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

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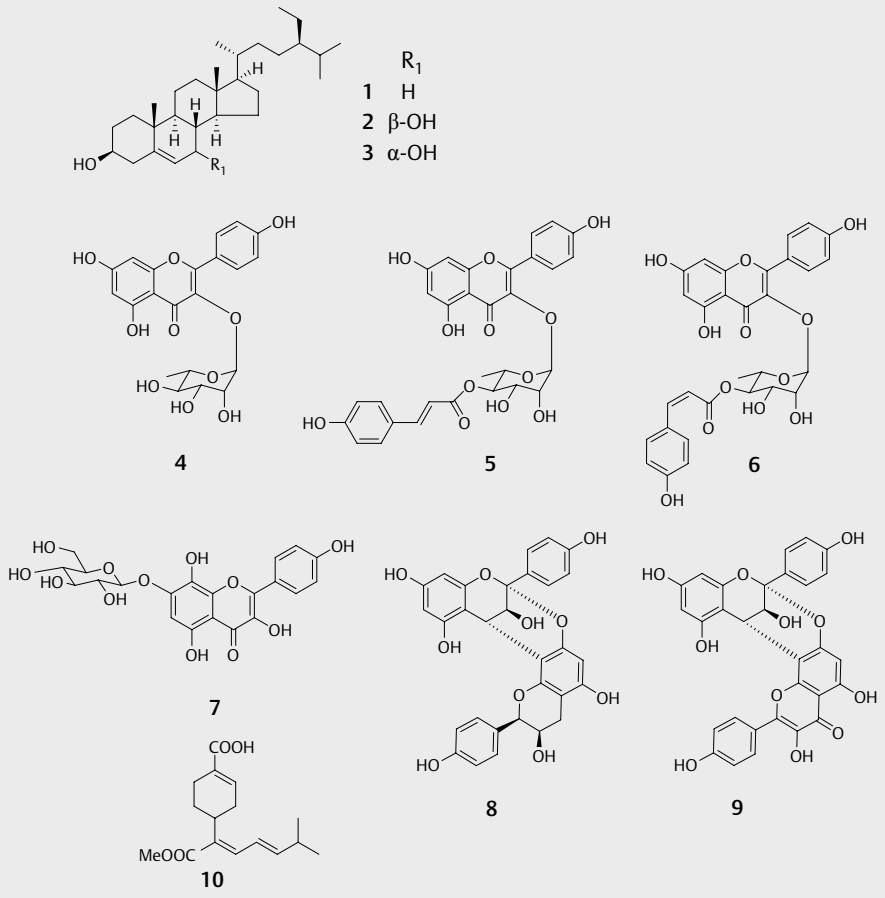
Abstract
Bioassay-guided fractionation of the MeOH extract of *Ephedra sinica* terrestrial stems, using a PPAR-γ ligand binding assay, resulted in the isolation of 10 compounds, including one new bisabolane-type sesquiterpenoid (**10**). The structure of the new compound was determined by extensive spectroscopic 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.

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 (**1**–**10**), including a new bisabolane-type sesquiterpenoid (**10**) (▶ **Fig. 1**). This paper is a report on the structural determination of the new sesquiterpene obtained by extensive spectroscopic analysis, including 2D NMR data. The isolated compounds were evaluated for their PPAR-γ ligand-binding activity.

Results and Discussion

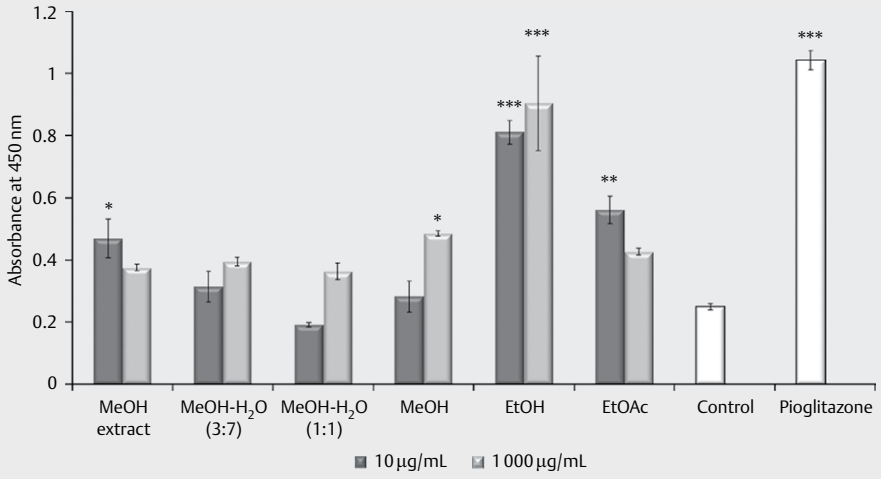
The terrestrial stems of *E. sinica* (5.4 kg dry weight) were extracted with MeOH at 50 °C. After removing the solvent, the MeOH extract (665 g) was applied to a porous polymer polystyrene resin (Diaion HP-20) column. The MeOH, EtOH, and EtOAc-eluted portions showed PPAR-γ ligand-binding activity (▶ **Fig. 2**). Thus, the MeOH and EtOH-soluble fractions were passed through column chroma-



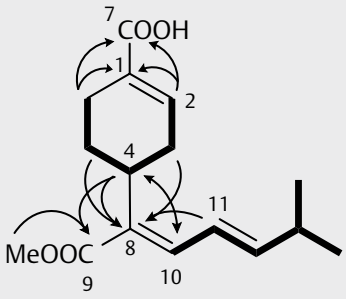
▶ **Fig. 1** Isolated compounds from *E. sinica*.

tography on silica gel and octadecylsilanized (ODS) silica gel producing compounds **1**–**10**. Compounds **1**–**9** were identified as *β*-sitosterol (**1**) [10], 7*β*-hydroxysitosterol (**2**) [11], 7*α*-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 ^{13}C NMR (16 carbon signals) data. The IR spectrum of **10** showed absorption bands of carbonyl groups at 1700 and 1644 cm^{-1}. The ^{13}C NMR spectrum exhibited two carbonyl carbon signals at δ_C 172.0 and 168.1, and the HMBC spectrum displayed a correlation peak between δ_C 168.1 and a methyl singlet signal at δ_H 3.74. On the other hand, treatment of **10** with bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine produced the corresponding trimethylsilyl (TMS) derivative of **10** ($C_{19}H_{30}O_4Si$; 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 ^{1}H NMR and ^{1}H-^{1}H COSY spectra disclosed the following spin-coupling correlations: δ_H 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 H_2–6 methylene protons showed long-range correlations with the quaternary olefinic carbon at δ_C 129.2 (C-1) and the carbonyl carbon of the carboxy group at δ_C 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 δ_H 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 H_2–3, H-4, H_2–5, and H-11 to the olefinic carbon at δ_C 131.8 (C-8) and from H-4 to the carbonyl carbon of the methyl carboxylate group at δ_C 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 = 15.0 Hz) and NOE correlations between H-10 and H-12 and between H-11 and H_2–3 allowed the geometry at C-8 and C-11 to be established as 8*E* and 11*E*. In ESI-TOF/MS-MS analysis of **10**, the prominent fragment ion-peak at *m/z* 207 originated from a loss of the $C_{(11)}H$-$C_{(12)}H$-$C_{(13)}H$-$Me_{(14)}$($Me_{(15)}$) 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-**



▶ **Fig. 2** PPAR-γ ligand-binding activity of the MeOH extract of *E. sinica* and fractions. The PPAR-γ ligand-binding activity of each fraction was measured at sample concentrations of 1000 and 10 µg/mL and that of pioglitazone at 5.0 µM. Data are represented as the mean ± SEM of three experiments performed in triplicate; $^{***}p < 0.0001$ vs. control, $^{**}p < 0.001$ vs. control, $^{*}p < 0.01$ vs. control.



▶ **Fig. 3** ^{1}H-^{1}H COSY and selected HMBC correlations of **10**.

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

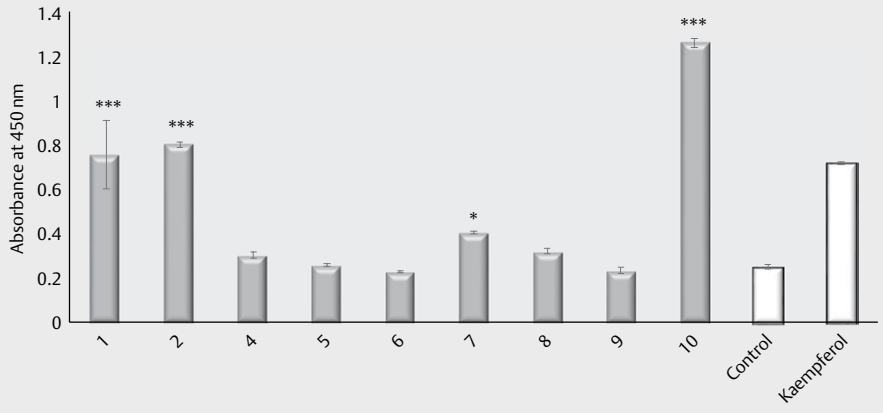
The instruments and experimental conditions were the same as those described in a previous paper [18]. All solvents used for extraction and isolation were high grade (> 99 %) (Fujifilm Wako Pure Chemical). Purities of all isolated compounds (> 95 %) were confirmed by NMR and TLC analysis.

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-H_2O (3:7), MeOH-H_2O (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-



▶ **Fig. 4** PPAR-γ ligand-binding activity of **1**, **2**, and **4**–**10**. PPAR-γ ligand-binding activities of **1**, **2**, **4**–**10**, and kaempferol were measured at a sample concentration of 0.5 mM. Data are represented as the mean ± SEM of three experiments performed in triplicate; $^{***}p < 0.0001$ vs. control, $^{*}p < 0.01$ vs. control.

▶ **Table 1** ^{1}H and ^{13}C NMR (500 and 125 MHz, $CDCl_3$) spectroscopic assignments of **10**.

Position	δ_H		*J* (Hz)	δ_C
1	—			129.2
2	7.16	br d	2.5	142.2
3	2.73	ddd	19.5, 11.2, 2.5	30.5
	2.20	m		
4	2.89	m		33.4
5	2.12	qd	12.4, 5.0	26.4
	1.74	br d	12.4	
6	2.54	ddd	16.9, 2.3, 2.0	24.7
	2.24	m		
7	–			172.0
8	–			131.8
9	–			168.1
10	7.16	br d	11.4	140.2
11	6.35	ddd	15.0, 11.4, 1.1	121.9
12	6.09	ddd	15.0, 7.1, 0.6	151.4
13	2.44	sd	7.1, 1.1	31.9
14	1.06	d	6.7	22.0
15	1.05	d	6.7	22.0
16	3.74	s		51.4

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. × 430 mm) eluted with MeCN-H_2O (1:3; 1:2; 1:1; 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**).

Compound **10**

An amorphous solid; $[\alpha]_D^{25}$ –0.22 (*c* 0.10, $CHCl_3$); IR ν_{max} (film) cm^{-1}: 3414 (OH), 1700 and 1644 (C = O); ^{1}H and ^{13}C NMR (500 and 125 MHz, $CDCl_3$), see ▶ **Table 1**; HR-ESI-TOF-MS (*m/z*: 301.1413 $[M + Na]^+$, calcd. for $C_{16}H_{22}NaO_4$: 301.1416).

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 cofactor 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 reagents 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

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Conflict of Interest

The authors declare that they have no conflict of interest.

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