



PRODUCTION OF HYDROXY FATTY ACIDS BY CELL SUSPENSION CULTURES OF THE MARINE BROWN ALGA *LAMINARIA SACCHARINA*

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Key Word Index—*Laminaria saccharina*; Laminariaceae; cell culture biosynthesis; hydroxy fatty acids.

Abstract—Photolithotrophic cell suspension cultures established from the microscopic gametophytic life phase of *Laminaria saccharina* produced three hydroxy fatty acids deriving from an ω -6 lipoxygenase (LOX) oxidation: 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), 13-hydroxy-6,9,11,15-octadecatetraenoic acid (13-HODTA), and 13-hydroxy-9,11-octadecadienoic acid (13-HODE). The yields of 15-HETE, 13-HODTA, and 13-HODE ranged from 100 to 1000 μ g product g^{-1} dry biomass. In the attempt to stimulate ω -6 LOX metabolism *in vitro*, three C_{18} polyunsaturated fatty acids, including linoleic, α -linolenic, and γ -linolenic acid were added separately to the culture medium after 20 days of cultivation and then allowed to incubate with the culture for 10 days. Linoleic and γ -linolenic acid increased the yield of all three hydroxy fatty acids from 2 to 4 times over controls, with 15-HETE showing the most significant increase. In contrast, α -linolenic acid was toxic to the culture at 200 $mg\ l^{-1}$. © 1997 Elsevier Science Ltd

INTRODUCTION

Macrophytic marine algae are an emerging source of bioactive compounds [1], including eicosanoids and related oxylipins [2, 3]. In particular, macrophytic brown algae within the genus *Laminaria* are known to contain several hydroxy fatty acids derived from ω -6 lipoxygenase (LOX) metabolism [4, 5]. In ω -6 LOX metabolism, regio- and stereo-specific oxidation of C_{18} or C_{20} polyunsaturated fatty acids (PUFAs) occurs at the n -6 terminus of the molecule. Specific bioactive hydroxy fatty acid products found in field-collected *Laminaria saccharina* sporophytes and their putative biosynthesis from fatty acid precursors are shown in Fig. 1. In Fig. 1, linoleic acid is a common precursor for three principal routes. In the first route, linoleic acid is oxidized directly to 13-HODE by ω -6 LOX. In the second route, linoleic acid is desaturated to γ -linolenic acid, which undergoes further desaturation and elongation steps to yield arachidonic acid in accordance with known pathways for fatty acid metabolism in marine algae [6]. Along this route, ω -6 LOX could catalyse the oxidation of γ -linolenic acid to 13-HOTE (γ) and arachidonic acid to 15-HETE. In the third route, linoleic acid undergoes two desatu-

ration steps to yield α -linolenic and stearidonic acid. Along this route, ω -6 LOX could catalyze the oxidation of α -linolenic acid to 13-HOTE and stearidonic acid to 13-HODTA.

Cell or tissue cultures established from macrophytic marine algae have the potential to biosynthesize pharmacologically active compounds in controlled quantities. Toward this end, we have developed novel cell and tissue culture systems for the brown alga *L. saccharina* and other marine algae, and determined how process parameters affect their biomass growth kinetics in various phototrophic bioreactor systems [7–10]. However, the ability of these macroalgal cell suspension cultures to produce bioactive compounds has never been reported. The *L. saccharina* gametophyte cell suspension culture system was our first candidate for biosynthetic studies involving bioactive hydroxy fatty acids. This cell culture system has been extensively characterized, and field-collected plants contained bioactive hydroxy fatty acids. However, it was not known if the cell culture system could produce hydroxy fatty acids *in vitro*. Therefore, the first objective of this work was to determine if the *L. saccharina* gametophyte cell suspension cultures produce bioactive hydroxy fatty acids, and if so, quantify their yields in the cellular biomass. The second objective was to determine if the yields could be enhanced by

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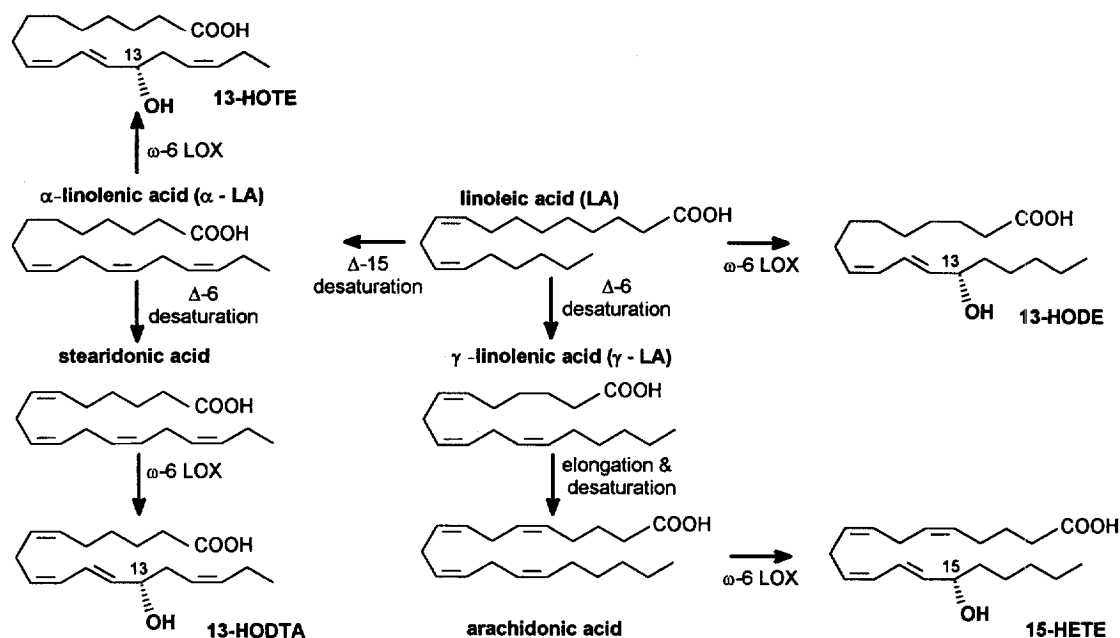


Fig. 1. Proposed biosynthesis of the hydroxy fatty acids from polyunsaturated fatty acids by ω -6 LOX.

adding C_{18} fatty acids (linoleic, α -linolenic, γ -linolenic acid) directly to the culture medium.

RESULTS AND DISCUSSION

This study reports that cell suspension cultures established from macrophytic marine algae (seaweeds) can produce bioactive natural products. In particular, three bioactive hydroxy fatty acids derived from ω -6 LOX metabolism—13-hydroxy-9,11-octadecadienoic acid (13-HODE), 13-hydroxy-6,9,11,15-octadecatetraenoic acid (13-HODTA), and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE)—were produced by gametophytic liquid cell suspension cultures of the macrophytic brown alga *Laminaria saccharina* in yields ranging from 100 to 1000 $\mu\text{g g}^{-1}$ of dry cell mass. The quantitative identification of these hydroxy fatty acids in the cell biomass is described below. The biomass yield, cell viability, and hydroxy fatty acid yields in response to the exogenous addition of fatty acids are also described.

Biomass yields

The addition of fatty acids to the culture lowered the final cell density in culture, as shown in Fig. 2. In all experiments, each fatty acid was added to the culture at the 20th day of cultivation to a concentration of 200 mg l^{-1} and allowed to incubate for 10 days. The biomass yields were based on pooled biomass from three replicate flasks averaged over two separate subculture cycles. The total cultivation time was 30 days. The final cell density for the control experiments was 600 $\text{mg dry cell weight (DCW) l}^{-1}$, but decreased to about 400 to 500 mg DCW l^{-1} if a given fatty acid

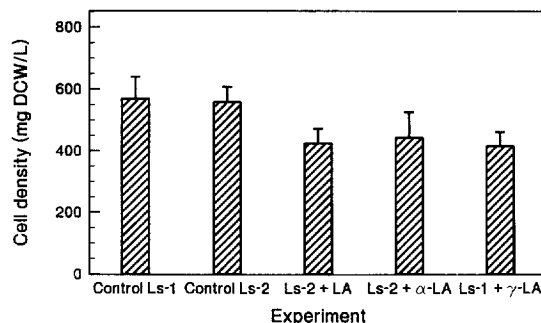


Fig. 2. Average biomass yields from *L. saccharina* gametophyte cultures separately containing 200 mg l^{-1} of either linoleic acid (LA), α -linolenic acid (α -LA), or γ -linolenic acid (γ -LA). A given fatty acid was added after 20 days of cultivation and remained in the culture for 10 days. The designations 'Ls-1' and 'Ls-2' represent two separately maintained culture lines of *L. saccharina* female gametophytic cells. Error bars represent 1 standard deviation (s.d.) based on two different subculture cycles.

(linolenic acid, α -linolenic acid, γ -linolenic acid) was added to the culture at the 20th day of cultivation.

Viability of *L. saccharina* in response to fatty acid addition

The viability of *L. saccharina* gametophyte cell suspension cultures in response to the exogenously added fatty acids is shown in Fig. 3. The maintenance of viability was dependent on both the type of fatty acid and its concentration in the culture medium. Concentrations of all fatty acids exceeding 1000 mg l^{-1} ultimately proved toxic to the culture within the first 10 days of incubation, whereas concentrations of 10

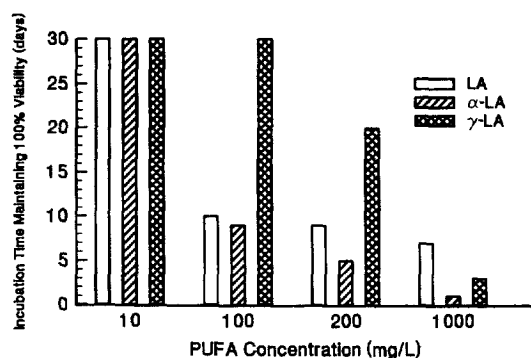


Fig. 3. Viability of *L. saccharina* gametophyte cell cultures to exogenous addition of fatty acids, in terms of incubation time necessary to maintain a nominal viability of 100%.

mg l⁻¹ did not significantly affect culture viability within this time. A concentration of 200 mg l⁻¹ was considered as the maximum acceptable fatty acid concentration that the *L. saccharina* cultures could withstand within a 10 day incubation period. Ethanol up to a concentration of 20 g l⁻¹ had no effect on culture viability.

Identification of oxylipin products in cell biomass

A representative total ion abundance chromatogram of the TMSi methyl ester derivatives of fatty acid compounds contained within the oxylipin rich fraction isolated from *L. saccharina* gametophytic cell culture biomass is shown in Fig. 4. This sample chromatogram represents a control cultivation experiment where no fatty acids were added to the culture. Oxylipin compounds were detected within the retention time window of 12 to 16 min. Table 1 lists all the compounds in the sample identified by matching MS spectra of the compound peaks with the MS spectra obtained for each pure standard compound. Compounds in the biomass samples were identified by their *m/z* signature fragments, not by the percentage of total matched fragments, as many of the peaks were not

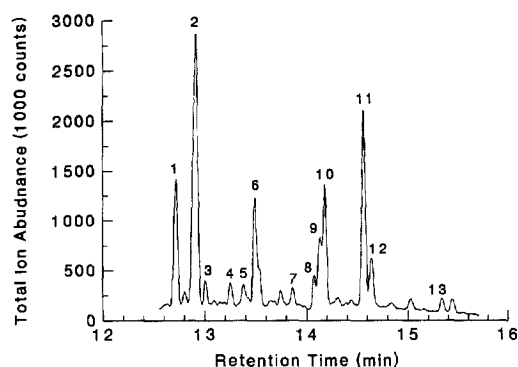


Fig. 4. Total ion abundance chromatogram of the partially purified oxylipin extract from *L. saccharina* gametophyte cell biomass (control culture). Oxylipins were found in the retention time window of 12.6 to 15.6 min. The compounds associated with the numbered peaks are given in Table 1.

resolved to baseline or partially overlapped with other substances, thus reducing the fragment matching percentage. Since these same compounds were also identified in field-collected *L. saccharina* plant tissue as detailed in previous work [5], it was determined that compound identity on the basis of *m/z* signature fragments was sufficient. Three hydroxy fatty acids deriving from ω -6 LOX metabolism were positively identified as their TMSi methyl esters, including 13-hydroxy-9,11-octadecadienoic acid (13-HODE), 13-hydroxy-6,9,11,15-octadecatetraenoic acid (13-HODTA), and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE). The *R* and *S* enantiomers of these compounds were not determined.

Two factors complicated quantitative analysis of 13-HODE, 13-HODTA, and 15-HETE. First, the TMSi methyl esters of each compound rearranged during separation by GC and formed two separate peaks. Peak rearrangement was verified with standards for 13-HODE, 13-HODTA, and 15-HETE both alone and in combination with the ricinelaidic acid internal standard. Second, 13-HODE co-chromatographed with ricinelaidic acid, the internal standard. Therefore, response factors used to quantify 13-HODE yield were based on peak areas obtained from mass-selective signal data at *m/z* 311 for 13-HODE and *m/z* 187 for the ricinelaidic acid internal standard to avoid signal overlap.

The hydroxy fatty acids 13-HODE, 13-HODTA, and 15-HETE found in gametophyte cell suspension cultures of *L. saccharina* were also found in field-collected sporophyte tissue [5]. The field-collected sporophyte tissue also contained 13-hydroxy-9,11,15-octadecatrienoic acid (13-HOTE), another putative product of ω -6 LOX metabolism deriving from α -linolenic acid (Fig. 1). However, 13-HOTE was not detected in the oxylipin-rich extracts from the gametophytic liquid cell suspension cultures. The hydroxy fatty acid 13-HOTE (γ) (13-hydroxy-6,9,11-octadecatrienoic acid), a putative product of ω -6 LOX metabolism deriving from γ -linolenic acid, was not detected in either the sporophyte tissue or the cultured gametophytic cell biomass. The detection limit for the hydroxy fatty acids by the GC-MS analysis technique used in this study was conservatively estimated to be 4 μ g hydroxy fatty acid g⁻¹ dry cell mass. If 13-HOTE was present in the culture samples, then the yield was below this threshold value.

Hydroxy fatty acid yields

Yields of the hydroxy fatty acids 13-HODE, 13-HODTA, and 15-HETE from flask cultures of *L. saccharina* gametophytic cell suspensions after 30 days in culture presented in Table 2. A given fatty acid feeding experiment and its associated control experiment are grouped together. Each hydroxy fatty acid yield was obtained from cell biomass pooled from three replicate flasks. The designations 'Ls-1' and 'Ls-2' represent the hydroxy fatty acid yields from two sep-

Table 1. Identified compounds within retention time window of 12.6 to 15.6 min for the total ion abundance chromatogram shown in Fig. 4

Peak	% Match*	Compound name	Signature fragments† (<i>m/z</i>)
1	99	13-hydroxy-6Z,9Z,11E,15Z-octadecatetraenoic acid (13-HODTA) methyl ester, TMSi ether	309 (100), 187(13)
2	81	ricinelaic acid methyl ester, TMSi ether and 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) methyl ester, TMSi ether	270(14), 187 (100) 311 (100), 171(15)
3	94	arachidic acid methyl ester	326(53), 283(38), 143(100)
4	86	13-HODTA methyl ester, TMSi ether (rearranged)	309 (100) 171(30)
5	10‡	13-HODE methyl ester, TMSi ether	311 (100), 171(42)
6	64	hexanedioic acid, diocyl ester	241(6), 129(100)
7	96	13-HODTA methyl ester, TMSi ether (rearranged)	309 (4), 171(100)
8	53	1,2-benzenedicarboxylic acid, butyl cyclohexyl ester	149(100), 167(37)
9	98	15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE) methyl ester, TMSi ether	335 (39), 225(100)
10	40	1,3,5-triazine-2,4-diamine, 6-(3-methylphenyl)	201(100)
11	90	2-ethylhexyl phthalate	279(10), 149(100)
12	12‡	15-hydroxy-5Z,8Z,11E,13E-eicosatetraenoic acid (15-HETE) methyl ester, TMSi ether	335 (35), 173(100)
13	68	15-HETE methyl ester, TMSi ether (rearranged)	335 (41), 173(100)

*% Match is percentage of all *m/z* signals found at the peak maxima relative to all *m/z* signals of the pure standard compound.

† Fragments highlighted in bold are the signature fragments for the oxylipins; relative intensities based on the actual sample profiled in Fig. 4, not pure compound standards.

‡ Low match with standard due to high relative intensity of background ions.

Table 2. Absolute and relative yields of 13-HODE, 13-HODTA, and 15-HETE from flask cultures *L. saccharina* female gametophyte cell suspensions after 30 days of cultivation

Cell line fatty acid added, and subculture cycle	Hydroxy fatty acid yield µg Product g ⁻¹ dry cell mass (% of control)			Mole ratio*
	13-HODE	13-HODTA	15-HETE	
Ls-1 control, cycle 17	939	476	229	4.4:2.3:1.0
Ls-1 + γ-LA, cycle 17	1880 (200)	1360 (286)	710 (310)	2.9:2.1:1.0
Ls-1 control, cycle 21	224	128	48	5.1:2.9:1.0
Ls-1 + γ-LA, cycle 21	330 (147)	193 (151)	85 (177)	4.2:2.5:1.0
Ls-2 control, cycle 15	669	216	83	8.8:2.9:1.0
Ls-2 + LA, cycle 15	1190 (178)	590 (273)	311 (375)	4.1:2.1:1.0
Ls-2 + α-LA, cycle 15	153 (23)	43 (20)	20 (24)	8.2:2.3:1.0
Ls-2 control, cycle 19	418	116	51	8.8:2.5:1.0
Ls-2 + LA, cycle 19	412 (99)	197 (170)	204 (400)	2.2:1.1:1.0
Ls-2 + αLA, cycle 19	106 (25)	66 (57)	40 (78)	2.9:1.8:1.0

*Mole ratio is 13-HODE:13-HODTA:15-HETE.

arately maintained culture lines of *L. saccharina* female gametophytic cells. The hydroxy fatty acid 13-HODE was always produced in the highest amount, followed in order by 13-HODTA and 15-HETE. Specifically, yields for 13-HODE ranged from 400–900 µg g⁻¹ dry cell mass, whereas yields for 13-

HODTA and 15-HETE ranged from 100–500 µg g⁻¹ and 50–200 µg g⁻¹ g dry cell mass, respectively. The absolute yields of the three identified hydroxy fatty acids varied significantly between experiment groups and consequently were not directly comparable to one another. The overall variability in the yield probably

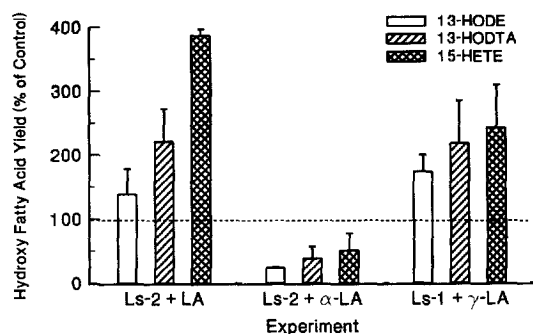


Fig. 5. Hydroxy fatty acid yields for the cultures incubated with a given fatty acid, normalized against the yield for each control culture. The normalized yields were averaged over two different subculture cycles. The error bars represent 1 standard deviation (s.d.) of the averaged value.

came from three sources: (1) the inherent variability in hydroxy fatty acid production associated with the culture itself as it passed through a series of subcultures; (2) the variability in the hydroxy fatty acid formation kinetics between subcultures; and (3) the experimental variability associated with all the sample analysis procedures, including the quantitative GC-MS analysis.

The response of the hydroxy fatty acid yield to the exogenous addition of fatty acids was brought out more clearly when the yield of a given fatty acid feeding experiment was normalized against the yield of its control experiment (Fig. 5). This form of data representation minimized the variability associated with the different subculture cycles. The normalized yield values reported in Fig. 5 were averaged over two separate subculture cycles. Figure 5 reveals that the exogenous addition of each C_{18} fatty acid to the culture non-selectively affected the level of all three hydroxy fatty acids relative to its control. The relative yield of 13-HODE was not significantly affected by the exogenous addition of fatty acids, as the yield increased less than 200% of the control cultures. However, relative yields of 13-HODTA and 15-HETE increased by 200 to 400% relative to controls in response to the exogenous addition of linoleic and γ -linolenic acids. The exogenous addition of α -linolenic acid depressed the yield of all three hydroxy fatty acids. A likely explanation for this last result that α -linolenic acid was much more toxic to the culture than linoleic or γ -linolenic acid at 200 mg l⁻¹ concentration.

The role of ω -6 LOX metabolism in the plant physiology of *L. saccharina* in either the gametophytic or sporophytic stages of the life cycle is unknown. However, in land plants, lipoxygenase metabolism plays a critical role in the biological defense response to stress [11–13]. A significant event in this stress response is the release of endogenous stores of 18:2 and 18:3 fatty acids [11, 12]. Our experiments suggest that the exogenous addition of a single fatty acid could stimulate the production of three hydroxy fatty acids associated with ω -6 LOX metabolism. Although

exogenously supplied fatty acids could serve as precursors for the ω -6 LOX oxidation to hydroxy fatty acids (Fig. 1), tracer studies were not performed to verify this process. However, exogenously supplied fatty acids did put a chemical stress on the *L. saccharina* gametophytic cell suspension cultures. It is possible that this chemical stress could have elicited the release of endogenous fatty acids available for enzymic oxidation to hydroxy fatty acids by ω -6 LOX. Further experiments would be needed to assess this hypothesis.

In conclusion, the production bioactive hydroxy fatty acids by *L. saccharina* gametophyte cell suspension cultures is possible at the flask scale. Our next step is to determine if these compounds are produced in phototrophic bioreactor culture.

EXPERIMENTAL

***Laminaria saccharina* cell cultures.** Female gametophytes of the macrophytic brown alga *Laminaria saccharina* were isolated from spores released from reproductive blade tissue using procedures described by Lüning [14] and Lüning and Neushul [15]. Further details are provided by Qi and Rorrer [8]. The photolithotrophic gametophytes were maintained on GP2 artificial seawater medium as modified by Steele and Thursby [16] at pH 8.5. The culture vol. was 100 ml of liquid cell suspension per 250 ml erlenmeyer flask. The culture flasks were incubated at 12° under white fluorescent light at an incident illumination intensity of 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ and photoperiod of 16 hr light/8 hr dark within an illuminated low-temp. incubator. The gametophytes grew as branched uniseriate filaments loosely packed into clumps of 0.5 to 2 mm in diameter. Every 30 days the liquid culture suspension was blended for 10 s on a Osterizer blender within a sterile blending cup. This procedure dispersed the filamentous cell mass to a uniform clumps between 300 and 400 μm in diameter (as determined by wet sieving) but did not comprise cell viability. The blended culture was subcultured at 25% into fresh GP2 medium. Sterile technique was used for all procedures. The cultures flasks were swirled for 10 s once day to disperse the culture and to re-equilibrate the dissolved gas concs (O_2 , CO_2) in the liquid medium.

Fatty acid feeding experiments. The polyunsaturated fatty acids (PUFAs) considered for culture feeding experiments were linoleic acid (9,12-octadecadienoic acid, 99% purity as free acid, Sigma #L1376), α -linolenic acid (9,12,15-octadecatrienoic acid, 98% purity as free acid, Sigma #L2376), and γ -linolenic acid (6,9,12-octadecatrienoic acid, 99% purity as free acid, ICN Biomedicals #55244). Stock solutions (10 g l⁻¹) were prepd by dissolving 1.00 g of the fatty acid into 10 ml of EtOH. This mixt. was slowly added to 90 ml H₂O under continuous stirring to bring the final vol. up to 100 ml. The pH of the final stock soln was adjusted to 7.5–8.0 with NaOH.

Each stock soln was sterile-filtered and stored frozen in the dark.

Flask cultures were allowed to achieve the late exponential phase of growth before a given fatty acid was added to the liquid to the liquid medium. In the present experiments, 100 ml of freshly subcultured liquid cell suspension was cultured for 20 days in a 250 ml Erlenmeyer flask at the environmental conditions described earlier. At the 20th day of cultivation, a specified vol. of stock soln containing a given fatty acid was added directly to the liquid suspension culture in each flask. Typically, 2 ml of 10 g l^{-1} stock soln was added to each culture flask to set the initial concn of a given fatty acid to 200 mg l^{-1} culture. The fatty acid stock soln was readily soluble in the artificial seawater culture medium at pH 8.5. The culture was incubated with the fatty acid for an additional 10 days to provide a total cultivation time of 30 days. Three flasks were set up for a given fatty acid incubation experiment. Three control flask cultures which did not contain any exogenously added fatty acids were also cultivated in parallel. For a given set of experiments, both the control flask cultures and the fatty-acid fed flask cultures were all initiated from a common culture inoculum stock in order to minimize the variability associated with subculture. At the 30th day of cultivation, the three replicate flask cultures were pooled together. The cell density, expressed as mg of dry cell weight per l of culture (mg DCW l^{-1}) was determined from a 40 ml aliquot of the pooled culture as described by Qi and Rorrer [8]. The remaining liquid suspension culture was filtered on a $20 \mu\text{m}$ nylon mesh under vacuum. The fr. wt of the retained biomass and the vol. of the filtrate were also measured. The dry wt% of a given biomass sample was determined from the ratio of the dry cell density to the fresh cell density. The filtered biomass was stored frozen in the dark for future analysis.

Viability screening experiments. The viability of the culture was sensitive to the exogenous addition of C_{18} fatty acids. Therefore, the toxicity of each fatty acid toward the *L. saccharina* liquid suspension culture was determined. Since biomass collection was not required, liquid cell suspensions were cultivated in 16 ml multi-well plates (8 ml of culture per well, duplicate samples) to increase the number of samples which could be screened simultaneously from a common inoculum source. The fatty acids were added to the culture wells at the 20th day of cultivation. Following the addition of fatty acids, aliquots were periodically removed from each well, stained with Evan's blue non-vital stain, and then observed microscopically at $\times 100$. The cell viability was semi-quantitatively assessed. The incubation time required to maintain the viability near 100% was selected to characterize the toxicity response of the *L. saccharina* cultures to exogenous addition of fatty acids.

Partial purification of oxylipins in cell biomass. The fr. containing the hydroxy fatty acids in the *L. saccharina* gametophytic cell culture biomass was iso-

lated by lipophilic extraction and TLC using the general procedures described by Proteau and Gerwick [5]. In the present study, 100 mg of filtered fresh biomass was extracted with 20 ml of CH_2Cl_2 -MeOH (2:1) with a Potter-Elvehjem tissue homogenizer at room temp. After the first extraction, $2 \mu\text{g}$ of ricinelaidic acid (*R*-12-hydroxy-9(*E*)-octadecenoic acid, 99% purity, Sigma #R7257) was added to the extract to serve as an int. standard for later quantitative analysis. The pulp was filtered, and the residual solids were re-extracted and then filtered twice more. The filtrates from each extraction were pooled together, and solvents were removed by rotary evapn at room temp. to yield a viscous green extract (typical wt 5 mg). This extract was then methylated in 2 ml CH_2N_2 at room temp. to yield the methyl-ester derivatives of the fatty acids. The methylated extract was partially purified by TLC using silican gel 60 F₂₅₄ on Al as the stationary phase and 25% EtOAc in hexane as the eluting solvent. Compounds were detected by UV illumination of the TLC plate or by visual inspection of charred spots after the TLC plate was sprayed with 50% wt H_2SO_4 soln and heated. Methylated standards of ricinelaidic acid, 13-HODTA, and 15-HETE were used to locate the R_f position of the partially purified hydroxy fatty acid fraction ($R_f = 0.26$ to 0.30). The hydroxy fatty acid spot was broadly scooped off the TLC plate (0.1 to $0.8 R_f$) and eluted with Et_2O . The final extract was blown down to dryness under flowing N_2 at room temp. to remove Et_2O .

Gas chromatography-mass spectrometry (GC-MS). The methyl esters of the hydroxy fatty acids in the artially purified oxylipin extract were derivatized to their corresponding volatile trimethylsilyl (TMSi) ethers by reacting the sample with a mixt. containing 2 drops hexamethyldisilazane (98% purity, Aldrich H1,000-2), 2 drops chlorotrimethylsilane (98% purity, Aldrich C7,285-4), and two drops pyridine for 2 hr at room temp. The reaction mixt. was dried *in vacuo*, re-suspended in hexane, and centrifuged. The hexane fr. was reduced by roto-evapn, and the re-suspended in hexane again so that the ricinelaidic acid int. standard was approximately 50 ng per μl . The TMSi methyl ester derivatives of the hydroxy fatty acids were sepd and identified by GCMS using a Hewlett-Packard HP 5890 Series II gas chromatograph and HP 5971 mass spectrometer at 70 eV total ionization energy. The sample ($1 \mu\text{l}$) was injected into a Hewlett-Packard HP-1 crosslinked methyl silicone capillary column ($12.5 \text{ m} \times 2 \text{ mm} \times 0.33 \mu\text{m}$) operating with He as the carrier gas at a temp. programme of 100° initial, $10^\circ \text{ min}^{-1}$ ramp, 240° final, 250° injector, and 280° detector.

The response factors for 13-HODE, 13-HODTA, and 15-HETE standards were determined separately with respect to the ricinelaidic acid int. standard. Relative response factors with respect to the ricinelaidic acid int. standards are presented in Table 3. The nominal limit for quantitative detection of each hydroxy fatty acid by GC-MS was 2 ng per μl injected (signal to noise ratio equal to 8). Standards for 13-HODE,

Table 3. Relative response factors of identified hydroxy fatty acids by GC-MS. Abbreviations: TIC (total ion current signal), RA (ricinelaidic acid internal standard)

Compound	Relative response factor
13-HODE	$0.102 \frac{\text{Area}_{m/z187,RA}}{\text{Area}_{m/z311,13-HODE}} \frac{\text{nmol 13-HODE std}}{\text{nmol RA std}}$
13-HODTA	$0.93 \frac{\text{Area}_{TIC,RA}}{\text{Area}_{TIC,13-HODTA}} \frac{\text{nmol 13-HODTA std}}{\text{nmol RA std}}$
15-HETE	$1.95 \frac{\text{Area}_{TIC,RA}}{\text{Area}_{TIC,15-HETE}} \frac{\text{nmol 15-HETE std}}{\text{nmol RA std}}$

13-HODTA, and 15-HETE (as their TMSi methyl ester derivatives) rearranged during GC-MS analysis, each forming two or three dominant peaks. Each rearranged peak had one signature m/z fragment unique to the hydroxy fatty acid compound: 13-HODE (m/z 311 for two peaks), 13-HODTA (m/z 309 for three peaks), 15-HETE (m/z 335 for three peaks). The ratio of peak areas for the rearranged peaks remained constant at different standard concs for a given hydroxy fatty acid. The ricinelaidic acid (as its TMSi methyl ester derivative) did not rearrange, as a TMS ether is not adjacent to a diene. Response factors for each hydroxy fatty acid were based on the sum of the areas of all rearranged peaks containing the signature m/z fragment unique to that fatty acid. The 13-HODE co-chromatographed with the ricinelaidic acid int. standard. Therefore, the response factor for 13-HODE was based on peak areas obtained from mass-selective signal data at m/z 311 for 13-HODE and m/z 187 for the ricinelaidic acid. The standards for 13-HODE, 13-HODTA, and 15-HETE were isolated as pure compounds from *L. saccharina* sporophyte tissue as described by Proteau and Gerwick [5]. With their identities verified by NMR and GC-MS [5]. The library MS spectra for each standard was constructed using these authenticated compounds. The final yield of each oxylipin in the sample was expressed as μg product per g dry cell mass using the amount of each oxylipin in the sample as determined by the GC-MS int. standard method and the dry cell mass in the sample.

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