Two Novel Cytotoxic Benzofuran Derivatives from Brazilian Propolis

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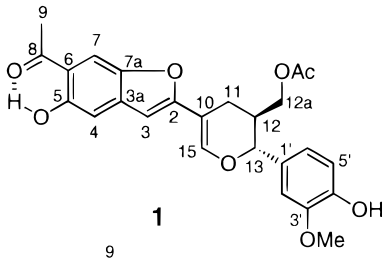
Two novel benzofuran derivatives, propolis-benzofurans A (**1**) and B (**2**), were isolated from the MeOH extract of Brazilian propolis, together with two known isoprenylated compounds (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid and (*E*)-3-{4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl]-5-prenylphenyl}-2-propenoic acid. The structures of these compounds were elucidated on the basis of spectral analysis. Both the new compounds exhibited mild cytotoxicity toward highly liver-metastatic murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells.

Propolis is a resinous hive product collected by honeybees from various plant sources. It has a long history of being used in traditional medicine and dates back at least to 300 B.C.[1] It has also been reported to possess various biological activities, viz. anticancer, antioxidant, antiinflammatory, antibiotic, and antifungal.[2,3] Recently, propolis has gained popularity as a health drink and has also been extensively used in food to improve health and prevent diseases such as inflammation, heart disease, diabetes, and even cancer. Because of its broad spectrum of biological activities and uses in health food and in folk medicine, there is a renewed interest in the composition of propolis. As a consequence, more than 160 constituents have been identified so far.[2,4] Propolis contains mostly sticky plant substances collected by honeybees, bee wax and other bee secretions. It has a pleasant aromatic odor and yellow-green to dark brown color depending on its source and age. The composition of propolis depends on the vegetation of the area from which it was collected. For example, propolis from temperate zones, especially European propolis, contains predominantly phenolic compounds, including several flavonoids.[4,5] The constituents of the propolis from tropical zones (i.e., South America) appear to be different from those of the propolis from temperate zones because of the difference in vegetation. A clerodane and several labdane-type diterpenoids, which are virtually absent in propolis from a temperate zone, were reported to be present in propolis from a tropical region.[6,7] Interestingly, despite the difference in their constituents, propolis from all regions, including the temperate and tropical zones, exhibit similar biological properties.[1−3] Thus, we undertook an examination of the bioactive components from Brazilian propolis and reported the isolation and hepatoprotective activities of four dicaffeoyl quinic acid derivatives from a water extract[8] and the isolation and cytotoxicities of 23 compounds, including diterpenes and phenolic compounds, from the MeOH extract.[9] Further work on the MeOH extract of Brazilian propolis led to the isolation of two novel benzofuran derivatives, propolis-benzofurans A (**1**) and B (**2**), together with two known isoprenylated compounds, (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid and (*E*)-3-{4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl]-5-prenylphenyl}-2-propenoic acid.[10] We report herein the structure determination of these two new benzofurans by spectroscopic analyses. Also, the cytotoxicity of these compounds to murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells is reported.



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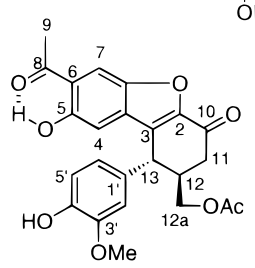

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Brazilian propolis was successively extracted with water, MeOH, and $CHCl_3$, and the cytotoxicity of the extracts was tested against highly liver-metastatic murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells. The MeOH extract, which showed comparatively strong cytotoxicity toward both cell lines, was partitioned into EtOAc-soluble and -insoluble fractions. The EtOAc-soluble portion, which showed the strongest cytotoxicity, was subjected to chemical investigation, and 23 compounds were isolated.[9] Further work on the same fraction led to the isolation of two novel benzofuran derivatives **1** and **2**, which were given the trivial names propolis-benzofuran A and propolis-benzofuran B, respectively. Two known isoprenylated compounds, (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid and (*E*)-3-{4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl}-5-prenylphenyl]-2-propenoic acid, which had been previously reported from Brazilian propolis, were also obtained from the MeOH extract.[10]

Propolis-benzofuran A (**1**) was isolated as a colorless amorphous solid, with $[\alpha]^{25}_D$ +111.9° (*c* 0.02, $CHCl_3$). The molecular formula of **1** was determined to be $C_{25}H_{24}O_8$ by HRFABMS. The IR spectrum of **1** shows absorption bands corresponding to a hydroxyl (3525 cm^{-1}), an ester carbonyl (1730 cm^{-1}), and a ketone carbonyl with an intramolecular hydrogen bond (1625 cm^{-1}). The ^1H NMR spectrum of **1**

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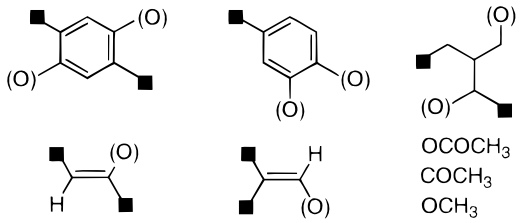

Figure 1. Partial structures for propolis-benzofuran A (**1**).

shows five aromatic proton signals corresponding to two phenyl rings [δ 7.95 (s), 6.92 (s), 7.07 (d, J = 2.0 Hz), 6.88 (dd, J = 8.0, 2.0 Hz), 6.84 (d, J = 8.0 Hz)]. The coupling constants indicated the phenyl rings are 1,3,4-trisubtituted and 1,2,4,5-tetrasubstituted. The ^{13}C NMR chemical shifts of four aromatic carbons at δ 148.5 (C-3′), 147.8 (C-4′), 159.7 (C-5), and 148.0 (C-7a) indicated that these carbons are oxygen-substituted. Moreover, signals for two singlet olefinic protons (δ 6.57 and 7.57), an acetyl group [δ_H 2.00 (s); δ_C 20.6, 170.9], a methoxy methyl [δ_H 3.87 (s); δ_C 56.3], and a methyl ketone [δ_H 2.71 (s); δ_C 26.9] also appeared, together with signals for two methine groups [δ 4.84 (d, J = 9.0 Hz), 2.55 (m)] and two methylene groups [δ_H 4.02 (dd, J = 12.2, 4.2 Hz), 3.82 (dd, J = 12.2. 5.8), δ_C 65.1, and δ_H 2.62 (m), 2.53 (m), δ_C 24.6]. Analyses of these signals by the ^{1}H−^{1}H COSY and HMQC spectra led to the partial structures depicted in Figure 1.

The partial structures were connected based on long-range correlations observed in the field gradient-pulsed HMBC spectrum (Table 1). Both the methyl protons at δ 2.71 (H_3-9) and the aromatic proton at δ 6.92 (H-7) had HMBC correlations with the ketone carbonyl carbon (δ 204.5) and the quaternary aromatic carbon (δ 116.3), suggesting the presence of a methyl ketone group on the 1,2,4,5-tetrasubstituted benzene ring. Moreover, a characteristic signal for a hydrogen-bonded hydroxyl proton (δ 12.29, s), having HMBC correlations with C-4, C-5, and C-6, indicated the presence of a hydroxy group at C-5. The olefinic proton at δ 6.57 (H-3), having HMBC correlations with C-3a, C-7a, and C-2, and the aromatic proton at δ 7.95 (H-4), with an HMBC correlation with C-3, led to the conclusion that **1** should have a benzofuran moiety. The connectivity of C-15 (δ 146.6) and C-11 (δ 24.6) with C-10 (δ 105.2) was established on the basis of the long-range correlations between the methylene protons H_2-11 and C-10 and between the olefinic proton H-15 and C-10 and C-11. Furthermore, HMBC correlations of C-2 with H-15 and H_2-11 suggested the connectivity between C-2 in the benzofuran moiety and C-10. Based on an HMBC correlation observed between the methoxy methyl and C-3′, the position of the methoxyl group was determined to be C-3′. Finally, the locations of the acetoxy group at C-12a, the 3-methoxy-4-hydroxybenzene at C-13, and the ether linkage between C-13 and C-14 were concluded from HMBC correlations between H_2-12a and the ester carbonyl carbon (δ 170.9); between H-13 and C-1′, C-2′, and C-6′; and between H-15 and C-13, respectively. The coupling constant for H-13 (9.0 Hz) indicated the diaxial relationship between H-12 and H-13, and thus both of the substituents, that is, the benzene ring at C-13 and CH_2OAc at C-12, should be in an equatorial position in the chair conformation. On the basis of this evidence, the structure of propolis-benzofuran A was elucidated as **1**.

Propolis-benzofuran B (**2**) was also obtained as a colorless amorphous solid with the molecular formula $C_{24}H_{22}O_8$, one carbon less than **1**. The IR spectrum of **2** shows absorption bands at 3525, 1730, 1680, and 1640 cm^{-1}, indicating the presence of hydroxy, ester carbonyl, cyclic ketone, and a ketone with an intramolecular hydrogen bond, respectively. The ^{1}H NMR spectrum of **2** was similar to that of **1**, except for the absence of the olefinic proton signals. The hydrogen-bonded hydroxyl proton signal (δ 11.81), together with two

Table 1. ^{1}H and ^{13}C NMR Data of Propolis-benzofurans A (**1**) and B (**2**)[a]

	1			**2**		
	δ_H	δ_C	HMBC[b]	δ_H	δ_C	HMBC[b]
2		162.5	3, 5, 11		151.7	13
3	6.57 s	99.8	4		135.0	9, 13
3a		138.3	3, 7		134.0	
4	7.95 s	106.9	OH-5	6.13 s	110.0	OH-5
5		159.7	4, OH-5, 7		158.2	4, OH-5, 7
OH-5	12.29 s			11.81 s		
6		116.3	4, OH-5, 9		120.5	OH-5, 9
7	6.92 s	112.4		8.21 s	115.5	
7a		148.0	3, 7		149.5	7
8		204.5	7, 9		205.8	9, 12
9	2.71 s	26.9		2.75 s	27.3	
10		105.2	3, 11, 15		187.5	11
11	2.62 m					
	2.53 m	24.6	13, 15	2.86 m (2H)	42.3	12a
12	2.55 m	37.8	11, 12a, 13	2.94 m	44.9	11, 12a, 13
12a	4.02 dd (12.2, 4.2)					
	3.82 dd (12.2, 5.8)	65.1		4.05 m (2H)	65.2	11, 13
13	4.84 d (9.0)	80.9	11, 15, 2′, 6′	4.33 d (9.9)	43.1	11, 12a, 2′, 6′
15	7.57 s	146.6	11			
1′		130.7	13		131.6	13, 5′, 6′
2′	7.07 d (2.0)	111.3	13, 6′	7.02 d (2.0)	112.9	13
3′		148.5	2′, OMe		148.9	5′, OMe
4′		147.8	2′		147.2	
OH-4′	7.73 br s			8.01 br s		2′
5′	6.84 d (8.0)	115.7	OH-4′	6.89 d (8.0)	116.1	
6′	6.88 dd (8.0, 2.0)	121.0	13, 2′	6.87 dd (8.0, 2.0)	122.5	13, 2′
OMe	3.87 s	56.3		3.73 s	56.3	
OAc	2.00 s	20.6		2.03 s	20.6	
		170.9	12a, OAc		170.8	12a, OAc

[a] The ^{1}H and ^{13}C NMR spectra were measured at 400 and 100 MHz, respectively, in acetone-d_6 and coupling constants (parentheses) are in Hz. [b] ^{1}H correlating with ^{13}C resonance.

aromatic protons in the para position (δ 6.13 and 8.21) and a methyl ketone (δ 2.75) in the ^{1}H NMR spectrum of **2**, were identical to those of **1**, suggesting the presence of the *o*-hydroxyacetobenzofuran moiety in **2**. Furthermore, the signals corresponding to the acetoxy methyl (δ 2.03 s), a 1,3,4-trisubstituted benzene ring [δ 7.02 (d, J = 2.0 Hz), 6.89 (d, J = 8.0 Hz), 6.87 (dd, J = 8.0, 2.0 Hz)], a methoxyl (δ 3.73 s), two vicinal methines [δ 2.94 (m), 4.33 (d, J = 9.9 Hz)], and two methylenes [δ_H 2.86 (m, 2H), 4.05 (m, 2H)] of **2** were also similar to those of **1** and indicated that **2** and **1** should differ only at C-10 to C-15.

The ^{13}C NMR spectrum of **2** displayed only two signals of a tetrasubstituted olefinic carbon at δ 151.7 and 135.0, indicating the presence of only one olefin. Moreover, an α,β-unsaturated ketone carbon (δ 187.5), having an HMBC correlation with the methylene protons (H_2-11), suggested the presence of the ketone group at C-10. A methine carbon of **2** (δ 43.1, C-13) appeared relatively highfield as compared to that of **1** (δ 80.9), suggesting that C-13 had no oxygen functionality. The HMBC correlations between H-13 and C-2 and C-3 have established the connectivity between C-13 and C-2. The location of the remaining parts of **2** were determined to be identical with those of **1**; that is, 1,3,4-trisubstituted benzene at C-13 and acetoxy group at C-12a. The benzene ring at C-13 and CH_2OAc at C-12 both were considered to be equatorial, based on the coupling constant for H-13 (9.9 Hz). From these data the structure of propolis-benzofuran B was determined as **2**.

Both propolis-benzofurans A (**1**) and B (**2**) represent unique structures, and both compounds possess mild cytotoxicity toward murine colon 26-L5 carcinoma and human HT-1080 fibrosarcoma cells. The ED_{50} values of **1** and **2** were 12.4 and 13.7 μg/mL toward colon 26-L5 carcinoma cells and 13.9 and 43.2 μg/mL toward HT-1080 fibrosarcoma cells, respectively. Both of these compounds possess stronger cytotoxicity than the previously isolated *o*-hydroxyacetobenzofuranone derivatives viscidone and 12-acetoxyviscidone,[9] which may be due to the presence of the additional phenolic group. Although the cytotoxicity of **1** and **2** were weaker than the flavones isolated from the same extract (betuletol, kaempferide, and ermanin, ED_{50} < 8 μg/mL),[9] they may contribute to the cytotoxicity of the MeOH extract (ED_{50} values toward colon 26-L5 and HT-1080 were 62.4 and 67.3 μg/mL, respectively) of Brazilian propolis. Two known compounds, (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid and (*E*)-3-{4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl}-5-prenylphenyl]-2-propenoic acid, possess no cytotoxicity against either cell line.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. UV spectra were obtained in $CHCl_3$ solution on a Shimadzu UV-160A UV−vis spectrophotometer, and IR specta were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solution. HRFABMS measurements were carried out on a JEOL JMS-700T spectrometer, and glycerol was used as a matrix. ^{1}H, ^{13}C, and 2D NMR were taken on a JEOL GX-400 spectrometer with tetramethylsilane (TMS) as an internal standard. Column chromatography was performed with normal-phase Si gel (Fuji Silysia, BW-820 MH). Analytical and preparative TLC were carried out on precoated Merck Kieselgel $60F_{254}$ plates (0.25 or 0.50 mm thickness).

Biological Material. Propolis (Yukari propolis) was collected in Brazil in 1995, and a voucher sample (P-2) is preserved in our laboratory.

Extraction and Isolation. Propolis collected from Brazil (1.8 kg) was successfully extracted to give the following extracts: water (130 g), MeOH (331 g), and $CHCl_3$ (315 g). The MeOH extract was then fractionated into EtOAc-soluble (271 g) and -insoluble (42 g) fractions and the EtOAc-soluble fraction was subjected to Si gel column chromatography with $CHCl_3$−MeOH gradient system to give seven fractions.[9]

Fraction 5 (20% MeOH−$CHCl_3$ eluate) was further chromatographed over Si gel with $CHCl_3$−MeOH gradient system to give 60 subfractions. Preparative TLC of subfraction 38 with toluene−EtOAc (1:1) gave (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-5-benzofuranyl]-2-propenoic acid (**3**, 13.2 mg) and (*E*)-3-{4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl]-5-prenylphenyl}-2-propenoic acid (**4**, 13.9 mg). Preparative TLC of subfractions 45 and 53 with $CHCl_3$-MeOH (9:1) gave propolis-benzofuran A (**1**, 7 mg) and propolis-benzofuran B (**2**, 18.2 mg), respectively.

Propolis-benzofuran A (1): yellow amorphous solid; $[\alpha]^{25}_D$ +111.9° (*c* 0.02, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 349 (3.8), 243 (3.2) nm; IR ($CHCl_3$) ν_{max} 3525, 1730, 1625, 1515, 1360, 1320, 1230, 1160 cm^{-1}; HRFABMS *m/z* 453.1514 (calcd for $C_{25}H_{25}O_8$ $[M + H]^+$, 453.1549); ^{1}H and ^{13}C NMR, see Table 1.

Propolis-benzofuran B (2): yellow amorphous solid; $[\alpha]^{25}_D$ +38.4° (*c* 0.08, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 310 (3.7), 243 (3.4) nm; IR ($CHCl_3$) ν_{max} 3525, 1730, 1680, 1640, 1510, 1430, 1360, 1320, 1230, 1030 cm^{-1}; HRFABMS *m/z* 439.1407 (calcd for $C_{24}H_{23}O_8$ $[M + H]^+$, 439.1393); ^{1}H and ^{13}C NMR, see Table 1.

Cytotoxic Assay. Cellular viability in the presence and absence of experimental agents were determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assays as described previously.[9]

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