J Wood Sci (2013) 59:238–242
DOI 10.1007/s10086-013-1328-4

ORIGINAL ARTICLE

Growth inhibition activities of Sugi bark components against *Heterosigma akashiwo*

Hiromi Saijo · Kazuya Tsuruta · Norihisa Kusumoto · Tatsuya Ashitani · Koetsu Takahashi

Received: 21 November 2012 / Accepted: 25 January 2013 / Published online: 16 February 2013

Abstract In our ongoing efforts to develop new uses for wood-based waste streams, the growth inhibition activities of extracts obtained from Sugi (*Cryptomeria japonica*) bark were examined against *Heterosigma akashiwo*, otherwise known as red tide plankton. The Sugi bark was separated into its outer and inner barks and then extracted sequentially with hexane, ethyl acetate, and methanol. Strong inhibitory activities against *H. akashiwo* were observed in the tests involving the hexane extract from the inner and outer barks, as well as the ethyl acetate extract from the inner bark. Gas chromatography mass spectroscopy (GC–MS) analysis revealed that cubebol, phyllocladanol, 6,7-dehydroferruginol, ferruginol, and sugiol were the main components in the active extracts. These components themselves were then tested for their growth inhibition activities against *H. akashiwo*. Cubebol and ferruginol showed potent inhibitory activities, whereas phyllocladanol, 6,7-dehydroferruginol, and sugiol were only weakly active. Taken together, these results suggested that the Sugi bark extracts could be used as inhibition reagents against red tide plankton.

H. Saijo (✉) · T. Ashitani · K. Takahashi
The United Graduate School of Agricultural Science, Iwate University, 18-8 Ueda 3-chome, Morioka, Iwate 020-8550, Japan
e-mail: abe18136@tds1.tr.yamagata-u.ac.jp

K. Tsuruta · N. Kusumoto · T. Ashitani · K. Takahashi
Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka, Yamagata 997-8555, Japan

N. Kusumoto
Institute of Wood Technology, Akita Prefectural University, 11-1 Kaieisaka, Noshiro, Akita 016-0876, Japan

Keywords Sugi bark · Extract · Inhibition activity · Red tide plankton · *Heterosigma akashiwo*

Introduction

The bark of Sugi (*Cryptomeria japonica* D. Don) has been recognized as one of the significant wood-based waste materials produced by the Japanese wood industry [1]. The development of new methods for the use of Sugi bark is required to allow for Japanese forest resources to be used more effectively. Several methods have been studied for the preparation of synthetic plastic materials from wood-based wastes such as polyurethane [2–5] and phenolic resin [6] through the liquefaction (Solvolysis) of the Sugi bark. There have also been several reports revealing that there are many bioactive components contained within Sugi bark [7–9]. In an effective cascade for the utilization of natural resources, in would be desirable for these functional bioactive components should be utilized prior to the preparation of the plastic materials mentioned above.

We previously reported that the essential oil obtained from the Sugi bark showed growth inhibition activities against *Skeletonema costatum*, known as one of the "red tide" planktons [10]. Red tide represents a serious environmental problem in field of marine research, and is generally caused by *S. costatum* [11, 12], *Heterosigma akashiwo* [13, 14], and *Chattonella antiqua* [15]. *H. akashiwo* in particular causes very serious problems because of the ichthyotoxic compounds produced by this alga [13, 14]. In our previous report [10], although essential oils were used in anti-algal tests, the extractive components of Sugi bark were found to consist predominantly of diterpenoids and phenolic compounds which were difficult to obtain in large amounts from the oil by distillation.

Furthermore, the yield of this bark essential oil was less than that of the wood and leaf parts. In addition, some of the components were observed to change during the hot water distillation process [10]. Thus, in this study, to find new uses for Sugi bark, we examined the inhibitory activities of the Sugi bark extracts obtained by solvent extraction and the major components contained within the extracts against *H. akashiwo*.

Materials and methods

Plant materials

Sugi bark was obtained from the Yamagata Field Science Center (Faculty of Agriculture, Yamagata University, Japan). The bark was separated into the inner and outer bark, and cut into pieces of about 1 cm^2.

Heterosigma akashiwo (NIES-293) was obtained from the National Institute for Environmental Studies (NIES, Japan), and was maintained under 2000 lux (12:12 LD cycle) at 20 °C in an NKsystem BIOTRON (Nippon Medical & Chemical Instruments Co., LTD, Tokyo, Japan). The cells were grown in f/2 medium according to a previously reported procedure [10].

Analysis of extract and compounds

GC/MS data were collected on a SHIMADZU QP-5000 GC–MS (Shimadzu, Kyoto, Japan) under the following conditions: DB-1 capillary column (0.32 mm i.d. × 30 m; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); column temperature from 50 °C (1 min) to 320 °C (5 min) at 5 °C/min; injection temperature of 250 °C; detection temperature of 250 °C; acquisition mass range of 50–450 atomic mass units (amu) using helium as the carrier gas with a flow rate of 3.6 mL/min). The majority of the components were identified by comparison of the experimental GC–MS data with those of the authentic compounds [7, 10]. NMR spectra were measured on a JEOL JNM-EX400 (^{1}H 400 MHz/^{13}C 100 MHz) spectrometer (JEOL Ltd., Tokyo, Japan).

Extraction and isolation

The inner and outer barks were extracted at ambient temperature for 7 days by successive extractions with hexane, ethyl acetate, and methanol. Each solvent was removed by evaporation to yield the extracts and the yields of each extract were calculated from the dry weights. The hexane extract of the inner bark was purified by column chromatography using silica gel (60 N, spherical 63–210 μm, neutral; Kanto Chemical Co., Inc., Japan). The column was eluted with a gradient of hexane:ethyl acetate (100:0 to 10:90, v/v) to provide pure cubebol, phyllocladanol, 6,7-dehydroferruginol, ferruginol, and sugiol.

Phyllocladanol, 6,7-dehydroferruginol, ferruginol, and sugiol were identified by comparison of their GC retention times and MS data with standard samples isolated during the course of a previous study [7, 10]. Cubebol was identified by comparison of its NMR and MS data with those previously reported [16].

Cubebol

Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.52 (1H, dddd, J = 12.20, 12.20, 12.49, 1.96 Hz), 0.75–1.08 (4H, m), 0.92 (3H, d, J = 6.89 Hz), 0.94 (3H, d, J = 7.13), 0.97 (3H, d, J = 6.71 Hz), 1.18–1.46 (2H, m), 1.29 (3H, s), 1.45–1.72 (5H, m), 1.84 (1H, ddd, J = 10.27, 10.69, 3.12); ^{13}C-NMR ($CDCl_3$) δ: 18.8 (CH_3), 19.7 (CH_3), 20.1 (CH_3), 22.6 (CH), 26.5 (CH_2), 27.9 (CH_3), 29.6 (CH_2), 30.9 (CH), 31.7 (CH_2), 33.4 (C), 33.7 (CH), 36.4 (CH_2), 39.1 (C), 44.1 (C), 80.3 (C).

Growth inhibition activity

The growth inhibition activities for *H. akashiwo* were measured according to the methods previously published in the literature [10]. A portion (1 mg) of each sample was measured, and 1 mL of acetone was added to the samples not containing any of the inner bark methanol extract. For the inner bark methanol extract, dimethylsulfoxide (DMSO, 1 mL) was added, because the extract was completely insoluble in acetone. A sample of this solution (1 mL) was then added to f/2 medium (5 mL) to provide a solution with a final concentration of 1 μg/mL. In the control test, 5 μL of pure solvent (acetone or DMSO) was added to the medium. *H. akashiwo* was added to each vial. The volume was adjusted with culture medium to 5 mL/vial. The cell count in each vial was measured daily for 14 days. Cells were counted microscopically using counting chambers (Hirschmann Laborgerate, Eberstadt, Germany). The number of cells (N) in 576 masses in the counting chambers was determined, and the counted result was converted to cell density per 1 mL (the counted volume of the counting chambers was 0.0025 mm^2 × 0.10 mm). The cell density (D) per 1 mL was defined according to the following Eq. (1). Inhibition activities (IA) were calculated by Eq. (2). A higher IA value indicated stronger growth inhibition.

$$D = (N/576) \times 1000/(0.0025 \times 0.10) \quad (1)$$

$$IA = 100 \times (1 - Ds/Dc) \quad (2)$$

Ds is the cell density of each sample vial on the final day and Dc is the cell density of control vial on the final day.

Statistical analysis

The test samples were compared using one-way analysis of variance (ANOVA), and the means were separated using Tukey–Kramer honestly significant difference (HSD) comparisons ($p < 0.05$; JMP version 9.0.3, SAS Institute Inc., Cary, NC, USA).

Results and discussion

Extraction and effects of bark extracts

The yields of the hexane, ethyl acetate, and methanol extracts are shown in Table 1. The total yields from the solvent extraction were 8.54 and 4.77 % from the inner and outer barks, respectively. The essential oil of the whole Sugi bark was obtained in a 0.15 % (1.5 g/kg) yield in our previous report [10]. Thus, for the use of the extractive compounds in Sugi bark, solvent extraction was more suitable than hot water distillation to obtain large amounts of the components.

The *H. akashiwo* growth curves obtained for the testing of each of the Sugi bark extracts at a concentration of 1 μg/mL are shown in Fig. 1. There was no significant difference between the DMSO and acetone controls ($p < 0.05$) on the final day (following 14 days). The hexane extracts from the inner and outer barks and the ethyl acetate extract from the inner bark strongly inhibited the growth of *H. akashiwo*, and there was a clear reduction in the cell densities from an early stage in the test period (that is, the following 1–5 days). The inhibition activities of each extract on the final day have been summarized in Fig. 2. Significant inhibition activities were observed in the hexane extracts from both bark samples, as well as the ethyl acetate extracts from the inner bark. These extracts showed inhibition activities of approximately 100 %. In contrast, the ethyl acetate extract from the outer bark and the methanol extracts from both the bark samples did not show any significant levels of activity. These results suggested that the most active components of the Sugi bark were contained within the least polar extracts.

Table 1 Yield (%) of Sugi bark extracts by solvent extraction

Solvent	Yield (%)	
	Inner bark	Outer bark
Hexane	3.91	1.29
Ethyl acetate	1.24	2.17
Methanol	3.39	1.30
Total	8.54	4.77

% based on dry weight

Analysis of active extracts

The total ion chromatograms of the active extracts of the Sugi bark analyzed by GC–MS are shown in Fig. 3. Cubebol, phyllocladanol, 6,7-dehydroferruginol, ferruginol, and

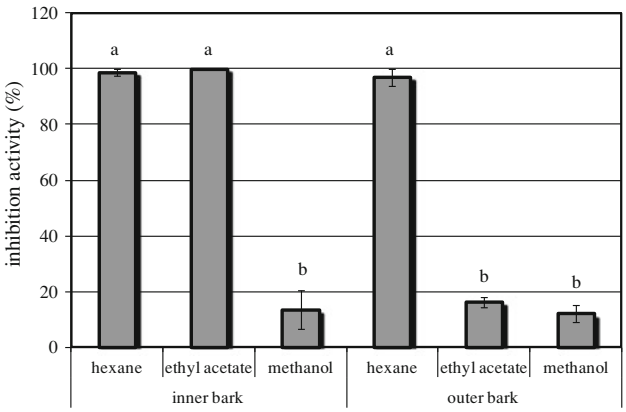

Fig. 2 Inhibitory activities of the Sugi bark extracts against *H. akashiwo* at 14 days. 0 % = control. Control tests were performed with acetone or DMSO (inner bark methanol extract) and without the sample. $N = 3$ replicates. Mean ± SE are given. The sample concentration was 1 μg/mL. *Common letters* denote no significant difference. Tukey–Kramer, $p < 0.05$

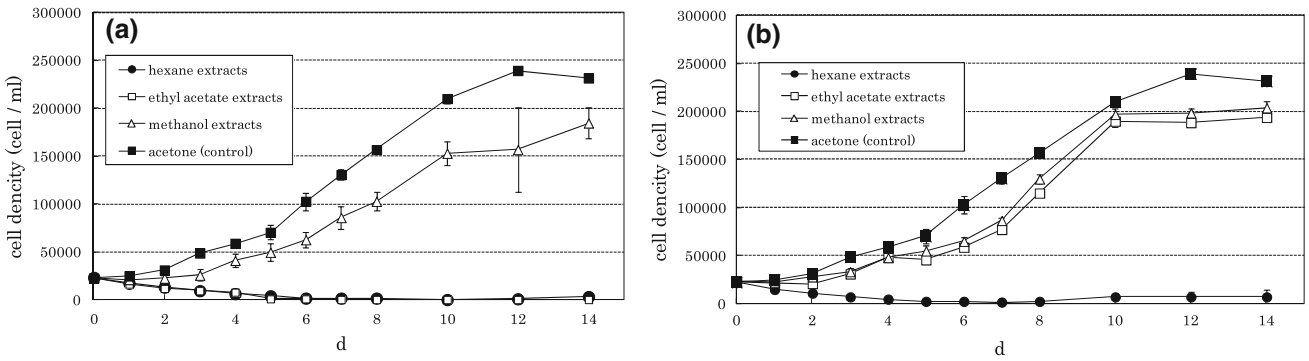

Fig. 1 Growth curves of *H. akashiwo* cells for 14 days in the tests of Sugi bark extracts at 1 μg/mL. **a** Inner bark extracts; **b** outer bark extracts

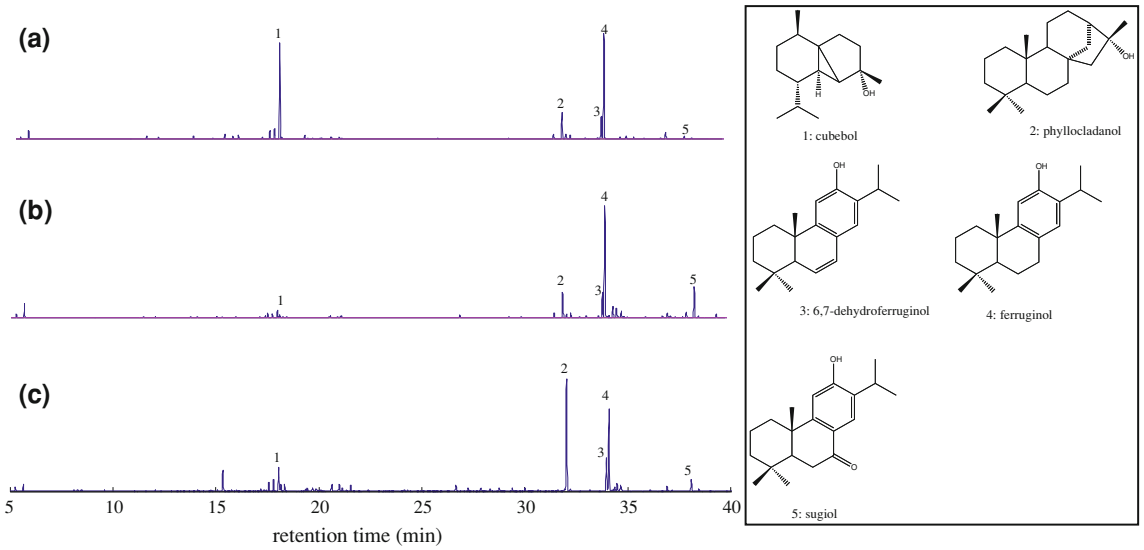

Fig. 3 Total ion chromatogram of the Sugi bark extracts exhibiting inhibitory activity against *H. akashiwo*. **a** Inner bark hexane extract; **b** inner bark ethyl acetate extract; **c** outer bark hexane extract. The conditions of the GC–MS analysis were described in the text part of the "Methods and materials" section

sugiol were detected by GC–MS analysis as the major components of the active extracts. Cubebol and phyllocladanol were strongly detected as the main components of the hexane extracts from the inner and outer barks, respectively. Cubebol in particular, which is a sesquiterpene alcohol, was observed as a weakly intense peak in the chromatograms of all of the extracts, with the exception of inner bark hexane extract. Cubebol and phyllocladanol were not detected in the essential oil, because of the occurrence of rearrangement and dehydroxylation pathways caused by the hot water distillation process [10].

Ferruginol and 6,7-dehydroferruginol were common to all of the extracts and detected as a strong peak in all of the chromatograms. Sugiol was clearly observed in the chromatogram from the inner bark ethyl acetate extract. These components have also detected and identified as characteristic of Sugi bark extracts in other reports in the literature [7]. Therefore, the activities of these five major components against *H. akashiwo* were investigated to identify the active compounds in the extracts.

Effects of the Sugi bark components against *H. akashiwo*

The results of the growth inhibition assays involving the five major components of the Sugi bark are shown in Fig. 4. Cubebol, phyllocladanol and ferruginol showed significant growth inhibition activities against *H. akashiwo* ($p < 0.05$). The two active components, cubebol and phyllocladanol, were the characteristic sesquiterpene and diterpene components in Sugi bark extracts [7]. Cubebol has been reported to exhibit repellent activities against the

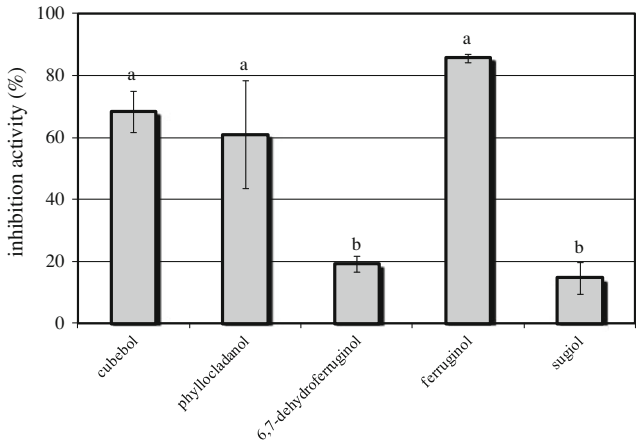

Fig. 4 Inhibitory activities of the Sugi bark components against *H. akashiwo* following 14 day. 0 % = control. Control tests were performed with acetone without the sample. $N = 3$ replicates. Mean ± SE are given. The sample concentration was 1 μg/mL. *Common letters* denote no significant difference. Tukey–Kramer, $p < 0.05$

crop pest snail (*Acusta despecta*) [17] and lethal levels of activity against the mosquito larvae (*Aedes aegypti*) [18], whereas phyllocladanol has been reported as an anti-termite compound [19]. This study has provided new information regarding the inhibitory activity of cubebol and phyllocladanol against red tide plankton. One of the other active compounds ferruginol has also been reported for its potent inhibitory activity against *S. costatum* [10]. It has been suggested that ferruginol would be a particularly effective reagents for protection against red tide. In contrast to the other components, 6,7-dehydroferruginol and sugiol did not show any obvious inhibitory activity against

H. akashiwo (Fig. 4). The 6,7-dehydroferruginol also exhibited low inhibitory activity against *S. costatum* [10]. In addition, the bioactivities of 6,7-dehydroferruginol and sugiol against wood decayed fungi and termites were lower than those reported for ferruginol in our previous reports [20, 21]. The 6,7-dehydroferruginol and sugiol could have been formed by oxidation of the ferruginol. This means that the ferruginol with high bioactivity changed to low bioactive compounds by oxidation. It would therefore be necessary to consider the preservation of ferruginol for the effective utilization of the Sugi bark components as bioactive reagents.

The inhibitory activities of active extracts against *H. akashiwo* were stronger than those of the active major components, cubebol, phyllocladanol, and ferruginol. These results suggested the existence particularly active minor components within the extracts or the occurrence of synergistic effects between the components in the Sugi bark extracts. Both of these possibilities will be investigated as part of our future work plans.

Conclusion

The inhibitory activities of Sugi bark extracts against *H. akashiwo* were investigated. Hexane extracts from the inner and outer barks and the ethyl acetate extract from the inner bark exhibited strong inhibitory activities against *H. akashiwo.* Cubebol, phyllocladanol, 6,7-dehydroferruginol, ferruginol, and sugiol were detected by GC–MS analysis as the common major components of the active extracts. Although cubebol, phyllocladanol, and ferruginol showed growth inhibition activity against *H. akashiwo*, 6,7-dehydroferruginol and sugiol did not show any discernible activity. It was therefore suggested that the Sugi bark extracts containing the aforementioned active components could be utilized as inhibition reagents against red tide plankton. The active extracts and components, however, were non-polar compounds and therefore poorly soluble in water. The activities of the bark components against fishes and other aquatic organisms remain unknown. Thus, the actual utilization of these extracts or components should be considered on a filter material, which could not interfere directly with any fish.

References

1. Ikami Y (2007) Current state and prospects of utilization of wood residue. Mokuzai Kogyo 62:50–55
2. Ueno T, Geng XL, Ashitani T, Oyadomari M, Sakai K (2001) Liquefaction of sugi (*Cryptomeria japonica*) bark by a polyethyleneglycol-bisulfite method. Mokuzai Gakkaishi 47:260–266
3. Ueno T, Ashitani T, Sakai K (2002) New method to determine the hydroxyl value in liquefied bark as polyurethane material. J Wood Sci 48:348–351
4. Ueno-Towatari T, Ashitani T, Tateishi U, Kurimoto Y, Fujita K, Sakai K (2007) Preparation and characterization of polyurethane foams prepared from liquefied Sugi bark. Mokuzai Kogyo 62:358–363
5. Ashitani T, Fujita K, Kusumoto N, Sekine N, Takahashi K, Kurimoto Y (2011) Preparation of polyurethane film from Sugi bark liquefied by PEG-bisulfite method. Mokuzai Kogyo 66:205–209
6. Saito T, Nakayama S (2009) Preparation of the adhesive from liquefaction mixture of Sugi bark. Rep Mie Ind Res Inst 33:104–106
7. Ashitani T, Ujike M, Nagahama S, Ueno T, Sakai K (2001) Characterization of sugi (*Cryptomeria japonica*) bark extracts. Mokuzai Gakkaishi 47:276–281
8. Kofujita H, Fujino Y, Sasaki T, Hasebe M, Ota M, Suzuki K (2001) Antifungal activity of the bark of *Cryptomeria japonica* and its relevant components. Mokuzai Gakkaishi 47:479–486
9. Kofujita H, Fujino Y, Ota M, Takahashi K (2006) Antifungal diterpenes from the bark of *Cryptomeria japonica* D. Don. Holzforschung 60:20–23
10. Tsuruta K, Yoshida Y, Kusumoto N, Sekine N, Ashitani T, Takahashi K (2011) Inhibition activity of essential oils obtained from Japanese trees against *Skeletonema costatum*. J Wood Sci 57:520–525
11. Tsuruta A, Ueno S, Ohgai M, Yamada M (1987) Seasonal and horizontal distributions of phytoplanktonic diatom *Skeletonema costatium* (Grev.) Cleve in Yatsushiro sea. Nippon Suisan Gakkaishi 53:141–144
12. Tang DL, Di BP, Wei G, Ni IH, Oh IS, Wang SF (2006) Spatial, seasonal and species variations of harmful algal blooms in the south yellow sea and east China sea. Hydrobiologia 568:245–253
13. Yamauchi S, Abe T (1984) Mechanisms to initiate a *Heterosigma akashiwo* red tide in Osaka Bay II Diel vertical migration. Mar Biol 83:255–261
14. Ono T, Khan S, Onoue Y (2000) Effects of temperature and light intensity on the growth and toxicity of *Heterosigma akashiwo* (Raphidophyceae). Aquacult Res 31:427–433
15. Shikata T, Sakurada K, Jomoto Y, Onji M, Yoshida M, Ohwada K (2010) Effects of temperature, and light irradiance on phytoplankton growth in the Yatsushiro sea. Nippon Suisan Gakkaishi 76:34–45
16. Matsushita Y, Sugamoto K, Miyakubo K, Kurogi C, Matui T, Oda H, Fujimoto H (2008) Chemical changes in terpenes of Sugi (*Cryptomeria japonica*) wood during steam drying in kiln at high temperature. J Wood Sci 54:476–482
17. Chen XH, Kim CS, Kashiwagi T, Tebayashi S, Horiike M (2001) Antifeedants against *Acusta despesta* from the Japanese Cedar, *Cryptomeria japonica* II. Biosci Biotechnol Biochem 65: 1434–1437
18. Gu HJ, Cheng SS, Huang CG, Chen WJ, Chang ST (2009) Mosquito larvicidal activities of extractives from black heartwood-type *Cryptomeria japonica*. Parasitol Res 105:1455–1458
19. Arihara S, Umeyama A, Bando S, Kobuke S, Imoto S, Ono M, Yoshikawa K, Amita K, Hashimoto S (2004) Termiticidal constituents of the black-heart of *Cryptomeria japonica*. Mokuzai Gakkaishi 50:413–421
20. Kusumoto N, Ashitani T, Hayasaka Y, Murayama T, Ogiyama K, Takahashi K (2009) Antitermitic activities of abietane-type diterpenes from *Taxodium distichum* cones. J Chem Ecol 35: 635–642
21. Kusumoto N, Ashitani T, Murayama T, Ogiyama K, Takahashi K (2010) Antifungal abietane-type diterpenes from the cones of *Taxodium distichum* Rich. J Chem Ecol 36:1381–1386