodine vapors, by spraying with 0.2% 2′,7′-dichlorofluorescein in methanol and viewing under UV light, or by spraying with 10 N H_2SO_4 followed by charring at 150°C for 1 hr. Radioactivity was determined as follows. The powder in each area was extracted in a glass-stoppered centrifuge tube with two 5-ml portions of either diethyl ether (for $AgNO_3$-containing plates) or absolute ethanol; extracts were evaporated to dryness under nitrogen in counting vials, and residues dissolved in 10 ml of 0.4% 2,4-diphenyloxazole–0.05% 1,4-bis[2-(5-phenyloxazolyl)]-benzene in toluene for radioassay in a Nuclear-Chicago liquid scintillation spectrometer.

GLC of fatty esters was performed in a Jarrell-Ash 28-700 instrument with argon ionization detector and 6 ft by 4 mm glass column of 15% diethyleneglycol succinate polyester on Gas-Chrom P (Applied Science Laboratories, Inc., State College, Pa.) at a column temperature of 185°C, a gas flow of 90 ml/min, and a sensitivity of 10^{-7} ma full scale. Peaks were quantified by means of a disc integrator.

Methyl esters of saturated fatty acids were prepared by treatment of 1–10 mg of material with 5 ml absolute

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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methanol–0.2 ml dimethoxypropane–0.25 ml concd H_2SO_4 in a sealed tube for 2 hr at 65°C; 5 ml of cold water was added and esters were extracted four times with 10 ml of *n*-hexane; pooled hexane extracts were washed once with an equal volume of water, dried over anhydrous $MgSO_4$, and evaporated to dryness in vacuo; residues were dissolved in appropriate solvents to yield 1–2% solutions. To minimize the possibility of isomerization of unsaturated compounds or dehydration of hydroxy compounds, we esterified such fatty acids (1–10 mg) using the BF_3–methanol reagent and the procedure of Metcalfe and Schmitz (3), with a 2 min heating period at 100°C.

The position of the double bond in hexadecenoic acid-1-^{14}C was established by modifications of the oxidative cleavage methods of von Rudloff (4, 5). In a final volume of 9 ml of water at pH 8.1 (adjusted with KOH), 6.5 μmoles of sodium hexadecenoate-1-^{14}C, 0.2 mmoles of $NaHIO_4$, 5.0 μmoles of $KMnO_4$, and 18.0 μmoles of K_2CO_3 were shaken in a sealed flask for 6 hr at room temperature. When fatty acid products were to be isolated, the reaction was stopped by addition of sodium metabisulfite (until the solution became colorless) and 1 ml of 10% H_2SO_4; the reaction mixture was extracted four times with 10 ml of petroleum ether (bp 30–60°C), the extracts were dried over anhydrous Na_2SO_4, and then evaporated to dryness in vacuo at 35°C. The residue was extracted five times with 1 ml of *n*-hexane, and the pooled extracts were evaporated to dryness under nitrogen in a screw-capped culture tube; the residue was then subjected to the saturated fatty acid esterification procedure described above in preparation for GLC. When it was desired to collect the radioactive carbon dioxide produced by the oxidative reaction, the reaction flask was sealed with a rubber serum cap from which was hung a glass center well. After the 6 hr reaction period, 0.3 ml of Hyamine-10X (Rohm and Haas, Philadelphia, Pa.) was injected into the center well and 0.2 ml of 10 N H_2SO_4 into the reaction mixture. Radioactive carbon dioxide was collected in the Hyamine during a 1 hr period of shaking at 37°C, and the center well and contents were transferred to 10 ml of liquid scintillation counting solution for radioassay.

Elemental analyses were performed by Chemco, Inc., Washington, D.C.

SYNTHETIC PROCEDURES AND ANALYTICAL RESULTS

3-Ketohexadecanoic Acid-1-^{14}C

Methyl 3-ketohexadecanoate-1-^{14}C was synthesized by the Stallberg-Stenhagen procedures (6) from 0.13 mole each of ethyl acetoacetate-1-^{14}C (0.75 mc, Nuclear Research Chemicals, Inc., Orlando, Fla.) and myristoyl chloride (Distillation Products Industries, Rochester, N.Y.). After recrystallization from acetone, the yield of product was 35% of theoretical; mp 40.0–40.5°C (40.1°C, reference 6).

Analysis: $C_{17}H_{32}O_3$(284.3);
calculated: C, 71.76; H, 11.34
found: C, 71.25; H, 11.31

The ester, in 0.7 mmole batches, was hydrolyzed to the free acid according to Mitz, Axelrod, and Hofmann (7). After recrystallization from acetone–water 4:1, the yield of product was 65% of theoretical; mp 98–99°C (98.0–99.5°C, reference 7).

The infrared spectrum of methyl 3-ketohexadecanoate-1-^{14}C (Fig. 1C) shows the characteristic ester carbonyl stretching band at 1170 cm^{-1}, the ester carbonyl absorption band at 1750 cm^{-1}, and the absorption band at 1715 cm^{-1} characteristic of a 3-keto carbonyl group (8, 9).

Fig. 2 is a photograph of a chromatoplate after TLC of methyl 3-ketohexadecanoate-1-^{14}C (lane 3) on Silica Gel G–$AgNO_3$; the R_f averaged 0.47, and this was reduced to 0.33 in the absence of $AgNO_3$; the corresponding R_f values for the free acid were 0.19 and 0.10, respectively. Only a single spot was detectable, and about 95% of the applied radioactivity was recovered from this spot.

Methyl 3-ketohexadecanoate could not be analyzed by GLC since it decomposed at elevated temperatures.

DL-*3-Hydroxyhexadecanoic Acid-1-^{14}C*

3-Hydroxyhexadecanoic acid-1-^{14}C was synthesized by the Reformatsky reaction (10) from 0.5 mole each of ethyl bromoacetate-1-^{14}C (3 mc, Volk Radiochemical Co., Chicago, Ill.) and tetradecanal (Distillation Products Industries, Rochester, N.Y.). After recrystallization from petroleum ether, the yield of product was 32% of theoretical; mp 84–85°C (83.0–83.5°C, reference 11).

Analysis: $C_{16}H_{32}O_3$ (272.4);
calculated: C, 70.52; H, 11.94
found: C, 70.40; H, 11.81

The product was optically inactive, and was, therefore a racemic mixture of the D- and L-optical isomers.

The free acid showed the strong infrared absorption band at 3530 cm^{-1} characteristic of secondary hydroxyl groups (Fig. 1B) (9).

The methyl ester had an average R_f of 0.19 on Silica Gel G–$AgNO_3$ TLC (Fig. 2, lane 2), and an average R_f of 0.12 in the absence of $AgNO_3$; the corresponding R_f values of the free acid were 0.13 and 0.03, respectively. The single spot obtained with TLC contained about 95% of the applied radioactivity.

GLC revealed that the methyl DL-3-hydroxyhexadecanoate-1-^{14}C contained no significant contaminants; its retention time, relative to that of methyl palmitate, was about 5.8.

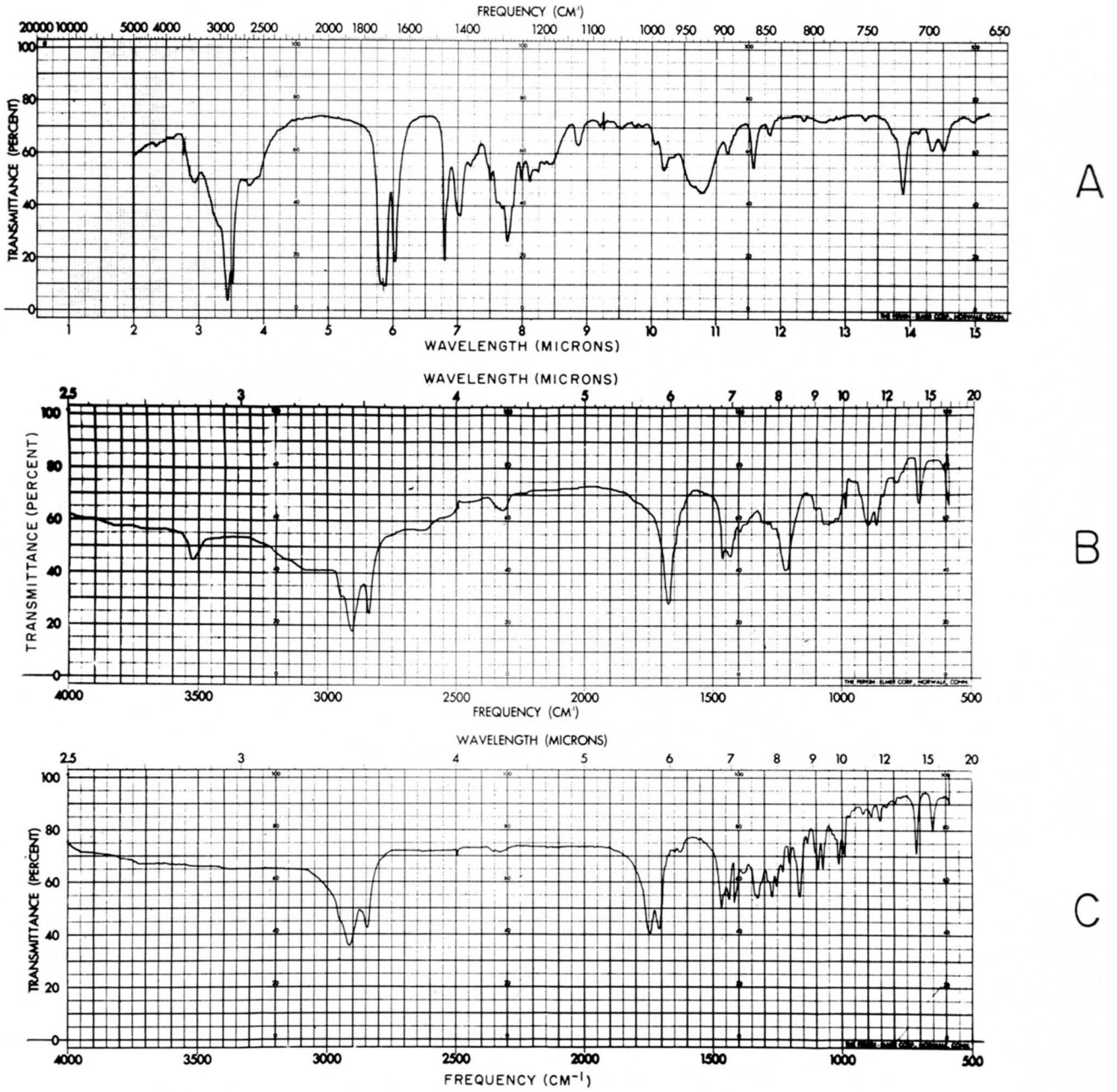

FIG. 1. Infrared absorption spectra: *A*, *trans*-2-hexadecenoic acid; *B*, DL-3-hydroxyhexadecanoic acid; *C*, methyl 3-ketohexadecanoate.

trans-2-Hexadecenoic Acid-1-^{14}C

2-Hexadecenoic acid-1-^{14}C was synthesized by a method devised by Dr. Carlo Colombini (now of the University of Padua, Italy) to whom we are grateful for supplying details of the method prior to publication. Equal weights of boric anhydride and dry DL-3-hydroxyhexadecanoic acid-1-^{14}C were heated under reduced pressure to effect dehydration and the product was then distilled off in vacuo and decolorized. Yield 38%; mp 44–45°C (45.0–45.5°C, reference 12).

Analysis: $C_{16}H_{30}O_2$ (254.2);
calculated: C, 75.51; H, 11.89
found: C, 75.00; H, 11.80

2-Hexadecenoic acid-1-^{14}C exhibited an infrared absorption band at 970 cm^{-1} characteristic of *trans* unsaturation, and an olefinic stretching band at 1750 cm^{-1} (Fig. 1A) (9).

As shown in Fig. 2 (lane 1), methyl hexadecenoate-1-^{14}C had an average R_f of 0.82 on TLC with Silica Gel G–$AgNO_3$. Its R_f in the absence of $AgNO_3$ was 0.67; the

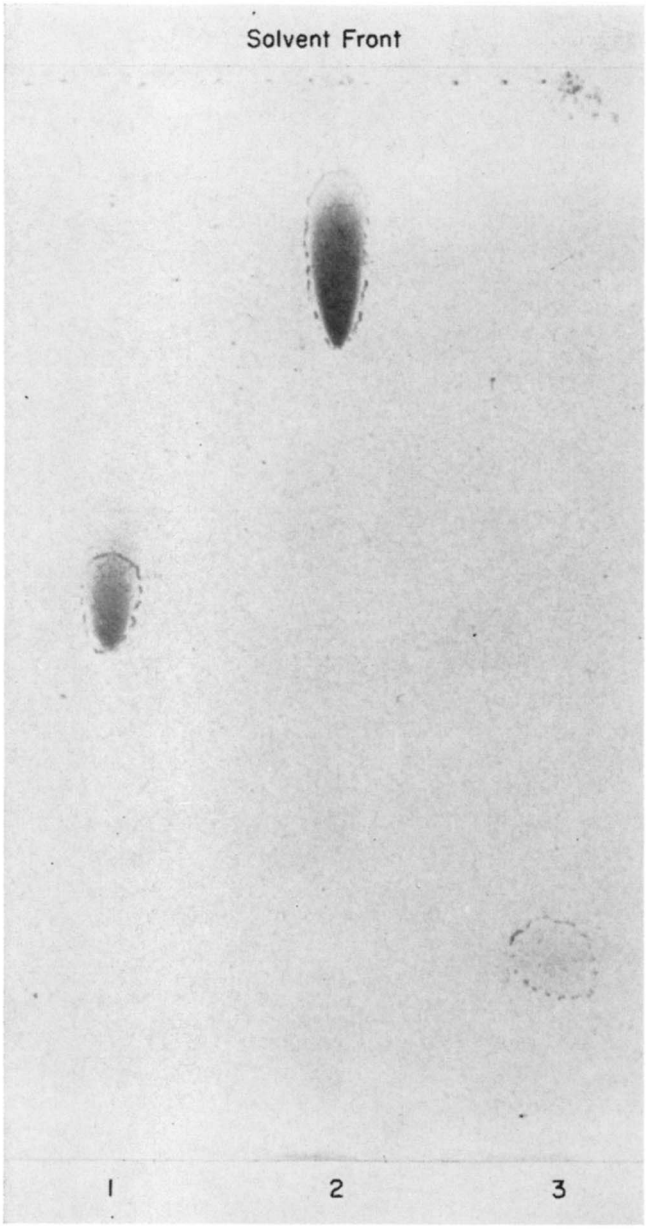

FIG. 2. TLC on Silica Gel G–silver nitrate: *1*, methyl *trans*-2-hexadecenoate-1-^{14}C; *2*, methyl DL-3-hydroxyhexadecanoate-1-^{14}C; *3*, methyl 3-ketohexadecanoate-1-^{14}C. For photography, chromatoplates were sealed between glass plates after being charred with 10 N H_2SO_4.

corresponding R_f values for the free acid were 0.37 and 0.35, respectively. Only a single spot was detectable under these conditions, and about 92% of the applied radioactivity was recovered from this spot. Since one spot was detectable for the unsaturated compound under conditions known to separate *cis* and *trans* geometric isomers of unsaturated fatty acids (13), it is apparent that only one of these isomers was synthesized; infrared data (Fig. 1A) indicated that the product was the *trans* isomer.

GLC of methyl *trans*-2-hexadecenoate-1-^{14}C revealed the presence of a single major (90%) peak with a retention time of 1.54 relative to methyl palmitate; one of the contaminants (6%) was probably 3-hexadecenoate. Retention times, relative to methyl stearate, of 0.93 for methyl *trans*-2-hexadecenoate and of 0.71 for methyl *cis*- and *trans*-3-hexadecenoate have been reported recently (14).

When *trans*-hexadecenoic acid-1-^{14}C (9130 cpm) was subjected to oxidative cleavage by periodate–permanganate, 9036 cpm were recovered as carbon dioxide; this is consistent with production of either malonic acid or oxalic acid from the original olefin, since under these conditions both dicarboxylic acids are decarboxylated to carbon dioxide (15, 16). However, the aliphatic long-chain monocarboxylic acid product of the oxidative cleavage of *trans*-hexadecenoic acid-1-^{14}C was identified by GLC as consisting of 92–97% myristate, with the remainder consisting of tridecanoate and lower acids. It was thus established that the double bond in the original olefinic acid was essentially entirely in the 2,3-position.

DISCUSSION

The procedure of Stallberg-Stenhagen (6) was adapted in its essentials to synthesize pure methyl 3-ketohexadecanoate-1-^{14}C in adequate yield, and the method of Mitz et al. (7) was utilized to obtain the free 3-keto acid without decomposition. Melting point, infrared data, and elemental analysis confirmed the purity and identity of the products, and TLC indicated their radiochemical purity.

The Reformatsky reaction (10) was used to prepare 3-hydroxyhexadecanoic acid-1-^{14}C in adequate yield. Optical rotatory data confirmed that the product was a racemic mixture composed of equal parts of the D- and L-optical isomers. Although the natural substrate for β-hydroxyacyl CoA dehydrogenase in the direction of oxidation is the L(+)-isomer (17, 18), the recent report (19) of a mitochondrial 3-hydroxyacyl CoA epimerase which epimerizes the D(−) form of long chain 3-hydroxyacyl CoA compounds to the L(+) enantiomorph would indicate that both the D- and L-3-hydroxyhexadecanoic acids-1-^{14}C can serve as substrates in mitochondrial β oxidation. Melting point, elemental analysis, and infrared data indicated the chemical purity and identity of the product. TLC and GLC demonstrated the radiochemical purity of the purified product.

2-Hexadecenoic acid-1-^{14}C resulted from the dehydration of DL-3-hydroxyhexadecanoic acid-1-^{14}C in the presence of boric anhydride. Melting point, elemental analysis, and infrared data established the identity and purity of the product. TLC demonstrated radiochemical purity; however, GLC indicated the presence of about 6% of the 3-hexadecanoic acid. In addition, infrared and

TLC data indicated that the unsaturated acid was of the *trans* configuration. Oxidative cleavage of the double bond in this olefin, followed by isolation of the resulting products, established the location of the double bond at the 2,3-position; only trace amounts of the 3,4-isomer were present. It is expected that this isomer would be the product of acyl CoA dehydrogenase activity upon palmitate (20); hydration of this geometric isomer would be expected to yield the L-(+)-3-hydroxy derivatives, by analogy with the behavior of the short (21, 22) and intermediate (19) chain length acids.

The three radioactive fatty acid derivatives described in the present report should prove to be of value in elucidating mitochondrial mechanisms of oxidation, interconversions, synthesis, and transport of long-chain fatty acids.

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