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Antioxidant Prenylated Flavonoids from Propolis Collected in Okinawa, Japan

SHIGENORI KUMAZAWA,*,† REIKA UEDA,† TOMOKO HAMASAKA,† SYUICHI FUKUMOTO,‡ TAKUNORI FUJIMOTO,§ AND TSUTOMU NAKAYAMA†

Laboratory of Functional Food Science and COE Program in the 21st Century, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan, Pokka Corporation, 45-2 Juuniso, Kumanoshou, Kitanagoya, Aichi 481-8515, Japan, and Japan Propolis Conference, 6-27-12 Honmachi, Nakano, Tokyo 164-0012, Japan

Propolis is a resinous substance collected by honeybees from various plant sources. The composition of propolis depends upon the vegetation at the site of collection. We previously isolated four prenylated flavonoids from propolis collected in Okinawa, Japan. In this study, further fractionation of the extracts of Okinawan propolis resulted in the isolation of a new prenylated flavonoid, prokinawan, and four known compounds. The structure of prokinawan was determined by MS and NMR spectroscopic methods. Furthermore, the antioxidant activity using 1,1-diphenyl-2-picryl-hydrazyl radical scavenging and β-carotene bleaching systems was investigated. The present study proved that the position of the geranyl or prenyl groups on the flavonoid skeleton plays an important role in exhibiting antioxidant activity.

KEYWORDS: Propolis; Okinawa; prenylflavonoid; antioxidant; radical scavenging

INTRODUCTION

Propolis is a natural substance collected by honeybees from the buds and exudates of certain plants, and it is stored inside their hives. Propolis has been used in folk medicines from ancient times in many regions of the world (*1*). Recently, it has been reported to have various biological activities such as antibacterial (*2*), antiviral (*3*), antiinflammatory (*4*), and anticancer (*5*) properties. For this reason, propolis is thought to prevent diseases such as inflammation, heart disease, diabetes, and cancer (*6, 7*).

Propolis is composed of 50% resin (composed of flavonoids and related phenolic acids and regarded as the polyphenolic fraction), 30% wax, 10% essential oils, 5% pollen, and 5% various organic compounds (*8*). The composition of propolis depends upon the vegetation at the site of collection (*8, 9*). Because of the geographical differences, propolis from Europe, South America, and Asia has different chemical compositions (*8–16*). Propolis from Europe and China contains many kinds of flavonoids and phenolic acid esters (*8–14*). In contrast, the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids (*17–20*).

Fujimoto et al. (*21*) studied the components in propolis from different geographic origins of Japan and reported that propolis from Okinawa has constituents not present in propolis from other regions. Okinawa is the southern-most prefecture of Japan and has a subtropical climate. We isolated and identified four prenylated flavonoids (**5**–**7**, and **9**) from propolis collected in Okinawa (*22*).

Further fractionation of the extracts of propolis from Okinawa resulted in the isolation of a new prenylated flavonoid, prokinawan (**3**), and four known compounds (**1**, **2**, **4**, and **8**). Here, we describe the structure determination of **1**–**4** and **8** and the antioxidant evaluation using two assay systems: the free radical scavenging activity on 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and the inhibition of linoleic acid oxidation by β-carotene bleaching.

MATERIALS AND METHODS

General Experimental Procedures. Butylated hydroxytoluene (BHT), β-carotene, linoleic acid, trifluoroacetic acid (TFA), and Tween 40 were purchased from Wako Pure Chemicals Industries (Osaka, Japan). α-Tocopherol was purchased from Sigma (St. Louis, MO).

Melting point (mp) data were recorded with a Bibby SMP 3 micromelting point apparatus and are uncorrected. Optical rotation values were determined with a Jasco DIP-1000 digital polarimeter. UV spectra were obtained using a Hitachi U-2000 spectrometer, IR spectra were recorded by a Jasco FT/IR-550 spectrometer, and CD (circular dichroism) spectra were recorded by a Jasco J-600 spectrometer. ^{1}H and ^{13}C NMR spectra were measured by a Jeol JNM-α400 (400 and 100 MHz, respectively), using TMS (tetramethylsilane) as an internal standard. FAB (fast atom bombardment) mass spectra were obtained by a Jeol JMS-700 spectrometer, using glycerol as a matrix.

Extraction and Isolation of Constituents from Propolis. Propolis collected in Okinawa was supplied by Aragaki Beefarm Inc. (Okinawa,

* To whom correspondence should be addressed. Tel: +81-54-264-5523. Fax: +81-54-264-5523. E-mail: kumazawa@smail.u-shizuoka-ken.ac.jp.
† University of Shizuoka.
‡ Pokka Corporation.
§ Japan Propolis Conference.

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Table 1. ^{13}C and ^{1}H NMR Data for Compound **3** in Acetone-d_6

position	δ_C	δ_H
2	79.9	5.36 (1H, dd, J = 12.7, 2.9 Hz)
3	43.7	2.71 (1H, dd, J = 17.1, 2.9 Hz)
		3.12 (1H, dd, J = 17.1, 12.7 Hz)
4	197.3	
5	162.3	12.47 (–OH, s)
6	109.0	
7	164.8	
8	95.3	6.03 (1H, s)
9	161.9	
10	103.1	
1′	131.7	
2′	114.7	7.03 (1H, s)
3′	146.0	
4′	146.3	
5′	116.0	6.86 (1H, s)
6′	119.1	6.86 (1H, s)
1″	21.5	3.27 (2H, d, J = 7.3 Hz)
2″	123.2	5.25 (1H, t, J = 7.3 Hz)
3″	135.4	
4″	16.1	1.76 (3H, s)
5″	41.0	1.94 (2H, t, J = 6.9 Hz)
6″	23.4	1.48 (2H, m)
7″	44.3	1.40 (2H, m)
8″	70.1	
9″	29.7	1.17 (3H, s)
10″	29.7	1.17 (3H, s)

Japan). The dried propolis sample (50 g) was extracted with 500 mL of ethanol at room temperature for 12 h while stirring and then concentrated under reduced pressure to give a crude extract (39.7 g). The extract was mixed with 20 g of silica gel (Merck, Silica gel 60, Germany) and concentrated to give the dried silica gel. The dried silica gel was placed on the top of a silica gel column (450 mm × 50 mm i.d.) and eluted with hexane/EtOAc and MeOH to give 21 fractions: fr. 1, hexane/EtOAc (9:1) eluate; fr. 2, hexane/EtOAc (8:2) eluate; fr. 3, hexane/EtOAc (7:3) eluate; frs. 4–12, hexane/EtOAc (6:4) eluate; fr. 13, hexane/EtOAc (5:5) eluate; fr. 14, hexane/EtOAc (4:6) eluate; fr. 15, hexane/EtOAc (3:7) eluate; fr. 16, hexane/EtOAc (2:8) eluate; fr. 17, hexane/EtOAc (1:9) eluate; fr. 18, EtOAc eluate; and frs. 19–21, MeOH eluate. Each fraction was collected by 100 mL. The parts of the fraction eluted with a hexane–EtOAc gradient (4:6–1:9) were rechromatographed by preparative HPLC on a 250 mm × 20 mm i.d. ODS column (YMC, Japan) with 0.1% TFA in CH_3CN–H_2O (6:4) at a 9 mL/min flow rate to give **1** (73 mg), **2** (8 mg), **3** (9 mg), **4** (13 mg), **5** (307 mg), **6** (231 mg), **7** (96 mg), **8** (101 mg), and **9** (142 mg) in their elution order.

Prokinawan (3). White powder; mp 97–100 °C; $[\alpha]_D^{26}$ +0.2° (*c* 0.2, MeOH). UV (MeOH) λ_{max} (log ϵ) 287 (4.29) nm. IR (KBr): ν_{max} 3430, 2970, 2940, 1680 cm^{-1}. CD (MeOH): $[\theta]_{253}$ + 12010, $[\theta]_{292}$ − 47480, $[\theta]_{336}$ + 25890. ^{1}H and ^{13}C NMR data are shown in **Table 1**. HR-FAB-MS obsd *m*/*z* 443.2067, calcd 443.2070 $[M + H]^+$.

Quantitative Analysis. The dried ethanol extracts of the propolis of Okinawa were dissolved in methanol at 5 mg/mL. The extracts were then filtered with a PTFE 0.45 μm membrane filter (Pall, NY). Different concentrations (0.0625, 0.125, 0.25, 0.5, and 1 mg/mL) of the standard compounds (**5**–**7** and **9**) were prepared to construct the calibration curve. These compounds were analyzed by HPLC using a 250 mm × 2 mm i.d. Capcell Pak ACR 120 ODS column (Shiseido, Tokyo, Japan). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was 20–60% B (0–60 min), 60–100% B (60–80 min), and 100% B (80–90 min) and a flow rate 0.2 mL/min. UV 270 nm was used for detection. The concentration of each compound in the propolis of Okinawa was determined by using the linear calibration curves based on peak area. The addition and recovery experiment that used quercetin as an internal standard revealed over 90% recovery.

Free Radical Scavenging Activity on DPPH. This assay was carried out according to the method of Chen and Ho with some modifications (*23*). The reaction mixture contained 2 mL of ethanol, 125 μM DPPH, and test samples (3.125, 6.25, 12.5, 25.0, 50.0, and 100 μM). After 1 h of incubation at room temperature, the absorbance was recorded at 517 nm. The control solution contained only ethanol and DPPH. The sample concentrations were plotted against absorption, and IC_{50} values were determined. The antioxidant activity was expressed in terms of IC_{50} (μg/mL, concentration required to inhibit the DPPH radical formation by 50%). BHT, α-tocopherol, and eriodictyol were used as positive controls.

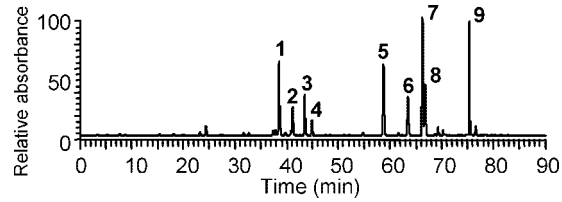

Figure 1. HPLC profile of the ethanol extracts of Okinawan propolis. HPLC conditions are described in the Materials and Methods.

Antioxidant Activity on Linoleic Acid Oxidation. This assay was carried out by the method of Emmons et al. with some modifications (*24*). In brief, β-carotene (3 mg) was dissolved in 30 mL of chloroform, and 3 mL of the solution was added to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was removed under a stream of nitrogen gas. Distilled water (100 mL) was added, and the solution was mixed well. Aliquots (3 mL) of the β-carotene/linoleic acid emulsion were mixed with 50 μL of the ethanol solution of the test samples (3.125, 6.25, 12.5, 25.0, 50.0, 100, and 200 μM) and incubated in a water bath at 50 °C. Oxidation of the emulsion was monitored spectrometrically by measuring the absorbance at 470 nm over a 60 min period. The control sample contained 50 μL of ethanol in place of the test sample. The antioxidant activity is expressed as percent inhibition relative to the control after 60 min of incubation. The sample concentrations were plotted against percent values, and IC_{50} values were determined.

RESULTS AND DISCUSSION

Identification of Prenylated Flavonoids from Propolis. **Figure 1** shows the HPLC profile of the ethanol extracts of Okinawan propolis. Previously, we isolated and identified four prenylated flavonoids (**5**–**7** and **9**) from this propolis (*22*). In the present study, we isolated another five compounds (**1**–**4** and **8**) and determined their structures.

Compounds **1**, **2**, **4**, and **8** were identified as propolin A [5,7,3′,4′-tetrahydroxy-2′-C-(7″-hydroxy-3″,7″-dimethyl-oct-2″-enyl)flavanone], propolin B [5,7,3′,4′-tetrahydroxy-5′-C-(7″-hydroxy-3″,7″-dimethyl-oct-2″-enyl)flavanone], propolin E [5,7,4′-trihydroxy-5′-C-(7″-hydroxy-3″,7″-dimethyl-oct-2″-enyl)-flavanone], and 5,7,4′-trihydroxy-3′-C-geranylflavanone (3′-geranyl-naringenin), respectively, by comparison of their spectroscopic data with those reported (*25*–*28*). Compounds **1**, **2**, **4**, and other prenylflavonoids (**5**–**7**) were also isolated from Taiwanese propolis by Chen et al. (*25*). Although compounds **5**–**7** were known (nymphaeol-B, isonymphaeol-B, and nymphaeol-A), Chen et al. named them propolins D (**5**), F (**6**), and C (**7**). Compound **8** has been isolated from the fruits of *Artocarpus nobilis* (*28*).

Compound **3** was obtained as a white powder. The molecular formula of **3** was determined to be $C_{25}H_{30}O_7$ by HR-FAB-MS. The IR spectrum of **3** indicated the presence of hydroxyl and carbonyl functions. The ^{1}H NMR spectrum of **3** in acetone-d_6 exhibited the signal for a phenolic OH at δ_H 12.47 (s, OH-5), which was a hydrogen strongly bonded to the 4-carbonyl group. The ABX system at δ_H 5.36 (1H, dd, J = 12.7, 2.9 Hz), 2.71 (1H, dd, J = 17.1, 2.9 Hz), and 3.12 (1H, dd, J = 17.1, 12.7 Hz) was diagnostic for H-2 and H-3 of a flavanone skeleton. The ^{1}H NMR chemical shifts of H-5′ and H-6′ in acetone-d_6 solution gave the same value (δ_H 6.86); thus, the coupling of these protons was not observed. However, in another solvent

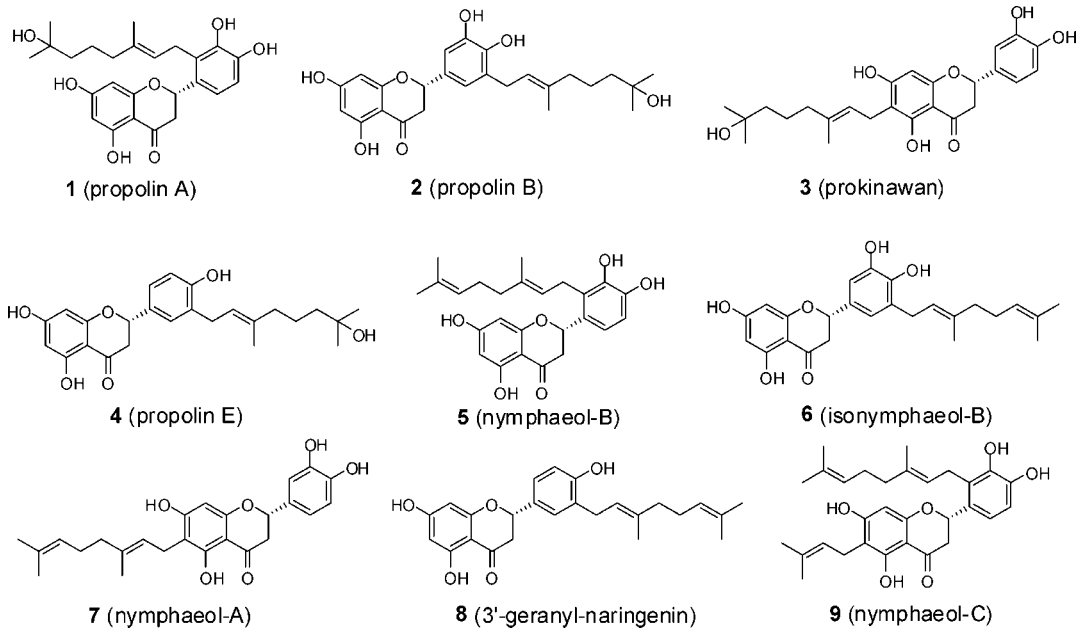

Figure 2. Structures of the prenylated flavonoids isolated from Okinawan propolis.

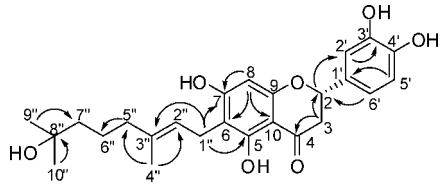

Figure 3. Key NMR HMBC correlations (H to C) for **3**.

such as chloroform, the chemical shifts of these protons gave different values (data not shown). The appearance of two methyl groups (δ_H 1.17), an olefinic methyl group (δ_H 1.76), six methylene protons (δ_H 1.40, 1.48, 1.94), two benzylic methylene protons (δ_H 3.27), and a vinyl proton (δ_H 5.25), indicated the presence of a hydroxyl dimethyloctenyl unit. The ^{13}C NMR spectrum of **3** contained 25 carbon signals. The signals in the ^{1}H and ^{13}C NMR spectra were assigned from the ^{1}H–^{1}H COSY, HSQC, and HMBC data. In the HMBC spectrum of **3**, the methylene signal at δ_H 3.27 (H-1″) was observed to be correlated with C-5 (δ_C 162.3) and C-7 (δ_C 164.8), indicating that the hydroxyl dimethyloctenyl unit was attached to C-5 (**Figure 3**). The configuration of the 2-position of **3** was determined to be *S* from the CD spectra of related compounds (*22*, *27*). Thus, **3** was determined to be 5,7,3′,4′-tetrahydroxy-6-C-(7″-hydroxy-3″,7″-dimethyl-oct-2″-enyl)flavanone, a new prenylated flavonoid, and named prokinawan. **Table 1** shows the complete ^{1}H and ^{13}C NMR assignments of **3**. Compound **3** was the structural isomer of propolins A and B from Taiwanese propolis (*26*).

Figure 2 shows all of the prenylflavonoids (nine compounds) from Okinawan propolis. We quantitatively analyzed the major components **5**–**7** and **9** in Okinawan propolis by HPLC. The calibration curve for each compound was obtained from replicate injections ($n = 3$) of known amounts of the corresponding standards. Linearity was in the range 0.1–10 mg/mL ($r^2 =$ 0.996–0.999). The detection limit was 0.05 mg/mL. The amount of each compound in the propolis was 127 (**5**), 105 (**6**), 135 (**7**), and 91 (**9**) mg/g. The contents of **5** and **7** in Taiwanese propolis have been reported to be 103.5 and 106.5 mg/g, respectively (*26*). These results indicate that the propolis of Okinawa origin is also a rich source of prenylated flavonoids.

Table 2. IC_{50} (μM) Values of the Compounds **1**–**9** in the DPPH and β-Carotene Assays[a]

compound	DPPH assay	β-carotene assay
1	9.0 ± 0.6	7.1 ± 0.8
2	10.9 ± 1.7	12.7 ± 1.3
3	5.2 ± 0.5	35.8 ± 1.2
4	62.6 ± 2.2	73.7 ± 2.3
5	7.1 ± 0.2	5.8 ± 0.2
6	8.5 ± 0.5	5.9 ± 0.4
7	6.5 ± 1.4	28.9 ± 1.9
8	64.2 ± 3.5	98.3 ± 3.3
9	9.8 ± 1.9	10.3 ± 0.4
BHT	16.8 ± 2.7	1.7 ± 0.1
α-tocopherol	11.4 ± 0.9	1.3 ± 0.3
eriodictyol	4.7 ± 0.7	16.0 ± 1.0

[a] Values are means of triplicate analyses ± SD.

The isolated compounds **1**–**9** were evaluated for antioxidant activity using the DPPH radical scavenging and the inhibition of linoleic acid oxidation by β-carotene bleaching systems (**Table 2**). Compounds **1**–**3**, **5**–**7**, and **9** showed the strong DPPH radical scavenging activity in comparison with those of BHT and α-tocopherol. The activity of these compounds may be related to the presence of two hydroxyl groups at the 3′- and 4′-positions. It has been reported that the neighboring hydroxyl groups are important for the antioxidant activity of flavonoids (*29*). Although **3** and **7** showed high antioxidant activity against the DPPH radical, those of **1**, **2**, **5**, **6**, and **9** showed higher activity than **3** and **7** in the β-carotene bleaching system (**Table 2**). Compounds **3** and **7** have a geranyl group on their A ring of the flavonoid skeleton, whereas **1**, **2**, **5**, **6**, and **9** have a geranyl group on their B ring. These results suggest that the difference of the position of a geranyl group affects the antioxidant activity. Especially in the β-carotene bleaching system, the antioxidant activity of the compounds with a geranyl group attached on their A ring was stronger than that of the compounds with a geranyl group on the B ring. In the antioxidant assay using β-carotene bleaching, linoleic acid exists as an emulsion in solution. Thus, the position of a geranyl group on the flavonoids affects the interaction with the emulsion of linoleic acid. The flavonoids with high antioxidant activity are

able to interact easily with the emulsion of linoleic acid. The present experiments proved that the position of the geranyl or prenyl groups on the flavonoid skeleton plays an important role in exhibiting antioxidant activity. No differences in the antioxidant activity were observed between **1** and **5**, **2** and **6**, **3** and **7**, and **4** and **8**. These results suggest that the hydroxylation of a geranyl group does not affect the antioxidant activity. We previously reported that Okinawan propolis had strong antioxidant activity (*14*). Major prenylated flavonoids **5**–**7** and **9** in Okinawan propolis had strong antioxidant activity described above; thus, these compounds are thought to contribute the total activity of Okinawan propolis.

The chemical composition of propolis reportedly depends on the specificity of the local flora at the site of collection (*30*, *31*). Thus, the composition of the plant origin determines the chemical composition of propolis. Various types of propolis and their plant origin have been studied. Bankova classified Okinawan and Taiwanese propolis as "Pacific propolis" (*31*). Several identical prenylated flavonoids have been found in Taiwanese propolis (*26*). Thus, the plant origin of Okinawan propolis might be the same as that of Taiwanese propolis. However, the origin plant(s) of these propolis are unknown. Thus, further study is needed to clarify the plant origin of Okinawan propolis.

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