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Agarotetrol: a source compound for low molecular weight aromatic compounds from agarwood heating

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Abstract Agarwood is known to generate a distinct fragrance on heating and is used as both a medicine and a fragrant wood. Low molecular weight aromatic compounds (LACs) such as benzylacetone are emitted from agarwood on heating and have a sedative effect on mice. These are detected exclusively in the headspace vapor of heated agarwood and are absent in the wood itself; hence, some compounds in agarwood are thought to be converted to LACs by the process of heating. In this study, different fractions obtained from agarwood were analyzed to reveal the source compounds of LACs. Some LACs detected in the resinous agarwood were absent from the non-resinous parts and confirmed as characteristic of the resinous parts. The essential oil and hydrosol of agarwood obtained by distillation were analyzed on GC-MS. Sesquiterpenes were detected in the essential oil, and sesquiterpenes and a variety of LACs were detected in the hydrosol. A hot water extract of agarwood remaining in the distillation flask after distillation was analyzed by HPLC, and agarotetrol was found to be the main compound. Purified agarotetrol was heated in a glass vial and its headspace vapor was analyzed by solid-phase microextraction-GC-MS. Benzylacetone and other LACs were detected. These results indicate that agarotetrol, a chromone derivative, contributes to the fragrance of agarwood through the generation of LACs on heating.

Key words agarwood · chromone derivatives · benzylacetone · hydrosol · essential oil · volatile compounds

Introduction

Agarwood is a resinous wood found in the trunks of *Aquilaria* and *Gyrinops* species [1]. It is used as a sedative in China, Korea, and Japan, and as an incense worldwide. Agarwood is formed in decaying or wounded trees. Phytochemical analyses of commercial agarwood pieces and agarwood oils have revealed that chromone derivatives, sesquiterpenes, and low molecular weight aromatic compounds (LACs) are the main constituents [2, 3, 4]. Chromone derivatives are non-volatile and non-fragrant compounds, while sesquiterpenes are volatile and fragrant. Chromone derivatives and sesquiterpenes are extracted by the organic solvents MeOH, hexane, and ethyl acetate. LACs such as benzylacetone and benzaldehyde are volatile and fragrant compounds and are detected exclusively in the headspace vapor of heated agarwood [4, 5]. Therefore, LACs are believed to contribute to the complex and distinct fragrance of agarwood on heating. In addition, inhalation of the vapor of some LACs has a sedative effect on mice, and benzylacetone in particular reduces mouse locomotor activity [2, 6]. This may partly explain the pharmaceutical use of agarwood. The amounts of LACs in agarwood essential oil increase when distillation is performed after soaking pieces of the wood in water for several days [3]. It is therefore inferred that certain compounds in agarwood are extracted with water and generate LACs when heated. Some studies have suggested that chromone derivatives change to LACs. However, this has not yet been demonstrated [4, 7, 8]. In this study, different fractions obtained from agarwood, especially water-soluble compounds, were analyzed to reveal the source compounds of LACs.

Materials and methods

Samples

Agarwood samples were offered by Shoeido incense Co., (Kyoto, Japan). Non-resinous samples of agarwood were collected from the Experimental Station for Medical Plants at the Graduate School of Pharmaceutical Science, Kyoto University, and cut into small pieces to dry. Essential oil and hydrosol samples for GC-MS analysis were prepared by hydro-distillation of agarwood pieces using a glass distillation apparatus (Herb oil maker laboratory type, Tokyo Seisakusho Co. Ltd., Japan), and the distilled oil and hydrosol fractions were collected. The oil fraction was dissolved in diethyl ether, and dehydrated with anhydrous sodium sulfate. Likewise, the hydrosol fraction was extracted with diethyl ether, and the diethyl ether fraction was dehydrated by anhydrous sodium sulfate. A hot water extract of agarwood was freeze-dried, dissolved in MeOH, filtered through filter paper (Tokyo Roshi Kaisha, Ltd., Japan) and evaporated. The resulting fraction was again dissolved in MeOH (1 mg mL^{-1}) and filtered (GL Chromatodisk 13P, 0.45 μm, GL Sciences, Tokyo, Japan) for HPLC analysis.

Collection of volatile compounds

The headspace (HS)-solid-phase microextraction (SPME) method was used in this experiment to collect the volatile compounds. Samples were put in glass vials (Supelco Inc., Bellefonte, PA), sealed with caps with holes and polytetrafluoroethylene-faced silicone septa (Supelco Inc.) and the vial containing sample was heated on a hot plate for 10 min at 190–200 °C. The surface of hot plate was measured with digital thermometer (CT-01, Custom corporation) fitted with thermocouple prove (TS-05, Custom corporation). After heating, an SPME fiber (polydimethylsiloxane 100μm, Supelco Inc.) was inserted into the headspace of each vial to adsorb the volatile compounds at room temperature for 10 min. Desorption was at 230 °C for 10 min through splitless injection in a GC-MS instrument (6850GC with 5975MDS, Agilent Technologies, Santa Clara, CA) equipped with a DB-WAX column (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies). The conditions for GC-MS analyses were as follows: carrier gas, helium; flow rate, 1 mL min^{-1}, column oven program 100°C initially, increasing by 4°C every min to 180°C, and maintained at this temperature for 30 min. Thereafter the temperature was increased by 5°C every min until it reached 240°C, and this was maintained for 18 min. The ionization voltage for MS was 15 eV. Peaks were identified by comparing the retention times and mass fragmentation patterns of the samples with those from the literature [9, 10] and the National Institute of Standards and Technology database.

Analyses of essential oil and hydrosol

Agarwood oil (3 μL) and hydrosol (1 μL) samples were analyzed by GC (G-5000, Hitachi Ltd., Tokyo, Japan) equipped with a InertCap-WAX column (60 m × 0.25 mm, film thickness 0.25 μm, GL Sciences). The split ratio was 99:1. The conditions for GC analysis were as follows: carrier gas, helium; flow rate, 1 mL min^{-}; injector temperature, 230°C; detector; flame ionization detector (FID), 250°C; column oven program, 100°C initially increased by 4°C every min until it reached 180°C, maintained at this temperature for 30 min, then increased by 5°C every min until it reached 240°C, and maintained for 18 min.

Analyses and identification of compounds contained in hot water extract of agarwood

The MeOH-soluble fraction of hot water extract of agarwood was analyzed by HPLC (pump, L-7100; column oven, L-7300; UV detector, L-7420, Hitachi Ltd.). Two conditions were set for the analysis: condition 1 covers a wide range of polarities and condition 2 is for the isolation of specific compounds.

Condition 1: YMC-Pack ODS/A column, 10 μm, 4.6 mm I.D × 250 mm; detection at 254 nm; solvent, MeOH-water; gradient from 37% (0 min) to 100% (30 min); flow rate, 1 mL min^{-1}; and injection volume, 3 μL.

Condition 2: $_5C_{18}$MS-II (COSMOSIL, Nacalai Tesque Inc., Kyoto, Japan) column, 4.6 mm I.D × 250 mm; detection at 254 nm; solvent, MeOH-water; gradient from 20% (0 min) to 30% (60 min); flow rate, 1 mL min^{-1}, and injection volume, 1 μL.

A Prominence series system (Shimadzu Corp., Kyoto, Japan) was used to isolate the target compound under the following conditions: Column, $_5C_{18}$MS-II (COSMOSIL, Nacalai Tesque Inc.), 20 mm I.D ×

250 mm; detection at 254 nm; solvent, MeOH-water; gradient, from 20% (0 min) to 30% (60 min); flow rate, 8 mL min^{-1}; and injection volume, 1 mL.

Fractionation and identification of target compound

The MeOH-soluble fraction from the hot water extraction was fractionated as follows: A solution of the hot water extraction was prepared by dissolving 50.6 mg of freeze-dried hot water extract in MeOH, filtered through filter paper, and evaporation to obtain 47.4 mg of extract. The extract was again dissolved in MeOH at 15 mg mL^{-1}, filtered (GL Chromatodisk 13P, 0.45 μm, GL Sciences), and separated by HPLC. Finally, 13.6 mg of the target compound was obtained.

^{1}H- and ^{13}C-NMR spectra were recorded on a 500 MHz spectrometer (JNMECA500KP, (JEOL Ltd., Tokyo, Japan). The compounds were identified by comparing the data with those published in the literature [11].

Results

Analyses of volatile compounds by heating agarwood

Ground agarwood pieces were analyzed by HS-SPME-GC-MS. Sesquiterpenes and LACs such as benzylacetone and benzaldehyde were detected in the headspace vapor of the resinous part on heating. On the other hand, some compounds such as vanillin and furfural, which are thought to be decomposition products of wood, were detected in the headspace vapor of the non-resinous part on heating, and LACs like benzylacetone were absent (Table 1). Area% of peaks in total ion chromatography of GC-MS were shown in Table 1 for reference.

Analyses of essential oil and hydrosol

The essential oil and hydrosol fractions were analyzed by GC and GC-MS. Sesquiterpenes were the main compounds detected in the essential oil. LACs were detected in the hydrosol in addition to sesquiterpenes (Table 2).

Analyses of hot water extract of agarwood and identification of target compound

Freeze-dried samples of hot water extracts of agarwood (4.0 mg) were analyzed by HS-SPME-GC-MS. LACs such as benzylacetone and benzaldehyde were detected. These compounds were also found in the MeOH-soluble fraction of hot water extract of agarwood. The MeOH-soluble fraction of hot water extract of agarwood was analyzed by HPLC. The HPLC chart showed a large peak that occupied approximately 60% of the total area of the chart (Fig. 1). NMR analysis showed that this compound was agarotetrol.

Analyses of volatile compounds from heated agarotetrol

Agarotetrol (6.84 mg) was heated to 190–200°C in a glass vial with a screw cap, and the volatile compounds in the headspace of the vial were adsorbed to an SPME fiber inserted through the septum of the cap. HS-SPME-GC-MS analysis showed the presence of benzylacetone, benzaldehyde, and benzenepropanoic acid methyl ester (Fig. 2). The contents in the vial were decreased to 6.70 mg after heating.

Discussion

Agarotetrol was the main compound found in a hot water extract of agarwood and generated LACs, especially benzylacetone, by heating. The amounts of LACs in the oil increase if distillation is performed after soaking pieces of the wood in water for several days [3]. In fact, the process of oil distillation in agarwood-producing countries often includes soaking agarwood in water for several

days before distillation. This process has been shown to contribute to increasing the amounts of LACs in essential oil as well as the production volume of the essential oil from limited agarwood resources.

The structure of agarotetrol was first elucidated by Yoshii et al. in 1978 [12] and was shown to be widely present in agarwood regardless of the species or grade [13, 14]. Some studies have investigated agarotetrol pyrolysis [5, 8, 15], but the relationship between agarotetrol in agarwood and the generation of benzylacetone in the volatile compounds of agarwood has not been clarified. The present experiment showed that LACs were detected when agarwood was heated to 190－200℃ and were also detected in the volatiles of agarwood and in the MeOH-soluble fraction of hot water extraction. According to these results, we suggest that agarotetrol contributes to the fragrance of agarwood.

Kishida et al. collected the essential oil of agarwood using hexane, and benzylacetone and benzaldehyde were detected in the oil fraction [3]. In this study, the essential oil and hydrosol of agarwood were collected without solvents and were analyzed separately. As a result, many kinds of polar LACs were detected in the hydrosol. The instrument for hydro-distillation was different from Clevenger type and the system was not a complete closed one. Thus, some LACs which were difficult to dissolve in water and easy to volatile, might vaporize into the air and weren't detected in hydrosol or essential oil. The hydrosol fraction of agarwood has barely been studied, but it turned out to be a good source of agarwood LACs because it contains some compounds that are difficult to collect by extraction methods using organic solvents.

Some of the LACs detected in the headspace vapor of resinous agarwood on heating were absent from the headspace vapor of non-resinous part. In a previous report, syringaresinol, a kind of lignin was detected in the non-resinous part, while chromones were detected in the resinous parts [16].
The detection of a series of chromone compounds during the cell cycle of cultured cells has been previously described: the amount of oxideagarochromones increases at an early stage and decreases as cell viability decreases. Thereafter, chromone derivatives such as agarotetrol and 6-methoxy-2-(2-phenylethyl) chromone are formed. Conversely, sesquiterpenes can be produced by stimulating cells with methyl jasmonate, and are thought to be phytoalexins [17]. The biosynthetic pathway of chromones has not yet been clarified but they are not formed by methyl jasmonate.

Because of its high economic value and popularity around the world, agarwood sources are becoming depleted due to over-exploitation and the decreasing number of tropical rainforests. For these reasons, all species of the origin plants that create agarwood are listed in Appendix II of the 2005 Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Therefore, there is considerable interest in the production of agarwood on cultivated *Aquilaria* spp trees, and many procedures and reagents have been attempted. However, few have been successful, and the quality of agarwood is variable. Studies into the mechanisms of resin accumulation in agarwood are of interest in terms of scientific research on the one hand, and the agarwood market on the other. Some compounds derived from agarwood such as benzylacetone are potent sedatives when inhaled [2, 6]. However, further investigation into the composition and synthetic mechanisms is needed to provide a scientific explanation for the effects of agarwood.

In this study, agarotetrol, a polar compound extracted from agarwood with water, was identified and shown to generate LACs on heating, especially benzylacetone. Agarotetrol and other chromone derivatives do not have a scent, although they are converted to LACs or other volatile compounds by heating to obtain the agarwood fragrance.

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Figure

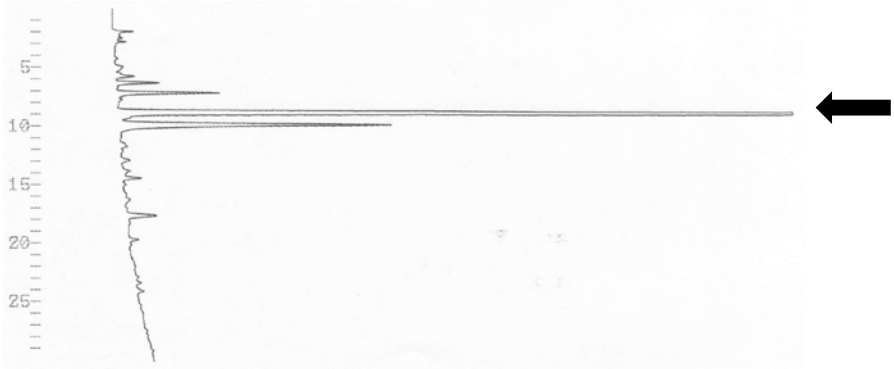

Fig.1 HPLC chart of MeOH soluble fraction of hot water extract of agarwood in condition 1.
⬅ = a large peak that occupied approximately 60% of the total area of the chart

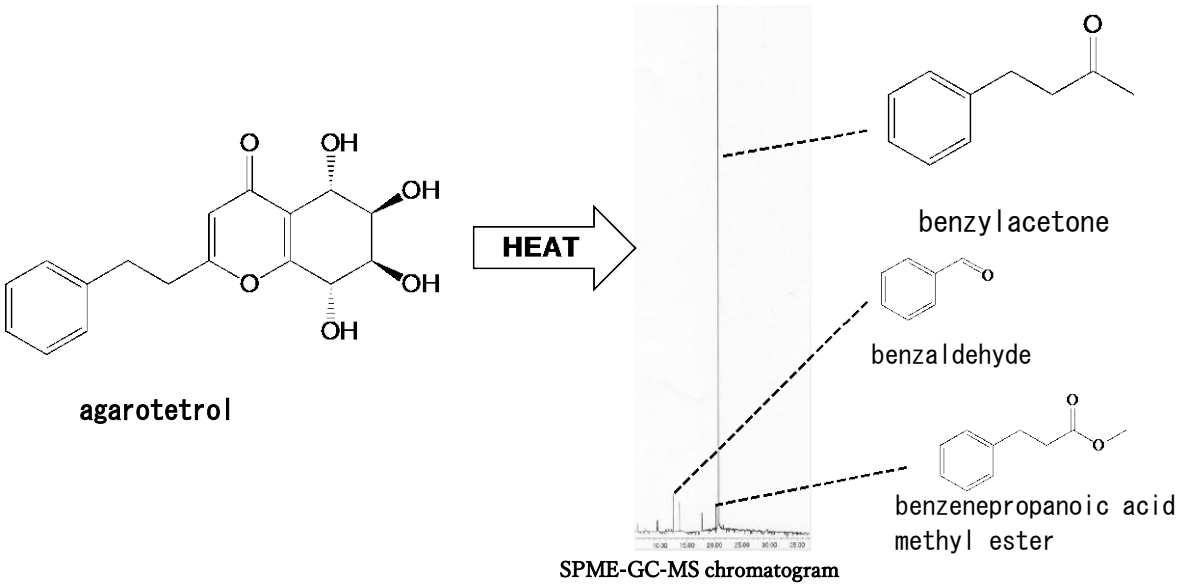

Fig.2 Low molecular weight aromatic compounds generated from agarwood on heating.

Table

Table 1 Compounds detected in the headspace vapor of resinous and non-resinous parts of agarwood.

Compound	RI	Resinous part*	Non-resinous part*
Furfural	1420	-	5.66
Benzaldehyde	1473	7.36	-
4-Ethyltoluene	1612	0.5	-
Acetophenone	1635	0.86	-
1-Furfurypyrrole	1816	-	2.99
Benzylacetone	1833	6.01	-
2-Acetylpyrrole	1887	-	0.61
Methyleugenol	2005	-	0.92
4-Methoxybenzaldehyde	2015	0.69	-
(E)-Cinnamaldehyde	2020	0.09	2.39
8-epi-γ-Eudesmol	2054	3.22	-
β-Eudesmol	2213	0.16	-
Hydrocoumarin	2231	0.14	-
Coumarin	2421	0.11	-
Vanillin	2548	-	0.56

Shaded boxes indicate LACs that were detected in only the headspace vapor of the part on heating.
RI = retention index on the DB-WAX column
*Data are shown as area% of peaks in total ion chromatograms of GC-MS.
- =absent

Table 2 Compounds detected in the essential oil and hydrosol of agarwood

		Hydrosol	Essential oil
Compound	RI	Area (%)**	Area (%)**
Furfural	1418	0.04	-
Benzaldehyde	1473	0.44	-
Acetophenone	1636	0.24	-
Isoborneol	1643	0.02	-
2-Hydroxybenzaldehyde	1650	0.01	-
endo-Borneol	1659	0.37	-
Benzylacetate	1671	tr	-
Benzylacetone	1834	9.32	-
Benzylalcohole	1839	0.08	-
4-Phenyl-2-butanol	2001	tr	-
4-Methoxybenzaldehyde	2016	tr	-
(E)-Cinnamaldehyde	2022	tr	-
[1R-(1α, 3α, 4β)]-4-ethyl-α, α, 4-trimethyl-3-(1-methylethenyl)-cyclohexanemethanol	2038	0.4	0.57
8-epi-γ-Eudesmol	2055	1.79	10.27
(E)-Benzalacetone	2056	tr	-
Eugenol	2081	0.11	-
γ-Eudesmol	2087	0.43	0.79
β-Eudesmol	2215	3.39	1.81

RI = retention index on the DB-WAX column

**Quantified by their FID peak are

- = absent

tr = trace