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Eucommicin A, a β-truxinate lignan from *Eucommia ulmoides*, is a selective inhibitor of cancer stem cells

Ayaka Fujiwara [a,1], Mayuko Nishi [b,1], Shigeo Yoshida [c], Morifumi Hasegawa [a], Chieko Yasuma [d], Akihide Ryo [b,*], Yoshihito Suzuki [a,*]

[a] Department of Bioresource Science, Ibaraki University, Inashiki-gun, Ibaraki 300-0393, Japan
[b] Department of Microbiology, Yokohama City University School of Medicine, Yokohama, Kanagawa 236-0004, Japan
[c] RIKEN, Wako, Saitama 351-0198, Japan
[d] Hekizan-en Co., Ltd., Aiko-gun, Kanagawa 243-0307, Japan

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ABSTRACT

Cancer stem cells (CSCs) constitute a small population of undifferentiated cells within a tumor that have the ability to self-renew and drive tumor formation, thus behaving as cancer-initiating cancer cells. Therapeutic interventions that eliminate CSCs are necessary to completely cure patients, since CSCs are a crucial source of tumor recurrence and metastasis. An induced CSC-like (iCSCL) model was recently established using induced pluripotent stem cells (iPSCs). In this study, a natural product—eucommicin A—was identified from *Eucommia ulmoides* leaves by screening for anti-CSC activity using the iCSCL model. Its structure was elucidated by spectroscopic methods as a quinic acid diester of 3,4,3′,4′-tetrahydroxy-β-truxinic acid. Eucommicin A exhibited selective anti-CSC activity and inhibited tumor sphere formation by iCSCL cells. The results of this study suggest that eucommicin A could serve as a lead compound in the development of drugs to abrogate the stemness and self-renewal ability of CSCs.

1. Introduction

Cancer stem cells (CSCs) are a small subpopulation of cancer cells with normal stem-cell-like properties. CSCs have self-renewal ability and multilineage potential, and thus behave as cancer-initiating cells. CSCs are resistant to conventional anticancer agents, since they are usually quiescent, have low metabolic activity, and express high levels of ATP-binding cassette (ABC) drug transporters. Furthermore, CSCs show prominent metastatic spreading with acquisition of mesenchymal properties, including enhanced motility and invasive ability. Therefore, CSCs are considered to be the main cause of cancer formation, as well as a crucial source of recurrence and metastasis.

One of the main goals in the field of anti-cancer therapy is to establish treatment strategies to eliminate CSCs within tumor tissues. Although anti-CSC agent screening requires routine preparation of large quantities of CSCs, separating a large number of CSCs from tumor tissue is not clinically practical. Our laboratory recently established a putative CSC model from human mammary epithelial MCF-10A cells, designated iCSCL-10A cells, using induced pluripotent stem cell technology (Nishi et al., 2014a). iCSCL-10A cells display a number of traits characteristic of CSCs, such as expression of cancer stem markers (CD44, CD133, and ALDH1), self-renewal, malignant phenotype in culture, and strong tumor-formation ability upon injection into immunocompromised mice. The usefulness of iCSCL-10As for screening agents targeting phenotypic traits of CSCs has been demonstrated (Nishi et al., 2014b).

Eucommia ulmoides Oliver (known as *Du-zhong* in Chinese) is a species of tree that is native to China and belongs to the monotypic family Eucommiaceae. The aqueous extract of *E. ulmoides*, commonly known as Du-zhong tea, is a popular drink in China and Japan and has been used as a functional food, especially for the treatment of hypertension (Nakazawa, 1997). Pharmacological studies have shown that the extract of *E. ulmoides* has antimicrobial and anti-inflammatory (Kim et al., 2009; Tsai et al., 2010), antioxidant (Yen and Hsieh, 2000; Qu et al., 2006; Xu et al., 2010), and antihypertensive properties (Kwan et al., 2003; Lang et al., 2005; Luo et al., 2010; Gu et al., 2011). The chemical constituents of *E. ulmoides* have been extensively studied, resulting in the identification of several types of compounds, including phenylpropanoids, flavonoids, iridoids, and lignans. Some of the constituent compounds of *E. ulmoides* have antihypertensive (Deyama et al., 2001; Xiao et al., 2009), neuroprotective (Hu et al., 2014), antibacterial (Li et al., 2014), antitumor (Isiguro

* Corresponding authors.
E-mail address: ayoshi@mx.ibaraki.ac.jp (Y. Suzuki).
[1] Co-first authors.

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Table 1
^{1}H, ^{13}C, COSY and HMBC correlation data of **1** in CD_3OD.

Position[a]	^{13}C shift (δ_C)	^{1}H shift (δ_H)	COSY correlations[c]	HMBC correlations via $^{2,3}J_{CH}$[d]
1, 1′	132.17, 132.17	–	–	H-5, H-7, H-8
2, 2′	116.46, 116.50	6.46 (2H, d, 1.7)	H-6, H-7	H-6, H-7
3, 3′	144.61, 144.65	–	–	H-2, H-5
4, 4′	145.77, 145.77	–	–	H-5
5, 5′	115.92, 115.94	6.56 (2H, d, 8.1)	H-6	
6, 6′	120.54, 120.54	6.37 (1H, dd, 8.1, 1.7), 6.38 (1H, dd, 8.1, 1.7)	H-2, H-5, H-7	H-2, H-5, H-7
7, 7′	45.95, 46.24	4.09 (1H, ddd, 10, 7.0, 1.0)[b], 4.16 (1H, ddd, 10, 7.0, 1.0)[b]	H-2, H-6, H-7′, H-8	H-2, H-6, H-7′
8, 8′	44.70, 45.12	3.79 (1H, ddd, 10, 7.0, 1.0)[b], 3.81 (1H, ddd, 10, 7.0, 1.0)[b]	H-7, H-8′[e]	H-7, H-8′
9, 9′	174.22, 174.45	–	–	H-7, H-8, H-$_q$3
1_q, 1_q′	76.32, 76.53	–	–	H-2_qαβ, H-5_q, H-6_q
2_q, 2_q′	38.91, 39.11	H-2_qα: 2.02 (1H, dd, 13, 10), 2.07 (1H, dd, 13, 10) H-2_qβ: 2.25 (1H, ddd, 13, 4.4, 2.2), 2.31 (1H, ddd, 13, 4.4, 2.2)	H-2_qα: H-2_qβ, H-3_q H-2_qβ: H-2_qα, H-3_q	H-6_qαβ
3_q, 3_q′	72.53, 72.68	5.32 (1H, ddd, 10, 8.9, 4.4), 5.35 (1H, ddd, 10, 8.9, 4.4)	H-2_qαβ, H-4_q	H-2_qαβ, H-4_q, H-5_q
4_q, 4_q′	73.50, 73.92	3.70 (1H, dd, 8.9, 3.5), 3.71 (1H, dd, 8.9, 3.5)	H-3_q, H-5_q	H-2_qαβ, H-3_q, H-5_q
5_q, 5_q′	71.53, 71.88	4.14 (2H, br. s)	H-4_q, H-6_qab	H-6_qαβ
6_q, 6_q′	38.13, 38.21	H-6_qα: 2.14 (1H, dd, 14, 2.5), 2.15 (1H, dd, 14, 2.5) H-6_qβ: 2.06 (2H, m)	H-6_qα: H-5_q H-6_qβ: H-5_q	H-2_qβ, H-5_q
7_q, 7_q′	177.07, 177.19	–	–	H-2_qαβ, H-6_qαβ

[a] Numbering of carbons as in Fig. 1.
[b] Coupling constants were determined by spin simulation (iNMR version 5.4.4).
[c] Correlations were shown only for protons attached to C-1 ~ C-9, and C-1_q ~ C-7_q.
[d] Correlations were shown only for carbon atoms C-1 ~ C-9, and C-1_q ~ C-7_q.
[e] Correlation between H-8 and H-8′ was not evident because of overlapping chemical shifts.

et al., 1986; Ikemoto et al., 2000; Zhang et al., 2001), antithrombotic (Suzuki et al., 2001), anti-inflammatory (Koo et al., 2004), and antioxidative activities (Shieh et al., 2000; Gao et al., 2001; Zhang et al., 2001; Ho et al., 2005).

In the present study, iCSCL-10A cells were used as a screening system to isolate promising anti-CSC agents from the leaf extract of *E. ulmoides*. Trial experiments indicated the presence of compounds with anti-iCSCL-10A activity in the extract of the dry powdered leaf of *E. ulmoides*, prompting us to identify the active agent (s) in the extract. Here, the isolation and structural determination of a novel phytochemical with anti-iCSCL-10A cell activity are described.

2. Results and discussion

Extract of the powdered leaves of *E. ulmoides* (126 g) was partitioned between water and 1-butanol, after which the aqueous fraction, which showed activity against iCSCL-10A cells, was fractionated by hydrophilic interaction liquid chromatography (HILIC). The activities of Fr. 3, Fr. 6, and Fr. 7 against iCSCL-10A cells were assessed. The anti-iCSCL-10A cell activity of Fr. 3 was abolished by subsequent purification. Fr. 6 and Fr. 7 were combined and roughly fractionated using ODS-HPLC (φ14 × 150 mm) to efficiently concentrate the active fraction by removing major impurities with higher polarity. The active fraction was more precisely fractionated on the same column with a solvent of weaker eluting power. Finally, purification was performed using a polar group-embedded ODS, after which the active compound (**1**) showed as a single peak (36.2 mg, a colorless oil). It should be noted that this compound did not show consistent behavior in the ODS HPLC assay when a small amount was injected; retention time varied and the peak exhibited abnormal tailing.

Negative electrospray ionization-mass spectrometry (ESI-MS) of **1** indicated a *m/z* 707 as a plausible ion for $[M-H]^-$, which was supported by the positive ESI-MS, which gave *m/z* 731 ($[M+Na]^+$) and *m/z* 747 ($[M+K]^+$). High resolution fast atom bombardment mass spectroscopy (FABMS) (negative) of **1** gave an ion for $[M-H]^-$ at *m/z* 707.1828, agreeing with the molecular formula $C_{32}H_{36}O_{18}$ (calculated for $C_{32}H_{35}O_{18}$ as 707.1824). The ^{13}C-NMR spectrum (Table 1) gave 29 signals, of which 3 at 145.77 ppm, 132.17 ppm, and 120.54 ppm were considered to be overlapped signals of two carbons due to their relatively strong intensities in comparison with those of others in the same region, agreeing with the presence of 32 carbons in the molecular formula as deduced by high resolution FABMS. DEPT analysis confirmed that, of the 32 carbons, 16 were methine groups, 4 were methylene groups, and 12 were quaternary carbons. The data also established the presence of two sets of similar signals, suggesting that structure **1** was a dimer. The four signals on the downfield side of the aromatic region (δ_C 144–146 ppm) suggested the presence of four carbons bearing an oxygen in the di-substituted phenyl rings. In the ^{1}H-NMR (in CD_3OD) spectrum (Table 1), the coupling patterns of six aromatic protons (δ_H 6.3–6.6 ppm) indicated the presence of two 3,4-disubstituted phenyl rings. The correlation of a carbon and a proton in each methine group was confirmed by the HSQC spectrum. The COSY and HSQC spectra indicated C-2_q/C-3_q/C-4_q/C-5_q/C-6_q connectivity, whereas the HMBC correlations (H-2_qβ → C-6_q, H-6_qαβ → C-2_q, H-2_qαβ → C-7_q, H-6_qαβ → C-7_q, H-2_qαβ → C-1_q, H-6_q → C-1_q) suggested the presence of quinic acid moieties. The downfield shift of H-3_q (5.33 ppm) in comparison with the corresponding signal of the free quinic acid at 3.96 ppm (in D_2O) and the HMBC of H-3_q with C-9 suggested acylation of quinic acid at the C-3_q position.

The identity of the quinic acid moiety was confirmed by recovering the quinic acid after hydrolysis of **1** in aqueous sodium hydroxide. The recovered candidate compound showed an ^{1}H-NMR spectrum identical to that of authentic quinic acid (data not shown), as well as a specific optical rotation that was equally negative (recovered sample, −43.3; authentic quinic acid, −43.8), thus confirming that identity of the quinic acid moiety as the naturally occurring (−)-form. Acetylation of 1 showed the presence of ten hydroxyl groups, which was explained by two monoacylated quinic acids and two dihydroxy phenyl rings. Given that two sets of these partial structures are included in **1**, the remaining structure required for a formula of $C_6H_4O_2$ should be composed of 4 methine groups (C-7, C-7′, C-8, and C-8′) and 2 quaternary carbons (C-9 and C-9′) and have three degrees of unsaturation. C-9 and C-9′ (δ_C 174.2 ppm and δ_C 174.4 ppm, respectively) were assigned to carbonyl carbons involved in quinic acid conjugation. Four methine groups at C-7, C-7′, C-8, and C-8′ should form a partial structure for the remaining one degree of unsaturation. A clear coupling

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was observed in the COSY spectrum since the difference in chemical shifts between H-7 and H-7′ was relatively large (4.09 ppm and 4.16 ppm) (Fig. 2), suggesting that bonding between C-7 and C-7′ is involved in the dimerization. COSY correlations between H-8 and H-8′ and H-7 and H-8′ were not evident because of the very close chemical shifts of H-8 and H-8′. Thus, the splitting patterns of H-7, H-7′, H-8, and H-8′ were analyzed by spin simulation software, after which the calculated spectrum was compared to the experimental spectrum. The obtained coupling constants ($^3J_{7,7'} = {}^3J_{8,8} = 10$ Hz, $^3J_{7,8} = {}^3J_{7',8'} = 7$ Hz, and $^4J_{7,8'} = {}^4J_{7',8} = 1.0$ Hz) were attributable to an AA′BB′ spin system on a cyclobutane ring. The ^{1}H-NMR spectrum and COSY correlations showed the presence of long-range coupling between H-7 and H-2/H-6, which indicated C-1/C-7 connectivity. HMBC analysis showed H-7 → C-1/C-2/C-6, H-8 → C-1, and H-7/H-8 → C-9 correlations, indicating C-1/C-7/C-8/C-9 connectivity (Fig. 2). Thus, compound **1** was determined to be a dimer of chlorogenic acid, with the possibility of some isomers, owing to the cyclobutane ring arrangement.

The NOESY correlations between H-2 and H-8 (H-2′ and H-8′), and H-6 and H-8 (H-6′ and H-8′) suggested a *trans* configuration of the H-7/H-8 and H-7′/H-8′ pairs (Fig. 3), constraining the structure to one of four ring arrangements: β-truxinic, δ-truxinic, ε-truxillic, or α-truxillic (Fig. 4) (Montaudo and Caccamese, 1973). Fragments produced from cyclobutane ring cleavage in the mass spectra allowed us to discriminate between head-to-head (truxinic) and head-to-tail (truxillic) types. The negative ESI-MS showed ions at *m/z* 463 and *m/z* 243 in addition to that measured at *m/z* 353, demonstrating the truxinic structure of compound **1**. Discrimination between β-truxinic and δ-truxinic structures was achieved by measuring the chemical shifts of H-8 and H-8′, which are subject to the anisotropic effects of the aromatic rings attached to C-7 and C-7′. It has been demonstrated that the H-8/H-8′ atoms of β-truxinic structures fall within the deshielding zone created by the aromatic rings, while those of δ-truxinic structures are subject to their shielding effect (Mahindru et al., 1993; Davis et al., 2007). Published ^{1}H-NMR data confirmed that all H-8/H-8′ chemical shifts of β-truxinic structures with acylated C-8/C-8′ atoms have been observed between 3.75 ppm and 4.23 ppm (Montaudo and Caccamese, 1973; Sudo et al., 2000; Dimberg et al., 2001; Krauze-Baranowska et al., 2013), while those of δ-truxinic structures have been observed between 3.24 ppm and 3.30 ppm

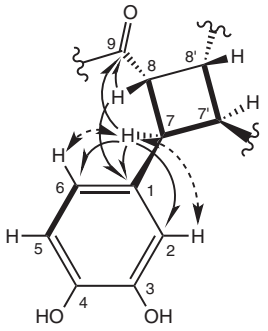

Fig. 2. COSY and HMBC correlations for the partial structure of **1** including a cyclobutane ring. ^3J couplings observed in COSY spectra are represented by bold lines, whereas long-range COSY correlations are represented by double-headed dotted arrows. HMBC correlations are represented by single-headed arrows.

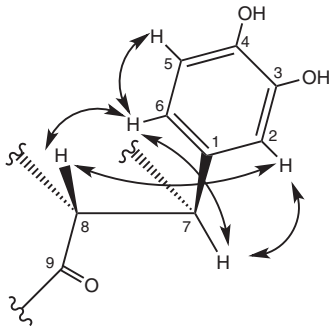

Fig. 3. NOESY correlations for the partial structure of **1**. NOESY correlations are represented by double-headed arrows.

(Montaudo and Caccamese, 1973; Deng et al., 2011; Wang et al., 2014). The chemical shifts of the H-8/H-8′ atoms of **1**, 3.79 ppm and 3.81 ppm, indicated that the ring arrangement of **1** was of the β-truxinic type. To confirm the ring arrangement, compound **1** was converted to a known compound, compound **2**, by solvolysis with methanol/sodium methoxide and methylation with

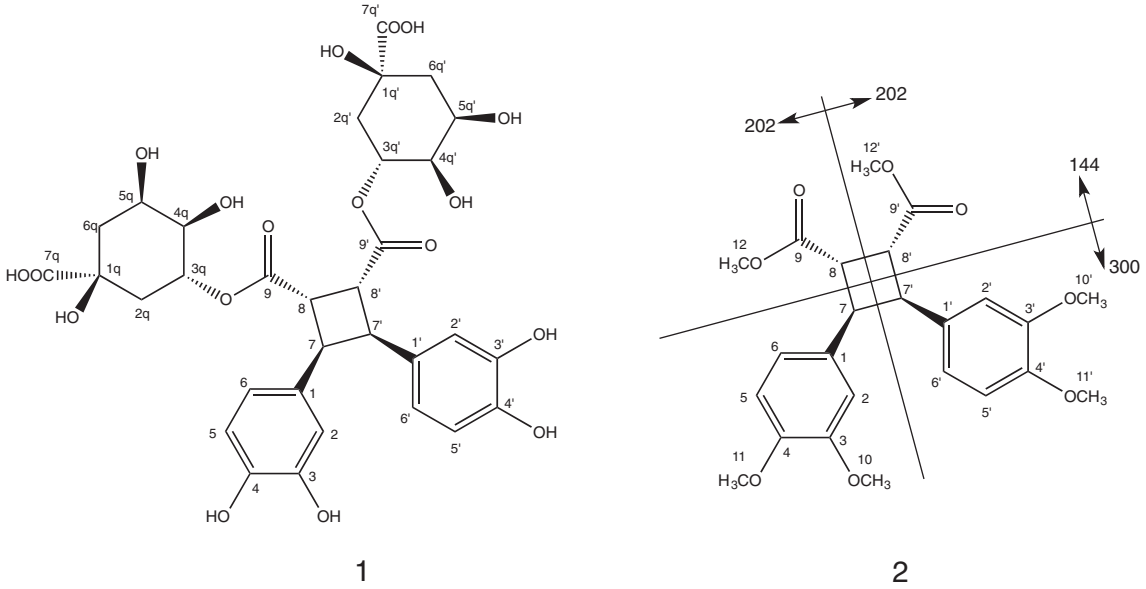

Fig. 1. Structure of eucommicin A (**1**) and 3,3′,4,4′-tetrahydroxy-β-truxinic acid (**2**). Masses of fragments arising from cleavage of the cyclobutane ring of **2** are shown.

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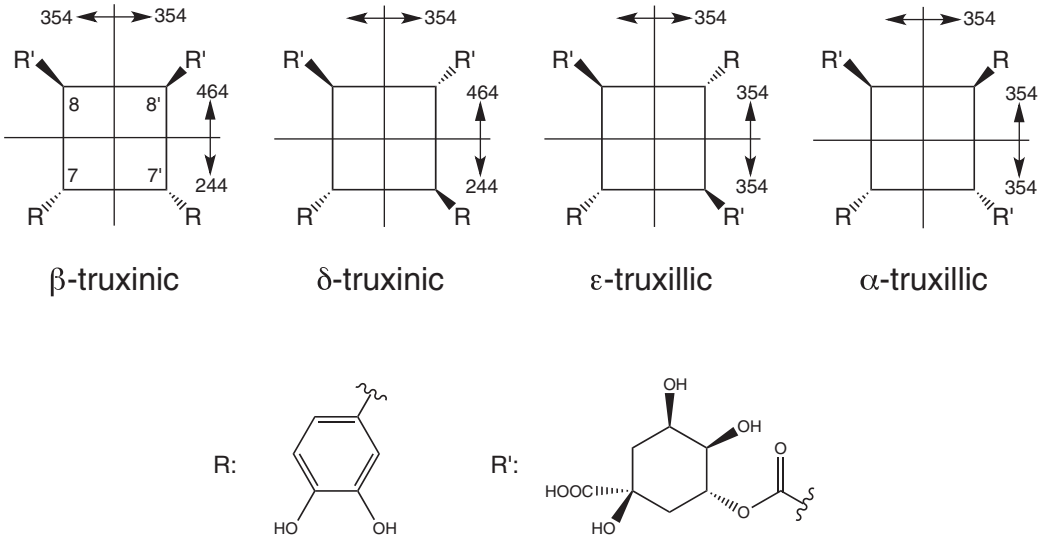

Fig. 4. Possible cyclobutane ring arrangements for **1**. Stereochemistry candidates for the cyclobutane ring in **1** are shown with the masses of fragments arising from cyclobutane ring cleavage.

diazomethane. Electron impact mass spectra (EI-MS) analysis revealed ions at *m/z* 300 and *m/z* 144 in addition to that observed at *m/z* 222 (Fig. 1), demonstrating a truxinic ring arrangement. The H-8/H-8′ signals of **2** at 3.91 ppm and 3.92 ppm fell within the range of the chemical shifts of the corresponding protons of β-truxinic type compounds. Moreover, all signals in the ^{1}H-NMR and ^{13}C-NMR spectra coincided with those described in the literature (Carmignani et al., 1999). Thus, compound **1** was determined to be a quinic acid diester of 3,4,3′,4′-tetrahydroxy-β-truxinic acid, which was named eucommicin A (Fig. 1).

The eucommicin A (**1**) production process might have artificially produced eucommicin A (**1**) since the compound was isolated from the powdered dry leaf of *E. ulmoides* produced as a commercial product. However, a compound isolated from freshly harvested *E. ulmoides* leaves by monitoring anti-iCSCL cell activity and liquid chromatography tandem-mass spectrometry (LC–MS/MS) gave spectra superimposable on those of eucommicin A (**1**). These results demonstrate that eucommicin A (**1**) is a naturally occurring compound.

The CSC-specific cytotoxicity of eucommicin A (**1**) was assessed using two different CSC clones, iCSCL-10A-1 and iCSCL-10A-2, and two breast cancer cell lines, MCF7 and MDA-MB231, which were plated in 96-well plates and cultured with different concentrations of eucommicin A (**1**) for 48 h. Eucommicin A (**1**) at a concentration of 50 μM reduced the viability of iCSCL-10A-1 and iCSCL-10A-2 cells (Fig. 5A).

Tumor sphere formation assays were performed to determine the effect of eucommicin A (**1**) on the self-renewal activity of CSCs. iCSCL-10A cells were cultured on ultra-low-attachment surface plates with growth media supplemented with various concentrations of eucommicin A (**1**), chlorogenic acid, or withaferin A as a positive control (Nishi et al., 2014b) (Fig. 5B). Fifty-percent cell viability inhibition (IC_{50} values) was determined by sigmoidal fitting of cell viability data. The IC_{50} of eucommicin A (**1**) was 55.0 μM, while chlorogenic acid produced no reduction in viability (IC_{50} = 6900 μM). Eucommicin A (**1**) was less potent than withaferin A (IC_{50} = 1.2 μM), a natural compound with anti-CSC activity. Eucommicin A (**1**), but not chlorogenic acid, significantly suppressed tumor sphere formation, demonstrating its capacity to abrogate the self-renewal ability of iCSCL-10A cells. These results also indicated that exposure to chlorogenic acid alone is insufficient to inhibit CSC function, suggesting that a unique structure containing a cyclobutane ring moiety is required to produce such an effect.

3. Concluding remarks

In the present study, a novel natural product, eucommicin A (**1**), was isolated from *E. ulmoides*. This is the first study to report the isolation of an anti-CSC agent from natural sources. Eucommicin A (**1**) could be a potential lead compound in the development of new carcinostatic agents targeting CSCs with low side effects, because of the long history of *E. ulmoides* leaf extract ingestion as Du-zhong tea.

4. Experimental

4.1. General experimental procedures

^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), and 2D-NMR were recorded on an AVANCE III FT-NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5-mm broadband fluorine observation (BBFO) probe. Chemical shifts (δ) were referenced to solvent signals ($C\underline{H}D_2OD$, δ_H 3.329; $\underline{C}D_3OD$ δ_C 49.03; $C\underline{H}D_2COCD_3$, δ_H 2.067; $\underline{C}D_3C\underline{C}D_3$, δ_C 29.81; $CD_3\underline{C}OCD_3$, δ_C 206.06). Coupling constants for H-7, H-7′, H-8, and H-8′ in compounds **1**, **2**, and acetylated **1** were determined by spin simulation using iNMR version 5.4.4 (nucleomatica, Molfetta, Italy; http://www.inmr.net). Specific optical rotations were measured with a P-2100 polarimeter equipped with a halogen lamp and a 589-nm filter (Jasco, Tokyo, Japan). High-resolution mass spectra were obtained using a JMS-BU25 (GCmate II) mass spectrometer (Jeol, Tokyo, Japan) in either the direct inlet EI mode (70 eV) or negative FAB mode using Ar gas and a glycerol matrix. LC–MS/MS analysis was performed using a 3200 QTrap LC–MS/MS device (AB Sciex, Foster City, CA, USA).

4.2. Plant material

E. ulmoides leaves were collected from the du zhong farm in Aiko, Kanagawa, Japan operated by Hekizan-en Co., Ltd. in October 2014. A voucher specimen is deposited in the Herbarium of the National Museum of Nature and Science, Japan (150908A). The *E.*

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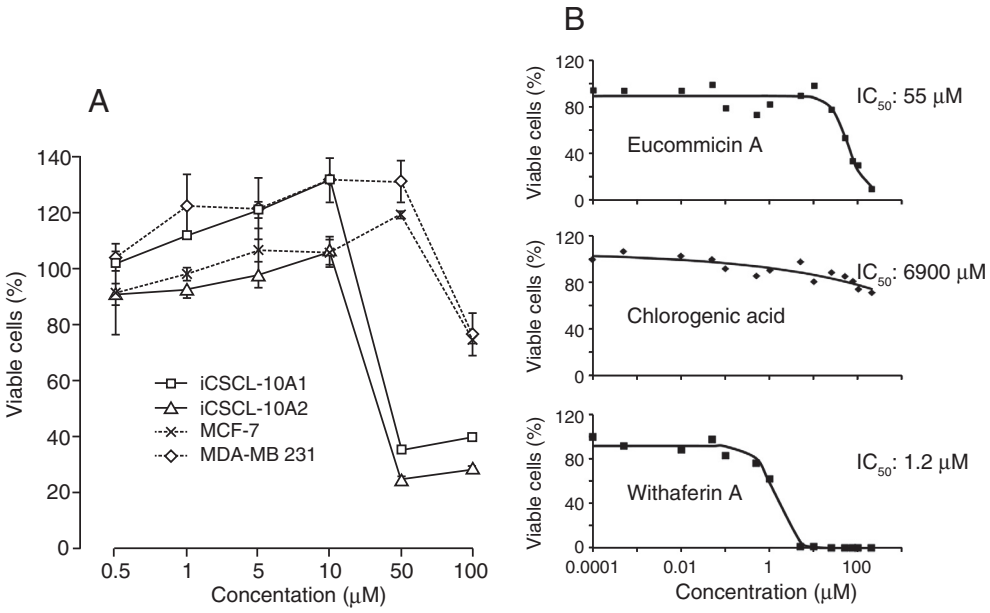

Fig. 5. Effects of eucommicin A (**1**) on cancer stem-like cells. (A) Two different clones of iCSCL-10A (iCSCL-10A-1 and iCSCL-10A-2) and two breast cancer cell lines, MCF7 and MDA-MB231, were plated in 96-well plates (5×10^3 cells/well in 100 μL). After 24 h, each test compound was added to the appropriate wells. Cell viability was measured after 48 h using WST-8 activity assays. (B). Quantification of tumor sphere formation. iCSCL-10A cells were plated on 96-well plates with an ultra-low-attachment surface in the presence of various concentrations of the indicated compounds. Tumor sphere formation was assessed after 48 h of exposure to the test compounds. IC_{50} values were determined by curve fitting with non-linear regression analysis (sigmoidal dose response).

ulmoides dry leaf powder used for isolation of eucommicin A is a commercial product known as *Hekizan* (product management number 14081455) produced by Hekizan-en Co., Ltd. (Kanagawa, Japan).

4.3. Isolation of eucommicin A (*1*) from dried powder of E. ulmoides leaves

Dried *E. ulmoides* leaf powder (126 g) was extracted with MeOH–H_2O (500 mL, 70:30, v/v), followed by extraction with hot H_2O (500 mL). The combined extract was concentrated under reduced pressure to produce an aqueous solution and extracted three times with a one-third volume of H_2O-saturated 1-BuOH. An aliquot of the remaining aqueous fraction equivalent to 18.5 g leaf powder was concentrated to dryness with diatomite filter agent (Celite 545, Junsei Chemical Co., Tokyo, Japan) and fractionated by hydrophilic interaction chromatography using DIOL MB100-75/200 (500 g, Fuji Silysia Chemical Ltd., Aichi, Japan) equilibrated with CH_3CN–H_2O (90:10, v/v). The column was eluted with CH_3CN–H_2O (2500 mL, 90:10, v/v) and CH_3CN–H_2O (2500 mL, 80:20, v/v). Eluate was collected every 625 mL, with each fraction doubled by repeating the fractionation with the same amount of the sample, equivalent to a total of 37 g of the leaf powder. The activities were detected in Fr. 3 (the third fraction eluted with CH_3CN–H_2O (90:10, v/v)), Fr. 6 (the second fraction eluted with CH_3CN–H_2O (80:20, v/v)), and Fr. 7 (the third fraction eluted with CH_3CN–H_2O (80:20, v/v)). Fractions 6 and 7 were combined and subjected to HPLC using an InertSustain C18 HPLC column (φ14 × 150 mm, 5 μm, GL Sciences) by employing a linear gradient elution from H_2O–AcOH (100:0.1, v/v) to MeOH within 20 min at 3 mL min^{-1}. The eluate was collected every 1 min. The active fraction eluted from 4 min to 5 min was fractionated again using the same column and eluted with CH_3CN–H_2O–AcOH (10:90:0.1, v/v) at 3 mL min^{-1}. The elution was monitored at 254 nm. The active fraction containing a peak eluted around 15.5 min was further purified with an Inertsil ODS-EP column (φ6 × 250 mm, 5 μm, GL Sciences) and eluted with CH_3CN–H_2O–AcOH (10:90:0.1, v/v) at 1 mL min^{-1}. Eucommicin A (**1**) was eluted at 13.3 min as a single peak (36.2 mg).

Eucommicin A (**1**) was obtained as a colorless amorphous solid. ESI-MS *m/z* (rel. int.): 191 (88), 243 (21), 353 (13), 463 (2.5), 707 ($[M-H]^-$, 100); FAB-HRMS (negative) *m/z* ($[M-H]^-$): calculated for $C_{32}H_{35}O_{18}$, 707.1824; found, 707.1828. For ^{1}H-NMR, ^{13}C-NMR, COSY, and HMBC (CD_3OD) spectroscopic data, see Table 1; [α] 20*D* −30.2 (*c* 0.136, MeOH).

4.4. Isolation of eucommicin A (*1*) from fresh leaves of E. ulmoides

Freshly harvested *E. ulmoides* leaves (40.3 g) were extracted and fractionated to isolate eucommicin A (**1**) following the purification steps that were used for powdered leaves. After partitioning of the extract with H_2O and 1-BuOH, the aqueous fraction was applied to DIOL MB100-75/200 (500 g) and eluted as described above. After confirmation that Fr. 6 showed inhibitory activity against iCSCLs and that the eucommicin A (**1**) included in the fraction could be detected by LC–MS/MS (mentioned below), eucommicin A (1.1 mg) was isolated by three successive HPLC purifications (twice with InertSustain C18 and once with Inertsil ODS EP) monitored by LC–MS/MS.

4.5. Quinic acid

Hydrolysis was performed by incubating eucommicin A (**1**) (2.7 mg) in 1 M NaOH (100 μL) at ambient temperature for 40 min. After acidification with 1 M HCl, the reaction mixture was concentrated to dryness, fractionated on an Inertsil ODS-EP column (φ6 × 250 mm, 5 μm), and eluted with 0.1% (v/v) AcOH at 1.0 mL min^{-1}. Quinic acid was recovered as a peak at 5.1 min. [α]20*D* −43.3 (*c* 0.02, H_2O).

4.6. Acetylation of eucommicin A (*1*)

Eucommicin A (**1**) (3 mg) was acetylated by incubation in a mixture of pyridine (200 μL) and Ac_2O (100 μL) for 2 days. The

reaction mixture was concentrated *in vacuo*, subjected to HPLC purification using an Inertsil ODS-EP column ($\varphi 6 \times 250$ mm, 5 μm, GL Sciences), and eluted by a linear gradient from H_2O–AcOH (100:0.1, v/v) to CH_3CN within 20 min, followed by elution in CH_3CN at 1 mL min^{-1}. Peracetylated eucommicin A (**2**) was recovered as a single peak at 20.0 min. ^{1}H-NMR (CD_3OD, 400 MHz) δ_H 2.0–2.1 ($4 \times 3H$, overlapped, s, 4_q-OAc, $4'_q$-OAc, 5_q-OAc, $5'_q$-OAc), 2.2 ($2 \times 3H$, 2s, 1_q-OAc, $1_q'$-OAc), 2.2 ($4 \times 3H$, overlapped, s, 3-OAc, 3′-OAc, 4-OAc, 4′-OAc), 2.03, 2.06 ($2 \times 1H$, m, H-$6_q\alpha$, H-$6_q'\alpha$), 2.42, 2.48 ($2 \times 1H$, dd, $^2J = 15.9$, $^2J_{2q}\alpha_{,3q} = 3.5$, H-2qα, H-$2'_q\alpha$), 2.60 ($2 \times 1H$, m, H-$6_q\beta$, H-$6'_q\beta$), 2.70 ($2 \times 1H$, m, H-$2_q\beta$, H-$2'_q\beta$), 3.87 (1H, ddd, $^3J_{8,8'} = 10$, $^3J_{7,8}$ (or $^3J_{7',8'}$) = 6.3, $^4J_{7',8}$ (or $^4J_{7,8'}$) = 1.0, H-8 or H-8′), 3.92 (1H, ddd, $^3J_{8,8'} = 10$, $^3J_{7,8}$ (or $^3J_{7',8'}$) = 7.6, $^4J_{7',8}$ (or $^4J_{7,8'}$) = 1.0, H-8 or H-8′), 4.27 (1H, ddd, $^3J_{7,7'} = 10$, $^3J_{7,8}$ (or $^3J_{7',8'}$) = 6.3, $^4J_{7',8}$ (or $^4J_{7,8'}$) = 1.0, H-8 or H-8′), 4.31 (1H, ddd, $^3J_{8,8'} = 10$, $^3J_{7,8}$ (or $^3J_{7',8'}$) = 7.6, $^4J_{7',8}$ (or $^4J_{7,8'}$) = 1.0, H-8 or H-8′), 5.08, 5.09 ($2 \times 1H$, dd, 9.9, 4.3, H-4_q, H-$4'_q$), 5.51, 5.56 ($2 \times 1H$, m, H-3_q, H-$3'_q$), 5.54 (2H, m, H-5_q, H-$5'_q$), 8.86, 8.88 ($2 \times 1H$, dd, 8.4, 2.0), 6.91, 6.95 ($2 \times 1H$, d, 2.0, H-2, H-2′), 6.97, 6.98 ($2 \times 1H$, d, 8.4, H-5, H-5′); ^{13}C-NMR (CD_3OD, 400 MHz) δ_C 20.4–20.5 (3-O(CO)$\underline{C}H_3$, 3′-O(CO)$\underline{C}H_3$, 4-O(CO)$\underline{C}H_3$, 4′-O(CO)$\underline{C}H_3$), 20.9–21.1 (4_q-O(CO)$\underline{C}H_3$, $4'_q$-O(CO)$\underline{C}H_3$, 5-O(CO)$\underline{C}H_3$, 5′q-O(CO)$\underline{C}H_3$), 21.4–21.5 (1_q-O(CO)$\underline{C}H_3$, $1'_q$-O(CO)$\underline{C}H_3$), 32.8, 33.0 (C-2_q, C-$2'_q$), 37.9, 38.0 (C-6_q, C-$6'_q$), 44.2, 44.3 (C-8, C-8′), 46.0, 46.1 (C-7, C-7′), 68.7, 69.0 (C-5_q, C-$5'_q$), 73.1, 73.5 (C-4_q, C-$4'_q$), 80.8, 80.9 (C-1_q, C-$1'_q$), 124 (overlapped, C-2, C-2′, C-5, C-5′), 127.2, 127.4 (C-6, C-6′), 138.3, 138.4 (C-1, C-1′), 142.3 (overlapped, C-3, C-3′), 143.3, 143.4 (C-4, C-4′), 169.8–169.9 (overlapped, 3-O($\underline{C}$O)CH_3, 3′-O($\underline{C}$O)CH_3, 4-O($\underline{C}$O)CH_3, 4′-O($\underline{C}$O)CH_3), 171.6, 171.7 (1_q-O($\underline{C}$O)CH_3, $1'_q$-O($\underline{C}$O)CH_3), 171.8–171.9 (overlapped, 4_q-O($\underline{C}$O)CH_3, $4'_q$-O($\underline{C}$O)CH_3, 5-O($\underline{C}$O)CH_3, $5'_q$-O($\underline{C}$O)CH_3), 172.6, 172.9 (C-9, C-9′), 173.8, 173.9 (C-7_q, C-$7'_q$).

4.7. Compound **2** (3,3′,4,4′-tetramethoxy-β-truxinic acid dimethyl ester)

Dried eucommicin A (**1**) (1.2 mg) was dissolved in MeOH 100 μL, added to excess ethereal diazomethane solution, and incubated for 1 h. After concentration to dryness, the methylated eucommicin A was treated with 0.1 M NaOMe in MeOH at ambient temperature for 1 h. The reaction mixture was acidified with 1 M AcOH and partitioned between H_2O and EtOAc. The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*, giving compound **2** as a white solid. EI-MS *m/z* (rel. int.): 144 (1.9), 222 (100, $[M/2]^+$), 300 (2.4), 351 (0.47, $[M-3 \times OMe]^+$), 382 (0.53, $[M-2 \times OMe]^+$), 413 (1.2, $[M-OMe]^+$), 444 (0.35, M^+). ^{1}H-NMR (CD_3OD, 400 MHz) δ_H 3.60 (6H, s, CH_3-12, CH_3-12′), 3.75 (12H, s, CH_3-10, CH_3-10′, CH_3-11, CH_3-11′), 3.91 (2H, detected as a doublet-like multiplet, ddd, $^3J_{8,8} = 11$, $^3J_{7,8} = 7$, $^4J_{7',8} = 1.0$, H-8, H-8′), 4.24 (2H, detected as a doublet-like multiplet, ddd, $^3J_{7,7} = 11$, $^3J_{7,8} = 7$, $^4J_{7,8'} = 1.0$, H-7, H-7′), 6.45 (2H, d, $^4J_{2,6} = 2.0$, H-2, H-2′), 6.67 (2H, dd, $^3J_{5,6} = 8.2$, $^4J_{2,6} = 2.0$, H-6, H-6′), 6.77 (2H, d, $^3J_{5,6} = 8.2$, H-5, H-5′); ^{1}H-NMR (acetone-d_6, 400 MHz) δ_H 3.63 (6H, s, CH_3-12, CH_3-12′), 3.70 (6H, s, CH_3-10, CH_3-10′), 3.70 (6H, s, CH_3-11, CH_3-11′), 3.92 (2H, detected as a doublet-like multiplet, ddd, $^3J_{8,8'} = 11$, $^3J_{7,8} = {}^3J_{7',8'} = 7.0$, $^4J_{7',8} = {}^4J_{7,8'} = 1.0$, H-8, H-8′), 4.25 (2H, detected as a doublet-like multiplet, ddd, $^3J_{7,7} = 11$, $^3J_{7,8} = {}^3J_{7',8'} = 7$, $^4J_{7,8'} = {}^4J_{7',8} = 1.0$, H-7, H-7′), 6.60 (2H, d, $^4J_{2,6} = 2.0$, H-2, H-2′), 6.69 (2H, dd, $^3J_{5,6} = 8.2$, $^4J_{2,6} = 2.0$, H-6, H-6′), 6.74 (2H, d, $^3J_{5,6} = 8.2$, H-5, H-5′); ^{13}C-NMR (CD_3OD, 400 MHz) δ_C 43.9 (C-8, C-8′), 45.6 (C-7, C-7′), 52.0 (C-12, C-12′), 56.0 (C-10, C-10′), 56.0 (C-11, C-11′), 112.2 (C-5, C-5′), 113.4 (C-2, C-2′), 120.9 (C-6, C-6′), 132.7 (C-1, C-1′), 148.9 (C-3, C-3′), 149.9 (C-4, C-4′), 173.6 (C-9, C-9′ [α]20*D* 0 (*c* 0.05, MeOH).

4.8. Assessment of anti-iCSC-10A activity

iCSCL-10A cells were generated and maintained as described previously (Nishi et al., 2014a). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell proliferation and cytotoxicity were evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). For the tumor sphere formation assay, cells were seeded in 96-well ultra-low-attachment surface plates (Corning) at a density of 5×10^3 cells/well and cultured in serum-free DMEM-Ham's F12 nutrient mixture (1:1, v/v) supplemented with 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 2% B27, and epidermal growth factor (20 ng/mL). Cell viability was evaluated using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. IC_{50} values were determined by fitting the data to a sigmoidal dose–response curve using ImageJ software (NIH, Bethesda, MD, USA).

4.9. LC–MS/MS detection of eucommicin A (**1**)

Eucommicin A (**1**) extracted from freshly-harvested leaves of *E. ulmoides* was detected by LC–MS/MS after each purification step. Each sample was separated using a Capcell Pak C_{18} MGIII column (2.0 mm i.d. × 250 mm, Shiseido, Tokyo, Japan) and eluted by varying the relative concentrations of solvents A (H_2O–AcOH (100:0.1, v/v)) and B (MeOH–AcOH (100:0.1, v/v)) in a linear gradient from 0% B to 50% B over a 10-min period, followed by a linear gradient from 50% B to 100% B over an 8-min period. The flow rate was 0.2 mL min^{-1}. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The ion source (Turbo V ion source) was operated in the negative ESI mode. The source parameters were set as follows: curtain gas, 40 psi; temperature, 500 °C; spray gas (GS1), 50 psi; dry gas (GS2), 80 psi; and ion spray voltage, −4500 V. The *m/z* 707 → 191 transition was monitored (collision energy, −56 V; collision cell exit potential, −2.0 V; declustering potential, −110 V). Both quadrupoles were set at unit resolution.

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