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Phytochemistry 63 (2003) 669–678
PHYTOCHEMISTRY
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Hydroperoxy-arachidonic acid mediated *n*-hexanal and (*Z*)-3- and (*E*)-2-nonenal formation in *Laminaria angustata*

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Received 29 October 2002; received in revised form 6 January 2003

Abstract

In higher plants, C6 and C9 aldehydes are formed from C18 fatty acids, such as linoleic or linolenic acid, through formation of 13- and 9-hydroperoxides, followed by their stereospecific cleavage by fatty acid hydroperoxide lyases (HPL). Some marine algae can also form C6 and C9 aldehydes, but their precise biosynthetic pathway has not been elucidated fully. In this study, we show that *Laminaria angustata,* a brown alga, formed C6 and C9 aldehydes enzymatically. The alga forms C9 aldehydes exclusively from the C20 fatty acid, arachidonic acid, while C6 aldehydes are derived either from C18 or from C20 fatty acid. The intermediates in the biosynthetic pathway were trapped by using a glutathione/glutathione peroxidase system, and subjected to structural analyses. Formation of (*S*)-12-, and (*S*)-15-hydroperoxy arachidonic acids [12(*S*)HPETE and 15(*S*)HPETE] from arachidonic acid was confirmed by chiral HPLC analyses. These account respectively for C9 aldehyde and C6 aldehyde formation, respectively. The HPL that catalyzes formation of C9 aldehydes from 12(*S*)HPETE seems highly specific for hydroperoxides of C20 fatty acids.

Keywords: *Laminaria angustata*; Laminariaceae; Brown algae; Lipoxygenase–hydroperoxide lyase pathway; (*S*)-12-Hydroperoxy arachidonic acid; (*S*)-15-Hydroperoxy arachidonic acid; *n*-Hexanal; Nonenal

1. Introduction

C6 and C9 aldehydes are volatile compounds that have a fresh green and cucumber-like flavor. In essential oil prepared from edible brown algae, *Laminaria* spp., C9 aldehydes such as (*E*)-2-nonenal (**1**) could be found in relatively large quantities, followed by C6 aldehydes, such as *n*-hexanal (**2**), (*Z*)-3- and (*E*)-2-hexenal (Kajiwara et al., 1996). In higher plants, C6 and C9 aldehydes are formed from unsaturated fatty acids, such as linoleic or linolenic acid through sequential reactions by lipoxygenase (LOX) and fatty acid hydroperoxide lyase (HPL) (Blée, 1998). This enzymatic pathway is a branch of a highly divergent biosynthetic pathway, called either the (phyto)oxylipin or octadecanoid pathway. The pathway starts from fatty acids, and various products such as jasmonates, hydroxylated fatty acids, keto-fatty acids, and long chain aldehydes, are formed in addition to the C6 and C9 aldehydes. In *Phaseolus vulgaris* (L.) leaves, C6 aldehydes are rapidly formed during hypersensitive resistance response against phytopathogenic *Pseudomonas syringae* (Croft et al., 1993). In *Arabidopsis*, it has been shown that the aldehydes can induce a subset of defense-related genes (Bate and Rothstein, 1998). From these results it has been established that the aldehydes have significant roles in resistance response of higher plants against abiotic and biotic attacks. The molecular mechanism of the pathway is now being clarified. Lipoxygenases form 13- and/or 9-hydroperoxides of linoleic or linolenic acid. Fatty acid hydroperoxide lyase, a novel type of cytochrome P450 (CYP74), acts on the hydroperoxides. From 13-hydroperoxides, C6 aldehydes and C12 oxo acids are formed, whereas from 9-hydroperoxides, C9 aldehydes and C9 oxo acids are formed. This type of HPL is sometimes called as a heterolytic HPL. Most HPLs characterized so

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doi:10.1016/S0031-9422(03)00026-8

far, show high substrate-specificity to 13-hydroperoxides. However, another type of HPL that can act on both the 13- and 9-hydroperoxides at almost equal efficiency has been found recently (Matsui et al., 2000). In addition, C5 or C8 compounds are also formed from 13-hydroperoxides, presumably through homolytic cleavage of the hydroperoxides. Thus, the HPL accounting for C5 or C8 formation is termed a homolytic HPL (Matsui, 1998). On the other hand, the C6 and C9 aldehyde-forming pathway in marine algae has not yet been fully studied. Recently, it has been shown that a diatom (*Thalasssiosira rotula*) can form short-chain aldehydes having C10 structures, such as (*E*, *Z*)-2,4-decadienal and (*E*, *Z*, *Z*)-2,4,7-decatrienal, which inhibit egg cleavage of copepods (Ban et al., 1997). They are thus believed to be involved in chemical defense in the diatom (Pohnert, 2002). C10 aldehydes are proposed to be formed from arachidonic acid through formation of 11-hydroperoxyeicosatetraenoic acid formed by LOX followed by cleavage by HPL (Pohnert and Boland, 1996; Pohnert, 2000). Because marine algae contain relatively high quantities of C20 and C22 fatty acids, and some can be used as substrates by LOX, it was thought that these C20 or C22 fatty acids would also serve as substrates of the lyase branch to form C6 and C9 aldehydes (Kajiwara, 1997). In this case, it is assumed that hydroperoxides of C20 and C22 fatty acids, should be formed (Gerwick, 1994). This accumulating knowledge led us to further attempt to elucidate the enzymatic process forming C6 and C9 aldehydes in marine algae. In this study we elucidated that C9 aldehydes, (*Z*)-3-nonenal (**3**) and (*E*)-2-nonenal (**1**), and C6 aldehyde, *n*-hexanal (**2**), could be formed from arachidonic acid (**4**) through formation of regio- and stereo-specific formation of hydroperoxides of the fatty acids. Detailed structural analyses of the hydroperoxides were also carried out.

2. Results and discussion

2.1. Enzymatic C6 and C9 aldehyde-formation in homogenized fronds

In order to investigate the enzymatic formation of C9 and C6 aldehydes, their amounts were quantified with SPME-GC–MS after incubating the homogenate of fronds at 5 °C for 80 min. With this method, volatiles were collected at 40 °C, so that very little pyrolysis could occur if heat-labile hydroperoxides were in the homogenate. In the SPME extract from the homogenate of *L. angustata,* (*E*)-2-nonenal (**1**) was found as the main product followed by (*E,Z*)-2,6-nonadienal, 1-octen-3-one, and (*E*)-2-nonen-1-ol (Table 1). C6 aldehydes, such as (*E*)-2-hexenal and *n*-hexanal (**2**) could also be found, as well as the other C7, C8, C12, and C14, which were aldehydes as previously found in simultaneous distillation extraction (Kajiwara et al., 1996). The aldehydes other than C6 and C9 are also thought to be formed from fatty acids; however, their biosynthetic pathway has not yet been completely elucidated. After incubation of the homogenized fronds at 5 °C for 80 min, the quantity of almost all compounds including (*E*)-2-nonenal (**1**) and *n*-hexanal (**2**) increased, which suggested that these compounds were enzymatically formed from endogenous substrates during incubation. In higher plants it is well established that these C6 and C9 aldehydes are formed from fatty acids such as linoleic and linolenic acids, through sequential catalyses by LOX and heterolytic HPL (Blée, 1998). Interestingly, the amounts of 1-octen-3-ol and 1-octen-3-one also increased after incubation. These C8 compounds are known to be formed via pyrolysis of hydroperoxides of arachidonic acid, and, in fungi and mushrooms, they are shown to be formed from 10-hydroperoxide of linoleic acid by homolytic HPL (Wurzenberger and Grosch, 1984a, b, 1986). Because heat was avoided during incubation and extraction, biosynthetic enzymes might be responsible for the increase of the C8 compounds in the homogenate. This suggests that heterolytic and homolytic HPLs are present in the fronds of *L. angustata*.

2.2. Unsaturated fatty acid precursors for the aldehyde branch

Previously it was suggested that C20 fatty acids, such as arachidonic acid (**4**), might be a precursor for the formation of C6 and C9 aldehydes in marine algae (Kajiwara, 1997). This is in contrast to the aldehyde-forming pathway in higher plants that predominantly uses C18 fatty acids, such as linolenic and linoleic acids (Hatanaka 1996; Matsui, 1998; Noordermeer et al., 2001). In order to confirm the substrates for C6 and C9 aldehyde formation in marine algae, both arachidonic

Table 1
Composition of volatile compounds identified by SPME-GC–MS from crude homogenate prepared from *L. angustata*[b]

Compounds	Content (μg/100 g fr. wt)	
	Without incubation	With incubation
n-Hexanal (**2**)	29.7	131
(*E*)-2-Hexenal	n.d.[a]	69.8
1-Octen-3-one	98.5	216
(*E*)-2-Octenal	44.7	114
1-Octen-3-ol	37.1	120
(*E, E*)-2, 4-Heptadienal	n.d.[a]	38.8
(*E*)-2-Nonenal (**1**)	868	1093
(*E, Z*)-2, 6-Nonadienal	175	242
(*E*)-2-Nonen-1-ol	82.7	119
(*E, E*)-2, 4-Dodecadienal	67.3	103
Tetradecanal	52.6	33.5

[a] n.d.: not detected.
[b] Homogenized fronds were incubated at 4 °C for 80 min.

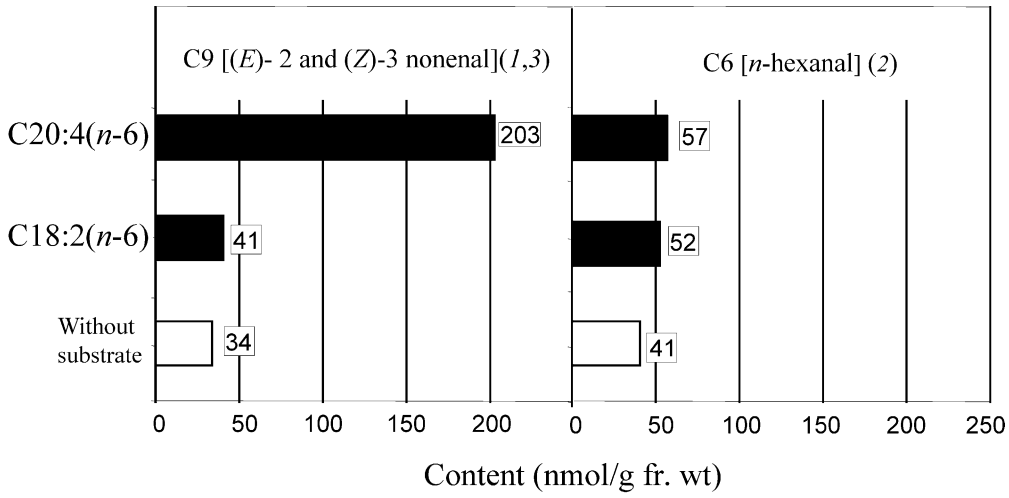

Fig. 1. Substrate specificity for *n*-hexanal (**2**), (*Z*)-3-nonenal (**3**) and (*E*)-2-nonenal (**1**) formation in the homogenate prepared from *L. angustata*. Reaction of crude enzyme (2.9 mg protein) with unsaturated fatty acid (700 nmol) was performed at 4 °C for 30 min. Under the condition employed, aldehyde-formation proceeded almost linearly until 30–40 min. Each reaction was done in duplicate, with triplicate HPLC analyses. The means of duplicates are shown. The amounts of aldehydes formed from with (■) and without (□) exogenous fatty acids were determined by reversed phase HPLC.

(**4**) and linoleic acid were added to the crude enzyme solution and the amount of C6 and C9 aldehydes were quantified. Formation of these aldehydes proceeded almost linearly until 30–40 min after addition of the fatty acid. As shown in Fig. 1, formation of C6 and C9 aldehydes was hardly detected without the crude enzyme solution, which excluded the possibility of nonenzymatic formation through autoxidation and subsequent non-enzymatic cleavage of the fatty acids. It was found that C9 aldehydes (**1** and **3**) were essentially formed from arachidonic acid (**4**), while the formation of C9 aldehydes (**1**,**3**) from linoleic acid was relatively low. In contrast, an activity to form *n*-hexanal (**2**) was low, although a very slight increase in the amount could be detected either from linoleic or arachidonic acid (**4**) with almost the same effectiveness.

2.3. Hydroperoxy fatty acids as the intermediates

According to current knowledge of the aldehyde-forming pathway in higher plants, the results shown above suggested that C9 aldehydes (**1** and **3**) might be formed from the 12-hydroperoxide of arachidonic acid (**5**), and at the same time, the 15-hydroperoxide of arachidonic acid (**6**) might also be formed to support the C6 aldehyde formation from arachidonic acid (**4**). It can be assumed that LOX(s) forms the hydroperoxides from arachidonic acid, and that they are further cleaved by heterolytic HPL(s). In order to examine whether hydroperoxides of arachidonic acid are formed as intermediates during C6 and C9 aldehyde formation, glutathione–glutathione peroxidase (GSH–GPx) system was used. This system is known to reduce lipid hydroperoxides to the corresponding hydroxides in mammalian cells (Bryant et al., 1982; Hatzelmann et al., 1989; Weitzel and Wendel, 1993; Schnurr et al., 1996; Sutherland et al., 2001). Since fatty acid hydroxides cannot be substrates for HPL, the addition of the system to the reaction mixture would inhibit formation of C6 and C9 aldehydes. This procedure has been successfully used to confirm the involvement of a fatty acid hydroperoxide as an intermediate in an oxylipin pathway (Hamberg et al., 1998, 1986; Hamberg and Gerwick, 1993; Brodowsky et al., 1992; Hombeck et al., 1999). When GSH was added to the reaction mixture, a slight increase in the amount of aldehyde was observed (data not shown). It seemed that GSH enhanced the aldehyde-forming reaction probably through protecting the enzymes from oxidative inactivation. This result also suggested that the activity of endogenous GPx in the homogenate, if any, was negligible. Addition of GPx showed essentially little effect on the activity, which in turn suggested that there was little endogenous GSH in the reaction mixture. As shown in Fig. 2, addition of GPx to the reaction mixture containing 3 mM GSH decreased the activity to form both the C6 and C9 aldehydes from arachidonic acid (**4**) in a concentration-dependent manner. This result strongly indicates that there must be the formation of hydroperoxides of arachidonic acid (**4**) as intermediates to form C6 and C9 aldehydes. This suggests that LOX–HPL system(s) exist in the marine algae.

2.4. Structural analyses of hydroxides of arachidonic acid

The previous experiment indicated that hydroperoxides of arachidonic acid (**4**) were formed as intermediate of the HPL branch in the oxylipin pathway in

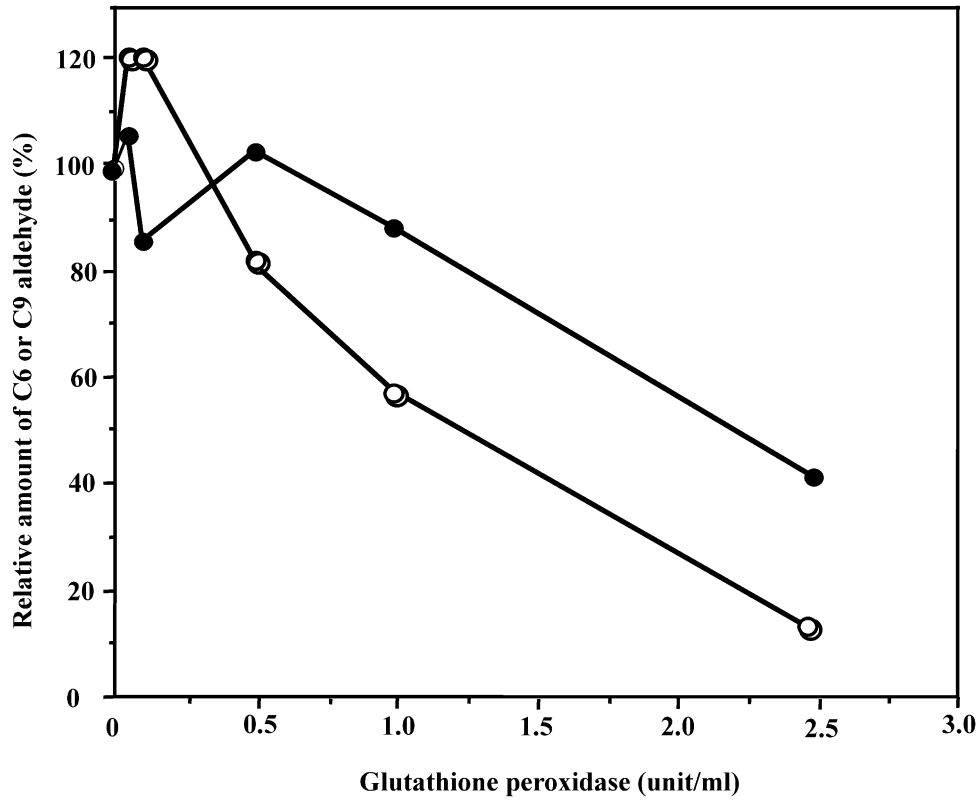

Fig. 2. Effect of glutathione–glutathione peroxidase on activities to form C6 and C9 aldehydes from arachidonic acid (**4**). Reaction of crude enzyme (2.6 mg protein) with arachidonic acid (**4**) (700 nmol) was performed at 4 °C for 30 min in the presence of a given amount of glutathione peroxidase (0–2.5 unit/ml) and 3 mM glutathione. Relative amounts (%, the amounts without the peroxidase was assigned as 100%) of *n*-hexanal (**2**) (○) and a sum of (*Z*)-3-nonenal (**3**) and (*E*)-2-nonenal (**1**) (●) are shown.

the marine algae. It would be plausible that, in the presence of GSH and GPx, the hydroperoxides formed as the intermediates must be trapped and converted into the corresponding hydroxides. Thus, arachidonic acid (**4**) was incubated with the crude enzyme solution in the presence of GSH and GPx. Fatty acids and their derivatives were then extracted and analyzed with normal phase HPLC employing a photodiode array detector. Two major absorbing compounds with an λ_{max} at 235 nm could be detected in the complete system (Fig. 3). These two compounds were thought to be the reduced form of the hydroperoxides formed as intermediates from arachidonic acid (**4**).

In order to analyze their structures, the hydroxides trapped by the GSH and GPx system were isolated in larger amounts, partially purified, and analyzed with reversed phase HPLC. Under these HPLC conditions, three unknown peaks, which were tentatively named as compounds I, II, and III, were resolved (Fig. 4, trace a) and all had λ_{max} values of 227, 234, and 234 nm, respectively. When authentic samples of 12-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid (12HETE) and 15-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (15HETE) were subjected to the HPLC analyses, they eluted at 11.5 and 12.7 min, respectively (Fig. 4, trace b) i.e. their retention times coincided with those of the peaks of compounds II and III found in the reaction mixtures. These coincidences were further confirmed by co-injection, in which compound II coeluted with 15HETE and compound III coeluted with 12HETE. In order to confirm the structure, the hydroxy fatty acids purified by HPLC were methylated, hydrogenated and converted into their TMSi ether derivatives, then they were subjected to GC–MS analyses. The mass spectrum of compound II (15HETE) showed prominent peaks at *m*/*z* (rel. int.): 173 (100), 343 (37.5), 367 [M-47] (7), 383 [M-31] (3.1), 399 [M-15] (1.5). Whereas compound III (12HETE) showed prominent peaks at *m*/*z* (rel. int.): 215 (100), 301 (90.6), 367 [M-47] (9.4), 383 [M-31] (3.1), 399 [M-15] (1.6). The characteristic ion peaks at 173 and 343 (*m*/*z*) and at 215 and 301 (*m*/*z*) are diagnostic for the hydroxyl groups of 15HETE and 12HETE, respectively (Boeynaems et al., 1980). These evidences supported that the two compounds are formed from the intermediates, 12-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid (**5**) and 15-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid (**6**). On the other hand, compound I is still unidentified.

To determine the stereochemistries of the HETE methyl esters, each was subjected to a chiral phase

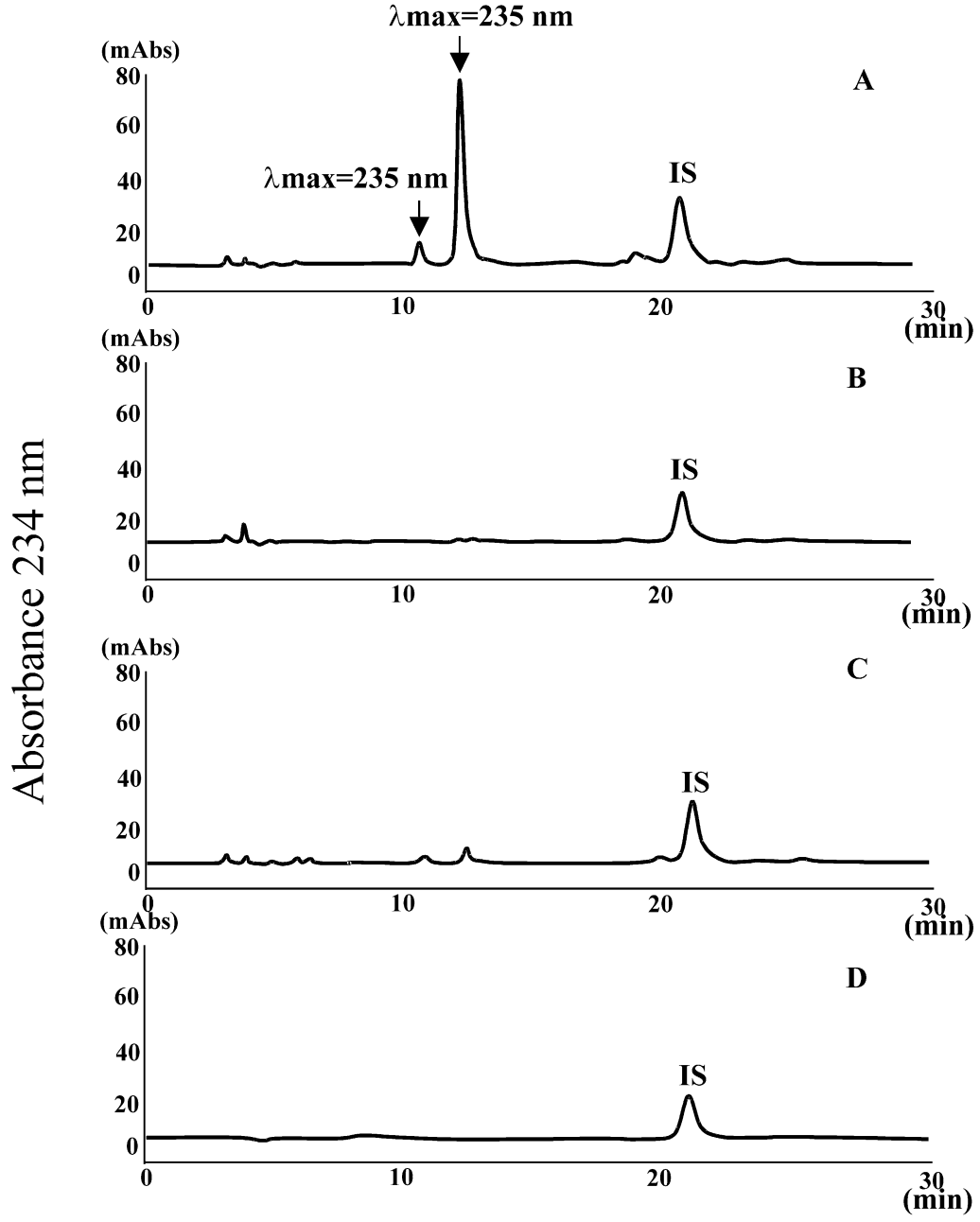

Fig. 3. Enzymatic generation of hydroxy-arachidonic acids in *L. angustata*. Reaction was composed of (A) crude enzyme (1.9 mg protein), arachidonic acid (**4**) (700 nmol), glutathione peroxidase (4 unit/ml) and glutathione (3 mM), (B) as in A but without arachidonic acid (**4**), or (C) as in A but without crude enzyme solution. Each reaction was performed at 4 °C for 30 min. A chromatogram with an internal standard (15-hydroxy-eicosadienoic acid) is showed in panel D.

HPLC system. Under the chiral HPLC condition employed, both the enantiomers of 12 and 15HETE could be baseline separated (Fig. 5). By using enantiomerically pure *S*-isomer of each hydroxide, it has been revealed that the (*R*)-isomer of each elutes faster than the corresponding (*S*)-isomer. When each hydroxide derived from the products formed by the algae were subjected to the HPLC system, one peak could be found respectively, and each retention time coincided with the respective (*S*)-isomer. This was again confirmed by co-injection, and these results indicated that 12HETE and 15HETE formed by the algal enzyme had their hydroxyl group in (*S*)-configuration with optical purities over 99% e.e.

3. Concluding remarks

C6 aldehydes [*n*-hexanal (**2**), (*Z*)-3- and (*E*)-2-hexenal] are ubiquitously found in higher plants. In general, they are formed from C18 fatty acids, such as linoleic or

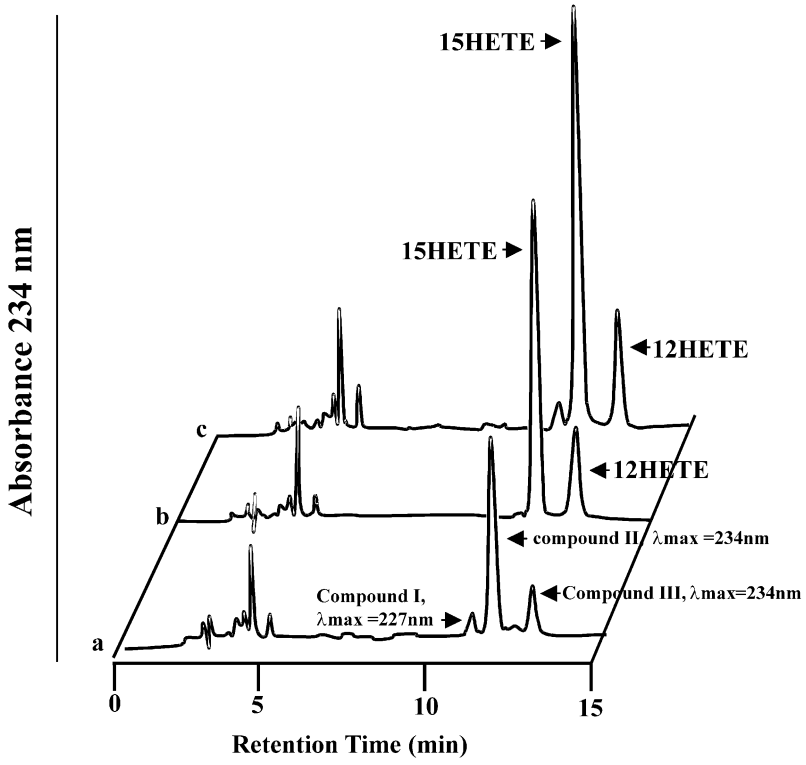

Fig. 4. Reversed phase HPLC analysis of hydroxy-arachidonic acids. Reaction was composed of crude enzyme (240 mg protein), arachidonic acid (**4**) (7 μmol), glutathione peroxidase (4 unit/ml) and glutathione (3 mM). The products isolated from the reaction mixture (trace a), and mixture of authentic 15HETE and 12HETE (trace b) were analysed. Trace c is a chromatogram obtained with co-injection of reaction mixture and authentic standards.

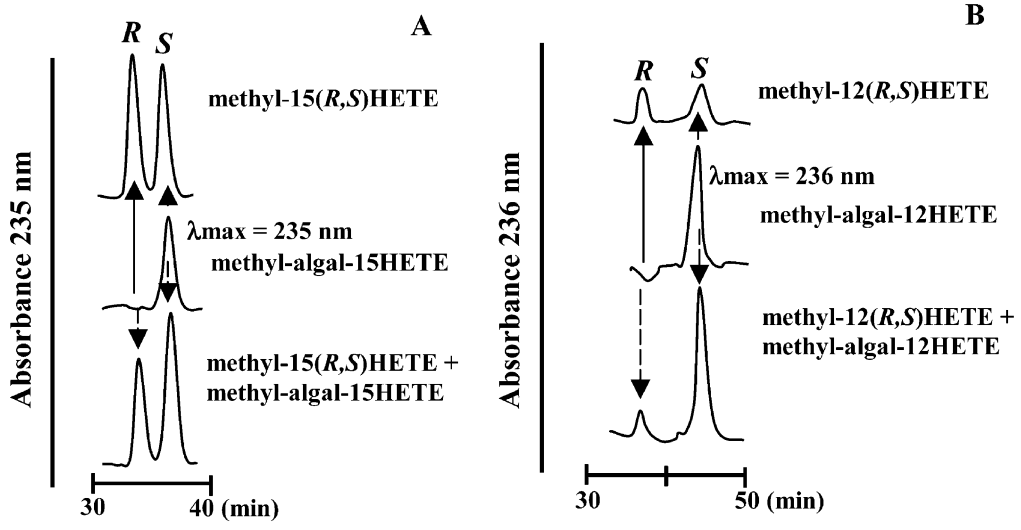

Fig. 5. Determination of configurations and optical purities by a chiral phase HPLC of methyl-15HETE (A) and methyl-12HETE (B) formed from arachidonic acid by crude enzyme solution. The peaks corresponding to authentic enantiomers were indicated with arrows.

linolenic acid, through the formation of 13-hydroperoxides, followed by their stereospecific cleavage by 13-HPL. Some plants, such as pear, cucumber, or melon can form both C9 and C6 aldehydes (Noordermeer, 2001). C9 aldehydes are also formed from C18 fatty acids through the formation of 9-hydroperoxides from the latter. It has been reported that some marine algae might also be able to form C6 and C9 aldehydes (Kajiwara et al., 1996). However, their biosynthetic pathway has not been elucidated fully. In this study, we show that *L. angustata* forms the aldehydes enzymatically. The algae forms C9 aldehydes (**1**, **3**) almost exclusively

from C20 fatty acid (**4**), arachidonic acid, through the formation of 12(*S*)HPETE (**5**), while C6 aldehydes (**2**) are derived from either C18 or C20 fatty acids, through formation of 13(*S*)-hydroperoxyoctadecadienoic acid (Boonprab et al., 2003) or 15(*S*)HPETE (**6**) (Fig. 6). The HPL that accounts for the formation of C9 aldehydes from 12(*S*)HPETE (**5**), seems to be the one highly specific to hydroperoxides of C20 fatty acids, because partially purified HPL, which is responsible for C6 aldehyde formation, shows little activity to hydroperoxides of C20 fatty acids (Boonprab et al., 2003).

4. Experimental

4.1. General

Chemicals: arachidonic acid (**4**) (⩾90% purity), linoleic acid (⩾95% purity), GSH and GPx were purchased from Sigma Chemical Co., USA. 12(*R,S*)HETE, 12(*S*)HETE, 15(*R,S*)HETE and 15(*S*)HETE were from Cayman Chemical Co., USA. Other chemicals were mostly from Wako Chemical Co., Japan. (*Z*)-3-Nonenal (**3**) was synthesized as described elsewhere (Kajiwara et al., 1975). 15-Hydroxyeicosadienoic acid (15HEDE) was prepared as described (Martini et al., 1994; Rustérucci et al., 1999). All solvents used in experiments were purified by distillation. For quantification experiments, treatments were prepared in duplicate, and HPLC analyses were repeated three times.

4.2. Plant materials

L. angustata was harvested at Charatsunai beach, Hokkaido (northern part of Japan) facing the Pacific Ocean during January and February, 2001. Fronds were kept at 4 °C immediately after the harvest and the temperature was maintained during shipping. For solid phase micro extraction analysis they were kept frozen at −80 °C. For biochemical experiments, they were kept at −80 °C as coarse powder prepared with liquid N_2. The whole powder was mixed prior to taking a sample (20 g) for each experiment in order to avoid individual differences.

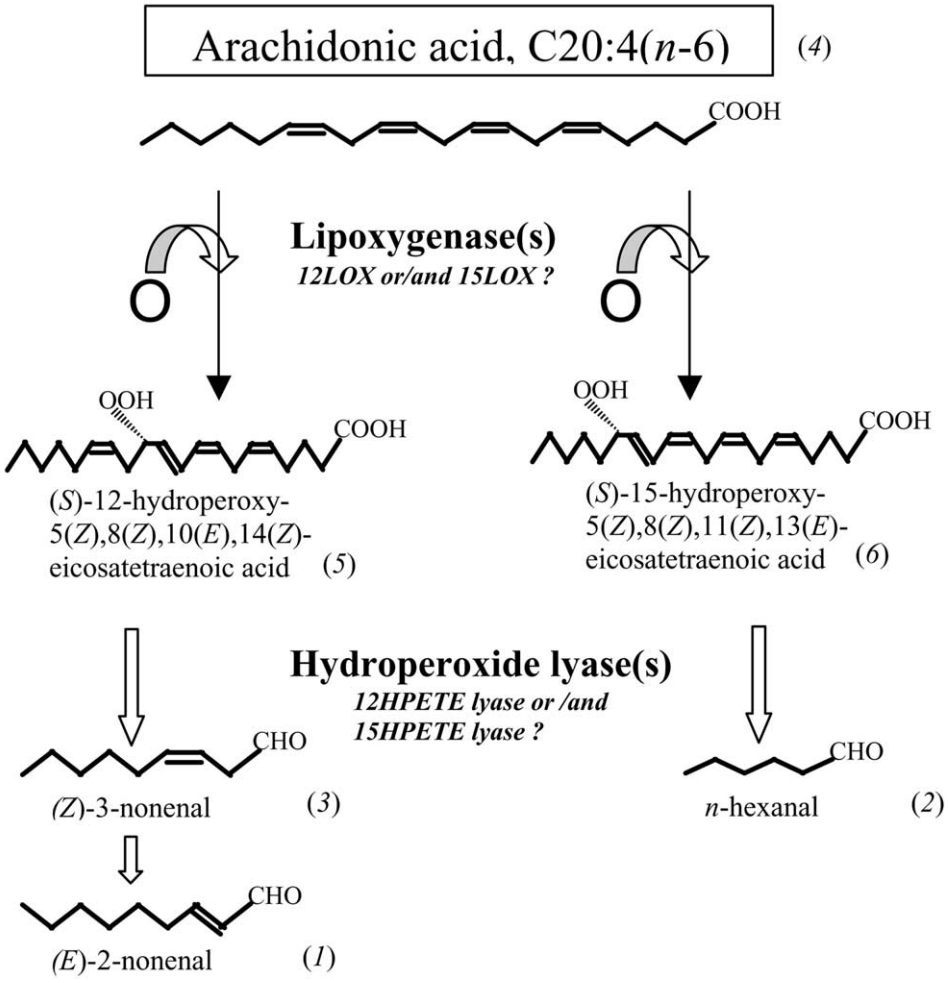

Fig. 6. The proposed pathway for C6 (*n*-hexanal (**2**)) and C9 [(*Z*)-3-nonenal (**3**) and (*E*)-2- nonenal (**1**)] formation in brown alga, *L. angustata*.

4.3. Solid phase micro extraction (SPME)

L. angustata (2 g fr. wt) was cut into small pieces and homogenized in 50 mM MES–KOH buffer (pH 5.5, 10 ml) with a Polytron mixer (Kinematca, Switzerland). The homogenate was incubated at 5 °C for 80 min, and then, solid $CaCl_2$ (660 mg) was added to inactivate enzymes. As a control, fronds were added to MES–KOH buffer (10 ml) containing $CaCl_2$ (660 mg). The mixture was then homogenized with a Polytron mixer. A portion of the mixture (20 ml) was transferred to a 22 ml screw-cap vial and 1.0 mM *n*-octanal (20 μl) was added as an internal standard. Sampling of the head space was performed by inserting a fiber (Carbowax-divinylbenzene, 65 μm, Supelco, Bellefonte, PA. USA) for 30 min at 40 °C. The adsorbed compounds were analyzed on GC–MS equipped with a fused silica capillary column (60 m×0.25 mm) coated with DB-WAX (0.25 μm), using helium as a carrier gas. The column temperature was programmed to increase from 40 °C (2 min hold) to 240 °C at 10 °C/min. The ionization energy was 70 eV.

4.4. Crude enzyme preparation

L. angustata was crushed into a fine powder under liquid nitrogen with a mortar and pestle, and subsequently with a Maxim homogenizer (Nihonseiki Kaisha Ltd., Japan). The powder was transferred into a glass bottle containing three volumes (v/w) of 0.1 M borate borax buffer pH 9.0, containing 2% Polyclar VT. The suspension was homogenized using a Polytron mixer to break down polysaccharides, then filtered through six layers of cheese cloth. The filtrate was centrifuged at 3076 *g* for 15 min at 4 °C to remove debris. The pH of the supernatant was readjusted to 6.9 on ice.

Protein contents were determined with the modified method of Lowry (Dulley and Grieve, 1975).

4.5. Analyses of aldehydes

A buffer (1 ml, 0.1 M MES-KOH, pH 6.9 prepared with seawater) and 700 nmol of arachidonic acid (**4**) (20 mM dissolved in EtOH) or linoleic acid (50 mM dissolved in EtOH) in a test tube was chilled on ice for 15 min then, crude enzyme solution (1 ml) was added to it. This mixture was incubated at 4 °C with gentle shaking. A preliminary experiment showed that the reaction was linear with time until 30–40 min; hence, in this study, the reaction was terminated by adding 1% 2,4-DNPH (in EtOH containing 0.5 M HOAc, 1.4 ml) and 1 mM *n*-heptanal (8 μl) as the internal standard after 30 min. The hydrazone derivatives were extracted with *n*-hexane (5 ml) and washed with satd. NaCl (2 ml). After removal of *n*-hexane with a vacuum centrifuge to yield a yellow powder, it was then re-dissolved in Et_2O (50 μl). The ether solution was subjected to a prep. TLC (Merck, silica gel 60 F-254, 10×20 cm, EtOAc:*n*-hexane 2:1). The yellow bands corresponding to the authentic compounds (R_f 0.6–1.0) were scraped off and extracted with distilled Et_2O (2×2 ml). The ether extract was filtered using a hydrophobic filter unit (0.5 μm DISMIC-3JP, Advantech TOYO, Japan), and evaporated with a vacuum centrifuge at 32 °C; the resultant residues were then dissolved in CH_3CN (50 μl) and subjected to quantification by reversed phase HPLC. Quantification of the aldehydes was carried out by using five points of a calibration standard curve constructed with various amounts of authentic aldehydes (*n*-hexanal (**2**), (*Z*)-3-nonenal (**3**) or (*E*)-2-nonenal (**1**)) based on the area ratio of the respective peak of aldehyde to that of the internal standard (*n*-heptanal). The linear regression of the three standard curves was $\geqslant 0.9950$. Reversed phase HPLC was performed on Zorbax SB C_{18} (250×4.6 mm, 5 μm) with a flow rate of 1 ml/min (CH_3CN:H_2O:THF 66:33:1(v/v/v)), and UV detection at 350 nm (Shimadzu, LC-9A, Japan). The activity to form aldehydes varied from fronds to fronds depending on the collection date, the site, and the developmental stage of the specimens (Boonprab et al., unpublished data; Kajiwara et al., 1993; Sekiya et al., 1984); however, the profiles (i.e., substrate and product specificities of enzyme system) shown in this paper were practically reproducible. Representative data are shown.

4.6. Analyses of hydroxy-arachidonic acids

To the chilled substrate solution, GSH and GPx were added to concentration of 3 mM and 4 unit/ml, respectively, from which crude enzyme solution (1 ml) was added. The enzymatic solution was then incubated at 4 °C for 30 min, following which 1 M citric acid (150 μl) was added to adjust the pH to 3–4. Oxygenated fatty acids were extracted with Et_2O (2×2 ml), with combined Et_2O extracts washed with satd. NaCl (2 ml), followed by drying over Na_2SO_4 at −10 °C for 3 h. The Et_2O solutions were transfered to a new tube and evaporated until dry under a gentle stream of N_2 gas, whereas 15HEDE (50 μl of 2.25 mM dissolved in *n*-hexane) was added to the residue. Separation of hydroxyeicosatetraenoic acid(s) [HETE(s)] was carried out by normal phase HPLC on Zorbax-SIL 250×4.6 mm with a flow rate of 1 ml/min [*n*-hexane:*iso*-PrOH:HOAc 987:12:1 (v/v)]. Detection was performed with a photodiode array detector (Shimadzu, LC-10AD*vp*, Japan).

4.7. Analyses of hydroxy-arachidonic acid for structure and stereoisomer identification

The reaction of crude enzyme and arachidonic acid in the presence of GPx was scaled-up five-fold, with derivatives of fatty acids being extracted using a Sep-pak

C_{18} silica cartridge (Water corporation Milford, Massachusetts USA) (Lehmann et al., 1992). The acids were next eluted by MeOH, and following evaporation of MeOH, the resultant yellow oil was re-dissolved in Et_2O and washed with satd. NaCl. The Et_2O layer was next dried (Na_2SO_4, −10 °C) for 12 h, with the Et_2O solubles removed, and the residue subjected to prep. TLC essentially as described above. HETE(s) were separated using a solvent system of *n*-hexane:Et_2O:HOAc 1:1:0.001, v/v. The spot at R_f 0.13–0.2 was scraped off, and HETE(s) were extracted from the gel with Et_2O (5×2 ml). After removal of ether, the residue was re-dissolved in Et_2O (5 ml) and washed with satd. NaCl soln. The ether layer was next dried (Na_2SO_4 at −10 °C for 12 h), with the solvent then removed and the residue reconstituted in EtOH (1 ml). The concentration of HETE(s) were estimated by reading A at 234 nm by using an extinction coefficient of 23200 M^{-1} cm^{-1} (Graff et al., 1990). The soln. was next diluted with EtOH to give 50 mM HETE and stored at −80 °C until needed. Reversed phase HPLC was performed using a Zorbax SB C_{18} (250×4.6 mm, 5 μm) column, eluted with (MeOH:H_2O:HOAc 80:20:0.01 (v/v/v)) at a flow rate of 1 ml/min, and UV detection at 234 nm (Shimadzu, LC-9A, Japan). Fractions containing 12HETE and 15HETE were collected following reversed phase HPLC and individually concentrated using a Sep-pak C_{18} cartridge. Aliquots were methylated with ethereal diazomethane for normal phase HPLC analyses, using a Zorbax-SIL 250×4.6 mm column eluted with *n*-hexane:*iso*-PrOH 100:3 (v/v) at a flow rate of 1 ml/min. Chiral phase HPLC was performed with Chiralcel-OD-H 250×4.6 mm at a flow rate of 0.5 ml/min (*n*-hexane: *iso*-PrOH 99:1 (v/v)) for 12HETE, or at a flow rate of 0.25 ml/min (*n*-hexane:*iso*-PrOH ; 100:3 (v/v)) for 15HETE.

4.8. MS Analyses of hydroxy-arachidonic acid

For MS analysis the procedure done by Lehmann et al. (1992) was followed. After purification of the HETE(s), they were methylated with ethereal diazomethane. The sample was re-dissolved in EtOH (800 μl), and platinum oxide (6 mg) was added. Hydrogen gas was bubbled through a sample soln. for 90 min, with platinum oxide being removed by filtration. The solvent was removed under stream of nitrogen, to which they then added BSTFA soln. (200 μl, Sigma-Aldrich). The mixture was next incubated for 60 min at 60 °C with constant stirring under argon. After cooling to room temperature, the reagents were removed under a gentle stream of nitrogen. The sample was re-dissolved in *n*-hexane (40 μl) and subjected to GC–MS analysis. GC–MS (GCMS-QP5050A, Japan) was equipped with a fused silica capillary column (60 m×0.25 mm) coated with DB-WAX (0.25 μm film thickness) using helium as a carrier gas. The column temperature was programmed to increase from 150 to 200 at 5 °C/min. Sample injection was carried out with a split ratio of 1/50. The ionization energy was 70 eV.

Acknowledgements

This work was performed under JSPS-NRCT Core University Program on "Development of thermotolerant microbial resources and their applications" under the cooperation of Japanese and Thai scientists, in association with Kasetsart University (Thailand) and Yamaguchi University (Japan). This study was supported in part by the San-Ei Gen Foundation for Food Chemical Research (2002).

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