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Studies on the Antarctic whale oils by gas-liquid chromatography using a hydrogen flame ionization detector. VIII. 7-Methyl-6-hexadecenoic acid in fin whale blubber oil.

By Yoshihiko Sano

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Studies on the Antarctic Whale Oils by Gas-Liquid Chromatography Using a Hydrogen Flame Ionization Detector. VIII.

(605)

7-Methyl-6-hexadecenoic Acid in Fin Whale Blubber Oil

Yoshihiko Sano

Research Laboratory, Miyoshi Oil & Fat Co., Ltd. (66-1, 4-chome, Horikiri, Katsushika-ku, Tokyo) Oil Chemistry (Abura Kagaku or Yu Kagaku) 16, 605-610 (1967)

(606)

An unsaturated branched-chain fatty acid, having an ECL[5] value of 16.9 on the PEGA column, was separated in a purity of 84.8% from the fatty acids of fin whale blubber oil by column chromatography on silicic acid impregnated with silver nitrate in combination with urea-complex fractionation technique. This acid was determined as 7-methyl-6-hexadecenoic acid by means of infrared analysis, high resolution nuclear magnetic resonance and mass spectrometry, and further by thin-layer and gas-liquid chromatographic analyses of the oxidative degradation products thereof.

In addition, nuclear magnetic resonance study showed that the acid has trans configuration, on the basis of the work of Bates and Gale[17].

The ECL value of the methyl ester of its hydrogenated product, 7-methylhexadecanoic acid, on the PEGA column was 16.4 and was different from that of *iso* or *anteiso* acid series.

The content of the acid in the fin whale blubber oil investigated was approximately 0.01% of the total weight of the acids.

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1. INTRODUCTION.

Previously the author reported that the Antartic fin whale blubber oil consisted of many different types of fatty acids, among which highly unsaturated, odd-numbered fatty acids were the major component, and poly-branched-chain fatty acids and unsaturated branched-chain fatty acids were two of the minor components.(1) The author also reported on the structures of three different poly-branched-chain fatty acids 2)-4).

In this report, an unsaturated branched-chain fatty acid, whose structure has not been determined, is chosen as the subject. It appears that the fin whale blubber oil contains at least two different fatty acids that belong to this group(1), and in this report, only the one that is contained in relatively large quantity and that has an ECL(Note 1) value of 16.9 is reported. The author could isolate the acid, and purified it to high purity by combination methods of isolation through addition-complex formation with urea and column chromatography using silicic acid impregnated with silver nitrate. The author confirmed its structure to be 7-methyl-6-hexadecenoic acid, that is hitherto unknown, by its infrared spectrum (IR), high resolution nuclear magnetic resonance spectrum (NMR), mass spectrum (MS), and by analyses of its potassium permanganate oxidation products using thin layer chromatography (TCL) and gas-liquid partition chromatography (GLC).

Note 1 - Abbreviation of the equivalent chain length 5)

2. EXPERIMENTS AND RESULTS.

fin whale blubber oil (1), whose fatty acid mixture was previously analyzed, was used as the sample.

2.1 Isolation of the Unsaturated Branched-chain Fatty Acids.

2.1.1 Preparation and Purification of the Acid Methyl Esters, and Fractionation of the Urea Complexes.

As previously reported(1), 433.2 g of Arctic fin whale blubber oil was neutralized in n-hexane with 20% potassium hydroxide solution, and the neutralized solution was passed through a silicic acid column, which produced 423.0 g of purified oil. The purified oil was treated with methanol and the methyl ester mixture was treated with a silicic acid column to remove highly unsaturated methyl esters and colored impurities (6), and 164.0 g of a colorless pure methyl ester mixture was obtained. Fractionation of urea-complex mixtures was repeated following the routine procedure, and each fraction was analyzed by GLC, and the fractions which contained the target product (the fraction with ECL value 16.9) were combined as the mixture fraction F-1 (279 mg).

2.1.2 Thin Layer Chromatography using Silica-gel-silver Nitrate, Fractionation by ($AgNO_3$-TLC), and GLC Analysis.

Since the mixture fraction F-1 contained many components

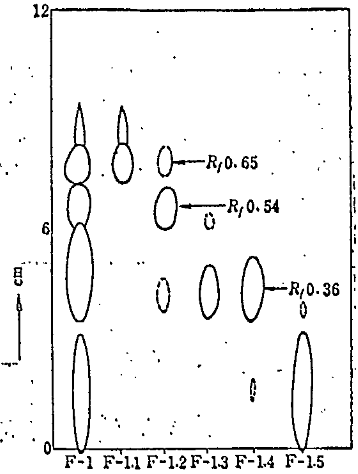

Plate : 5×20 cm, Silica gel G impregnated with 5 % silver nitrate, thin-layer, ca. 0.4mm.

Solvent : n-Hexane-Diethyl ether (80:20.vol/vol)

Development : Distance, 12 cm; Time.25 min; Temp., 25℃

Fig.-1 Thin-layer chromatograms of the fractions F-1-1, F-1-2, F-1-3, F-1-4 and F-1-5 from $AgNO_3$-TLC separation of the fraction F-1, containing small amounts of unsaturated branched-chain fatty acid methyl esters, obtained by repeated urea-complex fractionation.

other than the desired substance, the mixture fraction, F-1 (85 mg), was further divided into five fractions, F-1-1 (9 mg, the fraction at 85～100 mm from the sample spot), F-1-2 (21 mg, a fraction at 73～85 mm), F-1-3 (24 mg, a fraction at 60～73 mm), F-1-4 (12 mg, a fraction at 35～60 mm) and F-1-5 (5 mg, a fraction at 0～35 mm), using four Silica-gel-silver nitrate plates with the size of

5 cm x 20 cm x 0.4 mm prepared as previously reported(7). Figure 1 shows the chromatogram of these five fractions. (607) GLC analysis of each fraction showed that all except for the fraction F-1-5 had the same peak of identical retention time.

Therefore, each fraction was hydrogenated and the products were analyzed again by GLC. The desired substance (ECL value 16.9, γ_{18}(8) 0.738) was now found to have been fractionated in the F-1-1. Since the F-1-2 fraction also contained a small amount of the target substance, the spot with R_f 0.65 in figure 1 is the fraction that contains the object. The main component (R_f 0.54) of the F-1-2 was later found to be mono-en-acid methyl esters, and the main components of the F-1-3 and F-1-4 were di-en-acid methyl esters. The di-en-acid methyl esters were identified further as mainly octadeca-di-enoic acid methyl ester (18:2), but hexadeca-di-enoic acid methyl ester (16:2) was also identified clearly in the two* fractions (with γ_{18} values 0.719 and 0.742).

Figure 2 is the gas chromatogram of the fraction F-1-1, before and after the hydrogenation. The object substance with the ECL value 16.9 showed an ECL value of 16.4

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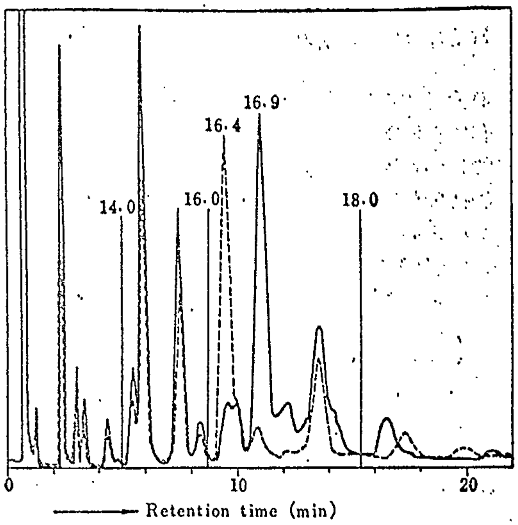
* The acid methyl ester, hexadeca-di-enoic acid may have been detected as <u>two</u> isomers with two γ_{18} values. This part is not clear. (Translator's Note)



GLC was performed on a Shimadzu model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector FID-1B

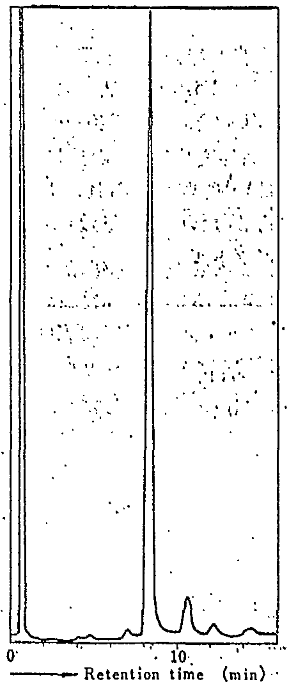
Column... 3mm i.d. x 185cm, 10% PEGA on Diabase B, 80-100 mesh, 210℃
Detector... FID-1B, H_2 : 25ml/min, Air : 0.8kg/cm^2, Sens. : 10^2, Range : 1.6 V
Carrier gas... He : 1.8kg/cm^2
Chart speed : 5mm/min

Fig.-2 Gas-liquid chromatograms of the $AgNO_3$-TLC fraction F-1-1 and its hydrogenated product (dotted line).

after hydrogenation, and therefore this eliminates the possible structure types, iso and anteiso. In this fraction, the author found the presence of non-straight-chain saturated compounds which were considered to be mainly branched-chain compounds.

2.1.3 Fractionation by Chromatography with Silicic Acid Impregnated with Silver Nitrate, and GLC Analysis

Since the afore-described $AgNO_3$-TLC analysis indicated that the object substance had a larger R_f value than straight-chain mono-ene acids, the author further attempted to obtain the substance in higher purity using a silver nitrate-silicic acid column. First, silicic acid impregnated with silver nitrate was prepared as follows; 10 g of silicic acid (Mallinckrodt product for chromatography, 100 mesh) was added to a solution of 1.0 g of silver nitrate in 25 ml of water, and the mixture was stirred well and then placed in a dish with an inside diameter of 20 cm. The mixture was heated at 105°C for 1.5 hours and then placed in the dark overnight. A column (inside diameter 20 mm, length 75 mm) was prepared using 2 g of silicic acid and 10 g of silicic acid impregnated with silver nitrate, and the prepared column was washed with 100 ml of n-hexane containing 1% of ether. A sample, 265 mg, containing 8.9% of the fraction with the ECL value 16.9, was added to the column wrapped with black paper to protect it from the light, and the column was eluted with 250 ml of n-hexane containing 1% of ether, which produced 25 mg of an eluate. GLC analysis of this eluate did not indicate presence of the object substance. The column was further eluted with an eluant, 250 ml of n-hexane containing 2% of ether, and the eluate was fractionated in a 25 ml portion, and each fraction was analyzed by GLC. The second fraction F-2 (7 mg) was shown to contain a considerably large amount of the object substance in fairly high purity (84.8%). (See figure 3). The next fraction (13 mg) contained the object substance in 12%, but no other fractions contained the object substance. Therefore, the sample before this chromatography contained the needed substance in about 3%, and about 2/3 portion of this sample with the ECL value 16.9 was found to be 16:2.



GLC conditions are the same as given in Fig.-2 except that column temp.: 216°C and He : 1.9kg/cm²

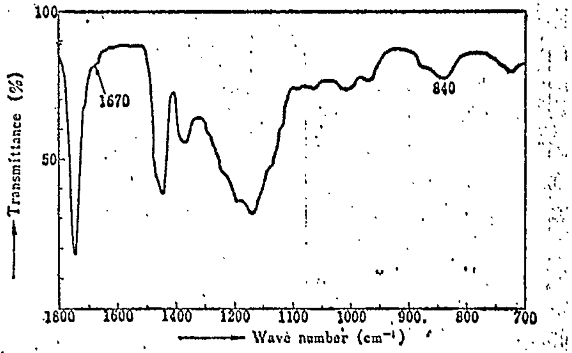
Fig.-3 Gas-liquid chromatogram of the unsaturated branched-chain fatty acid methyl ester fraction F-2 separated by column chromatography on silicic acid impregnated with silver nitrate.

2.2 The Structure of the Unsaturated Branched-chain Fatty Acid.

Using the unsaturated branched-chain fatty acid methyl ester isolated as described above, its IR, NMR, and MS (608) were determined, and TLC and GLC analyses were conducted on its potassium permanganate oxidation products, and based on this data, this unsaturated branched-chain fatty acid was found to be 7-methyl-6-hexadecenoic acid. The following sections describe the data and their interpretation.

2.2.1 Infrared Spectrum

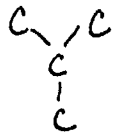
The determination was conducted on liquid films between rock-salt plates by using a self-recording infrared spectrophotometer, Model IR-S of Nippon Bunko (Japan Spectroscopic Co., Ltd.). The IR is shown in figure 4, which is typical of a fatty acid methyl ester, and shows absorption due to the $C-H$ out-of-plane deformation(9) of a tri-substituted double bond at near 840 cm^{-1} and a weak absorption due to $C=C$[☆] at 1,670 cm^{-1} but does not indicate the doublet[☆☆] at 1,380 cm^{-1}.



The analysis was made on thin films between rock-salt disks by using a twin-beam spectrophotometer model IR-S (Japan Spectroscopic Co., Ltd.)

Fig.-4 Infrared absorption spectrum of the unsaturated branched-chain fatty acid methyl ester fraction F-2.

[☆] Commonly $C=C$ stretching (Translator's Note)

[☆☆] Due to scissoring motion of  branching of saturated branched-chain. (Translator's Note)

2.2.2 Nuclear Magnetic Resonance Spectrum

In order to identify the structure near the branching chain, which was revealed by the IR analysis, and also to cumulate some knowledge on the partial structure, the NMR spectrum was determined using a high resolution nuclear magnetic resonance spectrometer, model JNM-4H-100 of Nippon Densi (Japan Electron Optics Co., Ltd.) (See figure 5). The sample was dissolved in tetrachloromethane, and tetramethyl silane (TMS) was used as an internal standard, and the chemical shift values were shown in δ (ppm). The conditions of determination are described in figure 5.

The signal near 0.87 ppm (δ) corresponds to three protons, by comparing its area with that of the carbomethoxy proton signal at 3.58 ppm (δ), and the signal is clearly, if not definitively, split into three peaks (10), which, therefore, is due to the terminal methyl group of a partial structure such as $CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2$ ...... The signal at 1.24 ppm (δ) is due to the ordinary methylene groups but not to the α or β methylene groups☆, and the signal corresponds to approximately 16 protons. Hashimoto and others(11) reported that the signal of the methylene group at a β position to a carboxyl group appeared at a lower field than the signals due to the methylene at a γ to or farther away from a carboxyl group, and that it usually appeared at

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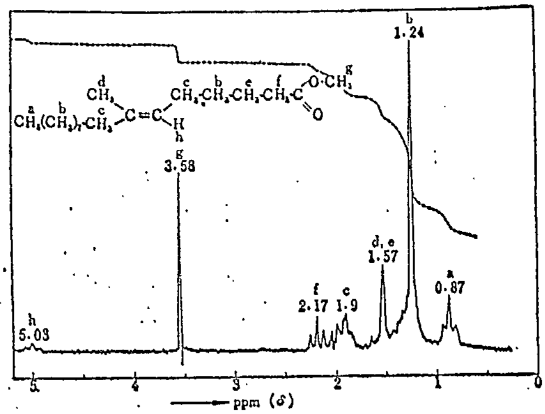
☆ The author meant α or β to the carboxyl groups, and allylic methylene protons are also to be excluded. (Translator's Note) a lower field than the signals due to the methylene at an γ to or farther away from a carboxyl group, and that it usually appeared at 1.6 ppm (δ) area, after determining NMR spectra of related compounds at 100 Mc. Somewhat broadened singlet at 1.57 ppm (δ) with small signals near its base corresponds to five protons, and this is perhaps overlapped signals of β- methylene group and of the branched methyl group(12) that is shifted to the lower field because of the double bond. The IR analysis indicated presence of a tri-substituted double bond, and, therefore, if this result and the presence of one vinyl tertiary proton of the double bond carbon at 5.03 ppm (δ) corresponding to one proton area can be combined we can assume a partial structure at the branching as $-CH_2-\overset{CH_3}{\overset{|}{C}}=CH-$. The triplet signal corresponding to two protons at 2.17 ppm (δ) is the



The measurement was made on a Japan Electron Optics model JNM-4H-100 high resolution nuclear magnetic resonance spectrometer operating at 100 Mc.
Solvent : CCl_4, Standard : TMS, S.F. Level : 40 db, R.F. Gain : 3, Record gain : 3-1, Response : 2, Sweep width : 9x1 ppm, Sweep time : 5min.

Fig.-5 Nuclear magnetic resonance spectrum of the unsaturated branched-chain fatty acid methyl ester fraction F-2 (cf., **Fig.-3**).

methylene at the α to the carbomethoxy group, and the methylenes adjacent to the double bond appear at 1.9 ppm (δ) corresponding to four protons but the signal is somewhat complicated and unresolvable.

The afore-described NMR analysis allows us to conclude that the component with the ECL value 16.9 has the partial structures, $CH_3.CH_2.CH_2.CH_2$ ...., $-CH_2-\overset{CH_3}{C}=CH-CH_2-$, and $-CH_2-CH_2-COO\ CH_3$, and therefore it is a mono-methyl-mono-ene carboxylic acid methyl ester. Thus the author confirmed the presence of an unsaturated branched-chain fatty acid in fin whale blubber oil.

2.2.3 Determination of the Position of the Double Bond by Potassium Permanganate Oxidation.

Since the NMR analysis verified that the double bond carried a methyl group at its olefinic carbon, the author decided to carry out a room temperature oxidation with potassium permanganate using acetone as the solvent, based on his experience of the synthesis of phytone from phytol(4). This oxidation is to establish the position of the double bond. The sample had 71.2% of purity (GLC analysis) and was prepared by urea-complex fraction and by chromatography using a column of silicic acid impregnated with silver nitrate. The largest single impurity of this sample was 8% of the total sample. To 11 mg of this sample, 2 ml of a saturated acetone solution of potassium permanganate (Ito Chemical Co., Ltd.; Best Grade Reagent[☆]) was added slowly under stirring, and the (609) solution was left at room temperature. The reaction solution was diluted with 5 ml of sodium chloride solution, and a very small amount of sodium bisulfite (Ito Chemical Co., Ltd.; First Grade Reagent[☆]) was added to decompose the excess potassium permanganate. The resulting colorless solution was extracted with petroleum ether (b.p. 35∼40°C). The extract was washed with sodium chloride solution, dried with anhydrous sodium sulfate, and evaporated under reduced pressure to yield a colorless oil, 5 mg. with strong odour, which appeared to be a ketone. TLC analysis of the oil, using 2-nonanone and 2-undecanone (both from Tokyo Kasei Co. Ltd., First Grade Reagent) as reference substances, showed that the R_f value of the main product corresponds to these two substances. A small amount of impurity was removed by a micro-column of silicic acid, and the n-hexane solution of the oil with 2-undecanone-like flavor was subjected to GLC analysis on the column for analysis of fatty acid methyl esters (refer to figure 2) using 2-nonanone, 2-undecanonone and 2-tridecanone (Tokyo Kasei Co. Ltd.; Best Grade Reagent) as reference substances. The column temperature employed was 120 and 140°C, and the retention time of the main peak

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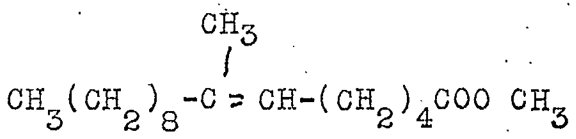
[☆] First Grade Reagent corresponds to A.R. of A.C.S. Best Grade Reagent is better than the First Grade Reagent and is available on a smaller scale. It carries individual assay result of the lot from which the bottled lot was taken. (Translator's Note)

[20.0 min (120°C), 9.0 min (140°C)] was confirmed to correspond to that of 2-undecanone.

The residual solution after extraction was basified with 2N sodium hydroxide solution, and condensed at reduced pressure. The condensate was acidified with 2N hydrochloric acid, and warmed, and, after cooling, extracted with ether. The ether extract was washed with saturated sodium chloride solution, dried with anhydrous sodium sulfate, and evaporated under reduced pressure to yield 2 mg of an oily substance. It was converted to a methyl ester with diazomethane, and the product was analyzed by TLC, which showed a main spot at the R_f value corresponding to dimethyl adipate. The product was further analyzed by a column for the analysis of fatty acid methyl esters (refer to figure 2) at the column temperature of 200°C. The main peak was confirmed to agree with that (Retention time 3.0 min) of dimethyl adipate. Therefore the substance under investigation should have the structure (I).

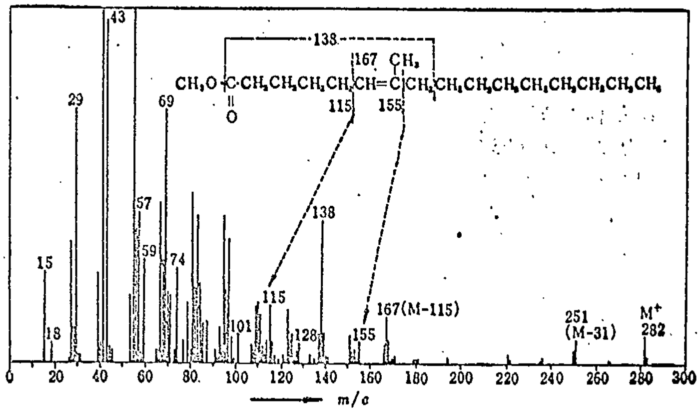


$$CH_3(CH_2)_8-\overset{\displaystyle CH_3}{C}=CH-(CH_2)_4COO\ CH_3$$

(I)

2.2.4 Mass Spectrum

For the determination, a mass spectrometer model RMU-6D of Hitachi Co., Ltd. was used. The spectrum had the molecular ion peak (M^{+}) at m/e 282 and the peak M-31 (m/e 251)



The measurement was made on a Hitachi RMU 6-D mass spectrometer

Fig.-6 Mass spectrum of the unsaturated branched-chain fatty acid methyl ester fraction F-2 containing 84.8 per cent of methyl 7-methyl-6-hexadecenoate having an ECL value of 16.9 on the PEGA column.

that was expected from the methyl ester was also detected clearly, as shown in figure 6. The molecular formula is $C_{18}H_{34}O_2$ which agrees with the structure (I). Peaks at m/e 115, 167 (M-115), 155 were also relatively intense, and particularly the strong peak at m/e 138 was significant. This peak was perhaps derived from the fragment, M-113-31, as illustrated in figure 6.

The analyses by IR, NMR, MS, and TLC and GLC of the oxidative degradation products determined the structure of the unsaturated branched-chain fatty acid as 7-methyl-6-hexadecenoic acid.

3. DISCUSSION

Relatively lately a few publications were made on unsaturated branched-chain fatty acids as constituents of natural oil and fat. Wolfe and others (1963)(13) detected a mono-enoic acid (16:1 br, 18:1 br) that was estimated to be an unsaturated branched-chain fatty acid in phosphatides of Orconectes rusticus, a species of fresh-water crawfish, and found that the acid had a retention time which was intermediate of those of straight-chain saturated acids and of straight-chain mono-enoic acids. Casal and Holman (1965)(14) reported an unsaturated branched-chain fatty acid (16:1 br) with the ECL value 16.60 (EGS Column) in a C_{15} fatty acid fraction isolated from the liver of a rat fed with starch-meal. They did not investigate the structure of the acid. On the other hand, Nicolaides and Ray (1965)(15) isolated thirteen iso- and four anteiso - unsaturated branched-chain fatty acids from the fat component of human skin surface lipid, and determined the position of double bonds by ozonization. They also reported five iso- and three anteiso - unsaturated branched-chain fatty acids of human fetuses. Very recently Etemadi and others (1967)(16) isolated a C_{80}-unsaturated branched-chain fatty acid from lipoids of Mycobacterium smegmatis, which is responsible for tuberculosis, and established its structure. In all these acids, the double bonds do not carry a branched methyl group, but the acid, 7-methyl-6-hexadecenoic acid, now isolated from the component fatty acids of fin whale blubber oil, has the methyl group branch (610) near the centre of the carbon chain, and this branching pattern resembles an isoprenoid group that has a double bond at its end. As a result, the effect of shortening of retention time by the branched methyl group and the effect of lengthening the same by the double bond compensated each other and the ECL value became 16.9 (PEGA Column)(Note 2). The hydrogenation product of the acid, i.e., 7-methylhexadecanoic acid had an ECL value 16.4, and this value enabled us to distinguish the iso-form and anteiso-form. This acid[☆] had nearly identical FCN value(Note 3) with that of 8-methyloctadecanoic acid with an ECL value (DEGS Column) 18.38(15). The decrease of the ECL value by 0.5 after hydrogenation indicated that the double bond at the end of methyl branching has greater, retention time - lengthening effect than the double bond in ordinary straight-chain mono-enoic acid. The conditions applied for the GLC analysis in this report could not distinguish 7-methyl-6-hexadecenoic acid from hexadeca-di-enoic acid (16:2) as they had nearly identical retention times, but they could be characterized separately by $AgNO_3$-TLC or by a column of silicic acid impregnated with silver nitrate.

[☆] 7-methyl-6-hexadecenoic acid or 7-methylhexadecanoic acid? (Translator's Note)

Note (2): The value was 16.8 under the conditions reported in Part III and also at the column temperature 200°C.

Note (3): Abbreviation of fractional carbon numbers.

In this structure elucidation, the author estimated presence of a tri-substituted double bond, and then conducted an NMR analysis, which confirmed the partial structure of unsaturated branched-chain that had one methyl group attached to the double bond. Since this partial structure suggested probably isolation of a methyl ketone, selection of reference materials for TLC and GLC analyses was easy.

The stereochemistry of the acid was studied referring to the analogous studies on the structure of isoprenoids by Bates and Gale (1960)(17) and on the stereochemistry of castaprenols by Wellburn and others(18) who also applied the principles of the former report(17). Thus the chemical shift, 1.57 ppm (δ), of the branched methyl establishes the trans structure of 7-methyl-6-hexadecenoic acid isolated from the fin whale blubber oil.

Further the NMR analysis proved that the number of total protons was thirty-four, indicating the sample to be a C_{17}-mono-methyl-mono-ene carboxylic acid methyl ester, and the MS analysis confirmed its molecular weight to be 282, which corresponded with a C_{17} unsaturated branched-chain fatty acid. However, since the MS analysis was not made on its hydrogenation product, the position of branching could not be clarified. Incidentally, the report(19) on the MS analysis of saturated branched-chain fatty acid methyl esters by Ryhage and Stenhagen (1960) seems to suggest probable identification of the position of the branching. Therefore, in future studies of unsaturated branched-chain fatty acids, it is important and valuable to obtain the MS data of hydrogenation products of the acids.

It appears that 7-methyl-6-hexadecenoic acid is contained in about 0.01% of the fin whale blubber oil used in this study. This same acid is also contained in spermwhale blubber oil(20), and the unsaponified material of this oil contained an alcohol corresponding to the acid, i.e. 7-methyl-6-hexadecenol. The author plans to report on the details of these findings elsewhere. Although 7-methyl-hexadecanoic acid has been synthesized by Weizel and Wojahn (1951)(21), this type[✲] of unsaturated branched-chain fatty acid seems to be a new acid found in nature.

Finally the author thanks Assistant Professor Nobutaka Takahashi and Research Assistant Asahi Murohashi of the Department of Agricultural Chemistry, College of Agriculture, Tokyo University for the determination of Nuclear Magnetic Resonance Spectra, and Mr. Akira Fujii of Nissei Sangyo Co. Ltd. for the determination of the Mass Spectrum. He also thanks the Research Director of this laboratory, Dr. Yozo Ishikawa for his permission of this research results.

[✲] Perhaps the authors meant fatty acids with vinylic methyl group. (Translator's Note).

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