AMP-activated protein kinase (AMPK) activators from *Myristica fragrans* (nutmeg) and their anti-obesity effect

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A R T I C L E   I N F O

Article history:
Received 25 February 2010
Revised 8 May 2010
Accepted 18 May 2010
Available online 10 June 2010

Keywords:
*Myristica fragrans*
Myristicaceae
Tetrahydrofuran lignans
AMP-activated protein kinase (AMPK)

A B S T R A C T

AMP-activated protein kinase (AMPK) is a potential therapeutic target for the treatment of metabolic syndrome including obesity and type-2 diabetes. As part of an ongoing search for new AMPK activators from plants, this study found that the total extract of *Myristica fragrans* (nutmeg) activated the AMPK enzyme in differentiated C2C12 cells. As active constituents, seven 2,5-bisaryl-3,4-dimethyltetrahydrofuran lignans, tetrahydrofurogualiacin B (1), saucernetindiol (2), verrucosin (3), neectarind B (4), nectarind A (5), fragransin C1 (6), and galbacin (7) were isolated from this extract. Among the isolates, compounds 1, 4, and 5 at 5 μM produced strong AMPK stimulation in differentiated C2C12 cells. In addition, the preventive effect of a tetrahydrofuran mixture (THF) on weight gain in a diet-induced animal model was further examined. These results suggest that nutmeg and its active constituents can be used not only for the development of agents to treat obesity and possibly type-2 diabetes but may also be beneficial for other metabolic disorders.

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Metabolic disorders, including obesity and type-2 diabetes, are becoming an epidemic in the developed and developing countries. Today, more than 1.1 billion adults worldwide are overweight, and 312 million of them obese. Unfortunately, the increase in the prevalence of obesity is closely linked to the upsurge in type-2 diabetes. Because the Western lifestyle, which involves decreasing physical activity and over-consumption of high energy foods, is a major contributing factor in obesity, exercise is beneficial for overcoming obesity by increasing the total energy expenditure. Recently, many studies suggested that AMP-activated protein kinase (AMPK) activators mimic or potentiate the exercise-related effects. AMPK, as a heterotrimeric complex, is activated when the AMP concentration is increased by the depletion of ATP in metabolic stress, and by upstream kinases, such as LKB1 and CaMKK. Activation of the AMPK enzyme switches off the ATP-consuming anabolic pathways, such as fatty acid synthesis, and switches on the ATP-generating catabolic pathways by fatty acid oxidation to supply energy to the cell. Therefore, the selective activation of AMPK is considered a potential drug target for the prevention and treatment of obesity and diabetes by regulating the whole-body glucose and lipid homeostasis.

*Myristica fragrans* Houtt. (Myristicaceae) is an aromatic evergreen tree cultivated in South Africa, India, and other tropical countries. After nutmeg, which refers to the dried kernels of this plant, was imported into Europe at the 12th century, it has long been used indigenously as a spice in many Western foods. Nutmeg is also prescribed for medicinal purposes in Asia to treat many diseases, such as rheumatism, muscle spasm, decreased appetite, and diarrhea. As part of an ongoing screening program to search for new AMPK activators from natural plants, the total extract of *M. fragrans* (nutmeg) was found to activate the AMPK enzyme in differentiated C2C12 cells.

The EtOH extract of *M. fragrans* was subjected to an HP-20 column (10 × 60 cm), eluted with a gradient of EtOH in H2O (60%, 80%, 90%, and 100%, each 3 L), and finally washed with acetone (2 L) to give five fractions. A bioassay of the five fractions on the AMPK activity revealed the 80% ethanol-eluted fraction to be most active. This active fraction was further subjected to a succession of chromatographic procedures, including silica gel chromatography, RP-C18, and preparative HPLC, to yield seven tetrahydrofuran-type lignans. The chemical structures of the isolated compounds were determined by based on physicochemical and spectroscopic data analyses (IR, UV, 1H and 13C-NMR, and MS data), as well as a comparison with those reported in the literature. Compound 1 was obtained as a colorless oil with the molecular formula, C20H24O5, as determined by high resolution mass spectroscopy ([HREIMS m/z 344.1624 (calcld for C20H24O5 344.1614)]. The 1H and 13C NMR spectra of compound 1 revealed the presence of 12 protons and 10 carbon atoms. This suggested the symmetric
nature of the molecular structure of compound 1 with each signal in the $^1$H and $^{13}$C NMR spectra corresponding to two identical sets of protons and carbon atoms, respectively. Furthermore, the NMR spectral data of compound 1 suggested the presence of a 3,4-dimethyl-2,5-disubstituted tetrahydrofuran moiety. The coupling to be saucernetidiol and verrucosin by $^1$H and $^{13}$C NMR spectroscopy from this plant for the first time (Fig. 1).

Among these, compounds at C-3 and C-4 were shifted down field in the literature. Among these, compounds were characterized as fragransin C1 (Fig. 1).

However, the two methyl groups at C-3 and C-4 were shifted down field [at $\delta_{\text{H}}$ 1.05 (6H, $d, J = 6.6$ Hz)] and the two protons at C-2 and C-5 were shifted up field [at $\delta_{\text{H}}$ 4.53 (2H, $d, J = 6.6$ Hz)] compared to those of compound 1. These observations indicated that H-2 and H-5 have a trans-configuration with the adjacent protons of H-3 and H-4, respectively. From the spectroscopic data and physiochemical values between compound 1 and the published data, it was determined that 4-lute and 4-methyl showed the presence of only twelve protons and ten carbon atoms. The molecular formula, which was confirmed to be C$_{20}$H$_{24}$O$_5$ by HREIMS [$m/z$ 344.1610 (calcd for C$_{20}$H$_{24}$O$_5$, 344.1624)]. A comparison of the $^1$H and $^{13}$C NMR spectral data with those published in the literature, the chemical and biological properties of the isolates indicated that the tetrahydrofuran lignans with symmetric structures (compounds 1, 4, and 5) had stronger stimulation effects on the AMPK activity than the asymmetrical ones (compounds 2, 3, and 6). Furthermore, the chemical structure of the isolates showed that all aromatic rings (compounds 1–6) were isolated from the plant for the first time (Fig. 1).

As AMPK has recently attracted attention as the next generation target for total metabolic control, it was suggested that AMPK activators can be used to treat type-2 diabetes and obesity. A part of an ongoing screening program for new AMPK activators from natural plants using cell-based assays, seven 2,5-bis-aryl-3,4-dimethylenetetrahydrofuran lignans were isolated from M. fragrans. The effect of the individual tetrahydrofuran-type lignans on AMPK and its downstream target ACCs (ACC1 and ACC2) in cultured skeletal cells were examined. Western blot analysis indicated that the phosphorylation levels of AMPKThr172 and its intracellular substrate, ACCs Ser79, were increased after the cells were treated with these compounds. Among them, compounds 1, 4, and 5 showed the most potential activation, exhibiting stimulation at a final concentration of 5 $\mu$M (Fig. 2). A detailed examination of the chemical and biological properties of the isolates indicated that the tetrahydrofuran lignans with symmetric structures (compounds 1, 4, and 5) had stronger stimulation effects on the AMPK activity than the asymmetrical ones (compounds 2, 3, and 6). Furthermore, the chemical structure of the isolates showed that all compounds had 4'- and 4''-OH and/or 3'- and 3''-OCH$_3$ in the two aromatic rings (compounds 1–6), with the exception of compound 7, which was replaced by two methylenedioxy moieties in the structure.

As the consumption of high levels of dietary fat is believed to be a major factor in promoting obesity in human and animals, the...
C57BL/6 animal model was used to examine the protective effect of THF against weight gain in high-fat diet (HFD)-induced mice. Figure 3A shows the phenotypic characterizations of the HFD-induced C57BL/6 mice treated with or without THF and non-diet (ND) mice. All groups of mice started with similar body weights (20.86 ± 1.10 g, n = 30), but the average body weight (31.41 ± 3.04 g, n = 10) of the HFD-induced mice after six weeks was increased by 10.55 g more than those of the ND mice. The groups administered THF showed significantly lower body weight (28.72 ± 3.23 g, n = 10) than the HFD-treated group without any difference in food intake between the two groups (Table 1). After the HFD and HFD + THF diets for six weeks, the epididymis fat content was compared. The epididymis fat content of the THF-fed mice (1.74 ± 0.53 g) (Fig. 3B and Table 1). The physiological changes in the C57BL/6 mice treated with or without THF and ND mice for six weeks were investigated further. The blood chemistry revealed a decrease in the plasma glucose level in the THF-fed mice (199.10 ± 29.89 mg/dL) compared to those of the HFD-treated mice (232.7 ± 23.39 mg/dL). The levels of TG and LDL, 79.70 ± 9.88 and 32.20 ± 3.97 mg/dL, respectively, in the blood collected from the THF-treated mice were significantly lower than those of the HFD-treated mice; 88.40 ± 14.83 and 32.20 ± 3.97 mg/dL, respectively. This was also accompanied by lower concentrations of cholesterol; 153.46 ± 22.06 and 170.91 ± 15.35 mg/dL for the THF-treated mice and HFD-treated mice, respectively (Table 1). The decrease (68.06 ± 56.29 mg/dL) in the level of glutamate pyruvate transaminase (GPT) in the THF-treated mice compared to those in HFD-treated mice (81.84 ± 67.63 mg/dL) shows that the administration of THF in the HFD-induced C57BL/6 mice offers some protection against the development and progression of liver damage induced by HFD in C57BL/6 mice.

M. fragrans has been used traditionally as a spice and for various medicinal purposes, such as stomachic, carminative, tonic, aphrodisiac, and a nervous stimulant. These results showed that final body weights and weight gain in the THF-treated mice were significantly lower than those of the HFD-induced obesity mice. THF prevented not only the increase in body weight and adipose tissue mass but also the increases in glucose and LDL levels in the THF-treated group compared to those of the HFD group. These effects may be due partly to whole-body energy regulation through the phosphorylation of ACCs by the AMPK activators in this extract. Therefore, the enriched extract with tetrahydrofuran-type lignans from M. fragrans can be used not only for the development of agents to treat obesity and possibly type-2 diabetes but may also be a new class of AMPK activators for treating metabolic disorders.

### Acknowledgments

This study was supported by Grants from the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084335) and the National Research Foundation of Korea (NRF) (RO1-2008-000-20253-0) funded by the Ministry of Education, Science and Technology (MEST).

### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.067.

### References and notes


### Table 1

<table>
<thead>
<tr>
<th>Blood chemistry</th>
<th>ND&lt;sup&gt;a&lt;/sup&gt; (n = 10)</th>
<th>HFD&lt;sup&gt;b&lt;/sup&gt; (n = 10)</th>
<th>HFD + THF&lt;sup&gt;c&lt;/sup&gt; (n = 10)</th>
</tr>
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<tbody>
<tr>
<td>GPT&lt;sup&gt;d&lt;/sup&gt; (mg/dL)</td>
<td>48.14 ± 15.22</td>
<td>81.84 ± 67.63</td>
<td>68.06 ± 56.29</td>
</tr>
<tr>
<td>BUN&lt;sup&gt;e&lt;/sup&gt; (mg/dL)</td>
<td>23.99 ± 3.09</td>
<td>23.89 ± 1.77</td>
<td>22.74 ± 1.89</td>
</tr>
<tr>
<td>GLUC&lt;sup&gt;f&lt;/sup&gt; (mg/dL)</td>
<td>159.33 ± 28.44</td>
<td>232.7 ± 23.39</td>
<td>199.10 ± 29.89 &lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHOL&lt;sup&gt;g&lt;/sup&gt; (mg/dL)</td>
<td>115.73 ± 17.16</td>
<td>170.91 ± 15.35</td>
<td>134.46 ± 22.06</td>
</tr>
<tr>
<td>HDLC&lt;sup&gt;h&lt;/sup&gt; (mg/dL)</td>
<td>90.64 ± 14.52</td>
<td>125.48 ± 5.40</td>
<td>125.98 ± 12.39</td>
</tr>
<tr>
<td>LDLC&lt;sup&gt;i&lt;/sup&gt; (mg/dL)</td>
<td>22.78 ± 2.54</td>
<td>32.20 ± 3.97</td>
<td>25.90 ± 2.47</td>
</tr>
<tr>
<td>TRIG&lt;sup&gt;j&lt;/sup&gt; (mg/dL)</td>
<td>99 ± 15.64</td>
<td>88.40 ± 14.83</td>
<td>79.70 ± 9.88</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.29 ± 0.03</td>
<td>0.31 ± 0.02</td>
<td>0.29 ± 0.02 &lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.04 ± 0.10</td>
<td>1.01 ± 0.06</td>
<td>0.95 ± 0.12 &lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymis fat (g)</td>
<td>0.46 ± 0.06</td>
<td>1.74 ± 0.53</td>
<td>1.24 ± 0.40 &lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>2.71 ± 0.08</td>
<td>2.24 ± 0.05</td>
<td>2.20 ± 0.07 &lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Glutamate pyruvate transaminase.<br><sup>b</sup> Blood urea nitrogen.<br><sup>c</sup> Glucose.<br><sup>d</sup> Cholesterol.<br><sup>e</sup> High-density lipoprotein cholesterol.<br><sup>f</sup> Low-density lipoprotein cholesterol.<br><sup>g</sup> Triglyceride.<br><sup>h</sup> Data were expressed as means ± SD.<br><sup>*</sup> P < 0.0001 versus ND.<br><sup>**</sup> P < 0.05 versus HFD.<br><sup>***</sup> P < 0.05 versus HFD.
Bioassay-guided isolation of active compounds: The dried semen of Myristica fragrans (nutmeg) was purchased at a folk medicine market in Gwangju city, Republic of Korea. The sample was identified by Professor YH Moon at Chosun University, and a specimen (No. 0010) was deposited at the Department of Pharmacy, Chosun University, Republic of Korea. The seed (nutmeg) of M. fragrans (3 kg) was extracted with 30% ETOH at room temperature for 7 days. The 30% ethanol-soluble extract was filtered, and placed directly onto a Diaion HP-20 column (10 × 60 cm), eluted with H₂O/ETOH (40:60, 20:80, 10:90, 0:100, each 3L), and finally washed with acetone (2 L) to give five fractions. A bioassay of the five fractions on AMPK revealed the 80% ethanol-eluted fraction to be most active. This was further chromatographed over silica gel (60:40:1), to yield five fractions (F.1–F.5) according to their TLC profiles. Compound 1 (370 mg) was purified from a part of fraction 2 by chromatography on a reversed phase ODS-A column (5.0 × 25 cm, 10 μm particle size) eluted with MeOH/H₂O (60:40); flow rate 2 mL/min; UV-detection at 205 nm /[using RS Tech OptimaPak C18 column (10 cm), 1.5 μm particle size] using a gradient of n-hexane/acetone (from 6:1 to 0:1), to yield five fractions (F.1–F.5) according to their TLC profiles. Compound 2 (12 mg) and 7 (8.5 mg), Purification of fraction 3 by semi-preparative Gilson HPLC systems ([using RS Tech OptimaPak C18 column (10 × 250 mm, 10 μm particle size); mobile phase MeOH/H₂O (60:40); flow rate 2 mL/min; UV-detections at 205 and 280 nm] resulted in the isolation of compounds 1 (28.9 mg, t₁/₂ 33.4 min), 6 (12.7 mg, t₁/₂ 37 min), 2 (0.7 mg, t₁/₂ 39.5 min), and 3 (7.8 mg, t₁/₂ 43.5 min).

Physicochemical and spectroscopic data of compounds 1–7: Tetrahydrofuroguaiacin B (1): colorless oil; HREIMS m/z 344.1624 (calcd for C₂₀H₂₄O₅, 344.1626); 1H and 13C NMR, see Supplementary Tables S1 and S2. Verrucosin (2): colorless oil; [δH] 0.88 (t, J 7.2, CH₂), 1.38, CHCl₃); HREIMS m/z 344.1610 (calcd for C₂₀H₂₄O₅, 344.1624); 1H and 13C NMR, see Supplementary Tables S1 and S2. Galbacin (3): colorless oil; [δH] 1.00 (s, 3H, CH₃); HREIMS m/z 344.1636 (calcd for C₂₀H₂₄O₅, 344.1636); 1H and 13C NMR, see Supplementary Tables S1 and S2. Myristica (4): colorless oil; [δH] 1.00 (s, 3H, CH₃); HREIMS m/z 344.1626 (calcd for C₂₀H₂₄O₅, 344.1626); 1H and 13C NMR, see Supplementary Tables S1 and S2. Cinnamaldehyde (5): colorless oil; [δH] 3.57 (s, 1H, CH); HREIMS m/z 340.1331 (calcd for C₁₇H₂₅O₃, 340.1322); 1H and 13C NMR, see Supplementary Tables S1 and S2.

Animal experiments: Thirty eight-week-old male C57BL/6 mice were housed in plastic cages in a temperature-controlled (22 ± 1 °C) room and maintained on a reverse 12 h light/dark cycle. The mice were divided randomly into three groups: normal diet group (ND), high-fat diet group (HFD) with a daily gavage of 200 mg/kg of tetrahydrofuran mixture-fed group (HFD + THF). The HFD (DIO series diets, Research Diets Inc., USA) was based on a modified Western diet and contained 21% (w/w) lard and 0.15% (w/w) cholesterol (Moon, H. S.; Lee, H. G.; Cho, I. Y.; Chung, C. S.; Kim, T. G.; Choi, Y. J.; Cho, S. J. Nutr. Biochem. 2009, 20, 187). The tetrahydrofuran mixture was administered for 6 weeks, and the weight gain was measured every week. The food intake was measured for three consecutive days per week by subtracting the food jar pre- and post-weights for 6 weeks, and the mean values were used as the daily food intake. At the necropsy, both sides of the inguinal and epididymal adipose tissues were removed and weighed, and the relative adipose tissue weight to body weight was calculated. All animal experiments were approved by the Institutional Animal Care and Use Committee, and were performed in accordance with the institutional guidelines at AmorePacific Corporation, Korea.

The AMPK assay by Western blot analysis: C2C12 myotubes were incubated with the appropriate concentration of the compounds for 30 min and then lysed in EBC lysis buffer [50 mM Tris–HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% NP-40, and 50 mM sodium fluoride]. The cell debris was removed by centrifugation at 12,000 rpm for 15 min, at 4 °C. The protein concentrations in the cell lysates were determined using a Bio-rad protein assay kit. Approximately 30 μg proteins of the total cell extracts were subjected to western blot analysis using anti-phosphospecific AMPKα Thr172, anti-phosphospecific ACC Ser79. β-Actin protein levels were used as a control for equal protein loading. The immunoreactive antigen was then recognized using a horseshard peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Lee, Y. S.; Kim, W. S.; Kim, K. H.; Yoon, M. J.; Cho, H. J.; Shin, Y.; Ye, J. M.; Lee, C. H.; Oh, W. K.; Kim, C. T.; Cordula, H. B.; Gosby, A.; Kraegen, E. W.; James, D. E.; Kim, J. B. Diabetes 2006, 55, 2256).