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Hydrogen, carbon and nitrogen isotopic fractionations during chlorophyll biosynthesis in C3 higher plants

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Abstract

We determined hydrogen, carbon and nitrogen isotopic compositions of chlorophylls *a* and *b* isolated from leaves of five C3 higher plant species (*Benthamidia japonica*, *Prunus japonica*, *Acer carpinifolium*, *Acer argutum* and *Querus mongloica*), and hydrogen and carbon isotopic compositions of phytol and chlorophyllides in the chlorophylls to understand isotopic fractionations associated with chlorophyll biosynthesis in these species. Chlorophylls are depleted in D relative to ambient water by ~189‰ and enriched in ^{13}C relative to bulk tissue by ~1.6‰. These data can be explained by the contribution of isotopic fractionations during phytol and chlorophyllide biosyntheses. Phytol is more depleted in both D (by ~308‰) and ^{13}C (by ~4.3‰), while chlorophyllides are less depleted in D (by ~44‰) and enriched in ^{13}C (by ~4.8‰). Such inhomogeneous distribution of isotopes in chlorophylls suggests that (1) the phytol in chlorophylls reflects strong D- and ^{13}C-depletions due to the isotopic fractionations during the methylerythritol phosphate pathway followed by hydrogenation, and (2) the chlorophyllides reflect D- and ^{13}C-enrichments in tricarboxylic acid cycle. On the other hand, chlorophylls are slightly (~1.2‰) depleted in ^{15}N relative to the bulk tissue, indicating that net isotopic fractionation of nitrogen during chlorophyll biosynthesis is small compared with those of hydrogen and carbon.

Keywords: *Benthamidia japonica*; *Acer carpinifolium*; Chlorophyll; Phytol; Chlorophyllide; δD; $\delta^{13}C$; $\delta^{15}N$; Isotopic fractionation; *Prunus japonica*; *Acer argutum*; *Querus mongloica*; Biosynthetic pathway

1. Introduction

Biomolecules in plants have large variations of stable isotopic compositions, particularly for hydrogen (Sessions et al., 1999, 2002; Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004a,b,c), which should be closely related to isotopic fractionation on the biochemical processes involved in their biosynthetic pathways (e.g. Schmidt, 2003; Schmidt et al., 2003). An understanding of the correlations between isotopic fractionations and biochemical processes is therefore essential for the elucidation of biosynthetic processes and for the discrimination of alternative pathways. Isotopic compositions of source-specific biomolecules, termed biomarkers, in geological samples such as soils and sediments as well as coal and petroleum would be a useful tool in resolving sources and in reconstructing paleoenvironments (e.g. Freeman et al., 1990; Hayes et al., 1990; Xie et al., 2000; Sauer et al., 2001; Huang et al., 2002). Furthermore, stable isotopic compositions of individual molecules have been widely applied to various fields including archaeology, ecology, and environmental chemistry (Meier-Augenstein, 1999; Lichtfouse, 2000).

Chlorophylls (**1**) and their related compounds are valuable molecules for use in the molecular isotopic

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studies for various reasons. Firstly, chlorophylls (**1**) are molecules directly related to the photosynthetic processes in all photoautotrophs, and their structural characteristics [e.g., chlorophylls *a* (**1a**), *b* (**1b**), and *c* (**1c**)] are taxonomically and evolutionally significant (e.g. Delwiche, 1999). Secondly, chlorophyll (**1**) biosynthesis involves two pathways potentially related to the evolution of photoautotrophs [Fig. 1, mevalonic-acid (MVA) (**2**) pathway or methylerythritol-phosphate (MEP) (**3**) pathway for phytol (**4**) biosynthesis and C5 pathway or Shemin pathway for chlorophyllide (**5**) biosynthesis], which can be discriminated by isotopic signatures (e.g. Beaumont et al., 2000). Thirdly, since chlorophylls (**1**) contain hydrogen, carbon and nitrogen, they could record multiple information such as hydrologic and nutrient cycles in the environment where the plants grow. Fourthly, diagenetic products of chlorophylls (**1**), geoporphyrins such as deoxophylloerythroetioporphyrins (DPEP) and etioporphyrins, are long preserved as geochemical fossils even in the coal and petroleum (e.g. Callot and Ocampo, 1999), and their isotopic signatures are useful for the estimation of sources and environment where they were formed (e.g. Boreham et al., 1989; Hayes et al., 1987; Ocampo et al., 1989).

Although the isotopic fractionation of carbon and nitrogen associated with chlorophyll (**1**) biosynthesis in various photoautotrophs has been reported in previous studies (e.g. Bidigare et al., 1991; Kennicutt et al., 1992; Madigan et al., 1989; Sakata et al., 1997; Sachs et al., 1999; Beaumont et al., 2000), that of hydrogen has never been reported. This study, therefore, aimed to determine hydrogen isotopic fractionations of chlorophylls *a* (**1a**) and *b* (**1b**) relative to ambient water in C3 higher plants and those of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**), as well as carbon and nitrogen isotopic fractionations relative to bulk carbon and nitrogen, respectively. Phytol (**4**) is related to the isoprenoid biosynthesis, while chlorophyllide (**5**) is related to the tricarboxylic acid (TCA) cycle (Fig. 1). Therefore, as an expansion of previous studies (Sessions et al., 1999, 2002; Chikaraishi et al., 2004a,b,c), this study provides fundamental information about isotopic

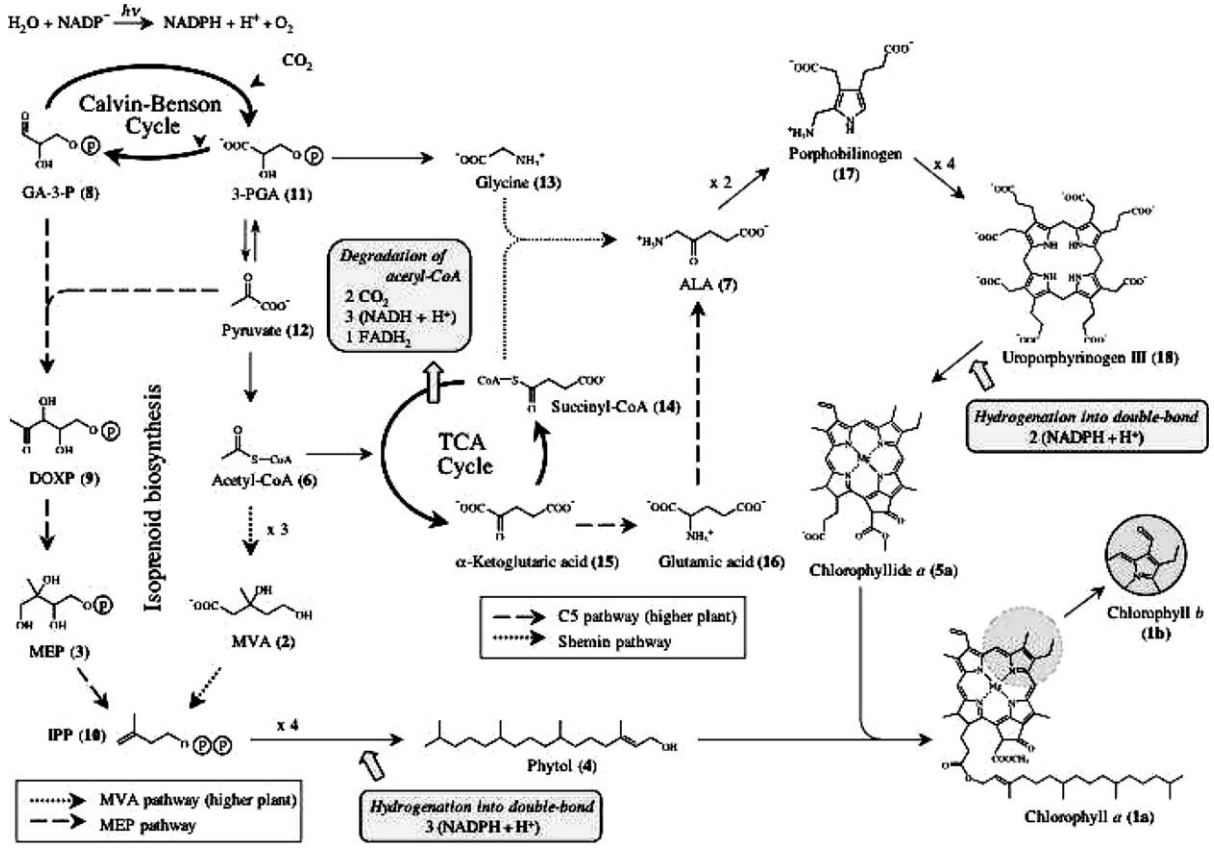

Fig. 1. Biosynthetic pathway of chlorophylls *a* (**1a**) and *b* (**1b**) (after Beale, 1978; Castelfranco and Beale, 1983; Reinbothe and Reinbothe, 1996; Lichtenthaler et al., 1997; Lichtenthaler, 1999). *Abbreviations*: acetyl-CoA, acetyl coenzyme-A (**6**); ALA, δ-aminolevulinic acid (**7**); GA-3-P, D-glyceraldehyde-3-phosphate (**8**); DOXP, 1-deoxy-D-xylulose-5-phosphate (**9**); IPP, isopentenyl pyrophosphate (**10**); MVA, mevalonic acid (**2**); MEP, 2-C-methyl-D-erythritol-4-phosphate (**3**); $NADP^+$, nicotinamide adenine dinucleotide phosphate; 3-PGA, 3-phosphoglyceric acid (**11**). IPP (**10**) is an intermediate precursor of phytol (**4**), which can be biosynthesized via alternative pathways: MVA (**2**) or MEP (**3**) pathway. ALA (**7**) is an intermediate precursor of chlorophyllide *a* (**5a**), which can be also biosynthesized via alternative pathways: the C5 pathway or the Shemin pathway.

fractionation in these biochemical processes, which is essential for the interpretation of isotopic records in geological samples.

2. Results and discussion

2.1. Samples

Fresh leaves from five terrestrial higher plant species are used in this study (Table 1). These plants are representative C3-angiosperms such as cherry, maple and oak, widely distributed in Japan as well as globally. We previously reported hydrogen isotopic compositions of cellulose nitrates, carbon isotopic compositions of bulk tissues, and hydrogen and carbon isotopic compositions of typical lipid molecules including solvent-extractable and saponifiable phytol (**4**) in these plants (Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004b). In this study, we determined hydrogen, carbon and nitrogen isotopic compositions of individual chlorophylls *a* (**1a**) and *b* (**1b**) isolated and purified from these plant leaves, and hydrogen and carbon isotopic compositions of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**). These higher plants were collected from suburb of Tokyo in Japan. The surfaces of leaves were washed with distilled water to removed contaminants, then stored at −20 °C until analysis.

2.2. Isotopic compositions of chlorophylls (1), and their bounded phytol (4)

In Table 2, we summarized the isotopic compositions of hydrogen (δD, ‰ relative to Standard Mean Ocean Water, SMOW), carbon (δ^{13}C, ‰ relative to Peedee Belemnite, PDB) and nitrogen (δ^{15}N, ‰ relative to Atmospheric Nitrogen, AIR) of chlorophylls *a* (**1a**) and *b* (**1b**) isolated from five plant leaves, and those of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**). For hydrogen and carbon, δ value of chlorophyllide (**5**) (δ_{chlide}) is calculated from that of chlorophyll (**1**) (δ_{chl}) and chlorophyll-bound phytol (**4**) (δ_{phy}), according to the isotopic mass balance mathematically expressed in the following equation:

$$\delta_{chlide} = (n_{chl}\delta_{chl} - n_{phy}\delta_{phy})/n_{chlide}, \quad (1)$$

where n is the number of element (i.e., hydrogen or carbon) atoms on chlorophyll (**1**) (n_{chl}), phytol (**4**) (n_{phy}) and chlorophyllide (**5**) (n_{chlide}). Chlorophylls (**1**) isolated from five plant leaves exhibit δD values ($^2\delta_{chl}$) ranging from −233‰ to −211‰, being composed of D-depleted phytol (**4**) ($^2\delta_{chl}$ of −345‰ to −330‰) and D-enriched chlorophyllides (**5**) ($^2\delta_{chlide}$ of −109‰ to −62‰). Little variation (<±11‰) in the $^2\delta_{chl}$ values between plant species suggests a similar hydrogen isotopic fractionation during chlorophyll (**1**) biosynthesis in these plants. δ^{13}C values of chlorophylls (**1**) ($^{13}\delta_{chl}$) range from −33.3‰ to −29.2‰, being composed of ^{13}C-depleted phytol (**4**) ($^{13}\delta_{phy}$ of −37.9‰ to −35.2‰) and ^{13}C-enriched chlorophyllides (**5**) ($^{13}\delta_{chlide}$ of −30.9‰ to −25.5‰). The $^{13}\delta_{chl}$ variation among five plant leaves is less than ±2.1‰. In contrast to $^2\delta_{chl}$ and $^{13}\delta_{chl}$ signatures, δ^{15}N values of chlorophylls (**1**) ($^{15}\delta_{chl}$) vary widely (−4.7‰ to +6.0‰). The large variation is also observed in the δ^{15}N values of bulk tissues ($^{15}\delta_{bulk}$) (Table 1), which could be related to the isotopic variation of nitrogenous nutrient sources for each plant species (e.g. Macko et al., 1987; Denton et al., 2001). Generally, plants growing under various nutrient sources have wide variation of δ^{15}N values (Werner and Schmidt, 2002). For $^2\delta_{chl}$, $^{13}\delta_{chl}$ and $^{15}\delta_{chl}$, no substantial difference is found between chlorophylls *a* (**1a**) and *b* (**1b**) within a single plant species, suggesting that the isotopic discriminations associated with the formation of chlorophyll *b* (**1b**) from chlorophyll *a* (**1a**) are insignificant in higher plants.

Table 1
Samples used in this study

Sample	Cellulose nitrate	Bulk plant leaves	
	δD (‰)[a]	δ^{13}C (‰)[b]	δ^{15}N (‰)
Benthamidia japonica	−64	−34.3	−2.0
Prunus jamasakura	−101	−32.0	+3.1
Acer carpinifolium	−83	−31.8	−2.8
Acer argutum	−105	−34.8	−2.7
Quercus mongolica	−92	−30.9	+7.4

[a] Reported in Chikaraishi et al. (2004b).
[b] Reported in Chikaraishi and Naraoka (2003).

2.3. Hydrogen and carbon isotopic fractionations during chlorophyll (1) biosynthesis

Hydrogen isotopic fractionation of chlorophyll (**1**), phytol (**4**) or chlorophyllide (**5**) relative to ambient water ($^2\varepsilon_{water}$) is defined in Eq. (2). Similarly, that of carbon isotopic fractionation relative to bulk tissue ($^{13}\varepsilon_{bulk}$) is defined in Eq. (3).

$$^2\varepsilon_{water} = 1000[(^2\delta_{chl,phy\ or\ chlide} + 1000)/(^2\delta_{water} + 1000) - 1], \quad (2)$$

$$^{13}\varepsilon_{bulk} = 1000[(^{13}\delta_{chl,phy\ or\ chlide} + 1000)/(^{13}\delta_{bulk} + 1000) - 1], \quad (3)$$

where δD value of ambient water ($^2\delta_{water}$) is available in the previous report (Chikaraishi and Naraoka, 2003), being $^2\delta_{water}$ of −42‰ as an annual mean value of precipitations. δ^{13}C values of bulk tissues ($^{13}\delta_{bulk}$) are summarized in Table 1. Calculated $^2\varepsilon_{water}$ and $^{13}\varepsilon_{bulk}$ values of chlorophylls *a* (**1a**) and *b* (**1b**), and those of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**) are

Table 2
Hydrogen, carbon and nitrogen isotopic compositions of chlorophylls (**1**) isolated from five higher plants, and those of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**)

Sample	δD (‰)			δ^{13}C (‰)			δ^{15}N (‰)
	Chlorophyll (**1**)	Phytol (**4**)	Chlorophyllide[a] (**5**)	Chlorophyll (**1**)	Phytol (**4**)	Chlorophyllide[a] (**5**)	Chlorophyll (**1**)
Benthamidia japonica							
Chlorophyll *a* (**1a**)	−232	−337	−109	−33.3	−37.5	−30.9	−4.3
Chlorophyll *b* (**1b**)	−233	−339	−99	−33.1	−37.1	−30.8	−4.5
Prunus jamasakura							
Chlorophyll *a* (**1a**)	−228	−338	−99	−29.8	−35.2	−26.7	3.3
Chlorophyll *b* (**1b**)	n.d.[b]	−339	n.d.	n.d.	−35.6	n.d.	n.d.
Acer carpinifolium							
Chlorophyll *a* (**1a**)	−219	−338	−79	−31.6	−37.0	−28.5	−4.6
Chlorophyll *b* (**1b**)	−222	−336	−79	−31.6	−37.9	−28.0	−4.7
Acer argutum							
Chlorophyll *a* (**1a**)	−224	−345	−81	−31.8	−37.4	−28.6	−2.4
Chlorophyll *b* (**1b**)	−226	−340	−83	−31.4	−37.3	−28.0	−2.4
Quercus mongolica							
Chlorophyll *a* (**1a**)	−211	−330	−70	−29.2	−35.7	−25.5	6.0
Chlorophyll *b* (**1b**)	−212	−332	−62	−30.0	−35.5	−26.9	5.7

[a] Isotopic composition of chlorophyllide (**5**) is calculated by isotopic mass balance between chlorophyll (**1**) and phytol (**4**) (see text).
[b] Not determined.

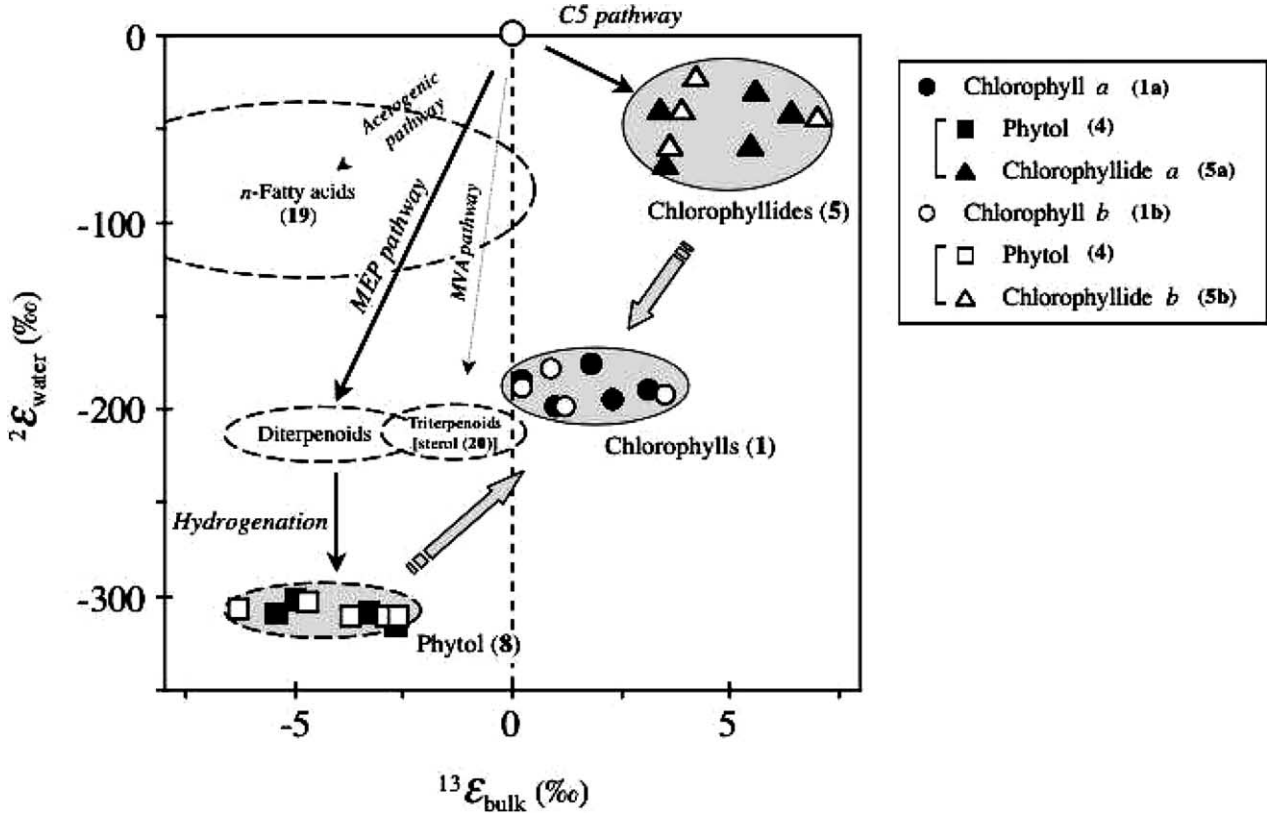

Fig. 2. The $^{2}\varepsilon_{water}$ and $^{13}\varepsilon_{bulk}$ of chlorophylls *a* (**1a**) and *b* (**1b**) isolated from five higher plants, and those of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**). Elliptic area with dashed line indicates hydrogen and carbon isotopic fractionations of the solvent-extractable and saponifiable lipid molecules including *n*-fatty acids (**19**) (acetogenic pathway), diterpenoids [MEP (**3**) pathway] triterpenoids, e.g., (**20**) [MVA (**2**) pathway] as well as phytol (**4**) [MVA (**3**) pathway with hydrogenation] in previous reports (Chikaraishi et al., 2004a, 2004b).

illustrated in Fig. 2. Chlorophylls (**1**) isolated from five higher plants have $^{2}\varepsilon_{water}$ values of $-189 \pm 8‰$ and $^{13}\varepsilon_{bulk}$ values of $+1.6 \pm 1.2‰$, being composed of isotopically lighter phytol (**4**) ($^{2}\varepsilon_{water} = -308 \pm 4‰$, $^{13}\varepsilon_{bulk} = -4.3 \pm 1.3‰$) and heavier chlorophyllides (**5**) ($^{2}\varepsilon_{water} = -44 \pm 16‰$, $^{13}\varepsilon_{bulk} = +4.8 \pm 1.4‰$).

In higher plants, phytol (**4**) is biosynthesized via MEP (**3**) pathway in the chloroplast (Fig. 1; e.g. Lichtenthaler et al., 1997; Lichtenthaler, 1999). The $^{2}\varepsilon_{water}$ and $^{13}\varepsilon_{bulk}$ values of phytol (**4**) in the chlorophylls (**1**) observed in this study are consistent with those of solvent-extractable ($^{2}\varepsilon_{water} = -313 \pm 7‰$, $^{13}\varepsilon_{bulk} = -3.9 \pm 1.3‰$) and saponifiable phytol (**4**) ($^{2}\varepsilon_{water} = -300 \pm 21‰$, $^{13}\varepsilon_{bulk} = -4.4 \pm 1.1‰$) reported in Chikaraishi et al. (2004b), indicating that the $^{2}\varepsilon_{water}$ and $^{13}\varepsilon_{bulk}$ values of phytol (**4**) in chlorophylls (**1**) result from hydrogen and carbon isotopic fractionations of MEP (**1**) pathway followed by hydrogen isotopic fractionation during hydrogenation to form phytol (**4**) (Fig. 2; Chikaraishi et al., 2004a). On the other hand, chlorophyllides (**5**) portion of the chlorophylls (**1**) are slightly depleted in D but somewhat enriched in ^{13}C relative to the ambient water and bulk carbon, respectively. These isotopic signatures of chlorophyllides (**5**) are different from those of lipid molecules such as fatty acids (**19**) and sterols (**20**) as well as phytol (**4**) in higher plants, which are substantially depleted in both D and ^{13}C (Fig. 2; Sessions et al., 1999; Chikaraishi et al., 2004a,b). These $^{2}\varepsilon_{water}$ and $^{13}\varepsilon_{bulk}$ values of chlorophyllides (**5**) suggest that specific D- and ^{13}C-enrichment processes should exist in the chlorophyllide (**5**) biosynthesis.

Previous studies suggested that the hydrogen isotopic compositions of biomolecules strongly reflect both the δD values of their sources and isotopic fractionations during biochemical redox reactions as well as hydrogen-exchange reactions (e.g. Sessions et al., 2002; Schmidt et al., 2003; Chikaraishi et al., 2004a). It has been reported that hydrogens removed during dehydrogenation and those incorporated during hydrogenation are substantially depleted in D due to the large kinetic isotope effects associated with the enzymatic biochemical reactions (Luo et al., 1991; Chikaraishi et al., 2004a,c). For example, Chikaraishi et al. (2004a) reported that the mean $^{2}\varepsilon_{water}$ value of hydrogens incorporated during the hydrogenation of three double-bonds to produce phytol (**4**) is expected to be approximately $-600‰$ in higher plants (*Cryptomeria japonica*). On the other hand, the hydrogen remaining on the reactant after dehydrogenation becomes significantly enriched in D as a result of the kinetically preferential ^{1}H elimination during dehydrogenation (Chikaraishi et al., 2004c). In case of chlorophyllide (**5**) biosynthesis, four hydrogens are incorporated during the hydrogenation of two double-bonds to produce chlorophyllide *a* (**5a**) (Fig. 1; Reinbothe and Reinbothe, 1996). Assuming that this double-bond hydrogenation implies a kinetic isotope effect as similar to that of phytol (**4**) biosynthesis (Chikaraishi et al., 2004a), four hydrogens derived from the double-bond hydrogenation in chlorophyllide (**5**) should be significantly depleted in D. This suggests that intermediate precursors of chlorophyllide (**5**) could be more enriched in D before the double-bond hydrogenation. In higher plants, chlorophyllide (**5**) is biosynthesized from δ-aminolevulinic acid (ALA) (7) as an intermediate precursor, which is produced through C5 pathway from glutamic acid (**16**) (e.g. Beale, 1978; Castelfranco and Beale, 1983; Reinbothe and Reinbothe, 1996). The glutamic acid (**16**) is produced by amination of α-ketoglutaric acid (**15**) formed in TCA cycle where disassimilates acetyl-CoA (**6**) into hydrogen and CO_2 with respect to the respiratory metabolism (Fig. 3). Although the specific mechanisms responsible for the D-enrichment of chlorophyllide (**5**) precursors have not been clarified from this study, these sequential biochemical reactions may contain several potential processes of D-enrichments (e.g., redox, and hydrogen-exchange). Particularly, in TCA cycle, hydrogen is in turn removed by the reductions of nicotinamide adenine dinucleotide (NAD^+) to form NADH and of flavin adenine dinucleotide (FAD) to form $FADH_2$. Kinetically preferential ^{1}H elimination could occur at such redox points in TCA cycle, which causes strong D-enrichment in the turned molecules [e.g., α-ketoglutaric acid (**15**)] in the cycle. When a part of the strong D-enriched hydrogen of the α-ketoglutaric acid (**15**) survives during the subsequent biochemical reactions to form chlorophyllide (**5**), the D-enrichment in TCA cycle is a likely explanation for the D-enriched chlorophyllide (**5**).

In case of carbon, the $^{13}\delta_{chlide}$ values should reflect the δ^{13}C values of their sources and isotopic fractionations during biochemical processes, particularly at branching points involved in the biosynthetic pathways (e.g. Hayes, 1993). For example, in a previous study on intramolecular ^{13}C distribution in chlorophylls (**1**) from a higher plant (nettle leaves), Bogacheva et al. (1980) reported that the methine bridge carbon between pyrrole rings is ~8‰ depleted in ^{13}C relative to the carbons in the pyrrole rings. This ^{13}C-depletion in the methine bridge carbon would be due to the isotopic fractionation associated with the condensation of porphobilinogen (**17**) to uroporphyrinogen III (**18**) (Fig. 1). Theoretically, such processes lead, however, to more ^{13}C-*depleted* products, which cannot explain the ^{13}C-enrichment in chlorophyllides (**5**) estimated in this study. Therefore, we think that the chlorophyllide (**5**) is biosynthesized from an intermediate precursor originally enriched in ^{13}C enough to buffer the ^{13}C-depletion during subsequent biochemical reactions. Previous studies on the carbon isotopic compositions of individual amino acids reported that the glutamic acid (**16**), an ultimate precursor of chlorophyllide (**5**), was strongly (~5–8‰) enriched in ^{13}C compared with bulk carbon (e.g.

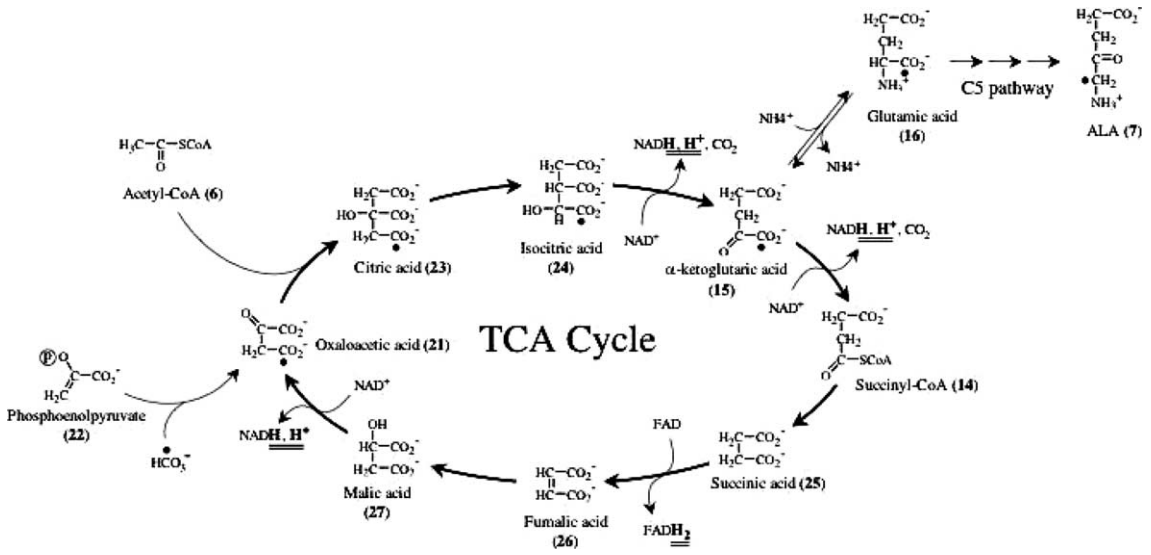

Fig. 3. Schematic pathway to ALA (**7**) from acetyl-CoA (**6**) showing the relationship between molecules with respected to hydrogen and carbon flows in the TCA cycle (after Avissar et al., 1989; Melzer and O'Leary, 1991; Przybyla-Zawislak et al., 1999; Hayes, 2001; Dufresne et al., 2003). The filled circle designates carbon from bicarbonate used to produce oxaloacetic acid (**21**) from phosphoenolpyruvate (**22**).

Macko et al., 1987). This ^{13}C-enrichment of the glutamic acid (**16**) has been explained by the contribution of ^{13}C-enriched bicarbonate used for the production of oxaloacetic acid (**21**) from phosphoenolpyruvate (**22**) (Fig. 3; Melzer and O'Leary, 1987, 1991), indicating that eight carbons in chlorophyllides (**5**) are derived from this ^{13}C-enriched bicarbonate. Therefore, we think that the $^{2}\varepsilon_{water}$ and $^{13}\varepsilon_{bulk}$ values of chlorophyllides (**5**) would be correlated with the D- and ^{13}C-enrichment processes in TCA cycle to produce α-ketoglutaric acid (**15**) as a primitive precursor.

2.4. Nitrogen isotopic fractionation during chlorophyll (*1*) biosynthesis

Nitrogen isotopic fractionation of chlorophyll (**1**) relative to bulk tissue ($^{15}\varepsilon_{bulk}$) is defined in Eq. (4), as similar to that of carbon.

$$^{15}\varepsilon_{bulk} = 1000[(^{15}\delta_{chl} + 1000)/(^{15}\delta_{bulk} + 1000) - 1]. \quad (4)$$

The δ^{15}N values of bulk tissues ($^{15}\delta_{bulk}$) are also summarized in Table 1. Although $^{15}\delta_{bulk}$ and $^{15}\delta_{chl}$ values vary widely (−2.8‰ to +7.4‰, and −4.7‰ to +6.0‰, respectively), $^{15}\varepsilon_{bulk}$ values of chlorophylls (**1**) are in a relatively narrow range (−2.5‰ to +0.2‰) with no substantial difference between chlorophylls *a* (**1a**) and *b* (**1b**) within a single plant species. This result is consistent with previous studies (Bidigare et al., 1991; Kennicutt et al., 1992), and suggests that nitrogen isotopic fractionation associated with chlorophyll (**1**) biosynthesis is largely independent from the δ^{15}N variation of nitrogenous nutrient sources. In higher plants, $^{15}\delta_{chl}$ value reflects δ^{15}N value of glutamic acid (**16**) as a nitrogen source and nitrogen isotopic fractionation during chlorophyllide (**5**) biosynthesis (Beaumont et al., 2000; Werner and Schmidt, 2002). This study clearly indicates that net nitrogen isotopic fractionation during chlorophyll *a* (**1a**) and *b* (**1b**) biosynthesis is thus small or slightly (∼1.2‰) negative in the terrestrial C3 higher plants, which suggests that $^{15}\delta_{chl}$ values can be used to predict that of bulk nitrogen in C3 plants.

2.5. Isotopic fractionation during chlorophylls (*1*) among various photoautotrophs

As the results from isotopic fractionations associated with phytol (**4**) and chlorophyllide (**5**) biosyntheses, chlorophylls (**1**) have $^{2}\varepsilon_{water}$ values of −189 ± 8‰ and $^{13}\varepsilon_{bulk}$ values of +1.6 ± 1.2‰ (Fig. 2). The $^{15}\varepsilon_{bulk}$ values of chlorophylls (**1**) (−1.2 ± 1.2‰) may reflect only the isotopic fractionation associated with chlorophyllide (**5**) biosynthesis. These $^{13}\varepsilon_{bulk}$ and $^{15}\varepsilon_{bulk}$ values of C3 plant chlorophylls (**1**) in this study are consistent with those reported in previous studies (Fig. 4; Bidigare et al., 1991; Kennicutt et al., 1992), suggesting that the kinetic isotope effects during MEP (**3**) pathway [for phytol (**4**)] and C5 pathway [for chlorophyllides (**5**)] have similar amplitudes in other C3 plants. However, the $^{13}\varepsilon_{bulk}$ and $^{15}\varepsilon_{bulk}$ values of chlorophylls (**1**) from other photoautotrophs such as C4 plants, phytoplankton, cyanobacteria and purple photosynthetic bacteria are not consistent with those of C3 plants (Fig. 4). Particularly, chlorophylls (**1**) from C4 plants have the $^{15}\varepsilon_{bulk}$ values widely range from −6.6‰ to −1.0‰ with considerable ^{15}N-depletion relative to those from C3 plants (Fig. 4; Bidigare et al., 1991), even though chlorophyllide (**5**) is also biosynthesized via C5 pathway in

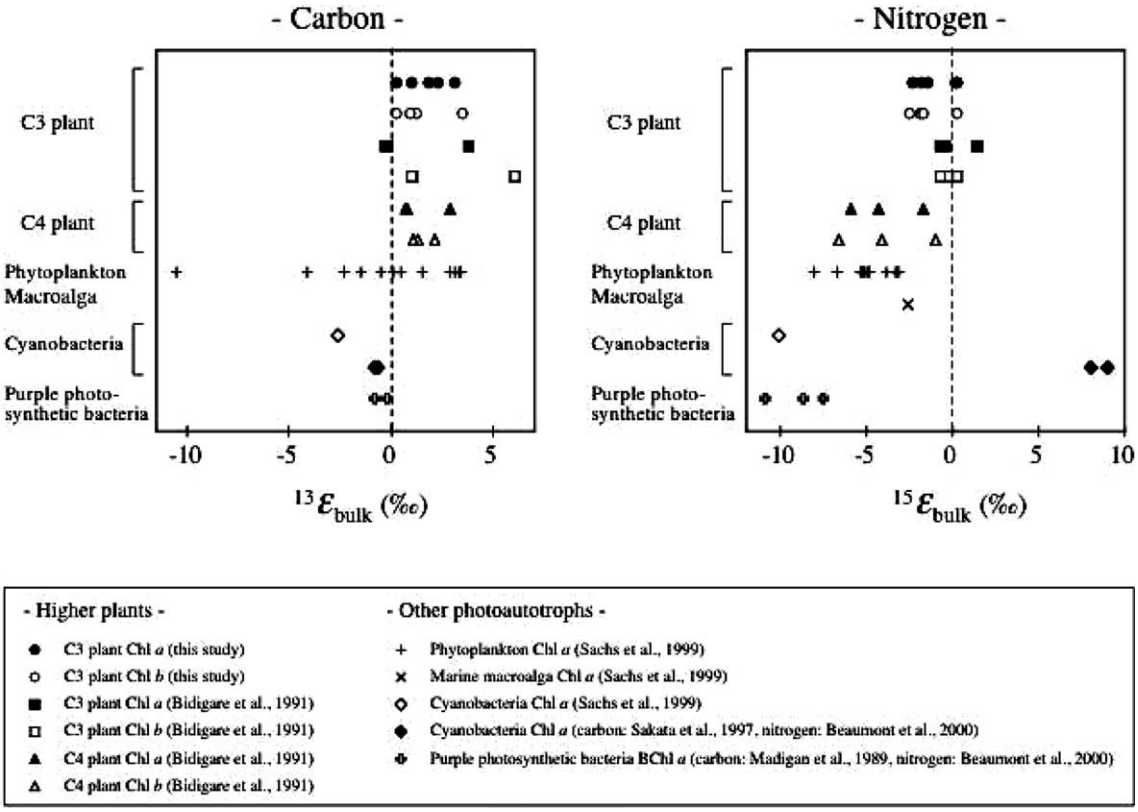

Fig. 4. The $^{13}\varepsilon_{bulk}$ and $^{15}\varepsilon_{bulk}$ of chlorophylls (**1**) in this study, with those of previous studies (Madigan et al., 1989; Bidigare et al., 1991; Sachs et al., 1999; Sakata et al., 1997; Beaumont et al., 2000). *Abbreviations*: Chl *a*, chlorophyll *a* (**1a**); Chl *b*, chlorophyll *b* (**1b**); BChl *a*, bacteriochlorophyll *a* (**1d**).

C4 plants. Furthermore, chlorophyll *a* (**1a**) from marine phytoplankton has a wide range of $^{13}\varepsilon_{bulk}$ values (−10.5‰ to +3.4‰; Fig. 4; Sachs et al., 1999), and bacteriochlorophyll *a* (**1d**) from purple photosynthetic bacteria has the $^{15}\varepsilon_{bulk}$ values of −10.9‰ to −7.5‰ being significantly depleted in ^{15}N relative to those from C3 plants (Fig. 4; Beaumont et al., 2000). This isotopic contrast of chlorophylls (**1**) between classes of photoautotrophs may be attributed to (1) the use of different biosynthetic pathways for phytol (**4**) and chlorophyllides (**5**), and (2) different degree of isotopic fractionations even in the same pathways. For example, phytol (**4**) in higher plants is biosynthesized via MEP (**3**) pathway while that in some algae such as Euglenophyta is biosynthesized via MVA (**2**) pathway (e.g. Lichtenthaler et al., 1997; Eisenreich et al., 2001). ALA (**7**), an intermediate precursor of chlorophyllide (**5**), in higher plants is biosynthesized via C5 pathway while that in purple photosynthetic bacteria is biosynthesized via Shemin pathway (e.g. Castelfranco and Beale, 1983; Avissar et al., 1989; Beaumont et al., 2000). Iida et al. (2002) also reported that Euglenophyta (*Euglena gracilis*) can biosynthesize ALA (**7**) by both pathways. It has been reported that molecules biosynthesized from MEP (**2**) pathway are more depleted in ^{13}C by ∼1–7‰ relative to those from MVA (**3**) pathways in higher plants (Fig. 2; Chikaraishi et al., 2004a,b). δ^{13}C and δ^{15}N differences between glycine (**13**) as a precursor of Shemin pathway and glutamic acid (**16**) as a precursor of C5 pathway are also reported (Abelson and Hoering, 1961; Macko et al., 1987: Fogel and Tuross, 1999). For example, Macko et al. (1987) reported that the glycine is more depleted in ^{13}C by ∼4–6‰ and ^{15}N by ∼1−5‰ relative to the glutamic acid in cyanobacteria. Further, it has been known that different classes of photoautotrophs utilize the same enzymes with different isotope effects (e.g. Estep et al., 1978; Goericke et al., 1994; Hayes, 2001). In fact, carbon isotopic fractionation of phytol (**4**) biosynthesis in C4 plants is larger (by ∼6‰) than that in C3 plants (Chikaraishi et al., 2004b). Moreover, different growth rate among photoautotrophs is also likely cause for variation of isotopic fractionation in the same pathways. Sachs et al. (1999) reported that chlorophyll *a* (**1a**) of slow-growing phytoplankton is enriched in ^{13}C (∼7‰) compared to that of rapidly growing phytoplankton. Thus, the use of either MVA (**2**) pathway or MEP (**3**) pathway for phytol (**4**) and of either C5 pathway or Shemin pathway

for chlorophyllide (**5**), as well as degree of isotopic fractionations in these pathways directly reflect the isotopic compositions of chlorophylls (**1**) among photoautotrophs.

3. Conclusions

Hydrogen, carbon and nitrogen isotopic compositions of chlorophylls *a* (**1a**) and *b* (**1b**), and those of phytol (**4**) and chlorophyllides (**5**) in the chlorophylls (**1**) were determined for five higher plants (C3 angiosperms), in order to understand the mechanism of isotopic fractionation associated with chlorophyll (**1**) biosynthesis in these species. Particularly, this is the first attempt to measure hydrogen isotopic fractionation associated with chlorophyll (**1**) biosynthesis in photoautotrophs. Isotopic compositions of plant chlorophylls (**1**) reflect isotopic fractionations associated with phytol (**4**) (D- and ^{13}C-depletion during MEP pathway followed by hydrogenation) and chlorophyllide (**5**) biosyntheses (D- and ^{13}C-enrichments in TCA cycle). Comparison with previous studies suggests that the isotopic fractionations during chlorophyll (**1**) biosynthesis vary with photoautotrophic classes, which is attributable to (1) the use of alternative biosynthetic pathways for phytol (**4**) [MVA (**2**) or MEP (**3**) pathway] and chlorophyllide (**5**) (C5 or Shemin pathway); and (2) the existence of distinct isotopic fractionations even in the same pathways. Since the alternative biosynthetic pathways and the distinct isotopic fractionations may be related to taxonomic differences among photoautotrophs, this study suggests that multiple isotope analysis of chlorophylls (**1**) and their components [i.e., phytol (**4**) and chlorophyllide (**5**)] is a useful means to assess photoautotroph evolution.

Chlorophylls (**1**) are present in all photoautotrophs and have been extensively used as a biomarker of their activities, in which their diagenetic derivatives are often preserved as stable geoporphyrins in geochemical fossils. Distinct isotopic compositions of chlorophylls (**1**) among photoautotrophs could be used as a quick means in resolving sources of chlorophylls (**1**) and their derivatives in geological samples. Moreover, their multiple isotopic compositions (δD, δ^{13}C and δ^{15}N) provides multiple information (e.g., hydrological and nutrient cycles) on the growth environment when they are biosynthesized. Thus, the isotopically-fractionated mechanisms of chlorophyll (**1**) biosynthesis presented in this study will be useful fundamental information to give a better interpretation of isotope records of chlorophylls (**1**) and their derivatives in geological samples, and to estimate various biological and geological history such as evolution of photoautotrophs and development of nutrient cycle with respect to paleoclimatic change.

4. Experimental

4.1. Chlorophyll (*1*) isolation and purification

Frozen fresh leaves were crushed to a fine powder before analysis. Chlorophyll (**1**) was isolated and purified according to previous studies (Watanabe et al., 1984; Bidigare et al., 1991; Sachs et al., 1999; Nakajima et al., 2003). In brief, powdered plant leaves were extracted with cold acetone by sonication for 2 min (×3), and the extracted pigments were subsequently reextracted by *n*-hexane from the acetone/H_2O layer to remove water. The residue of plant leaves was stored with acetone in a freezer (−20°C) for 24 h, and extracted until colorless. The combined solutions (*n*-hexane + acetone) were filtered through a Whatman GF/F filter (0. 7 μm pore-size), evaporated under a N_2 stream, and redissolved in MeOH. Chlorophylls (**1**) were precipitated by the sequential addition of 1,4-dioxane/H_2O (1/1, v/v) (Watanabe et al., 1984) and were extracted with *n*-hexane from MeOH/H_2O (17/3, v/v) (Sachs et al., 1999) to remove lipids and carotenoids.

Isolations and purifications of chlorophylls *a* (**1a**) and *b* (**1b**) were achieved using reversed phase high-performance liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry system (HPLC/APCI-MS; Agilent Technologies 1100 series) described in Nakajima et al. (2003). Baseline separation was performed by a ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm silica particle size) attached a ZORBAX SB-C18 analytical guard column (4.6 × 12.5 mm, 5 μm silica particle size). The solvent gradient was programmed from MeOH to acetone for 40 min, with the solvent flow of 1.0 ml min^{-1}. Chlorophylls (**1**) were identified by UV–Vis spectra and mass spectra. Chlorophylls *a* (**1a**) and *b* (**1b**) were eluted at c.a. 13 and 9 min, and collected into fraction collectors, respectively. Care was taken to collect the entire chlorophyll (**1**) peaks in order to prevent alteration of isotopic compositions (Bidigare et al., 1991). UV–Vis spectra and mass spectra revealed the purified chlorophylls (**1**) were free from contaminants. Purity of chlorophylls (**1**) is approximately 98% based on hydrogen, carbon and nitrogen contents [8.0 ± 0.4%H, 76.0 ± 3.6%C and 6.4 ± 0.2%N for chlorophyll *a* (**1a**), and 8.0 ± 0.3%H, 74.7 ± 3.5%C and 6.4 ± 0.4%N for chlorophyll *b* (**1b**)] determined by elemental analysis.

4.2. Hydrogen, carbon and nitrogen isotope analyses of chlorophylls (*1*)

Hydrogen isotope analysis of chlorophylls (**1**) was carried out using a high temperature conversion elemental analyzer (TCEA) coupled to an isotope ratio mass spectrometer (IRMS; Thermo Finnigan Delta plus XP) via a Conflo III interface. δD value was given in per

mil (‰) relative to SMOW, which was calibrated using Greenland Ice Sheet Precipitation (GISP, $\delta D = -190‰$) and Standard Light Antarctic Precipitation (SLAP, $\delta D = -428‰$). In the replicate analyses, the standard deviation of the δD values for purified chlorophylls (**1**) was always better than 7‰ (~3‰ in average) with the minimum sample amount of 5 μgH.

Carbon and nitrogen isotope analyses of chlorophylls (**1**) were carried out using a FLASH EA coupled to an IRMS (Thermo Finnigan Delta plus XP) via Conflo III interface. $\delta^{13}C$ and $\delta^{15}N$ values were given in per mil (‰) relative to PDB and AIR, respectively. Standard deviations of isotopic measurements for purified chlorophylls (**1**) were always better than 0.2‰ (~0.1‰ on average) for carbon and 0.4‰ (~0.1‰ on average) for nitrogen, with the minimum sample amount of 30 μgC and 30 μgN.

*4.3. Hydrogen and carbon isotope analyses of phytol (**4**) in chlorophylls (**1**)*

Purified chlorophylls (**1**) were saponified with 0.5 M KOH in MeOH/H_2O (95/5, w/w) at 75 °C for 4.5 h. After addition of water, phytol (**4**) was extracted with *n*-hexane/CH_2Cl_2 (2/1, v/v). Phytol (**4**) was acetylated using Ac_2O/pridine (1/1, v/v, 1 ml) at 75°C for 8 h (Chikaraishi et al., 2004b).

Hydrogen and carbon isotope analyses of phytol (**4**) were carried out using gas chromatography (GC; Agilent Technologies 6890N) interfaced an IRMS (Thermo Finnigan Delta plus XP) via pyrolysis or combustion furnace. The pyrolysis was performed in a microvolume ceramic tube with graphite at 1440 °C (Burgøyne and Hayes, 1998; Hilkert et al., 1999). The combustion was performed in a microvolume ceramic tube with CuO and Pt wires at 850 °C (Hayes et al., 1990). δD value was given in per mil (‰) relative to SMOW, and calibrated by GISP and SLAP. $\delta^{13}C$ value was given in per mil (‰) relative to PDB. Standard deviations of isotopic measurements for acetyl phytol (**4**) were always better than 4‰ (~2‰ on average) for hydrogen and 0.4‰ (~0.1‰ on average) for carbon, with the minimum sample amount of 5 ngH and 10 ngC, respectively. Contribution of hydrogen and carbon incorporated during acetylation were corrected by isotopic mass balance calculation (Chikaraishi et al., 2004b).

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