Studies on the Constituents of Brazilian Propolis

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Two new cinnamic acid derivatives along with twenty two known compounds, including five flavonoids, three phenolic compounds, six caffeoylquinic acids and eight cinnamic acid derivatives, were isolated from Brazilian propolis. New compounds were elucidated as (E)-3-(2,2-dimethyl-3,4-dihydroxy-8-prenyl-2H-1-benzopyran-6-yl)-2-propenoic acid and (E)-3-(2,3-dihydro-2-(1-hydroxy-1-methylcyclohexyl)-7-prenyl-benzofuran-5-yl)-2-propenoic acid, on the basis of spectral evidence. Three compounds; dihydrokaempferide, (+)-threo-1-C-guaiacylglycerol and 3-prenyl-4-(2-methylpropionyloxy)cinnamic acid were isolated from propolis at the first time.

Key words propolis; prenyl cinnamic acid; Brazil; Apis mellifera; Apidae; cinnamic acid

Propolis is a resinous hive product collected by honey bees, Apis mellifera (Apidae). It consists of secretions and/ or exudations from various plants, which are mixed by honey bees with bee wax to form a sealing material of a certain consistency. It has been considered that propolis is a protective wall against the enemies of honey bees. Propolis has been used as a folk medicine from ancient times in many regions of the world. In recent times, propolis and its constituents have been reported to possess various biological activities such as antimicrobial, antibacterial, antioxidant, anticancer, immunomodulatory and others. However, the chemical composition of propolis is very complex and is still not clear. In this paper, we describe the isolation of twenty four constituents including two new compounds, from Brazilian propolis.

Results and Discussion

Brazilian propolis was extracted with 75% ethanol at room temperature, and the extract was processed by the method described in the Experimental section. Twenty four compounds were isolated.

Compounds 1—5 were known flavonoids. They were identified as isosakuranetin (9—11), pinocembrin (9,11), dihydrokaempferide (3), kaempferide (9) and betuvelol (3) (5). Compounds 6—8 were known phenolic compounds. They were identified as (+)-threo-1-C-guaiacylglycerol (6), 3,4-dihydroxybenzoic acid (protocatechuic acid) (7) and 3-methoxy-4-hydroxybenzaldehyde (vanillin) (8). Compounds 9—14 were known caffeoylquinic acids. They were identified as 3-caffeoylquinic acid (chlorogenic acid) (9), 4-cafeoylquinic acid (10), 5-cafeoylquinic acid (11), 3,4-dicafeoylquinic acid (12), 3,5-dicafeoylquinic acid (13) and 4,5-dicafeoylquinic acid (14). Compounds 15—22 were known cinnamic acid derivatives. They were identified as 4-hydroxycinnamic acid (p-coumaric acid) (15), 3-(4-hydroxyphenyl)propanoic acid, 3,4-dihydroxypropanoic acid (rapuacin) (18), 3-prenyl-4-hydroxypropanoic acid (drupanin) (18), 3-prenyl-4-(2,3-dihydrocinnamo)glycinic acid (19), 3-prenyl-4-(2-methylpropiyloxy)cinnamic acid (20), 3,5-diprenyl-4-hydroxypropanoic acid (arpetillin (17,18) and 3-(2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-yl)-2-propenoic acid (22). These known compounds were identified by comparison of spectral data and specific optical rotation with values in the literature and/or authentic samples.

This is the first report of isolation of 3, 6 and 20 from propolis.

Compound 21 (18) was obtained as an amorphous powder, and afforded a [M]+ ion peak at m/z 298 (C19H22O3) in the positive-ion FAB-MS spectrum. In the 1H-NMR spectrum, a set of prenyl signals were observed at δ 5.26 (1H, br t), 3.26 (2H, d, J = 7.0 Hz), 1.74 (3H, s) and 1.73 (3H, s). Four olefinic signals at δ 6.55 (1H, d, J = 13.0 Hz), 2.74 (1H, d, J = 10.0 Hz), 6.32 (1H, d, J = 10.0 Hz), 6.32 (1H, d, J = 16.0 Hz) and 7.67 (1H, d, J = 16.0 Hz) were distinguished. The signals at δ 7.04 (1H, br s) and 7.18 (1H, br s) suggested the presence of an aromatic ring system. The other aliphatic signal in the 1H-NMR of 22 was a two methyl singlet at δ 1.44 (6H, s) on a tetrasubstituted carbon. The 13C-NMR spectrum of 22 showed a phenolic carbon at δ 153.2, an oxygenated carbon at δ 172.2 and a carbonyl group at δ 172.0. Assignment of the signals in the 1H- and 13C-NMR spectra were done by analysis of 1H-1H shift correlation spectroscopy (H-H COSY) and heteronuclear multiple-quantum correlation (HMOC) spectra, and comparison with the data of (2,2-dimethyl-2H-1-benzopyran-6-yl)-2-propenoic acid and 3,5-diprenyl-4-hydroxypropanoic acid (21) as shown in the Experimental section. In the heteronuclear multiple bond connectivity (HMBC) spectrum, the olefinic proton signal at δ 6.55 (H-3) showed correlations with a two methyl carbon signal at δ 28.2 (C-12, C-13), and the olefinic proton signal at δ 6.32 (H-4) correlated with the phenolic carbon signal at δ 153.2 (C-8a) and the oxygenated carbon signal at δ 177.2 (C-2). Other significant long-range correlations are shown in Fig. 1, and support the planar structure proposed for a cinnamic acid derivative having a prenyl group and benzopyran ring. Thus, the structure of 22 was concluded to be (E)-3-(2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-yl)-2-propenoic acid.

Compound 23 (18) was obtained as an amorphous powder, and afforded a [M]+ ion peak at m/z 316 (C19H24O3) in the positive-ion FAB-MS spectrum. Based on 1H- and 13C-NMR spectra, 23 was determined to be a cinnamic acid derivative similar to 22. Compound 23 exhibited a proton signal at δ 8.35 (1H, t, J = 6.0 Hz) on an alcoholic carbon, and two geminal methylene proton signals at δ 2.80 (1H, dd, J = 17.0, 6.0 Hz) and 3.06 (1H, dd, J = 17.0, 6.0 Hz). In the HMBC spectrum, the signal at δ 3.83 showed correlation with the carbon signals at δ 25.1 (C-12) and 22.2 (C-13), and with one aromatic carbon at δ 119.0 (C-4a). The aromatic proton signal at δ 7.12 (H-5) correlated with the methylene carbon signal at δ 31.4 (C-4).

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Therefore, the position of the hydroxy group in 23 was determined to be C-3. Other long-range correlations, depicted in Fig. 2 by arrows, indicated the presence of a prenyl group and a dihydrobenzopyran ring. This structure was also supported by comparison with the $^1$H- and $^{13}$C-NMR spectra of 22. Consequently, the structure of 23 was deduced to be (E)-3-[2,2-dimethyl-3,4-dihydro-3-hydroxy-8-prenyl-2H-1-benzopyran-6-yl]-2-propenoic acid.

Compound 24$^1$ was obtained as an amorphous powder, and afforded a [M]$^+$ ion peak at m/z 316 (C$_{18}$H$_{22}$O$_4$) in the positive-ion FAB-MS spectrum. Based on $^1$H- and $^{13}$C-NMR spectra, 24 was determined to be a cinnamic acid derivative similar to 22 and 23. 24 exhibited a proton signal at δ 4.67 (1H, t, J=9.0 Hz) on the carbon attached to an oxygen atom, and a methylene proton signal at δ 3.18 (2H, d, J=9.0 Hz). Those signals were observed at lower field shift compared with 23. The structure of 24 was determined by analysis of the H–H COSY spectrum, the HMOC spectrum and the HMBC spectrum (Fig. 3), and comparison with the data for (E)-3-[2,3-dihydro-2-(2-hydroxy-1-methylethyl)-7-prenylbenzofuran-5-yl]-2-propenoic acid (arteplin A)$^{19}$. Thus, the structure of 24 was concluded to be (E)-3-[2,3-dihydro-2-(1-hydroxy-1-methylethyl)-7-prenylbenzofuran-5-yl]-2-propenoic acid.

In this study, we found that the isolated compounds 1, 2, 18, 19, 20, and 21 were contained in some Baccharis species (Compositae)$^{10,11,17}$ which have been widely used as a folk medicine in Brazil. Thus, we speculate that Baccharis species are a possible plant sources of Brazilian propolis.

**Experimental**

Optical rotations were determined with a JASCO DIP-1000 digital polarimeter. UV spectra were measured on a Beckman DU 640 spectrophotometer. FAB-MS spectra were taken on a JEOL JMS-SX102 spectrometer. $^1$H- and $^{13}$C-NMR were recorded on JEOL GSX-500 (500 and 125.65 MHz, respectively), JEOL GSX-270 (270 and 67.80 MHz, respectively) and JEOL JNM-EX200 (400 and 100.40 MHz, respectively) spectrometers. Spectrometrical data were measured using tetramethylsilane (TMS) as an internal standard. Chemical shifts are given as δ values (ppm) and coupling constants (J) in hertz (Hz). The H–H COSY, HMOC and HMBC spectra were recorded with standard JEOL software. HPLC was run on a JASCO system 880 and JASCO system 987 instrument. Reversed-phase HPLC was carried out on a column of Deversol ODS-5 (Nomura Chemical Ltd.) Detection was by UV absorption at 280 nm. Daisen HQ-20 (Mitsubishi Chemical Industries Ltd.) was used for column chromatography with H$_2$O–MeOH gradient solvent elution.

**Propolis Material**

Brazilian propolis glue which was obtained from the state of Minas Gerais in Brazil, was supplied by Dai Ki Kenko-kan Co., Ltd.

**Extraction and Isolation**

Propolis (450 g) was extracted with 75% EtOH (1) at room temperature. Concentration of the 75% EtOH extract under reduced pressure gave a residue (70 g). The residue (173.6 g) was partitioned with AcOEt and H$_2$O. Concentration of each fraction gave 11.74 and 1.75 g of extracts, respectively. The AcOEt extract (1.50 g) was subjected to reversed-phase chromatography by preparative HPLC, using a gradient solvent of 2% AcOH in CH$_3$CN–H$_2$O (2:8) to (8:2), to give 76 fractions. They were rechromatographed by preparative HPLC [ODS: 2% AcOH in CH$_3$CN–H$_2$O and 1% AcOH in MeOH–H$_2$O] to give compounds 1 (1.9 mg), 2 (1.5 mg), 3 (9.1 mg), 4 (1.6 mg), 5 (1.9 mg), 15 (10.4 mg), 18 (7.7 mg), 19 (25.7 mg), 20 (3.5 mg), 21 (10.5 mg), 22 (6.6 mg), 23 (4.4 mg) and 24 (4.4 mg). The H$_2$O extract (1.75 g) was passed through a Daisen HP-20 column, and the adsorbed material was eluted with H$_2$O, 50% MeOH in H$_2$O and MeOH. The eluates were concentrated under reduced pressure to afford three fractions. The H$_2$O fraction (872 mg) was rechromatographed by preparative HPLC [ODS: 2% AcOH in CH$_3$CN–H$_2$O and 1% AcOH in MeOH–H$_2$O] to give compounds 6 (13.4 mg), 7 (1.9 mg), 9 (1.9 mg), 10 (1.6 mg), 11 (6.6 mg), 15 (7.7 mg), 16 (6.6 mg) and 17 (1.9 mg). In the same way, the 50% MeOH fraction (620 mg) was rechromatographed to give compounds 12 (6.4 mg), 13 (11.4 mg) and 14 (5.7 mg). The 100% MeOH fraction (114 mg) was rechromatographed to furnish compound 8 (1.9 mg). The known compounds were identified by comparison of spectral data and/or specific optical rotation with reported values and/or authentic samples.

**Compound 22 Amorphous powder: FAB-MS m/e: 298 [M]$^+$ UV 290.00 nm (log ε): 237 (4.31), 268 (4.39), 313 (3.20). $^{1}$H-NMR (CDCl$_3$) δ: 1.44 (6H, s, H-12, H-13), 1.73 (3H, s, H-5'), 1.74 (3H, s, H-4'), 3.26 (2H, d, J=7.0, H-1'), 5.26 (1H, Br H-2'), 6.65 (1H, d, J=10.0, H-3), 6.32 (1H, d, J=10.0, H-4), 6.33 (1H, d, J=16.0, H-10), 7.04 (1H, Br H-5), 7.18 (1H, Br H-7), 7.67 (1H, d, J=16.0, H-9). $^{13}$C-NMR (CDCl$_3$) δ: 17.9 (C-5'), 25.8 (C-4'), 28.1 (C-1'), 28.2 (C-12, C-13), 77.2 (C-2), 114.1 (C-10), 121.0 (C-2'), 122.0 (C-4'), 122.1 (C-4a), 124.4 (C-7), 126.3 (C-6), 129.7 (C-5), 130.0 (C-8), 131.0 (C-3), 132.7 (C-3'), 147.2 (C-9), 153.2 (C-9a), 172.0 (C-
Compound 23 Amorphous powder. FAB-MS m/z: 316 [M]+. UV λ_{max} nm (log ε): 218 (4.36), 235 (4.33), 314 (4.41). [α]_{D}^{20} +0.76° (c=0.53, EtOH).

1H-NMR (CDCl₃) δ: 1.33 (3H, s, H-12), 1.37 (3H, s, H-13), 1.72 (3H, s, H-5'), 1.75 (3H, s, H-4'). 2.80 (1H, dd, J=17.0, 6.0, H-4), 3.06 (1H, dd, J=17.0, 6.0, H-4), 3.28 (2H, d, J=7.0, H-1'), 3.49 (1H, s, C₂-OH), 3.83 (1H, t, J=6.0, H-3), 5.26 (1H, brt, H-2'), 6.27 (1H, d, J=16.0, H-10), 7.12 (1H, brs, H-5), 7.19 (1H, brs, H-7), 7.67 (1H, d, J=16.0, H-9). 13C-NMR (CDCl₃) δ: 17.8 (C-5'), 22.2 (C-13), 25.1 (C-12), 25.8 (C-4'), 28.4 (C-1'), 31.4 (C-4), 69.5 (C-3), 77.6 (C-2), 114.0 (C-10), 119.0 (C-4a), 122.0 (C-2'), 126.3 (C-6), 127.8 (C-7), 128.6 (C-5), 130.7 (C-8), 132.8 (C-3'), 147.1 (C-9), 153.2 (C-8a), 171.6 (C-11).

Compound 24 Amorphous powder. FAB-MS m/z: 316 [M]+. UV λ_{max} nm (log ε): 294 (4.19). [α]_{D}^{20} +0.38° (c=0.26, EtOH).

1H-NMR (CDCl₃) δ: 1.21 (3H, s, H-12), 1.34 (3H, s, H-13), 1.73 (3H, s, H-5'), 1.75 (3H, s, H-4'), 3.18 (2H, d, J=9.0, H-3), 3.28 (2H, m, H-1'), 3.49 (1H, s, C₂-OH), 4.67 (1H, t, J=9.0, H-2), 5.28 (1H, brt, H-2'), 6.25 (1H, d, J=16.0, H-9), 7.14 (1H, brs, H-4), 7.24 (1H, brs, H-6), 7.68 (1H, d, J=16.0, H-8). 13C-NMR (CDCl₃) δ: 17.8 (C-5'), 24.1 (C-12), 25.7 (C-4'), 25.9 (C-13), 28.2 (C-1'), 30.5 (C-3), 71.9 (C-11), 89.8 (C-2), 113.5 (C-9), 121.3 (C-2'), 122.4 (C-6), 123.7 (C-7), 127.2 (C-5), 127.8 (C-3a), 129.6 (C-4), 133.3 (C-3'), 147.3 (C-8), 160.2 (C-7a), 171.0 (C-10).

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References and Notes

1) A part of this work was presented previously: Tazawa S., Warashina T., Noro T., Miyase T., Kubukun H., Meeting of Tokai Branch, Pharmaceutical Society of Japan, Nagoya, Dec. 1997, Abstracts of Papers, p. 10.


