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New sesquiterpene from Vietnamese agarwood and its induction effect on brain-derived neurotrophic factor mRNA expression in vitro

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Abstract—Agarwood, one of the valuable non-timber products in tropical forest, is a fragrant wood, whose ethereal fragrance has been prized in Asia for incense in ceremony, as well as sedatives in traditional medicine. We separated the 70% EtOH extract of Vietnamese agarwood, which showed significant induction effect on brain-derived neurotrophic factor (BDNF) mRNA expression in rat cultured neuronal cells, to isolate a new compound and a 2-(2-phenylethyl)chromone derivative. The new compound was determined to be a spirovetivane-type sesquiterpene, (4*R*,5*R*,7*R*)-1(10)-spirovetiven-11-ol-2-one, by spectroscopic data and showed induction effect of BDNF mRNA.

1. Introduction

Agarwood,[1–4] also known as 'aloeswood,' 'agalloch,' or 'eaglewood' in English and 'jinko' in Japanese, is a fragrant wood and one of the valuable non-timber products in Asian tropical forest, which results from the action of damages on *Aquilaria* plants (Thymelaeaceae) and then infections by fungi such as *Fusarium* spp. Its ethereal fragrance has been prized in Asia for incense when performing ceremony of Islam or Buddhism since ancient age. Agarwood has been also used as ingredient in Kampo-formulae, such as 'rokushingan' and 'kiogan,' for sedative in Japanese and Chinese traditional medicine.

Agarwoods have been reported to contain sesquiterpenoids of eremophilane-,[5–7] spirovetivane-,[5,7] eudesmane-,[5,6] nor-guaiane-,[6] guaiane-,[8] and prezizaane-type,[5] 2-(2-phenylethyl)chromone derivatives,[9–11] etc. On physiological study of agarwood, on the other hand, only prolonging effect[7] of sleeping time in mice by jinkoh-eremol and agarospirol was reported.

Agarwood might have some effects toward central nervous system (CNS) such as higher brain function, from traditional use as a sedative. We attempted to study the components of agarwood using induction activity of BDNF mRNA expression as a guide of CNS effect. The BDNF is a member of the neurotrophin family and plays a key role in the survival, differentiation, and synaptic plasticity of neurons,[12] and also plays an important role in the postnatal development of the mammalian central nervous system.[13] In a preliminary examination, the 70% EtOH extract of Vietnamese agarwood (called 'Tram' in Vietnamese) with the highest grade significantly induced the brain-derived neurotrophic factor (BDNF) exon III–V mRNA expression in rat cortical cells at a concentration of 100 μg/mL (expression ratio, 2.6-fold increase; Fig. 1), though such a reagent was reported to be a synthetic type II pyrethroid insecticide, deltamethrin, which was used as a positive control in this study.[14] Therefore, we carried out separation of the EtOH extract to isolate a new

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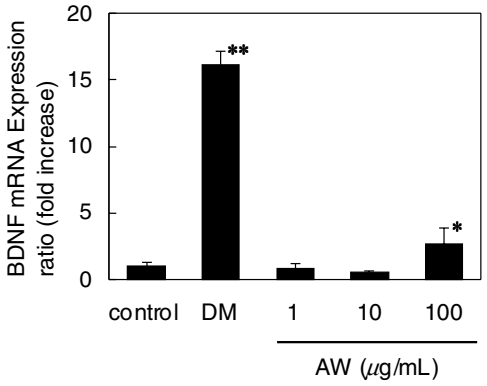
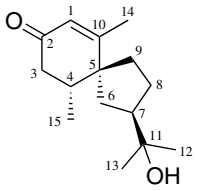


Figure 1. Expression of BDNF exon III–V mRNA in rat cortical neurons induced by agarwood extract. Cortical neuronal cells in culture were treated with deltamethrin (DM, 1 μM) or 70% EtOH extract of agarwood (AW, 1–100 μg/mL) and further incubated for 24 h before total cellular RNA was extracted for real-time RT-PCR. The ratio of mRNA expression to the control level is shown. The bars represent the mean ± SD from three independent experiments. $*p < 0.05$, $**p < 0.01$ compared to the control.



Scheme 1. Structure of compound **1**.

sesquiterpenoid (Scheme 1) along with a known 2-(2-phenylethyl)chromone derivative, and their activities of induce on BDNF expression were examined.

2. Results and discussion

AcOEt-soluble fraction, yielded from the EtOH extract of agarwood, was separated by normal-phase preparative TLC to isolate a new compound (**1**) and a known 2-(2-phenylethyl)chromone derivative (**2**). The known compound (**2**) was identified by spectroscopic analyses and comparisons with published data[9] to be AH_6, which was also reported as a constituent of agarwood.

The new compound (**1**) was obtained as a colorless amorphous solid. Its high-resolution EIMS (*m*/*z* 218.1682 $[M-H_2O]^+$) and ^{13}C NMR data indicated its molecular formula to be $C_{15}H_{24}O_2$. The IR (ν_{max} 1658 cm^{-1}) and UV (λ_{max} 240 nm) spectra showed the presence of a conjugated enone. The ^1H and ^{13}C NMR spectra of **1** revealed the signals of an α,β-unsaturated ketone (δ_H 5.74, δ_C 125.4, 167.4, 199.2), four methyls (δ_H 1.97, 1.25, 1.24, 1.00; δ_C 28.6, 28.4, 21.4, 16.2), and an oxygen-bearing quaternary carbon (δ_C 71.5), suggesting **1** to be a bicyclic sesquiterpene with an α,β-unsaturated ketone. In addition, analysis of the COSY and HMQC spectra indicated the partial structures depicted in Figure 2, while the HMBC spectrum confirmed the partial structures and connected them to spirovetivane carbon framework (Fig. 3).

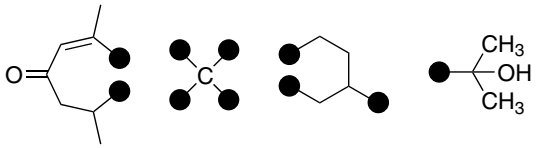


Figure 2. Partial structures for **1**.

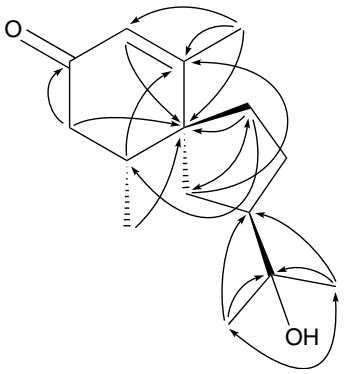


Figure 3. Key correlations in HMBC of **1**.

The relative stereochemistry on cyclohexenone ring was determined to be twisted chair with the C-4 methyl group in α-axial orientation, based on the coupling constants ($J_{3\alpha,4}$ = 5.1 Hz, $J_{3\beta,4}$ = 4.4 Hz). On the other hand, the configurations at C-5 and C-7 were determined by differential NOE experiments (Fig. 4). The NOEs from H_3-12 and H_3-13 to H-4, H_2-6, and H-7, from H-9β to H-3β, from H_3-14 to H-6α and H-7, and from H_3-15 to H-4 and H-6β indicated C-6 and C-9 in α-equatorial and β-axial orientations, respectively, and 1-hydroxy-1-methylethyl group at C-7 in β-orientation. Moreover, the absolute configuration at C-4 was determined to be *R*, the same as that of (−)-β-vetivane,[15] according to helicity rule[16] on the negative Cotton's effect ($[\theta]_{236}$ −3568) at $\pi \rightarrow \pi^*$ band in CD spectrum. Thus, **1** was concluded to be (4*R*,5*R*,7*R*)-1(10)-spirovetiven-11-ol-2-one.

The new compound (**1**) significantly induced the BDNF exon III–V mRNA expression at a concentration of 100 μg/mL (expression ratio, 15.7-fold increase; Fig. 5), though **2** did not. So, **1** is considered to play an important role in the inducible activity of agarwood.

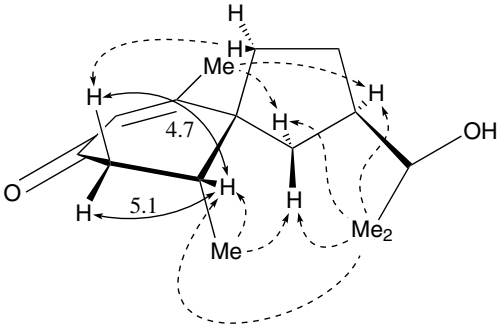


Figure 4. Coupling constants (*J* values in Hz, solid arrows) and NOEs (dotted arrows) for **1**.

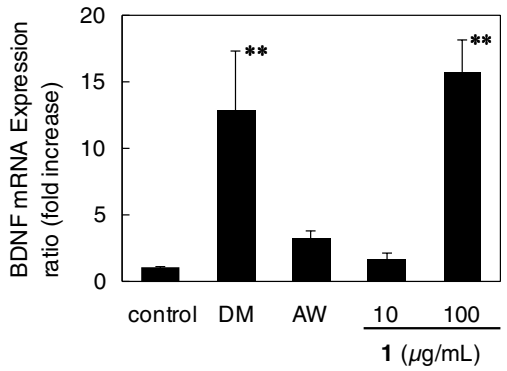


Figure 5. Induction of BDNF exon III–V mRNA expression in rat cortical neurons by **1**. Cortical neuronal cells in culture were treated with deltamethrin (DM, 1 μM), 70% EtOH extract of agarwood (AW, 100 μg/mL) or **1** (10, 100 μg/mL) and further incubated for 24 h before total cellular RNA was extracted for real-time RT-PCR. The ratio of mRNA expression to the control level is shown. The bars represent the mean ± SD from three independent experiments. $^{**}p < 0.01$ compared to the control.

This is the first report which spirovetivane-type sesquiterpene induce to present BDNF mRNA expression. Because the active constituent could be isolated from the highest-graded agarwood, **1** might be used as one of the indications of quality evaluation of agarwood. Thus, further investigations on the comparison of the content of **1** between the highest-graded agarwood and other lower-graded ones are now in progress, and a relationship between the content and the grades will be reported elsewhere.

3. Conclusion

In this study, we have described to isolate a new spirovetivane-type sesquiterpene along with a known 2-(2-phenylethyl)chromone from the highest-graded Vietnamese agarwood, which significantly induced BDNF exon III–V mRNA expression. The new sesquiterpene was determined as (4*R*,5*R*,7*R*)-1(10)-spirovetiven-11-ol-2-one by analysis of spectroscopic data and contributed to the induction of BDNF mRNA expression.

4. Experimental

4.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-140 digital polarimeter. UV, IR, and CD spectra were obtained on a Shimadzu UV-160A ultraviolet–visible spectrophotometer, a Shimadzu IR-408 infrared spectrophotometer, and a JASCO J-805 circular dichroism spectrophotometer, respectively. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard. EIMS and HREIMS measurements were carried out on a JEOL JMS-700T spectrometer, and glycerol was used as a matrix. Analytical and preparative TLC were carried out on precoated silica gel 60 F_{254} plates (0.25 or 0.5 mm thickness) or RP-18 F_{254S} (0.25 mm thickness) (both Merck, Darmstadt, Germany). Other reagents and solvents were of the highest grade available.

4.2. Material

The agarwood with the highest grade, Kyara 1st (K1), was purchased from the Department of Science Technology and Environment of Khanh Hoa Province, Vietnam, in December 2001.

4.3. Extraction and isolation

The agarwood (4.52 g) was crushed into powder and extracted under sonication for 15 min with Et_2O (150 mL, 3×), EtOH (150 mL, 3×), EtOH–H_2O (1:1, 150 mL, 3×), and H_2O (150 mL, 3×), successively. Each solution was evaporated under reduced pressure to give a Et_2O (452 mg), EtOH (1.05 g), EtOH–H_2O (345 mg), and H_2O (27.4 mg) extracts, respectively. A part of EtOH extract (216 mg) was partitioned between AcOEt-soluble fraction (156 mg) and AcOEt-insoluble fraction (45.1 mg). The AcOEt-soluble fraction (152 mg) was subjected to normal-phase preparative TLC ($CHCl_3$–Me_2CO, 25:1) to yield compound **1** (2.5 mg) and AH_6 (**2**, 1.1 mg).[9]

4.4. (4*R*,5*R*,7*R*)-1(10)-Spirovetiven-11-ol-2-one (1)

Colorless amorphous solid; $[\alpha]_D^{21}$ −13.2° (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 240 (3.6) nm; IR ($CHCl_3$) ν_{max} 1658 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 5.74 (1H, br s, H-1), 2.61 (1H, dd, *J* = 16.9, 4.4 Hz, H-3β), 2.20 (1H, dd, *J* = 16.9, 5.1 Hz, H-3α), 2.10 (1H, m, H-7), 2.09 (1H, m, H-4), 1.99 (1H, m, H-9β), 1.97 (3H, d, *J* = 1.0 Hz, H-14), 1.85 (1H, m, H-6α), 1.83 (1H, m, H-8), 1.76 (1H, m, H-8), 1.57 (1H, m, H-6β), 1.53 (1H, m, H-9α), 1.25 (3H, s, H-12 or -13), 1.24 (3H, s, H-13 or -12), 1.00 (3H, d, *J* = 7.1 Hz, H-15); ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.2 (C-2), 167.4 (C-10), 125.4 (C-1), 71.5 (C-11), 52.8 (C-7), 50.3 (C-5), 43.1 (C-3), 40.4 (C-4), 36.4 (C-9), 35.8 (C-6), 28.6 (C-12 or -13), 28.4 (C-13 or -12), 27.3 (C-8), 21.4 (C-14), 16.2 (C-15); CD (4.7×10^{-4} M, EtOH) $[\theta]_{236}$ −3568; EIMS (rel int, %) *m*/*z* 236 (2), 218 (13), 148 (100); HREIMS calcd for $C_{15}H_{22}O$ $[M-H_2O]^+$: 218.1671; found: 218.1682.

4.5. Primary culture of rat cortical cells

A primary culture of rat cortical cells was prepared from the cerebral cortexes of 17-day-old Sprague–Dawley (SD) rat (Japan SLC, Japan) embryos as described before.[17] The dissociated cells were suspended in DMEM (Invitrogen) containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin, and seeded at 2.5×10^6 cells in culture dishes (35 mm in diameter; Iwaki, Japan) that had been coated with polyethylenimine (Sigma). The cells were grown for 3 days, and then the medium was replaced with serum-free DMEM containing 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the mRNA analysis, the cells were stimulated with test specimen after 5 days in culture. Deltamethrin was used as positive control.[14]

4.6. RNA isolation and analysis

Total cellular RNA was extracted by the acid guanidine phenol–chloroform (AGPC) method. The isolation of RNA from cultured cells was previously described in detail.[18] In brief, total cellular RNA was isolated according to the ISOGEN protocol (Nippon gene, Japan) and quantified with a Beckman spectrophotometer. One microgram of RNA was used for reverse transcription with Superscript II (Invitrogen). Quantitative RT-PCR was conducted in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using $1 \times$ SYBR Green Master Mix (Applied Biosystems) containing 2 μL cDNA solution and 0.5 μM of primer pairs. For the amplification of rat BDNF exon III–V cDNA, BDNF exon III sense (5′-TCGGCCACCAAAGACTC-3′) and BDNF exon V antisense (5′-GCCCATTCACGCTCTCCA-3′) primers were used. For the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified using rat GAPDH sense (5′-TCCATGACA ACTTTGGCATCGTGG-3′) and rat GAPDH antisense (5′-GTTGCTGTTGAAGTCACAGGAGAC-3′) primers. The mRNA expression levels were computed from the C_T value and normalized to the concentration of GAPDH mRNA as an internal standard.

4.7. Statistics

All data were expressed as means ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Fisher's PLSD procedure for multiple comparisons as post hoc analysis.

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