(54) Title: COMPOUNDS ISOLATED FROM ANTRODIA CINNAMOMEA AND USE THEREOF

(57) Abstract: The present invention relates to novel compounds from Antrodia cinnamomea and their use.
COMPOUNDS ISOLATED FROM ANTRODIA CINNAMOMEA

AND USE THEREOF

Field of the invention

The present invention is related to a compound from the metabolite of Antrodin C isolated form *Antrodia cinnamomea*.

Background of the invention


**Summary of invention**

The present invention provides a compound having the formula

![Chemical structure 1](image1)

wherein R₁ is C₁-10 carboxylic acid or C₁-10 ester; R₂ is C₁-10 carboxylic acid or C₁-10 ester; R₃ is H, C₁-10 alkyl, C₂-10 alkenyl or C₂-10 alkynyl; and R₄ is H, C₁-10 alkyl, C₂-10 alkenyl or C₂-10 alkynyl.

The present invention also provides a composition comprising a compound having the formula

![Chemical structure 2](image2)

wherein R₁ is C₁-10 carboxylic acid or C₁-10 ester; R₂ is C₁-10 carboxylic acid or C₁-10 ester; R₃ is H, C₁-10 alkyl, C₂-10 alkenyl or C₂-10 alkynyl; and R₄ is H, C₁-10 alkyl, C₂-10 alkenyl or C₂-10 alkynyl.

**Brief description of the drawings**
Fig. 1  HMBC (a) and NOE (b) Correlations of M1.

Fig.2  HMBC Correlations of M2 and M3.

Fig.3 TIC of feces (a), bile (b) and plasma(c) sample after oral administration of Antrodin C at the dose of 50 mg/kg.

Fig.4 MS spectra (negative mode) of M1-M5 and Antrodin C in the rat feces, bile and plasma samples.

Fig.5 The structures of Antrodin C and its metabolites.

Fig.6 Concentration-time curve of M1 in bile samples after I.V. Antrocin C at the dose of 10 mg/kg (a) and P.O. 50 mg/kg (b).

Fig.7 The TIC of Bile and Plasma and the UV Spectra of Faeces after Administration of Antrodin C in Rats.

Fig.8 The UV spectra of the blank plasma samples and the plasma samples after i.v. of M1.

**Detail description of the invention**

In this invention, three maleic acid and two succinic acid derivatives (Antrodin A-E) were firstly isolated from the mycelium of *Antrodia cinnamomea*, and the cytotoxic activity against LLC cells of Antrodin C and B were confirmed (Nakamura N, *et al.* 2004 *J Nat Prod* 7: 46-48). Furthermore, Antrodin C, with the highest amounts in mycelium, exhibited protective effect against hepatitis model induced by LPS. Whereas the metabolism study on the compounds of *Antrodia cinnamomea* were never reported. In the present invention, the metabolites of Antrodin C in the rat bile and feces samples were identified by LC/MS-MS with electrospray ionization (ESI), and the pharmacokinetics of M1 in rat bile was performed after oral administration (50 mg/kg) and intravenous injection (10 mg/kg) of Antrodin C by PAD-HPLC.
The present invention provides a compound having the formula

\[
\begin{array}{c}
R_1 \quad R_2 \\
\quad R_3 \quad R_4
\end{array}
\]

wherein \( R_1 \) is \( C_{1-10} \) carboxylic acid or \( C_{1-10} \) ester; \( R_2 \) is \( C_{1-10} \) carboxylic acid or \( C_{1-10} \) ester; \( R_3 \) is H, \( C_{1-10} \) alkyl, \( C_{2-10} \) alkenyl or \( C_{2-10} \) alkynyl; and \( R_4 \) is H, \( C_{1-10} \) alkyl, \( C_{2-10} \) alkenyl or \( C_{2-10} \) alkynyl.

\( R_1 \) or \( R_2 \) of the compound is \( C_{1-6} \) carboxylic acid. In the preferred embodiment, \( R_1 \) or \( R_2 \) is COOH, \( R_3 \) is \( C_{1-6} \) alkyl and \( R_4 \) is isobutyl. In the more preferred embodiment, the compound is (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid, (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid 4-methyl ester or (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid 1-methyl ester.

The compounds are metabolites of Antrodin C in rats, and the Antrodin C is isolated from the mycelium *Antrodia cinnamomea*.

The present invention provides a composition comprising a compound having the formula

\[
\begin{array}{c}
R_1 \quad R_2 \\
\quad R_3 \quad R_4
\end{array}
\]

wherein \( R_1 \) is \( C_{1-10} \) carboxylic acid or \( C_{1-10} \) ester; \( R_2 \) is \( C_{1-10} \) carboxylic acid or \( C_{1-10} \) ester; \( R_3 \) is H, \( C_{1-10} \) alkyl, \( C_{2-10} \) alkenyl or \( C_{2-10} \) alkynyl; and \( R_4 \) is H, \( C_{1-10} \) alkyl, \( C_{2-10} \) alkenyl or \( C_{2-10} \) alkynyl.

In the preferred embodiment, the compound is (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid, (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-2-en-1-yl)oxy]phenyl}but-2-enedioic acid, (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid 4-methyl ester or (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-
-1-y1) oxy J phenyl } but- 2- enedioic acid 4-methyl ester or (2Z)-2-isobutyl-3-{4-

The present invention provides the compounds have possessing antioxidation,
antimicrobial, antibacterial actions, AChE inhibitory activity, antispasmodic or
vasorelaxant activities.

The compound of the invention can decrease systolic blood pressure or increase high
density lipoprotein. In addition, the same compound has central cholinergic agonism,
hepatoprotection, anti-inflammation or anti-tumor activity. Especially, the compound
of the invention can inhibit tumor from the cells or tissues selected from the group
consisting of liver, lung, intestine, bone, blood, lymph and breast. The subject
accepting the mixture of the invention includes but is not limited to human, mammal,
mouse, rat, horse, pig, chicken, duck, dog and cat.

The present invention also provides a composition, which comprises the compound of
the invention. The composition of the invention can decrease systolic blood pressure
or increase high density lipoprotein. In addition, the composition of the invention has
central cholinergic agonism, hepatoprotection, anti-inflammation or anti-tumor
activity. Especially, the compound of the invention can inhibit tumor from the cells or
tissues selected from the group consisting of liver, lung, intestine, bone, blood, lymph
and breast. The subject accepting the mixture of the invention includes but is not
limited to human, mammal, mouse, rat, horse, pig, chicken, duck, dog and cat.

Example

Chemicals and Reagents

General anaerobic medium (GAM) broth was purchased from Nissui Co. (Tokyo,
Japan). Liquid chromatographic grade solvents, triethylamine,
4-dimethylaminopyridine (4-DMAP), silica gel BW-820MH (Fuji Silysia), ODS DM 1020T (Fuji Silysia) for open column chromatography, Merck precoated Silica gel 60F$_{254}$ (0.25 mm) and Merck RP-18F$_{254}$ (0.25 mm) for TLC analysis were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

**Instruments**

Compounds were analyzed by $^1$H- and $^{^{13}}$C-NMR and 2D NMR using a Unity Plus 500 (varian) NMR spectrometer with tetramethylsilane as an internal standard, and chemical shifts are shown as $\delta$ values. Intestinal bacteria were anaerobically incubated using an EAN-140 (Tabai Co., Osaka, Japan). The HPLC instrument was an Agilent 1100 system (Agilent Technologies., Waldbronn, Germany) comprising an Agilent 1100 series binary pump with a photodiode array detector (PAD) and a series 7725i injector with a 20 $\mu$l loop. Data were acquired and intergrated using a ChemStation. The HPLC system was connected to an Esquire 3000$^{\text{plus}}$ mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an ESI source. All LC/MS-MS data were acquired using Esquire Control software and analyzed using software from by Bruker Daltonics.

**EXAMPLE 1**

**Synthesis of M1-M3**

Antrodin C (50 mg) was dissolved in 5 ml water, and 1N KOH (0.5 ml) was added stirring for 5 min. 1N HCl was used to adjust PH to 8. After filtration, the solution kept under room temperature overnight. After filtration again, the supernatant was lyophilized, and reconstituent by some MeOH, and then filtered and evaporated in vacuo., yield of M1 was 13 mg (26%).

The $^1$H and $^{^{13}}$C-NMR spectra of M1 (Table 1) was very similar to those of Antrodin C.
and showed the presence of isobutyl moiety, a 3-methyl-2-butenyloxy moiety, and a para-substituted benzene ring, which supported by $^1$H-$^1$H COSY - HMQC experiments. But carbonyl carbon ($\delta$ 178.9 : 1), methylene carbon ($\delta$ 39.7 : 1'), proton ($\delta$ 1.87 : 1') and methyne proton ($\delta$ 1.56 : 2') of isobutyl moiety, benzene carbon conjugated olefine ($\delta$ 131.6 : 1") and benzene proton next to that ($\delta$ 7.11 : 2", 6") were different from those of Antrodin C, these all the carbon of M1 were downfield shifted than those of Antrodin C and these all the proton of M1 were upfield shifted than those of Antrodin C. In the HMBC experiments, long-range correlations were observed as shown in Fig. 1 (a). We decided that Olefine coupling (2-C and 3-C) of M1 is Z because NOE was observed between 1'-H and 3'4'-H or 6''-H in the NOESY spectrum of M1 (Fig. 1 (b)). According to these results, M1 was defined as (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl) oxy]- phenyl} but-2- enedioic acid. Anhydride M4 (Antrodin A) and dicarboxylic acid M1 were converted each other by acid and base condition.

Antrodin A (500 mg) was dissolved in 1 ml MeOH, and triethylamine (0.2 ml, 1.6 mmol) and 4-dimethylaminopyridine (4-DMAP, 13.4 mg, 0.11 mmol) were added to the solution stirring for 20 h at 25°C. And then the mixture was chromatographed by a open ODS column eluting with methanol and water (30:70→100:0), the fraction containing M2 and M3 were evaporated in vacuo, and then analyzed by NMR and LC/MS. The data of $^1$H-NMR and $^{13}$C-NMR of M1-M3 were showed in Table1, 2. The $^1$H and $^{13}$C-NMR spectra of M2 and M3 were also similar to those of M1 except for methoxy groups and showed the presence of isobutyl moiety, a 3-methyl-2-butenyloxy moiety, and a para-substituted benzene ring. In the HMBC experiments, methylene proton of isobutyl moiety ($\delta$ 2.16 : 1') and carbonyl carbon ($\delta$ 174.0 : 1) of M2 showed long-range correlation, and methylene proton of isobutyl moiety ($\delta$ 2.14 : 1') and carbonyl carbon ($\delta$ 171.4 : 1) of M3 showed long-range
correlation (Fig. 2). The structure of M2 and M3 were defined as (2Z)-2-isobutyl-3-{4-[(3- methylbut-2-en-1-yl) oxy] phenyl} but-2- enedioic acid 4- methyl ester and (2Z)-2- isobutyl-3-{4-[(3- methylbut-2-en-1-yl) oxy] phenyl} but-2-enedioic acid 1-methyl ester, respectively.

Table 1  $^1$H-NMR Spectral Data of M1 (D$_2$O), M2 and M3 (CD$_3$OD) (δ ppm, $J$=Hz)

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'</td>
<td>1.87 (2H, d, $J$=6.5)</td>
<td>2.16 (2H, d, $J$=7.0)</td>
<td>2.14 (2H, d, $J$=7.0)</td>
</tr>
<tr>
<td>2'</td>
<td>1.56 (1H, m)</td>
<td>1.69 (1H, m)</td>
<td>1.69 (1H, m)</td>
</tr>
<tr>
<td>3', 4'</td>
<td>0.72 (6H, d, $J$=6.5)</td>
<td>0.81 (6H, d, $J$=7.0)</td>
<td>0.80 (6H, d, $J$=6.5)</td>
</tr>
<tr>
<td>2&quot;, 6&quot;</td>
<td>7.11 (2H, d, $J$=8.5)</td>
<td>7.14 (2H, d, $J$=9.0)</td>
<td>7.20 (2H, d, $J$=9.0)</td>
</tr>
<tr>
<td>3&quot;, 5&quot;</td>
<td>6.88 (2H, d, $J$=8.5)</td>
<td>6.91 (2H, d, $J$=9.0)</td>
<td>6.91 (2H, d, $J$=9.0)</td>
</tr>
<tr>
<td>1&quot;</td>
<td>4.50 (2H, d, $J$=6.5)</td>
<td>4.54 (2H, d, $J$=6.5)</td>
<td>4.54 (2H, d, $J$=6.5)</td>
</tr>
<tr>
<td>2&quot;</td>
<td>5.41 (1H, brs)</td>
<td>5.46 (1H, m)</td>
<td>5.46 (1H, m)</td>
</tr>
<tr>
<td>4&quot;</td>
<td>1.68 (3H, s)</td>
<td>1.77 (3H, s)</td>
<td>1.77 (3H, s)</td>
</tr>
<tr>
<td>5&quot;</td>
<td>1.64 (3H, s)</td>
<td>1.75 (3H, s)</td>
<td>1.75 (3H, s)</td>
</tr>
<tr>
<td>-OMe</td>
<td>—</td>
<td>3.82 (3H, s)</td>
<td>3.72 (3H, s)</td>
</tr>
</tbody>
</table>

Table 2  $^{13}$C-NMR Spectral Data of M1 (D$_2$O), M2 and M3 (CD$_3$OD) (δ ppm)

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>178.9</td>
<td>174.0*</td>
<td>171.4*</td>
</tr>
<tr>
<td>2</td>
<td>140.3</td>
<td>144.0</td>
<td>134.3</td>
</tr>
<tr>
<td>3</td>
<td>137.5</td>
<td>136.3</td>
<td>145.4</td>
</tr>
<tr>
<td>4</td>
<td>166.1*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>1'</td>
<td>39.7</td>
<td>40.0</td>
<td>40.0</td>
</tr>
</tbody>
</table>

8
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2'</td>
<td>27.2</td>
<td>29.0</td>
</tr>
<tr>
<td>3', 4'</td>
<td>22.2</td>
<td>22.8</td>
</tr>
<tr>
<td>1''</td>
<td>131.6</td>
<td>128.5</td>
</tr>
<tr>
<td>2'', 6''</td>
<td>130.5</td>
<td>131.4</td>
</tr>
<tr>
<td>3'', 5''</td>
<td>114.8</td>
<td>115.5</td>
</tr>
<tr>
<td>4''</td>
<td>157.0</td>
<td>160.0</td>
</tr>
<tr>
<td>1'''</td>
<td>65.3</td>
<td>65.9</td>
</tr>
<tr>
<td>2'''</td>
<td>118.4</td>
<td>121.1</td>
</tr>
<tr>
<td>3'''</td>
<td>141.6</td>
<td>138.7</td>
</tr>
<tr>
<td>4''''</td>
<td>25.1</td>
<td>25.9</td>
</tr>
<tr>
<td>5''''</td>
<td>17.3</td>
<td>18.2</td>
</tr>
<tr>
<td>-OMe</td>
<td>—</td>
<td>**</td>
</tr>
</tbody>
</table>

**EXAMPLE 2**

**Treatment of animals**

Male Wistar rats (9 weeks old) purchased from SLC Co. (Hamamastu, Japan), were fed with standard laboratory chow for one week, fasted overnight and given free access to water before drug administration. Urine and feces samples were collected while the rats remained isolated in metabolic cages. The animals were light anesthetized with diethyl ether during surgical procedures. Bile samples (n=5) was collected by cannulating a polyethylene tube (PE-10) into the rat bile duct at intervals of 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h after oral (50 mg/kg) and intravenous (10 mg/kg) administration of Antrodin C. The blood sample was collected from the inferior vena cava using a heparinized injector when the abdomen was exposed by a midline abdominal incision after administration. The blood samples were centrifuged at 8000×g for 15 min to separate the plasma, and then all samples were stored at
–20°C for later analysis.

Sample preparation for analysis

Thawed urine and bile samples (0.5 ml) dissolved in 3 volumes of acetonitrile, and then centrifuged at 8000×g for 15 min. The supernatant was passed through a 0.45 μm Millipore syringe filter (Nihon Millipore, Tokyo, Japan) for LC/MS-MS analysis. Plasma samples were passed through Solid Phase Extraction cartridges (Waters Co., Milford, U.S.A.) that had been washed with 3 ml of acetonitrile and equilibrated with 6 ml of water. The constituents were eluted with 2-3 ml of acetonitrile from the cartridge, then the eluate was evaporated under a stream of nitrogen at 35°C to leave a residue that was dissolved in 100 μl of acetonitrile for LC/MS-MS analysis. The bile samples for pharmacokinetic study, which containing M2, M3 and M4 were diluted by same volume water, and then incubated in 37 °C bath for 12h, the M2-M4 would thoroughly converted to M1. After treating the bile sample as described above, the amount of M1 was calculated by PAD-HPLC.

Identification of Metabolites in Rat feces, Bile and Plasma

The metabolites in feces, bile and plasma were analyzed by LC/MS-MS. The LC/MS-MS equipment comprised a column containing TSK gel ODS-80 Ts (particle size, 5 μm; 4.6×150 mm i.d., Tosoh Co., Tokyo, Japan). Samples were eluted through the column with 0.1% AcOH and acetonitrile (35: 65) at a flow rate of 1 ml/min at 30°C. The standard negative ion mode was selected under the following conditions: full scan range, 50-800 m/z; scan resolution, 13000 m/z /sec; nebulizer, 50.0 psi; dry gas, 10.0 l/min; dry temperature, 360°C. Full scanning in the region of m/z 50 to 600 assigned several peaks to Antrodin C and the metabolites in the TLC when compared with those of blank samples (Fig. 3). The MS spectra revealed intense ion peaks at m/z 331, 345, 345, 313, 312 and 328 [M-H] as M1, M2, M3, M4, M5 and Antrodin C,
respectively (Fig. 4, Table 3). In the feces, metabolites were M1-M3, M5 with original Antrodin C; in the bile were M2-4; and in the plasma were M1 with another unknown peak. There was neither metabolite nor Antrodin C in the urine sample. Comparing to standard materials and synthesized compounds, the peak in the MS profile at m/z 328 [M-H]− with retention time (tR) = 8.2 min was derived from Antrodin C (MW 329), and M1 (m/z 331 [M-H]−, tR = 4.4 min) was dicarboxylic acid by hydrolysis of Antrodin C; M2 and M3 (m/z 345 [M-H]−, tR = 7.7 and 8.4 min), which were 14 larger than those of M1, were two kinds of monomethyl esters of M1; M4 (m/z 313 [M-H]−, tR = 21.6 min) was Antrodin A and M5 (m/z 312 [M-H]−, tR = 11.5 min) was Antrodin B. The structures of Antrodin C and its metabolites were shown in Fig. 5.

Table 3 Retention time (tR) and MS spectra of Antrodin C and its metabolites

<table>
<thead>
<tr>
<th></th>
<th>tR (min)</th>
<th>MS spectra (Negative mode)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antrodin C</td>
<td>8.2</td>
<td>328, 259, 242, 216</td>
</tr>
<tr>
<td>M1</td>
<td>4.4</td>
<td>331, 287, 218, 132</td>
</tr>
<tr>
<td>M2, M3</td>
<td>7.7, 8.4</td>
<td>345, 313, 244, 232, 189</td>
</tr>
<tr>
<td>M4</td>
<td>21.6</td>
<td>313, 244</td>
</tr>
<tr>
<td>M5</td>
<td>11.5</td>
<td>312, 243, 200</td>
</tr>
<tr>
<td>Unknown metabolite</td>
<td>10.8</td>
<td>319, 301, 257, 179, 163, 135</td>
</tr>
</tbody>
</table>

**Metabolism of Antrodin C and metabolites by intestinal bacteria in vitro**

Mixtures of rat (RIB) or human (HIB) intestinal bacteria (5 g each) prepared as described (Xie LH, et al. 2003 Biol Pharm Bull 51: 378-384), together with Antrodin
C (5 mg) dissolved in Tween 20 (0.5 ml), M1 (5 mg) dissolved in water (1.0 ml) or rat bile samples (10 ml) with metabolites M2-M4, which were collected after oral administration of Antrodin C, were added to GAM broth (50 ml), and anaerobically incubated at 37°C for 3 d. The incubation mixture was extracted with 3 volumes of acetonitrile, and then passed through a 0.45 μm filter. Then, Antrodin C was converted to M5 (Antrodin B). Moreover, the metabolites (M2-M4) in bile samples, which were collected after oral administration of Antrodin C in rats, could absolutely transferred to M1 after 30 min incubation. Whereas M1 was not metabolized by intestinal bacteria flora, although prolonged the incubation time to 3 d.

Validation of M1 by PAD-HPLC

Linearity: M1 was dissolved in rat blank bile to prepare seven dilutions of standard solutions. Response linearity was determined for the seven concentrations after three injections for each level. The limit of detection (LOD) of the method for each constituent was established when the signal to noise ratio (S/N) was 5.

Accuracy: Intra-and inter-assay variability was determined by analyzing high, medium and low standard concentrations of rat bile five times on the same day and continuously for 5 d, respectively.

Recovery: Two standard concentrations were mixed with rat bile samples after the oral administration of Antrodin C with a known amount of M1, and recovery rates of the added amounts were calculated.

Stability: Three concentrations of bile samples that had been prepared for PAD-HPLC analysis were placed at room temperature for 12 h, or in a refrigerator at 4°C for 1, 3 and 5 d. The average peak areas of constituents in the samples and relative standard deviation (RSD) were calculated.
Validation of PAD-HPLC Quantitation

The regression equation of M1 in rat bile sample was $Y = 610.22X - 3.94$; $\gamma = 0.9998$; and the linearity range was 0.05-2.0 µg/ml. Intra-day and inter-day (n=5) variations of M1 in rat bile samples were shown in Table 4. The CV did not exceed 6%, and the accuracy rates were all within 85-110%. CV values of recovery rates were shown in Table 5, which were less than 10% at low and high concentrations with recovery rates of 93.4 and 99.6%. The stability test showed that relative standard deviation remained within 5% under all the conditions; therefore, the samples were stable during the test. Thus, the accuracy, recovery, and stability tests met the criteria for quantitative determinations in bile samples.

Table 4  Intraday and Interday (n = 5) Variations of M1 in Rat Bile

<table>
<thead>
<tr>
<th></th>
<th>Added (µg)</th>
<th>Found (µg)</th>
<th>Accuracy (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td>0.05</td>
<td>0.0456±0.0018</td>
<td>91.2</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.533±0.008</td>
<td>106.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.99±0.04</td>
<td>99.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Interday</td>
<td>0.05</td>
<td>0.0438±0.0026</td>
<td>87.6</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.443±0.009</td>
<td>88.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.98±0.06</td>
<td>99.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table 5 Recovery of M1 in Rat Bile

<table>
<thead>
<tr>
<th></th>
<th>Added (µg)</th>
<th>Found (µg)</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.0467±0.0045</td>
<td>93.4</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>
Pharmacokinetics of M1 in rat bile

The concentration-time data in rat bile (n=5) were computer fitted using a program of Pharmacokinetics 3p97 edited by the Mathematics Pharmacological Committee, Chinese Pharmacological Society. The following pharmacokinetic parameters were obtained: half-time of absorption phase (t1/2 (Ka)) and half-time of elimination phase (t1/2 (Ke)) in the bile samples after oral administration of Antrodin C at the dose of 50 mg/kg. The area under the concentration–time curve (AUC(i.v.) and AUC(p.o.)) was calculated by the statistical moment method of non-compartmental pharmacokinetic analysis. And then clearance (Clm, b) and absolute bioavailability (Fm, b) were calculated by the equations as following: Clm, b (ml/h·kg) = Dose (i.v.) / AUC (i.v.) and Fm, b (%) = AUC (p.o.) / [AUC (i.v.) · Dose (p.o.)]. Data were expressed as the mean and standard deviation (S.D.) for each group.

The concentrations of M1 in bile samples were calculated after P.O. administration of 50 mg/kg and I.V. 10 mg/kg of Antrodin C. The concentration-time curves of M1 were shown in Fig. 6. The pharmacokinetic parameters were shown in Table 6. After oral administration, t1/2(Ka) and t1/2(Ke) were 0.95 h and 12.64 h, respectively. AUC0-tim were 1.61 (P.O.) and 1.68 h mg/ml (I.V.), Clm,b. was 5.96 ml/h·kg and Fm,b. was 19.43(%). Accumulated excretion ratio of Antrodin C were 5.46±1.62 % (P.O.) and 56.85±13.40 % (I.V.). Therefore, Antrodin C was very quickly not only absorbed from gastrointestinal, but also metabolized in the liver. The mainly excretion was bile-feces pathway in rats.

Table 6 Pharmacokinetic parameters of M1 in rat bile samples after P.O. and I.V. of
Antrodin C

<table>
<thead>
<tr>
<th></th>
<th>P.O. (50 mg/kg)</th>
<th>I.V. (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2(\alpha)}$</td>
<td>$t_{1/2 (k_0)}$</td>
<td>$AUC_{0\rightarrow\infty}$</td>
</tr>
<tr>
<td>(h)</td>
<td>(h)</td>
<td>(h mg/ml)</td>
</tr>
<tr>
<td>0.95±0.07</td>
<td>12.64±2.24</td>
<td>1.61±0.58</td>
</tr>
</tbody>
</table>

**EXAMPLE 3**

Repeat example 2, results are shown in Fig.7 and Fig.8.
Claims

1. A compound having the formula

\[
\begin{array}{c}
R_1 \\
\text{O} \\
R_3 \\
\text{O} \\
R_2 \\
R_4
\end{array}
\]

wherein

- \( R_1 \) is C\(_{1-10}\) carboxylic acid or C\(_{1-10}\) ester;
- \( R_2 \) is C\(_{1-10}\) carboxylic acid or C\(_{1-10}\) ester;
- \( R_3 \) is H, C\(_{1-10}\) alkyl, C\(_{2-10}\) alkenyl or C\(_{2-10}\) alkynyl; and
- \( R_4 \) is H, C\(_{1-10}\) alkyl, C\(_{2-10}\) alkenyl or C\(_{2-10}\) alkynyl.

2. The compound of claim 1, wherein \( R_1 \) or \( R_2 \) is C\(_{1-6}\) carboxylic acid.

3. The compound of claim 1, wherein \( R_1 \) or \( R_2 \) is COOH.

4. The compound of claim 1, wherein \( R_3 \) is C\(_{1-6}\) alkyl.

5. The compound of claim 1, wherein \( R_4 \) is isobutyl.

6. The compound of claim 1, which is

- \((2Z)-2\text{-isobutyl}-3\text{-}[4\text{-}[3\text{-methylbut-2-en-1-yl}]\text{oxy}]\text{phenyl}]\text{but-2-enedioic acid,}
- \((2Z)-2\text{-isobutyl}-3\text{-}[4\text{-}[3\text{-methylbut-2-en-1-yl}]\text{oxy}]\text{phenyl}]\text{but-2-enedioic acid 4-methyl ester or}
- \((2Z)-2\text{-isobutyl}-3\text{-}[4\text{-}[3\text{-methylbut-2-en-1-yl}]\text{oxy}]\text{phenyl}]\text{but-2-enedioic acid 1-methyl ester.}

7. The compound of claim 6, wherein the compound is metabolites of Antrodin C in rats.

8. The compounds of claim 7, wherein the Antrodin C is isolated from the mycelium of \textit{Antrodia cinnamomea}.

9. A composition comprising a compound having the formula
wherein

R₁ is C₁₋₁₀ carboxylic acid or C₁₋₁₀ ester;
R₂ is C₁₋₁₀ carboxylic acid or C₁₋₁₀ ester;
R₃ is H, C₁₋₁₀ alkyl, C₂₋₁₀ alkenyl or C₂₋₁₀ alkynyl; and
R₄ is H, C₁₋₁₀ alkyl, C₂₋₁₀ alkenyl or C₂₋₁₀ alkynyl.

10. The composition of claim 9, wherein the compound is

(2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid,
(2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl} but-2-enedioic acid 4-methyl ester or (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl} but-2-enedioic acid 1-methyl ester.

11. The composition of claim 9, wherein compounds have possessing antioxidation, antituberculosis, antibacterial actions, AChE inhibitory activity, antispasmodic or vasorelaxant activities.

12. The composition of claim 9, which decreases systolic blood pressure or increases high density lipoprotein.

13. The composition of claim 9, which has central cholinergic agonism, hepatoprotection, anti-inflammation or anti-tumor activity.

14. The composition of claim 12, wherein tumor is from the cells or tissues selected from the group consisting of liver, intestine, bone, blood, lymph and breast.
Claims

1. A compound having the formula

\[ \text{R}_1 \text{C}_6 \text{H}_4 \text{O} = \text{C} = \text{O} = \text{C} \text{R}_2 \text{R}_3 \text{O} \]

wherein

- \text{R}_1 \text{ is C}_{1-10} \text{ carboxylic acid or C}_{1-10} \text{ ester;}
- \text{R}_2 \text{ is C}_{1-10} \text{ carboxylic acid or C}_{1-10} \text{ ester;}
- \text{R}_3 \text{ is H, C}_{1-10} \text{ alkyl, C}_{2-10} \text{ alkenyl or C}_{2-10} \text{ alkynyl; and}
- \text{R}_4 \text{ is H, C}_{2-10} \text{ alkyl, C}_{2-10} \text{ alkenyl or, C}_{2-10} \text{ alkynyl.}

2. The compound of claim 1, wherein \text{R}_1 \text{ or } \text{R}_2 \text{ is C}_{1-6} \text{ carboxylic acid.}

3. The compound of claim 1, wherein \text{R}_1 \text{ or } \text{R}_2 \text{ is COOH.}

4. The compound of claim 1, wherein \text{R}_3 \text{ is C}_{1-6} \text{ alkyl.}

5. The compound of claim 1, wherein \text{R}_4 \text{ is isobutyl.}

6. The compound of claim 1, which is

- (Z)-2-isobutyl-3-\{4-[(3-methylbut-2-en-1-yl)oxy]phenyl\}but-2-enedioic acid,
- (Z)-2-isobutyl-3-\{4-[(3-methylbut-2-en-1-yl)oxy]phenyl\}but-2-enedioic acid 4-methyl ester or (Z)-2-isobutyl-3-\{4-[(3-methylbut-2-en-1-yl)oxy]phenyl\}but-2-enedioic acid 1-methyl ester.

7. The compound of claim 6, wherein the compound is metabolites of Antrodin C in
8. The compounds of claim 7, wherein the Antrodin C is isolated from the mycelium of *Antrodia cinnamomea*.

9. A composition comprising a compound having the formula

![Chemical Structure](image)

wherein

- $R_1$ is C$_{1-10}$ carboxylic acid or C$_{1-10}$ ester;
- $R_2$ is C$_{1-10}$ carboxylic acid or C$_{1-10}$ ester;
- $R_3$ is H, C$_{1-10}$ alkyl, C$_{2-10}$ alkenyl or C$_{2-10}$ alkynyl; and
- $R_4$ is H, C$_{2-10}$ alkenyl, C$_{2-10}$ alkynyl or isobutyl.

10. The composition of claim 9, wherein the compound is

- (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid,
- (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid 4-methyl ester or (2Z)-2-isobutyl-3-{4-[(3-methylbut-2-en-1-yl)oxy]phenyl}but-2-enedioic acid 1-methyl ester.

11. The composition of claim 9, wherein compounds have possessing antioxidation, antimicrobial, antibacterial actions, AChE inhibitory activity, antispasmodic or vasorelaxant activities.
12. The composition of claim 9, which decreases systolic blood pressure or increases high density lipoprotein.

13. The composition of claim 9, which has central cholinergic agonism, hepatoprotection, anti-inflammation or anti-tumor activity.

14. The composition of claim 12, wherein tumor is from the cells or tissues selected from the group consisting of liver, intestine, bone, blood, lymph and breast.
Fig. 1
Fig. 2

M2

M3
Fig. 3

(a) Feces

(b) Bile

(c) Plasma
Fig. 4
Atrodirn C \[\xrightarrow{\text{intestinal bacteria}}\] M5 (Atrodirn B)

\[\xrightarrow{\text{liver}}\] M4 (Atrodirn A)

M3

M2

Bile

\[\xrightarrow{\text{intestinal bacteria}}\] M1

Fig. 5
Fig. 6
Fig. 7
Fig. 8
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

See the extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07C, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNPAT(Cprs), CTCMPD, CNKI Full-Text database, Chinese Medicine Abstract, WPI, EPODOC, PAJ, CA, Embase, Medline, STN; antrodia, antrodin, isobutyl, carboxylic, phenyl, oxy, but, enedioic, acid, methyl, ester

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>--- ---</td>
<td>5-8, 10</td>
</tr>
</tbody>
</table>

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "&" document member of the same patent family

Date of the actual completion of the international search
26 May 2008 (26.05.2008)

Date of mailing of the international search report
12 Jun. 2008 (12.06.2008)

Name and mailing address of the ISA/CN
The State Intellectual Property Office, the P.R. China
6 Xitucheng Rd., Jinen Bridge, Haidian District, Beijing, China 100088
Facsimile No. 86-10-62019451

Authorized officer
LEI, Yaolong
Telephone No. (86-10)62411119

Form PCT/ISA/210 (second sheet) (April 2007)
<table>
<thead>
<tr>
<th>CLASSIFICATION OF SUBJECT MATTER:</th>
</tr>
</thead>
<tbody>
<tr>
<td>C07C 69/734 (2006. 01)i</td>
</tr>
<tr>
<td>C07C57/34 (2006. 01)i</td>
</tr>
<tr>
<td>A61K 31/216 (2006. 01)i</td>
</tr>
<tr>
<td>A61K 31/194 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 39/06 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 31/00 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 31/04 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 25/28 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 21/02 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 9/08 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 9/00 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 25/00 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 1/16 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 29/00 (2006. 01)i</td>
</tr>
<tr>
<td>A61P 35/00 (2006. 01)i</td>
</tr>
</tbody>
</table>