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The Identification of *cis*-11,12-Methylene-2-hydroxyoctadecanoic Acid from *Thiobacillus thiooxidans**

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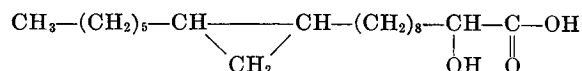
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SUMMARY

A polar fatty acid has been observed as a component of an ornithine-containing lipid of *Thiobacillus thiooxidans*. A comparison of thin layer chromatographic mobilities of reference compounds to those of the natural acid and its derivatives suggested that the acid was a 2-hydroxy fatty acid. The presence of a cyclopropane function in the acid was indicated by ^{14}C -labeling experiments and infrared spectroscopy. Mass spectrometry of the methyl ester and the acetylated methyl ester of the natural acid provided a molecular weight for the acid. Equivalent chain lengths were determined for the natural acid, the acid obtained by oxidative decarboxylation of the natural acid with permanganate, and the acids derived through reductive ring cleavage of the cyclopropane group in the ester of the oxidatively decarboxylated natural acid. The mass spectral data, the equivalent chain length determinations, and the permanganate oxidation study clearly indicated that the acid possessed an 18-carbon chain with a methylene bridge and a 2-hydroxyl function. The equivalent chain length determinations further suggested that the cyclopropane group had the *cis* configuration. Mass spectrographic analysis of the branched chain esters obtained by reductive cleavage of the ester which was in turn derived through oxidative decarboxylation of the natural acid allowed the assignment of the 11,12 position for the cyclopropane group. Based on these data, the polar acid is proposed to be *cis*-11,12-methylene-2-hydroxyoctadecanoic acid.

An ornithine-containing lipid has been recently isolated from *Thiobacillus thiooxidans* (1). During subsequent structural investigations, a polar fatty acid was observed as a component of the lip amino acid. The purpose of this paper is to report the identification of the polar acid as *cis*-11,12-methylene-2-hydroxyoctadecanoic acid.

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The acid appears to be bound to ornithine through an ester linkage and does not occur, to an appreciable extent, in the free form or as a component of other common lipids of the microorganism.

EXPERIMENTAL PROCEDURES

Methods—Methanolysis was performed by refluxing the sample for 1 hour in 2 to 3 ml of 0.02 N sodium methoxide. After the addition of a drop of glacial acetic acid, most of the methanol was evaporated, and the sample was partitioned between 2 ml of water and 2 ml of diethyl ether. The aqueous phase was extracted twice with equal quantities of diethyl ether. The combined extract was dried over anhydrous Na_2SO_4 and evaporated to a suitable working volume.

Free fatty acids were converted to their respective methyl esters according to the method of Schlenk and Gellerman (2). Acetylation reactions were carried out by refluxing samples in 2 to 3 ml of an acetic anhydride-pyridine (2:3, v/v) mixture for 30 min. Saponification of lipid samples was performed by refluxing the samples in 2 to 3 ml of 0.8 N NaOH in aqueous 86% ethanol for 2 hours. The 2-hydroxy fatty acids were oxidatively decarboxylated with KMnO_4 in acetic acid by the method of James and Webb (3). Reductive cleavage of the cyclopropane ring in fatty acid esters was accomplished by hydrogenation with Adams' catalyst under conditions similar to those of McCloskey and Law (4). All solvent evaporations were carried out under a stream of nitrogen.

Gas-liquid chromatography was performed with two columns which were identical except for their length. A 5-ft column was utilized for the separation of the methyl esters of acetoxy fatty acids, while a 12-ft column was employed for the analysis of nonhydroxylated fatty acid methyl esters. The 0.25-inch stainless steel columns were packed with 16% (w/w) diethylene glycol succinate (stabilized, Analabs, Inc., Hamden, Connecticut) on Anakrom AB, 90/100 mesh (Analabs, Inc.). The isothermal (185°) chromatograph used was equipped with an 80:1 effluent splitter and a β ionization detector. Other operating conditions and the trapping procedure employed have been reported (5).

In the determination of equivalent chain lengths (6-8), methyl nonanate and the methyl esters of fatty acids with even numbers of carbon atoms from 12 to 22 were used as standards for the 12-ft column. The esters of even numbered fatty acids from C-16 to

C-26 were used for the 5-ft column. Immediately following the analysis of an unknown methyl ester, a standard methyl ester with a similar equivalent chain length was also analyzed as a precaution.

Thin layer chromatography was accomplished with Silica Gel-G (Brinkman) plates which were prepared as described previously (5). In all cases the thin layer chromatography plates were developed with diethyl ether-heptane-glacial acetic acid (70:30:1, v/v/v). Lipids were located by spraying the plates lightly with 0.2% Rhodamine 6G (Allied Chemical Company) in 95% ethanol and viewing them under an ultraviolet lamp. Samples were recovered from thin layer chromatography plates by removing the adsorbent of the desired area from the plate and extracting it three times with chloroform.

A Perkin Elmer IR model 21 instrument was used to obtain infrared spectra of samples which were prepared as thin films on NaCl crystals. Mass spectra were obtained from a Hitachi model RMU-6D double focusing instrument with an ionizing potential at 80 eV and a maximum temperature of 180° for the inlet oven.

Materials—The source of material for the identification of *cis*-11,12-methylene-2-hydroxyoctadecanoic acid was the ornithine-containing lipid isolated from *T. thiooxidans*. The culture of the organism and the isolation of the lipid have been reported (1). The aminolipid, labeled with ¹⁴C, was obtained from cultures grown on a medium containing methionine-methyl-¹⁴C, as previously reported (9).

All fatty acids and esters were obtained from Sigma, except for 2-hydroxyhexadecanoic and 2-hydroxyoctadecanoic acids (Al-

drich) and those prepared synthetically. Ethyl 3-hydroxyoctadecanoate (melting point, 46–47°) was synthesized by the Reformatsky reaction (10) and converted to its methyl ester by methanolysis. Palmitaldehyde sulfite addition product and 2-bromoacetic acid were purchased from Aldrich. The *cis*- and *trans*-9,10-methyleneoctadecanoic acids were synthesized from oleic and elaidic acid, respectively, according to the procedure described by Christie and Holman (11). All other chemicals used were reagent grade and were used without further purification.

RESULTS AND DISCUSSION

Chromatographic Isolation and Characterization—When subjected to methanolysis, the ornithine-containing lipid of *T. thiooxidans* yielded a diethyl ether-soluble product (I) which exhibited thin layer chromatography properties that were identical to those of methyl esters of 2-hydroxy fatty acids. The mobility of Product I was the same as that of monohydroxy esters such as methyl 2-hydroxyhexadecanoate, methyl 3-hydroxyoctadecanoate, and methyl 12-hydroxy-9-octadecenoate, and it was slightly greater than that of the fatty alcohols hexadecanol and octadecanol. The acetate derivatives of Product I and of the same methyl esters mentioned above showed identical thin layer chromatography mobilities. However, only 2-hydroxy fatty acids were chromatographically similar to saponified Product I. Fig. 1, a tracing of a developed thin layer chromatography plate, illustrates the relative mobilities of various derivatives of Product I and 2-hydroxyhexadecanoic acid. For comparison, 3-hydroxyoctadecanoic acid is also included. The chromatographic trailing observed for the 2-hydroxy fatty acids is to be expected since their acidity is greater than the acetic acid of the solvent.

After extraction from the thin layer chromatography adsorbent, a portion of Product I was acetylated, isolated by thin layer chromatography, and subjected to gas-liquid chromatography. The tracing obtained is shown in Fig. 2. Although minor components were evident, the acetylated derivative of Product I appeared to contain one major component which exhibited an equivalent chain length of 25.2. The fatty acid represented by this component was tentatively designated as HFA.¹

Determination of Hydroxyl Position—With the same gas-liquid chromatography conditions used to determine the equivalent chain length of the acetylated derivative of methyl-HFA, methyl 2-acetoxyhexadecanoate and methyl 2-acetoxyoctadecanoate exhibited equivalent chain lengths of 21.4 and 23.4, respectively. Thus the 2-acetoxy group appears to increase the equivalent chain length of *normal* fatty acid methyl esters by 5.4 units in the gas-liquid chromatography system employed. This value is similar to the value (5.5 units) observed by Tulloch for the contribution of the 2-acetoxy group in 18-carbon esters when analyzed on a column with ethylene glycol succinate as the liquid phase (12).

When 2-hydroxyhexadecanoic acid was oxidized with potassium permanganate in acetic acid, the methyl ester of the pri-

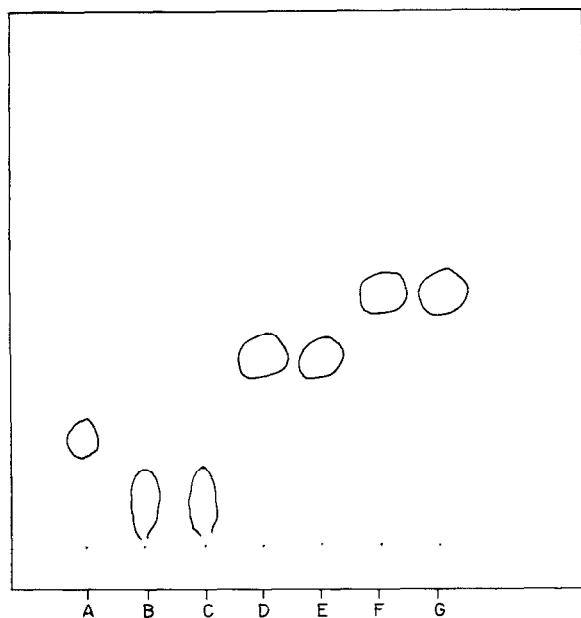


FIG. 1. A tracing showing the thin layer chromatographic mobilities of reference compounds and derivatives of the diethyl ether-soluble product released by methanolysis of an ornithine-containing lipid from *T. thiooxidans*. A, 3-hydroxyoctadecanoic acid; B, 2-hydroxyhexadecanoic acid; C, saponified methanolysis product; D, methyl 2-hydroxyhexadecanoic acid; E, methanolysis product; F, methyl 2-acetoxyhexadecanoate; G, acetylated methanolysis product. Chromatography was performed with a solvent of diethyl ether-heptane-glacial acetic acid (70:30:1, v/v/v). For detection of lipids the plate was sprayed with 0.2% Rhodamine 6G in 95% ethanol.

¹ In this paper HFA indicates that acid which was isolated as its acetylated methyl ester by gas-liquid chromatography. Steps prior to gas-liquid chromatography included: methanolysis of the ornithine-containing lipid, isolation of the hydroxy methyl ester by thin layer chromatography, acetylation and separation of the acetylated methyl ester by thin layer chromatography. Methyl-HFA represents the methyl ester of HFA.

mary product exhibited an equivalent chain length of 15, as expected. Minor components with equivalent chain lengths of 14, 13, and 12 units also resulted from the oxidation. HFA, obtained by saponification of its acetylated methyl ester, was oxidized under the same conditions. The gas-liquid chromatography tracing obtained for the methylated reaction products is shown in Fig. 3. The equivalent chain length of the major fatty acid was found to be 18.8. Adding 5.4 units for the acetoxy group, 1.0 unit for the carbon atom lost through decarboxylation, and 18.8 for this acid, one obtains 25.2 units. This value is identical to the equivalent chain length determined for the acetylated derivative of methyl-HFA. These results provided strong evidence for the conclusion that HFA is a 2-hydroxy fatty acid and indicated that olefinic linkages were absent in the molecule, since no dicarboxylic acids were detected among the oxidation products.

Identification of Cyclopropane Function—The ornithine-containing lipid isolated from bacteria grown on a medium containing methionine-methyl- ^{14}C was found to contain radioactivity. Isolation of HFA from the labeled aminolipid and subsequent liquid scintillation counting showed that radioactivity was associated with the acid. This observation, the absence of dicarboxylic acids among the oxidation products of HFA, and knowledge concerning the biosynthesis of cyclopropane and branched chain fatty acids in other organisms (13) led us to investigate the possible presence of a cyclopropane group or a methyl branched chain in HFA.

Following methanolysis of a quantity of the ornithine-containing lipid, the ether-soluble product (I) was isolated by thin layer chromatography and then acetylated. The acetylated product, also isolated by thin layer chromatography, obviously contained small quantities of other components in addition to the HFA derivative. However, because of the quantity of sample required, infrared spectroscopy of this material was performed. The spectrum exhibited a weak absorption band at $9.8\ \mu$, thus indicating the presence of a cyclopropane group.

Mass spectra of methyl-HFA and acetylated methyl-HFA gave discernible molecular ion peaks (M) of 326 and 368 m/e , respectively (Fig. 4, A and B). The presence of a ring structure such as a cyclopropane group in HFA seemed quite certain since the indicated molecular weights were two mass units less than that expected for a saturated, 19-carbon *normal* or branched chain, hydroxy, or acetoxy fatty acid. The absence of olefinic linkages had been previously indicated by the permanganate-oxidation studies. Even though the fragmentation patterns for the two derivatives did not permit definitive conclusions concerning their hydrocarbon structure, features characteristic of methyl branched carbon chains were absent. Supporting evidence for the presence of one hydroxyl group in the 2 position was obtained from the spectra. In methyl-HFA an intense peak (base peak in Fig. 4B) was observed at 267 m/e ($M - 59$). In the mass spectra of hydroxy fatty esters, this characteristic appears to be unique for the methyl esters of 2-hydroxy fatty acids. The mass spectral features directly attributed to the hydroxy function in methyl 2-DL-hydroxydocosanoate by Ryhage and Stenhagen (14) were observed in the spectrum of methyl-HFA, except for the relative intensity of the $M - 1$ peak which was much lower in the spectrum of methyl-HFA. For methyl-HFA, the magnitude of the $M + 1$ and $M + 2$ peaks, expressed as a percentage of the molecular ion peak (M), were 23.6 and 3.6%, respectively. Calculated values (15), based on an empirical formula of $\text{C}_{20}\text{H}_{38}\text{O}_3$

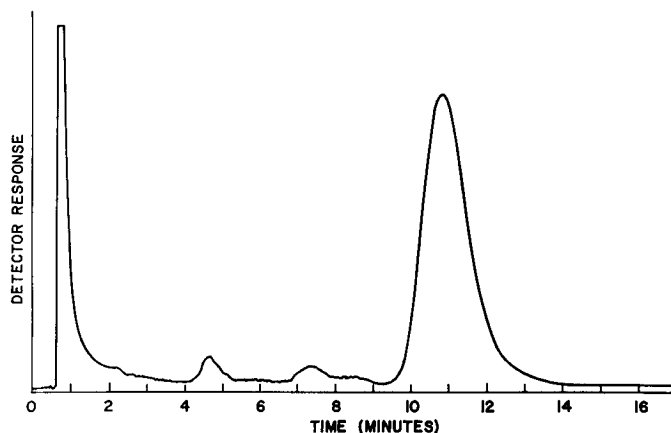


FIG. 2. The gas-liquid chromatography tracing of the derivative (G, Fig. 1) obtained by acetylation of the product released by methanolysis of the ornithine-containing lipid of *Thiobacillus thiooxidans*. The equivalent chain length of the major component was found to be 25.2. Chromatography was performed with a 5-ft column with diethylene glycol succinate as the liquid phase. Operating conditions are indicated in "Experimental Procedures."

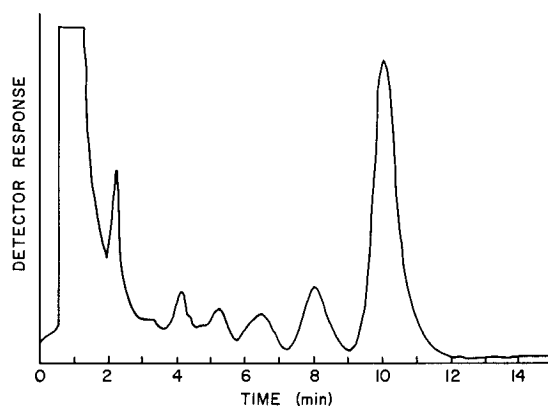


FIG. 3. The gas-liquid chromatography tracing of the methyl esters derived by the methylation of the products formed through oxidation of HFA with KMnO_4 in glacial acetic acid. HFA was obtained by saponification of the acetylated methyl ester which was isolated by trapping the major gas-liquid chromatographic component shown in Fig. 2. The equivalent chain length of the primary oxidation product was determined to be 18.8. Chromatography was performed with a 12-ft column with diethylene glycol succinate as the liquid phase. Operating conditions are described in "Experimental Procedures."

were $M + 1 = 22.3\%$ and $M + 2 = 3.0\%$. Methyl-HFA acetate yielded values of $M + 1 = 27.3\%$ and $M + 2 = 3.4\%$ while calculated values ($\text{C}_{22}\text{H}_{40}\text{O}_4$) were $M + 1 = 24.6\%$ and $M + 2 = 3.7\%$. Since the methyl esters usually exhibit isotopic abundance ratios which are slightly higher than calculated values (16), these results added credence to our molecular weight determinations.

Comparing the upper mass range of the methyl-HFA spectrum to that of methyl 2-hydroxydocosanoate, the fragmentation pattern is virtually the same if one assumes that in the methyl 2-hydroxydocosanoate spectrum the intense peak, assigned as $M - 1$ (369 m/e) should actually be a molecular ion peak (370 m/e). In the spectrum of methyl 2-hydroxydocosanoate, the ratios of the 369, 370, and 371 m/e peaks appear to be about that expected for 370, 371, and 372 m/e peaks. Such ratios could be possible but,

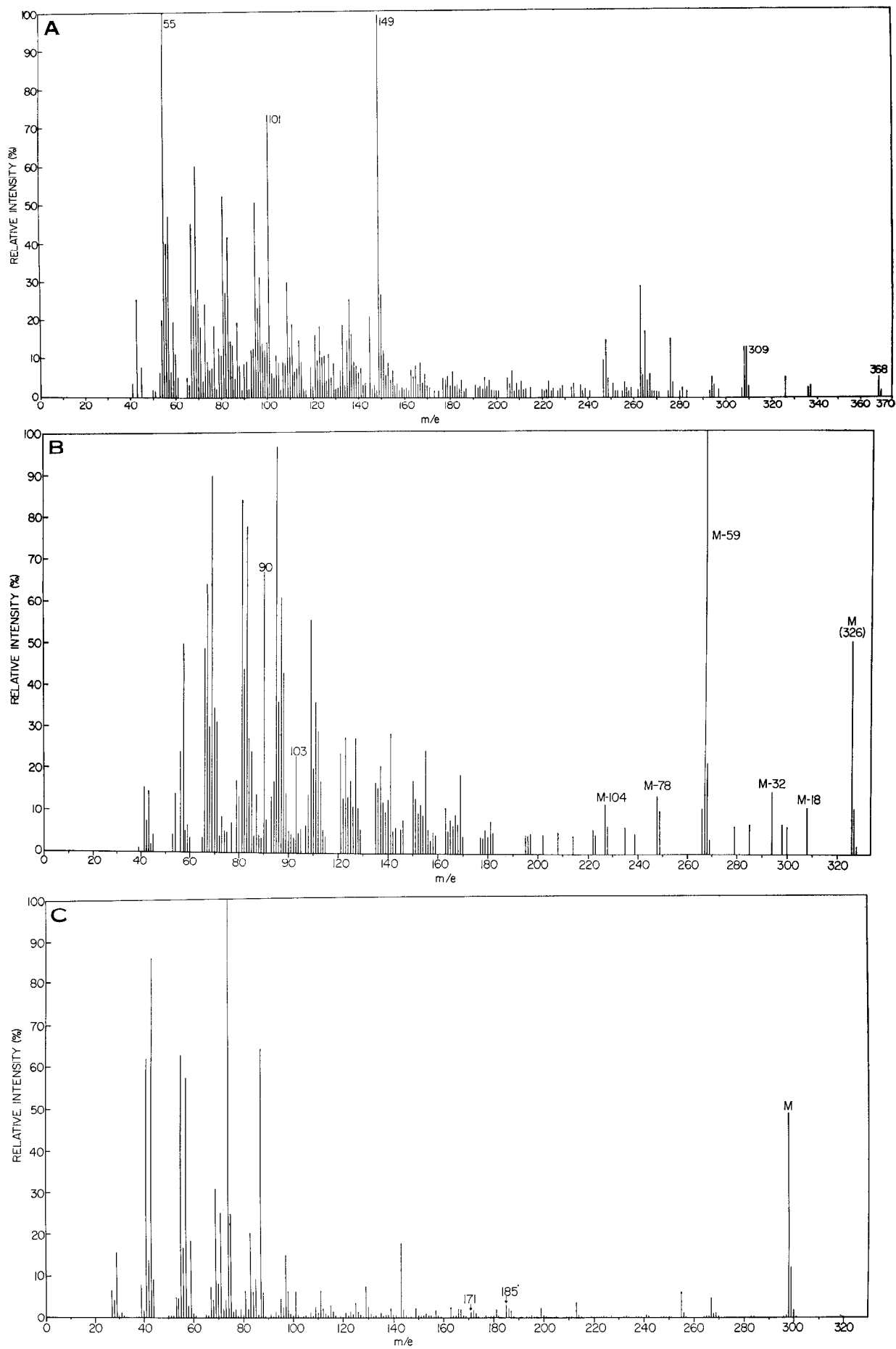


FIG. 4. A to C

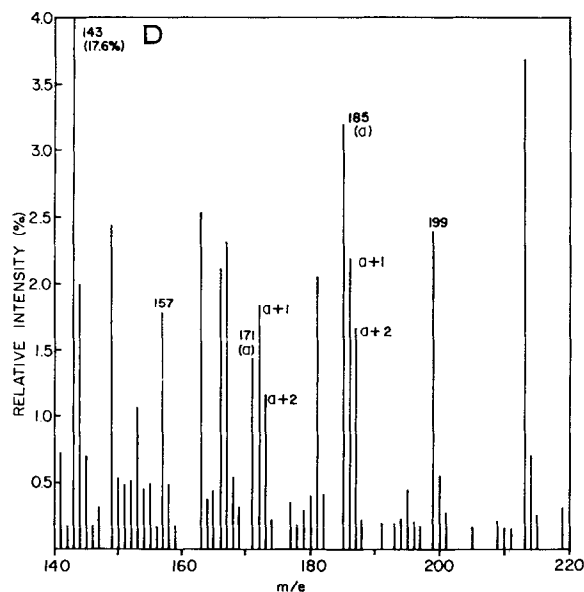


FIG. 4D

FIG. 4. Mass spectra of derivatives of HFA. *A*, acetylated methyl-HFA (the acetylated methyl ester isolated by trapping the major gas-liquid chromatographic component shown in Fig. 2); *B*, methyl-HFA (the methyl ester obtained by methanolysis of *A*); *C*, the branched chain methyl ester resulting from the reductive ring opening of the methyl ester of the primary HFA oxidation product shown in Fig. 3; *D*, the same as *C*, presented as an enlarged partial spectrum. In *A*, *B*, and *C* only those peaks which exceed 1.5% of the base peak are presented, while in *D* those above 0.15% of the base peak are shown.

from the appearance of the major fragment ions, would seem unlikely unless the loss of 1 hydrogen atom occurred exclusively and completely with those ions which did not undergo further fragmentation. To determine whether our mass assignments were correct and to observe the fragmentation patterns of other 2-hydroxy fatty esters, further mass spectrometry was performed with methyl-HFA, 2-hydroxyhexadecanoate, 2-hydroxyoctadecanoate, and a reference compound, heptacosafuorotributylamine. The reference compound exhibits a peak at 326 *m/e* (0.4%) and no other interfering peaks within 10 mass units of 326 with intensities greater than 0.03%. In the spectrum obtained when methyl-HFA and the reference compound were analyzed simultaneously, the ratio of the 326:327 peaks was much greater than the ratio observed in the spectrum of pure methyl-HFA. The same relationship was observed for the 326:327 ratio. This clearly indicated that our mass assignments were correct. Other mass assignments for methyl-HFA were supported by the mixed spectrum. The spectra of methyl 2-hydroxyhexadecanoate and 2-hydroxyoctadecanoate, obtained both in the presence and in the absence of the reference compound, exhibited very low intensity *M* - 1 peaks and intense molecular ion peaks. Other spectral features in the upper mass range were very similar to those of methyl-HFA and 2-hydroxydocosanoate. Since intense molecular ion peaks and minor *M* - 1 peaks were observed in all our spectra of 2-hydroxy fatty acid esters, it seems likely that the molecular ion peak for 2-hydroxydocosanoate (14) was improperly assigned.

Data concerning the contributions of cyclopropane groups to the equivalent chain length of fatty acid methyl esters suggested the presence of a *cis*-cyclopropane group in HFA (11). To investigate this possibility, methyl *cis*- and *trans*-9,10-methyl-

eneoctadecanoate were synthesized and analyzed by gas-liquid chromatography. Equivalent chain lengths of 19.8 and 18.9 were determined for the *cis* and *trans* forms, respectively. Thus the *cis*-cyclopropane group in the 9,10 position increased the equivalent chain length of methyl octadecanoate by 1.8 units. Adding this value to the contributions of a 2-acetoxy group and a normal 18-carbon fatty acid, one obtains the value observed for the equivalent chain length of the acetylated derivative of methyl-HFA ($1.8 + 5.4 + 18.0 = 25.2$). The methyl ester of the primary oxidation product of HFA exhibited an equivalent chain length of 18.8, a value expected for a heptadecanoic acid with a *cis*-cyclopropane function near the middle of the carbon chain. From available data, the position of a cyclopropane group does not appear to affect its contribution to the equivalent chain length of the methyl ester to a significant extent, except, possibly, when the function is close to either end of the hydrocarbon chain (11). However, the geometrical configuration of the cyclopropane does have a pronounced effect. From comparisons of the equivalent chain lengths of known compounds to the various HFA derivatives, it would appear that there was no interaction between the cyclopropane function and the acetoxy group which affected the equivalent chain length of acetylated methyl-HFA.

When either methyl *cis*-9,10-methylenecarboxylate (equivalent chain length = 19.8) or methyl *trans*-9,10-methylenecarboxylate (equivalent chain length = 18.9) was catalytically hydrogenated and subjected to gas-liquid chromatography, two resolvable components were observed. One, presumably a mixture of the methyl esters of 9- and 10-methyloctadecanoic acid, constituted about 80% of the total sample and exhibited an equivalent chain length of 18.2. According to its equivalent chain length of 19.0, the other component apparently was the normal chain fatty acid ester which would be expected from reductive ring opening of the cyclopropane function. Thus the reduction of methyl *cis*-9,10-methylenecarboxylate yielded products which exhibited equivalent chain lengths of 1.6 and 0.8 units less than the parent compound. For methyl *trans*-9,10-methylenecarboxylate the equivalent chain lengths of the reduction products were 0.7 unit less and 0.1 unit more than the parent ester. When the methyl ester of the oxidative decarboxylation product of HFA (equivalent chain length = 18.8) was hydrogenated and analyzed, two components with equivalent chain lengths of 17.2 and 18.0 were observed. The ratio of the two components was similar to that observed for the reduction products of the synthetic fatty esters. The equivalent chain lengths of the two components were 1.6 and 0.8 units less than the methyl ester from which they were derived, which is in agreement with the results obtained with methyl *cis*-9,10-methylenecarboxylate. These data substantiate the conclusion that a cyclopropane group is present in HFA and further indicate that it has a *cis* configuration.

Location of Cyclopropane Function—Because of the lack of definitive fragmentation patterns for cyclopropane fatty acid esters, the position of the cyclopropane group could not be confidently determined from the spectra of methyl-HFA and its acetylated derivative. Therefore, a portion of the acetylated methyl-HFA was saponified and the free acid was oxidized with potassium permanganate in acetic acid. The resulting products were methylated, separated by gas-liquid chromatography, and trapped. The methyl ester of the primary oxidation product (equivalent chain length = 18.8) was then subjected to catalytic hydrogenation. The reaction products were isolated by thin layer chromatography, resolved into two components by gas-liquid

uid chromatography, and trapped. The component representing a mixture of the two branched chain fatty esters (equivalent chain length = 17.2) was then analyzed by mass spectrometry. The spectrum, showing a molecular ion peak at 298 m/e , is presented in Fig. 4C. An enlarged partial spectrum (Fig. 4D) exhibited a small $M - 15$ peak which is suggestive of methyl branched chain fatty acids. As shown by McCloskey and Law (4), the location of the cyclopropane function may be unequivocally determined from the mass spectrum of the methyl branched esters, which are formed by reductive ring opening of the cyclopropane group. The position of the methyl branches may be established from the fragmentation ions which belong to the $73 + n$ [14] series (4). If $73 + n$ [14] is equal to a , then the intensity of the $a + 1$ and $a + 2$ peaks, relative to a , will be greater when cleavage of the carbon chain occurs α to the methyl branch on the ester side. As may be seen in Fig. 4D, the intensity of the peaks 172 and 173 m/e relative to 171 and the 186 and 187 m/e peaks relative to 185 is greater than other members of the $73 + n$ [14] series. Thus it would appear that these two methyl branched chain esters were derived from methyl 10,11-methyleneheptadecanoate by reductive ring opening. Since the latter was obtained through oxidative decarboxylation of HFA, HFA must be *cis*-11,12-methylene-2-hydroxyoctadecanoic acid.

The absolute configuration of *cis*-11,12-methylene-2-hydroxyoctadecanoic acid has not been determined. However, work in progress toward the synthesis of the 2-D and L-hydroxy derivatives of *cis*-11,12-methyleneoctadecanoic acid should aid in the configurational assignment of the hydroxyl group. The lipid extracts of *T. thiooxidans* appear to contain *cis*-11,12-methyleneoctadecanoic acid.² If this acid is lactobacillic acid (one of the optical isomers of *cis*-11,12-DL-methyleneoctadecanoic acid (17)), then it would seem likely that the absolute configuration of carbons 11 and 12 in *cis*-11,12-methylene-2-hydroxyoctadecanoic acid is the same as that in lactobacillic acid.

From reviews concerning the natural occurrence of 2-hydroxy fatty acids, it appears that 2-hydroxy acids may be widespread (18, 19). While they have not been isolated from a large number of sources, a variety of sources have been shown to contain at least small quantities of these acids, usually as components of complex lipids. Thin layer chromatographic analysis of the methyl esters released by methanolysis of the total lipid extract of *T. thiooxidans* did not indicate the presence of hydroxy fatty acids, yet *cis*-11,12-methylene-2-hydroxyoctadecanoic acid is the predominate fatty acid released upon methanolysis of the ornithine-containing lipid from which it was isolated. A structural determination of the ornithine-containing lipid has not been completed. However, it appears that the carboxyl group of *cis*-11,12-methylene-2-hydroxyoctadecanoic acid is involved in an ester linkage with the lipoamino acid since mild base-catalyzed methanolysis releases the 2-hydroxy fatty acid as its methyl ester. Whether the hydroxyl group of the acid is free in the complex lipid is not known.

Of the lipoamino acids which have been observed, the ornithine containing lipid isolated by Gorchein from *Rhodopseudomonas sphaeroides* (20) and the lysine-containing lipid of *Streptomyces sioyaensis* (21) resemble the ornithine-containing lipid from *T. thiooxidans* in several respects. These lipids do not contain glycerol or phosphorus and appear to possess both ester and amide linkages. However, the lipids of *R. sphaeroides* and *S. sioyaensis* release primarily fatty alcohols under transesterification or mild alkaline hydrolysis conditions, while methyl *cis*-11,12-methylene-2-hydroxyoctadecanoic acid is released from the lipid of *T. thiooxidans*.

The role of 2-hydroxy fatty acids in complex lipids and the function of lipoamino acids such as these mentioned may become more apparent when structures and variations in structures are completely established.

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² Unpublished observations from our laboratories.