J. Nat. Prod. **2003,** *66,* 743−751

Isolation of Halogenated Monoterpenes from Bioreactor-Cultured Microplantlets of the Macrophytic Red Algae *Ochtodes secundiramea* and *Portieria hornemannii*

Luis F. Barahona[†] and Gregory L. Rorrer*[,‡]

CICY-Biotecnologia, Apartado Postal 87, 97310 Cordemex, Merida, Mexico, and Department of Chemical Engineering, Oregon State University, Corvallis, Oregon 97331

Received December 31, 2002

Field collections of the red macroalgae *Ochtodes secundiramea* and *Portieria hornemannii* exhibit site-to-site variations in halogenated monoterpene (HMT) content. In contrast, microplantlets of *O. secundiramea* and *P. hornemannii* established through cell and tissue culture techniques had remarkably similar HMT profiles when cultivated in a photobioreactor under identical, controlled conditions. Both algae shared Apakaochtodene B (**6**) as the only cyclic HMT, 10*E*-bromomyrcene (**3**) and 10*E*-bromo-3-chloro-α-myrcene (**4**) as the dominant acyclic HMTs, and myrcene (**1**) as their common precursor. Furthermore, HMT yields were comparable between organisms (0.9−1.3 μmol/g dry mass of **6**; 3.4−4.4 μmol/g of **3**). *P. hornemannii* microplantlets also contained 7-chloromyrcene (**9**) as the dominant compound (37−73 μmol/g), suggesting additional chlorination capacity. Proposed pathways for HMT biosynthesis shared by *P. hornemannii* and *O. secundiramea* microplantlets possessed two common manifolds: (a) bromonium ion (Br^+)-catalyzed cyclization of **1**, followed by chlorination to yield **6**; (b) Markovnikov addition of Br^+ at $\Delta^{6,10}$ of **1** to yield **3** with a marked selectivity (>50:1) for the *E* isomer, followed by chlorination of **3** to **4**. This study demonstrated that bioreactor tissue culture is a new venue for bioprospecting and production of natural compounds from marine macroalgae under a controlled environment.

Halogenated monoterpenes are a prolific class of marine natural products found primarily in three genera of marine red macroalgae, particularly *Plocamium*, *Portieria*, and *Ochtodes*,[1] where they serve as a chemical defense to herbivory.[2] Although over 100 halogenated monoterpenes have been isolated and characterized from field collections of red macroalgae, their biosynthesis is poorly understood.[3] Naylor et al.[1] suggest that ocimene is the common precursor to all halogenated monoterpenes found in *Plocamium*, whereas myrcene is the common precursor to all halogenated monoterpenes found in *Portieria* and *Ochtodes*, both of which are within the family Rhizophyllidaceae.

Although halogenated monoterpene biosynthesis in both *Ochtodes* and *Portieria* may share myrcene as the common precursor, specific halogenated monoterpenes found in various field collections of these organisms differ markedly. For example, field collections of *Ochtodes* species contain cyclic halogenated monoterpenes,[4−6] all based on an ochtodane template that is presumably formed by bromonium-ion-promoted cyclization of acyclic monoterpenes.[7,8] In contrast, field collections of *Portieria hornemannii* contain a plethora of acyclic halogenated monoterpenes,[9−14] some of which exhibit potent and selective antitumor activity.[14,15] *P. hornemannii* also contains cyclic halogenated monoterpenes based upon the ochtodane template.[14−18] Interestingly, *P. hornemannii* contains monoterpenes that are only chlorinated,[9,13,15] whereas *O. secundiramea* does not, suggesting variations in halogenation enzyme activity.

Field collections of red macroalgae show considerable site-to-site and temporal variations in halogenated monoterpene content, particularly for *P. hornemannii.*[13,15,19,20] These variations have complicated efforts to understand halogenated monoterpene biosynthesis in these organisms and have hampered efforts to provide sufficient quantities of specific bioactive compounds for preclinical testing and product development.[14,21] In vitro culture systems could eliminate the need for collection of biomass from the field, as plant tissue could be grown up in a laboratory environment under controlled and axenic conditions. Toward this end, we recently developed a photosynthetic "microplantlet" tissue culture of *O. secundiramea*, using callus induction and shoot tissue regeneration techniques.[22] These microplantlets were amenable to bioreactor cultivation,[23] and GC−MS analyses confirmed that the biomass contained both myrcene and a variety of halogenated monoterpenes.[22,23] Furthermore, yields of some halogenated monoterpenes selectively increased in response to changes in the rate of nutrient perfusion, demonstrating that the halogenated monoterpene profile could be externally manipulated in vitro.[23] The *O. secundiramea* microplantlets also contained a novel myrcene synthase[24] and a bromoperoxidase,[25] two enzymes that may be necessary for biosynthesis of halogenated monoterpenes.

The overall objective of this study was to compare the halogenated monoterpenes produced by microplantlets of the red algae *O. secundiramea* and *P. hornemannii* that were cultured within a photobioreactor under identical conditions. Toward this end, the dominant cyclic halogenated monoterpene produced by both organisms in bioreactor culture was isolated and characterized. Furthermore, the yields of the dominant acyclic and cyclic halogenated monoterpenes shared by microplantlets of *O. secundiramea* and *P. hornemannii* were compared. Finally, a biogenic scheme for halogenated monoterpenes by cultured microplantlets of *O. secundiramea* and *P. hornemannii* was proposed that contained pathways shared by both organisms as well as pathways unique to each organism.

* Corresponding author. Tel: 541-737-3370. Fax: 541-737-4600. E-mail: rorrergl@che.orst.edu.
† CICY-Biotecnologia.
‡ Oregon State University.

10.1021/np0206007 CCC: $25.00 © 2003 American Chemical Society and American Society of Pharmacognosy
Published on Web 05/10/2003

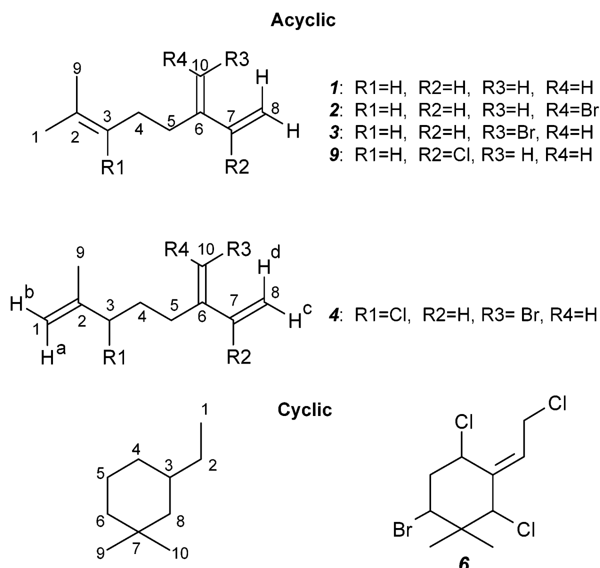

Figure 1. Carbon number assignments for acyclic and cyclic monoterpenes.

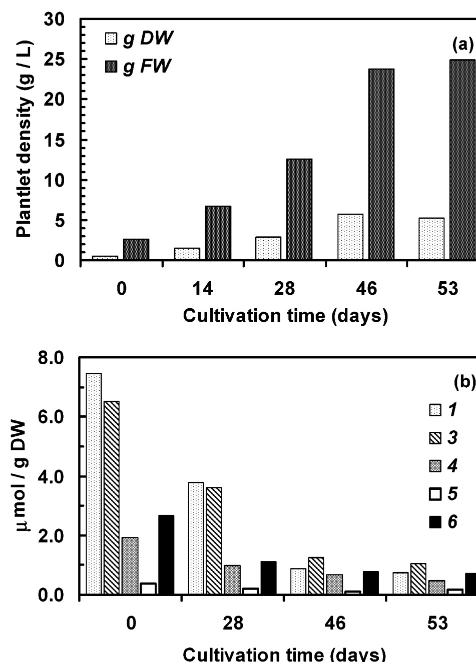

Figure 2. Biomass production and halogenated monoterpene content vs time during cultivation of *O. secundiramea* microplantlets for 53 days in a 3 L stirred-tank photobioreactor at the conditions given in Table 3. (a) Biomass production; (b) myrcene (**1**) and halogenated monoterpenes **3**–**6** in DCM extracts.

Results

Identification of Halogenated Monoterpenes by GC–MS. Halogenated monoterpenes in the dichoromethane (DCM) extracts of the cultured *O. secundiramea and P. hornemannii* microplantlets were readily identified by GC–MS (Tables 1 and 2). The carbon-numbering assignments used in the common names of the halogenated monoterpenes are presented in Figure 1. Five halogenated monoterpenes (compounds **2**–**6**) were always found in extracts of both *O. secundiramea* and *P. hornemannii* microplantlets. Each compound from both *O. secundiramea* and *P. hornemannii* possessed identical mass spectra. Myrcene (compound **1**, 7-methyl-3-methylene-1,6-octadiene) was the only nonhalogenated monoterpene shared by both cultures. In addition to myrcene, *P. hornemannii* also contained one more nonhalogenated monoterpene (**7**) with a putative molecular formula of $C_{10}H_{16}O$ (Table 2). Halogenated monoterpenes **2** and **3** with molecular formulas of $C_{10}H_{15}Br$ possessed mass spectra precisely matched with 10*E*-bromomyrcene (*E*-3-bromomethylene-7-methyl-1,6-octadiene) and its stereoisomer 10*Z*-bromomyrcene (*Z*-3-bromomethylene-7-methyl-1,6-octadiene), found previously in field collections of *P. hornemannii*[9,10] but not in field-collected *O. secundiramea.* 10*E*-Bromomyrcene was the dominant stereoisomer (57:1 in *O. secundiramea* and 5:1 in *P. hornemannii*). In addition to compounds **1**–**6**, *P. hornemannii* microplantlet extracts also contained several halogenated monoterpenes not found in *O. secundiramea* microplantlet extracts. Most importantly, *P. hornemannii* microplantlet extracts contained the monochlorinated monoterpene **9** ($C_{10}H_{15}Cl$) as the dominant compound. Compound **9** possessed a mass spectrum precisely matching 7-chloromyrcene (2-chloro-3-methylene-7-methyl-1,6-octadiene), identified previously in one field collection of *P. hornemannii.*[9] Furthermore, *P. hornemannii* microplantlet extracts contained two more isomers of bromomyrcene (compounds **8**, **12** as $C_{10}H_{15}Br$) and three additional chloromyrcene derivatives with molecular formulas of $C_{10}H_{14}Cl$ (compounds **10**, **13**) and $C_{10}H_{16}Cl$ (compound **11**). Halomon, a pentahalogenated acyclic monoterpene with antitumor activity,[15] was not found in DCM extracts of *P. hornemannii* or *O. secundiramea* microplantlets.

Identification of Sesquiterpenes and Diterpenes by GC–MS. DCM extracts of *P. hornemannii* microplantlets contained several nonhalogenated sesquiterpenes and diterpenes not found in bioreactor-cultured *O. secundiramea* microplantlets. *P. hornemannii* microplantlet extracts contained significant amounts of an oxygenated sesquiterpene (compound **15**), with a molecular formula of $C_{15}H_{18}O_4$ as firmly suggested by mass spectra (Table 2). Other sesquiterpenes found were α- and *β*-selinene (**16**, **17**). Minor amounts of two diterpenes (**18**, *E,E*-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene; **19**, *E,E,E*-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene) were also found. Compounds **16**–**19** were identified by the match (>90%) of their measured mass spectra to library mass spectra.

Production of Halogenated Monoterpenes in a Three-Liter Stirred-Tank Photobioreactor. Figure 2 presents biomass production and halogenated monoterpene content of the biomass for the long-term cultivation of *O. secundiramea* microplantlets within a 3 L stirred tank photobioreactor, operated at the conditions shown in Table 3. "Day 0" represents the halogenated monoterpene content of the flask-cultured tissue inoculum. Over a cultivation period of 53 days, biomass density increased 10-fold to yield 13.0 g of dry biomass (62.2 g wet biomass) at a specific growth rate of 0.052 ± 0.006 day^{-1}. The concentration of myrcene and halogenated monoterpenes with the biomass became constant over time as the biosynthesis of myrcene and halogenated monoterpenes adapted from the conditions of flask cultivation to the conditions of bioreactor cultivation. Myrcene (**1**) and 10*E*-bromomyrcene (**3**) contents were particularly susceptible to the perturbation from flask cultivation to bioreactor cultivation. Ultimately, compounds **3** and **6** were the dominant halogenated monoterpenes produced by bioreactor-cultured *O. secundiramea* microplantlets. Myrcene and halogenated monoterpenes were not detected in the liquid medium.

Figure 3 compares the myrcene and halogenated monoterpene content of *O. secundiramea* and *P. hornemannii* microplantlets after 28 days of cultivation in the 3 L stirred-tank photobioreactor at the same conditions (Table 3). There were two major differences in the yields of the

Table 1. Dominant Monoterpenes Shared by Cultured *O. secundiramea* and *P. hornemannii* Microplantlets

	GC retention time (min)		characteristic mass signals (*m*/*z*)			
compd	DB5 col.	HP5 col.	100% peak	M−X	M+	mol. formula
1	4.79	4.21	93	93 (100) X = 43	136 (5.0%)	$C_{10}H_{16}$ (acyclic)
2	7.05	6.31	69	93 (11%) X = Br+43−H 135 (14%) X = Br 171 (41%), 173 (41%) X = 43	214 (1.5%), 216 (1.3%)	$C_{10}H_{15}Br$ (acyclic)
3	7.31	6.66	69	93 (69%) X = Br+43-H 135 (36%) X = Br 171 (10%), 173 (10%) X = 43	214 (0.5%), 216 (0.5%)	$C_{10}H_{15}Br$ (acyclic)
4	8.86	8.16	133	133 (100%) X = Br+Cl+H 169 (65%), 171 (22%) X = Br 213 (4.6%), 215 (4.5%) X = Cl	248 (6.9%), 250 (8.8%), 252 (2.3%)	$C_{10}H_{14}BrCl$ (acyclic)
5	9.79	9.08	133	133 (100), X = 2Br+H 213 (10), 215 (11), X = Br 279 (8.9), 281 (11) X = CH_3	292 (8.8), 294 (15), 296 (8.7)	$C_{10}H_{14}Br_2$ (acyclic)
6	11.67	10.97	203	167 (94%), 169 (41%) X = Br+2Cl 203 (100%), 205 (66%) X = Br+Cl+H 239 (27%), 241 (27%), 243 (9.0%) X = Br 283 (7.4%), 285 (12%), 287 (5.2%) X = Cl	318 (0.7%), 320 (1.3%), 322 (0.8%)	$C_{10}H_{14}BrCl_3$ (cyclic)

Table 2. GC−MS of Additional Monoterpenes and Sesquiterpenes Found in Cultured Microplantlets of *P. hornemannii*

	GC retention time (min)		characteristic mass signals (*m*/*z*)			
compd	DB5 col.	HP5 col.	100% peak	M−X	M+	mol. formula
7	7.10	6.46	43	119 (28%) X = H_2O+CH_3 134 (11%)	152 (6%)	$C_{10}H_{16}O$
8	7.43	6.78	135	93 (42%) X = Br+43-H 135 (100%) X = Br	214(12%), 216(12%)	$C_{10}H_{15}Br$
9	6.34	5.72	69	127 (75%), 129(25%) X = 43 135 (8.4%) X = Cl	170(4.3%), 172(1.4%)	$C_{10}H_{15}Cl$
10	7.75	7.09	127	127 (100%), 129 (31%) X = 43-H 133 (19%) X = Cl+H	169 (6.9%), 171 (2.5%)	$C_{10}H_{14}Cl$
11	7.90	7.24	69	135 (44%) X = Cl+H	171 (12%), 173 (5.0%)	$C_{10}H_{16}Cl$
12	8.30	7.62	69	93 (73%) X = Br+43-H 135 (74%) X = Br 171 (5.7%), 173 (5.0%) X = 43	214, 216 (trace)	$C_{10}H_{15}Br$
13	8.41	7.74	67	127 (68%), 129 (23%) X = 43-H 133 (40%) X = Cl+H	169 (42%), 171 (14%)	$C_{10}H_{14}Cl$
14	8.54	7.87	69	135 (8.7%) X = 2Cl+2H 163 (0.7%) X = 43+2H 171 (1.5%), 173 (1.5%), X = Cl+2H	207 (9.4%), 209 (10%)	$C_{10}H_{18}Cl_2$
15	11.19	11.86	119	187 (35%) X = 43+2O 229 (19%) X = 2O+H	262 (50%)	$C_{15}H_{18}O_4$

Table 3. Cultivation Conditions for *O. secundiramea* and *P. hornemannii* Microplantlets

variable	stirred tank	bubbler flask
working volume	2500 mL	450 mL
vessel inner diameter	13 cm	10 cm (conical flask)
impeller speed	100 rpm	no impeller
aeration rate	1.0 L air L^{-1} min^{-1}	1.7 L air L^{-1} min^{-1}
nominal bubble size	2−3 mm	6−10 mm
CO_2 partial pressure	35.5 Pa (350 ppm)	35.5 Pa (350 ppm)
medium	natural seawater + ESS	natural seawater + ESS
medium replacement rate	100% every 14 days	100% every 14 days
nitrate concentration	1.41 mM	0.71 mM
pH	8.3−8.8 (before/after medium replacement)	8.3−8.8 (before/after medium replacement)
phosphate concentration	0.070 mM	0.037 mM
incident light intensity (two-sided illumination)	120 μE m^{-2} s^{-1}	100 μE m^{-2} s^{-1}
photoperiod (h ON/h OFF)	14:10 LD	14:10 LD
temperature	26 °C	26 °C

dominant halogenated monoterpenes produced by *O. secundiramea* and *P. hornemannii* microplantlets in bioreactor culture. First, *P. hornemannii* had a much higher myrcene (**1**) and 10*E*-bromomyrcene (**3**) content. Second, the dominant compound in extracts of *P. hornemannii* was 7-chloromyrcene (**9**), whereas *O. secundiramea* contained no measurable amount of 7-chloromyrcene or any of its isomers.

GC profiles of the dichoromethane extracts best revealed the diversity of secondary metabolites in the bioreactor-cultured microplantlets. The GC profiles of crude extracts from bioreactor-cultured *O. secundiramea* and *P. hornemannii* microplantlets are compared in Figure 4. All halogenated monoterpenes were found within a retention time window of 4−12 min. Beyond 12 min, only C_{15} and higher hydrocarbon compounds were detected.

Structural Determination of Compounds 4 and 6. After 10*E*-bromomyrcene (**3**), brominated metabolites **4** ($C_{10}H_{14}BrCl$) and **6** ($C_{10}H_{14}BrCl_3$) were present in the highest quantities within the bioreactor-cultured microplant-

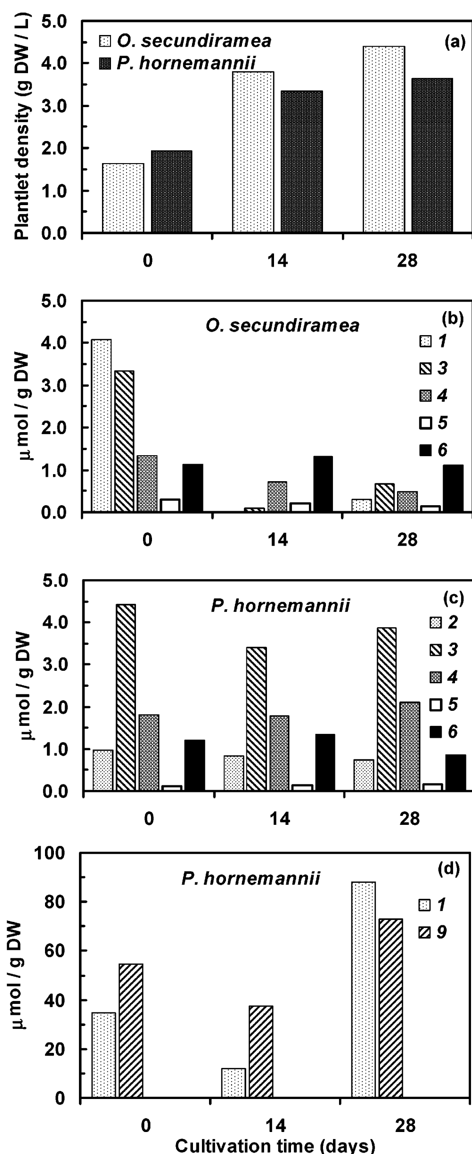

Figure 3. Comparison of halogenated monoterpene content of *O. secundiramea* and *P. hornemannii* microplantlets cultivated in a 3 L stirred-tank photobioreactor for 28 days at the conditions given in Table 3. (a) Dry cell density of *O. secundiramea* and *P. hornemannii*; (b) myrcene (**1**) and halogenated monoterpenes **3**−**6** from DCM extracts of *O. secundiramea*; (c) halogenated monoterpenes **2**−**6** from DCM extracts of *P. hornemannii*; (d) myrcene (**1**) and 7-chloromyrcene (**9**) from DCM extracts of *P. hornemannii*.

lets. Since the identities of **4** and **6** were not known, they were isolated by chromatographic methods and characterized by GC−MS and NMR. The carbon-numbering system used for structural characterization is provided in Figure 1.

The halogen regiochemistry of compound **4** isolated from *O. secundiramea* was deduced from ^{13}C and ^{1}H NMR spectra (Table 4) and complimentary mass spectra (Table 1). Key assignments: $\delta_C = 117.5$ ppm, $\delta_H = 5.36$ (dd) and 5.49 ppm (dd) assigned to C^8H^c/C^8H^d, H,H-COSY Y at C7−C8, suggesting a terminal double bond at C7−C8 with single H at C7; $\delta_C = 106.5$ ppm, $\delta_H = 6.14$ ppm (s) assigned to $C^{10}H$, suggesting placement of a single H and bromine (Br) at C10, with a terminal double bond at C6−C10; $\delta_C = 62.2$ ppm, $\delta_H = 4.34$ ppm (t), H,H-COSY at C3−C4, suggesting placement of a single H and chlorine (Cl) at C3; $\delta_C = 136.1$ ppm (no ^{1}H signal) assigned to C6, confirming the double bond at C6−C10, and $\delta_C = 133.1$ ppm (no ^{1}H signal) assigned to C2, confirming the double bond at C1−C2. Key peaks from ^{1}H NMR spectra mapped to these

Table 4. NMR Spectral Data for Compound **4**

carbon position (Figure 1)	10*E*-bromo-3-chloro-α-myrcene, Naya et al.[11]	compound **4** *O. secundiramea* microplantlets		
	δ_H (ppm, mult *J*, Hz)	δ_C (ppm)	δ_H (ppm, mult *J*, Hz)	H,H-COSY
1	5.00 (b) 4.88 (b)	113.7	5.39 (b) 5.19 (b)	
2	no H	133.1	no H	
3 (Cl)	4.36 (t, 7.0)	62.2	4.34 (t, 6.7)	C3−C4
4	1.90 (m)	27.6	2.18 (b)	C4−C3 C4−C5
5	2.30 (m)	33.2	2.31−2.38 (m)	C5−C4
6	no H	136.1	no H	
7	6.79 (dd, 18, 11)	133.8	6.83 (dd, 17.5, 11.0)	C7−C8
8	5.40 (dd, 18) 5.31 (dd, 11)	117.5	5.49 (dd, 17.6) 5.36 (dd, 11.0)	C8−C7
9	1.80 (d)	18.1	1.62 (s)	
10 (Br)	6.16 (b)	106.5	6.14 (b)	

halogen placements also matched ^{1}H NMR spectra for halogen placements on 10*E*-bromo-3-α-chloromyrcene, found in one field collection of *Chondrococcus japonicus* Harvey.[11] MS signal intensities and ^{1}H NMR spectra of compound **4** did not match those associated with any of the other previously reported $C_{10}H_{14}BrCl$ compounds found in one field collection of *P. hornemannii.*[9] Therefore, compound **4** was proposed to be 10*E*-bromo-3-chloro-α-myrcene (*E*-3-bromomethylene-6-chloro-1,7-octadiene).

Compound **6** was the only cyclic monoterpene found in DCM extracts of both *O. secundiramea* and *P. hornemannii* microplantlets. Characteristic signals from the mass spectrum of compound **6** from DCM extracts of bioreactor-cultured *O. secundiramea* and *P. hornemannii* microplantlets are presented in Table 1. Compound **6** ($C_{10}H_{14}BrCl_3$), with its four halogens, possessed mass spectra consistent with a cyclic halogenated monoterpene found in *P. hornemannii*;[17,18] however, it did not possess mass spectra consistent with an acyclic halogenated monoterpene of the same molecular formula.[10] Chemical shifts from ^{1}H and ^{13}C NMR analysis of compound **6** isolated from bioreactor-cultured *O. secundiramea* and *P. hornemannii* microplantlets are presented in Table 5. Compound **6** from both *O. secundiramea* and *P. hornemannii* microplantlets possessed identical ^{1}H and ^{13}C NMR spectra. Furthermore, the ^{1}H and ^{13}C NMR spectra precisely matched NMR spectra reported for the cyclic halogenated monoterpene apakaochtodene B, found previously in field collections of *P. hornemannii.*[18] Therefore, compound **6** from both *O. secundiramea* and *P. hornemannii* microplantlets was proposed to be apakaochtodene B, 6(*S**)-bromo-1,4(*S**),8(*R**)-trichloro-2(*E*)-ochtodene. Apakaochtodene A, its *Z* geometric isomer, was not found.

Discussion

The overall objective of this study was to compare the halogenated monoterpenes produced by cultured microplantlets of the red algae *O. secundiramea* and *P. hornemannii*. Prior to this study, halogenated monoterpene biogenesis had to be inferred from field collections of *Ochtodes* and *Portieria* species. For example, field collections of *O. secundiramea* and *P. hornemannii* share the cyclic halogenated monoterpenes ochtodene and chondrocole A.[4,16,19] These field-collected plants also contained other cyclic halogenated monoterpenes unique to each organism that appear to be associated with the site of collection. In contrast, in vitro microplantlets derived from *O. secundiramea* and *P. hornemannii* produced apakaochtodene B as the dominant cyclic halogenated monoterpene under

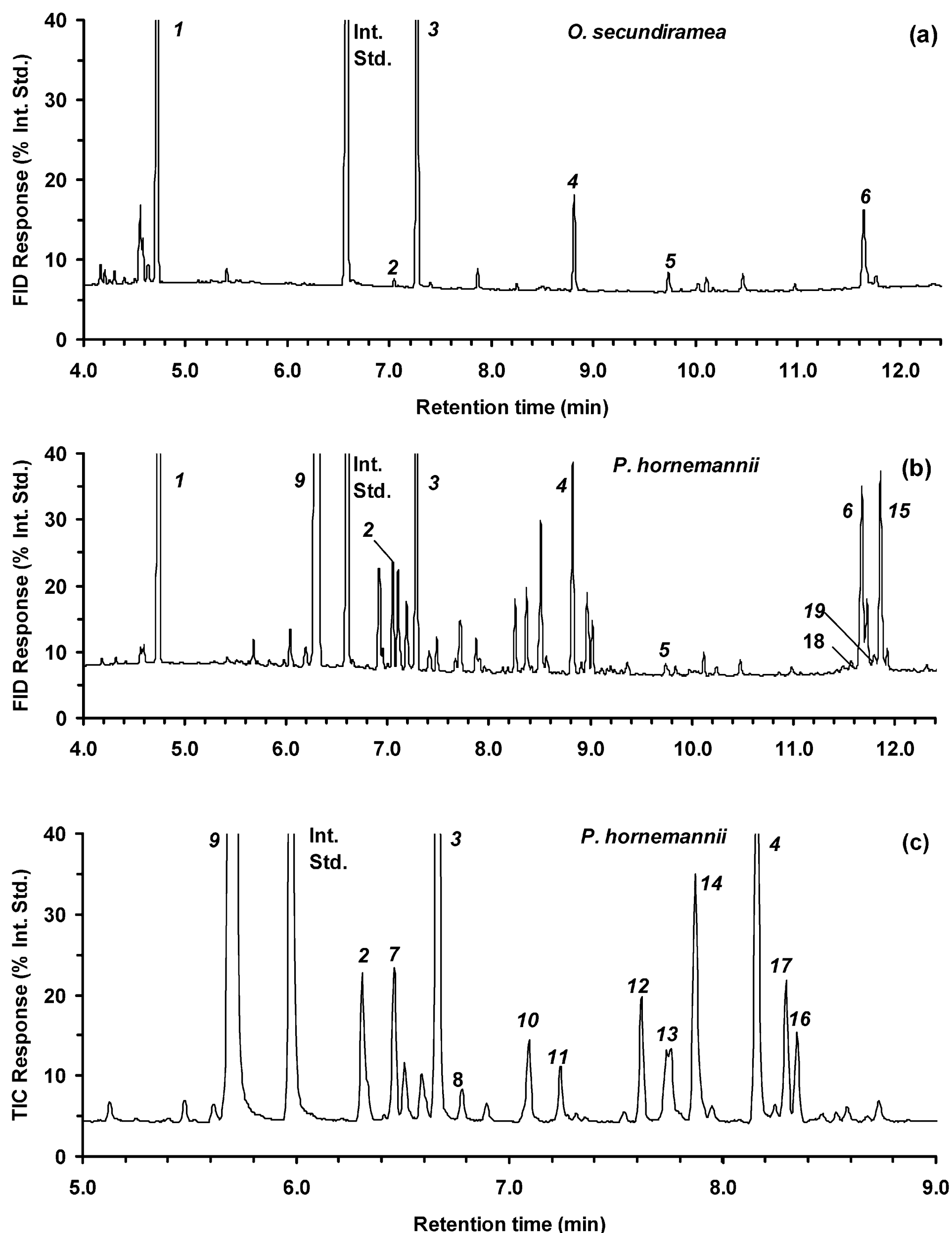

Figure 4. GC profiling of selected samples given in Figures 2 and 3. (a) GC-FID profile of *O. secundiramea* DCM extract on a DB-5 column, 28 days in culture; (b) GC-FID profile of *P. hornemannii* DCM extract on a DB-5 column, 14 days in culture; (c) GC-TIC profile of *P. hornemannii* DCM extract on a HP-5 column, 14 days in culture, focusing on acyclic halogenated monoterpene window.

Table 5. NMR Spectral Data for Compound **6**

carbon position (Figure 1)	apakaochtodene B, Gunatilaka et al.[18]		compound **6** *P. hornemannii*		compound **6** *O. secundiramea*	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1 (Cl)	4.04 (dd)	37.6	4.10 (dd)	38.0	4.10 (dd)	38.1
	4.18 (dd)		4.24 (dd)		4.24 (dd)	
2	5.95 (dd)	131.9	6.01 (dd)	132.2	6.01 (dd)	132.3
3	no H	137.8	no H	138.3	no H	138.3
4 (Cl)	4.97 (dt)	50.4	5.03 (dt)	50.9	5.03 (dt)	50.9
5	2.53 (ddd)	41.3	2.59 (ddd)	41.8	2.59 (ddd)	41.8
	2.68 (ddd)		2.73 (ddd)		2.73 (ddd)	
6 (Br)	4.83 (dd)	52.7	4.89 (d)	53.1	4.89 (d)	53.1
7	no H	41.4	no H	41.8	no H	41.8
8 (Cl)	4.38 (d)	70.0	4.44 (d)	70.4	4.44 (d)	70.4
C9	1.01 (s)	20.5	1.07 (s)	20.9	1.07 (s)	20.9
10	1.28 (s)	28.5	1.34 (s)	29.0	1.34 (s)	29.0

identical cultivation conditions. Although apakochtodene B and other halogenated monoterpenes isolated and characterized from microplantlets of *O. secundiramea* and *P. hornemannii* are not new compounds, their venue of production—in vitro bioreactor culture under controlled conditions—is unique and has significant implications for

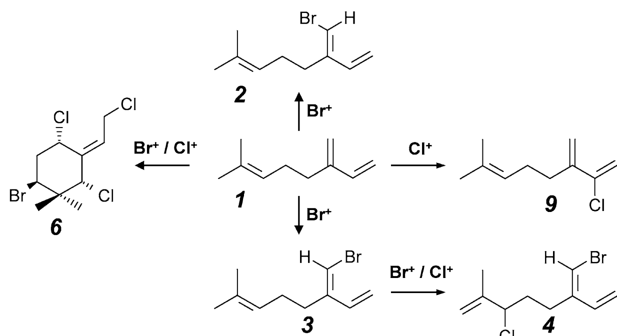

Figure 5. Halogenated monoterpene biosynthesis in bioreactor-cultured microplantlets of *O. secundiramea* and *P. hornemannii.*

marine natural product research. Consequently, it was not a focus of this study to compare the halogenated monoterpene profiles in field-collected plants versus those in tissue culture. Instead, this study compared halogenated monoterpene production in tissue cultures derived from *O. secundiramea* and *P. hornemannii* that were maintained under identical conditions.

A scheme for halogenated monoterpene biosynthesis in cultured *O. secundiramea* and *P. hornemannii* microplantlets is proposed in Figure 5. The common precursor to all halogenated monoterpenes is myrcene (**1**). *O. secundiramea* and *P. hornemannii* microplantlets share overall reaction steps for the production of acyclic halogenated monoterpenes **2**−**4** and the production of the single cyclic halogenated monoterpene **6**. *P. hornemannii* also possesses a reaction for the chlorination of myrcene to 7-chloromyrcene (**9**). This is the dominant reaction in *P. hornemannii,* but is absent in *O. secundiramea*. Evidently, microplantlets of *P. hornemannii* have augmented capacities for biosynthesis of myrcene and chlorination relative to *O. secundiramea* microplantlets. Otherwise, their chemistries are remarkably similar.

It is reasonable to propose that halogenation of monoterpenes is driven by the formation of halonium ions and their subsequent attack on a particular olefinic bond of myrcene, culminating in a Markovnikov addition of the halogen to the olefin. Previous models suggest that the bromonium ion (Br^+) generates a carbonium ion, and then chloride (Cl^-) adds to the carbonium ion.[1,26] Vanadium-dependent bromoperoxidases found in red macroalgae other than those studied here have often been implicated in the formation of brominated marine natural products via an enzyme-bond Br^+ intermediate,[27−30] but regio- and stereoselective halogenation of organic substrates by this enzyme has not been demonstrated. Furthermore, a halogenase capable of selective chlorination has never been found to date in red macroalgae. Nevertheless, the most direct pathway to BrCl compounds we found in *O. secundiramea* and *P. hornemannii* microplantlet cultures needs the generation of both bromonium (Br^+) and chloronium (Cl^+) ions. Specifically, a scheme leading to the formation of compound **6** by Br^+-promoted cyclization followed by a sequential Markovnikov addition of two Cl^+ across the conjugated olefinic bonds at $\Delta^{1,2}$ and $\Delta^{3,8}$ followed by attack of Cl^+ at $\Delta^{3,4}$and abstraction of H is presented in Figure 6a. A scheme leading to formation of compound **4** by Markovnikov addition of Br^+ at $\Delta^{6,10}$ and Cl^+ at $\Delta^{2,3}$ followed by removal of H and restoration of the olefinic bond is presented in Figure 6b.

Compounds **4** and **6** are the dominant BrCl compounds found in both *P. hornemannii* and *O. secundiramea,* and their yields with respect to each organism are comparable (Figure 3). Furthermore, yields of 10*E*-bromomyrcene (**3**) are nominally the same in each organism. Therefore, the biosynthesis of these compounds must proceed similarly in each organism. All evidence suggests that the $\Delta^{6,10}$ olefinic bond is the preferred site on the substrate for bromination of acyclic monoterpenes in both *O. secundiramea* and *P. hornemannii* microplantlets, with *O. secundiramea* showing a marked selectivity (>50:1) for the *E* geometric isomer over the *Z* isomer. The main differences in halogenated monoterpene biosynthesis appear in the upstream components of the biogenic scheme. If one assumes that reaction steps leading to compounds **2**−**4** and **6** in Figure 5 are the same for both *P. hornemannii* and *O. secundiramea*, and these steps are limited by formation of the Br^+ intermediate, then increasing the level of myrcene beyond its saturation level for the bromination enzyme will not have an effect on the yields of these products or their net rates of production. However, it appears that *P. hornemannii* has an augmented capacity for myrcene biosynthesis and may also possess a chlorination enzyme that does not require a bromonium ion intermediate. This would account for the observed high accumulation of myrcene and 7-chloromyrcene relative to *O. secundiramea*.

In summary, this study demonstrated that bioreactor culture of axenic tissues is a promising new venue for the bioprospecting of natural products from marine macroalgae. Furthermore, this study has demonstrated that it is feasible to produce natural compounds from marine macroalgae within a controlled environment. Current methods of bioprospecting are limited to field collection, which has two principal limitations: lack of a reliable supply of biomass bearing the target compound,[31] and an inability to "tune" the biosynthetic pathways with the organism to the target compounds. Bioreactor culture circumvents these limitations. In this regard, studies are underway in our laboratory to control the patterns of halogenated monoterpene biosynthesis in microplantlet suspension cultures of *O. secundiramea* and *P. hornemannii* through various secondary metabolite elicitation techniques.

Experimental Section

Materials. All solvents for extraction and chromatography were HPLC grade, including dichloromethane (Mallinckrodt 4879, bp 40 °C, density 1.3266 g cm^{-3} at 20 °C), *n*-hexane (Mallinckrodt 5167, bp 69 °C), ethyl acetate (Mallinckrodt 3442, bp 77 °C), and acetonitrile (Mallinckrodt 2856, bp 81.6 °C). HPLC and GC standards used were β-myrcene (Sigma M0382, 90% purity, bp 167 °C) and naphthalene (Fisher N134, bp 218 °C). All experimental work involving solvents was carried out within a fume hood.

Microplantlet Stock Cultures. Microplantlets of the macrophytic tropical red alga *Ochtodes secundiramea* (Montagne) Howe (Cryptonemiales, Rhizophyllidaceae) were established by callus induction and shoot tissue regeneration techniques as described in our previous work.[22,32] *O. secundiramea* plants used to establish the axenic, in vitro microplantlet culture were collected from the Discovery Bay West Fore Reef (1−2 m deep), Discovery Bay, Jamaica, during mid-February of 1995. Axenic microplantlet suspension cultures of *Portieria hornemannii* (Lyngbye) Silva (Gigartinales, Rhizophyllidaceae) were established using tissue culture techniques similar to *O. secundiramea*. *P. hornemannii* plants used to establish microplantlet cultures were collected from Double Reef NW, Guam, during late January of 1999.

O. secundiramea and *P. hornemannii* cultures were maintained on natural seawater (Hatfield Marine Science Center, Newport, OR) containing enriched seawater supplement (ESS) nutrients[33] adjusted to pH 8.0 with NaOH. The 100X ESS nutrient stock solution was sterile filtered on a 0.2 μm filter (cellulose acetate, Corning 430626), whereas natural seawater

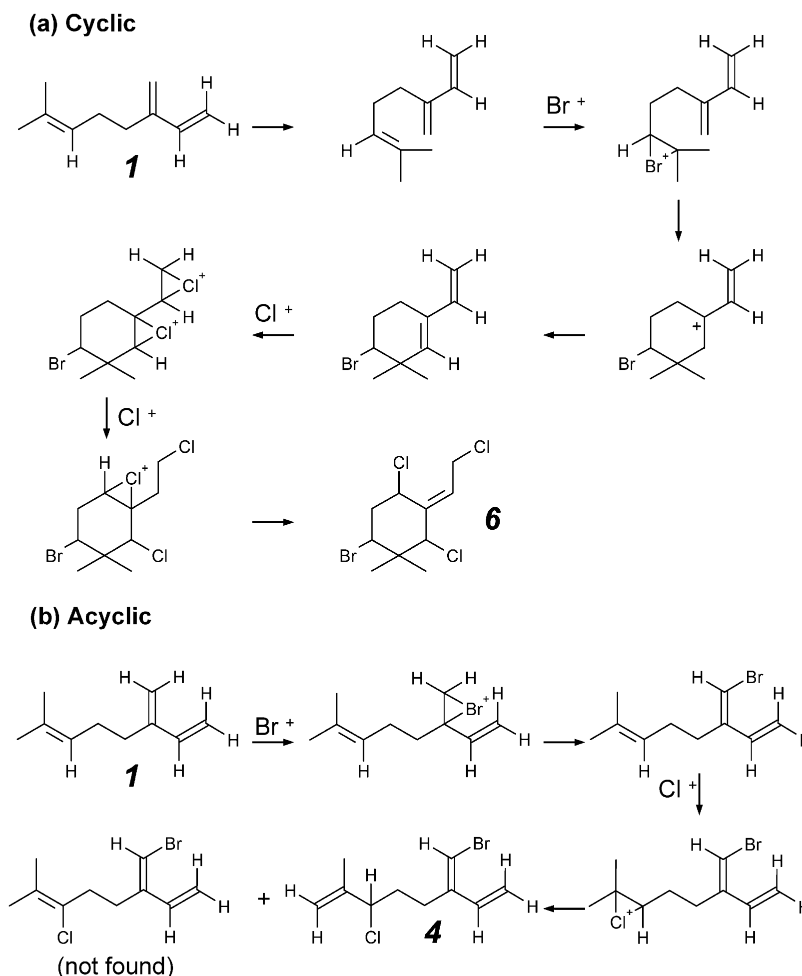

Figure 6. Proposed scheme of myrcene halogenation using bromonium (Br^+) and chloronium (Cl^+) ion intermediates. (a) Formation of cyclic compound **6**; (b) formation of acyclic compound **4**.

was autoclaved (121 °C and 15 psig for 30 min). The final composition of ESS nutrients in the natural seawater base medium was 0.706 mM $NaNO_3$; 0.037 mM $K_2HPO_4·3H_2O$; 0.0109 mM EDTA Fe–Na salt; 0.384 mM Na–HEPE buffer; 0.060 μM KI; 0.0032 μM $ZnCl_2$; 0.0291 μM $MnCl_2·4H_2O$; 0.00067 μM $CoCl_2·6H_2O$; 0.00725 μM $FeCl_3·6H_2O$; 0.0967 μM $Na_2EDTA·2H_2O$; 0.738 μM H_3BO_3; 0.00074 μM vitamin B_{12}; 0.00409 μM biotin ($C_{10}H_{16}N_2O_3S$); 0.296 μM thiamine-HCl; 0.812 μM nicotinic acid; 0.420 μM DL-pantothenic acid hemi Ca salt; 0.0729 μM *p*-aminobenzoic acid; 5.55 μM meso-inositol; and 0.793 μM thymine. Natural seawater contained 0.8 mM bromide and ca. 40 nM vanadate.[34]

Microplantlet suspension cultures of *O. secundiramea* or *P. hornemannii* were maintained in 500 mL Erlenmeyer bubbler flasks. At inoculation, each flask contained 0.5 g fresh weight tissue in 450 mL of ESS-natural seawater liquid medium. Sterile-filtered air was bubbled into each flask through a 6 mm diameter glass tube at 750 mL min^{-1}. Bubble flask cultures were grown under cool-white fluorescent light at 100 $\mu E\ m^{-2}·s^{-1}$, 14 h light/10 h dark photoperiod and a temperature of 26 °C within an illuminated incubator. Microplantlets were subcultured every 4 weeks, with medium exchange every 2 weeks. Prior to subculture, visibly healthy microplantlet suspensions from three 28 day old flasks were pooled, sterile-filtered on 100 μm nylon mesh, and rinsed with ESS-natural seawater medium. The filtered, rinsed tissue was loaded into a 500 mL glass vessel containing 500 mL of fresh ESS-natural seawater medium. The vessel was fitted with an Osterizer blender assembly (Sunbeam-Oster corporation) consisting of four pitched, stainless steel blades, and the microplantlet suspension within the vessel was chopped up on the "grind" setting of the blender for approximately 2 s. The chopped biomass suspension was sterile-filtered on 100 μm nylon mesh. The filtered tissue was transferred into eight 450 mL bubbler flasks (ca. 0.5 g FW per flask), with each flask containing 450 mL of ESS-natural seawater medium. After 4 weeks, the typical biomass harvest per flask was ca. 3 g FW.

O. secundiramea and *P. hornemannii* microplantlets possessed the same thallus morphology. At inoculation, the plantlets were typically about 2–3 mm in diameter. When the plantlets were cultivated as an agitated suspension in the bubbler flasks, the morphology of the plantlets consisted of branched shoot tissues emanating from a core thallus. About 4 weeks after subculture, the plantlets ultimately assumed a symmetrical, spherical shape of nearly 10 mm diameter, where several tiny shoots lined each branch. Tissues of *O. secundiramea* possessed a brown-red color, whereas tissues of *P. hornemannii* possessed a purple-red color.

Stirred-Tank Photobioreactor Cultivation. Liquid suspensions of *O. secundiramea* or *P. hornemannii* microplantlets were cultivated within an externally illuminated, 3 L stirred-tank photobioreactor equipped with a three-blade marine impeller of 4.5 cm diameter and 6 cm height. Details on the design and operation of this bioreactor for cultivation of macroalgal tissue suspension cultures are provided by Rorrer et al.[35] Typically, the bioreactor vessel was inoculated with 20 g fresh weight of blended, 28 day old flask-cultured tissue along with 2500 mL of sterilized natural seawater/ESS medium using the techniques described above. Conditions of photobioreactor cultivation are given in Table 3. Every 14 days, the biomass suspension was removed from the vessel and filtered under sterile vacuum. The biomass was weighed and then resuspended into fresh medium. A 1.0 g fresh weight sample of the filtered biomass was removed for later deter-

mination of dry cell mass and halogenated monoterpene content. All culture transfer procedures were carried out using sterile technique within a laminar flow hood.

Extraction and Column Chromatography of Selected Halogenated Monoterpenes. The microplantlet suspension from the bioreactor vessel at the end of the cultivation was vacuum filtered. Microplantlet tissues were blotted with a paper towel to remove entrained liquid medium and then ground within a 500 mL mortar and pestle in liquid nitrogen until all the liquid nitrogen evaporated. An aliquot of the ground biomass (20 g fresh weight) was extracted with 80 mL of dichloromethane (DCM) within a screw-cap 250 mL flask for 24 h at 22 °C under continuous mixing on an orbital shaker at 100 rpm. The solid residue was vacuum filtered and then re-extracted at the same conditions. For isolation of nonvolatile halogenated monoterpenes (compound **6**), the combined extract was evaporated down to dryness under flowing nitrogen gas at 22 °C. The resulting dark green oil was weighed and then dissolved in 20 mL of DCM. For isolation of semivolatile halogenated monoterpenes (compounds **3**, **4**, **5**) the concentrated crude extract was evaporated down to 20 mL. Typically, a combined extraction of 40 g FW biomass yielded 40 mg of nonvolatile crude DCM extract from *O. secundiramea* and 160 mg of crude extract from *P. hornemannii*.

Methanol was also considered as a solvent for extraction of polar compounds from microplantlet biomass at the same conditions detailed above. However, GC and HPLC profiling of the MeOH crude extract did not reveal any additional halogenated monoterpenes relative to the DCM crude extract. Furthermore, the use of methanol as an extraction solvent was suspected to generate methoxy derivatives of halogenated monoterpenes.[11] Therefore, 100% DCM was always used as the extraction solvent.

The DCM extract (20 mL) was loaded on a chromatography column (24 mm inner diameter) packed to a depth of 15 cm with 20 g of silica gel (Whatman 4791, 70−230 mesh, 60 Å pore size). Then, 20 mL portions of elution solvent were loaded onto the column, and the eluent was collected after each solvent addition. Each fraction was profiled for halogenated monoterpene content by GC analysis, and fractions bearing the same compounds of interest were pooled. From the concentrated DCM extracts of *O. secundiramea* microplantlets, a mixture of compounds **3**, **4**, and **5** was isolated in pooled fractions 1−3 under stepwise elution with 100% *n*-hexane. For the solvent-evaporated DCM extracts, the column was eluted with 100% *n*-hexane until the first compounds appeared, followed by stepwise elution with *n*-hexane/ethyl acetate. For the *O. secundiramea* DCM extract, compound **6** was isolated in pooled fractions 33−44 with 98:2 v/v *n*-hexane/ethyl acetate. For the *P. hornemannii* DCM extract, compound **6** was isolated in fractions 11−23 with 98:2 v/v *n*-hexane/ethyl acetate, and compound *S-1* was isolated in pooled fractions 61−67 with 95:5 v/v *n*-hexane/ethyl acetate. Each pooled fraction was evaporated down to dryness under flowing nitrogen gas at 22 °C.

HPLC. Halogenated monoterpenes from DCM crude extracts were profiled on a C18 reversed-phase HPLC column (Waters Symmetry WAT054275 analytical column, 250 mm × 4.6 mm, 5 μm packing, 100 Å pore diameter, Waters WAT054225 guard column) at 22 °C. The mobile phase was 90:10 v/v acetonitrile/water at 0.6 mL min^{-1}. Eluted peaks were detected by UV absorbance at 190, 225, or 254 nm. Selected peaks of interest were collected by an Isco Retriever II fraction collector. Retention times of identified compounds were as follows: hexadecanoic acid (4.9 min), $C_{10}H_{14}BrCl_3$ (**6**, 8.3 min), $C_{10}H_{14}BrCl$ (**4**, 9.2 min), β-myrcene (**1**, 12.0 min), $C_{10}H_{15}Br$ (**3**, 12.4 min), $C_{10}H_{14}Br_2$ (**5**, 12.8 min), $C_{10}H_{15}Cl$ (**9**, 13.7 min), $C_{15}H_{18}O_4$ (**15**, 16.9 min). Dichloromethane eluted the column at 5.0 min.

Halogenated monoterpenes in the collected fractions from silica gel column chromatography were purified by HPLC at the analysis conditions given above. To isolate at least 2 mg of a given purified compound for ^{13}C NMR analysis, up to 40 injections (20 μL per injection) were made. Collected peaks from HPLC containing the purified nonvolatile compound **6** at retention time of 8.3 min were pooled, evaporated down to dryness under flowing nitrogen gas at 22 °C, and then resuspended in $CDCl_3$. Collected peaks from HPLC containing purified compound **4** at retention time of 9.2 min were concentrated to 25% of their original liquid volume by evaporation. Anhydrous $MgSO_4$ was added to the concentrated sample to remove water, and the precipitate was filtered. The remaining acetonitrile in the sample was removed by evaporation under flowing nitrogen gas at 22 °C. The dried sample was immediately resuspended in $CDCl_3$. All samples were stored at −20 °C.

Compound **5** ($C_{10}H_{14}Br_2$) eluted as a shoulder of compound **3** in the retention time window of 12.5−13.0 min. Attempts to isolate compound **5** by repeated peak shaving and reinjection were not successful.

GC−MS. DCM extracts and selected purified compounds were analyzed by GC−MS using an Agilent 6890 gas chromatograph and Agilent 5973 mass selective detector. Compounds were profiled on a 30 m × 0.25 mm HP-5 capillary column (Hewlett-Packard, 5% phenyl methyl silicone film, 0.25 μm thickness) under the following program: injector temperature 250 °C, column oven 50 °C initial, 20 °C min^{-1} ramp to 320 °C, 6.5 min hold at 320 °C; He carrier gas at 10:1 split, 10 psig column head pressure. The ionization voltage of the MS detector was 70 eV. Sample injection volume was 1.0 μL. All mass spectra were obtained from the scan taken at the apex of the chromatographic peak corresponding to a given compound.

NMR. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AM400 FT-NMR, Department of Chemistry, Oregon State University. In all analyses, the solvent was $CDCl_3$ with δ_C = 77.4 ppm (t) and δ_H = 7.296 ppm. ^{1}H NMR spectra were obtained at 400.0 MHz. Proton-decoupled ^{13}C NMR spectra were obtained at 100.6 MHz. ^{1}H−^{1}H COSY, ^{1}H−^{13}C HETCOR, and HMQC analyses were also performed using standard pulse sequences. As needed, ^{1}H and ^{13}C NMR spectra were corrected for the trace presence of residual solvents.[36]

Quantitative Analysis by GC-FID. A 0.6 g fresh weight aliquot of plantlet tissue was ground to a powder in liquid nitrogen as described earlier, weighed to precision of ±0.1 mg, and then added to 2.0 mL of DCM containing 50 μg mL^{-1} naphthalene (internal standard) within a sealed 4.0 mL glass vial. The vial contents were vortexed for 10 s, allowed to stand at room temperature for 24 h, syringe filtered through a 0.22 μm Teflon filter (Osmonics, Cameo 13F), and then stored in a septum-capped vial at −20 °C. Just before GC analysis, the DCM extract was blown down to 0.5 mL under nitrogen gas flow at 22 °C to concentrate the sample. Control experiments showed that the loss of naphthalene and myrcene during sample concentration from 2.0 mL to 0.5 mL DCM at 22 °C was negligible. A separate 0.6 g fresh mass aliquot of tissue from the same sample was used for determination of dry mass to wet mass. Monoterpene and halogenated monoterpene yields were averaged from duplicate extractions of a given biomass sample; standard errors in yield were generally less than 15%. Re-extraction of the solid residue did not statistically improve the monoterpene yield.

Myrcene and halogenated monoterpenes identified by GC−MS were quantified by GC-FID. Specifically, DCM extracts were analyzed on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a flame-ionization detector (FID). Compounds were profiled on a 30 m × 0.25 mm DB-5 capillary column (J&W Scientific, 5% phenyl−95% methyl silicone film, 0.25 μm thickness) under the following program: injector 250 °C, detector 280 °C, column oven 50 °C initial, 20 °C min^{-1} ramp to 300 °C, 1.0 min hold at 300 °C, 40 °C min^{-1} ramp to 320 °C, 2.0 min hold at 320 °C; He carrier gas flowrate 1.3 mL min^{-1} (20:1 split); column head pressure 16 psig. Retention times for monoterpenes and halogenated monoterpenes on GC-FID were linearly correlated to their corresponding retention times on GC−MS. The GC-FID response factors for halogenated monoterpenes were scaled from the response factor for myrcene standard using well-established effective carbon number methods detailed by Grob.[37] The yield of a given compound within the biomass (μmol/g DW) was calculated by the internal standard method, using the

mass of the internal standard and equivalent dry mass of tissue in the extraction vial.

Acknowledgment. The authors gratefully acknowledge the National Science Foundation, Division of Bioengineering and Environmental Systems (Award No. BES-9810797), for supporting this research. The authors also thank Prof. Donald P. Cheney, Northeastern University, for providing initial tissue cultures of *P. hornemannii*, and Prof. Valerie J. Paul, University of Guam, for providing field-collected biomass of *P. hornemannii* used to initiate the cell and tissue culture system for this organism.

References and Notes

(1) Naylor, S.; Hanke, F. J.; Manes, L. V.; Crews, P. *Fortschr. Chem. Org. Naturst.* **1983**, *44*, 189–241.
(2) Paul, V. J.; Hay, M. E.; Duffy, J. E.; Fenical, W.; Gustafson, K. *J. Exp. Mar. Biol. Ecol.* **1987**, *114*, 249–260.
(3) Wise, M. L.; Croteau, R. Monoterpene biosynthesis. In *Comprehensive Natural Products Chemistry, Volume 2: Isoprenoids Including Carotenoids and Steroids*; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Elsevier: Amsterdam, 1999; pp 97–153.
(4) McConnell, O. J.; Fenical, W. *J. Org. Chem.* **1978**, *43*, 4238–41.
(5) Paul, V. J.; McConnell, O. J.; Fenical, W. *J. Org. Chem.* **1980**, *45*, 3401–07.
(6) Gerwick, W. H. *Phytochemistry* **1984**, *23*, 1323–1324.
(7) Fenical, W. *Recent Adv. Phytochem.* **1979**, *13*, 219–239.
(8) Fenical, W. *Science* **1982**, *215*, 923–928.
(9) Ichikawa, N.; Naya, Y.; Enomoto, S. *Chem. Lett.* **1974**, 1333–36.
(10) Burreson, B. J.; Woolard, F. X.; Moore, R. E. *Chem. Lett.* **1975**, 1111–1114.
(11) Naya, Y.; Hisose, Y.; Ichikawa, N. *Chem. Lett.* **1976**, 839–842.
(12) Woolard, F. X.; Moore, R. E.; Mahendran, M.; Sivapalan, A. *Phytochemistry* **1976**, *15*, 1069–1070.
(13) Wright, A. D.; König, G. M.; Sticher, O. *Tetrahedron* **1991**, *47*, 5717–5724.
(14) Fuller, R. W.; Cardellina, J. H.; Jurek, J.; Scheuer, P. J.; Alvarado-Lindner, B.; McGuire, M.; Gray, G. N.; Steiner, J. R.; Clardy, J.; Menez, E.; Shoemaker, R. H.; Newman, D. J.; Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1994**, *37*, 4407–4411.
(15) Fuller, R. W.; Cardellina, J. H.; Kato, Y.; Brinen, L. S.; Clardy, J.; Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1992**, *35*, 3007–3011.
(16) Burreson, B. J.; Woolard, F. X.; Moore, R. E. *Tetrahedron Lett.* **1975**, *26*, 2155–2158.
(17) Crews, P.; Myers, B. L.; Naylor, S.; Clason, E. L.; Jacobs, R. S.; Staal, G. B. *Phytochemistry* **1984**, *23*, 1449–1451.
(18) Gunatilaka, A. A. L.; Paul, V. J.; Park, P. U.; Puglisi, M. P.; Gitler, A. D.; Eggleston, D. S.; Haltiwanger, R. C.; Kingston, D. G. I. *J. Nat. Prod.* **1999**, *62*, 1376–1378.
(19) Puglisi, M. P.; Paul, V. J. *Mar. Biol.* **1997**, *128*, 161–70.
(20) Matlock, D. B.; Ginsburg, D. W.; Paul, V. J. *Hydrobiologia* **1999**, *398/399*, 267–273.
(21) Carte, B. K. *BioSci.* **1996**, *46*, 271–286.
(22) Maliakal, S.; Cheney, D. P.; Rorrer, G. L. *J. Phycol.* **2001**, *37*, 1010–1019.
(23) Polzin, J. P.; Rorrer, G. L. *Biotechnol. Bioeng.* **2003**, in press.
(24) Wise, M. L.; Rorrer, G. L.; Polzin, J. P.; Croteau, R. *Arch. Biochem. Biophys.* **2002**, *400*, 125–132.
(25) Tucker, M. P.; Rorrer, G. L.; Cheney, D. P.; Maliakal, S. *Biotechnol. Bioeng.* **2001**, *74*, 389–395.
(26) Faulkner, D. J. *Pure Appl. Chem.* **1976**, *48*, 25–28.
(27) Butler, A. *Curr. Opin. Chem. Biol.* **1998**, *2*, 279–285.
(28) Butler, A.; Tschirret-Guth, R. A.; Simpson, M. T. Reactivity of vanadium bromoperoxidase. In *ACS Symposium Series 711, Vanadium Compounds: Chemistry, Biochemistry, and Therapeutic Applications*; Tracey, A. S., Crans, D. C., Eds.; American Chemical Society: Washington, DC, 1998; pp 202–215.
(29) Butler, A. *Coord. Chem. Rev.* **1999**, *187(1)*, 17–35.
(30) Butler, A. Vanadium haloperoxidases. In *Bioinorganic Catalysis*, 2nd ed.; Reedijk, J., Bouwman, S., Eds.; Marcel Dekker: New York, 1999; pp 55–79.
(31) Rhouhi, A. M. *Chem. Eng. News* **1995**, *73* (47), 42–44.
(32) Huang, Y. M.; Maliakal, S.; Cheney, D. P.; Rorrer, G. L. *J. Phycol.* **1998**, *34*, 893–901.
(33) Kitade, Y.; Yamazaki, S.; Saga, N. *J. Phycol.* **1996**, *32*, 496–498.
(34) Butler, A. *Science* **1998**, *281*, 207–210.
(35) Rorrer, G. L.; Polne-Fuller, M.; Zhi, C. *Biotechnol. Bioeng.* **1996**, *49*, 559–567.
(36) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem.* **1997**, *62*, 7512–7515.
(37) Grob, R. L. *Modern Practice of Gas Chromatography*, 3rd ed.; Wiley-Interscience: New York, 1995.

NP0206007