The Separation and Structure Determination of an Eicosatrienoic and an Eicosadienoic Acid in Nagi Seed Oil[*1]

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The presence of an eicosatrienoic acid in the seed oil of Nagi, *Podocarpus Nagi*, has been reported in a previous study by Koyama and Toyama.[1] However, the structure of this acid has not yet been determined; it is only known that this acid, after isomerization with alkali, shows the characteristic absorption of conjugated dienes without showing that of conjugated trienes.

In this study, the presence of an eicosadienoic

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1) Y. Koyama and Y. Toyama, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zassi*), **78**, 1223 (1957). The eicosatrienoic acid of Nagi seed oil was carelessly named "podocarpic acid" by these authors, but this nomenclature should be withdrawn since the same name had already been given to a different resin acid (cf. A. C. Oudemans, *Ber.*, **6**, 1122, 1125 (1873)).

acid besides the eicosatrienoic acid in Nagi seed oil has been indicated by gas-liquid chromatography (GLC). For the separation of these two acids, the methyl ester of the fatty acids of Nagi seed oil was subjected to repeated urea-adduct segregation; a crude eicosatrienoate fraction and a fraction rich in eicosadienoate were thus separated. The crude eicosatrienoate fraction was purified further by column chromatography to give the eicosatrienoate fraction. The fraction rich in eicosadienoate was fractionally distilled to give the eicosadienoate fraction. The positions of the double bonds in the eicosatrienoate and in the eicosadienoate were determined by the oxidative ozonolysis of the respective methyl ester fractions and also, in the case of the eicosatrienoate, by the oxidative ozonolysis of the nonadecatrienyldimethylcarbinol and the hydroxyeicosadienoate derived from it.

Experimental

Properties of the Oil.—The Nagi seed oil used in this study was extracted with acetone from Nagi seeds collected in Nara in late November, 1960. The yield of the oil from 3 kg. of seeds was 270 g. It is a pale yellow liquid with a faint aroma. The characteristics of the oil and of its fatty acids are as follows:

Oil: n_D^{20} 1.4802, acid value 1.2, saponification value 188.0, iodine value 151.4, and nonsaponifiable matter 0.64%.

Fatty acids: n_D^{20} 1.4704, neutralization value 193.5, and iodine value 162.4.

Fatty Acid Composition.—The methyl ester of fatty acids, freed from nonsaponifiable matter, was prepared from the oil in the usual way. It had a refractive index (n_D^{20}) of 1.4634, a saponification value of 186.5 and an iodine value of 153.9, and it was analyzed by GLC. All GLC analyses were performed using a conventional GLC apparatus with a thermoconductivity detector. Column: 225 cm. in length and 6 mm. in diameter, packed with 60~80 mesh Celite 545 coated with 20% diethylene glycol succinate polyester. Temperatures: 230°C for the methyl ester fractions of fatty acids, 200°C for the methyl esters of the dibasic cleavage products of ozonolysis, and 170°C for the benzyl esters of monobasic cleavage products of ozonolysis. Helium flow rate: 80 ml./min. The peaks on the chromatogram were identified by the addition of reference samples or by a comparison of the retention times. With diethylene glycol succinate polyester as the fixed liquid phase, the retention time of the methyl eicosenoate is almost equal to that of the linolenate, whereas with propylene glycol succinate polyester as the fixed liquid phase, the retention times for these two methyl esters are significantly different.[2] The chromatogram of the fatty acid methyl esters of Nagi seed oil using propylene glycol succinate polyester as the fixed liquid phase showed the peak corresponding to the eicosenoate but no peak corresponding to the linolenate. The composition of the fatty acid methyl esters was calculated by the half-band width method. The results are shown in Table I.

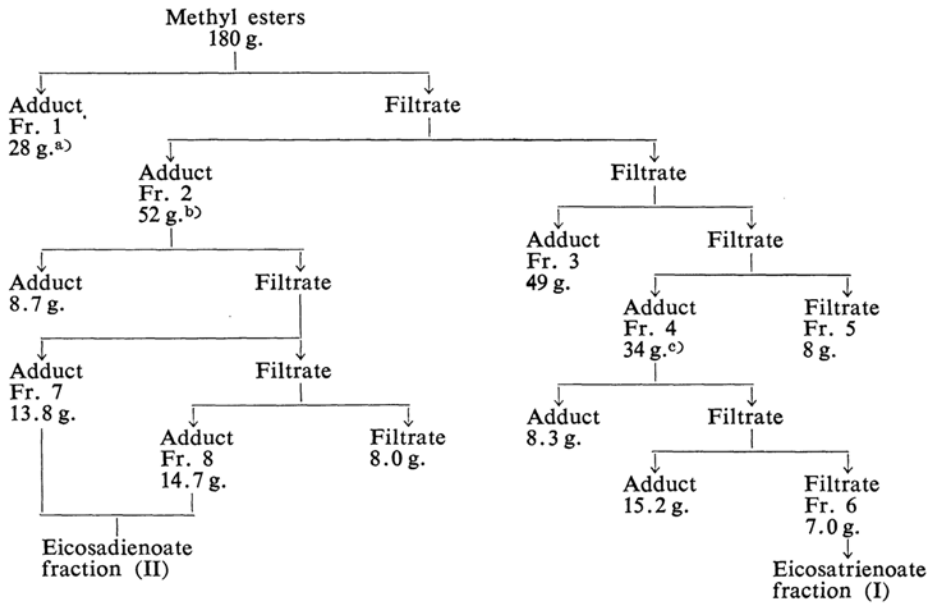

Fig. 1. Scheme of the urea-adduct segregation of methyl esters.

a) The yields for adducts and filtrates are those for methyl ester regenerated therefrom.
b) The succeeding segregation was performed with 46.5 g. of this fraction.
c) The succeeding segregation was performed with 32.0 g. of this fraction.

2) S. Ito and K. Fukuzumi, *J. Chem. Soc. Japan, Ind. Chem. Sec.* (*Kogyo Kagaku Zasshi*), **65**, 1963 (1962).

TABLE I. THE COMPOSITION OF THE FATTY ACID METHYL ESTERS OF NAGI SEED OIL

Methyl ester	Wt. %[a)]
Palmitate	3.8
Stearate	1.9
Oleate	21.4
Linoleate	37.8
Eicosenoate	1.8
Eicosadienoate	9.6
Eicosatrienoate	23.7

a) Corrected. Cf. Ref. 2.

The Urea-adduct Segregation of Methyl Esters. —Methyl esters were prepared by refluxing 200 g. of oil with 600 ml. of methanol and 0.6 g. of metallic sodium for 30 min., and then by extracting them with hexane after the solution had been cooled to room temperature. A 180 g. portion of the methyl esters thus obtained was treated with 180 g. of urea and 1350 ml. of methanol; the crystalline urea-adduct (Fr. 1) was then filtered off at room temperature and washed with hexane. The material recovered from the hexane washing was added to the methanol filtrate and then treated with another 150 g. of urea. The urea-adduct (Fr. 2) was filtered and washed as before. The filtrate was treated further with urea in a similar manner to give urea-adduct fractions (Fr. 3 and Fr. 4) and the final filtrate fraction (Fr. 5) as shown schematically in Fig. 1. The methyl ester fractions regenerated from Frs. 1—5 were analyzed by GLC to ascertain the relative distribution of the eicosatrienoate and of the eicosadienoate in these fractions. It was found that, although Fr. 2 contained more oleate and linoleate than eicosadienoate and eicosatrienoate, the proportion of the amount of eicosadienoate to eicosatrienoate in this fraction was relatively large. Fraction 4 contained eicosatrienoate and linoleate but no eicosadienoate. Fraction 5 appeared to contain almost exclusively the eicosatrienoate as a regular methyl ester, but oxidized and polymerized methyl esters and some other impurities were also concentrated in this fraction. Fractions 2 and 4 were subjected to further fractionation using urea, as Fig. 1 shows.

Methyl Eicosatrienoate and Eicosadienoate. —The methyl ester fraction regenerated from Fr. 6 in Fig. 1 was found by GLC to contain no methyl ester other than the eicosatrienoate. It was purified further by elution chromatography using hexane and hexane-ether (100 : 0.5) as the eluants and an adsorption column, 20.6 cm. in height and 3.0 cm. in diameter, packed with 70 g. of activated silica.

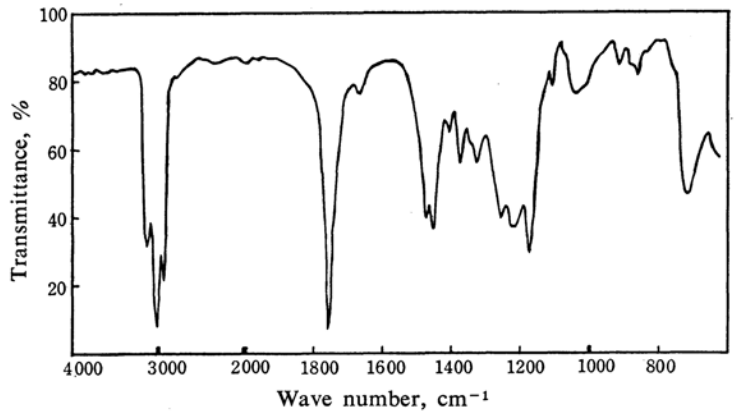

Fig. 2. Infrared spectrum of the fraction I (film on NaCl flats).

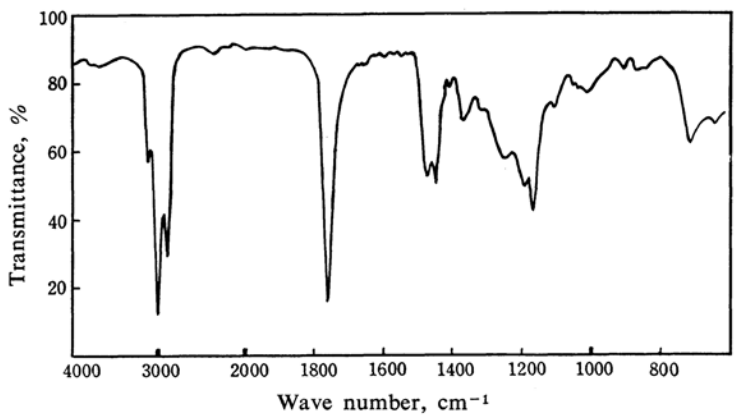

Fig. 3. Infrared spectrum of the fraction II (film on NaCl flats).

When the first and last eluates were removed, 6.1 g. of the methyl eicosatrienoate fraction (I) was obtained.

The methyl ester fractions regenerated from Fr. 7 and Fr. 8 in Fig. 1 were united, and the united fraction was fractionally distilled under 2 mmHg using a fractionating column, 90 cm. in height and 1.8 cm. in diameter, packed with stainless steel rings, 4 mm. in diameter. Thirteen fractions were separately collected. The lower boiling fractions consisted predominantly of the methyl ester of C_{18}-acids. The 12th fraction, 1.2 g., was used as the methyl eicosadienoate fraction (II) in this study. The chromatograms of GLC indicated that, while fraction I consists almost exclusively of methyl eicosatrienoate, fraction II is not a pure methyl eicosadienoate fraction but is contaminated more or less with other unsaturated methyl esters. The characteristics of fractions I and II are shown in Table II.

Table II. The characteristics of methyl eicosatrienoate (I) and eicosadienoate (II)

	I[a]	II[b]
n_D^{32}	1.4660	1.4572
Saponification value	174.9 (175.1)	175.6 (174.0)
Iodine value	235.5 (237.6)	154.7 (157.4)
Hydrogen absorption ml./mmol.	67.2 (67.5)	—

a) The figures in parentheses are the values calculated for $C_{21}H_{36}O_2$.
b) The figures in parentheses are the values calculated for $C_{21}H_{38}O_2$.

The hydrogenation of fractions I and II with palladium black in ether gave methyl arachidate. The infrared spectra of I and II exhibited no absorption at 965 cm^{-1} (Figs. 2 and 3). The ultraviolet absorptions of fractions I and II after isomerization with 21% potassium hydroxide in ethylene glycol at 180°C for 15 min. were measured. After the isomerization both fractions exhibited the characteristic absorptions of conjugated dienes, $k_{233}=85.0$ for fraction I and $k_{233}=71.5$ for fraction II. In the region of conjugated trienes, 268 mμ, fraction II after the isomerization exhibited no absorption, while fraction I after the isomerization exhibited only a minor absorption, one which is possibly to be ascribed to the presence of a small amount of preformed conjugated trienes in fraction I.

The Ozonolysis of Fractions I and II.—A 100 mg. portion of I was ozonized in 3 ml. of methylene chloride at −15°C by passing ozonized oxygen (4.5%) through the solution for 2 hr. at a flow rate of 80 ml. per min. Then, 2 ml. of 30% hydrogen peroxide and 5 ml. of a 5% aqueous solution of potassium hydroxide were added to the ozonide solution, and the mixture was gradually heated to 80°C in the course of 2 hr., during which period nitrogen bubbles were allowed to pass through the mixture. The reaction mixture was then distilled, and the distillate was neutralized with a 0.1 N solution of potassium hydroxide and evaporated to dryness. The residue was taken up with 20 ml. of ethanol, and the ethanol solution was concentrated to a volume of 2~3 ml. Finally, benzyl bromide in an amount equivalent to the amount of potassium hydroxide required to neutralize the distillate was added to the concentrated ethanol solution, and the mixture was refluxed for 20 min. After the mixture had cooled, the extraction of the reaction mixture with ether gave 94 mg. of benzyl esters. Benzyl formate, acetate and caproate were identified by GLC. Another 100 mg. portion of I was ozonized in the same way, and the ozonide was decomposed by gradually heating the ozonide solution with 2 ml. of 30% hydrogen peroxide and 5 ml. of water up to 90°C in the course of 3 hr., during which period nitrogen bubbles were allowed to pass through the mixture. The reaction product was then evaporated in vacuo for the removal of the bulk of the water, and the residue was taken up with ether. The ether solution was dried over anhydrous sodium sulfate. After distilling off the ether, the residue was esterified with sulfuric acid and methanol to give methyl esters of dibasic acids (68 mg.), in which methyl glutarate and adipate were identified as the main components by GLC.

A 100 mg. portion of II was ozonized, and the ozonide was treated in the same way as has been described for I. From the distillate, benzyl esters were obtained in a yield of 92 mg. Benzyl formate, acetate and caproate were identified by GLC. The distillation residue was extracted with ether, and the ether extract was converted to methyl esters with sulfuric acid and methanol. Yield: 63 mg. Methyl undecanedioate was identified as the main component by GLC.

The Preparation and Ozonolysis of Nonadecatrienyldimethylcarbinol. — A solution of 400 mg. of fraction I in 10 ml. of ether was added to a Grignard reagent prepared from 400 mg. of methyl iodide, 70 mg. of magnesium turnings, and 5 ml. of ether in the usual way. The mixture was then refluxed for 1 hr., and the reaction product was hydrolyzed by the successive addition of water and of dilute hydrochloric acid. The product was obtained by extraction with ether in a yield of 402 mg. It could be recognized as nonadecatrienyldimethylcarbinol by the pattern of the preparation and by the infrared spectrum shown in Fig. 4. This tertiary, unsaturated alcohol was subjected to ozonolysis, and the ozonide was treated in the same way as has been described for fraction I. From 200 mg. of alcohol 97 mg. of methyl esters of dibasic acids were obtained; in these esters methyl adipate and succinate, together with glutarate, were identified by GLC.

Methyl Hydroxyeicosadienoate.—A 4.0 g. portion of the fatty acid obtained from fraction I by saponification was dissolved in 30 ml. of ether and brominated at −15°C, yielding 1.35 g. of an ether-insoluble bromide which had a m. p. of 152~152.5°C (reported,[1] 152~152.5°C) after recrystallization from carbon tetrachloride and which was regarded as hexabromoarachidic acid. A solution of 1.10 g. of recrystallized hexabromide in 80 ml. of ethanol-ether (1 : 1) was neutralized with a solution of potassium hydroxide and refluxed for 4 hr. After 1.5 to 2 hr. from the beginning of this refluxing, potassium bromide was formed and precipitated

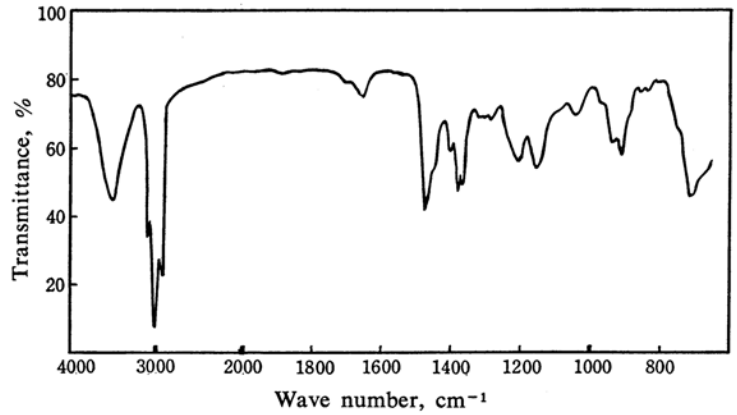

Fig. 4. Infrared spectrum of nonadecatrienyldimethylcarbinol derived from the fraction I (film on NaCl flats).

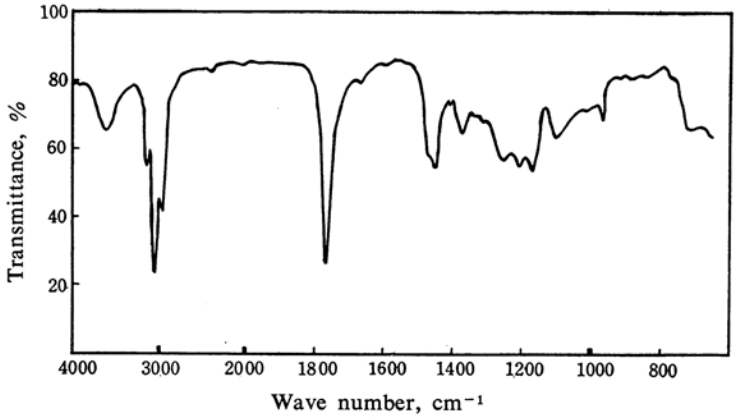

Fig. 5. Infrared spectrum of methyl hydroxyeicosadienoate derived from the fraction I (film on NaCl flats).

from the solution. The reaction product was extracted with ether, and the ether solution was washed with alkali to remove the unchanged hexabromoarachidic acid. After distilling off the ether from the ethereal solution, 634 mg. of a neutral substance (bromolactone) was obtained. Yield: 64% of theory. To 625 mg. of this substance were added 500 mg. of zinc powder and 13 ml. of methanol, and the mixture was refluxed for 1 hr. Then, 250 mg. of zinc powder, 7 ml. of methanol and 1 ml. of concentrated sulfuric acid were added, and the mixture was refluxed further for 1 hr. The reaction product obtained by extraction with ether was found to be free from bromine. Yield: 334 mg. The debromination product could be recognized as methyl hydroxyeicosadienoate by the pattern of the preparation and by the infrared spectrum (Fig. 5). It had a refractive index (n_D^{31}) of 1.4772 and an iodine value of 155.6 (calcd. for $C_{21}H_{38}O_3$: 149.9). The isomerization product obtained under the same conditions as have been described for fractions I and II exhibited the characteristic absorption of conjugated dienes, $k_{233}=70.8$.

A solution of 100 mg. of the debromination product in 3 ml. of methylene chloride was ozonized at $-15°C$ for 1.5 hr. Then, 10 ml. of a 5% solution of potassium hydroxide and 500 mg. of silver oxide were added to the ozonide solution, and the mixture was heated at 70°C for 3 hr. during which period nitrogen bubbles were allowed to pass through the mixture. The alkaline solution was filtered. The filtrate was acidified with diluted sulfuric acid and then distilled. The distillate was subjected to a procedure similar to that described for fractions I and II to give 103 mg. of benzyl esters. Benzyl formate, acetate and caproate were identified by GLC.

Discussion

Both methyl eicosatrienoate (I) and eicosadienoate (II), upon hydrogenation, give methyl arachidate. Accordingly, I and II have no branched chain. The ethylenic linkages in I and II are of the all-cis form, since the infrared spectra of I and II (Figs. 2 and 3) show no absorption at 965 cm^{-1}. After isomerization with alkali, I and II show the characteristic absorption of conjugated dienes at 233 mμ.,

indicating that both I and II have the $=CHCH_2CH=$ group of the divinylmethane type.

The ozonolysis of II gives formic, acetic and caproic acids as the monobasic acids and *n*-undecanedioic acid as the dibasic acid. Among these cleavage products, formic and acetic acids may be regarded as secondary decomposition products of malonic acid or of its precursor resulting from the $=CHCH_2CH=$ group. Accordingly, $CH_3(CH_2)_4CH=$ and $=CH(CH_2)_9COOCH_3$ are the terminal groups of II.

From these results, the free fatty acid corresponding to II may be concluded to be all-*cis*-11, 14-eicosadienoic acid.

The ozonolysis of I gives formic, acetic and caproic acids as the monobasic acids, and adipic and glutaric acids as the dibasic acids. Formic and acetic acids are considered to be secondary decomposition products originating from the $=CHCH_2CH=$ group. Hence, the free fatty acid of I is found to have the terminal group $CH_3(CH_2)_4CH=$ on the methyl side and either $=CH(CH_2)_4COOH$ or $=CH(CH_2)_3COOH$ as the terminal group on the carboxyl side. However, the oxidative ozonolysis of the nonadecatrienyldimethylcarbinol derived from I gives succinic acid in a large proportion, together with glutaric and adipic acids as the dibasic cleavage products. Hence, the terminal group on the carboxyl side should be $=CH(CH_2)_3COOH$. As for the arrangement of the two intermediate groups, $=CHCH_2CH=$ and $=CH(CH_2)_4CH=$, the results of the ozonolysis of hydroxydienoate (I') derived from I indicate that the $=CHCH_2-CH=$ group is adjacent to the terminal group, $CH_3(CH_2)_4CH=$, since the hydroxydienoate contains an alkali conjugatable diene, $=CHCH_2-CH=$, and since on ozonolysis it gives formic, acetic and caproic acids as the monobasic cleavage products. On the basis of these findings, the free fatty acid corresponding to I may be concluded to be all-*cis*-5, 11, 14-eicosatrienoic acid. The hydroxydienoate I' is methyl 5-hydroxy-11, 14-eicosadienoate.

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