Studies on the Constituents of Brazilian Propolis[1)]

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Two new cinnamic acid derivatives along with twenty two known compounds, including five flavoniods, 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-dihydro-3-hydroxy-8-prenyl-2*H*-1-benzo pyran-6-yl)-2-propenoic acid and (*E*)-3-[2,3-dihydro-2-(1-hydroxy-1-methylethyl)-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 beewax 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.[2)] In recent times, propolis and its constituents have been reported to possess various biological activities such as antimicrobial,[3)] antibacterial,[4)] antioxidant,[5)] anticancer,[6)] immunomodulatory[7)] and others.[8)] 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)] (**1**), pinocembrin[9,11)] (**2**), dihydrokaempferide[12)] (**3**), kaempferide[9)] (**4**) and betuletol[13)] (**5**). Compounds **6**—**8** were known phenolic compounds. They were identified as (+)-*threo*-1-*C*-guaiacylglycerol[14)] (**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)[7)] (**9**), 4-caffeoylquinic acid[7)] (**10**), 5-caffeoylquinic acid[7)] (**11**), 3,4-dicaffeoylquinic acid[5,7)] (**12**), 3,5-dicaffeoylquinic acid[5,7)] (**13**) and 4,5-dicaffeoylquinic acid[5,7)] (**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 (2,3-dihydro-*p*-coumaric acid)[15)] (**16**), 3,4-dihydroxycinnamic acid (caffeic acid) (**17**), 3-prenyl-4-hydroxycinnamic acid (drupanin)[16,17)] (**18**), 3-prenyl-4-(2,3-dihydrocinnamoyloxy)cinnamic acid[3,10)] (**19**), 3-prenyl-4-(2-methylpropionyloxy)cinnamic acid[10)] (**20**), 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C)[3,10,17)] (**21**) and 3-(2,2-dimethyl-8-prenyl-2*H*-1-benzopyran-6-yl)-2-propenoic acid[1,18)] (**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 **22**[1,18)] was obtaind as an amorphus powder, and afforded a $[M]^+$ ion peak at *m/z* 298 ($C_{19}H_{22}O_3$) in the positive-ion FAB-MS spectrum. In the ^{1}H-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 5.65 (1H, d, J=10.0 Hz), 6.32 (1H, d, J=10.0 Hz), 6.33 (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 ^{1}H-NMR of **22** was a two methyl singlet at δ 1.44 (6H, s) on a quaternary carbon. The ^{13}C-NMR spectrum of **22** showed a phenolic carbon at δ 153.2, an oxygenated carbon at δ 77.2 and a carbonyl group at δ 172.0. Assignment of the signals in the ^{1}H- and ^{13}C-NMR spectra were done by analysis of ^{1}H–^{1}H shift correlation spectroscopy (H–H COSY) and heteronuclear multiple-quantum correlation (HMQC) spectra, and comparison with the data of (2,2-dimethyl-2*H*-1-benzopyran-6-yl)-2-propenoic acid[3)] and 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C)[3,10,17)] (**21**) as shown in the Experimental section. In the heteronuclear multiple bond connectivity (HMBC) spectrum, the olefinic proton signal at δ 5.65 (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 77.2 (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-2*H*-1-benzopyran-6-yl)-2-propenoic acid.

Compound **23**[1)] was obtained as an amorphus powder, and afforded a $[M]^+$ ion peak at *m/z* 316 ($C_{19}H_{24}O_4$) in the positive-ion FAB-MS spectrum. Based on ^{1}H- and ^{13}C-NMR spectra, **23** was determined to be a cinnamic acid derivative similar to **22**. **23** exhibited a proton signal at δ 3.83 (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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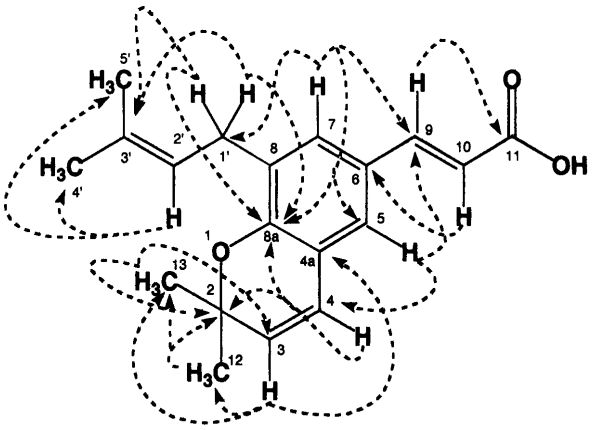

Fig. 1. HMBC Correlation of **22**

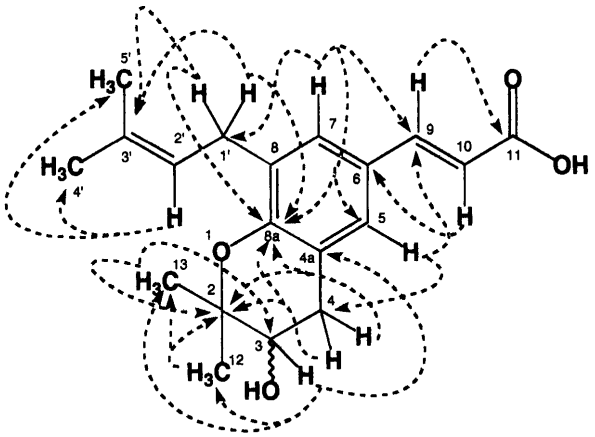

Fig. 2. HMBC Correlation of **23**

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-2*H*-1-benzopyran-6-yl)-2-propenoic acid.

Compound **24**[1] was obtained as an amorphus powder, and afforded a $[M]^+$ ion peak at *m/z* 316 ($C_{19}H_{24}O_4$) 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 δ4.67 (1H, t, J=9.0 Hz) on the carbon attached to an oxygen atom, and a methylene proton signal at δ3.18 (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 HMQC spectrum and the HMBC spectrum (Fig. 3), and comparison with the data for (*E*)-3-[2,3-dihydro-2-(2-hydroxy-1-methylethyl)-7-prenyl-benzofuran-5-yl]-2-propenoic acid (artepillin A)[19]. Thus, the structure of **24** was concluded to be (*E*)-3-[2,3-didihydro-2-(1-hydroxy-1-methylethyl)-7-prenyl-benzofuran-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.

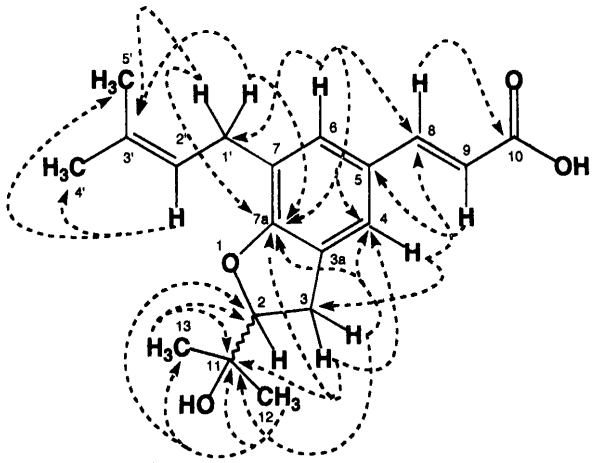

Fig. 3. HMBC Correlation of **24**

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 A-400 (400 and 100.40 MHz, respectively) spectrometers, 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, HMQC 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 Deverosil ODS-15/30 and Deverosil ODS-5 (Nomura Chemical Ltd.) Detection was by UV absorption at 280 nm. Diaion HP-20 (Mitsubishi Chemical Industries Ltd.) were used for column chromatography with H_2O–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 l) at room temperature. Concentration of the 75% EtOH extract under reduced pressure gave a residue (70 g). The residue (13.76 g) was partitioned with AcOEt and H_2O. 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_3CN–H_2O (2 : 8) to (8 : 2), to give 76 fractions. They were rechromatographed by preparative HPLC [ODS: 2% AcOH in CH_3CN–H_2O and 1% AcOH in MeOH–H_2O] 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** (105.3 mg), **22** (6.6 mg), **23** (4.4 mg) and **24** (4.4 mg). The H_2O extract (1.75 g) was passed through a Diaion HP-20 column, and the adsorbed material was eluted with H_2O, 50% MeOH in H_2O and MeOH. The eluates were concentrated under reduced pressure to afford three fractions. The H_2O fraction (872 mg) was rechromatographed by preparative HPLC [ODS: 2% AcOH in CH_3CN–H_2O and 1% AcOH in MeOH–H_2O] 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 comparision of spectral data and/or specific optical rotation with reported values and/or authentic samples.

Compound 22 Amorphous powder. FAB-MS *m/z*: 298 $[M]^+$. UV λ_{max}^{MeOH} 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, brt, H-2′), 5.65 (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, brs, H-5), 7.18 (1H, brs, 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-8a), 172.0 (C-

11).

Compound 23 Amorphous powder. FAB-MS *m/z*: 316 $[M]^+$. UV λ_{max}^{MeOH} nm (log ε): 218 (4.36), 235 (4.33), 314 (4.41). $[\alpha]_D^{23}$ +0.74° (c=0.53, EtOH). ^{1}H-NMR ($CDCl_3$) 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, C_3-OH), 3.83 (1H, t, J=6.0, H-3), 5.26 (1H, br t, H-2′), 6.27 (1H, d, J=16.0, H-10), 7.12 (1H, br s, H-5), 7.19 (1H, br s, H-7), 7.67 (1H, d, J=16.0, H-9). ^{13}C-NMR ($CDCl_3$) 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}^{MeOH} nm (log ε): 294 (4.19). $[\alpha]_D^{23}$ +0.38° (c=0.26, EtOH). ^{1}H-NMR ($CDCl_3$) 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, C_{11}-OH), 4.67 (1H, t, J=9.0, H-2), 5.28 (1H, br t, H-2′), 6.25 (1H, d, J=16.0, H-9), 7.14 (1H, br s, H-4), 7.24 (1H, br s, H-6), 7.68 (1H, d, J=16.0, H-8). ^{13}C-NMR ($CDCl_3$) 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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Refrences and Notes

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