Two New Cytotoxic Phenylallylflavanones from Mexican Propolis

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Two new phenylallylflavanones, (2*R*,3*R*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin (1) and (2*R*,3*R*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin 3-acetate (2) were isolated from a methanolic extract of Mexican propolis. Their structures were elucidated with spectroscopic analysis. Both compounds (1, 2) exhibited preferential cytotoxic activity against PANC-1 human pancreatic cancer cells in a nutrient-deprived medium with the concentration at which 50% cells died preferentially in NDM (PC_{50}) values of 17.9 μM and 9.1 μM, respectively.

Key words Mexican propolis; phenylallylflavanone; preferential cytotoxicity; PANC-1 cell

Propolis is the resinous substance collected by bees from various plants, and used for hive construction and repairs as well as for defense purposes.[1)] Humans have used propolis as a natural remedy for thousands of years because of its numerous health benefits.[1)] Propolis has been reported to have anticancer,[2,3)] antiviral,[4)] antifungal,[4,5)] antibacterial,[4,6)] antioxidant,[7)] immunomodulatory,[8)] and anti-inflammatory activity.[9)] Today, propolis is widely used in food, beverage, and pharmaceutical industries as a health supplement.[10)] As a continuation of our previous research on propolis from different geographic locations,[11—19)] we previously reported two new flavonoids, 8-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]galangin (**3**) and 8-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]chrysin (**4**), possessing a unique structural feature with a phenylallyl moiety attached to their flavone skeleton, from a methanol (MeOH) extract of Mexican propolis.[19)] Interestingly, they showed strong preferential cytotoxic activity against PANC-1 human pancreatic cancer cells (PANC-1) in a nutrient-deprived medium (NDM). Further work on the extract resulted in the isolation of two new phenylallylflavanones (**1**, **2**), shown in Chart 1. Herein we report the structural elucidation of these new phenylallylflavanones together with the results of our study of their *in vitro* preferential cytotoxicity against PANC-1 cells in NDM.

Compound **1** was obtained as a yellow amorphous solid with $[\alpha]_D^{22}$ −12.9 (c=0.1, CH_3OH), and its molecular formula was deduced to be $C_{25}H_{22}O_7$ by high resolution-fast atom bombardment-mass spectrometry (HR-FAB-MS). The infrared spectrum (IR) of **1** showed absorption bands of hydroxyl (3434 cm^{-1}) and conjugated-ketone carbonyl (1684 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** exhibited signals for a hydrogen-bonded hydroxyl group (δ_H 12.42), a phenyl group (δ_H 7.42—7.51, 5H), a 1,3,4-trisubstituted phenyl group (δ_H 6.78, d, J=1.6 Hz; δ_H 6.64, d, J=8.0 Hz; δ_H 6.56, dd, J=8.0, 1.6 Hz), a hydroxyl group (δ_H 5.87, d, J=6.1 Hz), two coupled oxymethines (δ_H 5.18, d, J=11.4 Hz; δ_H 4.64, dd, J=11.4, 6.1 Hz), an aromatic singlet (δ_H 5.99, H-8), a methine (δ_H 4.99, d, J=8.8 Hz), a vinyl group (δ_H 6.56, 5.09, 5.06), and an *O*-methyl group (δ_H 3.69) (Table 1), while its ^{13}C-NMR spectrum (Table 1) displayed 25 carbon signals including those corresponding to the above-mentioned groups, and for a ketone carbonyl carbon (δ_C 198.0). These data were similar to those of compound **3**, a phenylallyl-substituted flavone isolated from the

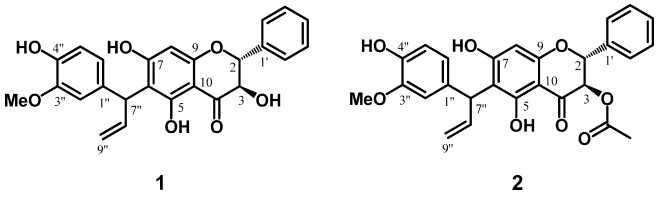

Chart 1. Structures of Compounds **1** and **2**

Table 1. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) Data for Compounds **1** and **2** in DMSO-d_6 (J Values in Parentheses)

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
2	5.18 d (11.4)	82.8	5.61 d (11.7)	79.9
3	4.64 dd (11.4, 6.1)	71.5	5.94 d (11.7)	72.0
4		198.0		191.5
5		160.7		160.6
6		110.3		110.7
7		164.5		160.2
8	5.99 s	94.8	6.02 s	95.3
9		160.4		160.2
10		100.3		100.3
1′		137.3		135.7
2′ 6′	7.51 d (7.6)	128.0	7.53 d (7.6)	127.7
3′ 5′	7.42 m[a)]	128.2	7.44 m[a)]	128.5
4′	7.42 m[a)]	128.6	7.44 m[a)]	129.3
1″		133.8		133.7
2″	6.78 d (1.6)	111.9	6.78 d (1.7)	112.0
3″		147.0		147.0
4″		144.6		144.6
5″	6.64 d (8.0)	115.0	6.64 d (8.3)	115.0
6″	6.56 dd[a)] (8.0, 1.6)	119.6	6.56 dd[a)]	119.6
7″	4.99 d (8.8)	42.9	5.00 d (8.6)	42.9
8″	6.56 m[a)]	139.4	6.56 m[a)]	138.7
9″	5.09 d (15.9)	115.1	5.09 d (17.1)	115.1
	5.04 d (9.5)		5.04 d (7.8)	
3-OH	5.87 d (6.1)			
3-O$\underline{C}$OCH$_3$				168.7
3-OCO$\underline{CH_3}$			1.94 s	20.0
5-OH	12.42 s		11.95 s	
3″-OMe	3.69 s	55.6	3.67 s	55.6

a) Chemical shifts were deduced based on the cross peaks in COSY and HMQC spectra.

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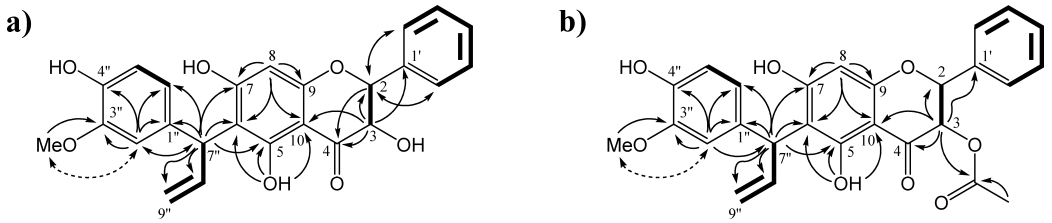

Fig. 1. COSY (Bold Lines) and Selected HMBC (Arrows: $^{1}H \rightarrow ^{13}C$) Correlations and Difference NOE (Dashed Arrows) in (a) **1** and (b) **2**

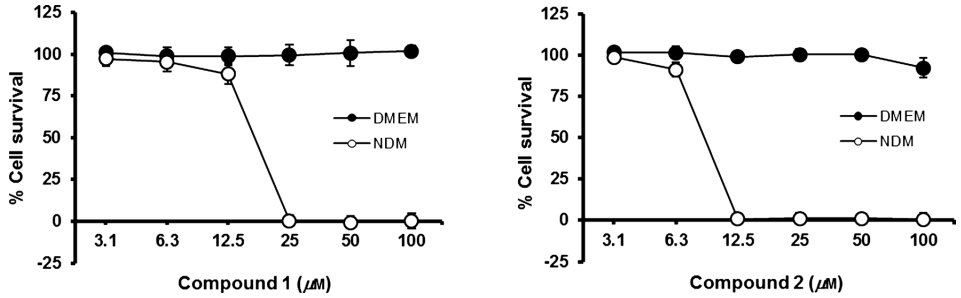

Fig. 2. Effect of **1** and **2** on the Survival of PANC-1 Cells in NDM and DMEM after 24 h Incubation at the Indicated Concentrations

Data expressed as mean±S.D., $n=3$.

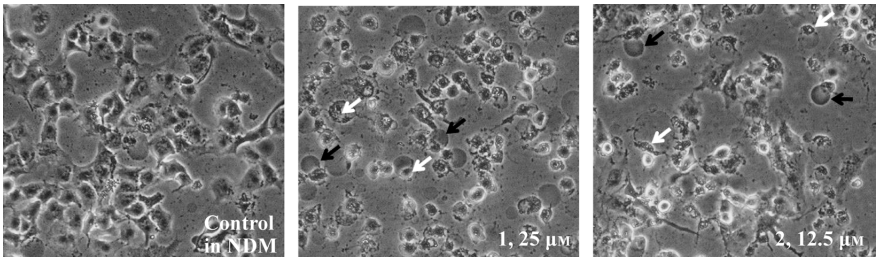

Fig. 3. Morphological Changes of PANC-1 Cells in NDM after 24 h Exposure with 25 μM of **1** or 12.5 μM of **2**

White arrow: nucleus fragmentation and condensation; black arrow: membrane bleb.

same extract in our previous study,[19] but differs due to presence of two coupled oxymethines (δ_H 5.18, 4.64; δ_C 82.8, 71.5) in **1** instead of the two olefinic signals (δ_C 145.8, 136.8) in **3**. This suggested **1** to be a flavanone derivative having a 1-(4′-hydroxyl-3′-methoxyphenyl)allyl moiety. The location of the 1-(4′-hydroxyl-3′-methoxyphenyl)allyl moiety was determined to be at C-6 based on the heteronuclear multiple bond coherence (HMBC) correlations of a methine proton at δ_H 4.99 (H-7″) with C-5 (δ_C 160.7), C-6 (δ_C 110.3), and C-7 (δ_C 164.5), and that of a hydrogen-bonded hydroxyl proton at δ_H 12.42 with C-5, C-6, and C-10 (δ_C 100.3) (Fig. 1a). Thus, the planar structure of **1** was deduced to be 6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin, which was further confirmed by analysis of the correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and HMBC spectra, together with the nuclear Overhauser effect (NOE) experiment (Fig. 1a). The typical absorptions at 293 and 327 nm as well as their corresponding cotton effects ($[\theta]_{293}$ −7699; $[\theta]_{327}$ +3471) observed in circular dichroism (CD) spectrum indicated a 2*R*-configuration in **1**.[20,21] Since a *trans* relationship between H-2 and H-3 was assigned on the basis of their coupling constant ($J_{2,3}=11.4$ Hz),[21] the absolute configuration at C-3 was determined to be *R*. However, the absolute configuration at C-7″ could not be determined because of the limited amount of available compound isolated. Thus, **1** was identified as (2*R*,3*R*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin.

Compound **2** was a yellow-brown amorphous solid having $[\alpha]_D^{22}$ −13.9 ($c=0.1$, CH_3OH) and its molecular formula was deduced to be $C_{27}H_{24}O_8$ by HR-FAB-MS. The IR spectrum of **2** indicated the presence of hydroxyl and carbonyl groups. The ^{1}H- and ^{13}C-NMR data of **2** (Table 1) closely resembled those of compound **1**, except for the presence of an additional signal because of an acetyl group (δ_H 1.94, s, 3H; δ_C 168.7, 20.0) in **2**. The location of the acetyl group was deduced to be at C-3 on the basis of a low field shift of H-3 (**2**, δ_H 5.94; **1**, δ_H 4.64) and the HMBC correlation between H-3 and the ester carbonyl carbon (δ_C 168.7) (Fig. 1b). The absolute configurations at C-2 and C-3 in **2** were determined to be the same as that of **1** on the basis of its CD spectrum. Thus, **2** was determined to be (2*R*,3*R*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin 3-acetate.

Both compounds (**1**, **2**) were then examined for their *in vitro* preferential cytotoxicity against PANC-1 cells utilizing the anti-austerity strategy, a novel approach in anticancer drugs discovery.[22—24] As shown in Fig. 2, each compound displayed the preferentially cytotoxicity in NDM in a concentration-dependent manner (the concentration at which 50% cells died preferentially in NDM (PC_{50}): 17.9 μM for **1**;

9.1 μM for **2**). Furthermore, PANC-1 cells exposed to **1** at 25 μM or **2** at 12.5 μM exhibited 100% cell death within 24 h of starvation (Fig. 2). The dying cells displayed morphological changes such as membrane blebbing, nuclear condensation, and fragmentation (Fig. 3). Arctigenin,[22] a potent anti-austerity strategy anticancer agent isolated from *Arctium lappa*, was used as a positive control in this study showed PC_{50} at 0.56 μM.

Experimental

General Experimental Procedures Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. CD measurements were carried out on a JASCO J-805 spectropolarimeter. UV spectra were obtained using a Shimadzu UV-160A, UV–visible recording spectrophotometer. IR spectra were measured with a Shimadzu IR-408 spectrophotometer. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as the internal standard, and chemical shifts are expressed in δ values (ppm). FAB-MS and HR-FAB-MS measurements were carried out on a JEOL JMS-700T spectrometer and glycerol was used as the matrix. Column chromatography (CC) utilized silica gel (silica gel 60N, spherical, neutral, 40—50 μm, Kanto Chemical Co., Inc., Japan) and reverse-phase silica gel (Cosmosil 75C_{18}-OPN, Nacalai Tesque Inc., Japan). Medium-pressure liquid chromatography (MPLC) was performed using a Buchi double pump module C-605 system. Preparative high-pressure liquid chromatography (HPLC) was carried out in a Discovery® C18 column (10×250 mm i.d., 5 μm particle size, Supelco, U.S.A.) using a Waters 600 pump (Waters, U.S.A.) and Waters 2998 photodiode array detector (Waters, U.S.A.).

Biological Material The propolis sample used in this study was collected in Caborca, Sonora, Mexico in May of 1999, and was stored at −40 °C. A voucher specimen (TMPW 26808) was deposited at the Museum of Materia Medica, Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and Isolation Mexican propolis (40.0 g) was extracted with MeOH under sonication (90 min, ×3) at room temperature to yield 15.8 g of extract. The MeOH extract (15.0 g) was chromatographed on silica gel with MPLC using hexane and then MeOH–$CHCl_3$ solvent systems to yield six fractions [fr. 1, hexane eluate, 2.8 g; fr. 2, $CHCl_3$ eluate, 254 mg; fr. 3, MeOH–$CHCl_3$ (1 : 24) eluate, 805 mg; fr. 4, MeOH–$CHCl_3$ (2 : 23) eluate, 4.5 g; fr. 5, MeOH–$CHCl_3$ (1 : 9) eluate, 2.4 g; fr. 6, MeOH–$CHCl_3$ (3 : 7) eluate, 1.9 g].

Fraction 6 (1.9 g) was separated by reversed-phase medium pressure liquid chromatography (RP-MPLC) using H_2O–acetone (9 : 1→8 : 2→7 : 3→5 : 5→1 : 9) to obtain five subfractions (fr. 6-1, 235 mg; fr. 6-2, 482 mg; fr. 6-3, 223 mg; fr. 6-4, 112 mg; fr. 6-5, 405 mg). Subfraction 6-3 was further subjected to preparative HPLC (Discovery® C18 column; 10×250 mm i.d., 5 μm particle size, Supelco, U.S.A.) with H_2O–CH_3CN (5 : 5) containing 0.01% trifluoroacetic acid (TFA) at a flow rate of 4 ml/min to yield (2*R*,3*R*,7″*S*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]-pinobanksin (**1**, 2.5 mg, t_R 8.7 min) and (2*R*,3*R*,7″*S*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin 3-acetate (**2**, 2.2 mg, t_R 17.2 min).

(2*R*,3*R*)-6-[1-(4′-Hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin (**1**): Yellow amorphous solid; $[\alpha]_D^{22}$ −12.9 (c=0.1, CH_3OH); IR (KBr) ν_{max} 3434 (br), 1684, 1634, 1510, 1449 cm^{-1}; UV λ_{max} (CH_3OH) nm (log ε): 210 (3.83), 293 (3.51); CD (c=2.3×10^{-4} M, EtOH) $[\theta]_{327}$ +3471, $[\theta]_{293}$ −7699, $[\theta]_{225}$ +9822; ^{1}H- and ^{13}C-NMR, see Table 1; HR-FAB-MS *m*/*z* 435.1480 (Calcd for $C_{25}H_{23}O_7$ $[M+H]^+$, 435.1444).

(2*R*,3*R*)-6-[1-(4′-Hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin 3-Acetate (**2**): Yellow-brown amorphous solid; $[\alpha]_D^{22}$ −13.9 (c=0.1, CH_3OH); IR (KBr) ν_{max} 3426 (br), 1752, 1632, 1513, 1449, 1230 cm^{-1}; UV λ_{max} (CH_3OH) nm (log ε): 214 (3.87), 296 (3.66); CD (c=2.09×10^{-4} M, EtOH) $[\theta]_{335}$ +7934, $[\theta]_{293}$ −28326, $[\theta]_{232}$ +14109; ^{1}H- and ^{13}C-NMR, see Table 1; HR-FAB-MS *m*/*z* 477.1546 (Calcd for $C_{27}H_{25}O_8$ $[M+H]^+$, 477.1549).

Preferential Cytotoxicity in NDM Preferential cytotoxicity was determined by a previously described procedure.[23] Briefly, PANC-1 human pancreatic cancer cells were seeded in 96-well plates (2×10^4 cells/well) and incubated in fresh Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) at 37 °C under 5% CO_2 and 95% air for 24 h. Then, the cells were washed with PBS (Nissui Pharmaceuticals, Tokyo, Japan), the medium was changed to either DMEM or NDM,[22] and serial dilutions of the test samples were added. Cell viability in each medium was then measured after 24 h of incubation. At the end of the incubation, the morphological changes were recorded by photomicrograph using a phase-contrast microscope under 200× magnification (Olympus D-340L/C-840L Digital Camera, Tokyo, Japan). Again, the cells were washed with PBS, and 100 μl of DMEM containing 10% WST-8 (Dojindo; Kumamoto, Japan) was added to the wells. The absorbance at 450 nm was measured after incubating 3 h. Cell viability was calculated from the mean values of data from three wells using the following equation:

$$(\%)\ \text{cell viability} = [(Abs_{(\text{test sample})} - Abs_{(\text{blank})})/(Abs_{(\text{control})} - Abs_{(\text{blank})})] \times 100$$

The preferential cytotoxicities were expressed as PC_{50} values (the concentration at which 50% cells died preferentially in NDM).

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References

1) Castaldo S., Capasso F., *Fitoterapia*, **73** (Suppl. 1), S1—S6 (2002).
2) Chen C. N., Weng M. S., Wu C. L., Lin J. K., *Evid. Based Complement. Alternat. Med.*, **1**, 175—185 (2004).
3) Tan-No K., Nakajima T., Shoji T., Nakagawasai O., Niijima F., Ishikawa M., Endo Y., Sato T., Satoh S., Tadano T., *Biol. Pharm. Bull.*, **29**, 96—99 (2006).
4) Kujumgiev A., Tsvetkova I., Serkedjieva Y., Bankova V., Christov R., Popov S., *J. Ethnopharmacol.*, **64**, 235—240 (1999).
5) Silici S., Koç N. A., Ayangil D., Cankaya S., *J. Pharmacol. Sci.*, **99**, 39—44 (2005).
6) Scazzocchio F., D'Auria F. D., Alessandrini D., Pantanella F., *Microbiol. Res.*, **161**, 327—333 (2006).
7) Kumazawa S., Ueda R., Hamasaka T., Fukumoto S., Fujimoto T., Nakayama T., *J. Agric. Food Chem.*, **55**, 7722—7725 (2007).
8) Orsatti C. L., Missima F., Pagliarone A. C., Bachiega T. F., Búfalo M. C., Araújo J. P. Jr., Sforcin J. M., *Phytother. Res.*, **24**, 1141—1146 (2010).
9) Paulino N., Teixeira C., Martins R., Scremin A., Dirsch V. M., Vollmar A. M., Abreu S. R., de Castro S. L., Marcucci M. C., *Planta Med.*, **72**, 899—906 (2006).
10) Banskota A. H., Tezuka Y., Kadota S., *Phytother. Res.*, **15**, 561—571 (2001).
11) Banskota A. H., Tezuka Y., Prasain J. K., Matsushige K., Saiki I., Kadota S., *J. Nat. Prod.*, **61**, 896—900 (1998).
12) Banskota A. H., Tezuka Y., Midorikawa K., Matsushige K., Kadota S., *J. Nat. Prod.*, **63**, 1277—1279 (2000).
13) Banskota A. H., Tezuka Y., Adnyana I. K., Midorikawa K., Matsushige K., Message D., Huertas A. A. G., Kadota S., *J. Ethnopharmacol.*, **72**, 239—246 (2000).
14) Usia T., Banskota A. H., Tezuka Y., Midorikawa K., Matsushige K., Kadota S., *J. Nat. Prod.*, **65**, 673—676 (2002).
15) Banskota A. H., Nagaoka T., Sumioka L. Y., Tezuka Y., Awale S., Midorikawa K., Matsushige K., Kadota S., *J. Ethnopharmacol.*, **80**, 67—73 (2002).
16) Awale S., Shrestha S. P., Tezuka Y., Ueda J. Y., Matsushige K., Kadota S., *J. Nat. Prod.*, **68**, 858—864 (2005).
17) Awale S., Li F., Onozuka H., Esumi H., Tezuka Y., Kadota S., *Bioorg. Med. Chem.*, **16**, 181—189 (2008).
18) Li F., Awale S., Zhang H., Tezuka Y., Esumi H., Kadota S., *J. Nat. Prod.*, **72**, 1283—1287 (2009).
19) Li F., Awale S., Tezuka Y., Esumi H., Kadota S., *J. Nat. Prod.*, **73**, 623—627 (2010).
20) Wu T. S., Hsu M. Y., Kuo P. C., Sreenivasulu B., Damu A. G., Su C. R., Li C. Y., Chang H. C., *J. Nat. Prod.*, **66**, 1207—1211 (2003).
21) Shrestha S. P., Narukawa Y., Takeda T., *Chem. Pharm. Bull.*, **55**, 926—929 (2007).
22) Awale S., Lu J., Kalauni S. K., Kurashima Y., Tezuka Y., Kadota S., Esumi H., *Cancer Res.*, **66**, 1751—1757 (2006).
23) Izuishi K., Kato K., Ogura T., Kinoshita T., Esumi H., *Cancer Res.*, **60**, 6201—6207 (2000).
24) Win N. N., Awale S., Esumi H., Tezuka Y., Kadota S., *Chem. Pharm. Bull.*, **56**, 491—496 (2008).