[Chem. Pharm. Bull. 27(12)3130—3136(1979)]

UDC 547.918.02.05 : 518.192

A New Acylated Flavonol Glycoside from *Cyathea contaminans* Copel. and Its Distribution in the Pterophyta[1)]

Atsushi Hiraoka[2a,b)] and Masaakira Maeda[2c)]

Department of Biology, Faculty of Science, Tokyo Metropolitan University[2a)] and Department of Biochemistry, Faculty of Science, Saitama University[2c)]

(Received July 7, 1979)

A new acylated flavonol glucoside was isolated from the fronds of a Philippine Pterophyta species, *Cyathea contaminans* Copel. (Cyatheaceae) and chemically characterized as kaempferol-7-(6″-succinyl)-glucoside. This is the first report of s succinyl ester of a flavonoid glycoside and this compound was named pteroflavonoloside. The distribution of pteroflavonoloside in 57 species of 11 genera belonging to the three representative families of pterophyta is also discussed from the viewpoint of chemotaxonomy and biochemical systematics.

Keywords——*Cyathea contaminans* Copel. (Cyatheaceae); pterophyta; acylated flavonol glycoside; kaempferol-7-(6″-succinyl)-glucoside; structure elucidation; distribution; chemotaxonomy; biochemical systematics

Many flavonoid glycosides have been identified as plant constituents, and some of them are acylated at a sugar hydroxyl group(s) *via* an ester linkage. However, most of these acyl groups are phenolic acids such as *p*-coumaric, caffeic or gallic acids *etc.*, and aliphatic ones such as malonic[3)] or tiglic acid[4)] are rare.

The present authors recently isolated a new acylated flavonol glycoside from the fronds of a Philippine pterophyta, *Cyathea contaminans* Copel. (Cyatheaceae), and identified it as a succinyl ester of kaempferol glucoside.[5)] This paper deals with its further chemical characterization and with its distribution in the pterophyta from the viewpoint of chemotaxonomy and biochemical systematics.

The fronds of *Cythea contaminans* Copel. contained an acylated kaempferol glucoside (I) in addition to kaempferol, kaempferol-3-glucoside (astragalin), kaempferol-3-rhamnoside (afzelin), kaempferol-3-sophoroside, kaempferol-7-rhamnoglucoside, vitexin and orientin.[5)] The flavonoid profile of this specimen qualitatively resembles those of *C. fauriei*, *C. mertensiana* and *C. leichhardtiana*, which are distinguishable from *C. podophylla* and *C. hancockii* on the basis of both morphological character[6)] and the glycosylation patterns of kaempferol glycosides in the fronds.[7)] Extraction and separation of the flavonoids were performed in a manner similar to that reported previously in connection with chemotaxonomical work on the genus *Cyathea*[7)] (summarized in Chart 1). As described previously,[7)] all the flavonoids except for I were isolated by paper partition chromatography (PPC) or thin layer chromatography (TLC) and identified on the basis of ultraviolet (UV) spectral data[8,9)] and PPC of either the

1) This reserch was submitted by A.H. in partial fulfillment of the requirements of Tokyo Metropolitan University for the degree of Ph.D in phytochemistry.
2) Location: *a*) *2-1-1*; *Fukazawa, Setagaya, Tokyo, 158, Japan*; *b*) Present address: *Laboratory of Phytochemistry, The Institute of Physical and Chemical Research, 2-1, Hirosawa, Wako, Saitama, 351, Japan*; *c*) *255, Shimo-ohkubo, Urawa, Saitama, 338, Japan.*
3) J.B. Harborn, C.A. Williams, and D.M. Smith, *Biochem. System.*, **1**, 51 (1973).
4) M. Ogawa and Y. Ogihara, *Yakugaku Zasshi*, **95**, 655 (1975).
5) A preliminary report of this work was presented at the 43rd Annual Meeting of the Botanical Society of Japan Chiba, Oct. 1978.
6) E.B. Copeland, "Genera Filicum," Ronald Press, New York, 1948, p. 98.
7) A. Hiraoka and M. Hasegawa, *Bot. Mag. Tokyo*, **88**, 127 (1975).
8) T.J. Mabry, K.R. Markham, and M.B. Thomas, "The Systematic Identification of Flavonoids," Springer-Verlag, Berlin, 1970.
9) L. Jurd and R.M. Horowitz, *J. Org. Chem.*, **22**, 1618 (1957).

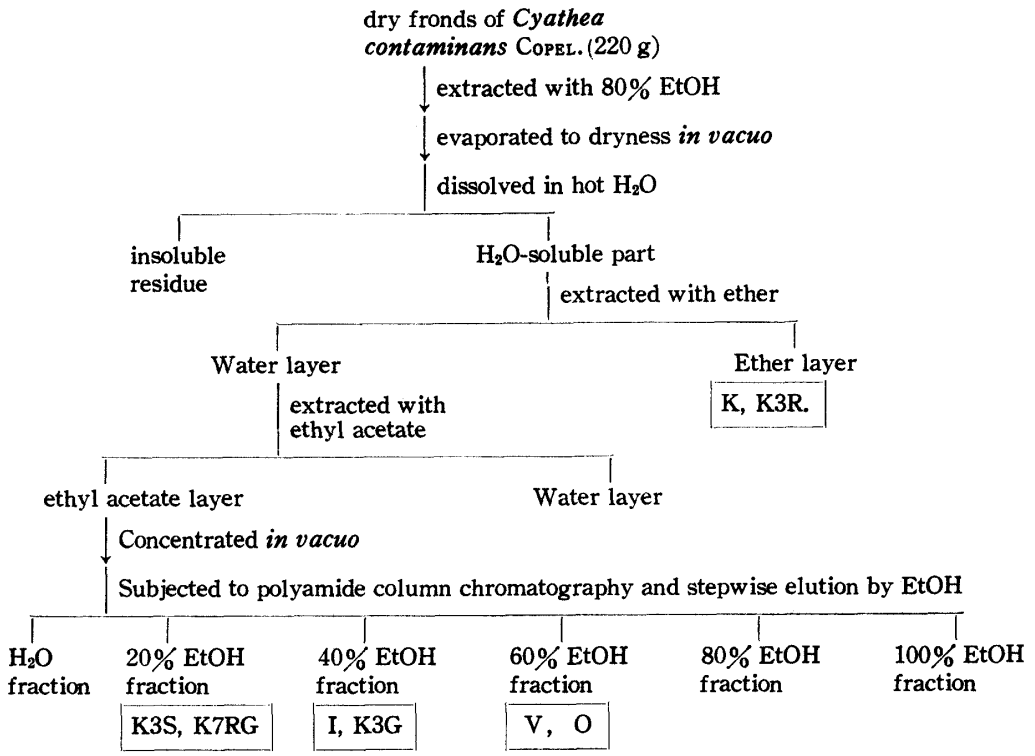


Chart 1. Procedures for the Isolation of the Pteroflavonoloside of *Cyathea contaminans* Copel.

The following abbreviations are used: K=kaempferol, K3R=kaempferol-3-rhamnoside, K3S=kaempferol-3-sophoroside, K7RG=kaempferol-7-rhamnoglucoside, K3G=kaempferol-3-glucoside, V=vitexin, O=orientin.

intact materials or their hydrolysis products. It has been shown that kaempferol glycosides including I are major flavonoids of this specimen by two-dimensional PPC of the ether and ethyl acetate soluble fraction using BuOH–AcOH–H_2O (4:1:2, v/v) and 15% AcOH as developing solvents.

After purification, I was obtained as yellow needles of mp 239—242° and determined to be $C_{25}H_{24}O_{14}$ by elemental analysis. I gave an orange-red coloration in the flavone reaction (Zn–HCl and Mg–HCl) and reacted greenish-brown with ferric chloride solution. The infrared (IR) spectrum of I showed absorptions at 3380, 1690, 1660, 1640, 1610, 1570 and 1510 cm^{-1} indicating the presence of hydroxyl, carbonyl and aromatic groups. It was similar to that of kaempferol, in the region of 1700—1600 cm^{-1}, though two characteristic absorption bands at 1690 and 1640 cm^{-1} were observed in addition to those at 1660 and 1610 cm^{-1} commonly observed in kaempferol. These data suggested the presence of a carbonyl group in addition to that of kaempferol in the molecule. The UV spectrum of I in MeOH showed peaks at 266 (log ε 4.35) and 364 nm (log ε 4.28), and the former (Band II) gave no bathochromic shift on addition of sodium acetate (NaOAc). The UV data indicated the absence of a free 7-hydroxyl in the flavonol.[8,9]

I gave kaempferol, D-glucose and succinic acid in a ratio of nearly 1:1:1 on acid hydrolysis. Kaempferol was obtained from the acid hydrolysate as crystals and was identified by PPC, as well as UV and IR spectroscopy. A mixed melting point test (mmp) with an authentic sample showed no depression. D-Glucose and succinic acid were estimated by the anthrone-sulfuric acid method[10] and the fluorometric method using resorcinol and conc. H_2SO_4,[11] respectively.

10) J.E. Hodge and B.T. Hofreter, "Methods in Carbohydrate Chemistry," Vol. I, p. 380, Academic Press, New York, 1962.
11) C.G. Barr, *Plant Physiol.*, **23**, 443 (1948).

Mild alkaline saponification (deacylation) of I afforded a yellow solid (II). The *Rf* values of II in PPC agreed with those reported for keampferol-7-glucoside from the petals of *Thespesia populnea*.[12] Attempts to crystallize it failed, but it gave one spot on PPC and was identified as kaempferol-7-glucoside by the standard method based on UV spectral analysis[8,9] and PPC of the hydrolysis products. Thus II was proposed as a partial structure of I.

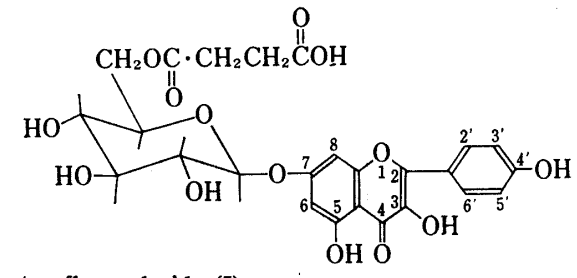
Enzymatic hydrolysis of I using β-glucosidase (emulsin) gave kaempferol and a white powder (III) which was detected on a paper chromatogram as a single spot with reagents such as aniline-hydrogen phthalate,[13] Bacon-Edelman reagent[14] and bromocresol green.[15] The IR spectrum of III showed absorptions not only at 3420 (OH), and 2880 (CH), which are typical of carbohydrate, but also at 1695, and 1640 cm^{-1} (C=O), which suggested the presence of a free carbonyl and an ester linked acyl group attached to the sugar residue. III gave D-glucose and succinic acid in a ratio of nearly 1 : 1 on mild alkaline saponification. Furthermore, enzymatic hydrolysis of III with esterase also gave D-glucose and succinic acid. Thus, a monosuccinyl ester of D-glucose was considered to be present in III, and I was considered to be degraded into kaempferol and glucose monosuccinate by the action of β-glucosidase.

Methylation of I using dimethyl sulfate and potassium carbonate, followed by acid hydrolysis of the methylated sample afforded pale yellow crystals (IV) of mp 278—280°. The mass spectrum (MS) of IV showed a molecular ion peak (M^+) at *m/e* 328, indicating that it was a kaempferol trimethyl ether. Fragment ions at *m/e* 313 ($M^+ - CH_3$) and 135, corresponding to a monomethoxylated aromatic ring formed from the B-ring of the flavonoid,[16] suggested that the 4′-hydroxyl of kaempferol was methylated. The proton magnetic resonance (PMR) spectrum of IV in CD_3OD solution exhibited three sharp singlets due to methoxyl groups at 3.70, 3.82 and 3.84 ppm, indicating that three out of four hydroxyls of kaempferol were methylated. The UV spectrum of IV showed peaks at 264 and 336 nm. A bathochromic shift of Band II was observed in NaOAc, but not in $AlCl_3$, in contrast to the case of I. This indicated the absence of free 3- or 5-hydroxyl groups and the presence of a free 7-hydroxyl in the flavonol.[8,9] Furthermore, no depression of its melting point was observed on admixture with an authentic sample of kaempferol-4′,3,5-trimethyl ether. Thus, in the original glycoside (I), all the kaempferol hydroxyls except 7-OH were free.

I was then permethylated by the method of Kuhn[17] in order to confirm the identification of succinic acid by combined gas chromatography–mass spectrometry (GC–MS) as its dimethyl ester and to determine the position of the ester linkage on the glucose residue. Complete permethylation was indicated by the absence of IR absorption for hydroxyl groups. Dimethyl succinate was isolated after deacylation of the permethylated I by mild alkaline methanolysis, and was identified by direct comparison with an authentic sample in GC–MS. On the other hand, the partially methylated D-glucose obtained from the acid hydrolysate of deacylated permethylated I showed Rg (*Rf* relative to 2,3,4,6-tetra-O-methyl-D-glucose) values identical with those of co-developed authentic 2,3,4-tri-O-methyl-D-glucose in PPC and TLC. Furthermore, after conversion to the corresponding partially methylated glucitol acetate, it was subjected to GC–MS and identified as 2,3,4-tri-O-methyl-1,5,6-tri-O-acetyl-glucitol by direct comparison of the fragmentation pattern with that of an authentic sample. These results suggested that succinic acid was substituted at the 6-hydroxyl of D-glucose.

These procedures, summarized in Chart 2, showed that I was kaempferol-7-(6″-succinyl)-glucoside. I was a new flavonoid glycoside, which we named pteroflavonoloside.

12) J.B. Harborne, "Comparative Biochemistry of Flavonoids," Academic Press, New York, 1968, p. 68.
13) S.M. Partridge, *Nature* (London), **164**, 443 (1948).
14) J.S.D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951).
15) J. Sherma and G. Zweig, "Paper Chromatography and Electrophoresis," Vol. II, Academic Press, New York, 1971, p. 191.
16) M.A.F. Jalal, K.H. Overton, and D.S. Rycroft, *Phytochem.*, **17**, 149 (1978).
17) R. Kuhn and H. Trischamann, *Angew. Chem.*, **67**, 32 (1955).



pteroflavonoloside (I)

acid hydrolysis → kaempferol + D-glucose + succinic acid

mild alkaline saponification → kaempferol-7-glucoside (II)

β-glucosidase → kaempferol + glucose monosuccinate (III)

mild alkaline hydrolysis → D-glucose + succinic acid

esterase → D-glucose + succinic acid

methylation with $(CH_3)_2SO_4$ and K_2CO_3 → acid Hydrolysis → kaempferol-4',3,5-tri-O-methyl ether (IV)

permethylation with Kuhn's method → mild alkaline methanolysis → dimethyl succinate + deacylated permethylated I

deacylated permethylated I → acid hydrolysis → 2,3,4-tri-O-methyl-D-glucose

Chart 2. Structure of Pteroflavonoloside and the Methods used for Structure Determination

The distribution of pteroflavonoloside in 57 species of 12 genera belonging to the three pterophyta families is summarized in Table I. This compound occurred in all the species tested in the genus *Cyathea*, *Matteuccia*, *Onoclea* and *Cyrtomium* and in the so-called *variae* group[18] of the genus *Dryopteris*. However, it was not found in the other species tested, except for *Polystichum lepidocaulon*, which was pointed out by Ching[19] to be morphologically related to *Cyrtomium*. Thus, it occurs widely in pterophyta and should be useful as a marker for chemotaxonomy. The presence of pteroflavonoloside may be a primitive character in Athyriaceae and the genus *Dryopteris* of Dryopteridaceae from the viewpoint of biochemical systematics, based on the generally accepted view that evolutionary advancement produces a simplification of flavonoid patterns within the taxa,[20] because it was detected in species having larger numbers of flavonoids than others tested within the taxa.[21]

The results of earlier flavonoid analysis of the materials listed in Table I (except for *Cyathea contaminans*) have been discussed in connection with their taxonomy and phylogeny.[7,21] In that work, however, I was misidentified as kaempferol-7-arabinoside by the usual method (UV spectral analysis,[8,9] and PPC of its hydrolysate), because PPC of the sugar component of the acid hydrolysate afforded a spot which was positive to the Bacon-

18) M. Tagawa, "Coloured Illustration of the Japanese Pteridophyta," Hoikusha, Osaka, 1974, p. 100.
19) R.C. Ching, *Acta Phytotaxonomica Sinica*, **6**, 265 (1957).
20) J.B. Harborne, *Biochem. System. Ecol.*, **5**, 7 (1977).
21) A. Hiraoka, *Biochem. System. Ecol.*, **6**, 171 (1978).

Table I. Distribution of Pteroflavonoloside in Ferns

Cyatheaceae		Dryopteridaceae	
Cyathea		*Cyrtomium*	
C. contaminans	+	*C. fortunei*	+
C. fauriei	+	*C. fortunei var. clivicola*	+
C. mertensiana	+	*C. falcutum*	+
C. leichhardtiana	+	*Polystichum*	
C. podophylla	+	*P. lepidocaulon*	+
C. hanckokii	+	*P. craspedosorum*	−
		P. tripteron	−
Athyriaceae		*P. polyblephurum*	−
Athyrium		*Arachniodes*	
A. niponium	−	*A. aristata*	−
A. otophorum	−	*A. pseudo-aristata*	−
A. vidalii	−	*A. ambilis*	−
A. shererii	−	*A. ambilis var. yakushimensis*	−
A. yokoscence	−	*A. standishii*	−
Diplazium		*Dryopteris*	
D. subsinuatum	−	Section *Dryopteris*	
D. wichurae	−	*D. atrata*	−
D. mettenisnum	−	*D. dickinsii*	−
D. mesosorum	−	*D. kominatoensis*	−
Deparia	−	*D. lacera*	−
D. japonium	−	*D. uniformis*	−
D. petersenii	−	*D. polylepis*	−
D. dimorphophyllum	−	*D. crassirizhoma*	−
D. conilii	−	*D. watanabei*	−
D. lobato-crenatum	−	Section *Erythro-variae*	
D. pycnosorum	−	*D. bissetiana*	+
D. okuboanum	−	*D. sordidipes*	+
Matteuccia		*D. sacrosancta*	+
M. orientales	+	*D. pacifica*	+
M. struthiopteris	+	*D. erythrosora*	−
Onolea		*D. nipponensis*	−
O. sensibilis	+	*D. hondoensis*	−
Woodsia		*D. championii*	−
W. manchuriensis	−	*D. gymnosora*	−
W. polystichoides	−	*D. gymnophylla*	−

Edelman reagent, and its *Rf* values (0.18 in BuOH–AcOH–H_2O (6:1:2) and 0.43 in BuOH–pyridine–H_2O (6:4:3) solvent) coincided with those of codeveloped authentic arabinose. Thus, although the spot color was rather darker than that of the authentic pentose sample, the sugar part of this compound was considered to be arabinose based on the PPC result. The misleading spot may have been produced by a reaction between the detecting reagent and barium succinate produced during the neutralization of 2 N H_2SO_4 used for acid hydrolysis with barium ions. Indeed, when succinic acid alone was dissolved in 2 N H_2SO_4 and treated in a similar manner, an identical spot was obtained with Bacon-Edelman reagent on a chromatogram. Miller and Kraemer[22] also reported the occurence of spots apparently resembling those of reducing sugars on paper chromatography with detection using the above reagent in the presence of inorganic ions.

A further question was raised, however, because the *Rf* values of I in two-dimensional PPC seemed to correspond to those of flavonol monoglycoside.[12] If it was really kaempferol-7-mono-arabinoside, acid hydrolysis of I should afford *ca.* 68.4% (w/w) of the aglycone relative to the glycoside, but in fact the ratio of aglycone/glycoside estimated by the spectrophotometric method using the increase of absorption at the longer wavelength maximum was only

22) H. Miller and D.M. Kraemer, *Anal. Chem.*, **24**, 1371 (1952).

ca. 54.5%. Thus, this work was intended to reinvestigate the structure of I. It was found to be not an arabinoside, but a new acylated kaempferol glucoside, pteroflavonoloside.

Experimental

Plant Materials——*Cyathea contaminans* COPEL. was collected in the Philippines in December, 1975.

Apparatus——All melting points were determined with a Mitamura Riken melting point apparatus or a Yanaco micro melting point apparatus (MP-3), and are uncorrected. IR and UV spectra were obtained on a Jasco IR-G or a Digilab FTS-14 Fourier transform IR spectrometer and a Hitachi 124 spectrometer, respectively. The determination of succinic acid by the fluorometric method[11] was performed with a Hitachi 204 fluorescence spectrometer. GC-MS was carried out with an LKB-9000 unit. Helium, at a flow rate of 30 ml/min, was used as a carrier gas. The PMR spectrum was obtained in CD_3OD at 100 MHz using a JEOL FX-100 spectrometer and the signals were given as chemical shifts in values (ppm) with TMS as an internal standard. The MS spectrum for IV was obtained with a Hitachi RMU-6MG machine by direct injection. The ion source temperature was maintained at 220°, with an electron energy of 20 eV and an accelerating voltage of 3.0 kV.

Chromatography——PPC was carried out on Whatman No. 1 paper, developing with A); BuOH–AcOH–H_2O (4:1:2), B); 15% AcOH, C); water-saturated phenol, D); BuOH–pyridine–H_2O (6:4:3), E); ether–AcOH–H_2O (13:3:1), F); ethyl acetate–AcOH–H_2O (3:1:1) and G); BuOH–EtOH–H_2O–NH_4OH (40:10:49:1). TLC was carried out on Silica Gel G (Merck), developing with $CHCl_3$–MeOH (20:3). All the ratios are given as v/v. The detecting reagents were 2% aqueous $FeCl_3$ for flavonoids, aniline-hydrogen phthalate[13] and Bacon-Edelman reagent[14] for both free and methylated sugars, and bromocresol green reagent[15] for succinic acid.

Pteroflavonoloside (I)——From 220 g of dry fronds of *C. contaminans*, 62.8 mg of I was obtained. Recrystallization from dilute EtOH provided yellow needles of mp 239—242°. *Anal.* Calcd. for $C_{25}H_{24}O_{14}$: C, 54.75; H, 4.41. Found: C, 54.98; H, 4.37. IR λ_{max}^{KBr} cm^{-1}: 3380 (OH), 1690, 1660, 1640 (C=O), 1610, 1570, 1510 (aromatic C=C). UV λ_{max}^{MeOH} nm (log ε): 266 (4.35), 364 (4.28), $\lambda_{max}^{MeOH+AlCl_3}$ nm: 274, 423., $\lambda_{max}^{MeOH+NaOAc}$ nm: 266, 385. PPC: *Rf* 0.83 (solv. A), 0.10 (solv. B), 0.60 (solv. C).

Acid Hydrolysis of I——I (9.4 mg) was dissolved in 2 N H_2SO_4 (20 ml) and refluxed on a boiling water bath for 2 hr. After cooling, yellow crystals (5.1 mg) precipitated and were filtered off. The material was identified as kaempferol by direct comparison with an authentic sample (PPC, UV, IR and mmp). PPC: *Rf* 0.83 (solv. A), 0.10 (solv. B), 0.58 (solv. C), UV λ_{max}^{MeOH} nm: 266, 365., IR λ_{max}^{KBr} cm^{-1}: 3300 (OH), 1660 (C=O), 1610, 1570, 1510 (aromatic C=C), mp 281° (no depression on admixture with authentic kaempferol). On the other hand, the filtrate of the acid hydrolysate was neutralized with $BaCO_3$, filtered and then subjected to ion exchange column chromatography using Amberlite IR-120 (H^+) and IR A-410 (CH_3COO^-). D-Glucose in the neutral fraction and succinic acid in the acidic fraction from the anion resin were identified by PPC and estimated by the anthrone–sulfuric acid method[10] and fluorometry using conc. H_2SO_4 and resolcinol,[11] respectively. PPC: *Rf* D-glucose 0.15 (solv. A), 0.35 (solv. D), succinic acid 0.66 (solv. A), 0.64 (solv. C), 0.68 (solv. E), 0.72 (solv. F). Determination: D-glucose 2.8 mg, succinic acid 1.7 mg. The molar ratio of kaempferol: glucose: succinic acid was estimated to be 100: 87: 81.

Mild Alkaline Saponification (Deacylation) of I——I (*ca.* 5 mg) was dissolved in ice-cold 0.2 N KOH–MeOH (4 ml) and stirred at room temperature for 1 hr. The mixture was then neutralized with dil. HCl followed by evaporation to dryness *in vacuo*. A small volume of EtOH was added to the residue and kaempferol-7-glucoside (II) was obtained as yellow solid from the cold EtOH-soluble portion: UV λ_{max}^{MeOH} nm: 266, 364, $\lambda_{max}^{MeOH+AlCl_3}$ nm: 274, 423, $\lambda_{max}^{MeOH+NaOAc}$ nm: 266, 385., PPC: *Rf* 0.54 (solv. A), 0.17 (solv. B), 0.62 (solv. C). These values were consistent with those reported for kaempferol-7-glucoside from the petals of *Thespesia populnea*[12] and the material was finally identified as kaempferol-7-glucoside on the basis of UV spectral analysis[8,9] and PPC of its acid hydrolysate.

Enzymatic Hydrolysis of I——I (*ca.* 16 mg) was dissolved in 0.5 M acetate buffer (pH 5.4, 25 ml) and then β-glucosidase (emulsin from almond, purchased from Wako Chemicals, *ca.* 15 mg) was added. The mixture was left to stand at room temperature overnight, acidified with a few drops of dil.HCl and then extracted with ethyl acetate. The ethyl acetate extract was washed with H_2O and evaporated to dryness *in vacuo*. The residue was treated with a small volume of H_2O and the water-insoluble yellow precipitate of kaempferol was filtered off. On the other hand, concentration of the filtrate afforded D-glucose monosuccinate (III) as white powder: IR λ_{max}^{KBr} cm^{-1}: 3420 (OH), 2880 (CH), 1695, 1640 (C=O), PPC: *Rf* 0.36 (solv. A).

Mild Alkaline Saponification of III——III was allowed to stand with 0.05 N NH_4OH in 50% (v/v) MeOH for 1 hr at room temperature, followed by evaporation to dryness *in vacuo* below 30°. The D-glucose and succinic acid thus obtained were identified by PPC and estimated by the method described previously.[10,11] From 4.9 mg of III, 2.4 mg of D-glucose and 1.36 mg of succinic acid were obtained: the molar ratio was calculated to be approximately 1: 1.

Enzymatic Hydrolysis of III——III (*ca.* 1.5 mg) was dissolved in 0.1 M borate buffer (pH 8.0, 5 ml), then, a small volume of concentrated esterase (carboxy esterase from hog liver, EC 3.1.1.1, purchased from

Sigma Chemicals) was added. The mixture was left at room temperature for 1 hr, followed by neutralization with dil. HCl and evaporation to dryness *in vacuo*. D-Glucose and succinic acid in the residue were detected by PPC.

Preparation of Kaempferol-4′,3,5-trimethyl Ether by Methylation of I with $(CH_3)_2SO_4$ and K_2CO_3 followed by Acid Hydrolysis——I (*ca.* 10 mg) was dissolved in anhydrous acetone (20 ml) and $(CH_3)_2SO_4$ (0.5 ml) and K_2CO_3 (2 g) were added. The mixture was heated at 50° for 6 hr with stirring until $FeCl_3$ gave no reaction with the solution. After cooling, the mixture was filtered to remove K_2CO_3 and the filtrate was concentrated *in vacuo*. The residue was treated with 1.5 N H_2SO_4 (20 ml) and heated on a boiling water bath for 90 min. The cooled acid hydrolysate afforded pale yellow crystals of kaempferol-4′,3,5-trimethyl ether (IV) which was recrystallized from EtOH. The mp was 278—280°, showing no depression on admixture with an authentic sample pf kaempferol-4′,3,5-trimethyl ether. UV λ_{max}^{MeOH} nm: 264, 336., $\lambda_{max}^{MeOH+AlCl_3}$ nm: 264, 336., $\lambda_{max}^{MeOH+NaOAc}$ nm: 280, 362., MS *m/e*: 328 (M^+), 313, 135., PMR: OCH_3 3.70(s), 3.82(s), 3.84(s)., aromatic H 6.36(s), 6.45(s), 7.00 (d, $J=9$ Hz), 7.99 (d, $J=9$ Hz).

Permethylation of I——I (*ca.* 20 mg) was dissolved in N,N-dimethyl formamide (DMF, 1 ml), then CH_3I (0.5 ml) and Ag_2O (1.5 g) were added. The mixture was stirred for 72 hr in the dark at room temperature, then Ag_2O was removed by filtration. The filtrate was extracted with $CHCl_3$ three times, then the combined $CHCl_3$ extract was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was redissolved in DMF (1 ml) and the process of permethylation was repeared again. The product after concentration of the final $CHCl_3$ extract was a pale yellow solid (permethylated I) whose IR spectrum showed no OH absorption.

Mild Alkaline Methanolysis (Deacylation) of Permethylated I——Permethylated I was left overnight in saturated methanolic Na_2CO_3 (10 ml) at room temperature. The mixture was then neutralized with dil. HCl, evaporated to dryness *in vacuo* and the residue was treated with a small volume of MeOH. The MeOH–soluble portion was concentrated and then a small volume of petroleum ether was added to the residue. A material insoluble in the mixture was separated by filtration.

Identification of Dimethyl Succinate——After removal of the solvent by evaporation *in vacuo* below 40°, the petroleum ether–soluble layer was subjected to GC–MS. GC was performed on a glass column packed with 1% OV-1 on Gaschrom Q at a column temperature of 108°. The MS spectrum was recorded at an inlet temperature of 250°, ionizing potential of 50 eV, ionizing current of 60 A and ion source temperature of 290°. Only one peak was detected on the chromatogram (t_R 2.6) and characteristic fragments appeared in the MS at *m/e* 115 (M^+-OCH_3), 59 ($CH_3OC{=}O-$), 31 (OCH_3), 27 (C_2H_3) and 15 (CH_3). The t_R and fragmentation were identical with those of an authentic sample of dimethyl succinate.

Preparation and Identification of 2,3,4-O-Trimethyl-D-glucose——The petroleum ether insoluble material from the mild alkaline methanolysate (deacylated permethylated I) was hydrolyzed with 2 N H_2SO_4 under the conditions used in the case of I. After removal of a precipitated oily solid by filtration, the cooled acid hydrolysate was extracted with $CHCl_3$. The $CHCl_3$ extract was evaporated to dryness *in vacuo* and the partially methylated glucose in the residue was analyzed by PPC (R_{TMG}[23] 0.86 in solv. G) and TLC (R_{TMG} 0.84). Its R_{TMG} value was the same as that of co-developed authentic 2,3,4-tri-O-methyl-D-glucose prepared from permethylated gentiobiose and the identification was confirmed by GC–MS after conversion to the corresponding partially methylated glucitol acetate by reduction with $NaBH_4$ followed by acetylation with acetic anhydride. The partially methylated alditol acetate thus obtained was analyzed by gas chromatography using glass column (2 m×3 mm *i.d.*) containing 1% OV-17 on Gaschrom Q (80—100 mesh) with isothermal column temperature at 180°. It was identified as 2,3,4-tri-O-methyl-1,5,6-triacetyl-glucitol based on the coincidence of its t_R (2.49) relative to 2,3,4,6-tetra-O-methyl-1,5-diacetyl-glucitol with that of an authentic sample of 2,3,4-tri-O-methyl-1,5,6-tri-acetyl-glucitol. Mass spectra were recorded at an inlet temperature of 300°, ion source temperature of 300°, ionizing potential of 70 eV and ionizing current of 60 amp. The characteristic primary mass fragmentation (*m/e*) and relative intensity (%) of 2,3,4-tri-O-methyl-1,5,6-triacetylglucitol were as follow: *m/e* 42(100), 45(26), 71(18), 75(14), 87(59), 99(62), 100(89), 117(84), 129(47), 159(9), 161(12), 174(7), 189(12), 205(11), 235(5).

Acknowledgement The authors wish to express their thanks to Prof. Seiichi Yoshida of Tokyo Metropolitan University and Prof. Masao Hasegawa of Tamagawa University for helpful discussions and to Prof. Kunio Iwatsuki of Kyoto University for collecting *Cyathea contaminans* COPEL. in the Philippines. They also thank Prof. Naokata Morita of Toyama Medical and Pharmaceutical University for performing a micro-scale mixed melting point test of IV with his authentic kaempferol-4′,3,5-trimethyl ether. Thanks are also due to Drs. Shigeo Ohta and Takao Yokota of Tokyo University, Dr. Ryohshi Ishiwatari of Tokyo Metropolitan University and Dr. Yuji Kamiya of The Institute of Physical and Chemical Research for spectral (IR, PMR, GC–MS, MS) measurements.

23) R_{TMG}: mobility relative to 2,3,4,6-tetra-O-methyl-D-glucose.