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CO_2 as main carbon source for isoprenoid biosynthesis via the mevalonate-independent methylerythritol 4-phosphate route in the marine diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis*

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Abstract

Isoprenoid biosynthesis was investigated in the two diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis* by labeling experiments performed in mixotrophic growth conditions with sodium [1-^{13}C]acetate, $^{13}CO_2$, [1-^{13}C]glucose, sodium [3-^{13}C]pyruvate and 1-deoxy-D-[5,5-$^{2}H_2$]xylulose. A clear dichotomy was found. Acetate was the preferred carbon source for the formation of the sterols in the cytoplasm via the mevalonate pathway. Carbon dioxide was the main source for phytol biosynthesis in the chloroplasts via the mevalonate-independent methylerythritol 4-phosphate pathway. The two diatoms showed the same compartmentation for isoprenoid biosynthesis as that previously found in higher plants, the red alga *Porphyridium cruentum* and the Chrysophyte *Ochromonas danica*.

Keywords: *Phaeodactylum tricornutum*; *Nitzschia ovalis*; Diatoms; Biosynthesis; Isoprenoids; Sterols; Phytol; Carbondioxide; Mevalonate; Deoxy-D-xylulose

1. Introduction

Isopentenyl diphosphate (**3**, Scheme 1) (IPP), the universal building block for all isoprenoids is formed by two different biosynthetic routes: the well-known acetate/mevalonate pathway (Qureshi & Porter, 1981) (Scheme 1, pathway **A**), that was long unanimously accepted as the sole biosynthetic route to IPP, and the mevalonate-independent pathway starting from pyruvate (**4**, Scheme 1) and from glyceraldehyde 3-phosphate (**5**) (Rohmer, Seemann, Horbach, Bringer-Meyer & Sahm, 1996) and yielding IPP via 1-deoxy-D-xylulose 5-phosphate (DXP, **6**) (Broers, 1994) and 2-*C*-methyl-D-erythritol 4-phosphate (MEP **7**) (Duvold, Bravo, Pale-Grosdemange & Rohmer, 1997a; Duvold, Cali, Bravo & Rohmer, 1997b) (Scheme 1, pathway **B**). The latter route shares DXP as common intermediate with the biosynthesis of thiamin diphosphate and pyridoxol phosphate (David, Estramareix, Fischer & Thérisod, 1981; Himmeldirk, Kennedy, Hill, Sayer & Spenser, 1996; Himmeldirk, Sayer & Spenser, 1998). As no other function than that of an isoprenoid precursor is known for MEP, this implies that the rearrangement of DXP (Rohmer, Knani, Simonin, Sutter & Sahm, 1993; Kuzuyama, Takahashi, Watanabe & Seto, 1998) and the concomitant reduction catalyzed by the recently identified isomero–reductase (Takahashi, Kuzuyama, Watanabe & Seto, 1998) might represent the first committed step of this pathway. This alternative metabolic route to isoprenoids was first detected in eubacteria (Flesch & Rohmer, 1988; Rohmer et al., 1993; Rosa Putra, Disch, Bravo & Rohmer, 1998) and later also found in green algae as well as in the plastids of higher plants, the red alga *Cyanidium caldarium* and the Chrysophyte *Ochromonas danica* (Schwender, Seemann, Lichtenthaler & Rohmer, 1996; Lichtenthaler,

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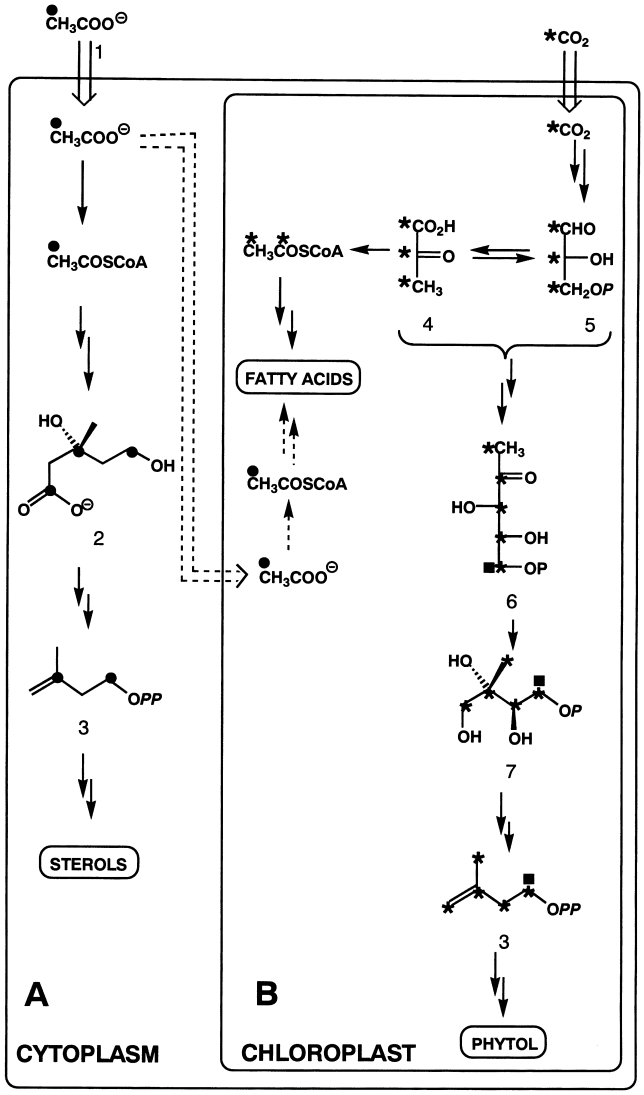

Scheme 1. Isoprenoids and fatty acids biosynthesis in the diatom *Phaeodactylum tricornutum*. A: mevalonate pathway. B: mevalonate-independent methylerythritol 4-phosphate pathway.

Schwender, Disch & Rohmer, 1997; Disch, Schwender, Müller, Lichtenthaler & Rohmer, 1998b). In plants, not only essential chloroplast isoprenoids related to the photosynthetic apparatus (carotenoids, phytol from chlorophylls, plastoquinone), but also plastid related secondary metabolites such as isoprene, mono- and diterpenes (Schwarz, 1994; Eisenreich, Schwarz, Cartayrade, Arigoni, Zenk & Bacher, 1998; Schwarz & Arigoni, 1999) are produced via the MEP pathway.

Diatoms (*Bacillariophyceae* subdivision of the Chrysophyta) belong to the most important primary producers in the oceans. Nothing is known about the early steps of isoprenoid biosynthesis in these organisms. The formation of two isoprenoids, phytol as representative of the chloroplast isoprenoids, and 24-epibrassicasterol, as representative of the cytoplasm isoprenoids, was investigated in the two marine diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis* by incorporation of ^{13}C or ^{2}H labeled precursors.

2. Results and discussion

Phaeodactylum tricornutum contained (22*E*,24*S*)-24-methylcholesta-5,22-dien-3β-ol (epibrassicasterol; **8**, Fig. 1) as the major sterol (>97%) and traces of cholesta-5,22-dienol, cholesterol, 24-methylcholesterol and 24-ethylcholesterol. This was in accord with previously reported results (Rubinstein & Goad, 1974; Wright et al., 1978). In *Nitzschia ovalis*, epibrassicasterol (**8**) and (22*E*)-cholesta-5,22-dien-3β-ol (**9**) were accompanied by minor Δ^5-sterols such as cholesterol, 24-ethylcholesta-5,22-dienol, 24-ethylcholesterol and traces of $\Delta^{5,7}$-sterols (cholesta-5,7-dienol and ergosta-5,7-dienol). Phytol (**10**) was isolated from both diatoms after hydrolysis of the chlorophylls. Major sterols and phytol were identified by ^{1}H- and ^{13}C-NMR, GC and GC/MS and comparison of the data with those found in the literature (Rubinstein, Goad, Clague & Mulheirn, 1976; Eggert, van Antwerp, Bhacca & Djerassi, 1976) or obtained from reference compounds available in the laboratory. Minor sterols were only identified by GC and GC/MS.

For the identification of the biosynthetic pathway to isoprenoids, epibrassicasterol and the phytyl side chain of chlorophylls were analyzed in both diatoms, and cholestadienol (**9**) in *N. ovalis* only. Carotenoids could not be isolated in amounts that were sufficient for ^{13}C-NMR analysis. Nearly all labeling experiments were performed under low light conditions in order to favor mixotrophic growth and to try to enhance the incorporation of the labeled carbon source. Such growth conditions were also expected to minimize recycling via photosynthesis of the ^{13}C labeled carbon dioxide released by the catabolism of the labeled precursor (Seemann, 1995). Neither *P. tricornutum*, nor *N. ovalis* were capable of growing heterotrophically on acetate or glucose in full darkness. Interestingly, the phytol concentration was almost two times higher in cells grown under mixotrophic conditions than that in autotrophic cells. Labeling with sodium [1-^{13}C]acetate was performed with *P. tricornutum* as well as *N. ovalis*. All additional labeling experiments were performed with *P. tricornutum* only, because it contained epibrassicasterol (**8**) as single major sterol.

2.1. Incorporation of [1-^{13}C]acetate

Sodium [1-^{13}C]acetate was directly incorporated into epibrassicasterol by *P. tricornutum* and into epibrassicasterol and cholesta-5,22-dienol by *N. ovalis* according to the MVA pathway: all carbon atoms corresponding to C-1 and C-3 of IPP were labeled

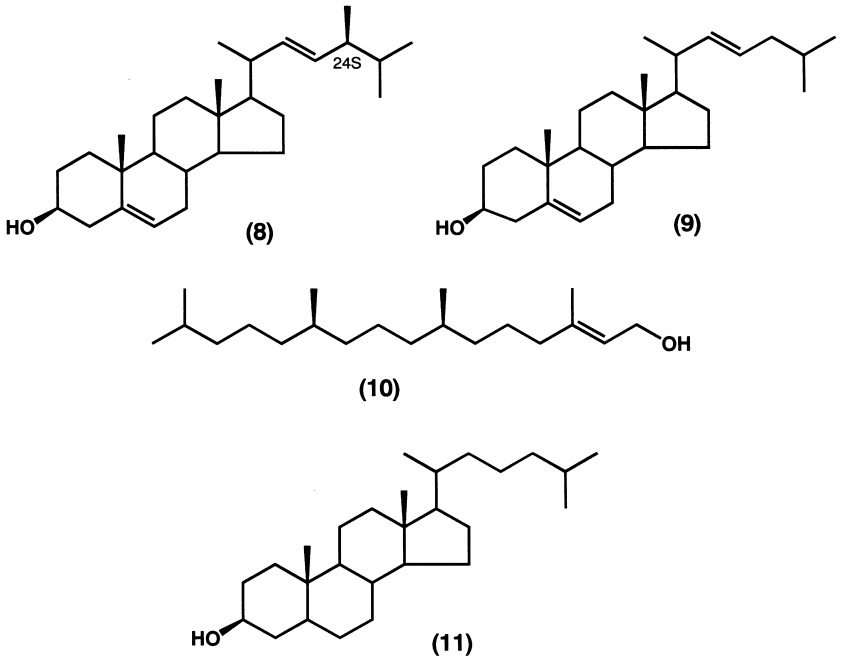

Fig. 1. Isoprenoids from the diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis*: epibrassicasterol (**8**), cholestadienol (**9**) and phytol (**10**). Cholestanol (**11**) was obtained after hydrogenation of cholestadienol (**9**).

(Table 1). In contrast, phytol was not labeled at all. Even by GC–MS, no trace of ^{13}C incorporation was found. This suggested that phytol was solely derived from another unlabeled carbon source which most probably was the carbon dioxide from air.

Earlier experiments already showed that the biosynthesis of plastidic isoprenoids was not accessible in plants to exogenously applied acetate, which was only a suitable precursor for sterols (Heintze et al., 1990). Such results were mainly explained by the difficulty or the impossibility of acetate penetration into the chloroplast. However, acetate was easily capable of crossing membranes, to be taken up by the plastids where it is activated to acetyl-CoA for fatty acid biosynthesis

Table 1
Isotopic abundances (%) of steryl acetates after feeding of sodium [1-^{13}C]acetate (20% isotopic abundance) to the diatoms *Phaeodactylum tricornutum* and *Nitzschia ovalis*

C atom[a]	Epibrassicasterol (**8**)			Cholestanol (**11**)		C atoms of IPP
	δ (ppm)	*P. tricornutum* (%)	*N. ovalis* (%)	δ (ppm)	*N. ovalis* (%)	
C-2	27.79	8.7	16.8	27.51	13.2	C-1
C-4	38.14	4.8	9.4	34.06	15.4	C-3
C-6	122.66	6.4	10.5	28.64	13.8	C-1
C-8	31.9	6.7	11.4	35.49	14.1	C-3
C-10	36.64	5.8	9.9	35.83[d]	13.9	C-3
C-11	21.04	4.7[c]	7.8	21.22	11.4	C-1
C-12	39.66	6.6	12.8	40.01	13.0	C-1
C-14	56.82	6.1	10.4	56.44	10.0	C-3
C-16	28.82	5.7	10.9	28.28	11.8	C-1
C-20	40.28	6.7	13.5	35.83[d]	13.9	C-3
C-23	131.85	6.6	12.2	23.85	15.5	C-1
C-25	33.24	6.7	14.4	28.04	14.0	C-3
MeCO[b]	170.55	20.0	20.0	170.75	20.0	–

[a] Signals of carbons with natural ^{13}C abundance (1.1%) were not detected on the NMR spectrum.

[b] The signal of the acetyl methyl group (isotopic abundance 20.0%) was used as reference for the evaluation of the isotopic abundances.

[c] Chemical shift of C-11 and C-21 are identical in the ^{13}C-NMR spectra of epibrassicasteryl acetate. As C-21 should not be labeled, the ^{13}C enrichment was assigned to C-11.

[d] In the ^{13}C-NMR spectrum of cholestanyl acetate, C-10 and C-20 are characterized by identical chemical shifts. As both should be labeled, the same isotopic abundance was assigned to each of these carbon atoms.

(Harwood, 1997). Several ^{14}C-labeling studies with green tissues from plants (Goodwin, 1965; Wieckowski & Goodwin, 1967; Threlfall & Whistance, 1971), leaf segments or isolated plastids (Heintze et al., 1990) showed that the plastidic isoprenoids were labeled preferentially and with high rates from $^{14}CO_2$. Efficient $^{14}CO_2$ incorporation was also observed in monoterpenes and sesquiterpenes from different plants, which are also believed to be synthesized in chloroplasts (Loomis & Croteau, 1973). The fast incorporation of $^{14}CO_2$ into the plastidic isoprenoids suggested that their synthesis was very closely connected to photosynthetic CO_2 fixation (Sharkey, 1996). All these data supported the hypothesis that in diatoms also phytol was formed from CO_2.

2.2. Diatom growth in the presence of $^{13}CO_2$ and unlabeled acetate

In order to check the level of CO_2 fixation by photosynthesis under mixotrophic growth conditions, *P. tricornutum* was grown in an atmosphere containing $^{13}CO_2$ (generated by H_2SO_4 treatment of $Ba^{13}CO_3$, 20% isotopic abundance) in the presence of unlabeled sodium acetate, under the same experimental conditions as those utilized for the [1-^{13}C]acetate incubation. Phytol was uniformly labeled (average isotopic abundance: $7.4\% \pm 0.9\%$), whereas only a slight uniform incorporation of $^{13}CO_2$ was observed into epibrassicasterol (average isotopic abundance: $1.8\% \pm 0.7\%$). The C-28 methyl group of epibrassicasterol, which directly arises from CO_2, presented the same isotopic abundance (7%) as those of the phytol carbon atoms. Indeed, CO_2 is incorporated into tetrahydrofolic acid and is the precursor for the transferred S-adenosyl methionin methyl group during the alkylation of the sterol side chain (Bramley, 1997; Wink, 1997). Mixotrophic growth on acetate and CO_2 showed the channeling in *P. tricornutum* of the carbon fluxes for two distinct isoprenoid families located in two different cell compartments. Acetate was the major carbon source for sterols in the cytoplasm, and $^{13}CO_2$ was utilized via photosynthesis for the formation of phytol. A similar behavior was found for fatty acids. According to GC–MS of the fatty acid methyl esters, [1-^{13}C]acetate was only weakly incorporated (not more than 3%), whereas they were uniformly labeled from $^{13}CO_2$ with an isotopic abundance of the same order of magnitude (4–6%, according to ^{13}C-NMR) as that found for phytol.

2.3. Labeling experiments with D-[1-^{13}C]glucose and [3-^{13}C]pyruvate

MVA and MEP routes for isoprenoid biosynthesis can be clearly differentiated by the labeling patterns observed in isoprenic units after incorporation of [1-^{13}C]glucose (Rohmer et al., 1993). After feeding *P. tricornutum* with [1-^{13}C]glucose, however, no labeling was observed in the isoprenoids, neither in phytol, nor in epibrassicasterol. The ^{13}C labeling could be lost by glucose catabolism via the oxidative pentose phosphate pathway, but if mixotrophic growth occurred, the resulting $^{13}CO_2$ would be recycled, giving uniformly labeled metabolites (Seemann, 1995; Lichtenthaler et al., 1997). As no labeling was detected neither in sterols, nor in phytol (which was uniformly labeled after incubation in the presence of $^{13}CO_2$), loss of ^{13}C via the oxidative pentose phosphate pathway seemed rather unlikely. Glucose was not utilized (or at very low level) by this diatom, at least for the biosynthesis of the isoprenoids, which were again most probably synthesized from unlabeled carbon dioxide from air.

In experiments performed with the phototrophic cyanobacterium *Synechocystis*, the level of incorporation of ^{13}C labeled glucose was influenced by the light conditions. When low stimulatory light was applied (Disch et al., 1998b), utilization of glucose as a carbon source for isoprenoid biosynthesis was much higher in this prokaryote than under continuous low light (Proteau, 1998). Consequently, an additional experiment was performed with [1-^{13}C]glucose under periodic light pulses of higher intensity. Again, the investigated isoprenoids were not labeled. Accordingly, diatoms are most probably not capable of utilizing glucose as carbon source for isoprenoid biosynthesis.

Characterization of the MEP pathway was also attempted by an incubation of [3-^{13}C]pyruvate. Pyruvate is a direct precursor of 1-deoxy-D-xylulose 5-phosphate. It was readily incorporated into isoprenoids by *Methylobacterium fujisawaense* and *Escherichia coli* (Rohmer et al., 1993), *Scenedesmus obliquus* (Disch et al., 1998b) and tobacco (Disch, Hemmerlin, Bach & Rohmer, 1998a). In the case of *P. tricornutum*, no label was found in phytol and in epibrassicasterol after feeding of [3-^{13}C]pyruvate, indicating again that isoprenoid biosynthesis was only supported by the carbon dioxide from air.

2.4. Incorporation of 1-deoxy-D-[5,5-$^2H_2$]xylulose

The above-described labeling experiments were usually suited to unambiguous detecting for the presence of the two biosynthetic routes towards the isoprenoids in phototrophic eukaryotes. In the two investigated diatoms, they allowed to characterize unambiguously the MVA route for sterol biosynthesis in the cytoplasm and to detect the dichotomy in the isoprenoid biosynthetic routes in the cytoplasm and in the plastids. They did not yield direct evidence for the presence of the MEP route in the chloroplast of these diatoms. An additional feeding experiment was, there-

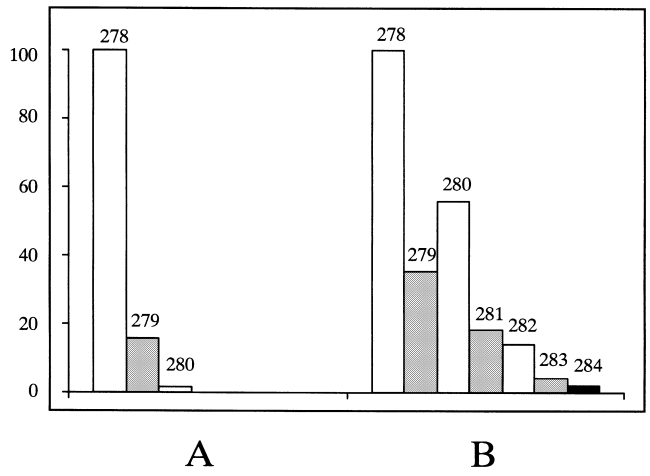

Fig. 2. GC/MS of phytyl acetate: section of the mass spectrum showing the fragment m/z 278 corresponding to the loss of acetic acid. (A) Natural abundance. (B) After incorporation of 1-deoxy-D-[5,5′-$^2H_2$]xylulose.

fore, performed with deuterium labeled 1-deoxy-D-xylulose, the first C_5 precursor in the MEP pathway. In most feeding experiments with this precursor, incorporation into isoprenoids of *E. coli* (Broers, 1994), algae (Schwender et al., 1997) or plants (Schwender et al., 1997; Arigoni, Sagner, Latzel, Eisenreich, Bacher & Zenk, 1997; Sagner, Latzel, Eisenreich, Bacher & Zenk, 1998; Piel, Donath, Banderner & Boland, 1998) synthesized via the MEP route was satisfactory. *P. tricornutum* was, therefore, grown in mixotrophic conditions on a medium containing 1-deoxy-D-[5,5-$^2H_2$]xylulose and unlabeled acetate. The isoprenoids from the cells grown in these conditions were analyzed by GC–MS after acetylation. Sterols were not labeled at all with deuterium. This confirmed the conclusions reached from previous experiments: sterols were solely synthesized via the MVA pathway. Phytol in contrast was significantly labeled from deoxyxylulose with about a 10% incorporation yield, the major isotopomer bearing two deuterium atoms as expected from the incorporation of intact deoxyxylulose in a single isoprenic unit of the phytyl chain (Fig. 2). This represented a direct evidence for the intervention of the mevalonate-independent MEP pathway for the formation of the phytyl side chain of chlorophylls in the diatom *P. tricornutum*.

Incorporation of 1-deoxy-D-[5,5-$^2H_2$]xylulose also resulted in an unusual and unexpected incorporation of a single deuterium from a doubly labeled precursor (Fig. 2). As the 1-deoxy-D-[5,5-$^2H_2$]xylulose used for this labeling experiment did not contain detectable amounts of monodeuterated 1-deoxy-D-[5-^{2}H]xylulose (as checked by ^{1}H- and ^{13}C-NMR), the presence of phytol isotopomers with one and three deuterium atoms, as shown by the significant intensities of the signals from the m/z 279, 281 and 279 ions (Fig. 2), could represent the signature for a partial degradation of the deuterated precursor and for the recycling of the resulting metabolites. Such a catabolism would yield DXP which contained only one deuterium and which would be re-incorporated into phytol. As sterols were not labeled, this deoxyxylulose metabolism apparently only occurred in the chloroplast. Such a phenomenon was never observed in earlier experiments. It was probably favored by the long incubation times required by the slow growth of the diatoms.

2.5. Conclusion

The two investigated diatoms showed the same dichotomy for isoprenoid biosynthesis as that previously found in higher plants (Lichtenthaler et al., 1997), the red alga *Cyanidium caldarium* and another Chrysophyte, *Ochromonas danica* (Disch et al., 1998b). According to the results of the performed labeling experiments (Table 2), the MVA route is involved in the biosynthesis of the cytoplasm isoprenoids such as the sterols, and the MVA independent MEP route is utilized for the biosynthesis of the chloroplast isoprenoids.

Very low incorporation of [1-^{13}C]acetate or [1,2-$^{13}C_2$]acetate was reported into the geranyl diphosphate derived prenyl moiety of domoic acid in the diatom *Nitzschia pungens* (Douglas, Ramsey, Walter & Wright, 1992), suggesting that this prenyl chain was

Table 2
Feeding experiments performed with *Phaeodactylum tricornutum*: isoprenoid labeling

Labeled substrate	Isoprenoid labeling	
	MVA pathway, epibrassicasterol	MEP pathway, phytol
[1-^{13}C]acetate	+	–
[1-^{13}C]glucose	–	–
[3-^{13}C]pyruvate	–	–
$^{13}CO_2$ + unlabeled acetate	–[a]	+
[5-$^2H_2$]DX + unlabeled acetate	–	+

[a] Very weak uniform labeling.

not derived from the MVA, but rather from the MEP route.

The labeling experiments performed with these diatoms directly pointed out the key role of CO_2 and photosynthesis in the MEP pathway for isoprenoid biosynthesis. The mixotrophic growth conditions shed light on the competition and the preferential use of two carbon sources (acetate or CO_2) in different cell compartments and for different biosynthetic routes. They also allowed a much closer approach to the isoprenoid biosynthesis under normal life conditions for many photosynthesizing organisms, without any perturbation induced by the addition to the culture medium of an intermediate (such as MVA or deoxyxylulose) which under normal growth conditions is only present in very low concentrations.

3. Experimental

3.1. Labeled compounds

Sodium [1-^{13}C]acetate (99% isotopic abundance) was obtained from Sigma Aldrich Chemicals (USA), D-[1-^{13}C]glucose (99% isotopic abundance) from Omicron Biochemicals (South Bends, IN, USA), sodium [3-^{13}C]pyruvate (99% isotopic abundance) from Eurisotop (Saint Aubin, France) and $Ba^{13}CO_3$ (99% isotopic abundance) from Cambridge Isotope Laboratories (USA). 1-Deoxy-D-[5,5′-$^2H_2$]xylulose was synthesized in the laboratory by the method of Broers (1994).

3.2. Culture conditions and labeling experiments

P. tricornutum 1052/1B and *N. ovalis* 1052/12 (Culture Collection of Algae and Protozoa, Ambleside, Scotland, UK) were grown at 18°C on a rotatory shaker (120 rpm) in conical flasks on Guillards medium (*P. tricornutum*) or on Guillard's medium containing in addition sodium metasilicate (*N. ovalis*) (Catalogue of Strains 1995, Culture Collection of Algae and Protozoa, Ambleside, Scotland, UK). A bulk culture of *P. tricornutum* (190 mg l^{-1}, dry weight, from 5.5 l) was autotrophically grown for 17 days under illumination (~100 μmol photon m^{-2} s^{-1}, light/dark cycle 16 h/8 h) in order to identify the sterols and to obtain an epibrassicasterol reference sample of natural ^{13}C abundance. In order to avoid recycling of $^{13}CO_2$ resulting from catabolism of the labeled carbon source via photosynthesis, mixotrophic cultures required for labeling experiments were grown under low illumination (< 10 μmol photon m^{-2} s^{-1}), with the exception of one experiment performed with ^{13}C labeled glucose.

Labeling experiments with sodium [1-^{13}C]acetate were performed with both diatoms, all other experiments with *P. tricornutum* only. The 5 and 7 l cultures containing sodium [1-^{13}C]acetate (1 g l^{-1}, 20% isotopic abundance) were maintained during 15 days affording enough material for ^{13}C-NMR analysis of *P. tricornutum* (60 mg l^{-1}, lyophilized cells) and *N. ovalis* (70 mg l^{-1}). $^{13}CO_2$ labeling was performed for 31 days with a 5 l culture of *P. tricornutum* (65 mg l^{-1}) in the presence of unlabeled sodium acetate (1 g l^{-1}) in an atmosphere of air enriched with $^{13}CO_2$. Cultures were performed in five Erlenmeyer flasks (2.5 l total volume), each containing the culture medium (0.5 l) and hermetically closed with a rubber stopper. CO_2 was released four times from $Ba^{13}CO_3$ (isotopic abundance 20%, 480 mg for each Erlenmeyer flask, placed in a small glass container suspended into the culture flask via the hole in the rubber stopper and closed with a rubber pipette bulb) by addition of 2 M H_2SO_4 with a syringe. 1-Deoxy-D-[5,5-$^2H_2$]xylulose (200 mg l^{-1}) was fed in the presence of unlabeled sodium acetate (1 g l^{-1}) to a 0.5 l culture for 21 days yielding 160 mg l^{-1} (lyophilized cells). For the incorporation of sodium [3-^{13}C]pyruvate (10% abundance isotopic, 1 g l^{-1}) and for a first labeling attempt with D-[1-^{13}C]gluglucose (20% isotopic abundance, 1 g l^{-1}), 5 l cultures of *P. tricornutum* were respectively grown for 24 days (yielding 60 mg l^{-1}, lyophilized cells) or 18 days (60 mg l^{-1}) under low illumination as described for the former cultures. An additional 5 l culture in the presence of D-[1-^{13}C]glucose was performed under different light conditions (about 70 μmol photon m^{-2} s^{-1} light/dark cycle: 10 min/100 min) yielding 60 mg l^{-1} (lyophilized cells) after 28 days.

3.3. Analytical methods and evaluation of isotopic abundances

NMR spectra were recorded on a Bruker WP 400 spectrometer in [^{2}H]chloroform solution. ^{13}C isotopic abundances were determined as previously described (Flesch & Rohmer, 1988). Cholesta-5,22-dienyl acetate obtained from *N. ovalis* was hydrogenated by stirring in ethyl acetate (0.5 ml) for 4 h at room temperature under atmospheric pressure, and the determination of the isotopic abundances was performed on the resulting cholestanyl acetate.

For phytyl acetate or steryl acetates, the methyl singlet of the acetoxy group (1.1% isotopic abundance, 21.1 or 171.2 ppm, respectively) was used as an internal standard for the determination of isotopic abundances. In the case of strong isotopic enrichment, the peaks of non-labeled carbons, including that of the acetoxy methyl group, were not visible. After isolation and preliminary estimation of the ^{13}C isotopic abundance, steryl acetates were, therefore, deacetylated with a 0.5% K_2CO_3 solution in MeOH (1 ml/1 mg) for 3 h at room temperature. After evaporation of the metha-

nol, the free sterols were directly isolated by TLC (dichloromethane, two migration) and reacetylated with a 40% solution of [1,1′-$^{13}C_2$]acetic anhydride in toluene (20% isotopic abundance, 200 μl) in the presence of pyridine (80 μl). The acetoxy carbonyl group served as a reference.

Deuterium incorporation from deoxyxylulose into phytol and fatty acid was determined by mass spectrometry. Peak ratios of labeled phytyl acetate or fatty acid methyl esters were compared with those of the same compounds of natural abundance. Peak contribution from natural ^{13}C abundance were deduced. GC–MS was performed on a Fisons MD 800 spectrometer. Acetylated compounds were separated on a DB1 silica capillary column (30 m × 0.1 mm, 0.32 μm film thickness) with the following temperature program: 50°C for 3 min, from 50 to 220°C at 20°C min^{-1} and from 220 to 300°C at 6°C min^{-1}. MS characteristics were as follows: 70 eV electron impact ionization energy, 170°C source temperature, 250°C transfer line temperature and 0.9 s scanning from 50 to 600 Da.

3.4. Isolation of isoprenoids

Most analytical procedures were as previously described (Disch et al., 1998a, 1998b). The lyophilized cells were extracted for 1.5 h under reflux with $CHCl_3$/MeOH (2:1, v/v, 3 × 40–50 ml). After removal of the solvents, the extract was saponified for 1.5 h under reflux with a 6% KOH solution in methanol (5–8 ml). After addition of water (2 volumes), the lipids were extracted with hexane. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The non-saponifiable lipids were separated by TLC (CH_2Cl_2) on silica gel yielding phytol ($R_f = 0.32$) and sterols ($R_f = 0.19$), which were acetylated overnight at room temperature with an acetic anhydride/pyridine mixture (1:1, v/v, 100 μl). After removal of the reagents under a nitrogen stream, the acetates were purified by TLC (cyclohexane/EtOAc, 9:1 v/v, $R_f = 0.61$). Steryl acetates from *N. ovalis* were further separated by argentation TLC (Aitzetmüller & Guaraldo Goncalves, 1990) (absolute $CHCl_3$, one migration) yielding (24*S*)-24-methylcholesta-5,22-dien-3β-yl acetate (epibrassicasteryl acetate, $R_f = 0.41$) and cholesta-5,22-dien-3β-yl acetate ($R_f = 0.31$). Under low light conditions, *P. tricornutum* afforded phytol (3.5 mg l^{-1}, lyophilized cells) and epibrassicasterol (0.6 mg l^{-1}), and *N. ovalis* phytol (2.4 mg l^{-1}), epibrassicasterol (0.24 mg l^{-1}), and cholestadienol (0.17 mg l^{-1}).

The residue left after hexane extraction was acidified with concentrated H_2SO_4 (pH = 1–2). The fatty acids were extracted with hexane and methylated with a diazomethane solution in ether. After catalytic hydrogenation, the saturated fatty acid methyl esters were purified by TLC (cyclohexane/EtOAc, 9:1 v/v, $R_f = 0.50$).

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