Isolation of Camelliaside C from "Tea Seed Cake" and Inhibitory Effects of Its Derivatives on Arachidonate 5-Lipoxygenase

Toshikazu SEKINE,[a] Yasuo ARAI,[a] Fumio IKEGAMI,*,[a] Yuichi FUJII,[b] Shoichiro SHINDO,[b] Toshihiko YANAGISAWA,[b] Yuko ISHIDA,[b] Siriporn OKONOGI,[c] and Isamu MURAKOSHI[a]

Faculty of Pharmaceutical Sciences, Chiba University,[a] Yayoi-cho 1–33, Inage-ku, Chiba 263, Japan, Tsumura Research Institute for Pharmacology,[b] Yoshiwara 3586, Ami-machi, Ibaraki 300–11, Japan, and Faculty of Pharmacy, Chiang Mai University,[c] Chiang Mai 50002, Thailand. Received November 26, 1992

A new flavonol glycoside, camelliaside C, was isolated from "tea seed cake" prepared from the defatted seeds of *Camellia sinensis* O. KUNTZE. The structure was determined as kaempferol 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside by spectroscopic methods (FAB-MS, UV, IR, ^{1}H- and ^{13}C-NMR) and the enzymatic transformation of camelliaside C to astragalin. Camelliaside C showed an inhibitory effect on the arachidonate 5-lipoxygenase of RBL-1 cells (IC_{50}: 1.4×10^{-4} M) as did camelliaside A and B isolated from the same product.

Keywords camelliaside C; arachidonate 5-lipoxygenase inhibitor; tea seed cake; *Camellia sinensis*; Theaceae

In a previous paper,[1] we have reported the isolation and structural determination of new flavonol glycosides, camelliaside A and B, from the "tea seed cake." This folk medicine used traditionally for skin diseases in tropical regions of Asia originates from the defatted seeds of tea (*Camellia sinensis* O. KUNTZE; *Thea sinensis* L. [Theaceae]).

During our ongoing studies on the biologically active products in medicinal plants,[2,3] further investigation of the same flavonoidal fraction of this folk medicine led to the isolation of a new flavonol diglycoside, camelliaside C, as a minor constituent.

This paper presents the isolation and structural determination of camelliaside C, and also describes the inhibitory effects of some flavonoidal derivatives on the arachidonate 5-lipoxygenase of RBL-1 cells.

Results and Discussion

The crushed "tea seed cake" was extracted with hot water and the aqueous extract was partitioned with ethylacetate and *n*-BuOH, respectively, as previously reported.[1] The *n*-BuOH fraction was chromatographed on Diaion HP-20, and then on Sephadex LH-20 with an H_2O–MeOH mobile phase, with TLC monitoring to give several flavonoidal fractions. In these fractions, camelliaside A and B were found to be major components.[1] During the purification of these compounds by re-chromatography, we noticed an unidentified minor component (compound **1**) besides camelliaside B on HPLC. This compound was finally isolated by repeated preparative HPLC [Inertsil ODS-2, H_2O–MeOH (1 : 1)], although the yield of **1** was two orders of magnitude less than that of camelliaside A or B. It gave a positive reaction to Mg–HCl and was expected to be a flavonoidal glycoside from its chromatographic behavior.

Compound **1** was obtained as a yellow amorphous solid, $[\alpha]_D$ −45.9° (MeOH), and its molecular formula was determined to be $C_{27}H_{30}O_{15}$ by FAB-MS and distortionless enhancement by polarization transfer (DEPT) measurements. It showed infrared absorption maxima at 3350 (br, OH), 2930 (C–H), 1660 (C=O), 1600 (C=C), and 1060 (C–O) cm^{-1}. On the basis of the UV spectral data, showing bathochromic shifts with diagnostic reagents, **1** was considered to be 3-*O*-monosubstituted kaempferol.[4,5] On acid hydrolysis, **1** liberated a kaempferol as an aglycone along with a glucose and a galactose as sugar components. Compound **1** showed a *quasi*-molecular ion $[M+H]^+$ at *m/z* 611 in the positive ion FAB-MS. In addition, two fragment ion peaks were observed at *m/z* 449 due to $[M+H-\text{hexose}]^+$ and at *m/z* 287 due to $[M+H-2\times\text{hexose}]^+$. The total acetate of **1** revealed a peak at *m/z* 331 due to a terminal hexose and another at *m/z* 286 due to an aglycone in its EI-MS. On the basis of this evidence, **1** was thought to be kaempferol 3-*O*-diglycoside, consisting of two hexose units.

In the ^{1}H-NMR spectrum for **1**, a set of 2H aromatic doublet peaks [δ 8.03 (H-2′, 6′), 6.90 (H-3′, 5′)] and two 1H singlet peaks [δ 6.35 (H-8), 6.15 (H-6)] were observed along with two anomeric hydrogen peaks [δ 5.40 (1H, d, J=7.3 Hz, Glc H-1), 4.78 (1H, d, J=6.2 Hz, Gal H-1)]. The ^{13}C-NMR spectrum showed 25 carbon signals due to the kaempferol skeleton and two monosaccharide units. The C-2 of the glucopyranosyl moiety resonated downfield 7.4 ppm, compared with the reported value of δ 74.8 ppm for methyl-β-D-glucopyranoside,[6] while all galactopyranosyl carbons resonated at normal positions, indicating that the terminal galactopyranosyl moiety is linked to the C-2 of the inner glucopyranosyl residue by an interglycosidic bond.

The structure of **1** was finally established by the following enzymatic study: Enzymatic hydrolysis of **1** with cellulase in sodium acetate buffer (pH 5) at 37 °C for 14 d afforded a partial hydrolysate (**2**) as a single product (Chart 1). The structure of **2** was identified as kaempferol 3-*O*-β-D-glucopyranoside (astragalin) by direct comparison with the physico-chemical and chromatographic data ($[\alpha]_D$, ^{1}H- and ^{13}C-NMR, HPLC analysis) of an authentic sample.[7] In addition, the physico-chemical data of a partial hydrolysate from the reaction of camelliaside A with crude hesperidinase are identical to those of **1**. From these results, the structure of **1** was established as kaempferol 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside. It should be clear that **1** is a natural product and not an artifact arising from camelliaside A during extraction. This is because no peak due to **1** was detected on heating the camelliaside A solution in the same manner as in the extraction method, and also because the extraction of "tea seed cake" in 80% MeOH at room temperature showed a peak due to **1** on HPLC. A

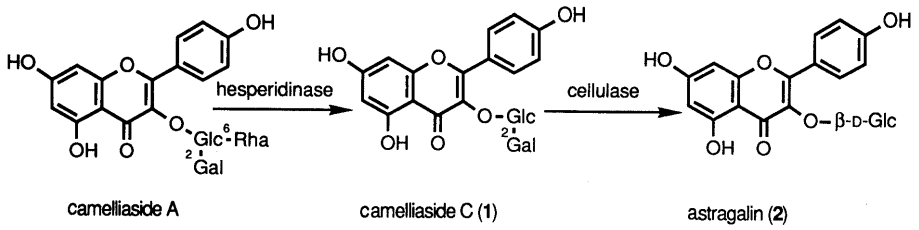
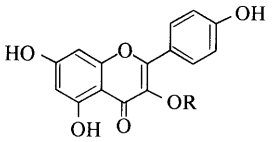


Chart 1

TABLE I. Inhibitory Effects of Flavonoidal Derivatives on Arachidonate 5-Lipoxygenase of RBL-1 Cells



R = Glc(6)–Rha(2)–Gal : camelliaside A
R = Glc(6)–Rha(2)–Xyl : camelliaside B
R = Glc–Gal : camelliaside C
R = H : kaempferol
R = Glc(6)–Rha : nicotiflorin
R = Glc(2)–Xyl : leucoside
R = Glc : astragalin

Compound	IC_{50} (M)
Kaempferol	1.0×10^{-6}
Camelliaside A	3.9×10^{-4}
Camelliaside B	1.0×10^{-3}
Camelliaside C	1.4×10^{-4}
Nicotiflorin	1.2×10^{-4}
Leucoside	2.9×10^{-4}
Astragalin	2.6×10^{-4}
Rutin	1.2×10^{-5}

careful literature survey revealed that this is the first report of camelliaside C (**1**) from a natural source.

It is well documented that flavonoids exhibit a variety of biological actions such as an anti-allergic[8,9] and an anti-inflammatory activity.[10-13] It has been also reported that polysaccharides[14] and a number of phenolic compounds[15,16] show anti-inflammatory activity. With this in mind, camelliaside C and its derivatives (camelliaside A and B, nicotiflorin, leucoside, astragalin and kaempferol as their aglycones, and rutin) were tested for inhibitory effects on arachidonate 5-lipoxygenase (Table I). The inhibitory effect was measured as previously reported[2a] based on the methods of Jakschik and Lee,[17] and Steinhoff *et al.*[18] IC_{50} values of camelliaside A and C, nicotiflorin, leucoside and astragalin were in the range of 1—4×10^{-4} M, while camelliaside B showed a lower activity (IC_{50} was 1×10^{-3} M). In contrast, the inhibitory effects of kaempferol (IC_{50}: 1.0×10^{-6} M) and rutin (IC_{50}: 1.2×10^{-5} M) were higher by one to two orders of magnitude than those of the camelliasides. The inhibitory effect of kaempferol was almost the same as that of a selective 5-lipoxygenase inhibitor, AA861,[19] but one order of magnitude less than that of a microbial metabolite, KF8940.[20] From these results, it was suggested that the structural difference in the type and the number of sugar components of these kaempferol glycosides was not closely related to the magnitude of the inhibitory effect on arachidonate 5-lipoxygenase. However, it seems likely that the flavonoidal skeleton—containing phenol goups, but not a sugar moiety—contributes to this inhibitory activity.

Flavonoids as taxonomic markers in *Camellia* species are considered to be one of the most important secondary metabolites from a chemotaxonomical point of view.[21] Up to now, more than thirty flavonoidal derivatives have been reported from tea (the literature is cited in ref. 1). Finger *et al.*[22] has recently isolated new flavonol triglycosides from tea leaves and determined the amount of these glycosides in Assam, Ceylon, Russian, Gorgian and Chinese teas. Much attention is being focused on the flavonoidal derivatives in teas as anti-asthmatic and anti-inflammatory agents.

Experimental

General The instruments used to obtain physico-chemical data and the experimental conditions for chromatography were the same as those described in our earlier paper.[1]

Isolation of Camelliaside C (1) "Tea seed cake" (1 kg) was crushed and macerated with boiling water (5 l) for 30 min. The aqueous extract was concentrated *in vacuo* to about 300 ml and partitioned with EtOAc (400 ml × 3) and *n*-BuOH (500 ml × 5). After evaporation of the solvent, an *n*-BuOH extract (132 g) was obtained. A portion of the *n*-BuOH extract (58 g) was chromatographed on Diaion HP-20 (1 kg) and eluted with H_2O (6.6 l), 50% MeOH (11 l) and MeOH (11 l), respectively. The 50% MeOH eluate fraction (3 g) was then rechromatographed on Sephadex LH-20 (100 g) using a gradient solvent system (H_2O–MeOH) to give five fractions (A—E). Fraction A (camelliaside A-rich fraction, 118 mg); fr. B (camelliaside A, 656 mg); fr. C (mixture of camelliaside A and B, 321 mg); fr. D (camelliaside B, 291 mg); fr. E (camelliaside B-rich fraction, 183 mg). Fraction E was then repeatedly subjected to prep. HPLC (Inertsil ODS-2, with H_2O–MeOH (1 : 1, v/v) to give camelliaside C (**1**, 2.5 mg). The *Rf* values on silica-gel TLC of camelliaside B and C were 0.40 and 0.52, respectively, using *n*-BuOH : MeOH : H_2O (4 : 1 : 4, upper phase) mixture as the developing solvent system.

Camelliaside C (1) Yellow amorphous solid. mp 200—202 °C $[\alpha]_D$ −45.9° ($c = 0.025$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 348.0 (4.19), 265.8 (4.30); (NaOH) 396.4, 324.4, 273.4; ($AlCl_3$) 394.4, 350.2, 303.2, 272.8; ($AlCl_3$ + HCl) 393.4, 346.4, 301.8, 273.8; (NaOAc) 369.0, 301.8, 272.8; (NaOAc–H_3BO_3) 350.0, 266.0, 210.2. IR ν_{max} cm^{-1}; 3350, 2930, 1660, 1600, 1060, 890, 820. Positive-ion FAB-MS [glycerol + *m*-nitrobenzylalcohol] *m/z* (rel. int.); 611 (10) $[M+H]^+$, 449 (4) $[M-Gal+H]^+$, 287 (40) [kaempferol + H]$^+$; [glycerol + *m*-nitrobenzylalcohol + NaCl]; 633 (26) $[M+Na]^+$. ^{1}H-NMR δ: 8.03 (2H, d, J = 8.8 Hz, H-2′, 6′), 6.90 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.35 (1H, s, H-8), 6.15 (1H, s, H-6), 5.40 (1H, d, J = 7.3 Hz, Glc H-1), 4.78 (1H, d, J = 6.2 Hz, Gal H-1), 3.8—3.2 (12H, m). ^{13}C-NMR δ: 158.5, 134.9, 179.6, 163.0, 100.1, 166.5, 94.9, 158.9, 105.6, 122.8, 132.4, 166.3, 161.5 (kaempferol; from C-1 to C-10 and from C-1′ to C-4′); 101.1, 82.2, 78.1,[a] 71.2,[b] 78.1,[a] 62.4[c] (glucose; from C-1 to C-6); 104.5, 75.4, 77.8, 71.0,[b] 77.8,[a] 62.5[c] (galactose; from C-1 to C-6). *a*—*c*) Assignments may be reversed.

Acid Hydrolysis of 1 Compound **1** (1 mg) in 3% H_2SO_4 (0.5 ml) was heated at 100 °C for 30 min. Extraction with EtOAc gave kaempferol as yellow needles; mp 274—276 °C. The water layer, neutralized with 5% NH_4OH, was concentrated *in vacuo*. The residue was trimethylsilylated with TMSCl and analyzed by GLC as described previously.[1]

Enzymatic Hydrolysis of 1 Compound **1** (20 mg) with cellulase (Onozuka R-10, 20 mg, Yakult Co., Ltd. Japan) in sodium acetate buffer (pH 5) was incubated at 37 °C for 14 d. The reaction mixture was diluted with H_2O and extracted with *n*-BuOH. The *n*-BuOH extract was subjected to silica-gel column chromatography with $CHCl_3$–MeOH–H_2O (6 : 4 : 1,

v/v) as the eluent to give astragalin (**2**, 5.3 mg); yellow needles (MeOH), mp 177—178 °C, (ref. 7, mp 175—177 °C). $[\alpha]_D$ −15.8° (MeOH, $c=0.04$). UV λ_{max}^{MeOH} nm (log ε): 348.7 (4.20), 265.5 (4.35). ^{1}H-NMR (DMSO-d_6) δ: 8.04 (2H, d, $J=8.8$ Hz, H-2′, 6′), 6.88 (2H, d, $J=8.8$ Hz, H-3′, 5′), 6.44 (1H, d, $J=2.0$ Hz, H-8), 6.21 (1H, d, $J=2.0$ Hz, H-6), 5.45 (1H, d, $J=7.4$ Hz, Gal H-1). ^{13}C-NMR (DMSO-d_6) δ: 159.9, 133.1, 177.4, 155.9, 98.6, 164.1, 93.6, 156.2, 103.9, 120.8, 130.8, 115.0, 159.9, 115.0, 130.8 (kaempferol; from C-1 to C-10 and from C-1′ to C-4′), 100.8, 74.1, 76.3, 69.8, 77.4, 60.8 (glucose; from C-1 to C-6).

Enzymatic Transformation of Camelliaside A into 1 A mixture of camelliaside A (50 mg) and crude hesperidinase (50 mg, Tanabe Pharm. Co., Ltd. Osaka, Japan) in citrate–phosphate buffer (pH 5, 5 ml) was incubated at 37 °C for 5 h. The reaction mixture was diluted with H_2O and extracted with *n*-BuOH. The *n*-BuOH extract was chromatographed on Sephadex LH-20, using H_2O as the eluent to give **1** (29 mg).

Biological Activity The inhibitory effects of flavonoidal derivatives on arachidonate 5-lipoxygenase of RBL-1 cells were measured as described previously[2a] based on the methods of Jakschik and Lee,[17] and Steinhoff *et al.*,[18] and then IC_{50} values of the test compounds were determined.

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