Chemical Constituents of the Terrestrial Stems of *Ephedra sinica* and their PPAR-γ Ligand-Binding Activity

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Key words
*Ephedra sinica*, Ephedraceae, sesquiterpene, bisabolane, flavonoid, PPAR-γ

Introduction
*Ephedra sinica* is described in the Japanese Pharmacopoeia (17th edition) as one of the original plants from which the crude drug Ephedra Herb has been obtained [1]. Ephedra Herb has long been used as an antitussive and expectorant in traditional Japanese medicine [2, 3]. The terrestrial stems of *E. sinica* are well known to contain ephedrine alkaloids, (-)-ephedrine and (+)-pseudoephedrine, and tannins ephedrannin A and B [4–6]. The ephedrine alkaloids in Ephedra Herb are mainly responsible for exerting pharmaceutical effects as well as adverse effects [7, 8]. In addition, only a few bioactive constituents of Ephedra Herb, except for ephedrine alkaloids, have been reported [9]. Pharmacological studies of minor constituents of Ephedra Herb, such as sesquiterpenes and monoterpenes, are needed. As part of our continuous search for bioactive secondary metabolites of traditional medicines, a MeOH extract of *E. sinica* was found to exhibit peroxisome proliferator-activated receptor (PPAR)-γ ligand-binding activity. To identify the secondary metabolites in *E. sinica* having PPAR-γ ligand-binding activity, bioassay-guided fractionation of the MeOH extract of *E. sinica* terrestrial stems was carried out. This led to the isolation of 10 compounds, including one new bisabolane-type sesquiterpenoid (10). The structure of the new compound was determined by extensive spectroscopic analysis, including two-dimensional (2D) NMR. Among the isolated compounds, the sitosterol derivatives (1 and 2), flavonoid glucoside (7), and the new sesquiterpenoid (10), showed significant PPAR-γ ligand-binding activity.

Results and Discussion
The terrestrial stems of *E. sinica* (5.4 kg dry weight) were extracted with MeOH at 50 °C. After removing the solvent, the MeOH extract (665 g) was applied to a porous polymer polystyrene resin (Diaion HP-20) column. The MeOH, EtOH, and EtOAc-eluted portions showed PPAR-γ ligand-binding activity (Fig. 2). Thus, the MeOH and EtOH-soluble fractions were passed through column chroma-
tography on silica gel and octadecylsilanized (ODS) silica gel producing compounds 1–10. Compounds 1–9 were identified as β-sitosterol (1) [10], 7β-hydroxysitosterol (2) [11], 7α-hydroxysitosterol (3) [11], kaempferol-3-O-rhamnoside (4) [12], kaempferol-3-O-(4′-trans-p-coumaroyl)-rhamnopyranoside (5) [13], kaempferol-3-O-(4′-cis-p-coumaroyl)-rhamnopyranoside (6) [14], herbacetin-7-O-glucopyranoside (7) [6], mahuanin A (8) [15], and ephedrannin A (9) [16], respectively (▶ Fig. 1).

Compound 10 (C_{16}H_{22}O_{4}) was obtained as a colorless oil, which was determined by high-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESI-TOF-MS, m/z 301.1413 [M + Na]^+ , calcd. for C_{16}H_{22}NaO_{4} 301.1416) and 13C NMR (16 carbon signals) data. The IR spectrum of 10 showed absorption bands of carbonyl groups at 1700 and 1644 cm⁻¹. The 13C NMR spectrum exhibited two carbonyl carbon signals at δC 172.0 and 168.1, and the HMBC spectrum displayed a correlation peak between δC 168.1 and a methyl singlet signal at δH 3.74. On the other hand, treatment of 10 with bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine produced the corresponding trimethylsilyl (TMS) derivative of 10 (C_{19}H_{30}O_{4}Si; GC/MS, m/z: 350.1 [M + TMS]). These data implied the presence of two carboxy groups in the molecule of 10, one is free and another is methyl ester. The 1H NMR and 1H-1H COSY spectra disclosed the following spin-coupling correlations: δH 7.16 (H-2)/ 2.73 (H-3a) and 2.20 (H-3b)/2.89 (H-4)/ 2.12 (H-5a) and 1.74 (H-5b)/2.54 (H-6a) and 2.24 (H-6b), and the H-2 olefinic proton and H₂-6 methylene protons showed long-range correlations with the quaternary olefinic carbon at δC 129.2 (C-1) and the carbonyl carbon of the carboxy group at δC 172.0 (C-7). These data provided evidence of the presence of a cyclohexene group bearing a carboxy group at C-1. The other the spin-coupling correlations δH 1.06 (Me-14) and 1.05 (Me-15)/2.44 (H-13)/6.09 (H-12)/6.35 (H-11)/7.16 (H-10) were consistent with a 4-methyl-2-pentene group. The HMBC correlations from H₂-3, H-4, H₂-5, and H-11 to the olefinic carbon at δC 131.8 (C-8) and from H-4 to the carbonyl carbon of the methyl carboxylate group at δC 168.1 (C-9) indicated that C-4 of the cyclohexene group, C-9 carbonyl group, and C-10 of the 4-methyl-2-pentene group were linked to the C-8 olefinic carbon, and a double bond was present between C-8 and C-10 (▶ Fig. 3). The spin-coupling constant of H-10/H-11 (J = 11.4 Hz) and H-11/H-12 (J = 15.0Hz) and NOE correlations between H-10 and H₂-3 allowed the geometry at C-8 and C-11 to be established as Z and E. In ESI-TOF/MS-MS analysis of 10, the prominent fragment ion-peak at m/z 207 originated from a loss of the C_{11}H_{10}O_{4} moiety of 10. Other minor peaks observed at m/z 245, 235, 233, 201, 179, and 163 corresponded to the proposed fragments as depicted in the Supporting Informa-
tion. Thus, the structure of 10 was established as a bisabolane-type sesquiterpenoid as shown in ▶ Fig. 1. Compound 10 showed no specific rotation and was assumed to be a racemate.

The isolated compounds 1, 2, and 4–10 were evaluated for their PPAR-γ ligand-binding activity using a nuclear receptor cofactor assay system (▶ Fig. 4). Kaempferol was used as a positive control. Compounds 1, 2, 7, and 10 exhibited significant PPAR-γ ligand-binding activity, among which the new bisabolane-type sesquiterpenoid 10 was the most potent. In the kaempferol derivatives (4–7), the glycosylation at the C-2 hydroxy group by a rhamnosyl moiety (4–6) reduced the PPAR-γ ligand activity.

In conclusion, a new bisabolane-type sesquiterpenoid (10) and nine known compounds (1–9) were isolated from the MeOH extract of E. sinica terrestrial stems. Compounds 1, 2, 7, and 10 showed significant PPAR-γ ligand-binding activity, among which the new bisabolane derivative 10 was the most potent. In a previous study, terpenoids with a cyclohexene ring were reported to act as a native retinoid X receptor agonist [17]. With expectation, 10 exhibited PPAR-γ ligand-binding activity.

Materials and Methods

General experimental procedures

The instruments and experimental conditions were the same as those described in a previous paper [18]. All solvents used for extraction and isolation were high grade (> 99%) (Fujifilm Wako Pure Chemical). Purities of all isolated compounds (> 95%) were confirmed by NMR and TLC analysis.

Plant material

The dried terrestrial parts (5.4 kg) of E. sinica Stapf (Ephedraceae) were collected from the Medicinal Plant Garden of the Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan on July 10, 2015. This genetic resource was introduced to the garden in December 1981. The plant material was authenticated by one of the authors (K. M.) and was also identified according to the ITS rDNA sequence [19]. A voucher specimen has been deposited in the Herbarium of Tokyo University of Pharmacy and Life Sciences (KS-2015–001).

Extraction and isolation

The terrestrial stems of E. sinica (5.4 kg) were extracted with MeOH (50 L) at 50 °C. After evaporation of MeOH under reduced pressure at 40 °C, the MeOH extract (665 g) was subjected to a Diaion HP-20 column (2200 g, 85 mm i.d. × 600 mm) and successively partitioned by eluting with MeOH-H2O (3:7), MeOH-H2O (1:1), MeOH, EtOH, and EtOAc (each 10 L) in order of decreasing polarity. The EtOH-eluted fraction (55 g) was passed through a silica gel column (2000 g, 85 mm i.d. × 600 mm) eluted with gradient mixtures of hexane-EtOAc (4:1; 3:1; 2:1; 1:1) and MeOH, which produced 9 fractions (Frs. A-I). Fr. C was separated by a silica gel column (1800 g, 80 mm i.d. × 400 mm) eluted with hexane-EtOAc (7:1; 4:1; 1:1) and, sequentially, an ODS silica gel column eluted with MeCN-
H_2O (3:1; 4:1; 5:1) to yield **1** (44.8 mg), **2** (1.5 mg), and **3** (4.3 mg). The MeOH-eluted portion (120 g) was chromatographed on silica gel (2700 g, 45 mm i.d. × 340 mm) eluted with MeCN-H_2O (1:3; 1:2; 2:1) to give 10 subfractions (Frs. a-j). Fr. a was subjected to a Sephadex LH-20 column (470 g, 25 mm i.d. × 300 mm) eluted with MeOH-H_2O (1:1; 2:1) to give **4** (58.0 mg), **7** (6.7 mg), and **8** (17.0 mg). Fr. e was subjected to a Sephadex LH-20 column (470 g, 25 mm i.d. × 300 mm) eluted with MeOH-H_2O (2:1) and a silica gel column eluted with CHCl_3-MeOH-H_2O (100:10:1; 50:10:1; 30:10:1) to yield **5** (25.2 mg), **6** (6.2 mg), and **9** (7.1 mg). Fr. i was subjected to a Sephadex LH-20 column (470 g, 25 mm i.d. × 300 mm) eluted with MeOH-H_2O (2:1) and an ODS silica gel column eluted with MeCN-H_2O (2:1) to yield **10** (14.9 mg). Separation and isolation were guided a PPAR-γ ligand-binding activity and TLC analysis of the fractions. (▶ Fig. 1S–8S).

**Table 1** 

<table>
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<th>Position</th>
<th>δ_H</th>
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<tr>
<td>1</td>
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<td>–</td>
<td>129.2</td>
</tr>
<tr>
<td>2</td>
<td>7.16</td>
<td>br d</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>2.73</td>
<td>ddd</td>
<td>19.5, 11.2, 2.5</td>
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<tr>
<td></td>
<td>2.20</td>
<td>m</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>2.89</td>
<td>m</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>2.12</td>
<td>qd</td>
<td>12.4, 5.0</td>
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<tr>
<td></td>
<td>1.74</td>
<td>br d</td>
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<td>6</td>
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<td>ddd</td>
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<td>m</td>
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<td>7</td>
<td>–</td>
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<td>8</td>
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<td>–</td>
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<tr>
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**Derivatization of 10**

A mixture of **10** (0.5 mg) and BSTFA (50 µL) in pyridine (50 µL) was sealed in a glass vial and placed at 60 °C for 3 h. The reaction mixture was evaporated under a gentle stream of nitrogen to give the TMS derivative of **10**.

**PPAR-γ ligand-binding activity**

PPAR-γ agonist activity was examined using a nuclear receptor co-factor assay system (EnBio RCAS for PPAR-γ; EnBioTec Laboratories) according to the manufacturer’s instructions. Briefly, a peptide of cyclic AMP response element-binding protein (CBP) was immobilized on the bottom of a microtiter plate. After adding the recombinant human PPAR-γ solution to the wells, DMSO (purity > 99.5 %) as a control, or a positive control or isolated compounds were added, respectively. The binding of the PPAR-γ ligand complex to the CBP on the plate was detected by measuring the absorbance at 450 nm. This assay involves a cell-free system using nuclear receptors and cofactors. Pioglitazone (purity > 98.0 %) and kaempferol (purity > 97.0 %) were used as positive controls (TCI). All other agents or solvents used were of biochemical reagent grade.

**Statistical analyses**

Data are represented as the mean ± standard error of the mean (SEM) of three experiments performed in triplicate. Dunnett’s test was used and the level of significance is indicated by p values.
Supporting information

1D and 2D NMR and MS/MS spectra of 10 are available as Supporting Information.

Acknowledgements

We thank Hirokazu Ando and Yohei Sasaki for identifying E. sinica according to the ITS rDNA sequence.

Conflict of Interest

The authors declare that they have no conflict of interest.

References