Synthesis of eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid-3-^{14}C and DL-3-hydroxy eicosa-8,11,14-all *cis*-trienoic acid-3-^{14}C

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ABSTRACT A general procedure for the synthesis of 2-*trans* polyenoic fatty acids and of DL-3-hydroxypolyenoic acids is described. The 2-*trans* acids are prepared by $LiAlH_4$ reduction of a suitable polyenoic fatty acid ester to the alcohol, formation of the tosylate, oxidation to the aldehyde, and Doebner condensation of the latter with malonic acid. The 3-hydroxy acids are obtained by reaction of the acyl chloride of a suitable polyenoic acid with the sodium enolate of methyl acetoacetate and sodium methoxide to give the 3-keto ester, the keto group of which is reduced with sodium borohydride to the alcohol. These procedures were applied to the synthesis of eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid-3-^{14}C and DL-3-hydroxy eicosa-8,11,14-trienoic acid-3-^{14}C.

KEY WORDS ^{14}C-labeled polyenoic acids · eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid · DL-3-hydroxy eicosa-8,11,14-trienoic acid

THE IN VITRO chain elongation of many 1-^{14}C-labeled polyenoic acids has been reported previously (1–6). The enzyme complexes responsible for chain elongation and olefin formation in the biosynthesis of polyenoic fatty acids are associated with the microsomal membrane fraction. The chain elongation of unsaturated fatty acids proceeds in a manner similar to the de novo synthesis of saturated fatty acids, i.e., malonyl-CoA donates the C_2 units for chain elongation and NADPH is required as a cofactor for reduction. Wakil (7) has described another system, associated with the mitochondria, which utilizes acetyl-CoA activated by pyridoxamine phosphate. However, when the efficiencies of the two systems are compared, the biological significance of the mitochondrial system becomes questionable.

In order to achieve further insight into the mechanism of the mitochondrial elongation reaction, we synthesized eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid and DL-3-hydroxy eicosa-8,11,14-all *cis*-trienoic acid, both labeled at C_3. The 2-*trans* and D(−) isomers of the 3-hydroxy acids have been shown to be intermediates in the chain elongation of octadeca-6,9,12-trienoic acid to eicosa-8,11,14-trienoic acid (8). The 2-*trans* acid and the 3-hydroxy acid also should be valuable substrates in studies of the mitochondrial β-oxidation of the saturated carboxylic end of the long-chain polyenoic acids. The reaction mechanism for the degradation of the double bond system has been elucidated and described recently (9, 10). The two acids have been labeled with ^{14}C in order to permit a reliable analysis after incubations.

It was most desirable to limit the synthesis to a few steps after the label had been introduced. Therefore the two acids were synthesized from the common intermediate: octadeca-6,9,12-trienoic acid-1-^{14}C. The synthesis of this acid has been described in detail previously (11, 12).

OUTLINE OF THE SYNTHESIS

Fig. 1 represents the scheme for the two syntheses. Octadeca-6,9,12-trienoic acid-1-^{14}C was synthesized by the reaction of $Na^{14}CN$ with 1-chloro-heptadeca-5,8,11-all *cis*-triene. The resulting nitrile was hydrolyzed under acid conditions to the ester and saponification of the ester yielded the labeled octadecatrienoic acid (11, 12). The free acid was purified by countercurrent distribution in the solvent system developed by Ahrens and Craig (13); 1250 transfers were sufficient to obtain a radio-gas chromatographically pure starting material.

Abbreviations: GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

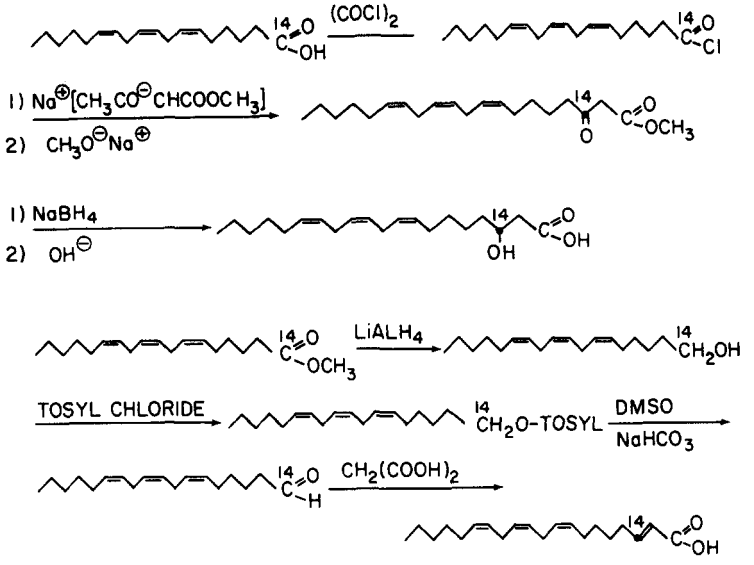

FIG. 1. Diagram of the synthesis of DL-3-hydroxyeicosa-8,11,14-trienoic acid-3-^{14}C (above) and eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid-3-^{14}C (below).

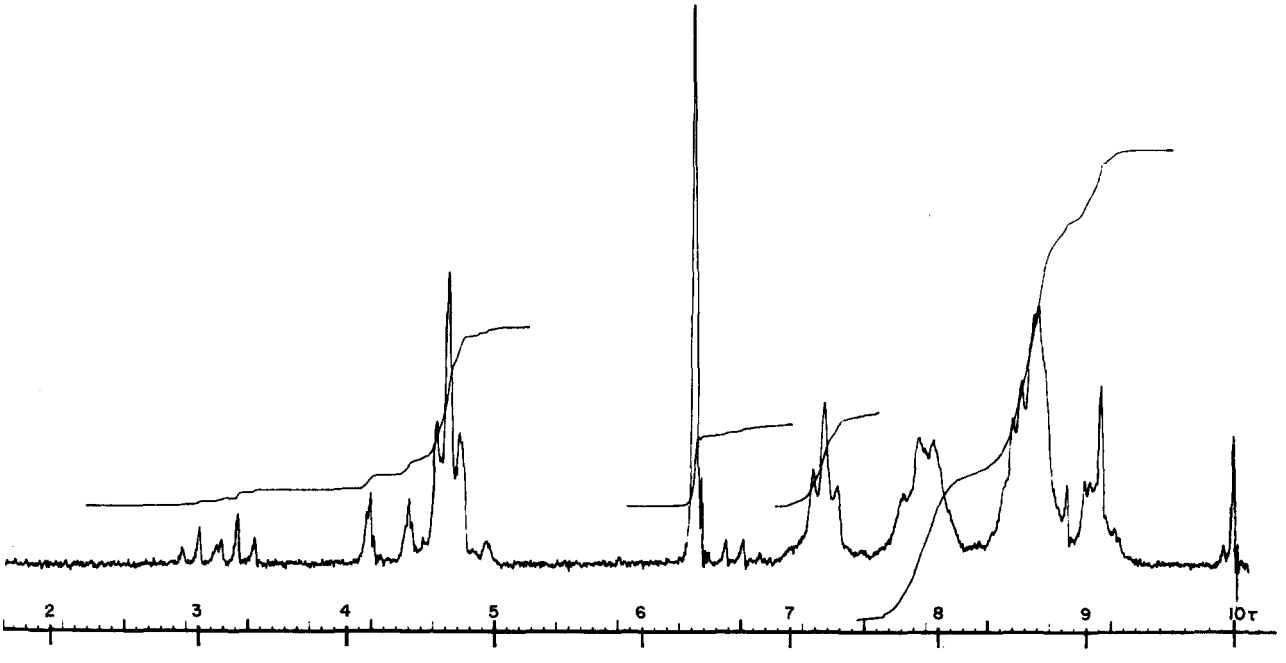

FIG. 2. NMR spectrum of eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid.

The reduction of the methyl ester with $LiAlH_4$ followed conventional procedures. The tosylate was prepared similarly to octadec-9-enyl tosylate (14). The tosylate of octadeca-6,9,12-trienol-1-^{14}C was oxidized by the general procedure of Kornblum, Jones, and Anderson (15), which yielded the octadeca-6,9,12-trien-1-al-1-^{14}C. This method has also been used by Mahadevan, Phillips, and Lundberg (16) for preparation of long-chain unsaturated aldehydes. The eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid-3-^{14}C was then obtained via Doebner condensation with malonic acid. This condensation yielded only the 2-*trans* acid as confirmed by NMR spectroscopy (Fig. 2) and by the symmetry of the GLC peak under different conditions. The IR spectrum of the methyl tetraenoate (Fig. 3) exhibited an absorption band at 970 cm^{-1}, which is characteristic of *trans* olefinic bonds.

For the synthesis of the DL-3-hydroxy eicosa-8,11,14-all *cis*-trienoic acid-3-^{14}C, octadeca-6,9,12-all *cis*-trienoic acid-1-^{14}C was first transformed into the acyl chloride (Fig. 1) by treatment with oxalyl chloride. The sodium

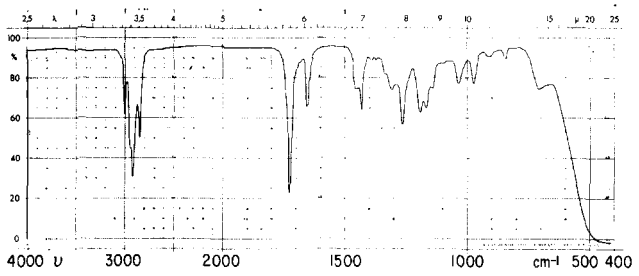

FIG. 3. IR spectrum of methyl eicosa-2-*trans*-8,11,14-all *cis*-tetraenoate.

salt of the methyl acetoacetate was then acylated with this acyl chloride and subsequently treated with sodium methoxide to yield methyl 3-keto eicosa-8,11,14-trienoic acid-3-^{14}C. The keto ester was reduced with $NaBH_4$ and saponified. The resulting racemic 3-hydroxy eicosa-8,11,14-trienoic acid was then purified by preparative TLC. All attempts to obtain the free 3-keto acid by acid hydrolysis according to Mitz, Axelrod, and Hofmann (17) or by mild alkaline hydrolysis failed. In each case eicosa-7,10,13-trien-2-one was produced.

Evidence in support of the fact that the products synthesized were those expected from the reaction sequences was obtained by IR, UV, and NMR spectroscopy, quantitative microhydrogenation, and oxidative ozonolysis.

The two reaction sequences outlined above have proved to be of general use for the synthesis of 2-*trans* and 3-hydroxy derivatives of polyenoic acids. In an analogous manner, eicosa - 2 - *trans* - 11,14 - *cis*,*cis*-trienoic acid, octadeca-2-*trans*-9,12-*cis*,*cis*-trienoic acid, 3-hydroxy eicosa-11,14-*cis*,*cis*-dienoic acid, all labeled at C_3, have been synthesized.

METHODS

TLC was carried out on 0.5 mm Silica Gel G layers in petroleum ether–ethyl ether–acetic acid 70:30:2. Spots were made visible by the charring technique (18).

Radio-gas chromatography was carried out on a Packard gas chromatograph coupled to a combustion furnace, flow cell, rate meter, and recorder. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter.

The number of double bonds was determined by quantitative catalytic microhydrogenation with 10–20-mg samples dissolved in glacial acetic acid (19). UV spectroscopy was carried out with a PMQ II Zeiss spectrometer; IR spectra were recorded with a Perkin-Elmer Model 125 IR spectrograph, and NMR spectra with a Varian A 60 model. The procedure of Holman and Burr (20) was followed for the alkali isomerization and that of Klenk and Bongard (21) for the oxidative ozonolysis. Hydroxy acids were acetylated according to Kishimoto and Radin (22).

SYNTHETIC PROCEDURES AND RESULTS

Eicosa-2-trans-8,11,14-all cis tetraenoic acid-3-^{14}C

Octadeca-6,9,12-all cis-trien-1-ol-1-^{14}C. 3 g (10.3 mmoles) of methyl octadeca-6,9,12-trienoate-1-^{14}C, dissolved in 75 ml of dry ethyl ether, was added dropwise to a suspension of 3 g of $LiAlH_4$ in 150 ml of dry ether under continuous stirring. After the reaction had continued for 4 hr at room temperature, the mixture was refluxed for an additional hour. The suspension was cooled to 0°C and the excess $LiAlH_4$ was decomposed by the careful addition of 10 ml of ethyl acetate. The precipitated salts were dissolved in 20% H_2SO_4, the ether phase was separated, and the aqueous phase was extracted with two 50 ml portions of ether. The combined extracts were washed with $NaHCO_3$ and water, dried over Na_2SO_4, and concentrated. The residue was treated with 25 ml of a 2% methanolic KOH solution and heated for 2 hr at 60°C. The alcohol was extracted with petroleum ether (bp 30–60°C), dried over Na_2SO_4, and concentrated. Short-path distillation yielded 2.58 g (9.8 mmoles) of octadeca-6,9,12-trien-1-ol-1-^{14}C, i.e., 95% of the theoretical value. $[n]_D^{21} = 1.4798$. The IR spectrum of the alcohol is shown in Fig. 4.

Octadeca-6,9,12-trien-1-ol p-toluenesulfonate. 5.5 g (21 mmoles) of octadeca-6,9,12-trien-1-ol was dissolved in 7 ml of dry pyridine, and 4.77 g (25 mmoles) of *p*-toluenesulfonyl chloride was added over a period of 30 min with continuous stirring. After the reaction had proceeded for 3 hr at room temperature, 0.2 ml of water was added. Then, after 10 hr at room temperature, the reaction mixture was diluted with 50 ml of 2 N H_2SO_4 and the aqueous phase was extracted with three 50 ml portions of ether. The combined extracts were washed with a saturated NaCl solution and water, and then dried over Na_2SO_4. The yield of crude product was 6.7 g.

Octadeca-6,9,12-all cis-trien-1-al-1-^{14}C. The tosyl ester was heated, together with 3 g of $NaHCO_3$ in 30 ml of dimethyl sulfoxide, under nitrogen and with vigorous stirring in a preheated oil bath at 155°C for 5 min. The reaction vessel was then cooled immediately and ice-water was added. The aldehyde was extracted with

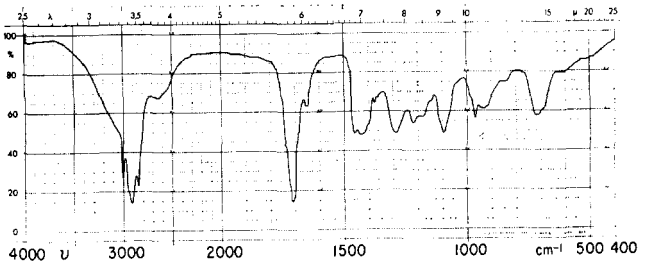

FIG. 4. IR spectrum of octadeca-6,9,12-trien-1-ol.

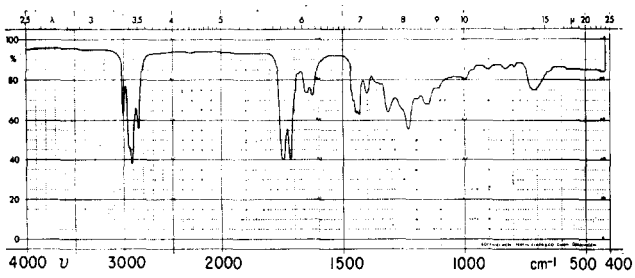

FIG. 5. IR spectrum of methyl 3-keto eicosa-8,11,14-all *cis*-trienoate.

pentane. The pentane solution was washed with water, dried over Na_2SO_4, and concentrated under nitrogen. The residual aldehyde was used immediately for the following synthesis.

Eicosa-2-trans-8,11,14-all cis-tetraenoic acid-3-^{14}*C.* The aldehyde was allowed to react with malonic acid in a Doebner condensation as follows. 3.2 g (30 mmoles) of malonic acid was added to 7 ml of dry pyridine in small portions over a period of 20 min. A solution of the aldehyde in 5 ml of pyridine was added, followed by 0.2 ml of piperidine. The mixture was allowed to react for 1 hr at 55°C and then for 5 hr at 80–90°C. The reaction vessel was constantly flushed with nitrogen. 7 ml of concentrated HCl in 25 ml of ice-water was then added, and the acid solution was extracted twice with 25 ml of ether. The combined ether extracts were washed four times with 10 ml of 2 N NH_4OH. The combined washings were acidified with 2 N H_2SO_4 and the acid was extracted with petroleum ether. The extracts were washed with water, dried over Na_2SO_4, and concentrated. The acid was purified by chromatography on a 20 g silicic acid column. The eluent was petroleum ether–diethyl ether 95:5. This method yielded 2.2 g (7.25 mmoles) of eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid-3-^{14}C, i.e., 35% of the theoretical value from octadeca-6,9,12-trien-1-ol. The material was proved pure by the criteria of radio-gas chromatography and TLC. Oxidative ozonolysis yielded the expected dicarboxylic acids: oxalic, malonic, and adipic acids. The equivalent weight was 304–306 (calculated, 304) and the specific activity 250,000 dpm/μmole. The methyl ester gave a single spot on TLC in petroleum ether–diethyl ether 90:10 on $AgNO_3$–silicic acid.

The UV spectrum obtained after alkali isomerization exhibits the following maxima, which are typical for tetraene systems: 234 mμ (13,400), 269 mμ (15,120), 279 mμ (14,050), 301 mμ (6230), 315 mμ (5320). Fig. 3 represents the IR spectrum of eicosa-2-*trans*-8,11,14-all *cis*-tetraenoic acid. The olefinic stretching vibrations are indicated by the band at 1650 cm^{-1}, the *trans* double bond at 970–980 cm^{-1}, and *cis* double bonds at 710 cm^{-1}.

Fig. 2 represents the NMR spectrum, which showed the following signals: singlet at 6.35τ (OCH_3); multiplets (AB-type) centered around 3.1τ and 4.3τ [2-*trans* protons (AB = 16 Hz)]; 4.7τ (*cis* olefinic protons); 7.2τ (diallylic protons); 7.9τ (monoallylic protons); 8.3–8.9τ (—$(CH_2)_n$—); and 9.1τ (CH_3 terminal group).

DL-*3-Hydroxy Eicosa-8,11,14-all cis-trienoic acid-3-*^{14}*C*

Octadeca-6,9,12-trienoyl chloride. 4.9 g (18 mmoles) of octadeca-6,9,12-trienoic acid-1-^{14}C and 10 ml of freshly distilled oxalyl chloride were dissolved in 10 ml of

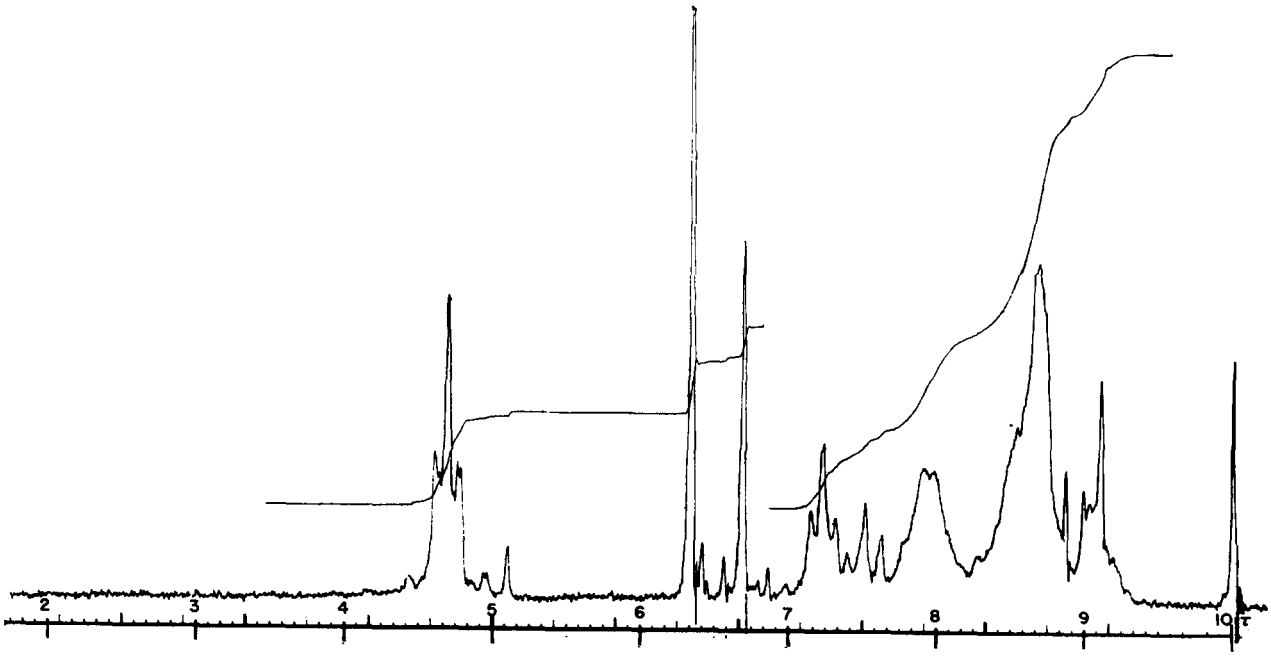

FIG. 6. NMR spectrum of 3-keto eicosa-8,11,14-all *cis*-trienoate.

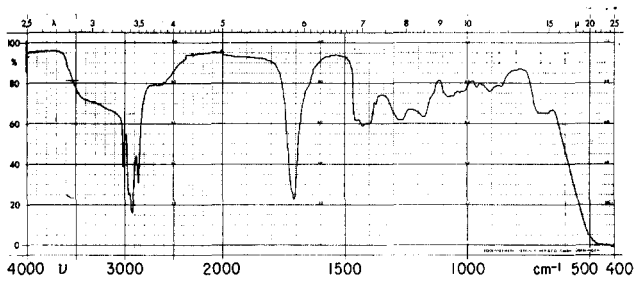

FIG. 7. IR spectrum of 3-hydroxy eicosa-8,11,14-all *cis*-trienoic acid.

dry benzene and heated at 40°C under anhydrous conditions for 8 hr. Benzene and excess oxalyl chloride were evaporated off in vacuo and the last traces of oxalyl chloride removed by flash evaporation with five 10 ml portions of benzene. The acid chloride was immediately used for the following synthesis.

Methyl 3-keto eicosa-8,11,14-all cis-trienoate. 2.5 g (19.2 mmoles) of methyl acetoacetate and an equivalent amount of sodium were refluxed in 20 ml of dry benzene for 10 hr. The mixture was cooled to 0°C and a solution of 5.0 g (16.5 mmoles) of octadeca-6,9,12-trienoyl chloride in 30 ml of dry benzene was added dropwise over a period of 15 min with continuous stirring. The reaction was allowed to proceed at room temperature for 2 hr. The mixture was then refluxed for 30 min. The benzene solution was washed with ethanol–water 1:1 and then with water–ethanol 9:1. The combined aqueous phases were extracted with ether, and the combined ether and benzene extracts were dried over Na_2SO_4. The solvent was evaporated and the residue made to react with sodium methoxide solution (400 mg of sodium dissolved in 20 ml of absolute methanol) for 15 hr at room temperature. 20 ml of 2 N H_2SO_4 was added to the reaction mixture and the product was extracted with ether. The ether extracts were dried over Na_2SO_4 and concentrated. The total yield was 2.75 g (6.75 mmoles), 37% of the theoretical value.

The IR spectrum of the material (Fig. 5) displays two carbonyl bands: at 1715 cm^{-1} for the 3-keto group and at 1745 cm^{-1} for the ester carbonyl group. All attempts to obtain the free 3-keto acid by acid hydrolysis (17) or mild alkaline hydrolysis were unsuccessful. In each case nonadeca-7,10,13-trien-2-one was obtained and characterized by the iodoform test, IR spectroscopy, and GLC. Unlike the 3-keto esters of saturated fatty acids, the 3-keto ester of the eicosa-8,11,14-trienoic acid seems to be unstable even at 0°C. Fig. 6 gives the NMR spectrum of the 3-keto-eicosa-8,11,14-all *cis*-trienoate. This NMR spectrum closely resembles that of the methyl eicosa-2-*trans*-8,11,14-all *cis*-tetraenoate, but it lacks the AB-system of the *trans* protons of the α,β-double bond. The additional singlet at 6.7τ is due to the protons at C_2, and the disturbed triplet at 7.5τ to the protons at C_4.

DL-3-*Hydroxy eicosa*-8,11,14-*all cis-trienoic acid*-3-^{14}C. 500 mg (1.5 mmoles) of methyl 3-keto eicosa-8,11,14-trienoate was dissolved in 50 ml of absolute methanol and 200 mg of $NaBH_4$ was added in small portions. The reaction mixture was stirred for 14 hr at room temperature, after which a solution of 600 mg of NaOH in 2 ml of water was added. The mixture was then refluxed for 2 hr under nitrogen. Unsaponifiable material (eicosa-8,11,14-triene-1,3-diol) was extracted with petroleum ether–ether 1:1. Then 100 ml of 2 N H_2SO_4 was added to the mixture after it had cooled to room temperature. The aqueous

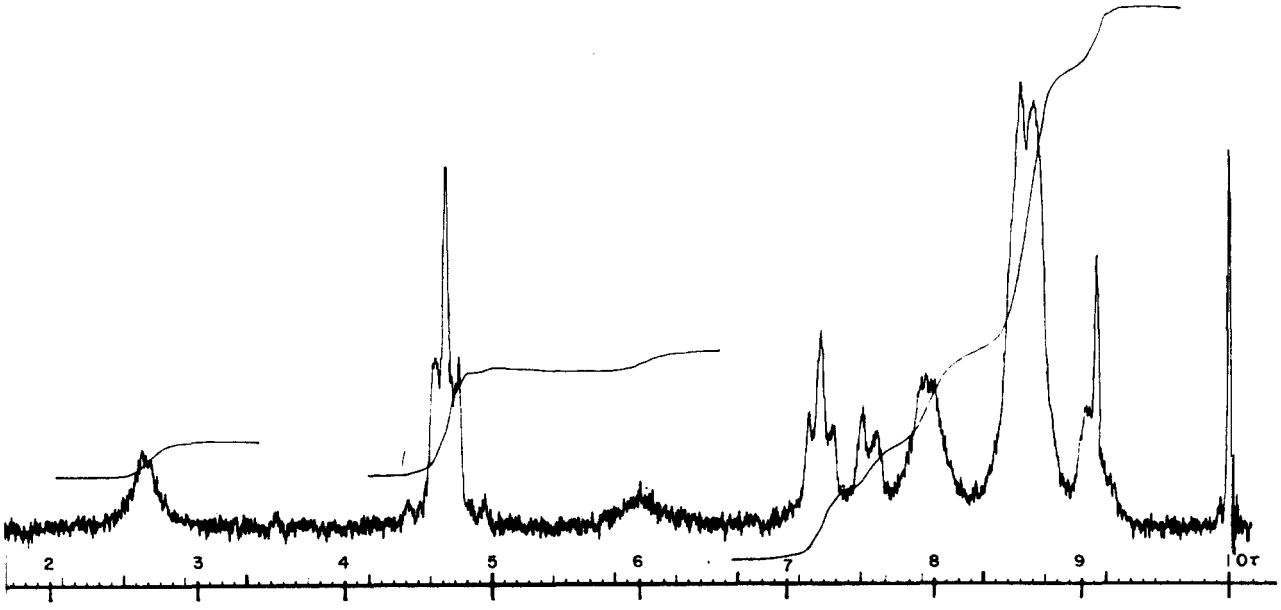

FIG. 8. NMR spectrum of 3-hydroxy eicosa-8,11,14-all *cis*-trienoic acid.

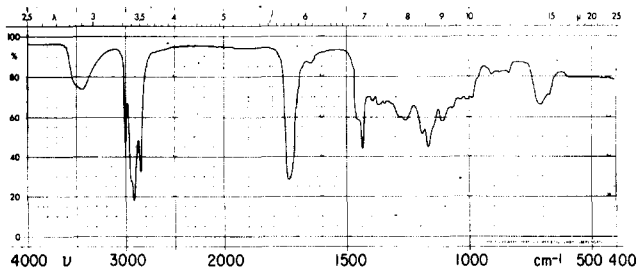

FIG. 9. IR spectrum of methyl 3-hydroxy eicosa-8,11,14-all *cis*-trienoate.

phase was extracted three times with ether. The combined ether extracts were washed with water and dried over Na_2SO_4. Evaporation of solvent yielded 355 mg (1.1 mmoles) of the colorless 3-hydroxy eicosa-8,11,14-trienoic acid-3-^{14}C, 73% of the theoretical value. The acetylated ester was proved homogeneous by GLC analysis. The acid has been characterized by oxidative ozonolysis, quantitative microhydrogenation, and UV, IR and NMR spectroscopy (Figs. 7 and 8). Fig. 9 shows the IR spectrum of the methyl ester. After alkali isomerization the UV spectrum was identical with that of the eicosa-2-*trans*-8,11,14-tetraenoic acid. This indicated that the 3-hydroxy acid had dehydrated under the conditions of the alkali isomerization. The absorption maxima and the molar extinction coefficients are: 237 mμ (11,560); 269 mμ (14,980); 301 mμ (5880); 315 mμ (5210). The NMR spectrum has eight multiplets. Those at 4.7τ, 7.2τ, and 7.9τ have been discussed before. The protons at C_2 couple with the proton at C_3 to yield a doublet centered around 7.6τ. The signal at 6.0τ is caused by the protons at C_3. The broad singlet at 2.6τ with intensity 2 is due to the OH proton of the COOH group and the —OH group at C_3.

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