NOTE

Characteristic Scent from the Tahitian Liverwort, *Cyathodium foetidissimum*

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Abstract: The volatile components of the Tahitian liverwort *Cyathodium foetidissimum* was analyzed using headspace solid phase micro-extraction (SPME) and GC-MS. Three volatile components, 4-methoxystyrene (24.4%), 3,4-dimethoxystyrene (28.7%), and skatole (15.9%) were identified as the major components from the fresh *C. foetidissimum*, along with several aliphatic aldehydes, *n*-octanal, *n*-nonanal, and *n*-decanal. However, (*E*)-2-nonenal recognized as aged malodor was not identified. In GC-O analysis, 2-aminoacetophenone was detected as one of the minor components with a strong aging note. In fact, *C. foetidissimum* showed the characteristic aging odor reminiscent the damp smell from old chest of drawers, or the civet like note with very strong feces and urine odor. The mixture consisted of 4-methoxystyrene, 3,4-dimethoxystyrene, and skatole in the detected ratio showed the sedative effect on CNV (contingent negative variation) measurement.

Key words: *Cyathodium foetidissimum*, 4-methoxystyrene, 3,4-dimethoxystyrene, skatole, aging odor

1 INTRODUCTION

The bryophytes, especially, liverworts produce a great number of terpenoids, aromatic compounds including flavonoids and polyketides, many of which possess new carbon skeletons^{1a-c}. Their secondary metabolites show not only interesting biological activities such as muscle relaxation, anti-obesity, antioxidant, anticancer, and antimicrobial, and antiviral activity^{1a-d} but also strong mushroomy, marine, milky, and moldy smell^{1b-d}. In fact, the liverwort, *Conocephalum conicum* elaborates (*R*)-1-octen-3-ol and its acetate as well as (*E*)-methyl cinnamate which are the most important aroma of the Japanese fungus, Matsutake (*Tricholoma matsutake*)^{1b-d}. When physiologically stressed, *Marchantia paleacea* subsp. *diptera* starts to biosynthesize (*S*)-(-)-perilladehyde². Liverworts produce not only pleasant but also very unpleasant odor, like stink bugs or feces smell^{1b-d}. A very tiny Malaysian thalloid *Asterella* (or *Mannia*) species (3 to 5 mm) produced skatole (3) as the major component which was responsible for feces and urine smell of this liverwort^{1b,d}. Another liverwort emitting feces and urine smell is the Ta-

hitian, *Cyathodium foetidissimum*, which contains a trace amount of skatole (3), and two sesquiterpenoids, bicyclogermacrene and isolepidozene, and one bibenzyl, lunularin were identified³. In the present paper, we reinvestigated the volatile components of the Tahitian *Cyathodium foetidissimum* using HS-SPME-GC/MS because it emitted pleasant aged "old person" smell or old "chest of drawers", or "nostalgic" odor, rather than feces and urine smell.

2 MATERIALS AND METHODS

2.1 Plant materials.

Cyathodium foetidissimum was collected from the wet soil near the cave close to E.H.P. Gauguin Museum, Tahiti, in 2016 by Y. Asakawa, Prof. Dr. J.-P. Bianchini, and Mrs. H. Asakawa. The species is the same as that collected in Ua fuka Island in Marquesas Islands in 2009. The sample was identified by Dr. A'Pham and YA, and was deposited in Corporate Research and Development Division, Takasago

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International Corporation, University Polynesia and Tokushima Bunri University.

2.2 HS-SPME-GC/MS

C. foetidissimum (0.10 g) was kept in a 20 mL vial which was completely closed. The headspace gas was absorbed on a PDMS/DVB type fiber (Supelco) at 60°C for 20 min. Then, the fiber was introduced into an injector of 7890A GC System (Agilent Technologies) equipped with a BC-WAX column (20 m × 0.18 mm i.d., 0.18 µm film thickness). The oven temperature program was set at 45°C with 1 min initial hold and then raised to 230°C at a rate of 20°C/min. The carrier gas was helium with a constant flow of 1 mL/min by split ratio of 10:1. A 5975C inert XL EI/CI MSD (Agilent Technologies) was operated under electron ionization at an ionization energy of 70 eV in scan mode. The injector and the ion source temperatures were set at 250°C and 200°C, respectively. The retention indices were calculated relative to C₈–C₂₇ *n*-alkanes. Compounds were identified using Mass Finder 2.3 program, NIST library, FFNSC 2 library, and mass spectra from the literature and our own library databases.

2.3 Electroencephalogram (CNV: contingent negative variation) measurement

For electroencephalogram measurement and recording, Polymate II AP1132 manufactured by TEAC was used. The sound stimuli employed consisted of a tone burst sound (S1) and visual stimuli (Red or Green small circle: S2) on display LCD were delivered from Multi Trigger System manufactured by Medical Try Systems. Subjects were asked to turn off the circle as quickly as possible by pressing a hand-held button if the red circle would turn on.

An experimental run consisted of twenty trials, each lasting approximately 2 min. The tone burst was sounded randomly and 2.3 sec later the circle on the LCD turn on. The design of odor conditions was 1. Blank, 2. Odor, 3. Odor, 4. Blank and resting period of 5 min were taken between run 2 and run 3.

Standard Ag/AgCl electrodes were placed at Fz and Cz, according to the 10-20 system and were referenced to the linked earlobe.

CNVs to each pair of odor and blank were averaged using EPLYZER II manufactured by KISSEI COMTEC Co. Ltd. The early component of CNV was determined as a negative variation between 400 msec to 1000 msec after S1. The totals of amplitude of the early component of CNV from Fz were calculated and the change ratio for odor condition to the blank condition in each subject was obtained.

2.4 Antimicrobial activity

The methanol extract (100 µg/mL) from *C. foetidissimum* was tested against a few microorganisms, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and

Klebsiella pneumoniae. Antibacterial activity of the methanol extract was determined by micro-broth dilution assay. The bacterial strains were cultured for 18 hours at 37°C and were standardized to a cell density of 1.5×10^8 CFU/mL equivalent to 0.5 McFarland Standard. About 1.0 mL of standardized bacterial strains were seeded in petri-plates containing MH agar by streaking entire MH media surface using sterilized cell spreader under luminous flame. The mixture was then allowed to dry for five minutes. This was followed by the application of plant extract filter paper discs which were placed over the numbered divided parts on culture media. A two-fold serial dilution was used. Concentration was made in range of 100 to 12.5 mL. Plates were covered to avoid contamination and were incubated for 24 hours at 37°C. DMSO impregnated into discs were used as the negative control and standard reference antibiotic streptomycin was used as a positive control. Zones of inhibitions of growth were measured in millimeters. Tests were done in triplicates and the average values were recorded. The minimum inhibitory concentration (MIC) was described as the lowest concentration of the test compounds that completely inhibited the growth of microorganisms.

3 RESULTS AND DISCUSSION

Although the liverwort, *C. foetidissimum* was kept in three layers' Ziploc bag, its characteristic smell was recognized through the double layer's bag after the outer bag was off. Previously Ludwiczuk *et al.*³⁾ reported that two ubiquitous sesquiterpene hydrocarbons in liverworts, bicyclogermacrene and its diastereomer, isoleidozene, and lunularin (= 3,4'-dihydroxylbibenzyl) were identified as the major volatile components from the same species, collected at Ua Fuka Island in Marquesas Islands in French Polynesia, while 4-methoxystyrene (1) and skatole (3) were also identified as the minor volatile components and *C. foetidissimum* has been reported as feces and urine smelling liverwort because of its unpleasant smell which contained 4-vinylphenol, phenol, and a small amount of skatole (3). In the present study, *C. foetidissimum* was applied to HS-SPME-GC/MS analysis. **Figure 1** showed the presence of 4-methoxystyrene (1) (24.4%), 3,4-dimethoxystyrene (2) (28.7%), and skatole (3) (15.9%) as the predominant compounds. Additionally, the volatiles also contained the saturated long chain alkyl aldehydes, from C₆ to C₁₀, as well as, linalool, menthol, and several sesquiterpene hydrocarbons as shown in **Table 1**. The characteristic odor of *C. foetidissimum* reminds of the aged, "old person" smell or old "chest of drawers", or "nostalgic" odor. Skatole (3) has been used as alternative aroma having civet scent after the animal origin aroma, civet was prohibited. Although 4-methoxystyrene (1) is easy to polymerize, it shows the charac-

1. 4-Methoxystyrene, 2. 3,4-Dimethoxystyrene, 3. Skatole

Fig. 1 HS-SPME-GC/MS (TICC) of volatile components from the Tahitian liverwort *Cyathodium foetidissimum*.

Table 1 Volatile compounds from *Cyathodium foetidissimum* by HS-SPME-GC/MS.

Compound Name ^{a)}	RI (BC-WAX)	Area %	Compound Name ^{a)}	RI (BC-WAX)	Area %
Hexanal	1095	0.06	Longifolene	1562	1.35
Isobutyl butyrate	1163	0.03	β -Elemene	1584	0.45
Methyl hexanoate	1185	0.07	Calarene	1588	4.80
Dodecane	1200	0.20	β -Caryophyllene	1589	0.50
Limonene	1207	0.74	Menthol	1626	0.50
1,8-Cineole	1220	0.15	4-Methoxystyrene	1656	24.36
Butyl butyrate	1214	0.12	β -Guaiene	1664	0.48
Ethyl hexanoate	1228	0.05	α -Terpineol	1677	0.24
Isoamyl butyrate	1257	0.77	β -Cadinene	1684	0.84
Octanal	1282	0.08	α -Selinene	1711	0.44
Isoamyl isovalerate	1293	0.11	Benzyl alcohol	1848	0.24
Tridecane	1300	0.15	Phenol	1975	0.15
6-Methyl-5-hepten-2-one	1323	0.09	3,4-Dimethoxystyrene	2004	28.73
Nonanal	1383	0.37	<i>p</i> -Cresol	2054	<0.01
Tetradecane	1400	0.36	<i>m</i> -Cresol	2064	<0.01
δ -Elemene	1468	2.78	2-Phenoxyethanol	2114	0.15
2-Ethylhexanol	1475	1.08	2-Aminoacetophenone	2196	0.02
Decanal	1488	1.28	4-Acetylacetophenone	2330	0.04
Pentadecane	1500	0.20	Veratraldehyde	2361	0.02
Linalool	1531	0.23	Skatole	2451	15.91

^{a)} sorted by elution order on a BC-WAX

teristic aroma of *Ayrshire splendens*, *Ambridge Rose* classified as myrrh-type⁴⁾. 4-Methoxystyrene (1) was also reported as the characteristic aroma of *Rosa hurtula*

(white rose) having the 1,3-dimethoxy-5-methylbenzene (DMMB)-like odor. Besides, 4-methoxystyrene (1) showed 10 times stronger anti-melanin production than kojic acid,

and thus it was claimed as a whitening effect⁵⁾. 3,4-Dimethoxystyrene (2) is almost odorless, and firstly isolated from the Brazilian propolis as the natural product⁶⁾. The Malaysian *Asterella* (or *Mannia*) species retained feces smell because of having 3,4-dimethoxystyrene and skatole^{1b,d)}. We could confirm that the pleasant and nostalgic old person's odor of *C. foetidissimum* is due to the presence of an appropriate proportion of 4-methoxystyrene (1) to skatole (3) and 3,4-dimethoxystyrene (2). A few alkyl aldehydes were detected in *C. foetidissimum*, however, (*E*)-2-nonenal, possessing the characteristic pleasant aged "old person" smell, was not identified in the volatiles. GC-O analysis of the volatiles of *C. foetidissimum* showed the presence of 2-aminoacetophenone, which strongly contributed to the characteristic scent of this rare liverwort as well as skatole (3). Very recently the volatile components of the same liverwort *C. foetidissimum*, found in Costa Rica was analyzed using GC/MS and large level of skatole (3) was detected accounting for around 50% in GC analysis⁷⁾. However, it has not been clear why this liverwort *C. foetidissimum* captured so much skatole (3) in its oil bodies.

By the way, we tried to measure the contingent negative variation (CNV) in the diluted condition. If the concentration of the skatole (3) was high, this smell was very strong malodor like feces and a fart and would be a negative response as a subject's opinion, but when it was diluted with solvent or oils, that is to say, it becomes a suitable concentration, the volunteer's response would be "its smell was nostalgic". The 0.05 % of triethyl citrate solution of the mixture consisted of these three compounds 1, 2, and 3 in the detected ratio was used on the contingent negative variation (CNV) measurement for five volunteers. Then this mixture showed the sedative effect for the human beings as shown in Fig. 2.

Finally, the antimicrobial activity of the methanol extract (100 µg/mL) of *C. foetidissimum* was tested against a few microorganisms, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, however, no activities were observed.

4 CONCLUSION

4-Methoxystyrene (1), 3,4-dimethoxystyrene (2), and skatole (3) were identified as the major components from the fresh Tahitian liverwort *C. foetidissimum*. In GC-O analysis, 2-aminoacetophenone was detected as one of the minor components with a strong aging note. Then, the mixture of the compounds 1–3 showed the sedative effect on CNV measurement in the low concentration.

Fig. 2 CNV : The change ratio of early component.

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