

**In Vitro Inhibitory Effects of Pulvinic Acid Derivatives Isolated from Chinese Edible Mushrooms, *Boletus calopus* and *Suillus bovinus*, on Cytochrome P450 Activity**

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Increasing attention has been focused on food-drug interactions. We have investigated the inhibitory effect of Chinese edible mushrooms, *Boletus calopus* and *Suillus bovinus*, on cytochrome P450 (CYP) 1A2, 2C9, 2D6, and 3A4, the main drug-metabolizing enzymes. Three pulvinic acid derivatives, atromentic acid (1), variegatic acid (2), and xerocomic acid (3), isolated from *Boletus calopus* and *Suillus bovinus*, revealed nonspecific inhibitory effects on all four CYPs. Using these compounds, the maximum IC$_{50}$ values obtained with CYP3A4 in vitro were atromentic acid (1), 65.1 ± 3.9 μM; variegatic acid (2), 2.2 ± 0.1 μM; and xerocomic acid (3), 2.4 ± 0.1 μM. Variegatic acid (2) and xerocomic acid (3) were effective inhibitors, comparable to cimetidine, dicoumarol, erythromycin, safrole, and uniconazole.

Key words: atromentic acid; variegatic acid; xerocomic acid; cytochrome P450 (CYP) inhibition; ferryl heme reductant.

Foods contain some constituents that can induce or inhibit drug-metabolizing enzymes in the body. Food components provide a rich source of secondary metabolites with unusual chemical structures and interesting biological activities. The co-administration of certain foods and drugs often cause adverse effects.1-3 Mushrooms, for example, have been consumed for centuries as both foods and folk medicines (i.e., drugs). The mushrooms, *Boletus calopus* and *Suillus bovinus* which are edible mushrooms favored around the world, have a high oxidative capacity, as evidenced by their rapid discoloration after harvesting and cooking. Because of their high oxidative capacity, intake of these mushrooms may be a possible risk factor for pharmacokinetic interactions with co-administered conventional drugs. In the present study, the chemical structure of each component, compounds 2 and 3 obtained from *Boletus calopus* and compounds 1, 2, and 3 from *Suillus bovinus*, was identified.

Cytochrome P450s (CYPs), a family of drug-metabolizing enzymes, are monoxygenases containing a heme group that has a major role in the redox of molecular oxygen. The oxidation mechanism of CYPs involves the binding of substrates with the CYPs to form a complex. In the complex, a ferric heme loses an H$_2$O molecule and is immediately reduced by an electron to form ferrous heme. The ferrous heme of the new complex then reacts with one O$_2$, one electron, and two H$^+$ to produce a ferryl heme. Finally, the substrate is metabolized by the ferryl heme of the CYP, and the CYP stable ferric heme with a H$_2$O molecule is regenerated (Scheme 1).4,5 It was recently revealed that the reduction of ferryl heme to ferric heme is involved in the inhibitory effects of CYP oxidation.

CYPs are not inhibited by antioxidants such as ascorbic acid and α-tocopherol. Compounds 2 and 3 have a catechol moiety, and have therefore been thought to act as antioxidants that do not affect CYPs. Surprisingly, however, both compounds do in fact inhibit CYP1A2, CYP2C9, CYP2D6, and CYP3A4. The inhibitory effect may be due to a difference in the oxidative processes between compounds 2 and 3, and catechol that are mediated by the iron in the CYPs. To analyze this potential difference, the novel conversion from ferryl myoglobin (Fe$^{IV}$) to ferric myoglobin (Fe$^{III}$) has been studied by spectrophotometry in the Soret band region. The reason for selecting CYP1A2, CYP2C9, CYP2D6, and CYP3A4 for the present study is that these enzymes account for 80% of the total hepatic metabolism.7 CYP3A4, one of the most ubiquitous human isozymes, is involved in the phase I metabolism of more than 50% of current pharmaceuticals. For example, furanocoumarins, which are important CYP3A4 inhibitors, are thought to be the primary components responsible for grapefruit juice-drug interactions.8-14 Compounds 2 and 3 contain a catechol moiety, but furanocoumarins do not, suggesting that different mechanisms underlie their inhibitory effects on CYP3A4.

The aim of present study was to investigate the mechanisms of the inhibitory properties of compounds 1, 2, and 3, which are pulvinic acid derivatives isolated from *Boletus calopus* or *Suillus bovinus*, on human CYP isozymes.

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Abbreviations: CHC, 3-cyano-7-hydroxycoumarin; CYP, cytochrome P450; EDTA, ethylenediamine tetraacetic acid; HPLC, high performance liquid chromatography; HR-ESIMS, high resolution electron spray ionization mass spectra; IR, infrared; NMR, nuclear magnetic resonance; PIH, pyridoxal isocotinyl hydrazone
Materials and Methods

**Chemicals.** Drug metabolizing enzyme kits were obtained from Invitrogen Corporation (Carlsbad, CA). α-Naphthoflavone, dicoumarol, sulfaphenazole, cimetidine, quinidine, Ketocanzole, caffeic acid, and horse heart myoglobin were from Sigma Chemical (Tokyo). Ethylenediamine tetraacetic acid (EDTA) was from Dojindo Laboratories (Kumamoto, Japan). α-Tocopherol was from Kanto Chemical, (Tokyo). Pyridoxal isonicotinyl hydrazone (PIH) was from Calbiochem (Madison, WI). Saffrole was from Tokyo Kasei Kogyo (Tokyo). Ascobic acid, erythromycin, uncionezole, and other reagents were from Wako Pure Chemical Industries (Osaka, Japan).

**Mushrooms.** The dry fruiting bodies of *Boletus calopus* and *Suillus bovinus* were from the Kunning Edible Mushroom Institute (Kunning, China).

**Instruments.** Nuclear magnetic resonance (NMR) spectra were obtained using a Jeol JNM-ECA600 spectrometer or a Jeol JNM-A400 spectrometer. Semipreparative high performance liquid chromatography (HPLC) was performed on a Jasco PU-2080. High resolution electron spray ionization mass spectra (HR-ESIMS) were determined using a Jeol AccuTOF JMS-T700LICR. Infrared (IR) spectra were obtained using a Nicolet 380 Fourier transform-IR. Optical spectra were measured using a Hitachi U-4000 spectrophotometer. CYP activities were determined using a GEMINI EM fluorescence plate reader (Molecular Devices, Sunnyvale, CA).

**Purification and isolation of active compounds.** The dry fruiting bodies (500.0 g) of *Boletus calopus* were soaked in 80% acetone (10.0 liters) at room temperature for 3 d. The resulting filtrate was evaporated to an aqueous concentrate in vacuo, and extracted with equal volumes of ethyl acetate (EtOAc) at pH 3.0 to give EtOAc extracts (10.9 g). EtOAc extract (2 g) was applied to a Sephadex LH-20 column (30 mm ID × 700 mm), using CH₂OH as the eluent. Thirty 50-ml fractions were collected to obtain two active sub-fractions A (nos. 9–11) and B (nos. 12–18). Sub-fraction A (29.5 mg) was rechromatographed on a preparative HPLC (column: Shiseido Capcell pak C18 UI210, 15 mm ID × 250 mm; solvent system, 0.15% KH₂PO₄ (pH 3.5)/CH₂CN (80:20); detection, UV at 254 nm; flow rate, 8.8 ml/min) to give an active compound, compound 1 (4.8 mg). Sub-fraction B (133.9 mg) was rechromatographed on preparative HPLC (column: Shiseido Capcell pak C18 UI210, 15 mm ID × 250 mm; solvent system, 0.15% KH₂PO₄ (pH 3.5)/CH₂CN (85:15); detection, UV at 254 nm; flow rate, 8.8 ml/min) to give two active compounds, compound 2 (83.3 mg) and 3 (6.8 mg).

**Physico-chemical analyses of compounds 1, 2, and 3.** Compound 1 was isolated as a yellow amorphous powder with the molecular formula C₂₁H₂₂O₅, based on HR-ESIMS (neg.) at m/z 339.05071 (M−H)⁻ (calcd. 339.05048); IR ʋₘₐₓ (ATR) cm⁻¹: 3174, 1739, 1677, 1587, 1512, 1489, 1465, 1431, 1366, 1240, 1174, 1103, 1056, 962, 919, 827, 743, 704; ¹H-NMR (CDCl₃, 600 MHz) δ: 6.83 (2H, d, J = 8.8 Hz, H-3′, 5′), 6.85 (2H, d, J = 8.1 Hz, H-3, 5), 7.21 (2H, d, J = 8.8 Hz, H-2′, 6′), 8.08 (2H, d, J = 8.1 Hz, H-2, 6); ¹³C-NMR (CDCl₃, 150 MHz) δ: 101.0 (C-2′), 115.0 (C-3′), 115.0 (C-5′), 115.7 (C-7), 117.5 (C-4′), 123.6 (C-1), 126.8 (C-1′), 129.1 (C-2′), 132.6 (C-2′), 132.6 (C-6′), 154.6 (C-4′), 157.1 (C-4), 157.7 (C-6), 161.0 (C-3′), 168.3 (C-1′), 171.9 (C-6′).

**Compound 2** was isolated as an orange-yellow amorphous powder with the molecular formula C₂₁H₂₂O₅, based on HR-ESIMS (neg.) at m/z 371.04036 (M−H)⁻ (calcd. 371.04031); IR ʋₘₐₓ (ATR) cm⁻¹: 3351, 1739, 1677, 1598, 1516, 1430, 1375, 1112, 1057, 1000, 902, 816, 776, 741, 701, 663; ¹H-NMR (CDCl₃, 600 MHz) δ: 6.74 (1H, dd, J = 2.2, 8.1 Hz, H-6), 6.84 (1H, d, J = 8.1 Hz, H-5′), 6.80 (1H, d, J = 8.3 Hz, H-5), 6.89 (1H, d, J = 2.2 Hz, H-2′), 7.59 (1H, d, J = 2.0, 8.3 Hz, H-6), 7.74 (1H, d, J = 2.0 Hz, H-2); ¹³C-NMR (CDCl₃, 150 MHz) δ: 103.5 (C-2′), 115.2 (C-2), 115.3 (C-5′), 115.9 (C-5), 117.1 (C-1′), 118.5 (C-5′), 120.7 (C-6′), 123.0 (C-1), 123.2 (C-6′), 126.2 (C-1′), 145.0 (C-3′), 145.4 (C-3), 145.9 (C-4′), 146.0 (C-4′), 155.0 (C-4′), 161.4 (C-3′), 167.4 (C-1′), 173.6 (C-6′).

**Compound 3** was isolated as an orange-yellow amorphous powder with the molecular formula C₂₁H₂₂O₅, based on HR-ESIMS (neg.) at m/z 355.04594 (M−H)⁻ (calcd. 355.04539); IR ʋₘₐₓ (ATR) cm⁻¹: 3329, 1739, 1676, 1599, 1558, 1513, 1432, 1364, 1243, 1109, 1057, 960, 877, 821, 746, 707; ¹H-NMR (CDCl₃, 600 MHz) δ: 6.85 (1H, d, J = 8.3 Hz, H-5), 6.86 (2H, d, J = 8.8 Hz, H-3, 5), 7.23 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.61 (1H, dd, J = 2.0, 8.3 Hz, H-6), 7.76 (1H, d, J = 2.0 Hz, H-2); ¹³C-NMR (CDCl₃, 150 MHz) δ: 102.6 (C-2′), 115.0 (C-2′), 115.2 (C-3′), 115.2 (C-5′), 115.8 (C-5), 117.1 (C-3′), 120.5 (C-6′), 123.4 (C-1′), 126.2 (C-1′), 132.6 (C-2′), 132.6 (C-6′), 145.3 (C-3′), 145.5 (C-4′), 154.9 (C-4′), 157.9 (C-4′), 162.0 (C-3′), 167.7 (C-1′), 172.8 (C-6′).

**Scheme 1.** Catalytic Cycle of Cytochrome P450 (CYP).
CYP inhibition assay. The inhibitory effects of compounds 1, 2, and 3 on CYP activity were determined using black 96-well microtiter plates (Sumitomo Bakelite, Tokyo) based on the formation of fluorescent metabolites by the CYP enzymes. CYP inhibitory activities were assayed using the Vivid CYP Blue Substrate. 7-Ethoxyxymethylx-3-cyanocoumarin (EOMCC) was the substrate used with CYP1A2 and CYP2D6. 7-Benzoxymethylx-3-cyanocoumarin (BOMCC) was the substrate used with CYP2C9 and CYP3A4. The positive control was safranal (5) for CYP1A2, dicoumarol (6) for CYP2C9, cimetidine (7) for CYP2D6, erythromycin (8) for CYP3A4, and uniconazole (9) for all of the CYPs. We also examined the antioxidant agents ascorbic acid and α-tocopherol, and the chelating agents EDTA and PIH to further investigate the inhibitory mechanism of the compounds.

Compounds 1, 2, and 3 (1, 2, 3, 5, or 10 μM) or positive controls were dissolved in methanol. Briefly, 40 μL of the obtained solution was added to 50 μL of enzyme/NADPH/CYP reductase mixture in a 96-well microtiter plate, and pre-incubated for 20 min at room temperature. The reaction was initiated by the addition of 10 μL substrate/NADP+ mixture for 15 min at room temperature. The reaction was terminated by the addition of 10 mL of each stop solution (3 μM of α-naphthoflavone for CYP1A2, 30 μM of sulfaphenazole for CYP2C9, 30 μM of quinidine for CYP2D6, and 3 μM of ketocazole for CYP3A4). CYP1A2 and CYP2D6 activity was assessed by monitoring the metabolism of EOMCC to 3-cyano-7-hydroxycoumarin (CHC). CYP2C9 and CYP3A4 activity was assayed by monitoring the metabolism of BOMCC to CHC. Fluorescence was monitored using a fluorescence plate reader at an excitation wavelength of 409 nm and an emission wavelength of 460 nm. Activity was measured as the rate of fluorescent metabolite production over the course of the reaction. The IC50 values were calculated by linear interpolation.

Measurement of rate constant for ferryl decay. Horse heart myoglobin was prepared in 200 mM potassium phosphate (pH 8.0). The chemicals were fully oxidized to the ferric form by the addition of excess potassium ferricyanide [K₃Fe(CN)₆]. Ferri-ferrocyanide was extracted using a Sephadex G-25 column at 4 °C and the resulting concentrate was measured by optical spectra at 409 nm. Ferric myoglobin (20 μM) in 200 mM potassium phosphate (pH 8.0) was reacted with H₂O₂ (185 μM) for 40 s at room temperature, which induced an immediate change of ferric myoglobin to ferryl myoglobin. Catalase (2 μM) was added to remove excess H₂O₂ and this mixture was left to react for an additional 1 min. Each of the experimental compounds 1, 2, and 3 (10 μM) was then added to 200 mM potassium phosphate (pH 8.0) in a 1:1 volume ratio so that the final concentration of myoglobin was 10 μM. The optical spectrum was followed until the reaction was complete. Absorbance changes were measured by the difference in absorbance between 406 nm and 425 nm. The time course (ΔA₉₀₉₉ = ΔA₄₀₆nm – ΔA₄₂₅nm) was fitted to a single exponential function using the least squares method. Each set of rate constants resulting from the reduction of myoglobin was then plotted as a function of the reductant concentration.

Results

Isolation and identification of compounds 1, 2, and 3. HR-ESIMS (neg.) established the molecular formulas of compounds 1, 2, and 3 as C₁₈H₁₂O₇, C₁₈H₁₂O₆, and C₁₈H₁₂O₅ respectively. Data for each compound obtained with IR, ¹H-NMR, and ¹³C-NMR, was in agreement with compound 1 (atromentic acid), compound 2 (variegatic acid), and compound 3 (xerocomic acid), consistent with the literature (Fig. 1).²⁰⁻²²

Effects of atromentic acid (1), variegatic acid (2), and xerocomic acid (3) on CYP3A4 activity

Atromentic acid (1), variegatic acid (2), and xerocomic acid (3) inhibited CYP3A4 enzyme activity in vitro. All three compounds showed dose-dependent inhibitory effects on CYP3A4 activity (Fig. 2) with IC₅₀ values of 65.1 ± 3.9 μM for atromentic acid (1); 2.2 ± 0.1 μM for variegatic acid (2); and 2.4 ± 0.1 μM for xerocomic acid (3) (Table 1). In particular, variegatic acid (2) and xerocomic acid (3) had far more inhibitory strength than the positive control uniconazole (IC₅₀ = 25.1 ± 2.1 μM). Another positive control, erythromycin, (IC₅₀ = 1.2 ± 0.1 μM) produced the highest value in this system. CYP1A2, CYP2C9, and CYP2D6 were then examined.

Inhibitory effects of atromentic acid (1), variegatic acid (2), and xerocomic acid (3) on CYP1A2, CYP2C9, and CYP2D6

We next investigated the inhibitory effects of atromentic acid (1), variegatic acid (2), and xerocomic acid (3) on CYP1A2, CYP2C9, and CYP2D6. The IC₅₀ values of these three compounds on CYP1A2, CYP2C9, and CYP2D6 are shown in Table 1. Their inhibitory profiles on CYP1A2, CYP2C9, and CYP2D6 were similar to those on CYP3A4, revealing that variegatic acid (2) and xerocomic acid (3) also had the most potent inhibitory effects. These findings indicate that these compounds have inhibitory effects on all four CYP isozymes tested, suggesting that atromentic acid (1), variegatic acid (2), and xerocomic acid (3), all act as antioxidants in the reaction mixtures. Five positive controls, cimetidine, dicoumarol, erythromycin, safranal,
Uniconazole

Erythromycin

Dicoumarol

Safrole

Xerocomic

Variegatic

CYPs Activities

Table 1. Inhibitory Effects of Compounds Atromentic Acid (1), Variegatic Acid (2), Xerocomic Acid (3), and Each Positive Control on CYPs Activities

<table>
<thead>
<tr>
<th>Compounds</th>
<th>1A2</th>
<th>2C9</th>
<th>2D6</th>
<th>3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atromentic acid (1)</td>
<td>80.4 ± 4.2</td>
<td>120.1 ± 4.3</td>
<td>259.0 ± 9.5</td>
<td>65.1 ± 3.9</td>
</tr>
<tr>
<td>Variegatic acid (2)</td>
<td>3.3 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Xerocomic acid (3)</td>
<td>3.9 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>7.3 ± 0.2</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Safrole</td>
<td>22.1 ± 1.7</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Dicoumarol</td>
<td>&gt;50</td>
<td>0.9 ± 0.1</td>
<td>44.4 ± 1.3</td>
<td>40.2 ± 2.9</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>&gt;250</td>
<td>84.6 ± 2.6</td>
<td>21.3 ± 1.6</td>
<td>41.7 ± 2.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Uniconazole</td>
<td>10.2 ± 0.8</td>
<td>34.2 ± 2.6</td>
<td>37.9 ± 3.6</td>
<td>25.1 ± 2.1</td>
</tr>
</tbody>
</table>

Mean ± SD (n = 3)

Table 2. Inhibitory Effects of Antioxidant and Chelator on CYPs Activities

<table>
<thead>
<tr>
<th>Compounds</th>
<th>1A2</th>
<th>2C9</th>
<th>2D6</th>
<th>3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>&gt;250</td>
<td>58.3 ± 0.1</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>EDTA</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>PIH</td>
<td>&gt;50</td>
<td>27.5 ± 2.2</td>
<td>44.1 ± 1.2</td>
<td>19.5 ± 2.1</td>
</tr>
</tbody>
</table>

Mean ± SD (n = 3)

and uniconazole, also had inhibitory effects on the respective CYP enzymes (Table 1).

Inhibitory effects of antioxidants and chelators on CYP1A2, CYP2C9, CYP2D6, and CYP3A4

The antioxidants ascorbic acid, α-tocopherol, and caffeic acid as well as the chelators EDTA and PIH are classical agents used to conduct redox studies. We examined the inhibitory effects of these antioxidants on CYP1A2, CYP2C9, CYP2D6, and CYP3A4 in this system (Table 2). Ascorbic acid, α-tocopherol, and EDTA did not inhibit any of the CYPs at concentrations of less than 250 μM. The IC_{50} values of PIH were 27.5 ± 2.2 μM (CYP2C9), 44.1 ± 1.2 μM (CYP2D6), and 19.5 ± 2.1 μM (CYP3A4). In addition, caffeic acid also gave the specific IC_{50} value at 58.3 ± 0.1 μM against CYP2C9. These data indicate that PIH and caffeic acid acted in the CYP catalytic cycle, but that ascorbic acid, α-tocopherol, and caffeic acid did not.

Effects of variegatic acid (2) on the reduction of ferryl myoglobin

In the CYP inhibitory system, our data indicated that variegatic acid (2) and xerocomic acid (3) had stronger inhibitory effects than atromentic acid (1) (Table 1). In addition, variegatic acid (2), whose chemical structure is closely related to xerocomic acid (3) (Fig. 1), had a stronger inhibitory effect than xerocomic acid (3). Therefore, we used variegatic acid (2) to examine the conversion from ferryl myoglobin to ferric myoglobin in an effort to elucidate whether the CYP inhibitory mechanism is an oxidative process by spectrophotometry.

The addition of variegatic acid (2) (10 μM) to ferryl myoglobin led to optical changes assigned to the Soret bands from ferryl myoglobin (425 nm) to ferric myoglobin (406 nm; Figs. 3 and 4).

These optical changes fit a single exponential function, indicating pseudo-first-order kinetics (Fig. 4). The
Inhibitory Effects of Edible Mushrooms on CYP Activity

optical changes following the addition of variegatic acid (2) (10μM) to the ferryl derivative of myoglobin are shown in Fig. 3A. These changes, showing a decrease in the absorbance shoulder at 425 nm and an increase in the absorbance at 406 nm, are typical of the reduction of ferryl myoglobin to ferrie myoglobin. Change was also observed in the visible region, namely an increase in absorbance at 630 nm, indicative of high spin ferric protein. Similar results were obtained with atromentic acid (1) and xerocomic acid (3) (data not shown).

Discussion

Detailed spectroscopic analyses including 1H- and 13C-NMR and HR-ESIMS led to the identification of active compounds, isolated from the two Chinese edible mushrooms, as three known pulvinic acids: atromentic acid (1), variegatic acid (2), and xerocomic acid (3) (Fig. 1).20-22

CYP3A4 plays a pivotal role in oxidative drug metabolism. The three compounds, atromentic acid (1), variegatic acid (2), and xerocomic acid (3), inactivated CYP3A4 in a dose-dependent manner, by oxidative metabolism from BOMCC (non-fluorescent) to CHC (highly fluorescent) (Table 1 and Fig. 2). The potent inhibitory effects of both variegatic acid (2) and xerocomic acid (3) were comparable to those of the positive control erythromycin (Fig. 1). This effect of variegatic acid (2) and xerocomic acid (3) is more likely due to the presence of a catechol moiety with a hydroxy group at C-3 and C-4 (Fig. 1) rather than the catechol ring from C-1’ to C-6’ (Fig. 1).

CYP1A2, CYP2C9, CYP2D6, and CYP3A4 catalyze transformation of existing pharmaceuticals into active metabolites. For example, CYP3A4 has specific inhibitory effects on bergamottin, the major furanocoumarin in fresh grapefruit juice.23,24 To estimate the inhibitory effect of atromentic acid (1), variegatic acid (2), and xerocomic acid (3) on these CYP enzymes, in vitro inhibition experiments were performed with five positive controls. The results indicated that variegatic acid (2) and xerocomic acid (3) strongly inhibited all CYP isozyme activities (Table 1). Of the five positive controls used, uniconazole with an azole ring binds to the heme-iron atom of CYP through an azole nitrogen atom.25 Variegatic acid (2) and xerocomic acid (3), like uniconazole, highly suppressed all enzyme activities. Thus, the mechanism of nonspecific CYP isozyme inhibition may be related to an effect on the heme iron. The chemical structures of variegatic acid (2) and xerocomic acid (3) indicate that they have a catechol moiety, therefore the catechol moiety may exert an inhibitory effect on CYP activity, but uniconazole does not have a catechol moiety. Surprisingly, the inhibitory effects of variegatic acid (2) and xerocomic acid (3) were 3 to 10-fold higher than that of uniconazole (Table 1). Therefore, we initially assumed that the inhibitory effects of variegatic acid (2) and xerocomic acid (3) were due to the presence of the catechol moiety in their chemical structures. Catechol-containing compounds are known to have strong antioxidant activity, reducing capacity of the catechol moiety essentially transfers the ferryl heme to ferrie heme, leading to the inactive state of heme iron in CYPs.4,5 We realized later, however, that we should have focused on the ferryl heme reduction.

To examine the possibility that the isolated compounds mediate ferryl heme reduction, optical transitions from ferryl myoglobin to ferrie myoglobin following treatment with variegatic acid (2) (10μM) were measured.6 The optical changes of the initial ferryl spectrum were typical of the reduction of the ferryl form to the ferric form (Fig. 3); the absorbance of the Soret bands at 406 nm (ferric form) gradually increased and that at 425 nm (ferryl form) gradually decreased. The time-dependent profile of the difference values (Δabs = Δ406nm − Δ425nm) demonstrated a pseudo-first-order reaction fitted to a single exponential function, as is the case with deferriprone.6 The reducing properties of variegatic acid (2) on ferryl heme may be the mode of nonspecific inhibitory action on CYP enzymes. The oxygen atom of the ferryl heme may have changed into an H2O molecule by 2 hydrogen atoms from the catechol moiety in variegatic acid (2) and inactive ferric heme bound the H2O molecule then regenerated (Fig. 5). PIH, which is a heme enzyme inhibitor, had a nonspecific and moderate inhibitory effect and caffie acid, which is a catechol compound, was a moderate specific CYP2C9 inhibitor (Table 2). Representative antioxidants (ascorbic acid and α-tocopheryl) and another chelating agent (EDTA) had no inhibitory activity. These observations suggest that the strong

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Fig. 5. Proposed Mechanisms of Inhibitory Action of Variegatic Acid (2) and Xerocomic Acid (3) on CYPs.
inhibition effect of variegatic acid (2) and xerocomic acid (3) on CYP enzyme activity depends on their structural properties.

Food-drug interactions can be critically important. CYP enzymes, including CYP3A4, CYP1A2, CYP2C9, and CYP2D6, are mainly expressed in the human liver and gastrointestinal tract and metabolize many pharmacologic agents and chemicals. Inhibition of CYP enzymes changes the bioavailability of clinically used drugs. Patients taking medicines must be aware of the interactions between the drug being taken and the foods they consume, including foods containing CYP enzyme inhibitors. On the other hand, some CYP enzymes, such as CYP1A1, CYP19, and CYP17, have recently attracted attention as new targets of anti-cancer drug development. Studies of the clinical implications of CYP enzyme inhibitors are, therefore, very important.

Our findings indicate that two pulvinic acid derivatives, variegatic acid (2) and xerocomic acid (3), isolated from two Chinese edible mushrooms, are potent CYP inhibitors with ferryl heme reducing properties. These ferryl reductants may have protective activities against in vivo oxidative stress. Further detailed studies of the mechanisms of inhibition that are mediated by ferryl heme reduction in association with competitive, noncompetitive, and/or mechanism-based pathways, are necessary.

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