

Volatile Components in Protoplasts Isolated from the Marine Brown Alga *Dictyopteris prolifera* (Dictyotales)

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The volatile compounds released from protoplasts of *Dictyopteris prolifera* were extracted by a closed looping head space (CLHS) procedure. The volatile compounds in the extract were identified by GC and GC-MS. The major characteristic volatile compounds in both the protoplast and the intact plant, were dictyopterenes. Dipropyl disulfide was detected only in the protoplast, whereas cubenol was detected only in the intact plant. These volatile compounds were shown to be produced by the algal enzyme and not by attached bacteria or other epiphytes and then released into Provasoli's enriched seawater modification (PESI) medium. This suggests that a protoplast preparation is a useful tool for research of volatile compounds and their biogeneses in marine algae.

Introduction

Many marine algae are known to produce a wide variety of chemical compounds, some of which have important biological and pharmaceutical application¹⁻⁶⁾. For example, dimethyl sulfide which has "seashore smell" is produced by several kinds of multicellular algae^{7,8)} and unicellular algae⁹⁾. The odor of *Dictyopteris* species can frequently be detected in the air around beaches. Known as *limu lipoa* (seaweed gathered from the deep) in Hawaii, the freshly chopped seaweed is used as a condiment with raw fish and other foods¹⁰⁾. The characteristic odoriferous non-isoprenoid C₁₁ hydrocarbons, namely dictyopterenes were isolated as "ocean smell" from the Hawaiian *D. plagiogramma* and *D. australis*¹¹⁾. Some C₁₁ hydrocarbons are sexual pheromones such as male gamete-attracting substances of the odoriferous oil in the marine brown algae¹²⁾. The Caribbean *D. delicatula* also produces the chemical compounds that deter feeding by reef fishes and amphipods¹³⁾. The occurrence of the dictyopterenes has been confirmed in the essential oil of the Japanese *D. prolifera*¹⁴⁾.

Recently, there has been active study of the plant biotechnology using higher plant tissue culture and cell culture for developing valuable commercial compounds such as medicines, foods, cosmetics, flavors, fragrances and vitamins, which are mainly secondary metabolites and alkaloids, pigments, terpenoids, steroids, oils and fats¹⁵⁻¹⁷⁾. These techniques are important tools for production of commercially useful substances as well as for physiologically and morphologically basic researches. Whereas few papers have been published on this subject for the development of valuable substances

or the production of secondary metabolites in seaweed tissue culture and cell technology^{18,19} many have been written on the improvement of the breed for somatic hybridization in edible seaweeds.

Due to living in a marine environment seaweeds in general have adhesive and symbiotic relationships to various epiphytes and bacteria. Thus, care must be taken when determining the origins of trace components (bioactive compounds, volatile compounds *etc.*) extracted from seaweeds. We reported the production of long chain aldehydes as characteristic flavor compounds by plants that regenerated from protoplasts of the marine green alga *Ulva pertusa*¹⁸ and their production of the higher yields by immobilized-living cells¹⁹. However, no one has reported on the marine brown algae for the production of useful secondary metabolites using seaweed biotechnology.

In the present report, the production of volatile compounds released from living protoplasts of the marine brown alga *D. prolifera* during incubation under sterile conditions was examined by a closed looping head space (CLHS) technique.

Materials and Methods

1. Plant material

D. prolifera was collected from the Yoshimo coast of Shimonoseki, Yamaguchi, Japan.

2. Protoplast isolation

The protoplasts were isolated according to the method described previously²⁰. The protoplasts were isolated from the thalli of *D. prolifera* using a mixed enzyme solution of the oyster enzyme, the abalone enzyme and commercial enzymes such as Cellulase Onozuka R-10, Macerozyme R-10, Driselase, Bigalase M and Sumizyme X.

3. Volatile compounds analysis

The volatile compounds released from living protoplasts and the intact plant of *D. prolifera* were harvested by the CLHS apparatus (**Fig. 1**) modified technique of Grob and Zürcher²¹. Prior to use, all parts of the apparatus were autoclaved and air from the pump was sterilized by filtration through a 0.20 μm Dismic-25 disposable syringe filter unit (Advantec Toyo Kaisha Ltd., Tokyo, Japan). Then, the apparatus was made up in a clean hood. The compounds from plant materials (2 g fresh weight) in 200 ml of the antoclaved PESI medium²² were adsorbed onto activated

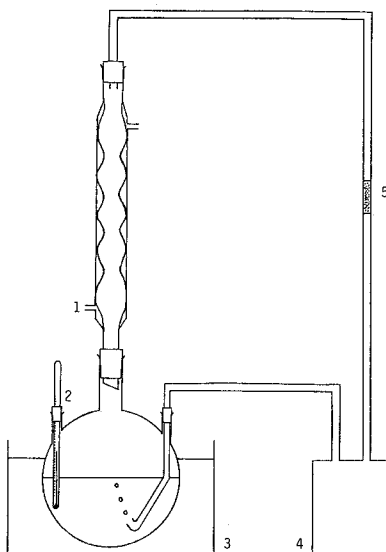


Fig. 1 Closed Looping Head Space apparatus.

1, Cool water; 2, Thermometer; 3, Water bath; 4, Pump; 5, Charcoal.

charcoal (100 mg, 30/60 mesh, Gasukuro Kogyo Inc., Tokyo, Japan) for 24 hr by the CLHS procedure, while the cultures were incubated at 18–20 °C under white fluorescent light (14 hr cycle of 20 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The adsorbed compounds were desorbed from the charcoal with dichloromethane (3 ml). The desorbed compounds were removed from the solvent by concentration. The resultant solutions (6 μl each) were subjected to GC and GC-MS analysis under the conditions described previously¹⁸. The volatile compounds were identified by retention indices²³ against GC retention time of C_5 – C_{25} *n*-paraffins and by mass spectra of GC-MS using authentic reference standards.

Results

The volatile compounds released from protoplasts and from the intact plant of *D. proliferata* were identified by comparing their retention indices on GC and mass spectra of GC-MS with those of authentic specimens. The contents of 10 typical compounds were quantitatively determined in the protoplasts and in the intact plant, respectively (**Table 1**). The major characteristic of the volatile compounds from the protoplast were various non-isoprenoid C_{11} hydrocarbons (*ca.* 99%) such as

Table 1 Production of volatile compounds in protoplast and in intact plant of *Dictyopteris proliferata*.

Compound	RI ^{*1}	Peak area (%)	
		Protoplast	Intact plant
3-Butyl-4-vinylcyclopentene (5)	1063	0.93	0.94
1-[(1 <i>Z</i>)-Hexenyl]-2-vinylcyclopropane (6)	1081	tr. ^{*2}	0.42
Dipropyl disulfide (10)	1092	0.79	— ^{*3}
Dictyopterene A (1)	1105	19.08	18.67
4-[(1 <i>E</i>)-Hexenyl]-cyclopentene (7)	1114	3.18	6.62
Dictyopterene D' (4)	1147	4.62	8.91
6-[(1 <i>E</i>)-Butenyl]-1,4-cycloheptadiene (8)	1156	2.27	3.95
Dictyopterene B (2)	1161	15.82	16.00
Dictyopterene C' (3)	1165		
Dictyoprolene (9)	1378	9.12	10.17
Cubenol (11)	1642	—	3.49

^{*1} Retention index found on DB-1 column. ^{*2} trace (<0.01%).

^{*3} Not detected.

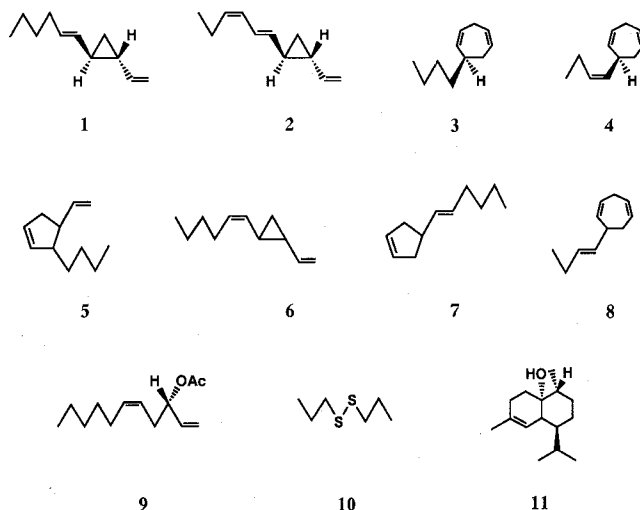


Fig. 2 Structures of volatile compounds.

dictyopterences which were present in the intact plant. The dictyopterene compounds consisted of dictyopterene A [1-[(1*E*)-hexenyl]-2-vinylcyclopropane] (**Fig. 2-1**), dictyopterene B [1-[(1*E*, 3*Z*)-hexadienyl]-2-vinylcyclopropane] (**2**), dictyopterene C' [6-butyl-1, 4-cycloheptadiene] (**3**) and dictyopterene D' [6-[(1*Z*)-butenyl]-1, 4-cycloheptadiene] (**4**). Compound **1**: RI 1105; MS *m/z* (%) 150 (*M*⁺, 2), 121 (2), 107 (6), 105 (1), 93 (30), 91 (17), 80 (43), 79 (100), 77 (17), 67 (28), 66 (21), 55 (13), 41 (28). **2**: RI 1161; MS *m/z* (%) 148 (*M*⁺, 9), 133 (4), 119 (22), 117 (5), 105 (34), 91 (77), 80 (14), 79 (100), 77 (24), 67 (28), 66 (51), 55 (14), 41 (57). **3**: RI 1165; MS *m/z* (%) 150 (*M*⁺, 10), 121 (4), 107 (12), 93 (65), 91 (28), 80 (52), 79 (100), 77 (28), 67 (21), 66 (19), 55 (9), 41 (28). **4**: RI 1147; MS *m/z* (%) 148 (*M*⁺, 9), 133 (4), 119 (23), 117 (5), 105 (34), 91 (76), 80 (17), 79 (100), 77 (22), 67 (28), 66 (46), 55 (14), 41 (58). Then, their related compounds such as 3-butyl-4-vinylcyclopentene (**5**), 1-[(1*Z*)-hexenyl]-2-vinylcyclopropane (**6**), 4-[(1*E*)-hexenyl]-cyclopentene (**7**), 6-[(1*E*)-butenyl]-1, 4-cycloheptadiene (**8**) and dictyoprolene (**9**) were also identified. Compound **6** and **8** are the (*Z*)-isomer of dictyopterene A (**1**) and the (*E*)-isomer of dictyopterene D' (**4**), respectively²⁴. On the other hand, dipropyl disulfide (**10**) was found in the protoplast, but was not detected in the intact plant. Compound **10**: RI 1092; MS *m/z* (%) 152 (3), 150 (*M*⁺, 32), 110 (2), 108 (25), 74 (4), 73 (3), 66 (7), 47 (3), 43 (100), 41 (19). With sesquiterpene, cubenol (**11**) was found in the intact plant, but not in the protoplast. Compound **11**: RI 1642; MS *m/z* (%) 222 (*M*⁺, 2), 204 (41), 179 (63), 161 (100), 135 (8), 119 (72), 105 (46), 95 (19), 81 (31), 69 (16), 55 (17), 43 (23).

Discussion

The dictyopterenes (**1-4**) have been known as odoriferous constituents responsible for the "ocean smell"¹¹ and sexual pheromones as male gamete-attracting substances in the marine brown algae¹². Dictyopterene B (**2**) and dictyopterene D' (**4**) were named hormosirene and ectocarpene, respectively, as the sex-pheromones of the brown algae *Hormosira banksii* and *Ectocarpus siliculosus*¹². In the biogenesis of the brown algal pheromones, ectocarpene was probably derived from the *cis*-disubstituted isomer of hormosirene²⁵. Hormosirene was also isomerized to ectocarpene through thermal Cope rearrangement in the heating condition of GC analysis²⁶. While dipropyl disulfide (**10**) is the first identification in the marine algae, this sulfur containing organic compound is a major odoriferous component in *Allium* species such as onion and garlic²⁷. The sulfur compound was also discovered to be a sulfur-containing feeding attractant promoting the striking behavior in goldfish *Carassius auratus*, carp *Cyprinus carpio* and crucian carp *Carassius auratus cuvieri*²⁸. Diisopropyl disulfide related to **10** was only observed from *Microcystis flos-aquae* and used as a chemical marker for this species in the aquatic ecosystem because different *Microcystis* species do not produce the compound²⁹. Also, cubenol (**11**) was for the first time identified in commercial cubeb oil³⁰ and in the wood of *Cedrela toona* Roxb.³¹ in higher plants. Further, the cadinane-type sesquiterpene alcohol was found in the brown alga *D. divaricata*³² and cultured red tide of *Gymnodinium nagasakiense*³³. The sesquiterpene alcohol was shown to cause cell-destroying activity against the red tide planktons³³.

It was confirmed that dictyopterenes (**1-4**) and related compounds (**5-9**) and dipropyl disulfide (**10**) can be extracted from living protoplasts without any damage and released into a liquid medium under sterile culture conditions in the CLHS apparatus. The results suggest that these intrinsic volatile compounds of *D. prolifera* were produced by the algal enzyme, not by various adhesive organisms such as epiphytes and bacteria. The extraction technique of volatile compounds by the CLHS apparatus may also be used to investigate secretions in very dilute solutions under natural conditions and their metabolism in living organisms. The typical volatile compounds from

D. prolifera might be biosynthesized through two different metabolic pathways: fatty acids and mevalonic acids. The hypothetic biosynthesis of dictyopterenes *via* (3*Z*, 6*Z*)-dodecadienoic acid or (3*Z*, 6*Z*, 9*Z*)-dodecatrienoic acid from unsaturated fatty acids has been proposed by Boland and Mertes²⁵). On the other hand, cubenol (**11**) is biosynthesized *via* (2*Z*, 6*E*)-farnesyl pyrophosphate from mevalonic acid³⁴). With the protoplast the biosynthetic pathway from fatty acids was operative, while the mevalonic acid pathway to the cadinane-type sesquiterpene did not work. This may be the reason that the mevalonic acid pathway has not led to the sesquiterpene due to the poor accumulation of lower terpenoids in the undifferentiated protoplast. In most undifferentiated plant cell cultures, the production of mono- and sesqui-terpenes in the desired quality and quantity has not been achieved³⁵). The primary reason for the poor accumulation of lower terpenoids in undifferentiated plant cells is considered to be the general absence of specialized secretory vesicles in which lower terpenoids are stored³⁶). However, it is possible to utilize immobilization of the protoplast as a technique to enhance the production of desired secretions from *D. prolifera*. Further, as an example, bioreactors packed with immobilized-living protoplasts are expected to make mass production of the dictyopterenes possible by adding various substrates, *e. g.*, natural and synthetic unsaturated fatty acids. These results indicate that the protoplast isolated from *D. prolifera* might provide a promising source for research on the production and biosynthesis of volatile compounds.

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《和文要約》

海産褐藻ヘラヤハズからのプロトプラストの揮発性成分

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海産褐藻アミジグサ科ヘラヤハズからのプロトプラストによる揮発性成分は、細胞を生きたままで培養しながら抽出する閉環式ヘッド・スペース (CLHS) 法, GC および GC-MS によって分析し、母藻体のそれと比較した。それらの両者は、共にほぼ共通の揮発性成分をもち、なかでも主要な特徴的成分のディクテオブテレン類は、CLHS 法により PESI 培地中に放出されていることが確認された。このことは、この化合物群が藻体そのものに起源をもち、その表面に付着する微小生物由来ではないことを示した。一方、ジプロピルジスルフィドは、プロトプラストのみに、キューベノールは、母藻体のみにみいだされた。すなわち、プロトプラスト化は、海藻の揮発性成分を研究する上で有用な手法であることが示唆された。