

# Comparison of the Biological Activity and Constituents in Japanese Ambers

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**How to cite this paper:** Shimizu, E., Shimoda, N., Kawamura, T., Ueda, N. and Kimura, K. (2020) Comparison of the Biological Activity and Constituents in Japanese Ambers. *Advances in Biological Chemistry*, 10, 99-112.

<https://doi.org/10.4236/abc.2020.103008>

**Received:** May 27, 2020

**Accepted:** June 27, 2020

**Published:** June 30, 2020

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## Abstract

**Background/Aim:** Kuji amber is an interesting natural source for drug discovery because a new anti-allergic compound, named kujigamberol and several new compounds have been isolated from it. It was important to evaluate the yield, biological activities and constituents of each methanol extract of Kuji, Iwaki, Choshi, Mizunami and Ube ambers in Japan in order to establish if additional new compounds could be identified in these ambers. **Materials and Method:** Biological activities of each extract were evaluated using growth-restoring activity of the mutant yeast strain involving Ca<sup>2+</sup>-signal transduction and inhibition activity of degranulation in rat basophilic leukemia (RBL)-2H3 cells. Constituents of each extract were analyzed by high performance liquid chromatography (HPLC). **Results:** All ambers except Ube amber have growth-restoring activity against the mutant yeast. Both Kuji and Iwaki ambers inhibited the degranulation of RBL-2H3 cells induced by the calcium ionophore A23187 in a dose dependent manner. The main biologically active compound in Kuji amber, kujigamberol, was also isolated from Iwaki amber and analyzed by mass spectrometry (MS) and nuclear magnetic resonance (NMR). **Conclusion:** Kuji and Iwaki ambers appeared to have the same origin. Choshi, Mizunami, and Ube ambers are valuable sources for biologically active compounds which are different from those of Kuji amber.

## Keywords

Japanese Ambers, Kujigamberol, Growth Restoration of a Mutant Yeast, Inhibition of Degranulation, RBL-2H3 Cells

## 1. Introduction

Amber is polymerized and fossilized tree resin which is found worldwide and is

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important as a source of new biologically active compounds [1] [2]. Kuji amber from Japan [90 - 86 million years ago (Ma) (Prof. Hisao Ando, Ibaraki University, personal communication): Late Cretaceous] is a particularly unique amber, and we have isolated and identified five kinds of new biologically active compounds and one unusual known compound from it using the mutant yeast involving  $\text{Ca}^{2+}$ -signal transduction (*zds1Δ erg3Δ pdr1/3Δ*) [1]-[8]. Biologically active compounds from Baltic (56 - 34 Ma, Poland and Russia) [1], Dominican (45 - 30 Ma and 20 - 15 Ma, Dominican Republic) [9] and Burmese (99 Ma, Burmese) [10] ambers have been isolated and identified. Biomarkers for Cretaceous ambers, amberene and 1-methylamberene without biological activities were also isolated and identified in Kuji amber and they were also detected in Burmese amber [11].

A main new compound from Kuji amber, named kujigamberol, has potent anti-allergic activity against a rhinitis model through the inhibition of  $\text{Ca}^{2+}$ -influx and leukotriene C4 production in rat basophilic leukemia (RBL)-2H3 cells [12]. Thus, ambers from Japan such as Kuji amber are not only fascinating natural sources for the study of organic geochemistry, but also for drug screening. A question that arises is why we can isolate new compounds from Kuji amber but isolate only known compounds from Baltic, Dominican and Burmese ambers. As the environment of the earth on the Cretaceous-Paleogene (K-Pg) (formerly Cretaceous-Tertiary, K-T) boundary (65 Ma) changed to destroy most of plants and dinosaurs, the environment for the formation of Kuji amber that is older than the K-Pg boundary may be crucial for isolating new compounds. In contrast, both Baltic and Dominican ambers are younger than the K-Pg boundary. Additionally, no new compound, such as kujigamberol, has been isolated and detected from Burmese amber that is older than Kuji amber [10]. Recently it was reported that kujigamberol was not detected in the Upper Albian-Lower Albian amber deposit of the Peñacerrada II (Basque-Cantabrian Basin, Spain) [13]. Is the environment of the earth around Japan different from other regions? Thus, it is important to examine the biological activity and their biologically active constituents in other Japanese ambers such as Iwaki (Late Cretaceous), Choshi (Early Cretaceous, 110 Ma), Mizunami (Pleistocene Quaternary, 16 - 15 Ma) and Ube ambers (Cenozoic Paleogene Oligocene, 30 Ma) as compared to Kuji amber [Figure S1(a)].

In this study, we examined the biological activities and their constituents of Iwaki, Choshi, Mizunami and Ube ambers and compared them to those from Kuji amber.

## 2. Materials and Methods

**General experimental procedure:** Kuji amber was excavated from mines of Kuji Kohaku Co. Ltd. located in the upper part of the Tamagawa Formation of Kuji Group of Kuji city, Iwate Prefecture. The other Japanese ambers were excavated in small quantity from each area (Figure S1(a)). The yeast strain is a de-

rivative of strain W303-1A and is YNS17 (*MATa zds1::TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) [3] [4]. Difco® YPD broth and YPD agar were obtained from Becton Dickinson (Franklin Lakes, NJ, U.S.A.). FK506 (tacrolimus) was kindly provided by the Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc., Tokyo, Japan). Unless otherwise stated, chemicals used were of the best commercially available grade.

**Biological activities of each ambers against the mutant yeast:** All ambers were ground to a powder and extracted with methanol (MeOH) for three days at 23 °C. After evaporation of the MeOH, each dried extract was weighed. Extracted samples of 10 mg/ml in MeOH were prepared for subsequent assays. Screening was carried out using the YNS17 strain and 5 µl of each sample was added to a plate as described previously [4]. The inhibitory activity of the Ca<sup>2+</sup>-signal transduction was determined by way of the strength and/or distinction of the yeast growth zone. An immunosuppressive drug, FK506 (2.5 ng/spot) was used as a positive control.

**Biological activities of each ambers against the degranulation of RBL-2H3 cells:** RBL-2H3 cells (ATCC CRL-2256, Manassas, VA, USA) were maintained in DMEM supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc., Logan, UT, USA) and antibiotics [penicillin (50 units/mL)-streptomycin (50 µg/mL), Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA]. Cell viability was determined using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Dojindo Lab., Kumamoto, Japan). RBL-2H3 cells were plated in triplicate at a concentration of  $3 \times 10^4$  cells/well in a 96-well plate and incubated overnight prior to treatment with various concentrations of each MeOH extract for 48 h. Cytotoxicity was measured as described previously. RBL-2H3 cells were grown overnight in 96-well plates ( $3 \times 10^4$  cells/well) and the degranulation activity of each MeOH extract against them stimulated with the calcium ionophore A23187, was determined as described previously [12].

**Isolation and identification of kujigamberol from Iwaki amber:** Powdered Iwaki amber (18.22 g) was extracted with MeOH for 3 days at 23 °C and the extract [0.80 g (4.37%)] was diluted with water, followed by two extractions with one volume of ethyl acetate (EtOAc). After evaporation of the EtOAc, the organic layer [0.56 g (3.06%)] was subjected to silica gel TLC (hexane:EtOAc = 3:1 as solvent). Active fractions ( $R_f = 0.45$ ) were collected and concentrated under reduced pressure to afford crude material [49.3 mg (0.27%)] which was further purified using high performance liquid chromatography (HPLC) [mobile phase MeOH:H<sub>2</sub>O = 85:15, flow rate 1 ml/min, Capcell Pak C<sub>18</sub> column (4.6 mm i.d. × 150 mm; Shiseido, Tokyo, Japan)]. Pure compound **1** [1.0 mg (0.054%)] was obtained as a colorless oil [1].

Identification of **1** was performed using high resolution electron impact mass spectrometry (HREIMS) (JEOL JMS700) and nuclear magnetic resonance (NMR) (JEOL AL-400), including <sup>13</sup>C NMR and <sup>1</sup>H NMR. Ultra violet (UV)

spectra in MeOH were measured on a UV mini 22 spectrophotometer (Shimadzu Co. Ltd., Kyoto, Japan). Compound **1** was compared with those of authentic kujigamberol [**1**] (**Table S1**).

Compound **1**: Colorless oil. HREIMS ( $m/z$ ): 260.2138 [M]<sup>+</sup> (Calcd. for C<sub>18</sub>H<sub>28</sub>O: 260.2140); <sup>1</sup>H-NMR and <sup>13</sup>C NMR (**Table S1**).

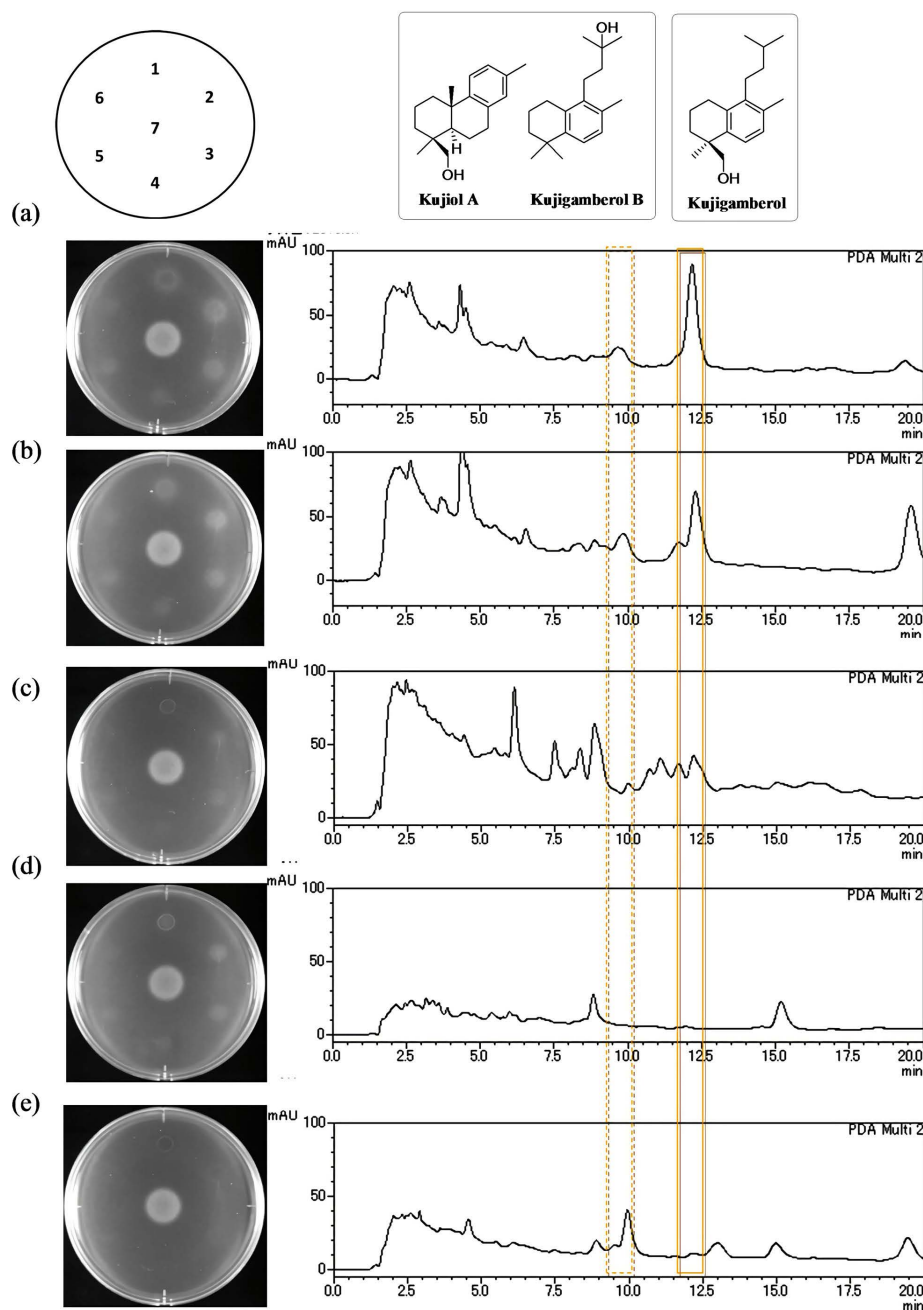
**HPLC analysis of kujigamberol, amberene and 1-methylamberene:** HPLC analysis was performed using a mobile phase [MeOH:H<sub>2</sub>O (80:20) for kujigamberol and (90:10) for amberene and 1-methylamberene] at a flow rate of 1 ml/min, with a Capcell Pak C<sub>18</sub> column [4.6 mm i.d. × 150 mm (Shiseido)], pump (PU-2080) and a photodiode array detector (MD-2018, JASCO Co., Tokyo, Japan).

### 3. Results and Discussion

**The weight of the MeOH extract of each amber:** The yield of the MeOH extract of Kuji, Iwaki, Choshi, Mizunami and Ube was 3.4%, 4.4%, 1.1%, 26.7% and 33.8%, respectively. The yield of the MeOH extract from their domestic ambers is roughly proportional to each reported age [**Figure S1(b)**]. The MeOH extractable materials indicate non-polymerized (non-mature) amber.

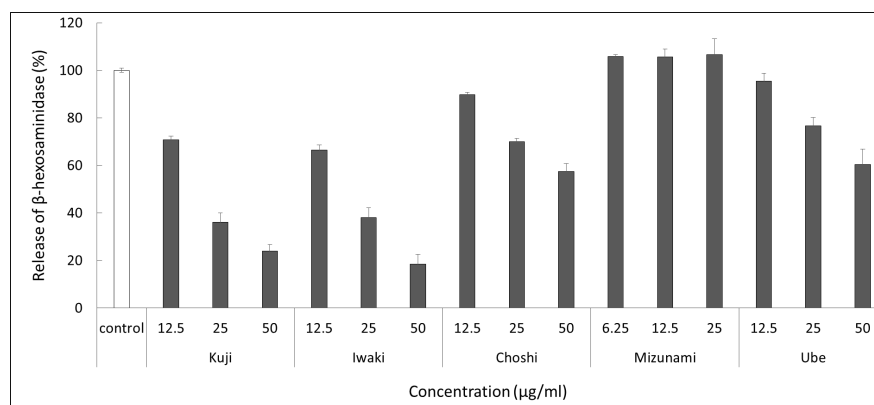
**Biological activity and its constituents:** Comparison of the main constituents with biological activity (kujigamberol) and/or without biological activity (amberene and 1-methylamberene) in ambers by HPLC analysis indicated that Iwaki amber is similar to Kuji amber, but the growth-restoring activity and the HPLC analytical patterns of Choshi, Mizunami and Ube ambers were different from those of Kuji amber (**Figure 1**). The analytical HPLC pattern of three kinds of new biologically active compounds in Kuji amber (kujigamberol [**1**], kujiol A and kujigamberol B [**5**]) was almost the same as those of Iwaki amber. Although the MeOH extract of Choshi and Mizunami ambers had biological activity, those two peaks including three compounds were not detected [**Figure 1(c)** and **Figure 1(d)**]. These results suggest that biologically active compounds in Choshi and Mizunami ambers are different from those of Kuji amber and it is therefore worth isolating and identifying them further. However, there was no growth-restoring activity in the MeOH extract of Ube amber [**Figure 1(e)**].

The MeOH extract of Kuji amber (MEKA) inhibited the degranulation of RBL-2H3 cells by stimulation of thapsigargin (IC<sub>50</sub> = 15.0 µg/ml) and A23187 (IC<sub>50</sub> = 19.6 µg/ml) without cytotoxicity, but not by stimulation of immunoglobulin E (IgE) + 2,4-dinitrophenol (DNP)-bovine serum albumin (BSA) (IC<sub>50</sub> > 50.0 µg/ml) [**12**]. Thus, the degranulation activity stimulated by A23187 was measured in each MeOH extract. Both MeOH extracts of Kuji and Iwaki amber showed almost the same inhibition activity against the degranulation (**Figure 2**). However, both the MeOH extract of Choshi and Ube ambers showed less activity than those of Kuji and Iwaki amber (**Figure 2**). Ube amber had inhibition activity against the degranulation, although it had no growth-restoring activity



**Figure 1.** Growth-restoring activity against the mutant yeast and HPLC analytical pattern of each Japanese amber [(a) Kuji amber, (b) Iwaki amber, (c) Choshi amber, (d) Mizunami amber, (e) Ube amber]. The growth-restoring activities of each MeOH extract against YNS17 strain (*zds1Δ erg3Δ pdr1/3Δ*) were measured in the presence of 0.3 M CaCl<sub>2</sub> [1: 5 μg/spot, 2: 2.5 μg/spot, 3: 1.25 μg/spot, 4: 0.625 μg/spot, 5: 0.31 μg/spot, 6: 0.16 μg/spot, 7: 2.5 ng/spot (FK506)]. HPLC analysis utilized a Capcell pak C<sub>18</sub> (4.6 × 150 mm) column with 80% MeOH, flow rate at 1 ml/min, 100 μg of each amber extract and a photodiode array (PDA) (205 nm). The solid line box shows a peak of kujigamberol and the dotted line box shows a peak of both kujiol A and kujigamberol B.

against YNS17 strain (Figure 1 and Figure 2). Although the MeOH extract of Mizunami amber showed growth-restoring activity against YNS17 strain, it had



**Figure 2.** Degranulation activity of each MeOH extract against RBL-2H3 cells. RBL-2H3 cells were sensitized with 10 µL A23187 (20 µM) for 30 min. Inhibition (%) of β-hexosaminidase release by the sample was measured as absorbance at 405 nm.

no inhibition activity against the degranulation (**Figure 2**).

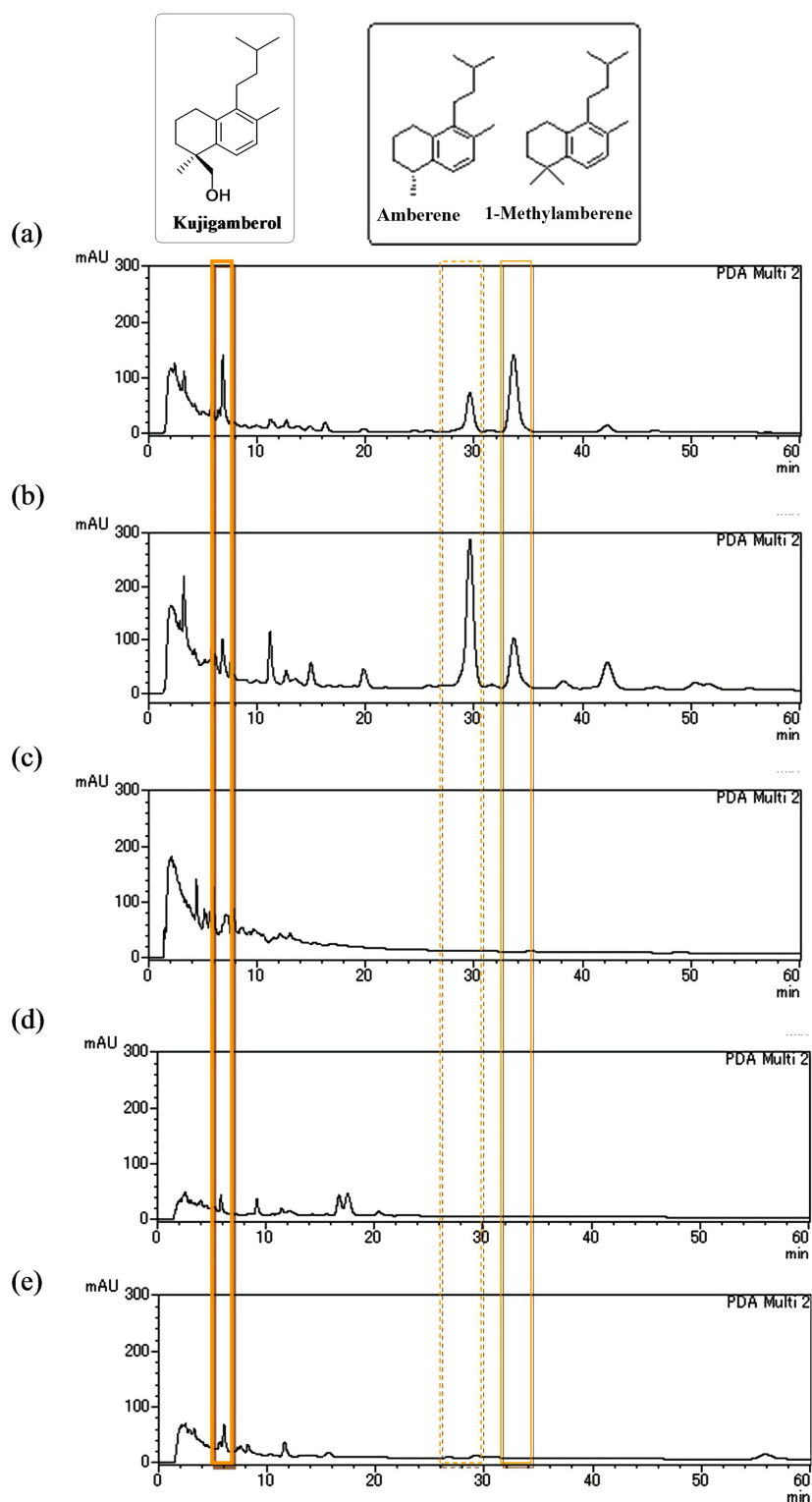
**Biologically active compounds and biomarkers in each amber:** The peaks of kujigamberol, Kujiol A and kujigamberol B in MEKA were detected by HPLC of Iwaki amber. Both HPLC profiles showed a similar pattern. However, both HPLC traces of Choshi, Mizunami, and Ube ambers were different from those of Kuji and Iwaki ambers (**Figure 1**). These results suggested that Kuji amber and Iwaki amber are from the same origin. The biologically active compounds in Choshi, Mizunami and Ube ambers are different from each other and different from those of Kuji and Iwaki ambers.

Amberene is one of biomarkers of Cretaceous amber and is detected in Kuji and Burmese ambers which are Late Cretaceous ambers [10]. It was detected in Iwaki amber by HPLC, but not identified in other ambers (**Figure 3**). This result was identical with each reported age.

**Isolation and identification of kujigamberol from Iwaki amber:** The main biologically active compound **1** in Iwaki amber was isolated from the MeOH extract for the first time as a colorless oil. The retention time (**Figure S2**), the UV spectrum (**Figure S3**) and the chemical shifts (**Figures S4-S6**, **Table S1**) of compound **1** and authentic kujigamberol showed the same values. They also showed clear and identical growth-restoring zones on the plate in a dose-dependent manner (from 0.5 to 0.031 µg/spot) [**Figure S7(a)**]. Kujigamberol [**Figure S7(b)**] was also isolated and identified from Iwaki amber and this suggested that it is from the same origin as Kuji amber.

## 4. Conclusion

The yield of methanol soluble fraction decreased with the age of amber. All ambers except Ube amber have growth-restoring activity against the mutant yeast strain involving Ca<sup>2+</sup>-signal transduction. Both Kuji and Iwaki ambers inhibited the degranulation of RBL-2H3 cells induced by A23187 in a dose dependent manner. Kuji and Iwaki ambers appeared to have the same origin, because amberene



**Figure 3.** HPLC analytical pattern of amberene and 1-methylamberene in each MeOH extract [(a) Kuji amber, (b) Iwaki amber, (c) Choshi amber, (d) Mizunami amber, (e) Ube amber]. HPLC analysis utilized a Capcell pak  $C_{18}$  ( $4.6 \times 150$  mm) column with 80% MeOH, flow rate at 1 ml/min, 100  $\mu$ g of each amber extract and a photodiode array (PDA) (205 nm). The solid line box shows a peak of kujigamberol, the dotted line box shows a peak of amberene and a solid line box shows a peak of 1-methylamberene.

and 1-methylamberene were identified in both by HPLC. Additionally, the main biologically active compound in Kuji amber, kujigamberol, was also isolated from Iwaki amber and analyzed by MS and NMR. Choshi, Mizunami, and Ube ambers are therefore valuable sources for biologically active compounds which are different from those of Kuji amber.

## Acknowledgements

We are grateful to Ms. Shizuko Nakajo from the Center for Regional Collaboration in Research and Education of Iwate University for HREIMS; to Prof. Hisao Ando, Ibaraki University for personal communications about Kuji amber; and to Emeritus Professor Tokichi Miyakawa of Hiroshima University for providing the YNS17 strain. We would like to thank Emeritus Professor Don R Phillips, La Trobe University for English language editing. This work was partially supported by Sanriku Fund, Japan Science and Technology Agency (JST) and the New Energy and Industrial Technology Development Organization (NEDO).

## Conflicts of Interest

The authors declare no competing financial interest.

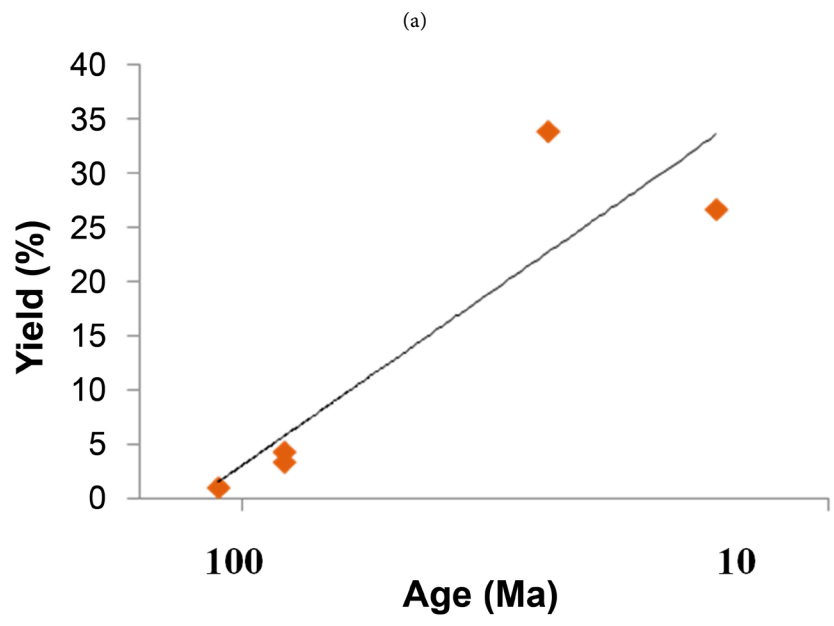
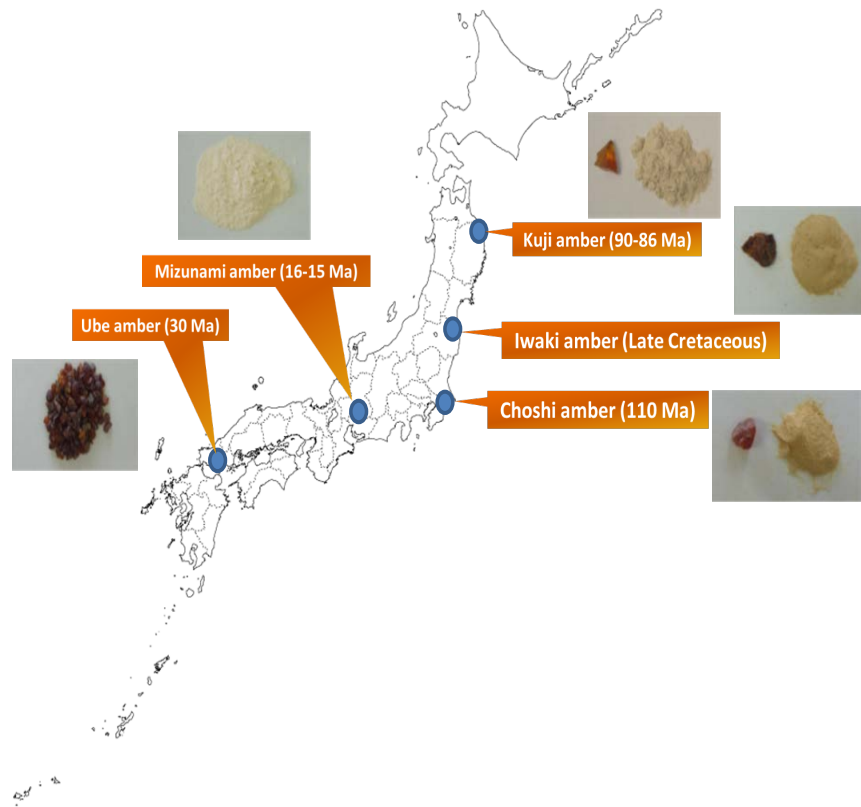
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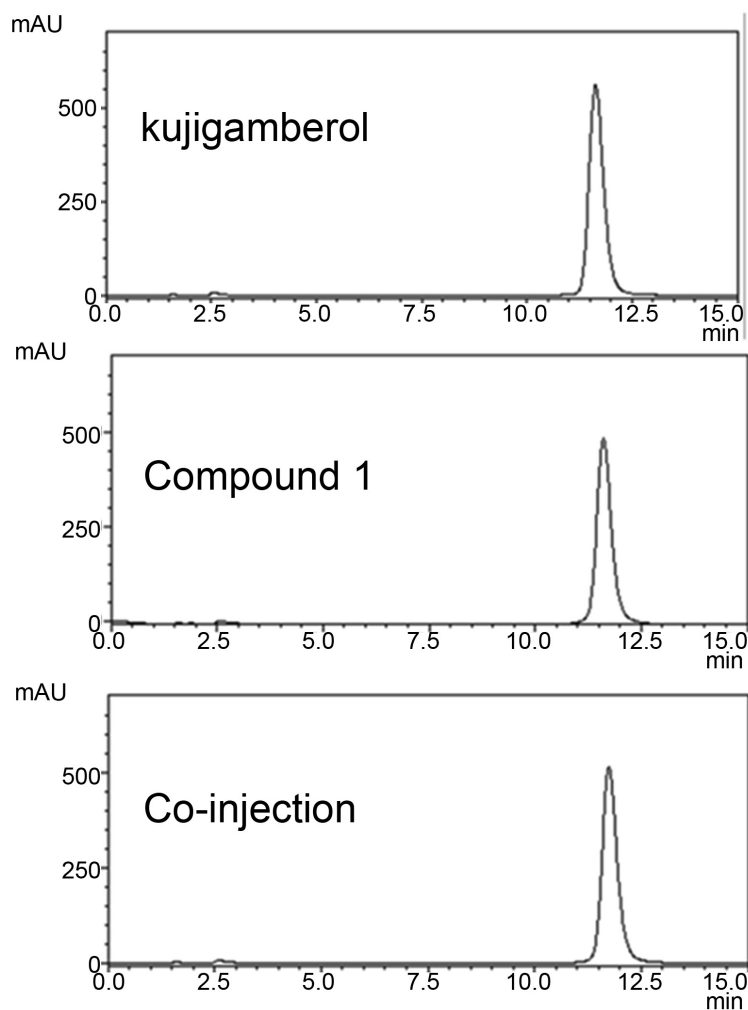


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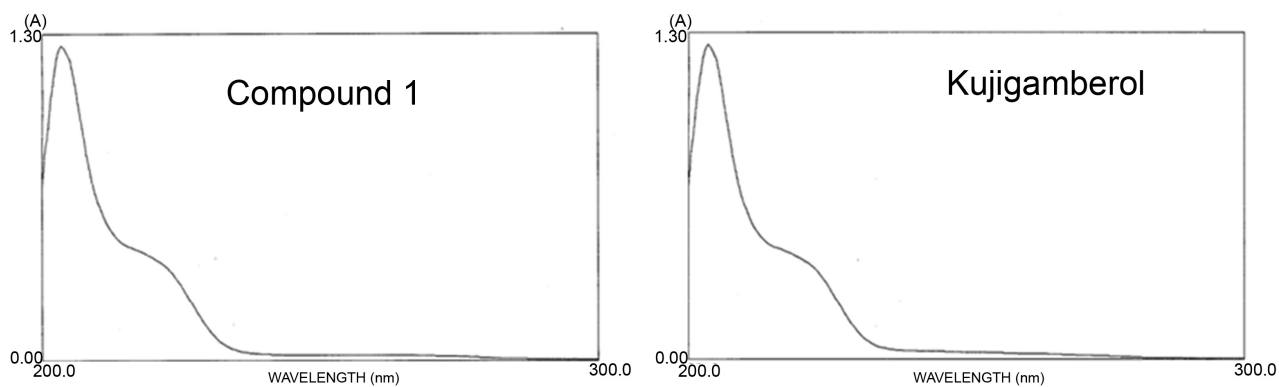
## Appendix



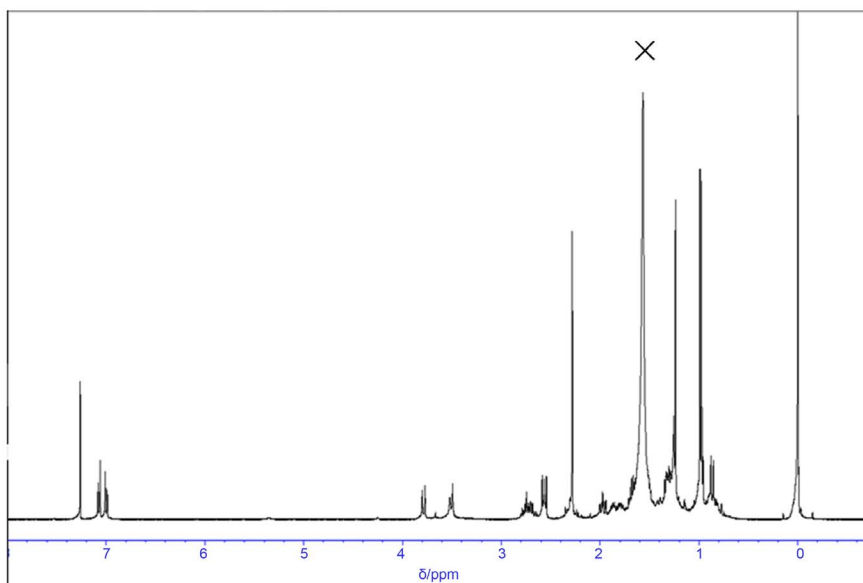
**Figure S1.** Location of Japanese ambers (a) and the relationship between the yield of MeOH extract and the age of the amber (b).



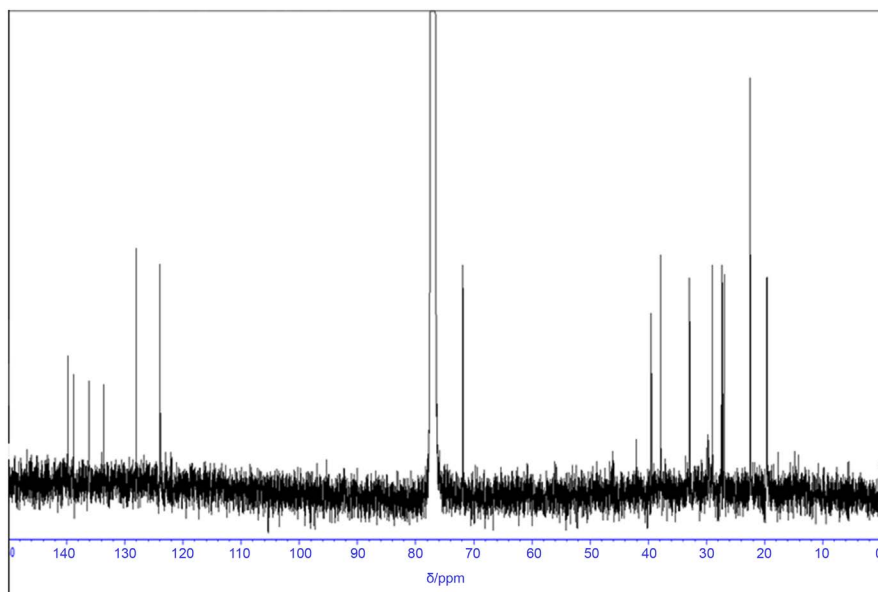
**Figure S2.** Co-injection with kujigamberol and compound 1 analyzed by HPLC. Column: Capcell Pak C18 (ODS 4.6 mm  $\times$  150 mm, TYPE UG 120 Å, 5  $\mu$ m), Flow rate: 1.0 ml/min, UV: PDA (205 nm), Injection: 2.5  $\mu$ g (co-injection: 1.25  $\mu$ g + 1.25  $\mu$ g).



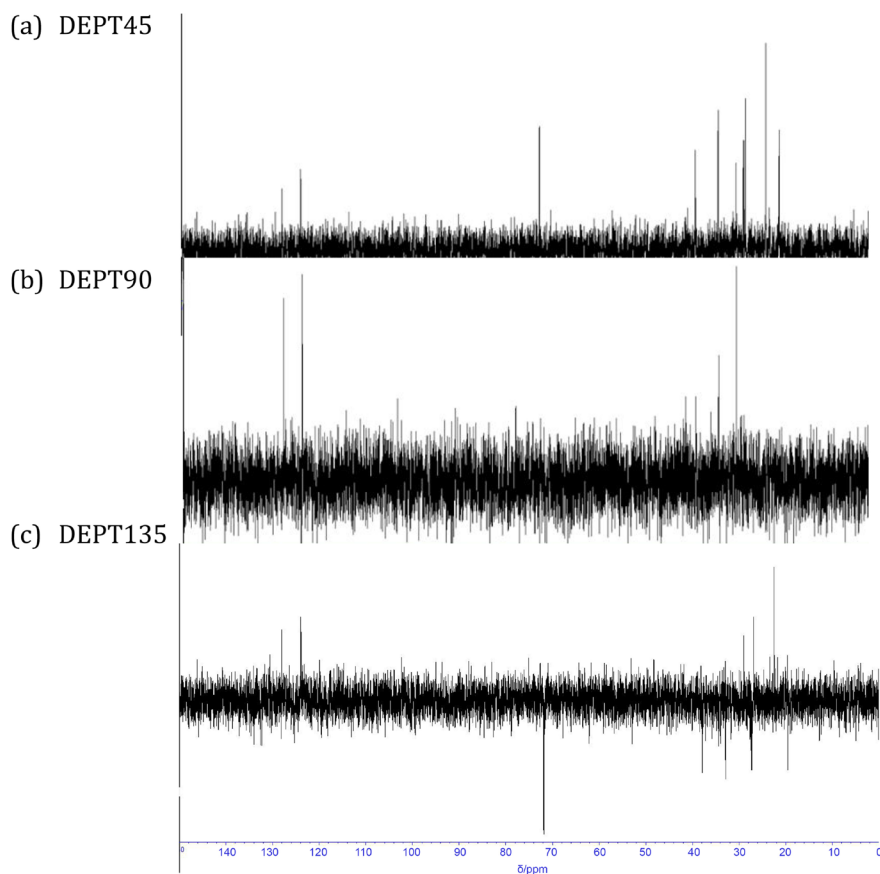
**Figure S3.** UV spectrum of compound 1 and kujigamberol (100  $\mu$ g/ml in MeOH).



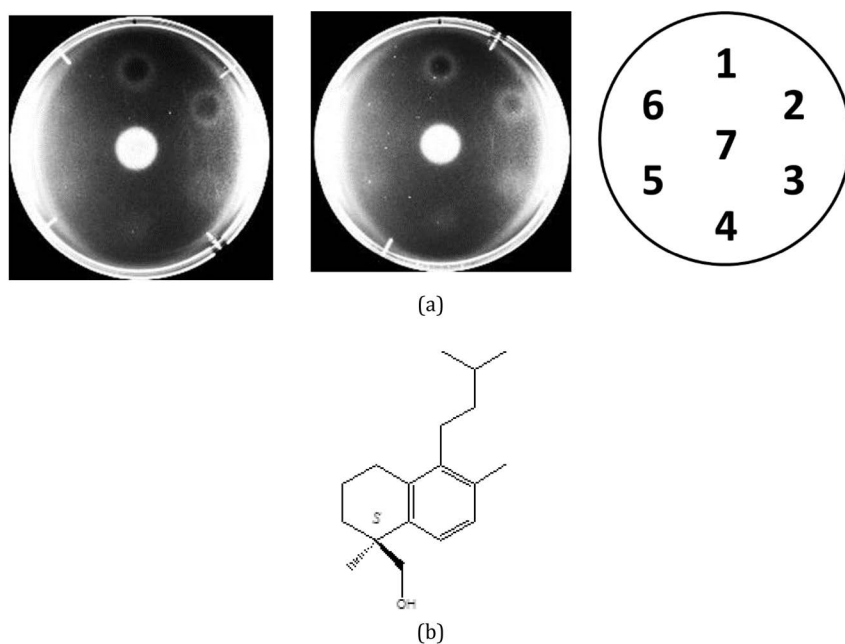
**Figure S4.**  $^1\text{H}$  NMR spectrum of compound **1** ( $\text{CDCl}_3$ , 400 MHz).



**Figure S5.**  $^{13}\text{C}$  NMR spectrum of compound **1** ( $\text{CDCl}_3$ , 100 MHz).



**Figure S6.** DEPT spectrum of compound **1** ( $\text{CDCl}_3$ ).



**Figure S7.** Growth-restoring activity and structure of **1**. (a) Growth-restoring activities of **1** and kujigamberol against YNS17 strain (*zds1Δ erg3Δ pdr1/3Δ*) in the presence of 0.3 M  $\text{CaCl}_2$ . 1: 0.5  $\mu\text{g/spot}$ , 2: 0.25  $\mu\text{g/spot}$ , 3: 0.125  $\mu\text{g/spot}$ , 4: 0.0625  $\mu\text{g/spot}$ , 5: 0.031  $\mu\text{g/spot}$ , 6: 0.016  $\mu\text{g/spot}$ , 7: 2.5 ng/spot (FK506). (b) The structure of **1** (kujigamberol).

**Table S1.** Comparison of the chemical shifts between kujigamberol and compound **1**.

	Kujigamberol <sup>1)</sup>			Compound <b>1</b>		
	$\delta_c$ (ppm)	$\delta_H$ (ppm, <i>J</i> in Hz)		$\delta_c$ (ppm)	$\delta_H$ (ppm, <i>J</i> in Hz)	
1	27.2	2.76	(H, ddd, 16.5, 5.9, 5.9)	27.2	2.72	(H, m)
		2.68	(H, ddd, 16.5, 8.2, 5.5)		2.70	(H, m)
2	19.5	1.88	(H, m)	19.5	1.90	(H, m)
		1.79	(H, m)		1.80	(H, m)
3	32.9	1.52	(H, m)	32.9	1.47	(H, m)
		1.97	(H, ddd, 13.0, 10.0, 3.1)		1.96	(H, m)
4	39.5			38.5		
5	138.8			138.8		
6	123.9	7.07	(H, d, 8.2)	123.9	7.07	(H, d, 7.8)
7	128.0	7.00	(H, br, d, 8.2)	128.0	7.00	(H, d, 7.8)
8	133.6			133.7		
9	139.8			139.8		
10	136.1			136.1		
11	27.4	2.57	(2H, m)	27.4	2.57	(2H, m))
12	37.9	1.32	(2H, m)	37.8	1.33	(2H, m)
13	29.0	1.68	(H, m)	29.0	1.66	(H, m)
14	22.5	0.98	(3H, d, 6.9)	22.5	0.98	(3H, d, 6.6)
15	22.5	0.98	(3H, d, 6.9)	22.5	0.98	(3H, d, 6.6)
16	19.6	2.28	(3H, s)	19.6	2.28	(3H, s)
17	26.9	1.24	(3H, s)	26.9	1.24	(3H, s)
18	71.9	3.78	(H, d, 11.0)	71.9	3.79	(H, d, 11.0)
		3.51	(H, d, 11.0)		3.51	(H, d, 11.0)