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Studies on Bisflavones in the Leaves of *Podocarpus macrophylla* and *P. nagi*[1]

Hiroshi Miura, Takahide Kihara, and Nobusuke Kawano

Faculty of Pharmaceutical Sciences, Nagasaki University[2]

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From the leaves of *Podocarpus macrophylla* were separated hinokiflavone (III), neocryptomerin (VI), sciadopitysin (VII), podocarpusflavone A, $C_{31}H_{20}O_{10}$ (VIII), and podocarpusflavone B, $C_{32}H_{22}O_{10}$ (IX). The latter two of them are new compounds and their structures were deduced by comparison of the chemical shifts of the methyl protons of their acetates. VIII and isoginkgetin (XII) were isolated from the leaves of *P. nagi*. VII was newly isolated from the leaves of *Ginkgo biloba*.

It has been reported by Sawada[3] that kayaflavone (I) is contained in the leaves of *Podocarpus macrophylla* D. Don (Podocarpaceae, inumaki in Japanese) and *P. nagi* Zoll. et Moritz (nagi in Japanese) in his studies on the taxonomic distribution of bisflavones in gymnosperms. However, further studies by thin-layer chromatography (hereafter, TLC) disclosed[4] that they contain several bisflavones. In this paper, we wish to report new bisflavones from these plants.

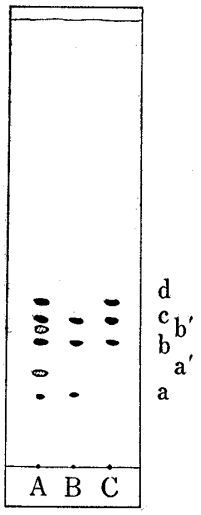


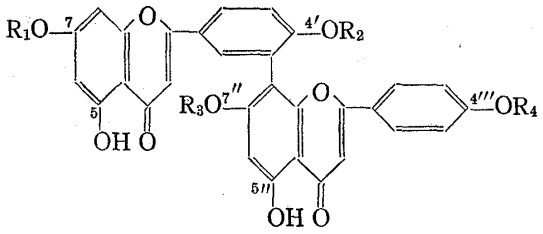
Fig. 1. TLC of Impure Bisflavone Mixtures before Refluxing with Ethanol

A: *Podocarpus macrophylla*
B: *P. nagi*
C: *Ginkgo biloba*

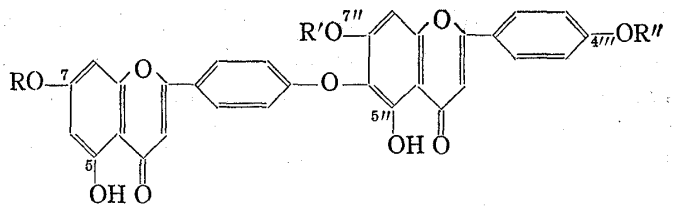
a: amentoflavone (0.16)
a′: hinokiflavone (0.21)
b: a. monomethyl ether (0.28)
b′: h. monomethyl ether (0.31)
c: a. dimethyl ether (0.33)
d: a. trimethyl ether (0.37)

When a solution of ferric chloride is sprayed on a TLC plate, amentoflavone (II) and its methyl ethers show greenish brown spots while hinokiflavone (III)[5] and its methyl ethers exhibit pale brown spots. By this method of TLC a crude bisflavone mixture obtained from *P. macrophylla* leaves proved to be a mixture of amentoflavone (trace), its mono, di, and trimethyl ethers, hinokiflavone, and its monomethyl ether (Fig. 1). Similarly, a bisflavone mixture obtained from *P. nagi* leaves seems to consist of amentoflavone (trace), and its mono and dimethyl ethers.

1) The preliminary report of this work appeared in *Tetrahedron Letters*, **1968**, 2339.
2) Location: *4–23 Bunkyo-machi, Nagasaki.*
3) T. Sawada, *Yakugaku Zasshi*, **78**, 1023 (1958).
4) N. Kawano, H. Miura, and H. Kikuchi, *Yakugaku Zasshi*, **84**, 469 (1964).
5) K. Nakazawa, *Tetrahedron Letters*, **1967**, 5223.



	R_1	R_2	R_3	R_4
I	H	Me	Me	Me
II	H	H	H	H
VII	Me	Me	H	Me
VIII	H	H	H	Me
IX	Me	H	H	Me
X	H	Me	H	H
XI	Me	Me	H	H
XII	H	Me	H	Me



	R	R′	R″
III	H	H	H
IV	H	H	Me
V	H	Me	H
VI	Me	H	H

The extraction of bisflavones from the leaves of *P. macrophylla* was carried out along the procedure shown in Chart 1 and described in the experimental part. Treatment of the bisflavone mixture with ethanol was effective to separate both type of bisflavones from each other. Each type of bisflavones was acetylated, recrystallized, and hydrolysed for purification. After this process no spot corresponding to amentoflavone could be detected by TLC. Then, they were subjected to countercurrent distribution method[6] between ethyl methyl ketone

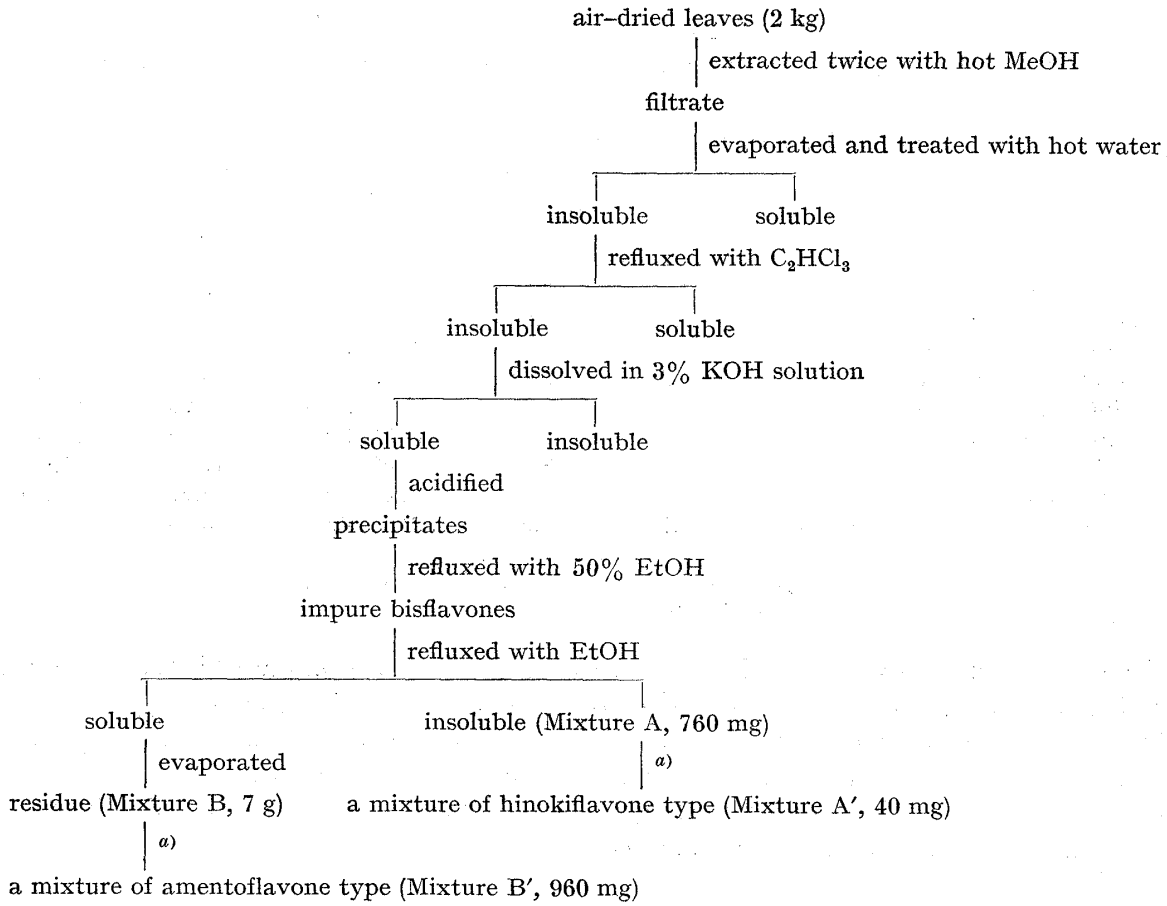


Chart 1. Extraction of Bisflavones from *P. macrophylla*

a) Means acetylated, recrystallized from EtOAc, hydrolyzed and recrystallized from pyridine–methanol.

6) W. Baker, A.C.M. Finch, W.D. Ollis, and K.W. Robinson, *J. Chem. Soc.*, **1963**, 1477.

and a borate buffer solution of pH 9.8. A mixture of hinokiflavone type bisflavones (Mixture A') gave hinokiflavone (III) and its monomethyl ether, which is identical with neither cryptomerin A (IV)[7] nor isocryptomerin (V)[8] but with neocryptomerin (VI)[9], a partially demethylated product obtained from hinokiflavone pentamethyl ether. This is the first report on the isolation of neocryptomerin from natural source.

On the other hand, a mixture of amentoflavone type bisflavones (Mixture B') gave sciadopitysin (VII)[10] and two new bisflavones, named podocarpusflavone A, $C_{31}H_{20}O_{10}$(VIII), mp 322—324° (decomp.) and podocarpusflavone B, $C_{32}H_{22}O_{10}$ (IX), mp 286° (decomp.). Their IR spectra were given in Fig. 2. On methylation with dimethyl sulfate and alkali both podocarpusflavone A and B produced amentoflavone hexamethyl ether. The nuclear magnetic resonance (NMR) spectra of podocarpusflavone A pentaacetate, $C_{41}H_{30}O_{15}$, mp 256—257° and B tetraacetate, $C_{40}H_{30}O_{14}$, mp 264—265° showed that they are amentoflavone monomethyl and dimethyl ether respectively. In comparison of the chemical shifts of their methyl protons with those of known biflavonyl methyl ether acetates it is deduced that podocarpusflavone A and B have the structure VIII and IX respectively as shown in Table I.

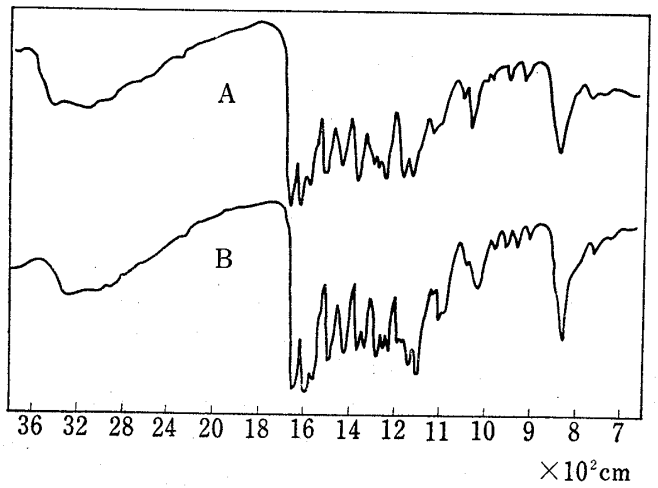


Fig. 2. IR Spectra of Podocarpusflavone A and B

Table I. NMR Signals (δ ppm) of Methyl Protons in Pyridine

Compounds	Assigned position in bisflavone nucleus 4′	4‴	5 5″	7	7″
Podocarpusflavone A (VIII) acetate	(2.05)	3.56	(2.44, 2.52)	(2.22)	(2.16)
Podocarpusflavone B (IX) acetate	(2.06)	3.55	(2.48, 2.54)	3.73	(2.16)
Bilobetin (X) acetate	3.72	(2.15)	(2.47, 2.51)	(2.24)	(2.10)
Ginkgetin (XI) acetate	3.73	(2.14)	(2.52, 2.52)	3.73	(2.10)
Isoginkgetin (XII) acetate	3.76	3.61	(2.49, 2.55)	(2.26)	(2.13)
Sciadopitysin (VII) acetate	3.76[a)]	3.58	(2.50, 2.53)	3.73[a)]	(2.10)
Kayaflavone (I) acetate	3.73	3.60	(2.47, 2.58)	(2.24)	3.80
Amentoflavone (II) acetate	(2.03)	(2.14)	(2.45, 2.52)	(2.22)	(2.11)
Amentoflavone hexamethyl ether	3.75	3.59	3.87 4.06	3.75	3.82

Figures in parentheses show the chemical shifts of acetyl protons.
a) Assignment is tentative.

The leaves of *P. nagi* were similarly extracted. Since the impure bisflavone mixture treated with 50% ethanol contained no compound belonging to hinokiflavone type they were purified through acetates as above described without refluxing with ethanol and subjected to countercurrent distirbution to give amentoflavone monomethyl and dimethyl ethers. The former was identical with podocarpusflavone A (VIII) and the latter was identified with isoginkgetin (XII) through their infrared (IR) and NMR spectra.

7) H. Miura, N. Kawano, and A. C. Waiss, Jr., *Chem. Pharm. Bull.* (Tokyo), **14**, 1404 (1966).
8) H. Miura and N. Kawano, *Chem. Pharm. Bull.* (Tokyo), **15**, 232 (1967); H. Miura, *Yakugaku Zasshi*, **87**, 871 (1967).
9) H. Miura and N. Kawano, *Chem. Pharm. Bull.* (Tokyo), **16**, 1838 (1968).
10) N. Kawano, *Chem. Pharm. Bull.* (Tokyo), **7**, 698, 821 (1959).

For the purpose of above identification isoginkgetin was obtained from the leaves of *Ginkgo biloba* according to the procedure reported formerly.[6] In this procedure, besides bilobetin (X), ginkgetin (XI), and isoginkgetin (XII) a considerable amount of sciadopitysin (VII) was newly separated from the leaves of *Ginkgo biloba*.

Experimental[11]

Extraction of Bisflavones from the Leaves of *P. macrophylla*——Air-dried leaves (2 kg) were extracted with boiling MeOH (15 liters) two times for 3 hr each. Combined MeOH solution separated while hot was concentrated at atmospheric pressure to about 3 liters and then *in vacuo* to give black extract, which was treated with hot water three times to remove water-soluble brownish substances and refluxed with trichloroethylene (1 liter) for 1.5 hr. Insoluble parts were collected by the aid of Hyflosuper-cel (Johns-Manville), washed with trichloroethylene until washings are almost colorless, dissolved in 3% KOH solution, and filtered. Filtrate was acidified to yield dark precipitates, which were refluxed with 50% EtOH (300 ml) for 2 hr and cooled to give impure bisflavones, which were then refluxed with EtOH (150 ml) for 3 hr and cooled to give a crystalline bisflavone mixture (Mixture A, 760 mg). The ethanol solution was evaporated *in vacuo* to dryness to give crude crystals (Mixture B, *ca.* 7 g).

Purification of Bisflavone Mixtures through Acetates——Mixture A (760 mg) was acetylated with Ac_2O (8 ml) and AcONa (0.8 g) in usual way, recrystallized from ethyl acetate, hydrolysed with 3% KOH solution, and recrystallized from pyridine-methanol to give bisflavone mixture A′ (40 mg). Mixture B (*ca.* 7 g) was also acetylated with Ac_2O (50 ml) and AcONa (5 g), recrystallized to give acetates and treated similarly to give bisflavone mixture B′ (960 mg).

Thin-Layer Chromatography (TLC)——Kieselgel G nach Stahl (Merck) was used drying at 110° for 1 hr. Solvent system: toluene-ethyl formate-formic acid (5:4:1). Sample was applied to a plate as a pyridine solution and dried. The mixture of bisflavones before refluxing with 50% ethanol showed six spots, *Rf*: 0.16, 0.21, 0.28, 0.31, 0.33, and 0.37, two (0.21 and 0.31) of which are pale brown after spraying $FeCl_3$ solution while the other four spots are greenish brown. Mixture A′ showed two spots, 0.21 and 0.31 while mixture B′ gave three spots, 0.28, 0.33 and 0.37.

Isolation of Pure Bisflavone by Countercurrent Distribution (CCD) Method——a) The above mixture A′ (40 mg) was subjected to CCD between ethyl methyl ketone (10 ml, equilibrated) and a borate buffer (Clark-Lubs, pH 9.8, 10 ml). After 30 transfers the following fractions were collected, acidified with HCl, and ethyl methyl ketone was distilled off to give pale yellow precipitates; fraction 1 (tubes 1—5, hinokiflavone detected by TLC, 19 mg); fraction 2 (tubes 13—25, hinokiflavone monomethyl ether, 10 mg).

b) The mixture B′ (300 mg) was dissolved in the first tube and subjected to similar CCD to give the following fractions (70 transfers): fraction 3 (tubes 17—37, amentoflavone monomethyl ether, 110 mg); fraction 4 (tubes 57—70, a mixture of amentoflavone di- and tri-methyl ethers, 135 mg). The precipitates (370 mg) obtained from fraction 4 were again separated by another CCD between ethyl methyl ketone and a phosphate buffer (Kolthoff, pH 12.0) into the following fractions (40 transfers): fraction 5 (tubes 12—19, amentoflavone trimethyl ether slightly contaminated with a dimethyl ether, 80 mg); fraction 6 (tubes 23—37, amentoflavone dimethyl ether, 166 mg). The fraction 5 (80 mg) was recrystallized from pyridine-methanol to give a pure trimethyl ether.

Hinokiflavone (III)——Fraction 1 (19 mg) was recrystallized from pyridine-methanol, acetylated with Ac_2O and NaOAc, and recrystallized from ethyl acetate to give hinokiflavone pentaacetate (7 mg), mp 225—226°, which was identified with an authentic sample by mixed mp and comparison of their NMR spectra.

Neocryptomerin (VI)——The monomethyl ether (10 mg) obtained from fraction 2 was recrystallized from ethanol to give neocryptomerin (6 mg), mp 299—300° (decomp.), which was identified with an authentic sample by mixed mp and comparison of their IR spectra.
IR ν_{max}^{KBr} cm^{-1}: 1654, 1605, 1590, 1555, 1537, 1500, 1460, 1440, 1425, 1365, 1299, 1255, 1230, 1197, 1176, 1158, 1125, 1110, 1096, 1037, 945, 920, 915, 907, 868, 835, 810, 799, 775.

Sciadopitysin (VII)——The trimethyl ether obtained from fraction 5 melted at 288—289° and was identified with an authentic sample of sciadopitysin by comparison of their IR spectra and mixed mp. Acetates, mp 268—269° were prepared and identified by mixed mp and NMR spectrum.

Podocarpusflavone A (VIII)——The monomethyl ether obtained from fraction 3 was recrystallized from pyridine-methanol to give pale yellow crystals, mp 322—324° (decomp.). TLC, *Rf*: 0.28. *Anal.* Calcd. for $C_{31}H_{20}O_{10}\cdot 1\frac{1}{2}H_2O$: C, 64.25; H, 3.97. Found: C, 64.63; H, 3.80. IR ν_{max}^{KBr} cm^{-1}: 1655, 1610, 1570, 1500, 1430, 1356, 1289, 1264, 1239, 1178, 1160, 1112, 1046, 1029, 997, 985, 945, 912, 834.

Podocarpusflavone A Pentaacetate——VIII (128 mg) was acetylated with Ac_2O (1.5 ml) and NaOAc (120 mg) and recrystallized three times from ethyl acetate to give colorless prisms (109 mg), mp 256—257°.

11) All melting points were not corrected. NMR spectra were taken on Hitachi H-60 instrument with tetramethylsilane as internal standard.

NMR (in pyridine) δ ppm: 3.56 (3H, OCH_3), 2.05, 2.16, 2.22, 2.44, 2.52 (3H each, O-$COCH_3$). *Anal.* Calcd. for $C_{41}H_{30}O_{15}$: C, 64.56; H, 3.96. Found: C, 64.44; H, 3.97.

Podocarpusflavone B (IX)——The dimethyl ether (166 mg) obtained from fraction 6 was recrystallized from pyridine–methanol to give pale yellow crystals (110 mg), mp 286° (decomp.). TLC, *Rf*: 0.33. *Anal.* Calcd. for $C_{32}H_{22}O_{10}\cdot 1\frac{1}{2}H_2O$: C, 64.75; H, 4.25. Found: C, 65.13; H, 4.00. IR ν_{max}^{KBr} cm^{-1}: 1655, 1605, 1502, 1425, 1372, 1340, 1309, 1288, 1253, 1200, 1188, 1180, 1160, 1112, 1079, 1055, 1037, 990, 964, 937, 912, 834. (Fig. 2).

Podocarpusflavone B Tetraacetate——IX (90 mg) was acetylated with AcONa (100 mg) and Ac_2O (1 ml) and recrystallized from ethyl acetate to give colorless prisms (50 mg), mp 264—265°. NMR (in pyridine) δ ppm: 3.55, 3.73 (3H each, OCH_3), 2.06, 2.16, 2.48, 2.54 (3H each, O-$COCH_3$). *Anal.* Calcd. for $C_{40}H_{30}O_{14}\cdot\frac{1}{2}H_2O$: C, 64.60; H, 4.20. Found; 64.76; H, 3.99.

Methylation of Podocarpusflavone A and B——VIII (70 mg) was methylated with dimethyl sulfate (1 ml) and 30% KOH solution and recrystallized from methanol to give amentoflavone hexamethyl ether (30 mg), mp 225—226°, which was identified with an authentic sample by mixed mp and comparison of their IR spectra. IX (70 mg) was also methylated to amentoflavone hexamethyl ether (54 mg), which was similarly identified.

Extraction of Bisflavone from the Leaves of *P. nagi*——Air-dried leaves (2.4 kg) were extracted in the same procedure with the case of *P. macrophylla*. Treatment with 50% ethanol (300 ml) yielded an insoluble part, which was acetylated and recrystallized from ethyl acetate to give colorless crystals (434 mg). Hydrolysation with 2.5% KOH solution (80 ml) followed by recrystallization from pyridine–methanol yielded a mixture of bisflavones (94 mg), which was subjected to CCD between ethyl methyl ketone and a borate buffer of pH 9.8 (80 transfers): tubes 15—27, amentoflavone dimethyl ether (54 mg); tubes 33—44, amentoflavone monomethyl ether (15 mg).

Podocarpusflavone A——The monomethyl ether (15 mg) above described was recrystallized from pyridine–methanol to give podocarpusflavone A (7 mg), mp 320—323° (decomp.), which was identified by mixed mp and comparison of its IR spectrum.

Isoginkgetin (XII)——The dimethyl ether (54 mg) from tubes 15—27 was also recrystallized from pyridine–methanol to give yellow crystals (26 mg), IR spectrum of which was same with that of isoginkgetin obtained from the leaves of *Ginkgo biloba*. The NMR spectrum of its acetate was also same with that of isoginkgetin tetraacetate described below.

Extraction of Bisflavones from the Leaves of *Ginkgo biloba*——Air-dried yellow leaves (1.2 kg) were treated as described in the extraction of the leaves of *P. nagi* to give a mixture of bisflavones (660 mg), which was subjected to a similar CCD (100 transfers): tubes 4—13, amentoflavone monomethyl ether (30 mg); tubes 18—34, dimethyl ether (49 mg); tubes 60—80, dimethyl ether (105 mg); tubes 88—100, trimethyl ether (113 mg).

Sciadopitysin (VII)——Above trimethyl ether (113 mg) was recrystallized from pyridine–methanol to give pale yellow crystals (40 mg), mp 286—287°, IR spectrum of which was same with that of aciadopitysin. NMR spectrum of its acetate, mp and mixed mp 266—267° was also same with that of sciadopitysin triacetate.

Bilobetin (X), Ginkgetin (XI), and Isoginkgetin (XII)——These were isolated along the reported procedure.[6] The monomethyl ether (30 mg) obtained from tubes 4—13 corresponding to bilobetin was acetylated and recrystallized from ethyl acetate to give bilobetin pentaacetate (7 mg). NMR (in pyridine) δ ppm: 3.72 (3H, OCH_3), 2.51, 2.47, 2.24, 2.15, 2.10 (3H each, O-$COCH_3$). The dimethyl ether (105 mg) obtained from tubes 60—80 was recrystallized from ethanol to give pale yellow crystals (50 mg), IR spectrum of which was same with that of synthetic ginkgetin.[12] The dimethyl ether (49 mg) obtained from tubes 18—34 was also recrystallized from ethanol to give isoginkgetin (25 mg), which was acetylated to isoginkgetin tetraacetate, mp 251—252°. NMR (in pyridine) δ ppm: 3.76, 3.71 (3H each, OCH_3), 2.55, 2.49, 2.26,2.13 (3H each, O-$COCH_3$).

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12) K. Nakazawa and M. Ito, *Chem. Pharm. Bull.* (Tokyo), 11, 283 (1963).