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Isolation and synthesis of TNF-α release inhibitors from Fijian kawa *(Piper methysticum)*

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Summary

Two unique evidence that cancer incidence rates in Fiji were unusually low, compared with those of another Pacific islands and that green tea beverage is an acknowledged cancer preventive in Japan, allowed us to study a local beverage in Fiji, kawa (kava kava) or yangona *(Piper methysticum)* belonging to Piperaceae. We isolated five known kawapyrones (kavapyrones) (1~5) and a new additional kawapyrone, 7,8-epoxyyangonin (6), from kawa MeOH extract and subjected them to TNF-α (tumor necrosis factor-α) release assay from BALB/3T3 cells treated with okadaic acid, a tumor promoter. 5,6-Dehydrokawain (desmethoxyyangonin)(1) and yangonin (4) significantly inhibited TNF-α release with IC_{50} values of 17 μM and 40 μM; a potency as great as (–)-epigallocatechin gallate (EGCG) isolated from green tea extract. Among the experiments with 1~5, dihydrokawain (2) was unique in showing the strongest inhibitory activity against TNF-α release in mice, but the weakest activity in the cells. We synthesized 5,6-dehydrokawain (1) and yangonin (4) *via* three steps from the dianion of ethyl acetoacetate achieving a good yield and determined their conformations by high resolution NMR and x-ray crystallographic analysis.

Key words: Fijian kawa, *Piper methysticum*, kawapyrone, desmethoxyyangonin, yangonin, TNF-α release inhibitors, total synthesis

Introduction

Tumor necrosis factor-α (TNF-α) known as cachectin as well was at first discovered by its antitumor activity in 1975. But, TNF-α is now recognized as one of the most pleotropic cytokines acting as a host defence factor in immunologic and inflammatory responses (Vassalli, 1992; Tracy and Cerami, 1994; Habtemariam, 2000). TNF-α is also effective on vascular endothelium which upregulates various cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leucocyte adhesion molecule-1 (ELAM-1) (Bevilacqua et al., 1994). The expression of these adhesion molecules and their counter-receptors on leucocytes result in the adhesion and extravasation of white blood cells in the process of inflammation. It is generally accepted that overproduction of TNF is related to development of various diseases. We previously reported that okadaic acid, a tumor promoter mimics TNF-α/interleukin-1 (IL-1), in inducing phosphorylation of the same proteins, and that TNF-α also induces similar expression of early-response genes in human fibroblasts, and activate NF-κB in Jurkat cells (Guy et al., 1992, Fujiki and Suganuma, 1993). Applying the concept of okadaic acid pathway of tumor promotion in human carcinogenesis, we found a possible link between tumor promoters and TNF-α (Fujiki and Suganuma, 1993). A single application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or okadaic acid induced TNF-α gene expression in mouse skin (Suganuma et al. 1996), and treatment with TPA or okadaic acid induced TNF-α re-

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lease from various cells including BALB/3T3, HL-60 and KATO-III cells (Fujiki and Suganuma, 1993). Furthermore, we demonstrated using TNF-α-deficient mice that TNF-α is an endogeneous tumor promoter and a central mediator of cancer development (Suganuma et al., 1999). As for green tea beverage, we found that (–)-epicatechin gallate (ECG), (–)-epigallocatechin gallate (EGCG), and (–)-epigallocatechin (EGC) dose-dependently inhibited TNF-α release from a human stomach cancer cell line, KATO-III cells treated with okadaic acid (Fujiki, 1999; Okabe et al., 1999). Since various structurally different cancer inhibitors, such as 9-*cis*-retinoic acid, sulindac, the active form of vitamin D_3, and tamoxifen, also inhibited TNF-α release (Suganuma et al., 1996), we think that the reduction of levels of TNF-α and similar cytokines in the tumor development process is the common, key criterion for cancer inhibitors and cancer-preventive agents. Cryptoporic acid E (Asakawa et al., 1992, Hashimoto et al., 1989, 1998) isolated from the fungus *Cryptoporus volvatus* inhibited tumor-promotion of okadaic acid in a two-stage carcinogenesis experiment on mouse skin and also inhibited mouse TNF-α release from BALB/3T3 cells, while cryptoporic acid D (Asakawa et al., 1992; Hashimoto et al., 1989, 1998) isolated from the same fungus enhanced both tumor promotion and TNF-α release from the same cells. Based on these studies, we think that TNF-α is a reasonable biomarker for tumor promotion and/or tumor inhibition.

In 1985, B. E. Henderson's group (Henderson et al.) reported unusually low rates of many cancers, such as stomach, breast, lung and prostate in Fijian Melanesians and Indians, compared with those of Polynesians in Micronesia and New Caledonia. However, the reasons for this difference were not elucidated. It is well known that people in Fiji are used to drink a local beverage, kawa (kava kava) or yangona (*Piper methysticum* Forst) belonging to *Piperaceae* (Keller et al., 1963). In particular, people in Fiji use kawa extract as analgesic and traditional medicine (Singh, 1992; Singh and Blumenthal, 1997). The kawapyrones isolated from kawa are thought to be pharmacologically active compounds associated with anxiolytic (Holm et al., 1991), anticonvulsive (Kretzschmar et al., 1969, 1970), analgestic (Jamieson and Duffied, 1990), central muscle relaxing (Meyer and Kretzschmar, 1966), $GABA_A$ receptor ligand binding (Boonen and Häberlein, 1998) and cyclooxygenase enzyme inhibitory activity (Wu et al., 2002).

During our investigation on cancer preventing agents derived from natural sources, we became interested in isolation of cancer preventing compounds from kawa . This paper reports the isolation and structural elucidation of a new kavapyrone, 7,8-epoxyyangonin (**6**), TNF-α release inhibitory activity and chemical synthesis of kavapyrones.

Materials and Methods

General experimental procedures

IR spectra were measured on a Jasco FT-IR 500 spectrophotometer. ^1H and ^{13}C NMR were recorded on Varian Unity 600 (^1H; 600 MHz, ^{13}C; 150 MHz) or Varian Unity 200 (^1H; 200 MHz, ^{13}C; 50 MHz) spectrometers. The solvent used for NMR spectra was $CDCl_3$. Mass spectra were measured on either JEOL JMS HX-100 or a JEOL AX-500 spectrometer. The specific rotation and the UV spectra were taken on a JASCO DIP-140 polarimeter and a Hitachi U-3000 spectrometer, respectively. Silica gel 60 for column chromatography was purchased from Merck.

TNF-α release inhibitory assay by kawapyrones *in vitro*

BALB/3T3 cells (2×10^5/ml) were preincubated with various concentrations of kawapyrones for 1 h, and then treated with 0.2 mM okadaic acid for another 24 h, resulting in induction of TNF-α release. The concentration of released TNF-α in the medium was measured using an enzyme-linked immunosorbent assay (ELISA) kit for mouse TNF-α (Genzyme, Cambridge, MA), as described previously (Komori et al., 1993). Okadaic acid-induced TNF-α release without pretreatment was expressed as 100%. The results were means of the two experiments. BALB/3T3 cells provided by the Japanese Cancer Research Resources Bank, Tokyo,were cultured in MEM medium containing fetal bovine serum.

TNF-α release inhibitory assay *in vivo*

BALB/cAnNCrj male mouse was purchased at 6 weeks of age, and the mouse within 30g of body weight was used. Each kawapyrone and kawa powder, which was prepared from air dried root of *Piper methysticum* and ground mechanically were suspended with carboxymethyl cellulose sodium salt (CMC-Na) at a concentration of 40 mg/kg. The lipopolysaccharide (LPS) solution was prepared in the physiological saline at 250 μg/ml. Each sample was subjected to intraabdominal administration of mice (n = 6) at 10 mg/kg. The control group was similarly conducted in mice by intraabdominal administration at 10 ml/kg capacity of CMC-Na. After *i.p.* administration of kawapyrones and kawa powder, the LPS solution (0.2 ml; 50 mg/mouse) was given intracecally. Blood was collected from mouse orbit for 90 minute after treatment, and then left for 1 hr at room temperature. Serum was collected after centrifugation at 11000 r.p.m. for 5 min, followed by the procedure of the ELISA kit.

Extraction from *Piper methysticum* root

Dried and ground root (1.0 kg) of *Piper methysticum* collected in Fiji were extracted with MeOH (3 l) for 16

days at rt . After filtration, the brown colored solution was evaporated *in vacuo* to afford a brown residue (152.0 g), which was partitioned between H_2O and EtOAc. The EtOAc layer was evaporated *in vacuo* to afford a residue (76.17 g) (m HPLC of EtOAc extract). The residue was further subjected to column chromatography (CC) on silica gel (1kg) with a solvent system of *n*-hexane-EtOAc increasing the amount of 5% portions EtOAc in a stepwise gradient to give 150 fractions (20 ml/fraction). Crude crystals (1.48 g) obtained from fr. 65~74 were recrystallized with EtOAc/*n*-hexane to afford 5,6-dehydrokawain (desmethoxyyangonin) (**1**) (Rezende et al., 1971) (1.218 g) as pale yellow needles. Crude crystals (3.78 g) from fr. 75~82 were recrystallized with EtOAc and *n*-hexane to afford dihydrokawain (**2**) (Dutta et al., 1972) (3.513 g) as colorless needles. Crude crystals (5.36 g) from fr. 86~94 were recrystallized from EtOAc/Et_2O to afford kawain (**3**) (Haensel et al. 1968; Dutta et al., 1972) (4.744g) as colorless prisms. Crude crystals (1.27 g) from fr. 97~102 were recrystallized with EtOAc/Et_2O to afford yangonin (**4**) (Chmielewska et al., 1958; Beak et al., 1962; Dutta et al., 1972) (0.847 g) as pale yellow prisms. Crude products (8.21 g) from fr. 103~115 were subjected to CC on silica gel (300 g) with a gradient solvent system of CHC_3-Et_2O increasing the amount of 5% portions Et_2O stepwise. Crude crystals (1.68 g) from 25% Et_2O-$CHCl_3$ eluate were recrystallized with EtOAc/Et_2O to afford yangonin (**4**) (1.322 g) as colorless needless. A crude crystal (1.68 g) from 30% Et_2O-$CHCl_3$ eluate was recrystallized with EtOAc/Et_2O to afford methysticin (**5**) (Klohs et al., 1959a, b; Dutta et al., 1972) (5.661 g) as pale yellow prisms. A crude product (385 mg) from fr. 138~142 was chromatographed on Sephadex-LH-20 with $CHCl_3$-MeOH=1:1 to afford 7, 8-epoxyyangonin (**6**) (59 mg) as colorless amorphous powders.

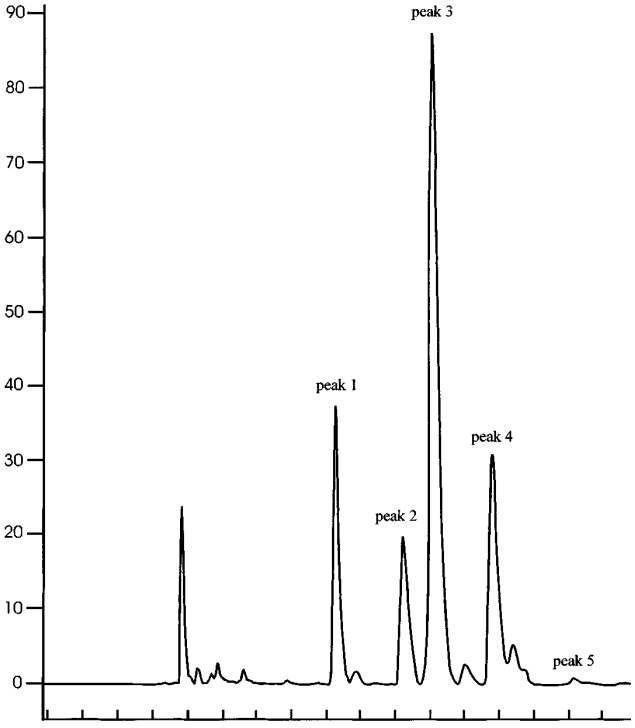
Table 1. ^{1}H NMR data of kawapyrones **4** and **6**[a].

	compd. **4**		compd. **6**	
	^{1}H	^{13}C	^{1}H	^{13}C
2		164.1 (*s*)		164.2 (*s*)
3	5.19 (*d*, 2.2)	88.3 (*d*)	5.22 (*d*, 2.2)	87.6 (*d*)
4		171.2 (*s*)		170.5 (*s*)
5	6.24 (*d*, 2.2)	110.4 (*d*)	5.73 (*d*, 2.2)	101.2 (*d*)
6		159.0 (*s*)	158.6 (*s*)	
7	6.44 (*d*, 15.7)	116.3 (*d*)	4.20 (*d*, 3.0)	42.9 (*d*)
8	7.45 (*d*, 15.7)	135.4 (*d*)	4.36 (*d*, 3.0)	45.5 (*d*)
1′		127.9 (*s*)		129.4 (*s*)
2′	7.44 (*d*, 8.8)	128.9 (*d*)	6.82 (*d*, 8.7)	128.4 (*d*)
3′	6.90 (*d* 8.8)	114.3 (*d*)	7.25 (*d*, 8.7)	113.8 (*d*)
4′		160.7 (*s*)		158.5 (s)
5′	6.90 (*d*, 8.8)	114.3 (*d*)	7.25 (*d*, 8.7)	113.8 (*d*)
6′	7.44 (*d*, 8.8)	128.9 (*d*)	6.82 (*d*, 8.7)	128.4 (*d*)
4-OMe	3.82 (*s*)	55.8 (*q*)	3.77 (*s*)	55.8 (*q*)
4′-OMe	3.81 (*s*)	55.3 (*q*)	3.69 (*s*)	55.1 (*q*)

[a] Chemical shifts from TMS in $CDCl_3$ and assignments from ^{1}H-^{1}H COSY, HMQC and HMBC spectra

7,8-Epoxyyangonin (6); 4-methoxy-6-[2-(4-methoxyphenyl)oxirane]-2H-pyran-2-one

White amorphous powder; $[a]_D^{18}$ +13.0° (c 1.51, $CHCl_3$); EI-MS: *m/z* 274 (M^+, 5%), 258 (100%), 230 (64%), 187 (66%); HR-MS: *m/z* 274.0851, $C_{15}H_{14}O_5$ requires 274.0841; FT-IR (KBr) $n_{max}cm^{-1}$: 2940, 1721 (C=O), 1645 (C=C), 1613, 1566, 1252 (C-O-C), 1181;^{1}H and ^{13}C NMR ($CDCl_3$) (Table 1).



Supplement. HPLC chromatogram of EtOAc extract prepared from Kawa *(Piper methysticum)*.
HPLC condition:
Column: Waters 5SL-II (SiO_2); Column size: 10 × 250 mm; Mobile phase: *n*-hexane:EtOAc = 1:1; Flow rate: 1.0 ml/min; Detection: UV 254 nm; Chart speed: 2.5 mm/min; Sample volume: 0.02 ml; a solution of kawa EtOAc extract (10 mg) in hexane:EtOAc = 1:1 (1 ml).
Retention time (Rt):
peak 1 (Rt. 34 min) = 5,6-Dehydrokawain **(1)** and (+)-Dihydrokawain **(2)**
peak 2 (Rt. 42 min): Yangonin **(4)**
peak 3 (Rt. 46 min) = (+)-Kawain **(3)**
peak 4 (Rt. 52 min): (+)-Methysticin **(5)**
peak 5 (Rt, 61 min): 7,8-Epoxyyangonin **(6)**

Chemical conversion of (+)-kawain (3) into 5,6-dehydrokawain (1)

A solution of (+)-kawain (**3**) (100 mg) in dry benzene (20 ml) was refluxed with DDQ (150 mg) for 2 hr. The reaction mixture was filtered over a short column packed with celite, and the solvent was evaporated *in vacuo* to afford the crude oil (262 mg), which was chromatographed on silica gel with *n*-hexane-EtOAc gradient, followed by recrystallization from EtOAc/n-hexane to afford 5,6-dehydrokawain (**1**) (88 mg; 88.8%) as colorless needles. mp 138–140 °C.

Catalytic reduction of (+)-kawain (3)

(+)-Kawain (**3**) (120 mg) was hydrogenated over 10% Pd-C (50 mg) in MeOH (5 ml) and EtOAc (10 ml) at r.t for 1 hr. After removal of the catalyst, the filtrate was concentrated *in vacuo*. The residue (125 mg) was subjected to CC on silica gel with *n*-hexane-EtOAc gradient to afford (+)-dihydrokawain (**2**) (109 mg; 90.1%) as colorless needles. mp 58–60 °C.

Synthesis of 4-hydroxy-6-(α-*trans*-styryl)-5,6-dihydro-2-pyrone (9)

The dianion of ethyl acetoacetate (2.30 g) was prepared in dry THF (50 ml) with 60% NaH (700 mg) and *n*-butyllithium solution (10 ml) at 0 °C according to the method of Huckin and Weiler (1971). A solution of cinnamaldehyde (**7**) (1.00 g) in dry THF (10 ml) was added dropwise for 10 min and stirred for 10 min at 0 °C. The reaction mixture was then added dropwise to ice water (300 ml) and stirred at rt for 3 hr. The alkaline solution was extracted with ether (3 × 100 ml). The aqueous phase was cooled with ice water and acidified to pH 1.0 with conc. HCl to afford a crystalline material, which was recrystallized from Et_2O/MeOH to afford 4-hydroxy-6-(α-*trans*-styryl)-5,6-dihydro-2-pyrone (**9**) (0.847 g; 78.4%) as colorless needles. mp 128–131 °C (decomp.) (Reffstrup and Boll, 1976).

Synthesis of (±)-kawain (11)

A solution of **9** (7.0 g) in dry acetone (150 ml) was mixed with dimethylsulfate (5.0 ml) and anhydrous K_2CO_3 (18 g). After a reaction mixture was reflux for 7 hr, the mixture was cooled and filtered. The filtrate was evaporated *in vacuo* to afford the crude crystal (7.25 g), which was recrystallized with Et_2O/MeOH to afford (±)-kawain (**11**) as colorless needles (4.931 g; 66.1%), 143–145 °C (Reffstrup and Boll, 1976).

Synthesis of 5,6-dehydrokawain (1)

A solution of (±)-kawain (**11**) (300 mg) in dry benzene (30 ml) was refluxed with DDQ (451 mg) for 2 hr. The reaction mixture was filtered over a short column packed with celite for 7 hr and evaporated *in vacuo* to afford the crude oil (725 mg) which was subjected to CC on silica gel with *n*-hexane-EtOAc gradient, followed by recrystallization from EtOAc/*n*-hexane to give 5,6-dehydrokawain (**1**) as colorless needles (245 mg; 82.3%), mp 138–140 °C (Rezende et al., 1971).

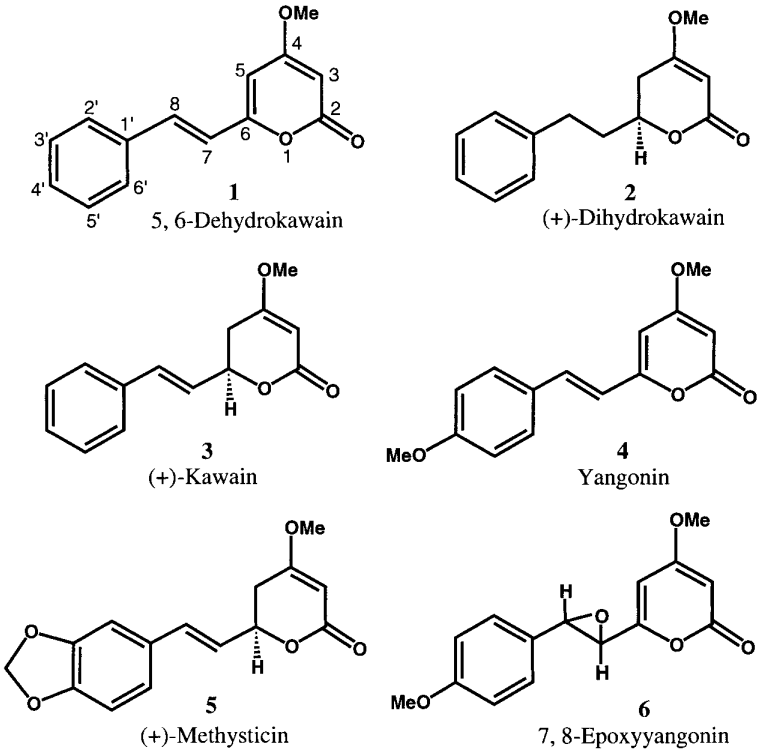


Fig. 1. Kawapyrones (**1–6)** isolated from *Piper methysticum.*

Synthesis of 4-hydroxy-6-(α-*trans*-4-methoxystyryl)-5,6-dihydro-2-pyrone (10)

The solution of *p*-methoxycinnamaldehyde (**8**) (9.00 g) in dry THF (30 ml) was added dropwise for 10 min to the dianion of ethyl acetoacetate (2.30 g), which was prepared in dry THF (100 ml) with 60% NaH (8.58 g) and *n*-butyllithium solution (117 ml) at 0 °C. The reaction mixture was treated by the same procedure as described above to afford 4-hydroxy-6-(α-*trans*-4-methoxystyryl)-5,6-dihydro-2-pyrone (**10**) as colorless needles (10.332 g; 75.6%), mp 127–130 °C (Reffstrup and Boll, 1976).

Synthesis of 5,6-dihydroyangonin (12)

A solution of **10** (5.0 g) in dry acetone (100 ml) was refluxed with dimethylsulfate (4.2 ml) and anhydrous K_2CO_3 (15 g) for 5 hr. The reaction mixture was treated by the same procedure as described above to afford 5,6-dihydroyangonin (**12**) as colorless needles (3.176 g; 60.1%), mp 120–123 °C (Reffstrup and Boll, 1976).

Synthesis of yangonin (4)

A solution of 5,6-dihydroyangonin (**12**) (300 mg) in dry benzene (30 ml) was refluxed with DDQ (450 mg) for 2 hr. The crude product was subjected to CC on silica gel with *n*-hexane-EtOAc gradient, followed by recrystallization from EtOAc/Et_2O to furnish yangonin (**4**) as yellow needles (245 mg; 82.4%), mp 153–155 °C (Chmielewska et al., 1958; Beak et al., 1962; Dutta et al., 1972).

The crystal data for yangonin (4)

$C_{15}H_{14}O_4$; Monoclinic; space group *Pc*; a = 7.323 (0) Å, b = 7.484 (0) Å, c = 23.761 (0) Å, b = 94.45 (0) Å, V = 1298 (0) $Å^3$, Z = 4, final *R* value was 0.044 for 1438 reflections. The structure was solved by direct method (Monte-Carlo Multan) and refined by full-matrix least-squares techniques. Diffraction data were obtained using a Mac Science MXC18 diffractometer at rt. All diagrams and calculations were performed using CRYSTAN (Mac Science, Japan).

Results and Discussions

Isolation of kawapyrones (1~6)

In order to isolate TNF-α release inhibitors from kawa, the MeOH extract from dried material of *Piper methysticum* was subjected to column chromatography using silica gel (n-hexane-EtOAc gradient and $CHCl_3$-Et_2O gradient) and Sephadex LH-20 ($CHCl_3$:MeOH = 1:1) to afford five known kawapyrones, 5,6-dehydrokawain (**1**) (isolated yield; 0.12% for dried powder), dihydrokawain (**2**) (0.35%), kawain (**3**) (0.47%), yangonin (**4**) (0.22%) and methysticin (**5**) (5.66%) along with a new kavapyrone, 7, 8-epoxyyangonin (**6**) (0.0059%) (Fig. 1).

Structural elucidation of 7,8-epoxyyangonin (6)

7,8-Epoxyyangonin (**6**) {$[\alpha]_D$ +13.04° ($CHCl_3$)} has a molecular formula of $C_{13}H_{14}O_3$ (M^+; *m/z* 274.0851) possessing one more oxygen than yangonin (**4**) as shown by high resolution EI mass (HR-EIMS) spectrum. The IR spectrum of **6** indicated the presence of an ester carbonyl group (1721 cm^{-1}) and a benzene ring (1613 cm^{-1}). The ^1H and ^{13}C NMR spectra (Table 1) of **6** in $CDCl_3$ were very similar to those of **4** except the chemical shifts of C-7 and C-8 . The ^1H and ^{13}C NMR spectra showed the presence of an epoxy ring [4.20 (1H, d, J = 3.0 Hz, H-7), 4.36 (1H, d, J = 3.0 Hz, H-8); 42.9 (d, C-7), 45.5 (d, C-8)]. The structure of **6** was deduced as 7, 8-epoxyyangonin by analysis of the 2D NMR spectra including HMBC and NOESY .

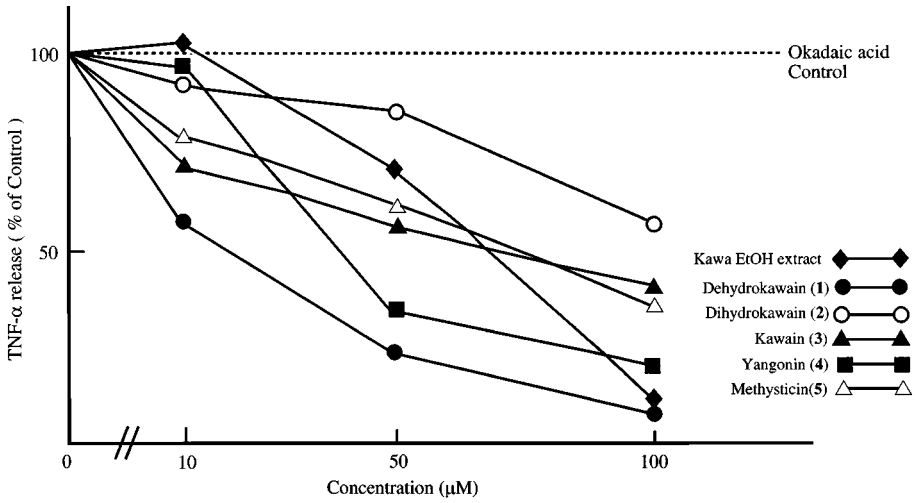


Fig. 2. Inhibition of TNF-α release by kawa-ethanol extract and kawapyrones **1–5** *in vitro*.

Inhibitory activity of TNF-α release from BALB/3T3 cells by kawapyrones

The ethanol extract of kawa dose-dependently inhibited TNF-α release from BALB/3T3 cells treated with okadaic acid as shown in Fig. 2. Five kavapyrones (**1**~**5**) inhibited TNF-α release from the same cell line. As shown in Fig. 2, TNF-α release inhibitory activities of 5,6-dehydrokawain (**1**) and yangonin (**4**) were much more stronger than those of compounds **2**, **3** and **5**. Among TNF-α inhibitory activities of compounds **1**~**3**, compound **1** most strongly inhibited TNF-α release from the cells. Thus, we suggest that TNF-α release inhibitory activity is associated with increase of the conjugated system of the kawapyrones. Compound **1** (IC_{50} 17 μM) significantly inhibited TNF-α release with a potency as great as (–)-epigallocatechin gallate (EGCG) (IC_{50} 20 μM) isolated from green tea extract.

TNF-α inhibitory activity of kawapyrones *in vivo*

Although TNF-α is mainly released from macrophages, TNF-α derived from fat cells is assumed to be related to insulin resistance. When chronic hyperglycemia was maintained in diabetic animals, TNF-α production was enhanced (Tanaka et al., 1992; Nishimura et al., 1997) Thus, crisis control of diabetes mellitus is considered by depressant drug of TNF-α with regards to diabetes and TNF-α. The TNF-α release inhibitory test of kawapyrones (**1**~**5**) and kawa powder was carried out *in vivo* using diabetic mice (n = 6). The production of TNF-α in blood of diabetic mice stimulated by lipopolysaccharide (LPS) was significantly suppressed by kawa powder and kawapyrones (**1**~**5**), while TNF-α in the control group was 494 ± 76 pg/ml, as shown in Figure 3. It is noteworthy that inhibitory activity of dihydrokawain (**2**) *in vivo*

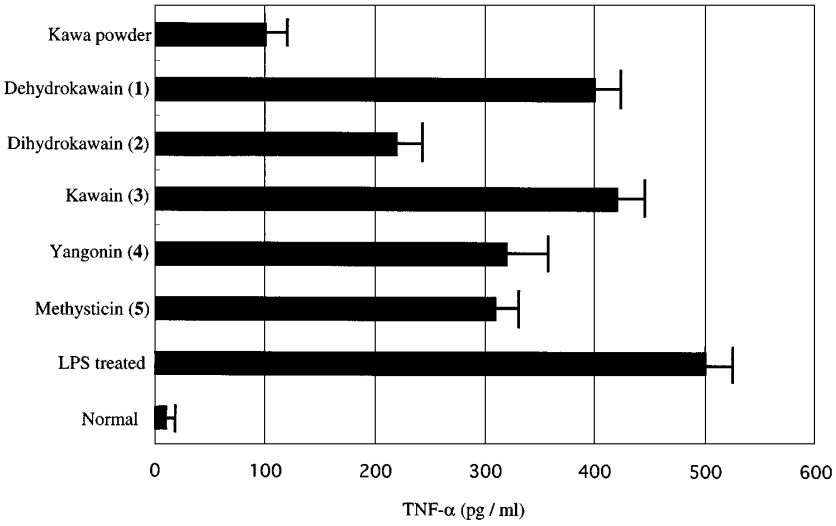


Fig. 3. TNF-α inhibitory activity (± standard deviation; n = 6) by kawapyrones **(1–5)** and kawa powder of LPS intraperitoneally administered mice.

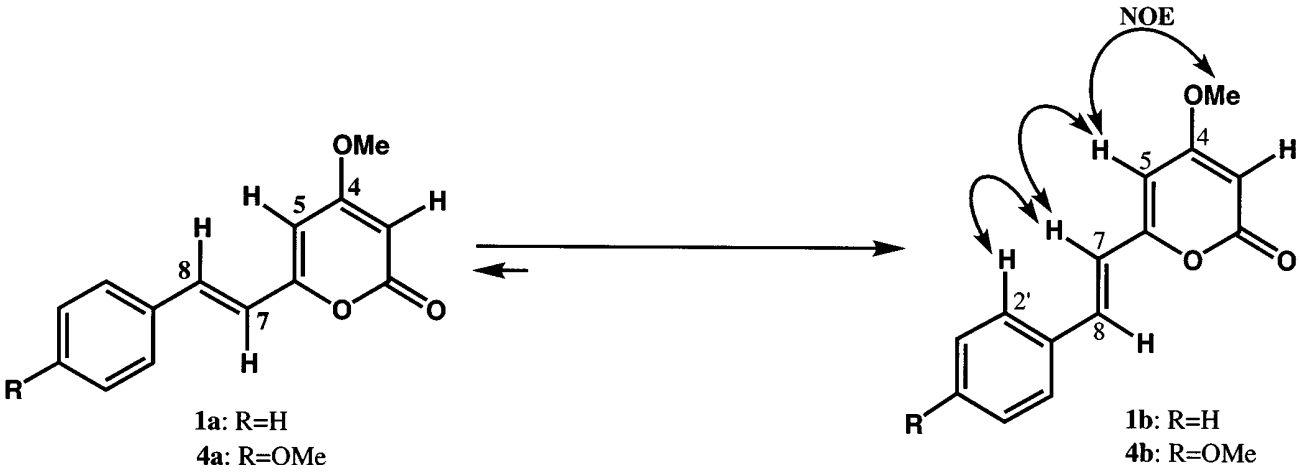


Fig. 4. Conformations of 5,6-dehydrokawain **(1)** and yangonin **(4)**.

was strongest among compounds **1**~**5**, whereas the activity *in vitro* was much lower than that of another kawapyrones. The difference in the activities between *in vivo* and *in vitro* is not easy to be elucidated. Considering synergistic effect of kawapyrones with each other and possible presence of compounds having more stronger activity, the kawa powder may be the beverage associated with the strongest activity. We could not carry out the biologically active test of 7,8-epoxyyangonin (**6**) isolated from kawa, because of insufficient amount available.

Chemical conversion of kawain (3) into 5,6-dehydrokawain (1) and dihydrokawain (2), and total synthesis of 5,6-dehydrokawain (1) and yangonin (4)

(+)-Kawain (**3**) which is a major component of *P. methysticum*, was converted into minor components, 5,6-dehydrokawain (**1**) and dihydrokawain (**2**) possessing the strongest TNF-α inhibitory activity *in vitro* and *in vivo*, respectively by chemical reactions. A solution of (+)-kawain (**3**) in benzene was refluxed with DDQ for 2 hr to afford desmethoxyyangonin (**1**) in a good yield (89.6%). Even if (+)-dihydrokawain (**2**) was refluxed with DDQ for further 48 hr, a reaction did not proceed and the starting material was recovered. Catalytic reduction of (+)-kawain (**3**) over 10%Pd-C in MeOH and AcOEt for 1 hr afforded dihydrokawain (**2**) in good yield (90.1%).

As *Piper methysticum ovinus* can not be obtained easily in Japan, the total synthesis of kawapyrones, 5,6-dehydrokawain (**1**) and yangonin (**4**) was conducted from a commercially available cinnnamaldehyde (**7**) and *p*-methoxycinnamaldehyde (**8**), and made it possible to test their biological activity *in vivo*. Reffstrup and Boll (1976) reported that kavapyrones, 5,6-dehydrokawain (**1**) and yangonin (**4**) were synthesized *via* four steps from the dianion of ethyl acetoacetate as shown in Scheme 1. Thus, aldol condensation of the dianion of ethyl acetoacetate with cinnamaldehyde (**7**) or *p*-methoxycinnamaldehyde (**8**) afforded 4-hydroxy-6-

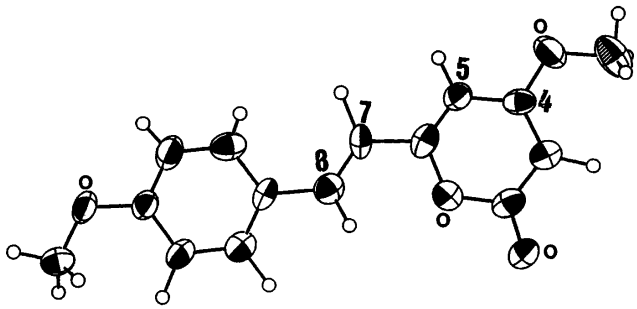
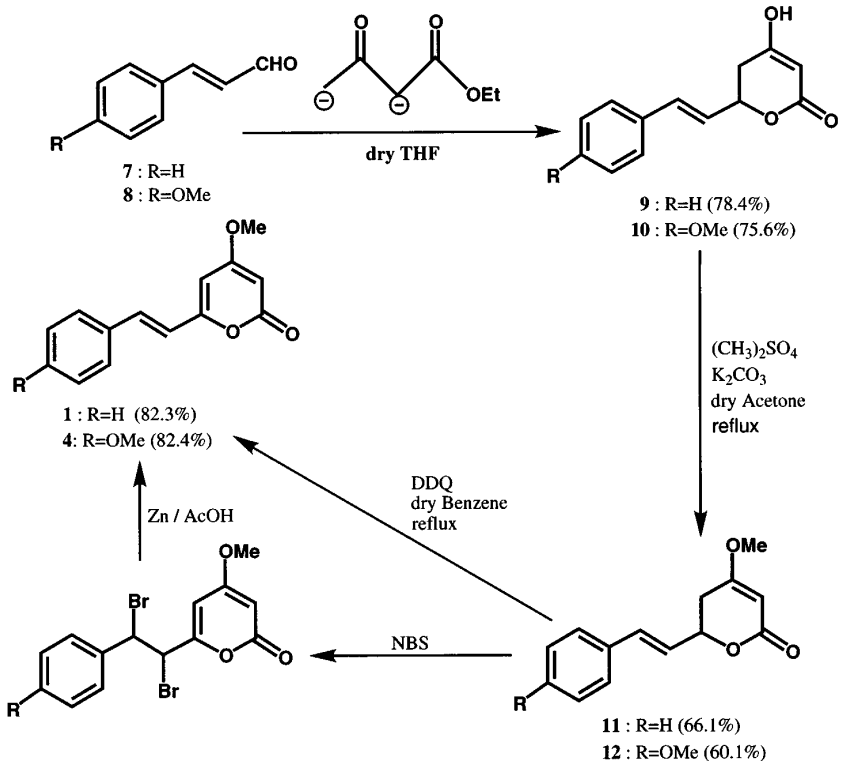


Fig. 5. X-ray crystallographic analysis (stereoview) of yangonin **(4)**.



Scheme 1. Synthetic pathway of 5,6-dehydrokawain **(1)** and yangonin **(4)**.

(α-*trans*-styryl)-5,6-dihydro-2-pyron (**9**) or 4-hydroxy-6-(α-*trans*-4-methoxystyryl)-5,6-dihydro-2-pyrone (**10**), followed by methylation with $(CH_3)_2SO_4$ to afford (±)-kawain (**11**) and 5,6-dihydroyangonin (**12**). Although compounds **11** and **12** were converted into 5,6-dehydrokawain (**1**) and yangonin (**4**) *via* two steps (i. Br_2; ii Zn/AcOH), the converting yield was relatively low (33%). We found that (+)-kawain (**3**) in benzene was refluxed with DDQ to afford compound **1** in good yield as described above. Compounds **11** and **12** obtained *via* two steps from the dianion of ethyl acetoacetate were converted into 5,6-dehydrokawain (**1**) and yangonin (**4**) *via* one step with DDQ in good yield (82.3% from **11**; 82.4% from **12**) as shown in Scheme 1.

Conformational analysis of 5,6-dehydrokawain (1) and yangonin (4)

Kagechika (1989a, b) and Yamakawa (1990) reported structure-activity relationship between differentiation-inducing activity on human promyelocytic leukemia cell line HL-60 and retinobenzoic acids such as stilbene-4-carboxylic acids, and found that the conformation of retinobenzoic acid was important for the activity. In order to examine the correlation between conformation and TNF-α release inhibitory activity, we analyzed conformations of 5,6-dehydrokawain (**1**) and yangonin (**4**) in solution and crystalline states. The conformations of **1** and **4** in the solution state were clarified by analysis of NOESY spectrum of two-dimensional NMR. In NOESY correlations of **1** and **4** (Fig. 4), the NOEs were observed between (i) H-5 and 4-OMe and (ii) H-5 and H-7. On the basis of the above mentioned results, the conformations of **1** and **4** in the solution state took **1b** and **4b** preferentially. The conformation of **4** in the crystalline state was determined as **4b** form by X-ray crystallographic analysis (Fig. 5). We concluded that the conformations of **1** and **4** preferentially took **1b** and **4b** than **1a** and **4a** in both solution and crystalline states.

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