Phytochemistry 107 (2014) 42–49

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Arachidonic acid-dependent carbon-eight volatile synthesis from wounded liverwort (*Marchantia polymorpha*)

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ARTICLE INFO

Article history:
Received 8 March 2014
Received in revised form 25 July 2014
Available online 28 August 2014

Keywords:
Common liverwort
Marchantia polymorpha
Marchantiaceae
Bryophyte
Acetylation
Oxidoreduction
1-Octen-3-ol
1-Octen-3-yl acetate
Octan-3-one

ABSTRACT

Eight-carbon (C8) volatiles, such as 1-octen-3-ol, octan-3-one, and octan-3-ol, are ubiquitously found among fungi and bryophytes. In this study, it was found that the thalli of the common liverwort *Marchantia polymorpha*, a model plant species, emitted high amounts of C8 volatiles mainly consisting of (*R*)-1-octen-3-ol and octan-3-one upon mechanical wounding. The induction of emission took place within 40 min. In intact thalli, 1-octen-3-yl acetate was the predominant C8 volatile while tissue disruption resulted in conversion of the acetate to 1-octen-3-ol. This conversion was carried out by an esterase showing stereospecificity to (*R*)-1-octen-3-yl acetate. From the transgenic line of *M. polymorpha* (*des6*^{KO}) lacking arachidonic acid and eicosapentaenoic acid, formation of C8 volatiles was only minimally observed, which indicated that arachidonic and/or eicosapentaenoic acids were essential to form C8 volatiles in *M. polymorpha*. When *des6*^{KO} thalli were exposed to the vapor of 1-octen-3-ol, they absorbed the alcohol and converted it into 1-octen-3-yl acetate and octan-3-one. Therefore, this implied that 1-octen-3-ol was the primary C8 product formed from arachidonic acid, and further metabolism involving acetylation and oxidoreduction occurred to diversify the C8 products. Octan-3-one was only minimally formed from completely disrupted thalli, while it was formed as the most abundant product in partially disrupted thalli. Therefore, it is assumed that the remaining intact tissues were involved in the conversion of 1-octen-3-ol to octan-3-one in the partially disrupted thalli. The conversion was partly promoted by addition of NAD(P)H into the completely disrupted tissues, suggesting an NAD(P)H-dependent oxidoreductase was involved in the conversion.

1. Introduction

Eight-carbon (C8) volatiles, such as 1-octen-3-ol (**5**), octan-3-one (**1**), and octan-3-ol (**4**) (Fig. 1), are ubiquitously found among fungi and bryophytes (Combet et al., 2006; Croisier et al., 2010; Asakawa et al., 2013). C8 volatiles are important flavor constituents in mushrooms (Combet et al., 2006), while they are also responsible for off-flavors in beverages like wine (Steel et al., 2013). C8 volatiles attract, or in some cases repel, arthropods (Combet et al., 2006). Since some vector insects carrying human disease are attracted by C8 volatiles, there is the potential for chemo-attraction. This raises the expectation that controlling the behavior of insects with C8 volatiles could lead to novel strategies to control certain diseases (Carlson and Carey, 2011). Recently, it has been reported that **5** may cause dopamine neuron degeneration in *Drosophila* that might result in Parkinson's disease (Inamdar et al., 2013). C8 volatiles are also known to possess bioactivity properties toward plants, including induction of oxidative burst, inhibition of growth, and induction of a subset of defense genes in *Arabidopsis thaliana* (Splivallo et al., 2007; Kishimoto et al., 2007). Conidiation of *Trichoderma* spp. was induced by **5**, **1**, and **4** (Nemcovic et al., 2008), but these volatiles also inhibited germination of *Penicillium paneum* and *Aspergillus nidulans* conidia at high concentrations (Chitarra et al., 2004; Herrero-Garcia et al.,

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http://dx.doi.org/10.1016/j.phytochem.2014.08.008
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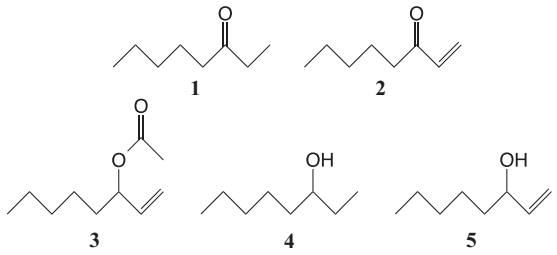

Fig. 1. Structures for compounds **1**–**5**.

2011). Because of these bioactivities, it has been assumed that C8 volatiles perform a signaling function when in the vapor phase.

Oxygenation of polyunsaturated fatty acids and subsequent rearrangement of the resulting hydroperoxides (HPOs) lead to the formation of a large family of oxygenated products, collectively known as oxylipins (Feussner and Wasternack, 2002). C8 volatiles are oxylipins formed from the di-oxygenation of linoleic acid (**6**) or arachidonic acid (**7**) to yield the respective HPO followed by cleavage (Combet et al., 2006; Croisier et al., 2010). Within fungi, a C18 fatty acid such as **6** is converted into its 10(*S*)-HPO and then cleaved to yield (*R*)-**5** and the C10 oxo acid (Wurzenberger and Grosch, 1982). Very recently, it was shown that a cyanobacterium (*Nostoc punctiforme*) forms **5** from **6** via cyclooxygenase- and catalase-related enzymes (Brash et al., 2014). The diatom (*Thalassiosira rotula*) also has an ability to dioxygenize and subsequently cleave fatty acids to form 6-hydroxy-7-octenoic acid (**8**), which is structurally related to **5**, from hexadecatrienoic acid (Barofsky and Pohnert, 2007). Bryophytes have pathways to form oxylipins from either C18 or C20 fatty acids (Croisier et al., 2010). In the moss *Physcomitrella patens*, the precursor **7** was converted to **5** through the formation of its respective 12-HPO (Wichard et al., 2005). In spite of these observations, the enzymes involved in **5** formation have not yet been identified with the exceptions of the cyclooxygenase/catalase from *N. punctiforme* and a lipoxygenase (LOX) from *P. patens*. When recombinant LOX derived from *P. patens* was expressed in *Escherichia coli*, **5** as well as (*Z*)-2-octen-1-ol (**9**) were formed from **7** *in vitro* (Senger et al., 2005). Furthermore, little is known about the pathways employed to form C8 volatiles other than **5**, such as 1-octen-3-yl acetate (**3**), **1**, 1-octen-3-one (**2**), or **4**.

In this study, the metabolic pathway responsible for the formation of C8 volatiles was examined in *Marchantia polymorpha* (liverwort). *M. polymorpha* is an emerging model plant suitable for molecular genetics, as it can be used for the preparation of isogenic lines for molecular and biochemical experiments, is easily transformed, and its genome is currently being sequenced (Kubota et al., 2013).

2. Results

2.1. Emission of volatiles after mechanical wounding of thalli grown in the field

It has been reported that some liverworts emit carbon 8 (C8) volatiles as well as terpenoids (Suire et al., 2000; Asakawa et al., 2013). However, it is not known whether the C8 volatiles are emitted when liverworts are grown in their natural habitat. In this study, volatile compounds in the headspace of thalli from *M. polymorpha* grown in its natural habitat were collected using a headspace SPME-GC/MS method.

Only minimal emission of volatiles was detected from the intact thalli of *M. polymorpha* (Fig. 2). When the thalli were mechanically wounded with forceps to form wounds on *ca.* 10% of the thalli, massive formation of volatiles was observed. Compounds **1** and **5** were the predominant C8 volatiles, while **2**, **3**, and **4** were detected as minor C8 volatiles. The MS profiles of other peaks with retention times ranging from 12 to 16 min suggested that they were C15 sesquiterpenoids with the chemical formulae $C_{15}H_{24}$ (*m*/*z* 204, with peaks a–j) and $C_{15}H_{22}$ (*m*/*z* 202, with the peak j); (Asakawa et al., 2013; Suire et al., 2000, Supplemental Fig. S1); however, these were not identified. The amounts of C8 volatiles were quantified by using calibration curves made with standards (inset of Fig. 2). The amounts of **1** and **5** increased *ca.* three fold after mechanical wounding and reached approximately 15 nmol m^{-2} h^{-1}.

The time course of **1** and **5** emission from the thalli after mechanical wounding was followed. Because solid-phase microextraction (SPME) collection is rather static and unsuitable for estimation of dynamic changes of volatiles, a dynamic charcoal collection system was used that involved pulling air through a charcoal cartridge using an air pump. The amounts of **1** and **5** increased within 40 min after mechanical wounding; thereafter, the values gradually decreased, and returned back to their original levels after 160 min (Fig. 3).

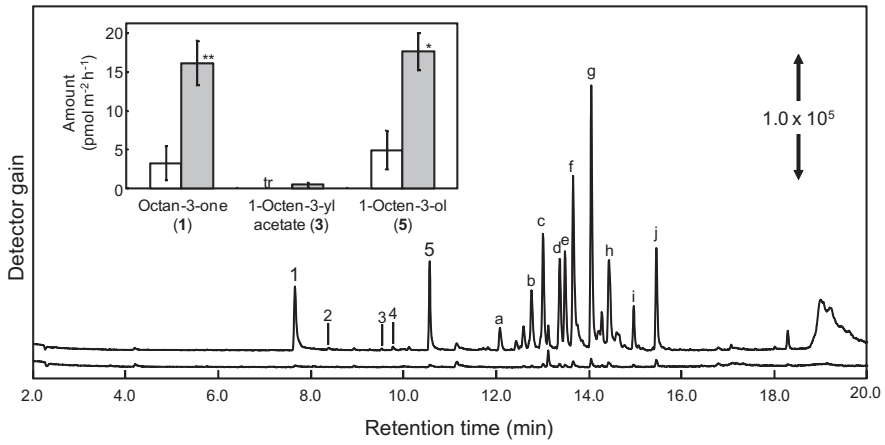

Fig. 2. Effect of mechanical wounding on volatiles emitted from field-grown *M. polymorpha* thalli. A mat of intact thalli (58.1 cm^2) was gently covered with a plastic cup, and volatiles accumulated in the headspace were collected using a SPME fiber for 30 min (intact, lower trace). The thalli were wounded with forceps, and then covered with the cup for SPME collection (wounded, upper trace). The inset shows the amounts of volatiles from intact (white bars) and wounded (black bars) thalli. Average ± s.e. (n = 5) is shown. Asterisks indicate significant differences for the noted compound (Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$). **1**: octan-3-one, **2**: 1-octen-3-one, **3**: 1-octen-3-yl acetate, **4**: octan-3-ol, **5**: 1-octen-3-ol. Putative C15 sesquiterpenoids with the chemical formulas $C_{15}H_{24}$ (*m*/*z* 204, with peaks a–i) and $C_{15}H_{22}$ (*m*/*z* 202, with the peak j) were tentatively identified based on their MS profiles (Supplemental Fig. S1); however, they are not yet identified.

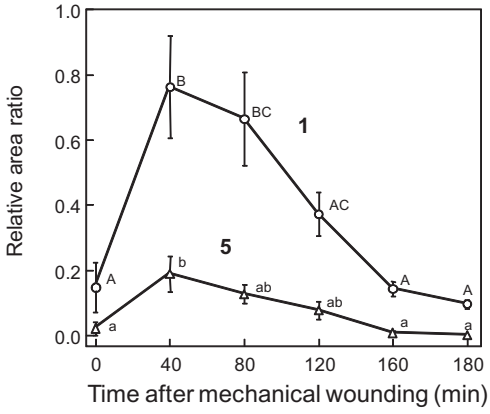

Fig. 3. Time course of emission of **1** and **5** after mechanical wounding of *M. polymorpha* thallus grown in a field. Average ± s.e. (n = 6) is shown. Different letters for each compound refer to significant differences (ANOVA, Bonferroni, P < 0.01).

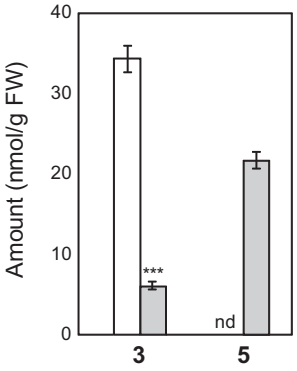

Fig. 5. Effect of incubation after tissue disruption on C8 volatile formation. The thalli were frozen and the volatiles were extracted (white bars), or the frozen thalli were thawed, and incubated for 5 min at 24 °C to facilitate the enzyme reaction (gray bar). Average ± s.e. (n = 4) is shown. Asterisks indicate significant differences for the noted compound (Student's t-test, $^{***}P$ < 0.001).

2.2. Volatiles in tissues

Rapid formation of volatile oxylipins after mechanical wounding is similar to C6 green leaf volatile formation from vascular plants (Matsui, 2006; Matsui et al., 2012). In vascular plants, the amounts of green leaf volatiles in intact tissues are usually low, but after disruption of the plant tissues, they are rapidly formed from endogenous substrates such as free fatty acids and glycerolipids through successive catalysis by LOXs and HPO lyases (Nakashima et al., 2013). Accordingly, it was assumed that C8 volatiles are formed *de novo* from C18 or C20 fatty acids through oxygenation and subsequent cleavage after disruption of the tissues (Wichard et al., 2005; Combet et al., 2006). On the other hand, it should be noted that liverworts contain oil bodies that most probably accumulate diversified mixtures of terpenoids and/or aromatic compounds (Suire et al., 2000; He et al., 2013). Thus, it is also possible that the C8 volatiles accumulate in certain cells or organelles such as oil bodies, and that they are simply released because the walls of the reservoirs are disrupted (Tissier, 2012).

To distinguish between these two possibilities, volatiles from intact thalli were extracted with an organic solvent, which would help to eliminate any enzymatic reactions that might be promoted after mechanical damage to the plant tissues. In the intact thalli, the accumulation of compounds was found with the retention times ranging from 21 to 28 min and from 29 to 35 min (Fig. 4). The peaks k–p, and the peaks q and r were tentatively identified as sesquiterpenoids and oxidized sesquiterpenoids, respectively, based on their MS profiles (Supplemental Fig. S2); however, these were not investigated further. Oxidized sesquiterpenoids were probably present because solvent extraction was used as a separation method instead of SPME-GC/MS (Fig. 4). Among the C8 volatiles, only **3** was detected in intact thalli. When frozen thalli were thawed and incubated for 5 min at 24 °C to disrupt the tissues, the amount of **3** decreased while that of **5** increased (Figs. 4 and 5). The amounts and compositions of putative sesquiterpenoids and oxidized sesquiterpenoids, however, remained essentially unchanged from those detected from intact thalli after incubation of freeze-disrupted thalli at room temperature.

2.3. Hydrolysis of *3* to *5*

It was expected that formation of **5** after mechanical wounding would be dependent on the hydrolysis of **3** stored in the thalli. To confirm this, crude enzyme extract from the thalli were prepared and added to **3**, and it was efficiently hydrolyzed to yield **5**. The highest activity was observed from pH 7.0 to 8.0, and the amount of **5** formed was dependent on the amount of crude enzyme soln. added to the reaction mixture (Supplemental Fig. S3). Furthermore, the activity disappeared when the crude extract was heat-denatured. These results suggested that the thalli possess an esterase

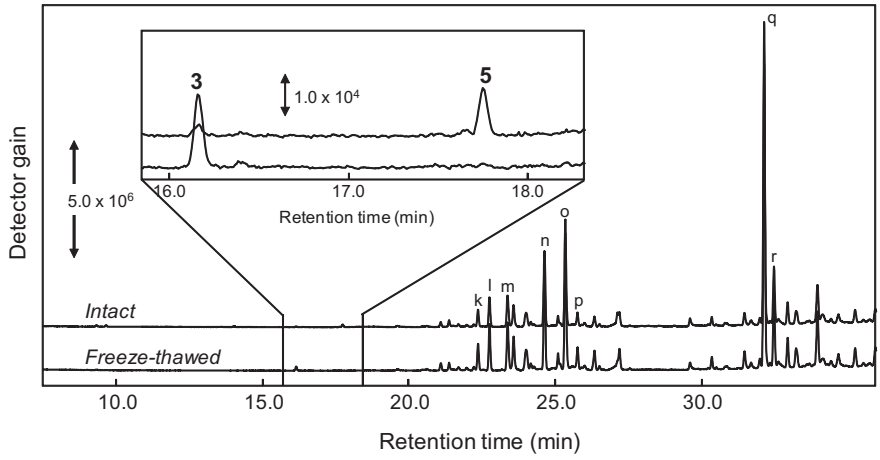

Fig. 4. Effect of incubation after tissue-disruption on the amounts of endogenous volatiles. Volatiles were extracted with methyl *tert*-butyl ether from intact thalli (lower chromatogram) or from freeze–thaw treated thalli (upper chromatogram), and analyzed using GC–MS. Inset shows the enlarged chromatogram from 15.8 to 18.4 min. C15 sesquiterpenoids with the chemical formulas $C_{15}H_{24}$ (m/z 204, with peaks k to p) and $C_{15}H_{26}O$ (m/z 222, with the peaks q and r) were tentatively identified based on their MS profiles (Supplemental Fig. S2).

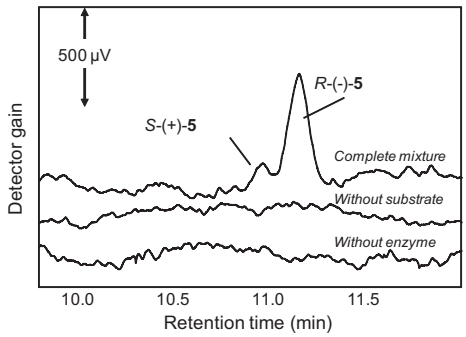

Fig. 6. Resolution of enantiomers of **5** formed from racemic **3**. The crude enzyme extract prepared from *des6*^{KO} thalli was reacted with racemic **3**, and the **5** formed by enzyme catalyzed hydrolysis was subjected to chiral phase GC analysis (upper chromatogram). **5** was not detected with the reaction mixture prepared without substrate (middle chromatogram) or without enzyme (lower chromatogram).

capable of hydrolyzing **3** to yield **5**. When a racemic mixture of **3** was used as substrate, the esterase showed a slight preference for the (*R*)-enantiomer, and yielded (*R*)-**5** with an enantiomeric excess (e.e.) value of 64% (Fig. 6). When the frozen powder was thawed in the absence of oxygen (in the presence of Ar), the amount of **5** formed was almost equivalent to the amount formed in the presence of oxygen. Thus, **5** formation after tissue disruption was independent of molecular oxygen.

2.4. Effect of the absence of arachidonic acid (*7*)

It has been reported that linoleic acid is the substrate from which **5** is formed in mushrooms, fungi, and cyanobacteria (Wurzenberger and Grosch, 1982; Combet et al., 2006; Brash et al., 2014). However, it was also recently reported that **7** was converted into **5** in most bryophytes (Croisier et al., 2010; Senger et al., 2005). *M. polymorpha* thalli contain a substantial amount of **7** (3.1 mol% of total fatty acids), as well as **6** (6.4 mol%) (Kajikawa et al., 2008). Therefore, both **6** and **7** may act as substrates for the formation of **5** and its derivatives in *M. polymorpha*. Recently, a Δ6-desaturase gene (*MpDES6*) was knocked out that is involved in the desaturation steps to form γ-linolenic acid (18:3$\Delta^{6,9,12}$) (**10**) from **7** and stearidonic acid (18:3$\Delta^{6,9,12,15}$) (**11**) from α-linolenic acid (18:3$\Delta^{9,12,15}$) (**12**), respectively, (Kajikawa et al., 2008) through a targeted gene-disruption technique (Ishizaki et al., 2013; Kubota et al., 2014). In the transgenic line (*des6*^{KO}), both the *n*-6 pathway and *n*-3 pathway were terminated at the Δ6-desaturase step, thus, **6** and **12** accumulated and no formation of **7** and eicosapentaenoic acid (20:5$\Delta^{5,8,11,14,17}$) (**13**) was observed (Fig. 7). When the thalli of the wild type strain was used for volatile analysis, 30.4 ± 1.08 nmol g fr wt^{-1} of **3** was detected in intact thalli tissues and 26.3 ± 0.58 nmol g fr wt^{-1} of **5** was detected in freeze–thawed thalli after solvent extraction. In contrast, these C8-volatiles were only minimally detected in intact or freeze–thaw-treated thalli of the *des6*^{KO} plant (see Table 1). This result suggested that **7** and/or **13** but not **6** was the substrate for **5** formation in *M. polymorpha* thalli.

2.5. Conversion of *5 to 3*

Because **5** is the primary product of the oxylipin pathway starting from fatty acids (Wurzenberger and Grosch, 1982; Wichard et al., 2005), its acetate was assumed to be formed from **5** via the activity of an acetylation enzyme. In the green leaf volatile pathway in vascular plants, an acetyl CoA transferase is involved in the formation of (*Z*)-3-hexen-1-yl acetate from (*Z*)-3-hexen-1-ol (D'Auria et al., 2007). Therefore, the ability of *M. polymorpha* thalli

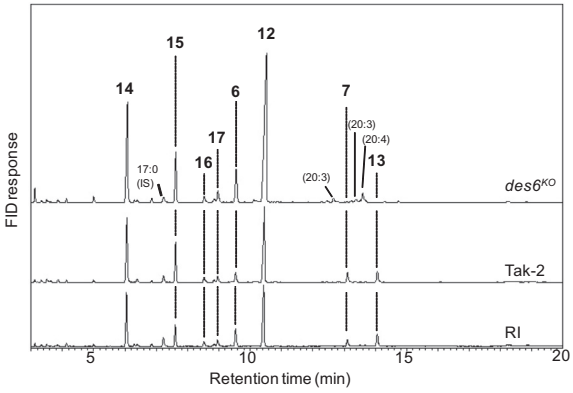

Fig. 7. Fatty acid profiles of thalli from wild type (accession Tak-2 and RI) and mutant (*des6*^{KO}) *M. polymorpha*. Total lipids were extracted from thalli of Tak-2 (Takaragaike-2) (a female parent of *des6*^{KO}), RI (a cross between Tak-1 and Tak-2), and *des6*^{KO}, and their fatty acid compositions were analyzed with GC–MS. The positions of double bonds in 20:3 and 20:4 fatty acid species detected only with *des6*^{KO} thalli (shown in parentheses) were not determined. **6**: linoleic acid (18:2$\Delta^{9,12}$), **7**: arachidonic acid (20:4$\Delta^{5,8,11,14}$), **12**: α-linolenic acid (18:3$\Delta^{9,12,15}$), **13**: arachidonic acid (20:5$\Delta^{5,8,11,14,17}$), **14**: palmitic acid (16:0), **15**: hexadecatrienoic acid (16:3$\Delta^{7,10,13}$), **16**: stearic acid (18:0), **17**: oleic acid (18:1Δ^{9}).

Table 1
Effect of exposing *des6*^{KO} thalli to **5** on the amounts of C8 volatiles produced.

	Control	Exposure of **5**
	(nmol g FW^{-1})	
1	nd*	27.3 ± 5.34
2	nd	nd
3	nd	18.7 ± 3.97
5	nd	5.48 ± 2.82

The amounts are given as means ± SE (n = 4).
* nd: not detected.

to form **3** from **5** was investigated. For this, the *des6*^{KO} mutant was used that lacked the ability to form **5** and its derivatives because conversion of **5** to its acetate could be easily followed in the absence of endogenous C8 volatiles. After exposing the mutant thalli to the vapor of **5** for 18 h, accumulation of **3** as well as **5** and **1** was detected while nothing was detected when thalli were exposed to the carrier solvent (Table 1). This indicated that *M. polymorpha* absorbed **5** from the atmosphere and converted it into **3**.

2.6. NAD(P)H-dependent oxidation/reduction of *5*

In the field experiment, partially wounded thalli emitted a substantial amount of **1** (Fig. 2), whereas **1** was only minimally detected in intact thalli or completely disrupted thalli (Fig. 3). Partial wounding, but not complete disruption, might be the prerequisite to form **1**. This was reminiscent of green leaf volatile metabolism where complete disruption of leaf tissues resulted in the formation of the primary product, hexenal in this case, while partial wounding of the leaf tissues resulted in the conversion of hexenal to the corresponding hexen-1-ol in the presence of NADPH which served in maintaining intact tissues located alongside the disrupted tissues (Matsui et al., 2012).

From intact *M. polymorpha* thalli, emission of the C8 volatiles was generally undetectable, but partial mechanical wounding facilitated the emission of **1** (Fig. 7). When the thalli were completely homogenized without addition of any cofactors, emission of **1** was largely suppressed, while **3** and **5** were the prominent C8 volatiles emitted into the headspace (Fig. 7). When NADH was added during disruption, the amount of **5** was significantly

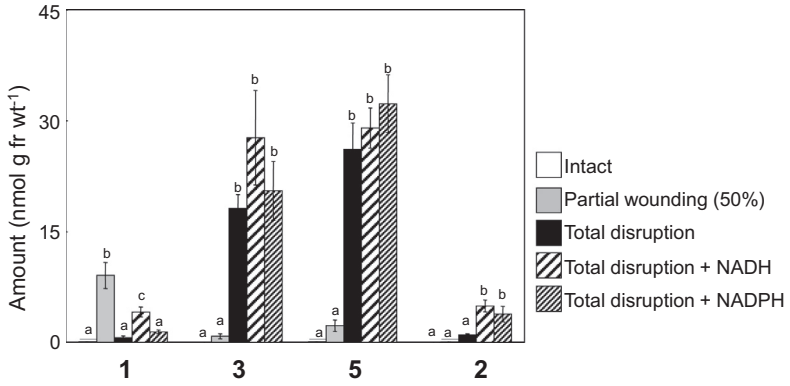

Fig. 8. Effect of addition of NAD(P)H on the C8 volatiles emitted from *M. polymorpha* thalli. C8 volatiles extracted from intact (white bars), partially wounded (50%, gray bars), totally disrupted (black bars), totally disrupted in the presence of NADH (coarse stripe), and totally disrupted *M. polymorpha* thalli in the presence of NADPH (dense stripe) were quantified. The average ± s.e. (n = 4–5) is shown. Different letters for each compound refer to significant differences (one way ANOVA, Fisher, $P < 0.05$).

increased while levels of **3** and **5** were largely unchanged. Addition of NADH or NADPH significantly increased the amount of **2** (Fig. 8). Addition of either NAD^+ or $NADP^+$ did not change the amount and composition of C8 volatiles emitted from the homogenate.

An enzyme assay was next performed using a crude enzyme soln. prepared from *M. polymorpha* thalli with **5** as a substrate. In the absence of cofactors, formation of **2** from **5** was detected (Table 2). When either NADH or NADPH was added to the reaction mixture, formation of **2** was entirely suppressed, while **1** was formed from **5**.

3. Discussion

The results described herein indicated that in *M. polymorpha* thalli (*R*)-**5** is formed from **7** or **13**. (*R*)-**5** is stored in the tissues as **3** after acetylation. Upon tissue wounding, the acetate was hydrolyzed to yield (*R*)-**5**. A portion of **5** remaining in the tissues was converted into **1** via **2** (Fig. 8). As a result, (*R*)-**5** and **1** were released from the wounded thalli of *M. polymorpha* (Fig. 9).

Even though **5** is widely distributed among plant and fungal kingdoms, the enzyme system involved in its biosynthesis has not yet been identified. In mushrooms (*Agaricus bisporus*), **6** was converted to (*R*)-**5** via a LOX-like reaction from its 10(*S*)-HPO and in turn, the subsequent HPL-like reaction on the 10(*S*)-HPO (Wurzenberger and Grosch, 1982). In the moss *P. patens*, **7** was converted into (*R*)-**5** through formation of its 12(*S*)-HPO (Wichard et al., 2005). By using a knock-out mutant lacking the desaturase enzyme essential to form **7** and **13**, it can be suggested that these fatty acids are the substrates used for the formation of **5** in the liverwort *M. polymorpha*. One of the LOXs identified in *P. patens*, PpLOX1, catalyzed the formation of **5** and (*Z*)-2-octen-1-ol from **7** *in vitro*. Based on this, it was assumed that PpLOX1 was primarily responsible for the formation of **5** in the moss (Senger et al., 2005). This agrees with the known chemistry of the β-scission reaction toward the alkoxy radical derived from the 12-HPO of **7**, where both **5** and (*Z*)-2-octen-1-ol are the products (Delcarte et al., 2001). However, this result does not agree with the composition of C8 volatiles observed in wounded *M. polymorpha* thalli, where (*Z*)-2-octen-1-ol was undetectable. Three *LOX* genes, *MpLOX1* to *3*, have been examined to date and shown to form the 15(*S*)-HPO of **7** (Kanamoto et al., 2012). Nine additional genes homologous to LOX were identified in the EST database of *M. polymorpha*. Further examination of the substrate and product specificities of the MpLOXs other than MpLOX1 to 3 should be conducted to identify the enzyme(s) involved in **5** formation from **7** and **13** in *M. polymorpha*.

Table 2
Effect of NADH and NADPH on the conversion of **5** to **1** or **2** in the presence of crude enzyme prepared from *M. polymorpha* (wild type) thalli.

	1	2
	(nmol mL^{-1} min^{-1})	
No addition	nd*	1.14 ± 0.414
NADH	1.94 ± 0.244	nd*
NADPH	2.39 ± 0.380	nd*

The amounts are given as means ± SE (n = 4).
* nd: not detected.

Rapid emission of volatile compounds after mechanical wounding is observed with a wide array of plants and fungi (Arimura et al., 2009). There are two types of responses that induce volatile emission. One is the up-regulation of genes coding enzymes involved in the synthesis of volatiles, a process often observed for terpenoid synthesis induced after mechanical wounding of vascular plants and occurs over several hours (Arimura et al., 2009). The other is the rapid emission of volatiles without gene up-regulation that occurs within seconds to minutes. In the latter case, rapid volatile emission is supported by *de novo* synthesis from endogenously reserved precursors with pre-existing enzymes, or via release of volatile compounds indigenous to a special reservoir in plant tissues. Here, it was found that rapid emission of **5** and **1** occurred after mechanical wounding of *M. polymorpha* thalli, this being supported by the observation that hydrolysis of **3** accumulating in the tissues yielded **5**, and that by subsequent oxido-reduction of a portion of **5** to **1**. Tissue disruption of the fruiting bodies of *A. bisporus* (Combet et al., 2009) and the mycelium of *Penicillium* sp. (Kermasha et al., 2002) promotes rapid formation of **5** from **6** through an oxygenation/cleavage reaction. Formation of green leaf volatiles is also induced in a similar way through the oxygenation/cleavage of fatty acids upon disruption of vascular plant tissues (Matsui, 2006). In contrast, soybean seeds (Matsui et al., 2011) and some plants belonging to the Lamiaceae accumulated **5** in their tissues and released their volatiles upon tissue disruption. Even though rapid emission of **5** after mechanical wounding is rather common among fungi and plants, the mechanisms by which it occurs are diversified, which suggests that the mechanism employed by liverworts was acquired independently and probably in a convergent manner from the ones used by fungi and vascular plants. The prompt formation of **5** might enhance the ability of *M. polymorpha* to defend itself against enemies in a low light and high humidity habitat. Some flowering plants lack the ability to form **5** and thus, it is likely that in these cases, the emission of **5** is not essential for plant survival.

Because of the range of bioactivities reported for **5**, especially against arthropods, it is assumed that rapid formation of **5** at wound sites in mosses and liverworts is necessary for defense

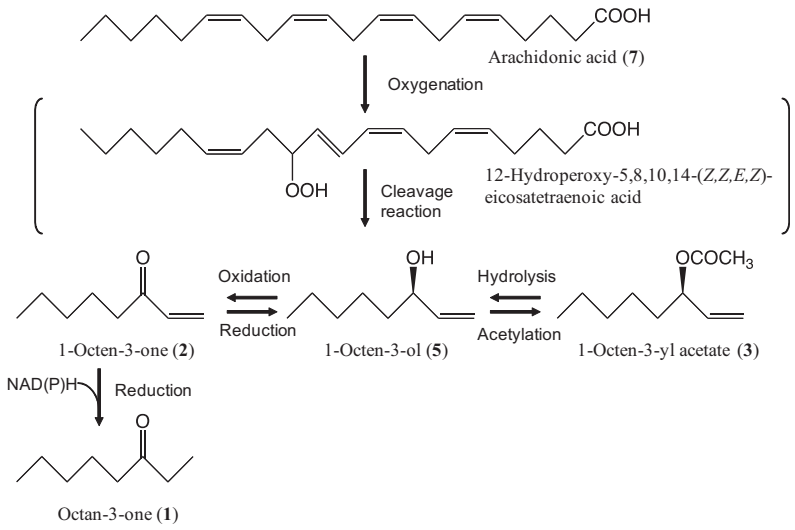

Fig. 9. Proposed biosynthetic pathway to form C8 volatiles in *M. polymorpha* thalli.

against herbivores and pathogens (Wichard et al., 2005). However, it is unknown what the advantage is for *M. polymorpha* to metabolize the beneficial volatile compound **5**. Here it was found that **5** in the vapor phase was absorbed by the intact thalli of *M. polymorpha*, and converted into its acetate probably intracellularly, where the other substrate, acetyl-CoA, was available. In plant leaves, (*Z*)-3-hexenol exogenously supplied as a vapor was efficiently converted into (*Z*)-3-hexenyl acetate by acetyl CoA:(*Z*)-3-hexen-1-ol acetyltransferase (D'Auria et al., 2007; Matsui et al., 2012). This acetylation is believed to be important to abrogate the bioactivity of (*Z*)-3-hexenol, which induces defense responses in plants (Matsui et al., 2012). **5** is also able to induce an oxidative burst and expression of a subset of defense genes in plants (Splivallo et al., 2007; Kishimoto et al., 2007). Therefore, it might be important to modulate the bioactivities of **5** through acetylation at the expense of acetyl-CoA, which is a precious carbon source that could otherwise be used for growth and development.

Complete disruption of the thalli tissues resulted in predominant formation of **5**, while partial disruption facilitates conversion of **5** to **1**. The intact tissues surrounding the wounded and disrupted tissues should help reduce any secondary bioactivities of **5**. In this case, **5** participates in oxido-reduction rather than acetylation. The genotoxic potential of **1** for mammalian cells is lower than that of **5** (Kreja and Seidel, 2002). **1** is also much less reactive than **2**, which harbors an α,β-unsaturated carbonyl structure and is prone to participate in a Michael addition reaction (Schultz et al., 2005). Accordingly, **1** is the most inert C8 volatile, and, contrary to **3**, it does not accumulate in thallus tissues. It is assumed that **5** formed in the wounded tissues subsequently diffuses into the neighboring intact tissues where it is converted into **1** to suppress its bioactivities, and thereafter, **1** is discarded into the atmosphere. Addition of NAD(P)H made the C8 metabolism in the completely disrupted tissues partly similar to that observed in partially disrupted tissues, by converting **5** to **2** and **1**. Oxidation of the *sec*-alcohol, which may be catalyzed by alcohol dehydrogenase, is reversible (Edegger et al., 2006), while reduction of the double bond, which may be catalyzed by alkenal reductase (Yamauchi et al., 2011; Wu et al., 2013), is irreversible. Thus, the overall reaction can be determined by the rate of reduction of the double bond that is dependent on NAD(P)H. However, increase in the amounts of **2** during complete disruption of thalli in the presence of NAD(P)H could not be explained only with this proposal. Involvement of other metabolic pathway could be suggested, and further study is definitely needed in order to dissect the C8 volatile metabolism completely in wounded thallus of *M. polymorpha*.

4. Experimental

4.1. Plant materials

M. polymorpha L. collected at the Yoshida campus (E131°29', N34°11') of Yamaguchi University, Yamaguchi, Japan, was used in all experiments except for the experiment with *des6*^{KO} plant. For some experiments, the harvested thalli were further grown in a growth chamber at 21 °C under continuous fluorescent light supplemented with light from an incandescent bulb (16–33 μmol m^{-2} s^{-1}) or on a half strength Gamborg-B5 plate at 21 °C under continuous fluorescent light (43–77 μmol m^{-2} s^{-1}). Under low intensity light, the formation of gemma, archegoniophore, and antheridiophore was suppressed. The *des6*^{KO} strain was prepared through a targeted gene-disruption procedure with F1 sporelings derived from crosses between male and female accessions, Takaragaike-1 (Tak-1) and Takaragaike-2 (Tak-2), respectively (Ishizaki et al., 2013; Kubota et al., 2014), and was aseptically grown on a half strength Gamborg-B5 plate at 21 °C under continuous fluorescent light.

4.2. Volatile collection in the field

Volatiles emitted from thalli in their natural habitat at the campus of Yamaguchi University were collected on December 5 and 19, 2012. It was occasionally cloudy, and essentially no wind was noted on those dates. Collection was done from 10:00 to 17:00. The average temperature was 6.3 to 6.7 °C and the relative humidity was 43–70%. To induce mechanical wounding, the thalli were pinched with forceps on *ca.* 10% of the area. For the SPME collection, a lawn of thalli was gently covered with a round-shaped polystyrene cup (basal area, 58.1 cm^2; height, 5 cm). A SPME fiber (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA, USA) was exposed to the headspace in the hood for 30 min. After collection, the fiber was inserted into the insertion port of a GC–MS (QP-5050, Shimadzu, Kyoto, Japan) equipped with a Stabilwax column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness, Restek, Bellefonte, PA). The column temperature was programmed as follows: 40 °C for 2 min, increasing by 10 °C min^{-1} to 200 °C for 2 min. The carrier gas (He) was delivered at 86.1 kPa. The glass insert was an SPME Sleeve (Supelco). Splitless injection with a sampling time of 1 min was used. The fiber was held in the injection port for 10 min to fully remove any compounds from the matrix. The temperatures of the injector and interface were 240 and 200 °C,

respectively. The mass detector was operated in the electron impact mode with an ionization energy of 70 eV.

To identify each compound, retention indices and MS profiles of corresponding authentic specimens were used. To construct calibration curves for quantification, a cotton swab containing given amounts of **1** (Wako Pure Chemicals, Osaka, Japan), **3** (Sigma–Aldrich, St. Louis, MO), **4** (Tokyo Chemical Industry Co., Tokyo, Japan), **2** (Tokyo Chemical Industry), **5** (Alfa Aesar, Ward Hill, MA), and caryophyllene (Sigma–Aldrich) was placed approximately 2–3 cm above ground with almost the same soil composition as the collection site but without any plants; then, the ground was covered with the hood for SPME collection. An aqueous soln. of each compound was prepared by suspending it in a small amount of Tween 20. In the absence of addition of the volatile compounds, the emission of the volatiles was not detected.

To follow the time course after mechanical wounding, the lawn of thalli was gently covered with a round-shaped polystyrene cup (basal area, 58.1 cm^2; height, 5 cm) immediately after mechanical wounding to 10% of the surface, and the headspace inside of the cup was continuously passed through a charcoal cartridge (ORBO 32 Small Activated Coconut Charcoal (20/40), 100/50 mg, Sigma–Aldrich) at 1000 mL min^{-1} with an air-pump (N86KT.18, KNF Japan Co., Tokyo, Japan). After collecting volatiles for 40 min, the adsorbed compounds were eluted three times with CH_2Cl_2 (250 μL containing 1 μg mL^{-1} cyclohexanol). A portion of the extract was subjected to GC–MS analysis essentially as shown above. Injection was performed using a split-mode with a ratio of 10. The column temperature was programmed as follows: 40 °C for 2 min, increasing by 5 °C min^{-1} to 150 °C, by 10 °C min^{-1} to 200 °C, then held at 200 °C for 2 min. The carrier gas (He) was delivered at 86.1 kPa.

4.3. Solvent extraction

The thalli collected from the campus and grown in a growth chamber for 3 months were used for solvent extraction. Three hours before analysis, the thalli were collected and placed on a wet towel to avoid wound-responses brought about during collection. The thalli were snap-frozen with liquid N_2, and powdered with a mortar. Volatiles were extracted from a portion (0.2 g fr wt) with 1 mL of methyl *tert*-butyl ether (MTBE) containing nonanyl acetate (1 μg mL^{-1}) (Tokyo Chemical Industry). Another portion (0.2 g fr wt) was thawed in a water bath, and then incubated for an additional 5 min at 24 °C to facilitate the enzyme reaction. The enzyme reaction was terminated by adding MTBE (1 mL) containing nonanyl acetate. The MTBE mixture was dispersed with the aid of an ultrasonic washer for 10 s, then mixed vigorously. The mixture was centrifuged at 210×*g* for 10 min at 25 °C (8100 Centrifuge, Kubota, Tokyo, Japan). A portion of the extract was subjected to GC–MS analysis essentially as described above. Injection was performed using a split-mode with a ratio of 2. The column temperature was programmed as follows: 40 °C for 2 min, increasing by 5 °C min^{-1} to 200 °C for 2 min. The carrier gas (He) was delivered at 26.7 cm s^{-1}. For quantification of **1**, **2**, **3**, **4**, and **5**, each standard was suspended in 0.2% Tween 20 at 10 mg mL^{-1}, and the suspension was diluted to prepare a series of aq. solutions, extracted with MTBE containing nonanyl acetate, and then subjected to GC–MS analysis to construct calibration curves.

4.4. Esterase assay

The thalli (wild type or *des6*^{KO}, 5 g fr wt) were homogenized with 50 mM Na phosphate (10 mL, pH 7.0 containing 2.5% Polyklar VT (w/v)) (Wako Pure Chemicals) in a mortar with the aid of sea sand on ice. The homogenized soln. was centrifuged at 10,000×*g* for 30 min at 4 °C, and the resultant supernatant was used as the crude enzyme soln. The reaction was initiated by mixing the crude enzyme soln. (200 μL) with 10 mg mL^{-1} **3** (10 μL) suspended in 0.2% Tween 20 in 50 mM Na phosphate, pH 7.0 (total volume of 1 mL) in a shaking water bath at 24 °C for 30 min. After the reaction, the C8 compounds were extracted with MTBE and analyzed using GC.

Resolution of each enantiomer was carried out using a GC equipped with a 0.25 mm × 30 m α-DEX 120 column (Supelco). The column temp. was maintained at 100 °C. Injector and detector (FID) temperature was set at 200 °C. N_2 gas was used as carrier gas at 20.0 cm s^{-1}. Under these conditions, (*S*)-**5** and (*R*)-**5** (Acros Organics, Geel, Belgium) eluted at 11.0 and 11.2 min, respectively.

4.5. Acetylation activity

To examine the acetylation activity responsible for forming **3** from **5**, the *des6*^{KO} mutant strain was used. The *des6*^{KO} thalli grown on a half-strength Gamborg-B5 plate were exposed to the vapor of **5** by hanging a sheet (7 × 7 mm) of filter paper containing 10 mg mL^{-1} **5** soln.(20 μL) in 0.2% Tween 20 for 18 h under continuous light at 21 °C. As a control, 0.2% Tween 20 (20 μL) was used. After exposure, the thalli were collected from the plate, and the volatiles in the tissues were immediately extracted with MTBE as described in Section 4.3.

4.6. Oxidoreductase assay

The thalli were collected and incubated on wet paper towel for 3 h at 24 °C to recover from the response caused by wounding during collection. The thalli (ca. 0.1 g fr wt) were mixed with an equal volume of distilled H_2O with or without 5 mM NADH or NADPH, and then were thoroughly homogenized in a mortar. The homogenate was transferred into a glass vial (22 mL, Perkin Elmer, Waltham, MA, USA), sealed tightly with a butyl rubber stopper and a crimp-top seal (National Scientific, Rockwood, TN, USA), and the volatiles in the headspace were collected using a SPME fiber at 25 °C for 30 min. To analyze volatiles from partially wounded thalli, about 50% of the surface area of thalli were mechanically wounded with forceps, and then placed in the vial for SPME collection. Intact thalli were used as the control. The volatiles were analyzed as described in Section 4.2. Aq. solutions of standard compounds were prepared from 10 mg mL^{-1} suspensions in 0.2% Tween 20 and used to construct calibration curves.

To estimate the oxidoreductase activity toward **5**, crude enzyme soln. (100 μL) prepared as described in Section 4.4. was mixed with 10 mg mL^{-1} **5** (10 μL) suspended in 0.2% Tween 20, and incubated at 27 °C for 5 min in 50 mM sodium phosphate (pH 7.0) with or without 5 mM NADH or NADPH. After the reaction, the C8 compounds were extracted with 1 mL of MTBE containing nonanyl acetate (1 μg mL^{-1}), and quantitatively analyzed using GC–MS essentially as described in Section 4.4 but with the modified column condition [40 °C (5 min) to 200 °C (2 min) at 8 °C min^{-1} with He as a carrier gas at 26.7 cm s^{-1}].

4.7. Fatty acid analysis

Approximately 2 g (FW) of *M. polymorpha* thalli were ground at room temperature and extracted with $CHCl_3$: MeOH (9 mL, 1:2 v/v) for 20 min. After adding 5 mL each of $CHCl_3$ and H_2O, the organic phase was recovered by centrifugation at 1860×*g*, at 4 °C for 20 min. After addition of EtOH (−15 mL), the extract was freeze-dried, and fatty acids were trans-methylated with 10% (v/v) H_2SO_4 in MeOH (2.5 mL) at 80 °C for 2 h. Fatty acid methyl esters were extracted with hexane (2.5 mL), and a portion was analyzed with GC–MS (Shimadzu GCMS-QP2010 Ultra) equipped with a quadrupole mass detector (EI, 70 eV) and a DB-23 capillary column

(30 m × 0.25 mm, film thickness 0.25 μm; Agilent), the carrier gas: He (0.89 mL min^{-1}), oven temperature: first kept at 160 °C for 1 min, elevated to 210 °C (4 °C min^{-1}), kept at 210 °C for 6.5 min.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 23580151 and Yobimizu Project from Yamaguchi University, and also partly by JST, ALCA (to HF). We thank Sakiko Ishida for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.008.

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