Isolation of Antioxidative Compounds from Brazilian Propolis: 3,4-Dihydroxy-5-prenylcinnamic Acid, a Novel Potent Antioxidant

Katsuhiko HAYASHI, Sadaaki KOMURA, Noriko ISAI, Nobuko OIHISHI, and Kunio YAGI*

Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Gifu 505-0116, Japan.
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One new and 11 previously known antioxidative compounds were isolated from Brazilian propolis. The new compound was determined as 3,4-dihydroxy-5-prenylcinnamic acid (3-[3,4-dihydroxy-5-(3-methyl-2-butenyl)phenyl]-2-(E)-propenoic acid) by various physical analyses (MS, IR, 1H-NMR, 13C-NMR, and 2D-NMR). The inhibitory activity of each compound against peroxidation of linoleic acid in a micelle solution was measured. We found that the novel compound possessed the highest potency (IC_{50}, 0.17 μM) among them and was more effective than butylated hydroxytoluene (BHT; IC_{50}, 0.36 μM) under the experimental conditions employed. Among the isolated antioxidative compounds, 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C; IC_{50}, 0.44 μM) was found to be most abundant in Brazilian propolis.

Key words  3,4-dihydroxy-5-prenylcinnamic acid; propolis; antioxidant; 3,5-diprenyl-4-hydroxycinnamic acid; lipid peroxidation; linoleic acid

Propolis is a resinous material that honeybees produce from exudates of various plants and beeswax in a beehive, and has been used for folk medicines and foods since ancient times in many parts of the world.1) Propolis has been known to possess antibacterial,2) antiviral,3) antitumoral,4) antioxidative,5,6) immunomodulatory,7) and other beneficial properties.1,6) These biological and pharmaceutical effects and chemical constituents of propolis have been mainly studied on samples of Central and Eastern European propolis.1,8) On the other hand, Brazilian propolis recently has been widely marketed in various foods and beverages with the intention of preserving or improving human health. There are many preparations with regional differences that may be attributed to different flora. Investigations on chemical and biological properties of Brazilian propolis started within the last 5—6 years,9) in comparison with the earlier ones on European propolis.

In the present report, we describe the isolation and characterization of antioxidative agents in Brazilian propolis in terms of a lipid peroxidation assay, according to the method of Kharasch6) except that 2,2'-azobisisobutyronitrile (AAPH) is used as oxidant.11) Especially we report the isolation, elucidation of structure, and antioxidative activity of a new compound, 3,4-dihydroxy-5-prenylcinnamic acid (3-[3,4-dihydroxy-5-(3-methyl-2-butenyl)phenyl]-2-(E)-propenoic acid).

Results and Discussion

Isolation of Antioxidative Compounds from Propolis

We preliminarily examined various ethanol-water mixtures for their extraction efficiency and inhibition of peroxidation of linoleic acid. Among them, use of 70% ethanol as a solvent for extraction afforded a high extract yield (44.5%), expressed as a percent of the amount of dried extract to that of the applied crude propolis (44.5%), and high antioxidative activity (IC_{50}, 0.4 μg/ml). Moreover, resinous compounds in the extract derived from propolis, that were not desirable for our study, were decreased under this condition. On the other hand, the water extract gave a lower yield (11.1%), even though the inhibitory activity (IC_{50}, 0.4 μg/ml) was similar to that of 70% ethanol extract. Therefore, 70% ethanol was selected as a solvent in the present experiment.

Propolis was cut into small pieces and extracted with 70% ethanol for one day under stirring at room temperature. After filtration and concentration in vacuo of the extract, the residue was partitioned with ether and water to give an ether extract. The ether extract was chromatographed on a silica gel column to give 3 fractions (Fr.1—Fr.3), and then these fractions were repeatedly separated by a combination of different kinds of chromatographies, assessing antioxidative activity by monitoring the inhibitory activity against peroxidation of linoleic acid to give the 12 compounds. Fraction 1 afforded 4',6-dimethoxy-3,5,7-trihydroxyflavone (betulnetol, 1),11,15) kaempferide (2), 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C, 3),10,18,19) 4-dihydroxycinnamoyl-3-prenylcinnamic acid (4),10) and 6-(2-carboxyethenyl)-2,2-dimethyl-2H-1-benzopyran (5).10,21) 4-Hydroxy-3-prenylcinnamic acid (drupanin, 6),19,22) 4-hydroxy-3-prenylbenzoic acid (7),23) and 6-methoxykaempferol (8)18,24) were obtained from fraction 2. Finally, separation of fraction 3 gave 4-hydroxy-3-(E)-(4-hydroxy-3-methyl-2-butenyl)-5-prenylcinnamic acid (capillartemin A, 9),25) 3-methylkaempferol (10),26) kaempferol (11), and a new compound, 3,4-dihydroxy-5-prenylcinnamic acid (12) (Fig. 1). Two known compounds 2 and 11 were identified by direct comparison of their spectroscopic properties with each authentic sample. Other known compounds (1, 3—10) were identified by comparison of their physical data (mp, HPTLC, IR, UV, 1H- and 13C-NMR) with data on them in the literature. In order to clarify the chemical structures of all of the above isolated compounds, we employed 2D-NMR spectra [13C—1H correlation spectroscopy (13C—1H COSY), heteronuclear multiple bond correlation spectroscopy (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)].

Structure of Compound 12

Physical data of compound 12 obtained as a pale yellow powder are as follows: mp 178—180 °C; FAB-MS (positive mode) m/z: 249 (M+H)+; HPTLC Silica gel 60 F254 Rf: 0.19, chloroform—methanol (10:1); IR (KBr) ν_{max} cm^{-1}: 3551, 3290, 2978, 2916, 1668, 1607, 1592; UV λ_{max} (MeOH) nm (ε): 322 (16200), 223 (17600); 1H-NMR (CD_{3}OD) δ: 7.48 (1H, d, J = 15.8 Hz, H-7), 6.89 (1H, d, J = 2.0 Hz, H-2), 6.79 (1H, d, J = 2.0 Hz, H-6), 6.79 (1H, d, J = 2.0 Hz, H-6),

* To whom correspondence should be addressed.
6.17 (1H, d, J=15.8 Hz, H-8), 5.31 (1H, m, H-11), 3.29 (2H, m, H-10), 1.74 (3H, s, H-13), 1.72 (3H, s, H-14); \(^1^3^C\)-NMR (CD\(_3\)OD) \(\delta\): 171.6 (C-9), 147.4 (C-4), 147.2 (C-7), 146.3 (C-3), 133.3 (C-12), 129.9 (C-5), 127.0 (C-1), 123.7 (C-11), 123.3 (C-6), 115.6 (C-8), 112.4 (C-2), 29.1 (C-10), 26.0 (C-13), 17.9 (C-14).

In the \(^1^H\)- and \(^1^3^C\)-NMR spectra of compound 12, the occurrence of the following moieties was confirmed: a trans-cinnamic acid moiety [\(\delta_{H}\) 7.48 (H-7), 6.89 (H-2), 6.79 (H-6), and 6.17 (H-8); \(\delta_{C}\) 171.6 (C-9), 147.4 (C-4), 147.2 (C-7), 146.3 (C-3), 129.9 (C-5), 127.0 (C-1), 123.3 (C-6), 115.6 (C-8), and 112.4 (C-2)] and a prenyl group [\(\delta_{H}\) 5.31 (H-11), 3.29 (H-10), 1.74 (H-13), and 1.72 (H-14); \(\delta_{C}\) 133.3 (C-12), 123.7 (C-11), 29.1 (C-10), 26.0 (C-13), and 17.9 (C-14)]. The connectivities of the quaternary carbons in 12 were characterized by correlation spectroscopy via the HMBC spectrum (Fig. 2A). The HMBC spectrum shows that the H-2 proton and H-10 protons correlate with the carbon at \(\delta_{C}\) 147.4 (C-4), suggesting that the \(\delta_{C}\) 147.4 carbon lies at the fourth position in the aromatic ring. The observations that the H-10 protons correlate with the C-4 and C-5 carbons and the H-6 proton correlates with the C-10 carbon indicate that the prenyl group connects with the aromatic ring at the fifth position. These findings led us to decide the structure of compound 12 as \(3\\{3\{4\{dihydroxy-5\{3\{methyl\{2\{butenyl\}phenyl\}\}propenoyl\}\}\}\}propenoic acid (3,4-dihydroxy-5-prenylcinnamic acid) as shown in Fig. 1. Furthermore, the observation of NOE correlations between the protons of compound 12 (Fig. 2B) supports this structure.

Inhibition of Peroxidation of Linoleic Acid by Compounds 1–12

The inhibitory activity against peroxidation of linoleic acid in a micelle solution was examined for compounds 1–12. Table 1 summarizes their inhibitory potency. We found that compound 12 possessed the highest activity (IC\(_{50}\) 0.17 \(\mu\)M) among them and was more effective than butylated hydroxytoluene (BHT; IC\(_{50}\) 0.36 \(\mu\)M) under the experimental conditions employed. Among other isolated compounds, compounds 3 and 9 were also strong antioxidants, of which the antioxidative potency was first demonstrated in this study. Since compound 12 is a derivative of 4-hydroxycinnamic acid, its antioxidative activity was compared with that of various 4-hydroxycinnamic acid analogues. Table 1 also indicates that compound 12 was the strongest antioxidant among them and exhibited antioxidative potency higher than that of the well-known antioxidant ellagic acid. As to the structure–activity relationship of 4-hydroxycinnamic acid analogues, we found that the antioxidative potency was high when the hydroxyl, alkyl, or methoxy group occurs at the \(\alpha\)-position of the 4-hydroxy group. Further, the data in Table 1 indicates that 4-hydroxycinnamic acid derivatives were more effective than 4-hydroxybenzoic acid derivatives. Several authors have shown that \(\alpha\)-substitution with an electron donor, alkyl or methoxy group, increased the stability of the arylxy radical through electron delocalization and its antioxidative action and that further \(\alpha\)-diolidryx substitution allowed metal chelation in addition to strong radical stabilization.\(^{27,28}\) Concerning the difference between the C=C–COOH group and the COOH group, it has been proposed that the former group participates in stabilizing the arylxy radical by resonance.\(^{29,30}\) Our observations support this suggestion and can explain the fact that the novel compound 12, possessing both the electron donor \(\alpha\)-prenyl group and \(\alpha\)-diolidryx groups, exhibits strong antioxidative potency. Among the isolated flavonoids kaempferide 2 and kaempferol 11 were effective. Comparing the structure–activity relationship of these flavonoids, the introduction of the hydroxyl group at the third position increased the activity of radical scavenger. These results agree with those reported previously.\(^{29,30}\)
Inhibitory activity against peroxidation of linoleic acid is given. Experimental conditions are described in the text. a) The percent of lipid peroxidation inhibition at the described condition is shown because of the weak inhibition.

Table 1. Inhibition of Peroxidation of Linoleic Acid by Antioxidative Compounds from Propolis in Comparison with 4-Hydroxycinnamic Acid and Phenolic Acid Analogues

<table>
<thead>
<tr>
<th>Compounds from propolis</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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<tbody>
<tr>
<td>1</td>
<td>2.2</td>
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<tr>
<td>2</td>
<td>0.72</td>
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<tr>
<td>3</td>
<td>0.44</td>
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<tr>
<td>4</td>
<td>160</td>
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<td>5</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>2.9</td>
</tr>
<tr>
<td>7</td>
<td>(24 μM: 6% inhibition)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>1.4</td>
</tr>
<tr>
<td>9</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
</tr>
<tr>
<td>11</td>
<td>0.92</td>
</tr>
<tr>
<td>12</td>
<td>0.17</td>
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4-Hydroxycinnamic acid analogues

4-Hydroxycinnamic acid (p-coumaric acid) 17
4-Hydroxy-3-methoxycinnamic acid 2.1
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid) 0.80
3,4-Dihydroxycinnamic acid (caffeic acid) 0.41
Phenolic acid analogues

p-Hydroxybenzoic acid (50 μM: 15% inhibition)<sup>a</sup> 90
p-Hydroxyphenylpropionic acid 1.2
Protocatechuic acid 1.2

Reference samples

n-Propyl/gallate 0.42
Chlorogenic acid 0.28
Ellagic acid 0.23
BHT 0.36
Quercetin 0.68

Experimental

Materials

Brazilian propolis was purchased from MN EXPORTAÇÃO E REPRESENTAÇÃO LTDA in Brazil. Kaempferide, kaempferol, and quercetin were obtained from Extrasynthese S.A., Genay, France; AAPH, BHT, p-coumaric acid, and other phenolic acids, from Wako Pure Chemicals, Osaka. Lubrol PX came from Naicali Tesque, Kyoto; and linoleic acid from Sigma Chemicals, St. Louis, MO.

For column chromatographies, latex-beads-80/100, 75—150 μm (for silica gel column A) was purchased from Iatron Laboratories, Tokyo; and Silica gel 60 No.7734, 63—200 μm (for silica gel column B), from E. Merck, Darmstadt, Germany. Silica gel BW-127Z, 53—150 μm (for silica gel column C), Microbeads MB-4B, 75—150 μm (for silica gel column D), Chromatorex DM1020, 75—150 μm and Chromatorex DM2035MT, 44—75 μm (for ODS column A and B, respectively) were obtained from Fuji Silysis Chemicals, Kasugai, Japan. Sephadex LH-20, 27—163 μm, was from Amersham Pharmacia Biotech, Uppsala, Sweden.

Silica gel 60 F<sub>254</sub> precoated plate No. 5642 and RP-18W F<sub>254</sub> precoated plate No.13124 for HPTLC, and Silica gel 60 F<sub>254</sub> precoated plate No.13895 for preparative TLC were obtained from E. Merck.

Instruments for Chemical Identification

The following instruments were used to obtain physical data: melting points, Yanagimoto micro-melting point apparatus MP-S3 (Yanagimoto Shoji, Kyoto); IR spectra, Hitachi 270-30 infrared spectrophotometer (Hitachi, Tokyo) or Shimadzu FTIR 8300 infrared spectrophotometer (Shimadzu, Kyoto); UV spectra, Shimadzu MPS-2000 spectrophotometer (Shimadzu); NMR spectra, JEOL EX-270 spectrometer (270 MHz for 1H-NMR and 68 MHz for 13C-NMR, Tokyo); MS spectra, JEOL-Mation JMS700 spectrometer (JEOL); HPLC spectra, LC-4A liquid chromatograph equipped with SPD-2A5 UV detector (Shimadzu). Separation by HPLC was performed on a reverse-phase TSKgel ODS-80TM column (15 cm x 4.6 mm i.d., 5 μm, Tosoh, Tokyo) with water-methanol-acetic acid (32:65:3) as a mobile phase at a flow rate of 1 ml/min at 36°C, and absorbancy of the eluate was monitored at 275 nm. For HPTLC, detection was achieved by illumination with a UV lamp at 254 nm.

Isolation of Constituents from Propolis

Brazilian propolis (216 g) was extracted with 70% ethanol (3.2 l), and the extract was concentrated in vacuo. The residue (103 g) was suspended in water and extracted with ether (4 l x 3) to give an ether extract (71 g). The ether extract was chromatographed on a silica gel column B with chloroform and then with chloroform-methanol (60:1—10:1) to give 3 fractions (Fr. 1—Fr. 3).

Fraction 1 (30 g) was rechromatographed on a silica gel column B with chloroform and then with chloroform (40:1—10:1) to give 3 fractions (Fr. 1-1, Fr. 1-2, and Fr. 1-3). Fraction 1-1 (4.3 g) was purified by silica gel column B chromatography with chloroform-methanol (50:1—10:1) and washing with chloroform–methanol (3:1) to give compound I (90 mg).

Fraction 1-2 (10 g) was chromatographed on a silica gel column B with chloroform–methanol (60:1—10:1 to give Fr. 1-2 and Fr. 1-2). Fraction 1-2-1 (1.1 g) was washed with chloroform–methanol (100:1) to give compound II (120 mg).

Fraction 1-3 (1.0 g) was successively subjected to silica gel column C chromatography with chloroform–methanol (20:1) and DMS column chromatography with 60—80% methanol to give Fr. 1-3-1 and Fr. 1-3-2. Fraction 1-3-1 (430 mg) was applied onto a DMS column with 65—70% methanol, and then twice onto an ODS column A with 80% methanol to give compound 4 (87 mg).

Repeated ODS column A chromatography of Fr. 1-3-2 (410 mg) using 75% methanol afforded compound 5 (170 mg).

Fraction 2 (14 g) was chromatographed on a silica gel column C with chloroform–methanol (50:1—10:1) to give Fr. 2-1 and Fr. 2-2. Purification of Fr. 2-1 (3.7 g) was carried out by silica gel column B, DMS column, and ODS column A chromatographies in a similar way to give compound 6 (220 mg).

Fraction 2-2 (6.6 g) was chromatographed on an ODS column A with 65% methanol to give Fr. 2-2-1 and Fr. 2-2-2. Fraction 3-2-1 (83 mg) was successively subjected to silica gel column D chromatography with chloroform–methanol (30:1), preparative TLC with chloroform–methanol (10:1), and ODS column B chromatography with 65% methanol to give compound 7 (28 mg). Washing Fr. 2-2-2 (68 mg) with methanol provided compound 8 (15 mg).
Fraction 3 (17 g) was chromatographed on a silica gel column C with chloroform-methanol (40: 1—10:1) to give Fr. 3-1 and Fr. 3-2. Fraction 3-1 (5.0 g) was subjected to DMS column chromatography with 55—90% methanol to give Fr. 3-1-1 and Fr. 3-1-2. Fraction 3-1-1 (500 mg) was successively subjected to ODS column A chromatography and Sephadex LH-20 column chromatography with 70% methanol to give 9 (54 mg). Fraction 3-1-2 (250 mg) was purified in the same manner as for compound 9 to give compounds 10 (18 mg) and 11 (7 mg). Fraction 3-2 (2.8 g) was successively subjected to DMS column chromatography with 55—90% methanol, ODS column A chromatography with 70% methanol, silica gel column B chromatography with chloroform-methanol (20:1—5:1), and Sephadex LH-20 column chromatography with 70% methanol to afford compound 12 (24 mg).

Among the 11 known compounds, detailed data of compounds 7 and 10 were obtained first and are described here. Physical data of the new compound 12 were described in Results and Discussion.

**Compound 7:** 4-Hydroxy-3-prenylnitrobenzoic Acid

Colorless amorphous solid. mp 99—100°C. HR-FAB-MS (positive mode) m/z: 207.0355 (+M+H+) (Calcd for C10H10O4, 207.0211). HPTLC RP-18W F254 Rf: 0.69, 75% methanol. IR (KBr) νmax cm⁻¹: 3500—2800 (OH), 1684, 1610 (C=O). 1H-NMR (CDCl3, δ): 0.94 (3H, d, J= 7.8 Hz, H-5), 2.35 (H, d, J= 8.8 Hz, H-4), 7.22 (2H, H-6, H-7), 7.41 (1H, d, J= 3.2 Hz, H-11), 7.50 (1H, s, H-2), 7.63 (1H, d, J= 3.2 Hz, H-3). 13C-NMR (CDCl3, δ): 106.2 (C-2), 132.6 (C-13), 132.3 (C-12), 133.1 (C-3), 129.3 (C-14), 126.1 (C-15), 125.6 (C-8), 115.3 (C-5), 29.1 (C-7), 26.0 (C-11), 17.9 (C-12).

**Compound 10:** 3-Methylkaempferol

Yellow needles, mp 283—287°C. HR-FAB-MS (positive mode) m/z: 301.0712 (+M+H+) (Calcd for C15H15O6, 301.0712). HPTLC silica gel 60 F254 Rf: 0.50, chloroform-methanol (10: 1). IR (KBr) νmax cm⁻¹: 3500—3060 (OH), 1660, 1615. 1H-NMR (DMSO-d6, δ): 12.7 (1H, brs, OH-5), 7.94 (2H, d, J= 8.6 Hz, H-2', 6'), 6.95 (2H, d, J= 8.9 Hz, H-3', 5'), 6.44 (1H, d, J= 2.0 Hz, H-8), 6.20 (1H, d, J= 2.0 Hz, H-6), 3.78 (3H, s, H-11). 13C-NMR (DMSO-d6, δ): 177.8 (C-4), 164.1 (C-7'), 161.2 (C-5), 160.1 (C-4'), 126.3 (C-9), 125.5 (C-2), 137.5 (C-3), 130.0 (C-2', 6'), 120.5 (C-1'), 115.5 (C-3', 5'), 104.1 (C-10), 98.5 (C-6), 93.6 (C-8), 59.6 (C-11).

**Inhibition of Peroxidation of Linoleic Acid**

Linoleic acid solution was prepared by adding 0.35 ml of 600 mM linoleic acid in ethanol to 62 ml of 0.3% Lubrol PX-20 mM sodium phosphate buffer (pH 7.4) and then sonicating the mixture for 3 min. The reaction mixture (1 ml) contained 0.89 mM of linoleic acid solution, 0.01 ml of test sample dissolved in dimethylsulfoxide, and 0.1 ml of 5 mM AAPH in 0.3% Lubrol PX-20 mM sodium phosphate buffer (pH 7.4). The final concentration of each component was as follows: linoleic acid, 100 µM; AAPH, 2.5 mM; dimethylsulfoxide, 1% and ethanol, 0.5%. After the mixture had been incubated at 37°C for 1 h, the reaction was stopped by the addition of 1 ml of methanol; and then 1 ml of the mixture was mixed with 4 ml of 70% methanol. Amounts of linoleic acid hydroperoxide were measured in terms of their conjugated diene with a UV spectrophotometer at 234 nm. As a control, 0.01 ml of dimethylsulfoxide was used.

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**References**