報 文

Cis-5-Olefinic Nonmethylene-Interrupted Fatty Acids in Lipids of Seeds, Arils and Leaves of Japanese Yew

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Fatty acids from the lipids of the Japanese yew *Taxus cuspidata* seeds collected at various districts all contained rich amounts of *cis*-5, *cis*-9-octadecadienoic acid (5,9-18:2) (16.1~18.9%) with small amounts of 5,9,12-18:3 (1.8~3.4%) and 5,11,14-20:3 (1.0~2.8%) as *cis*-5-olefinic nonmethylene-interrupted (NMI) acids. On the other hand, fatty acids from the neutral lipids of the arils and leaves contained appreciable amounts of 5,11,14-20:3 (leaves 8.1% and arils 1.2%) and 5,11,14,17-20:4 (leaves 6.2% and arils 1.5%), but 5,9-18:2 was not detected or detected only in a trace amount in them. Fatty acids from the galactolipids of the leaves also contained small amounts of the NMI acids (5,9,12,15-18:4 0.3%, 5,11,14-20:3 0.4%, 5,11,14,17-20:4 1.8%), in addition to a large amount of linolenic acid (72.8%). The 5-olefinic NMI acids were generally more rich in neutral lipids than in polar lipids. The yew seeds showed nearly the same fatty acid composition during maturation.

1 Introduction

The occurrence of unusual monoenoic and nonmethylene-interrupted (NMI) polyenoic fatty acids with a 5:6 double bond has been found in the plants belonging to some families¹⁾. Taxaceae is one of them, and the NMI polyenoic acids have been found in the tissues of two species of it, the seeds²⁾ and leaves³⁾ of the yew *Taxus baccata* collected in USA²⁾ and Scotland³⁾, and the seeds of *Torreya nucifera* collected in Japan⁴⁾ and Korea⁵⁾. The fatty acids from *T. baccata* seed oil contained a noticeable amount of *cis*-5, *cis*-9-octadecadienoic acid (5,9-18:2) with small amounts of 5,11-20:2 and 5,11,14-20:3²⁾. On the other hand, the fatty acids from *T. baccata* leaf lipids mainly contained 5,11,14-20:3 and 5,11,14,17-20:4 as the NMI acids³⁾. *T. nucifera* seed oil contained only one NMI acid, 5,11,14-20:3^{4),5)}.

The present work reports the compositions of the fatty acids from the lipids in the seeds, arils and leaves of the Japanese yew *Taxus cuspidata*.

2 Experimental

2.1 Extraction, Fractionation and

Methanolysis of Lipids

Fruits collected from yew trees were immediately separated to seeds and arils, and lipids were extracted by the method of Bligh and Dyer⁶⁾ after several days. Total lipids were separated to neutral and polar lipids by column chromatography using silicic acid (Silica Gel 60, Merck) and chloroform, acetone and methanol for development. Lipids were converted to methyl esters by transesterification with 2% sodium methoxide in methanol by refluxing for 20 min.

2.2 Chromatography

Gas-liquid chromatography (GLC) of methyl esters was carried out with glass capillary columns (60 or 70 m×0.28 mm i.d.) coated with Silar 5 CP⁷⁾. The carrier gas was nitrogen at 1 or 0.4 ml/min. The splitting ratio was 1/25 or 1/200. The column temperature was 170 or 190°C isothermally. The detector (FID) and injector temperatures were maintained at 230°C.

Thin-layer chromatography (TLC) of methyl esters was carried out on 15% silver nitrate impregnated silicic acid (Silica Gel G, Merck) plates (20×20 cm, 0.5 mm thick) using ethyl acetate-hexane (19:1 vol/vol) for development. Bands were located under UV after spraying with 2',7'-dichlorofluorescein ethanol solution

and were recovered by extraction with ether.

2.3 Reductive Ozonolysis

Reductive ozonolysis was carried out by the ordinary method using triphenylphosphine⁹⁾. One ml of a dichloromethane solution containing 10 mg methyl esters was cooled in acetone-dry ice bath and ozone-air mixture was bubbled through at 30 ml/min. After bubbling for a few minutes, a few crystals of triphenylphosphine was added to the solution, and the reaction products were analyzed by GLC. The GLC columns were glass tubes (1.5 m × 3 mm i.d.) packed with 5% Silar 10 C on 100~120 mesh Gas Chrom Q. The column temperature was programmed from 80 to 270°C at 2°C/min. The detector (FID) and injector temperatures were maintained at 300°C. The carrier gas was nitrogen at 40 ml/min.

2.4 Nuclear Magnetic Resonance (NMR)

A JEOL FX-200 spectrometer (Nippon Den-shi Co., Tokyo) in Fourier transform mode at 199.50 MHz for ¹H-NMR and 25.00 MHz for ¹³C-NMR was used to obtain NMR spectra of methyl esters in deuteriochloroform.

2.5 Mass Spectra (MS)

MS of the pyrrolidides of unsaturated fatty acids were taken for determination of the double bond positions with a JEOL mass spectrometer JMS-D 300 operated at an ionization potential 70 eV and ion source temperature 170°C.

Fatty acid pyrrolidides were prepared on a microscale⁹⁾. Methyl esters (10 mg) were dissolved in freshly distilled pyrrolidine (1 ml) and acetic acid (0.1 ml). The mixture was kept at 100°C in a screw-cap centrifuge tube for 30 min under nitrogen. After addition of water (3 ml), the products were extracted twice with dichloromethane (each 3 ml). The solution was washed with dilute hydrochloric acid, and then with water until neutral. After drying over anhydrous magnesium sulfate, the solvent was removed by nitrogen jet. Conversion to pyrrolidides was checked by GLC using glass columns (1.5 m × 3 mm i.d.) packed with 3% Silar 10 C on 100~120 mesh Gas Chrom Q. The column temperature was programmed from 60 to 260°C at 2°C/min.

2.6 Partial Hydrogenation with Hydrazine

Partial hydrogenation of unsaturated fatty

acids was carried out according to the method described previously¹⁰⁾. A mixture of 10 ml of 10% (vol/vol) hydrazine hydrate in methanol and 10 mg fatty acids was stirred in air at 50°C for 30 min by a magnetic stirrer. The products were extracted with hexane and converted to methyl esters with 7% boron trifluoride in methanol by refluxing for 30 min. Methyl esters were analyzed by the GLC using glass capillary columns under the conditions described in 2.2.

3 Results and Discussion

3.1 Unusual Fatty Acids

In GLC of the methyl esters from the lipids of seeds, arils and leaves of *T. cuspidata*, 26 peaks have been observed on the chromatograms (Fig.-1). In them, 20 peaks were identified

Fig.-1 Gas chromatograms of methyl esters of fatty acids from Japanese yew *T. cuspidata* seed lipids on a glass capillary column coated with Silar 5 CP.

as fatty acids having usual structures by comparison of the retention times with reference specimens⁷⁾, but 6 peaks (peaks 14, 16, 18, 21, 23 and 25) showed unusual retention data.

3.2 Identification of NMI acids

GLC peaks 21, 23 and 25 were identified as 5,11-20:2, 5,11,14-20:3 and 5,11,14,17-

20 : 4, respectively, by the agreement of their retention data with those of the reference specimens obtained from the lipids of sea urchins¹¹⁾, *Podocarpus nagi* seeds¹⁰⁾ and *Cedrus deodra* seeds¹²⁾. GLC peak 18 was tentatively identified as 5,9,12,15-18 : 4 from the retention data of NMI and usual fatty acid methyl esters¹²⁾.

On argentation TLC plates, R_f values of the methyl esters from *T. cuspidata* lipids decreased in the following order : 9-18 : 1 and 11-20 : 1, 9,12-18 : 2 and 11,14-20 : 2, GLC peaks 14 and 21 (I), 9,12,15-18 : 3, GLC peaks 16 and 23 (II), and GLC peaks 18 and 25. By the TLC, the component of GLC peak 14 (TLC fraction I) was isolated from the neutral lipids of the seeds in purity of more than 96%. GLC of the ozonolysis products of this component showed the peaks of C_5 aldehydic-acid ester (28.2%), C_9 aldehyde (60.6%) and C_4 dialdehyde (11.2%). These products indicate double bonds in positions 5 and 9.

aldehyde dialdehyde aldehydic acid ester

GLC of the ozonolysis products of TLC fraction II from the neutral lipids of the seeds showed C_5 aldehydic-acid ester (26.0%), C_9 aldehydic acid ester (16.0%), C_6 aldehyde (57.1%) and a trace amount of C_4 dialdehyde. These products indicate double bonds in positions 5, 9, and 12. The C_9 aldehydic acid ester was produced from 9,12,15-18 : 3 contaminated in this fraction.

The retention data of GLC peak 16 were in agreement with those of 5,9,12-18 : 3 obtained from *Pinus koraiensis*¹²⁾.

Reference specimens for the GLC identification of the ozonolysis products were prepared by the ozonolysis of methyl ester of 5,11,14-20 : 3 from *P. nagi* seed lipids¹⁰⁾ for C_5 aldehydic acid ester, 1,5-cyclooctadiene for C_4

Fig.-2 Mass spectrum of pyrrolidide derivative of GLC peak 14 (5,9-18 : 2).

dialdehyde and methyl oleate for C_9 aldehydic-acid ester. Reagent grade aldehydes (C_6 and C_9) were also used for the identification.

MS of the pyrrolidide derivative of GLC peak 14 is shown in **Fig.-2**. The irregular ion fragments for each carbon atom were as follows : C_4 (m/e 140, intensity 0.8% to parent fragment) - C_5 (m/e 152, 0.4%) and C_8 (m/e 194, 0.5%) - C_9 (m/e 206, 0.3%). The molecular ion fragment appeared at m/e 333 (4.8%). These fragments indicate the 5,9-18 : 2 structure for GLC peak 14.

IR and UV spectra of each TLC fraction showed no absorptions of *trans* and conjugated double bonds, respectively. ^1H and ^{13}C -NMR spectra of GLC peak 14 are shown in **Fig.-3**. The carbon chemical shifts (ppm) are as follows :

Figures in ^{13}C -NMR spectrum indicate the locant of carbon atoms.

Fig.-3 NMR spectra of GLC peak 14 (5,9-18 : 2)

The presence of 5 : 6 double bond is indicated by the difference in chemical shifts for two allylic carbon atoms. The differential shifts of monoenoic acids were 4.30 for 4 : 5, 0.70 for 5 : 6, 0.40 for 6 : 7, 0.25 for 7 : 8, and 0.10 for 8 : 9 and 9 : 10¹³⁾. Therefore, the difference 0.71 of the chemical shifts 27.39 and 26.68 in our spectrum indicates that the first double bond is in the position 5. The chemical shift 32.00 (C₁₆) means that a second double bond is in the positions 8, 9, or 10¹³⁾. The ¹H-NMR

spectrum (Fig.-3) had no absorptions at 2.77~2.81 ppm for the CH₂ hydrogen of =CHCH₂CH=. The absorptions at 5.35~5.37 ppm (intensity 4H) show the CH hydrogen of -CH=CH-. These results support the 5, 9-18 : 2 structure for GLC peak 14.

GLC of the partial hydrogenation products of GLC peak 14 showed the following composition : 18 : 0 15.4%, 5-18 : 28.9%, 9-18 : 1 20.1% and GLC peak 14 35.6%. Complete separation was obtained between 5- and 9-18 : 1 peaks.

Table-1 Fatty acid composition of yew lipids*

Place Date Part Lipid class	Sapporo Sep. 18 1979 Seed T	Nanae Oct. 18 1979 Seed T	Hiroshi- ma Oct. 6 1980 Seed T	Hakodate						
				Oct. 25 1978				Oct. 1980		
				Seed		Aril		Leaf		
				N	P	N	P	N	G	P
Lipid%	—	20.8	15.5	16.3	1.69	0.21	0.22	0.95	1.48	0.76
12 : 0	—	—	—	—	0.04	0.02	0.05	—	Tr.	Tr.
13 : 0	—	—	—	—	0.54	—	—	—	—	Tr.
14 : 0	0.09	0.06	0.10	—	0.70	4.37	2.06	3.16	0.35	Tr.
15 : 0	—	—	—	—	0.07	0.23	0.24	0.38	—	Tr.
16 : 0	3.07	2.99	3.40	2.65	22.82	15.93	30.45	8.59	2.87	30.62
16 : 1 <i>n</i> -9	0.04	—	0.08	Tr.	0.14	0.27	0.12	0.49	0.11	Tr.
<i>n</i> -7	0.08	0.06	0.07	0.04	0.07	1.08	0.75	0.22	0.15	Tr.
16 : 2 <i>n</i> -6	Tr.	Tr.	—	Tr.	0.07	Tr.	0.24	Tr.	0.79	0.17
17 : 0	Tr.	Tr.	0.04	Tr.	0.38	Tr.	0.20	Tr.	Tr.	0.40
16 : 3 <i>n</i> -3	Tr.	Tr.	—	Tr.	1.23	0.54	0.67	1.61	11.67	0.56
18 : 0	1.16	1.40	1.42	0.80	3.98	1.40	1.72	1.19	0.18	1.17
18 : 1 <i>n</i> -9	37.85	35.26	48.34	37.10	31.17	15.52	6.79	17.72	1.52	6.54
<i>n</i> -7	0.25	0.27	0.07	—	—	0.23	1.38	Tr.	Tr.	0.28
18 : 2 5, 9	18.47	18.93	17.89	16.06	5.26	—	—	Tr.	Tr.	—
<i>n</i> -6	30.42	32.45	23.32	32.96	10.15	28.10	21.83	14.36	4.67	8.07
18 : 3 5, 9, 12	2.83	2.94	1.75	3.37	0.30	0.13	0.13	Tr.	Tr.	—
<i>n</i> -3	1.46	1.33	1.07	1.58	17.43	28.56	27.05	31.18	72.83	48.56
18 : 4 5, 9, 12, 15	0.16	0.16	0.08	0.24	Tr.	0.18	—	0.80	0.29	—
20 : 0	Tr.	Tr.	Tr.	Tr.	0.66	0.38	1.26	1.66	1.19	Tr.
20 : 1 <i>n</i> -9	1.55	1.47	0.97	1.44	1.00	0.37	1.41	1.29	0.38	Tr.
20 : 2 5, 11	Tr.	0.16	0.08	Tr.	0.38	Tr.	—	1.13	Tr.	Tr.
<i>n</i> -6	0.61	0.72	0.34	0.95	0.65	—	—	—	—	—
20 : 3 5, 11, 14	1.96	1.80	0.98	2.81	1.42	1.18	0.68	8.10	0.36	1.89
20 : 4 5, 11, 14, 17	—	—	—	—	0.54	1.51	1.28	6.24	1.79	1.74
22 : 0	—	—	—	—	1.00	—	1.68	1.88	0.88	—

*T; Total lipids, N : Neutral lipids, P : Polar lipids, and G : Galactolipids. Yields were obtained without drying of the seeds. Nanae is in the suburbs of Hakodate. A trace amount of 20 : 3 *n*-3 was detected additionally.

The peaks were identified by mixed injection with a reference specimen containing 5-18 : 1 from sea urchin lipids¹¹⁾. These results also give an evidence for the 5,9-18 : 2 structure.

3.3 Fatty Acid Composition

The fatty acid composition of the lipids from the seeds, arils and leaves of the Japanese yew *T. cuspidata* are shown in **Table-1**. The fatty acid composition of the seeds collected at the different places in northern Japan resembled one another. The content of 5,9-18 : 2 in the seed lipids (16.1~18.9%) was significantly higher than that of the American yew *T. baccata* seed lipids (12.2%)²⁾. The 5,9-18 : 2 was also rich in the seeds collected at Hiroshima in southern Japan. This NMI acid, however, was not detected or detected in a trace amount from the aril and leaf lipids (**Table-1**). *T. cuspidata* is one of the most popular garden trees in northern Japan and its seeds are easily available in fairly large amounts. *T. cuspidata* seed lipids, therefore, are a favorable source for preparation of 5,9-18 : 2.

The 5-olefinic C₁₈ NMI acids found in *T. cuspidata* lipids seem to be formed from usual unsaturated acyl moieties by the 5-desaturase as follows:

The conversion to 5,9-18 : 2 from 9-18 : 1 occurs exclusively in the seeds. The 5-olefinic C₂₀ NMI acids are more rich in the arils and leaves than in the seeds (**Table-1**). Therefore, the 5-desaturase in the arils and leaves seems to selectively dehydrogenate 11,14-20 : 2 and 11,14,17-20 : 3 produced by C₂ elongation of 9,12-18 : 2 and 9,12,15-18 : 3, respectively, as follows:

It is noteworthy that the 5-desaturase in each part of *T. cuspidata* has different selectivities depending on the carbon number and number of double bonds of the substrates. It is generally known that lipids are not transported among each part of plants¹⁴⁾. The distinctive difference of the distribution of the NMI acids among each part of *T. cuspidata* supports this common idea. The first report concerning biosynthesis of 5-olefinic acids in oilseeds was presented recently. The 5-olefinic acids were formed by incubation of ¹⁴C-labeled substrates with the

Table-2 Fatty acid composition of the lipids from maturing seeds of *T. cuspidata*.

Date	Sep. 22, 1979			Sep. 1, 1980	Aug. 10, 1980
Color of fruits Lipid %	Red —	Yellow 20.6	Green 16.9	Green 7.00	Green 2.35
14 : 0	0.04	—	—	0.03	0.43
16 : 0	2.53	2.60	2.71	2.92	5.71
16 : 1 <i>n</i> -9	0.01	0.03	0.01	Tr.	0.11
<i>n</i> -7	Tr.	0.02	0.03	Tr.	0.06
16 : 2	Tr.	Tr.	Tr.	Tr.	0.21
18 : 0	1.41	1.41	1.54	1.04	1.13
18 : 1 <i>n</i> -9	38.12	37.94	38.08	33.65	27.17
<i>n</i> -7	Tr.	Tr.	Tr.	Tr.	Tr.
18 : 2 5,9	17.74	17.76	17.08	17.79	12.24
<i>n</i> -6	31.51	31.84	33.13	35.30	38.46
18 : 3 5,9,12	2.92	2.90	2.77	3.56	3.43
<i>n</i> -3	1.11	1.24	1.22	2.11	5.81
18 : 4 5,9,12,15	0.17	0.15	0.24	0.25	0.46
20 : 1 <i>n</i> -11	1.45	1.51	1.40	1.15	0.96
20 : 2 5,11	0.08	0.09	0.06	—	0.10
<i>n</i> -6	0.78	0.65	0.39	0.58	0.65
20 : 3 5,11,14	2.13	1.79	1.43	1.62	2.54
20 : 4 5,11,14,17	—	0.07	Tr.	—	0.53

developing seed slices or with a cell-free homogenate of the seeds of meadowfoam *Limnanthes alba*¹⁵⁾. Probably, *T. cuspidata* also contains 5-desaturase.

Fatty acid composition during the maturation of *T. cuspidata* seeds are shown in **Table-2**. Although the lipid contents increased remarkably as the seeds mature, the fatty acid compositions did not change, except for the earlier period of maturation having small amounts of lipids. Probably, this is due to the rapid accumulation of the acyl groups into triacylglycerols which are deposited in the seeds in inactive form. Thus, the fatty acids biosynthesized in the seeds showed nearly the same composition throughout maturation.

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日本産いちい種子, 仮種皮, 葉脂質中の *cis*-5 NMI 脂肪酸

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各地で採集した日本産いちい種子脂質はすべて著量の 5,9-18:2 (16.0~18.4%) と少量の 5,9,12-18:3 (1.7%3.4%) と 5,11,14-20:3 (1.0~2.8%) を *cis*-5 NMI 脂肪酸として含有した。これと対照的に仮種皮, 葉中の中性脂質からの脂肪酸はかなりの量の 5,11,14-20:3 (葉 8.0%, 仮種皮 1.2%) と 5,11,14,17-20:4 (葉 6.2%, 仮種皮 1.5%) を含有したが 5,9-18:2 は検出されたとしても微量であった。葉糖脂質からの脂肪酸は主にリノレン酸 (72.8%) を含有し, その他少量の他の NMI 酸 (5,9,12,15-18:4, 0.3%, 5,11,14-20:3, 0.4%, 5,11,14,17-20:4 1.8%) を含有した。NMI ポリエン酸は一般に極性脂質より中性脂質に多かった。いちい種子脂質の脂肪酸は種子の成熟の過程でほとんど同じ組成を示した。