KOREAN J. FOOD SCI. TECHNOL.
Vol. 23, No. 1, pp. 68~75(1991)

Presence of cis-11, 12-Methylene Octadecanoic Acid in the Oils of *Ternstroemia gymnanthera*

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후피향 종실의 cis-11, 12-Methylene Octadecanoic acid에 관한 연구

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Abstract

The seed oil of *Ternstroemia gymnanthera*, a species of the *Ternstroemiaceae*, is mainly composed of triglyceride(92.4%), followed by polar lipids(5.9%), sterol(1.2%) and pigments(0.5%). This oil contains 4.8% of cis-11, 12-methylene octadecanoic acid(lactobacillic acid) in the fatty acid composition of the total oil. This identification is based on information from non-urea inclusion formation, silver nitrate impregnated silica gel column and gas liquid chromatography, ^{1}H-& ^{13}C-nuclear magnetic resonance and mass spectroscopy. Smaller amounts(0.1%) of presumptive 9, 10-methylene hexadecanoic acid(dihydro malvalic acid) is also detected. The major fatty acids in this oil are C18 : 1(36.1%), C18 : 2(30.9%), C16 : 0(15.1%), C16 : 1(7.6%) and C18 : 0(3.4%).

Key words : cis-11, 12-methylene octadecanoic acid, lactobacillic acid, *Ternstroemia gymnanthera*, cyclopropanic fatty acid, cyclopropenic fatty acid

Introduction

Cyclopropenic fatty acids(CPEFA) such as malvalic (8, 9-methylene heptadec-8-enoic) and sterculic(9, 10-methylene octadec-9-enoic) acid are present in many species of the *Bombacaceae*[1-3], *Malvaceae*[1,4], *Sterculiaceae*[1,4], *Sarcolaenaceae*[5] and *Tiliaceae*[5] families of the *Malvales* order, the *Sapindaceae*[5] family of the *Sapindales* order, and the *Gnetaceae*[5] family of the *Gnetales* order.

Recent works[5,6] show that the oils containing CPEFA are frequently accompanied by smaller proportions of cyclopropanic fatty acids(CPAFA), such as dihydromalvalic and dihydrosterculic acids, which are the dihydro analogs of CPEFA. In some cases, CPAFA are the major component in the oils ; dihydrosterculic acid occurs as a major component in *Litchi sinensis* seed oil[7] and in the oil of *Euphoria longana*[8], both species being in the family *Sapindaceae*.

CPAFA are also found in bacteria and protozoa ; Hofmann[9-11] isolated a cyclopropanic acid, lactobacillic acid(cis-11, 12-methylene octadecanoic acid) from the bound lipids extracted from cultured cells of *Lactobacillus arabinosus, L. casei* and *Agrobacterium tumefaciens*. Cis-9, 10-methylene hexadecanoic acid(dihydromalvalic acid) occurs as a major fatty acid with lactobacillic acid in the phospholipids from *Escherichia coli*[12]. Meyer[13] also indicated that cis-9, 10-methylene octadecanoic acid(dihydrosterculic acid) is present(10.6%) in the phospholipids from a species of protozoa, *Crithidia fasciculata*. An investigation of the seed oil of *Byrsocarpus coccineus* disclosed cis-11, 12-methylene octadecanoic acid[11].

Ternstroemia gymnanthera belongs to the *Ternstromiaceae* family and is grown in the southern parts of Korea[15]. The bark of the tree has been used as a substitute for red brown pigment[15]. The seed is known to have antimicrobial activity, but little information about this activity and its proximate compostition is available.

In an analysis of the fatty acid methyl esters of the oil from *T. gymnanthera* seeds, we found an unusual constituent which eluted in gas-liquid chromatography (GLC) between the methyl esters of C18 : 2ω6 and C18 : 3ω3 fatty acids.

This paper describes the characterization of this co-

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mponent as cis-11, 12-methylene octadecanoic acid (lactobacillic acid).

Materials and Methods

Lipid composition and GLC of fatty acid methyl esters

T. gymnanthera seeds were collected in the area of Pusan. The lipids were extracted with chloroform/methanol(2 : 1, V/V)[16] and some portions of whole lipids were classified into neutral lipids, glycolipids and phospholipids on a silica gel column with chloroform, acetone and methanol, respectively[17]. The neutral lipids were fractionated into subclasses by column chromatography with the solvent system of hexane-diethyl ether. The other portions were dissolved in toluene to which 1% sulfuric acid in methanol was added. After the mixture was refluxed for 2 hours, 5% sodium chloride solution was added, and then the methyl esters of fatty acids were extracted with hexane[18]. The methyl esters of fatty acid were purified by column chromatography on silica gel with the mixture of hexane-diethyl ether[19], and were analyzed on GLC by the methods described in a previous paper[20].

Urea inclusion formation and silver nitrate column chromatography of fatty acid methyl esters

For separation of the methyl esters into saturated and unsaturated ones, urea adduct formation was carried out according to the method of Christie[21]. The fraction enriched with the unknown methyl ester was mounted on a silica gel column impregnated with 16% silver nitrate and were eluted step-wise with the solvent systems of hexane-benzene[22].

High performance liquid chromatography(HPLC) of triglyceride

A Waters chromatograph equipped with a differential refractometric detector and a stainless steel tubing(3.9 mm×30.0 cm) packed with μ-Bondapak C18 was used. Triglyceride obtained from a silica gel column was run isocratically using the mixture of acetonitrile-acetone-methanol-chloroform(3 : 3 : 3 : 1, by volume). The flow rate was 0.8 ml/min. The column was kept at 30℃ by water circulation and detector attenuation was 32X[23].

Picolinyl ester preparation

The methyl ester of the unknown fatty acid eluted from 16% silver nitrate-silica gel column was hydrolyzed and the free fatty acid was recovered as described in the text book[24]. The acid(60−70 mg) dissolved in diethyl ether(2 ml) was converted to the anhydride derivative by reaction with trifluoroacetic anhydride (0.8 ml) at 50℃ for 1 hr. The excess reagent was removed under a stream of nitrogen. A 10% solution of 3-(hydroxy methyl)-pyridine in tetrahydrofuran (0.8 ml) was added to the anhydride and the mixture was kept at 50℃ for 1 hr. The picolinyl ester was recovered in diethyl ether(20 ml) and hexane(10 ml). The combined extracts were washed with water(5 ml), 1 M hydrochloric acid(3 ml, three times) and water(5 ml, three times). The organic solvents were evaporated under reduced pressure. The picolinyl ester obtained was purified on a Florisil column with the eluting solvent of hexane-diethyl ether(1 : 1, v/v)[25].

IR spectroscopy

IR analysis was performed on a Perkin Elmer 683 Spectrophotometer. The ester was analyzed as a film on a NaCl disk.

Nuclear magnetic resonance(NMR) spectroscopy and mass spectrometry

NMR spectra of the methyl ester were recorded on a Brüker AM 300 NMR spectrometer, and all chemical shifts are reported relative to an internal TMS standard. The picolinyl derivative of the unknown fatty acid was injected directly into the ion source of a Hewlett Packard 5970 Mass Selective Detector operated at an ionization energy of 70 eV.

Results and Discussion

Oil content of the seed is 36.7%. The oil is a reddish fluid which crystallizes in a refrigerator(at 4~5℃), and mainly consists of triglycerides(92.4%), followed by polar lipids(5.9%), sterol(1.2%) and unknown pigments(0.5%).

GLC analysis of the fatty acid methyl esters from this oil revealed a component between C18 : 2ω6 and C18 : 3ω3 with an equvalent chain length(ECL) of 19.3 on a column packed with 15% DEGS on Chromosorb W(Table 1). From the ECL it is postulated that this component is C19 monoenoic ester since the ECL is one unit longer than that of C18 : 1 methyl ester[26]. Major fatty acid components of the seed oil are C18 : 1 (36.1%), C18 : 2(30.9%), C16 : 0(15.1%), C16 : 1(7.6%) and C18 : 0(3.4%). A minor component with an ECL of 17.2 is tentatively identified as dihydromalvalic acid

Table 1. Fatty acid composition of total lipids from *Ternstroemia gymnanthera* Seeds

Fatty Acid	ECL[a]	Area percent
C14 : 0	14.0	0.3
C16 : 0	16.0	15.1
C16 : 1	16.2	7.6
C17-CPAFA[b]	17.2	0.1
C18 : 0	18.0	3.4
C18 : 1	18.3	36.1
C18 : 2	18.9	30.9
LBA[c]	19.3	4.8
C18 : 3	19.7	1.1
C20 : 0	20.0	0.3
C20 : 1	20.3	0.3

[a] ECL : equivalent chain length
[b] Cis-9, 10-methylene hexadechanoic acid (dihydromalvalic acid)
[c] Lactobacillic acid (cis-11, 12-methylene octadecanoic acid)

by running with a standard[12].

HPLC of the triglycerides of the oil seems to substantiate this postulation since triglyceride with odd partition number was observed. This analysis also indicates that the unusual acyl group is a triglyceride constituent (Fig. 1).

When urea is allowed to crystallize in the presence of certain long-chain aliphatic compounds, it forms hexagonal crystals with a channel, into which the aliphatic compounds may fit, if they do not contain functional groups that increase their bulk. The non-urea inclusion fraction obtained which represents 70% of the original esters is enriched in the unusual ester as well as in the unsaturated compounds. This fact indicates that this acid has special functional group(s) on the carbon chain.

For separation of this fatty acid, the methyl esters recovered from the non-urea inclusion fraction were eluted on a silica gel column pregnant with silver nitrate by solvents of hexane-benzene by increasing the ratio of benzene to hexane. The unusual ester was isolated in a relatively pure state (up to 97% on GLC) from the mixture esters with the lowest polar solvent systems of hexane-benzene (99.5/0.5~98.5/1.5, v/v) with which saturated esters can be eluted. It is well known that cyclopropene fatty acids are easily destroyed by silver nitrate chromatography, but cyclopropane and saturated branch-chain esters co-chromatograph with normal saturated straight-chain compounds[27].

The methyl ester of the unknown fatty acid gave

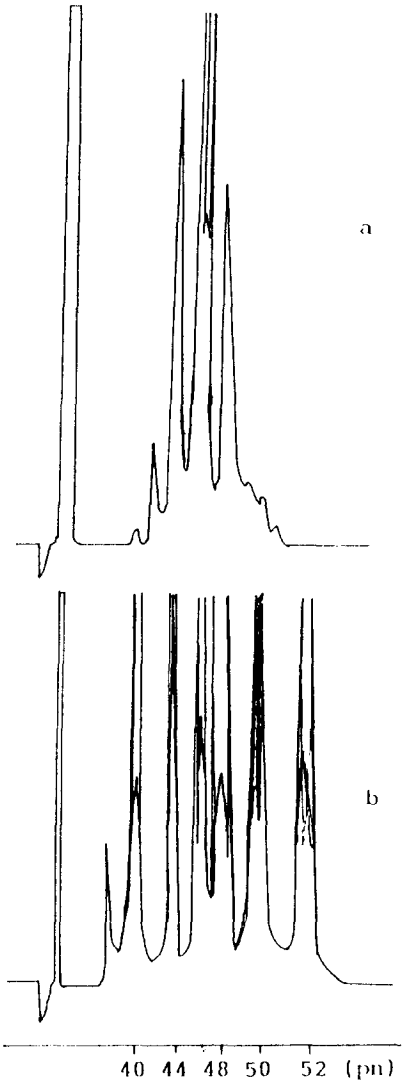

Fig. 1. HPLC of triglycerides from *T. gymnanthera* seed oil
a : sample, b : standard pn : partition number

characteristic absorption bands at 1020 and 3050 cm^{-1}[18,12,28,29], which are indicative of a cyclopropane ring.

From the experimental results obtained, the unusual acid in question seems to have at least one cyclopropane ring in the molecule.

The interpretation of the NMR signals of protons at cyclopropane ring of cis-1, 2-disubstituted cyclopropanic acid had been in disputed and is now apparently settled[18,29,30].

Longone[30] insisted that the peak at 10.3 τ(δ−0.3,

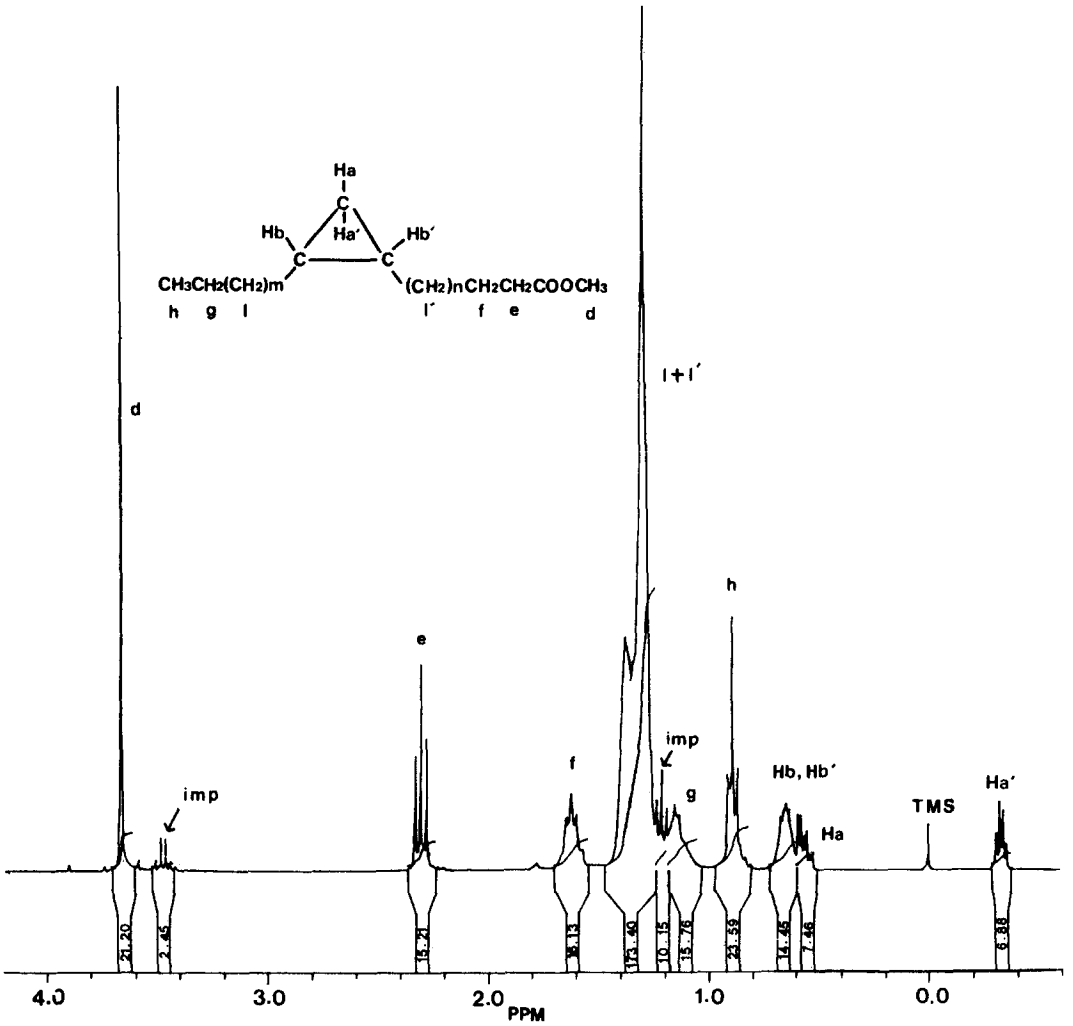

Fig. 2. ^{1}H-NMR spectra of the unknown fatty acid methyl ester from *T. gymnanthera* seed oil

$\delta = 10 - \tau$) is due to the cis proton $H_{a'}$, shielded strongly by long alkyl chains of cis-alkyl substituted cyclopropanes. After examining the NMR signals of 16 isomers of cis-methylene octadecanoate. Christie[29] concluded that for most isomers there was a multiplet at 10.3 τ(proton $H_{a'}$), and a broad band at 9.6 τ(protons H_a, H_b and $H_{b'}$), and for the three isomers with cyclopropane ring closest to the ester group the signal for $H_{a'}$ moved downfield to 10.23 τ(4, 5-isomer), 10.14 τ(3, 4-isomer), and 10.53 τ(2, 3-isomer).

The ^{1}H-NMR spectrum of the unknown fatty acid methyl ester is shown in Fig. 2. The ^{1}H-NMR spectrum was characterized by a quartet $\delta - 0.33$ ppm($J_{a,a'} = -5.0$, $J_{a,b} = 9.0$, 1 proton) assigned to the $H_{a'}$ cis to the two alkyl substituents absent in the trans isomer and by a multiplet at δ 0.56 of the H_a proton which had not been resolved in the previous works[8,29,31], and by a broad band at δ 0.64(2 protons) of the H_b, $H_{b'}$ protons. Signals centered at δ 0.88, 1.13, 1.60 and 2.30 correspond to the protons attached to ω1, ω2, C3 and C2 of the fatty acid chain. A multiplet resonated at δ 1.23~1.33 can be ascribed to the protons of the rest methylene radicals.

The assignment of ^{13}C-NMR signals to carbon atoms of the alkyl chain are listed in Table 2. The methylene and methine ring carbons of the cyclopropanic acid are strongly shielded to resonate at δ 10.84, 15.70 and 15.71 ppm, respectively(Fig. 3-A, B). These results are in good agreement with those of cis-9, 10-methylene octadecanoic acid[32]. The two carbon atoms, c and c'

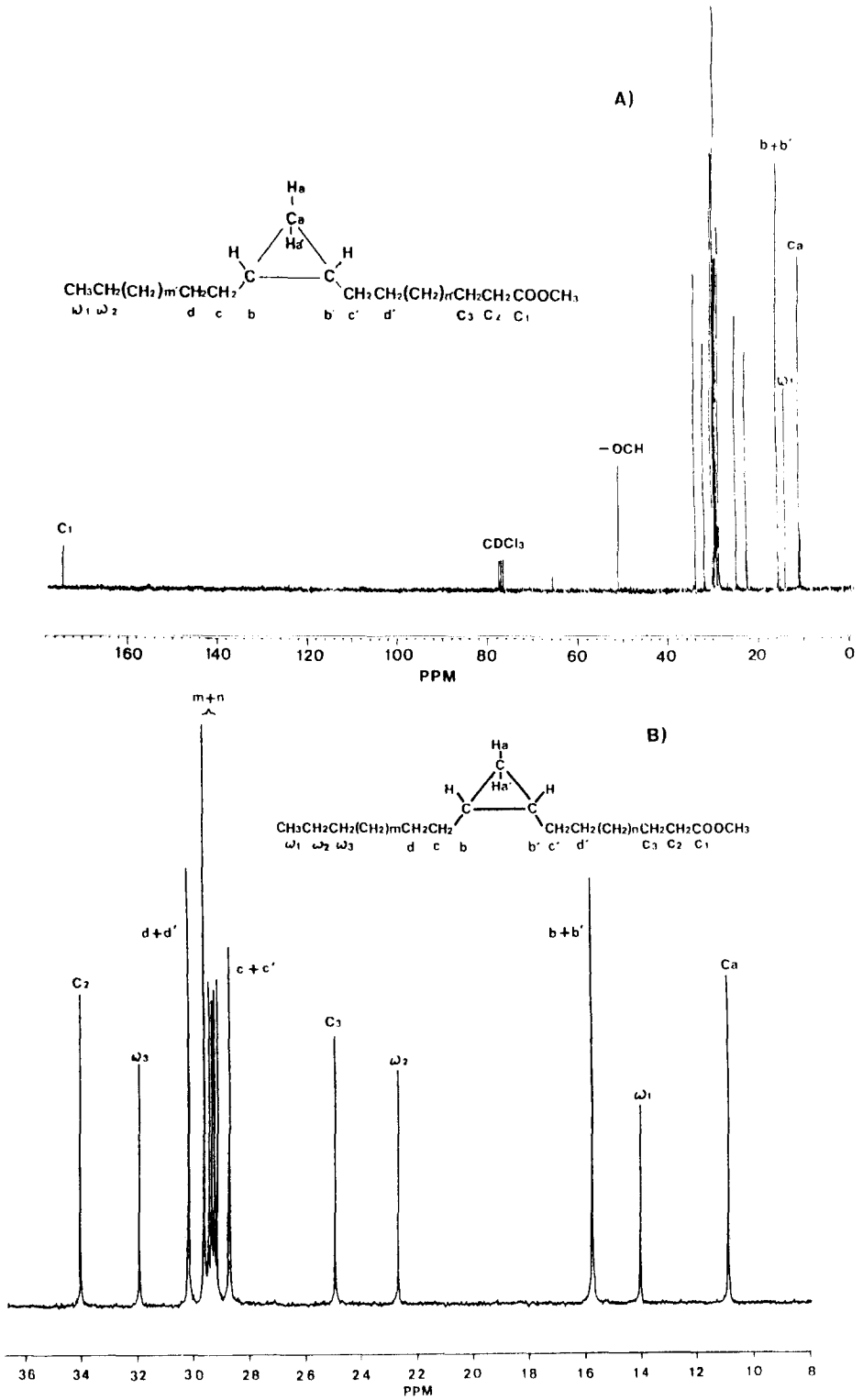

Fig. 3. ^{13}C-NMR spectra of the unknown fatty acid methyl ester from *T. gymnanthera* seed oil
A) Absorption signals from 200 to 0 ppm, B) Enlargement of absorption signals from 50 to 0 ppm

Table 2. Assignment of ^{13}C-NMR signals to each carbon atom of the methyl ester derivative of the unknown fatty acid from *T-gymnanthera*

Chemical shift[a]	Location of C atom
174.11	C_1
51.24	$-OCH_3$
34.01	C_2
31.90	ω_3
30.14	d and d'(probably both carbon signals overlapped)
29.58	
29.41	(m+n) methylene envelopes(not assigned), one or more of the signals may represent two carbon atoms
29.30	
29.21	
29.11	
28.67	
28.66	c and c'
24.90	C_3
22.63	ω_2
15.71	
15.70	b and b'
14.01	ω_1
10.84	a

[a] Chemical shift values in ppm relative to TMS as an internal standard. The spectrum obtained in $CDCl_3$.

(Fig. 3-B), resonated at relatively high field, δ 28.66 and 28.67 ppm. One of the most significant features of the ^{13}C-NMR chemical shift is the so called γ effect observed in alkanes ; an upfield shift is produced by steric compression of hydrogen when 1, 4 carbons(γ carbons) are in gauche conformation. The carbon c and c' to the ring which are in γ-juxtaposition with one another, experience the largest steric shifts in cyclopropanic fatty acids. The signals of two carbons d and d' probably overlap at δ30.14 ppm. The assignments to ω1(14.01), ω2(22.63), ω3(31.90) and C1(174.11), C2(34.01), C3(24.90) carbons of the chain can be predicted from the equation of alkanes proposed by Grant and Paul[33,34]. The chemical shifts at δ29.11, 29.21, 29.30, 29.41 and 29.58 correspond to those of the methylene carbons in the (m+n) envelopes(Fig. 3-B), one or more of which may represent two carbon atoms.

Methyl ester derivatives of cyclopropanic fatty acids are not readily distinguished from those of monoenoic acids with a similar total number of carbon atoms by mass-spectrometry, because on ionization the cyclopro-

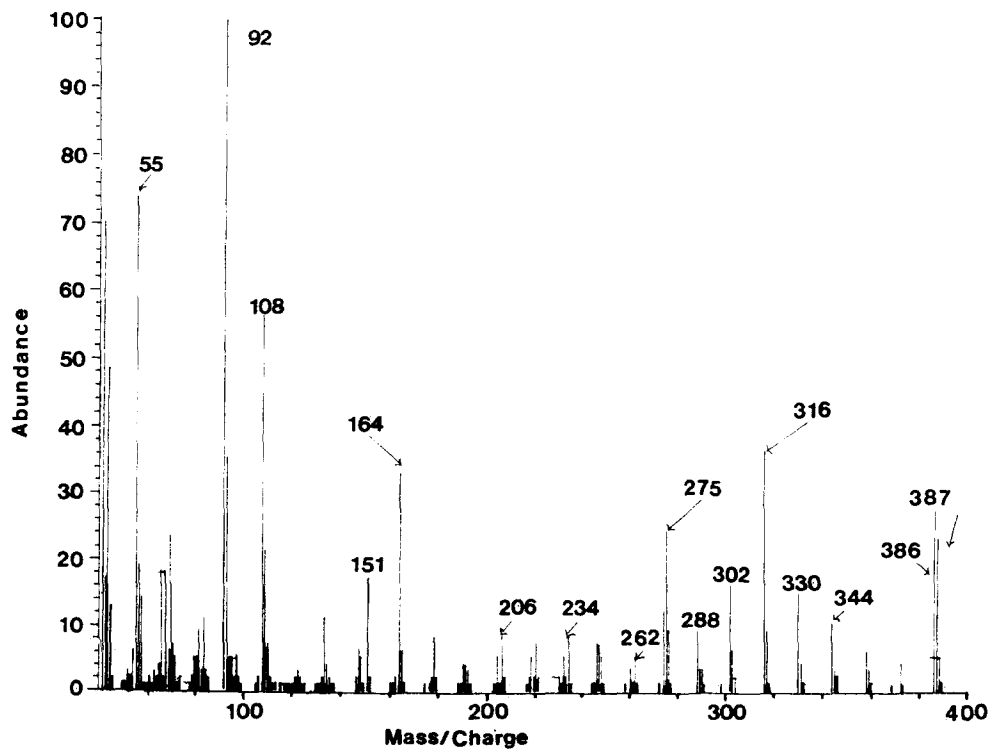

Fig. 4. The mass spectrum of the picolinyl ester derivative of unknown fatty acid

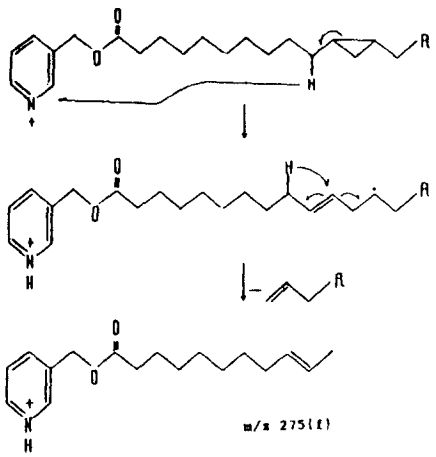

Fig. 5. A scheme for the formation of ion m/z 275(f)

pane ring opens up to form fragments with a double bond[35,36]. Pyrrolidine derivatives of cyclopropanic fatty acids also do not give the diagnostic spectra to locate the cyclopropane ring on the alkyl chain[37-40].

Recentyl, Christie[25] and Harvey[35] stressed the utility of picolinyl esters for locating the cyclopropane ring on the aliphatic chain and for discriminating between cyclopropanic acid and an unsaturated one with the same carbon number.

The spectrum of picolinyl ester of the unknown fatty acid has abundant molecular ion(m/z 387), prominent ions at m/z 92(base peak), 93, 108, 151, 164 and a series of ions produced by radical-induced cleavage at each carbon-carbon bond following random hydrogen abstraction from the chain(Fig. 4).

The most striking feature is the presence of the abundant ion of odd mass(m/z 275) in the spectrum of the fatty acid ester, although all of the major ions in the spectra of saturated or unsaturated straight chain fatty acid derivatives are even masses. The ion at m/z 275(f) is produced by formal cleavage through the cyclopropane ring and can be rationalized by the mechanism shown in Fig. 5. The major ions in the adjacent ion groups at both higher and lower mass are separated by only 13 mass units(m/z 288 and 262) from the ion f. This structural feature locates the position of the cyclopropane ring on the aliphatic chain. Another structural feature serving to disclose the position of the cyclopropane ring is the high abundance of the ion m/z 316 containing three carbon atoms more than the ion f.

The unusual fatty acid found in *Ternstroemia gymnanthera* seed is thus established as cis-11, 12-octadecanoic lactobacillic acid.

The absence of any significant number of acyl groups with the cyclopropene function in *T. gymnanthera* seed oil was demonstrated by a negative Halpen test[41] and by no IR absorption bands at 1850[41] and 1008 cm^{-1}[13].

요 약

차과의 일종인 후피향의 종실유에는 트리글리세리드가 92.4%로 대부분을 차지하고 있었으며, 소량성분으로 극성지질이 5.9%, 스테롤이 1.2%, 색소가 0.5%였다. 지방산 조성을 보면 C18 : 1(36.1%), C18 : 2(30.9%), C16 : 0 (15.1%), C16 : 1(7.6%) 및 C18 : 0(3.4%)가 중요한 성분이었으며, 매우 흥미롭게도 cyclopropane 고리가 탄소 11, 12에 위치한 cis-11, 12-methylene octadecanoic acid가 4.8% 함유되어 있었다.

Acknowledgment

This investigation was supported by the Korea Research Foundation(1990~1991 year). We thank Dr. F. D. Gunstone, Honorary Research Professor at the University of St. Andrews for commenting on the ^{1}H-& ^{13}C-NMR signals of the sample, and Dr. W.W. Christie at the Hannah Research Institute, Ayr, KA6 5HL, Scotland, for measuring the mass spectrum of the picolinyl ester.

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(Received Oct. 12, 1990)