Cyclodextrin-Enclosed Substances of Brazilian Propolis

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By using β-cyclodextrin-inclusion as a unique technique, an efficient separation of pharmacologically active phenolic compounds from Brazilian propolis was achieved to provide one new compound, 3-(3-hydroxy-3-methyl-butyl)-5-prenyl-4-hydroxycinnamic acid, together with two common cinnamic acid derivatives, artepillin C and capillartemisin A, and two known flavanols, aromadendrin and 3,5,7-trihydroxy-4′-methoxyflavanol.

Key words Brazilian propolis; β-cyclodextrin-inclusion; 4-hydroxycinnamic acid derivative

Propolis is a resinous hive product collected by honeybees from various plant sources.[1] It is extensively used in food and beverages and in folk medicine for the treatment of different ailments, and is reported to have a broad spectrum of pharmacological activities such as anti-microbial activity, antioxidant, anticancer and as an immune stimulant in addition to other pharmacological effects.[2,3]

Concerning the chemical composition of propolis, it turned out to be very complex, and more than 200 compounds have been isolated so far.[2] The most important constituent appears to be phenolics, which form more than *ca.* 50% of the propolis composition.[4] Since isolation of the bioactive substances is accompanied by many difficulties owing to their complexity and scarce amounts, we devised a method to obtain the aromatic compounds by using β-cyclodextrin-inclusion. In the present paper we describe the isolation and structural elucidation of some phenolic compounds isolated from Brazilian propolis by using β-cyclodextrin-inclusion as a selective method for the isolation of the phenolic constituents from the propolis extract.

Brazilian propolis was suspended in dist. water containing β-cyclodextrin. After the suspension was subjected to sonication, the insoluble propolis was filtered off. The water-soluble filtrate was concentrated by evaporation under reduced pressure to provide β-cyclodextrin-propolis inclusion, to which ethanol was added and vigorously stirred. The insoluble ethanol portion containing β-cyclodextrin was filtered off to give the ethanol solution, which was then concentrated to give the propolis extract. The propolis extract was subjected to Diaion HP-20, Sephadex LH-20, ODS and HPLC (ODS and TSK gel-120A) to give compounds **1**—**5**. Compounds **1**, **2**, **4**, and **5** were identified with artepilin C,[5] capillartermisin A,[6] aromadendrin[7—9] and 3,5,7-trihydroxy-4′-methoxyflavanol,[10] respectively, by spectroscopic measurements.

Compound **3** was obtained as a white powder showing $[\alpha]_D$ −14.8° (MeOH). It showed a molecular peak at *m/z* 318 $[C_{19}H_{26}O_4]^+$ in the electron impact (EI-MS). In the ^{1}H-NMR spectrum, a set of prenyl signals was observed at δ 5.58 (1H, t-like), 3.66 (2H, d, J=7.3 Hz), 1.69 and 1.68 each (3H, s). It also showed the presence of two olfenic protons at δ 6.91 and 8.10 each coupled with a large coupling constant (1H, d, J=15.9 Hz), suggesting it to be a *trans*-double bond in a side chain. The ^{13}C-NMR spectrum of **3** indicated the presence of 19-carbon atoms composed of four methyls (δ 17.8, 25.8, 2×29.8), three methylenes (δ 25.9, 29.6, 44.4), ten sp^2 carbons (δ 116.8, 123.8, 128.3, 127.0, 128.2, 130.1, 131.4, 132.5, 145.3, 156.3) and one carbonyl carbon (δ 169.8). The two dimensional (2D)-NMR spectra of proton–proton chemical shift correlation spectroscopy (^{1}H–^{1}H COSY), heteromolecular multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) were observed for the assignment of ^{1}H and ^{13}C signals. The result of the HMBC spectrum is illustrated in Fig. 1. That is, the structure of **3** was a derivative of 3,5-disubstituted 4-hydroxycinnamic acid, and a tertiary hydroxyl group was attached to C-3′ on the isopropyl moiety. Consequently, **3** was characterized as 3-(3-hydroxy-3-methyl-butyl)-5-prenyl-4-hydroxycinnamic acid, which has not yet been reported.

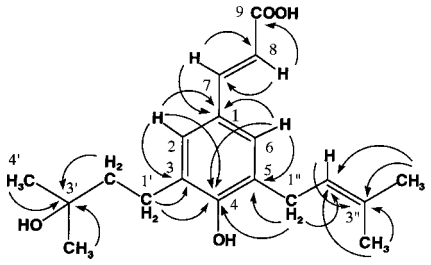

Fig. 1. Structure and Key HMBC of Compound **3**

Experimental

Optical rotations were determined on a JASCO DIP-1000 KUY polarimeter (l=5 cm). EI-MS was obtained using a JEOL α-500 spectrometer, and chemical shifts were referenced to tetramethylsilane (TMS). Column chromatography was carried out with silica gel 60 (230—400 mesh, Merck), Sephadex LH-20 (25—100 mm, Pharmacia Fine Chemicals), MCI gel CHP-20P (75—150 mm, Mitsubishikasei), Chromatorex ODS (30—50 mm, Fuji Silysia Chemical, Ltd.), and TLC was performed on precoated silica gel 60F_{254} (0.2 mm, Merck) and RP-18 F254S (Merck). HPLC was performed on ODS gel (TSK gel-120A, Tosoh Co., Ltd. Φ=7.7 mm, L=300 mm).

Isolation of Compounds 1—5 from Brazilian Propolis Using β-Cyclodextrin-Inclusion Brazilian propolis (10 g), which is regarded to be

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collected mainly from *Baccharis dracunculifolia*, Compositae, in the Minas Gerais region in Brazil, was suspended in 1 l of dist. water, which contained 10 g of β-cyclodextrin. After the suspension was subjected to sonication for 4 h, the insoluble propolis was filtered off. The water-soluble filtrate was concentrated by evaporation under reduced pressure to provide β-cyclodextrin-propolis inclusion, to which 700 ml of ethanol was added and vigorously stirred. The insoluble ethanol portion containing β-cyclodextrin was filtered off to give the ethanol solution, which was then concentrated to give the propolis extract. The propolis extract (1.054 g) was subjected to Diaion HP-20, and successively flowed out with 20, 30, 50, 80 and 100% MeOH–H_2O to give 4 fractions. Fraction 2 (0.467 g) was subjected to Sephadex LH-20 column chromatography using methanol to give two main fractions. Fraction 2.1 was chromatographed through ODS column chromatography using MeOH–H_2O (50, 60, 70%, successively) to give 2 fractions. Fraction 2.1.2 was further chromatogarphed through HPLC (ODS) using the MeOH–H_2O system (60%) to give Compound **1** (21.3 mg). Fraction 2.2 was also chromatographed through ODS by MeOH–H_2O (50, 60%) to give 2 fractions. Fraction 2.2.1 was further chromatographed by HPLC (ODS) using 50% MeOH–H_2O to give compound **4** (11.2 mg). Fraction 3 (0.128 g) was chromatographed through Sephadex LH-20 using MeOH to give 2 main fractions. Fraction 3.1 was chromatographed by ODS column chromatography and MeOH–H_2O systems (60, 70, 80%) gradiently to give 2 fractions. Fractions 3.1.1 and 3.1.2 were further chromatographed through HPLC [column TSK gel-120A] using a 70% MeOH–H_2O system to give compounds **2** (13.4 mg) and **3** (10.4 mg), respectively. Fraction 3.2 was chromatographed through ODS column chromatography using the MeOH–H_2O system (60, 70%) to give fraction 3.2.1, which was further chromatographed through HPLC (ODS) using a 60% MeOH–H_2O system to give compound **5** (9.4 mg).

Compound 1 ^{1}H-NMR (in pyridine-d_5) δ: 1.71 (6H, s, H_3-5′, 5″), 1.74 (6H, s, H_3-4′, 4″), 3.65 (2H, d, J=7.3 Hz, H2-1′, 1″), 5.62 (2H, t-like, H-2′, 2″), 6.86 (1H, d, J=15.9 Hz, H-8), 7.67 (2H, s, H-2, 6), 8.15 (1H, d, J=15.9 Hz, H-7). ^{13}C-NMR (in pyridine-d_5) δ: 17.8 (C-5′, 5″), 25.8 (C-4′, 4″), 29.2 (C-1′, 1″), 116.0 (C-8), 123.3 (C-2′, 2″), 126.6 (C-1), 127.9 (C-2, 6), 129.9 (C-3, 5), 132.3 (C-3′, 3″), 144.9 (C-7), 158.9 (C-4), 169.8 (C-9). Compound **1** was identified with artepillin C.[5)]

Compound 2 ^{1}H-NMR (in pyridine-d_5) δ: 1.68 (6H, s, H_3-4″, 5″), 1.90 (3H, s, H_3-5′), 3.68 (2H, d, J=6.7 Hz, H_2-1″), 3.79 (2H, d, J=7.3 Hz, H_2-1′), 4.32 (2H, s, H_2-4′), 5.56 (1H, t, H-2″), 6.14 (1H, t, H-2′), 6.88 (1H, d, J=15.9 Hz, H-8), 7.55 (2H, d, J=2.4 Hz, H-2, 6), 8.14 (1H, d, J=15.9 Hz, H-7). ^{13}C-NMR (in pyridine-d_5) δ: 14.1 (C-3′), 17.8 (C-5″), 25.7 (C-4″), 29.3, 29.6 (C-1′, 1″), 67.8 (C-4′), 117.1 (C-8), 123.1 (C-2′), 123.8 (C-2″), 127.1 (C-1), 128.1, 128.3 (C-2, 6), 129.9, 130.0 (C-3, 5), 145.2 (C-7). 169.8 (C-9). Compound **2** was identified with capillartemisin A.[6)]

Compound 3 A white powder, $[\alpha]_D$ −14.8° (c=0.25, MeOH), EI-MS (m/z): 318 [M, $C_{19}H_{26}O_4$]$^+$, ^{1}H-NMR (in pyridine-d_5) δ: 1.42 (6H, s, H_3-4′, 5′), 1.68 (3H, s, H_3-5″), 1.69 (3H, s, H_3-4″), 2.06 (2H, m, H_2-2′), 3.19 (2H, m, H_2-1′), 3.66 (2H, d, J=7.3 Hz, H_2-1″), 5.58 (1H, t, H-2″), 6.91 (1H, d, J=15.9 Hz, H-8), 7.54 (2H, s, H-2, 6), 8.10 (1H, d, J=15.9 Hz, H-7). ^{13}C-NMR (in pyridine-d_5) δ: 17.8 (C-5″), 25.8 (C-1′), 25.9 (C-4″), 29.6 (C-1″), 29. 8 (C-4′, 5’), 44.4 (C-2′), 69.9 (C-3′), 116.8 (C-8), 123.8 (C-2″), 128.3 (C-2), 127.0 (C-1), 128.2 (C-6), 130.1 (C-5), 131.4 (C-3), 132.5 (C-3″), 145.3 (C-7), 156.3 (C-4), 169.8 (C-9). *Anal.* Calcf for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23; Found: C, 71.69; H, 8.21.

Compound 4 ^{1}H-NMR (in pyridine-d_5) δ: 4.73 (1H, d, J=11.6 Hz, H-3), 5.28 (1H, d, J=11.6 Hz, H-2), 5.87 (1H, d, J=2.4 Hz, H-6), 5.90 (1H, d, J=2.4 Hz, H-8), 7.25 (2H, d, J=8.5 Hz, H-3′, 5′), 7.74 (2H, d, J=8.5 Hz, H-2′, 6′). ^{13}C-NMR (in pyridine-d_5) δ: 84.6, 73.3, 198.9, 165.1, 97.5, 168.8, 96.2, 163.1, 101.7, 128.9, 130.2, 116.0, 159.6, 128.9, 130.2. Compound **4** was identified with aromadendrin.[7—9)]

Compound 5 ^{1}H-NMR (in pyridine-d_5) δ: 4.98 (1H, d, J=11.0 Hz, H-3), 5.45 (1H, d, J=11.0 Hz, H-2), 6.38 (1H, d, J=1.8 Hz, H-6), 6.52 (1H, d, J=1.8 Hz, H-8), 7.06 (2H, d, J=7.9 Hz, H-3′, 5′), 7.74 (2H, d, J=8.5 Hz, H-2′, 6′). ^{13}C-NMR (in pyridine-d_5) δ: 84.3, 73.3, 198.5, 165.1, 97.5, 168.8, 96.3, 163.8, 101.6, 130.5, 130.0, 114.3, 160.5, 130.0, 55.3. Compound **5** was identified with 3,5,7-trihydroxy-4′-methoxyflavanol.[10)]

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