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DIFFERENTIAL RESPONSE OF MICROALGAE TO THE SUBSTITUTED PYRIDAZINONE, SANDOZ 9785, REVEAL DIFFERENT PATHWAYS IN THE BIOSYNTHESIS OF EICOSAPENTAENOIC ACID

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Key Word Index—Microalgae; *Monodus subterraneus*; *Porphyridium cruentum*; fatty acid biosynthesis; eicosapentaenoic acid; molecular species; SAN 9785.

Abstract—Treating the red alga, *Porphyridium cruentum*, with the herbicide, SAN 9785, an inhibitor of chloroplastic ω3-desaturation, resulted in a decrease in the proportion of eicosapentaenoic acid (EPA), especially in monogalactosyldiacylglycerols (MGDG). However, when the eustigmatophyte, *Monodus subterraneus*, was treated with this herbicide, the proportion of EPA in MGDG surprisingly increased from 54 to 81%. Molecular species analysis of MGDG, showed that the prokaryotic molecular species 20:5/16:0 was almost eliminated, while the eukaryotic 20:5/20:5 dominated. These findings indicate that EPA biosynthesis in *M. subterraneous* is different from that in *P. cruentum*. We suggest that in *M. subterraneus* there are at least two pathways involving ω3-desaturase activity which lead to the production of EPA. The chloroplastic pathway, which is inhibited by SAN 9785, and the cytoplasmic pathway, which presumably uses phospholipid-bound fatty acids and is not affected by this inhibitor. The product of the latter pathway is exported through the chloroplast membrane resulting in the production of 20:5/20:5 MGDG. We further suggest that, by analogy with higher plants, microalgae can be categorized according to their respective EPA biosynthetic pathways.

INTRODUCTION

Accumulated knowledge of the biosynthetic pathways of polyunsaturated fatty acids (PUFAs) in higher plants has been outlined by Sommerville and Browse [1] using *Arabidopsis thaliana* as a model. According to this scheme, 18:2 and 18:3ω3 are formed by sequential desaturation of 18:1 which is already esterified to a specific glycerolipid. Two pathways have been proposed for the biosynthesis of 18:3ω3 in higher plants and microalgae [2, 3]. By the prokaryotic scheme, monogalactosyldiacylglycerol (MGDG) is first synthesized as the 18:1/16:0 species within the chloroplast and is subsequently desaturated at both positions to form 18:3ω3/16:3ω3 MGDG. In the eukaryotic pathway, 18:1-CoA, synthesized in the chloroplast, is exported across the chloroplast envelope into the endoplasmic reticulum, where it is utilized in the synthesis of 18:1/18:1 phosphatidylcholine (PC). After desaturation to 18:2/18:2 PC, the diacylglycerol (DAG) backbone 18:2/18:2 is transported back to the chloroplast, where it is galactosylated to form 18:2/18:2 MGDG, which can be further desaturated by an ω3-desaturase to the 18:3ω3/18:3ω3 species. However, it appears, that various plant species have developed variations of this scheme. Thus, in pea leaves, 18:3ω3 is synthesized only via the eukaryotic pathway and mostly in the endoplasmic reticulum. In spinach leaves, in contrast, both the prokaryotic and the eukaryotic pathways are operative, but 18:3ω3 is produced mostly in the chloroplast [4].

While the biosynthetic pathways leading to 18:3ω3 are apparently similar in algae and higher plants, very little is known about the biosynthesis of C_{20} PUFAs which are found in many algae but not in higher plants [5]. Recently, Arao and Yamada [6] reported that, in *Phaeodactylum tricornutum*, eicosapentaenoic acid (20:5ω3, EPA) is synthesized by various pathways that take place in the cytoplasm utilizing phospholipid-bound fatty acids. Similarly, Schneider and Roessler [7] suggested that EPA biosynthesis in *Nannochloropsis* is entirely cytoplasmic. The elucidation of the pathways

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Abbreviations: in the shorthand numbering system used for identifying fatty acids, the figure preceding the colon indicates the number of carbon atoms in the fatty acid, while that following the colon represents the number of double bonds present. Pairs of numbers representing the fatty acids, when separated by a slash, designate the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species (except for 20:4/20:5 which is a mixture of two isomers).

Table 1. Effect of SAN 9785 on fatty acid composition of major lipids of *Monodus subterraneus*

Lipid class	SAN 9785	Fatty acid composition (% total fatty acids)												
		14:0	16:0	16:1 ω11	16:1 ω7	18:0	18:1 ω9	18:1 ω7	18:2	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
Total lipids	−	3.3	20.6	3.3	26.1	0.7	5.9	0.8	0.9	1.2	0.5	0.5	3.7	32.6
Total lipids	+	3.1	16.2	2.7	23.3	0.9	2.1	0.5	2.4	0.3	0.5	0.2	3.2	42.7
MGDG	−	5.4	13.5	1.5	13.4	1.3	4.3	0.8	1.8	0.6	0.3	0.9	2.2	54.2
MGDG	+	2.7	4.3	*t*	5.7	0.2	0.8	0.2	0.4	0.2	0.7	0.2	3.3	81.3
PC	−	1.0	33.1	—	13.2	2.2	7.4	0.9	7.3	15.4	—	0.4	4.4	13.9
PC	+	2.1	21.3	—	21.1	2.9	12.2	0.4	18.0	0.2	—	*t*	5.7	14.6

t, traces.

leading to 18:3 was based in part on studies using the substituted pyridazinone, SAN 9785 (BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone) [8–10]. This herbicide inhibits the chloroplastic but not the cytoplasmic desaturation of 18:2 to 18:3ω3 in both the prokaryotic and eukaryotic pathways in higher plants and algae [8]. We have shown that EPA biosynthesis in the red microalga, *Porphyridium cruentum*, was inhibited by SAN 9785 [11], whilst in the eustigmatophyte, *Monodus subterraneus*, this herbicide, unexpectedly, produced an increase in the proportion of EPA. Similarly, EPA production in the marine microalgae, *Chroomonas salina* and *Nannochloropsis oculata*, was not inhibited by SAN 9785 [12].

In the present work, we have compared the effect of SAN 9785 on the lipids of *M. subterraneus* and *P. cruentum*, in an attempt to gain a better understanding of the different pathways of EPA biosynthesis operating in microalgae. The data we have obtained suggest that ω3-desaturation can take place in the chloroplast, in the cytoplasm or at both sites.

RESULTS

Growing *M. subterraneus* for three days in the presence of 0.16 mM SAN 9785 resulted in a growth inhibition of 65%. The fatty acid composition of the biomass surprisingly displayed an increase in the proportion of the major PUFA, EPA, from 32.6 to 42.7% of total fatty acids (Table 1). In control cultures, most of the EPA is found in galactolipids and especially in MGDG [13]. We have therefore compared the fatty acid composition of MGDG, the major galactolipid, in treated and untreated cultures. In the presence of the inhibitor, the proportion of EPA in MGDG increased from 54.2 to 81.3%, while that of most other fatty acids and especially that of 14:0, 16:0 and 16:1 decreased (Table 1). In comparison, similar treatment of the red alga, *P. cruentum*, resulted in a significant decrease in EPA production; in MGDG, EPA decreased from 72.5 to 53.4% (Table 2).

In the control culture of *M. subterraneus*, the main molecular species of MGDG were 20:5/20:5 (57%), 20:5/14:0, 20:5/16:1, 20:5/16:0 and 20:4ω6/16:0 (Table 3). In the presence of SAN 9785, 20:5/20:5 became the predominant molecular species comprising 80% of the total. The proportion of all other molecular species decreased with the exception of an unidentified peak that increased from 1 to 3%. In *P. cruentum*, equal amounts of prokaryotic and eukaryotic molecular species were produced under control conditions. The inhibitor produced a decrease in the share of eukaryotic molecular species to 29% of total MGDG, primarily due to the decrease from 46 to 27% in the proportion of

Table 2. Effect of SAN 9785 on fatty acid composition of MGDG in *Porphyridium cruentum*

Lipid class	SAN 9785	Fatty acid composition (% total fatty acids)										
		16:0	16:1 ω7	18:0	18:1 ω9	18:1 ω7	18:2	18:3 ω6	20:2 ω6	20:3 ω6	20:4 ω6	20:5 ω3
Total lipids	−	29.9	2.9	0.4	0.4	0.3	5.4	0.6	0.6	0.6	14.3	44.6
Total lipids	+	38.3	4.3	0.5	1.0	0.5	5.7	0.5	0.6	1.0	15.9	31.9
MGDG	−	18.7	—	0.6	—	—	1.7	—	—	—	6.0	72.5
MGDG	+	27.0	—	0.6	0.7	—	3.5	—	—	0.3	14.5	53.4

Table 3. Effect of SAN 9785 on molecular species composition of MGDG in *Monodus subterraneus*

SAN 9785	Molecular species composition (%)					
	20:5/20:5	20:5/14:0	20:5/16:1	X	20:5/16:0	20:4/16:0
−	57.2	12.7	14.1	1.3	5.9	8.9
+	80.1	5.4	5.8	3.0	4.4	1.5

X, Unidentified: 20:5 and 20:4 stands for 20:5ω3 and 20:4ω6, respectively.

Table 4. Effect of SAN 9785 on molecular species composition of MGDG in *Porphyridium cruentum*

	Molecular species composition (%)					
SAN 9785	20:5/20:5	20:5/20:4	20:4/20:4	20:5/16:0	20:4/16:0	18:2/16:0
−	46.1	4.4	0.8	34.6	1.5	12.6
+	27.0	2.6	t	58.1	5.3	7.0

20:5/20:5 MGDG. The proportion of 20:5/20:4ω6 was also reduced from 4.4 to 2.6%, while that of 20:5/16:0 and 20:4ω6/16:0 increased from 34.6 and 1.5% to 58.1 and 5.3%, respectively (Table 4).

In PC, 18:3ω6 was almost eliminated; concurrently, its precursors 18:2 and 18:1 sharply increased. A shift from 16:0 to 16:1 was also noted (Table 1).

DISCUSSION

We hypothesized that, in *P. cruentum*, in line with the biosynthesis of 18:3ω3 in higher plants, EPA is produced by a pathway that include a chloroplastic ω3-desaturation. As a means for testing this hypothesis, we have used the herbicide, SAN 9785, which is known to inhibit the chloroplastic, but not the cytoplasmic, ω3-desaturation of 18:2 to 18:3ω3. As expected, SAN 9785 brought about a decrease in the proportion of EPA in this alga, especially in the galactolipids (Table 2). Recently, using radiolabelled fatty acid precursors, we have found that 20:4ω6-bound galactolipids can be desaturated in the chloroplast of *P. cruentum* to 20:5ω3 (Cohen *et al.*, unpublished data) (Fig. 1). EPA is predominantly concentrated in the galactolipids of both *M. subterraneus* [13] and *P. cruentum* [14]. However, in *M. subterraneus*, SAN 9785, surprisingly caused an increase in the proportion of EPA, primarily in MGDG (Table 1). Similarly, in another eustigmatophyte, *N. oculata*, SAN 9785 did not decrease the proportion of EPA in the galactolipids [12]. Indeed, Schneider and Roessler [7] have demonstrated that EPA biosynthesis in this alga is entirely cytoplasmic.

We have shown that, in *P. cruentum*, 18:2-PC is sequentially converted in the ω6-pathway to 20:4ω6-

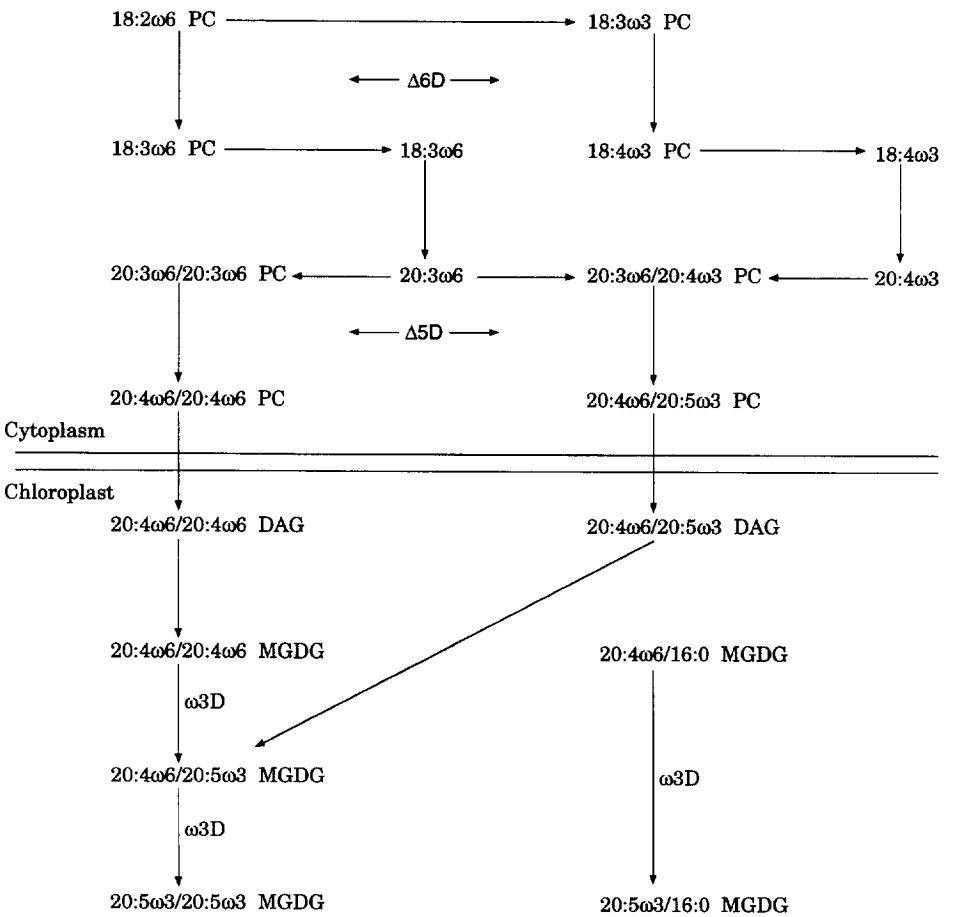

Fig. 1. Suggested scheme for EPA biosynthesis in *Porphyridicum cruentum*. Δ6D-Δ6 Desaturase; Δ5D-Δ5 desaturase; ω3D-ω3 desaturase.

containing molecular species of PC which are the major source of 20:4ω6 for both the prokaryotic and the eukaryotic molecular species of MGDG (Cohen *et al.*, unpublished) (Fig. 1). Chloroplastic EPA is primarily produced by ω3-desaturation of the 20:4ω6/20:4ω6 and 20:4ω6/16:0 molecular species of MGDG. In *M. subterraneus*, 20:5/20:5 was the major molecular species of MGDG even under control conditions. Nevertheless, we could not detect either 20:4ω6/20:4ω6 or 20:4ω6/20:5, its presumed precursors, while 20:4ω6/16:0 was quite abundant. The differential effect of SAN 9785 on MGDG in *M. subterraneus* by which the eukaryotic molecular species 20:5/20:5 increased at the expense of the prokaryotic ones (Table 3) thus pointed to the possible existence of two pathways leading to the eukaryotic and prokaryotic molecular species, respectively.

In the presence of the inhibitor, the proportion of 18:3ω6-PC was sharply reduced: however, that of EPA-PC was not affected (Table 1). Apparently, in the presence of the inhibitor, chloroplastic desaturation of 20:4ω6 that is produced in the cytoplasm by the ω6-pathway would be severely inhibited. The organism may thus diminish the flux in this pathway by reducing the Δ6-desaturation of 18:2-PC. Some of the accumulated 18:2 might be ω3-desaturated, in PC or another phospholipid, eventually resulting in 20:5. However, we cannot rule out, at the moment, that the decrease in 18:3ω6 results from a direct inhibition by the herbicide.

We infer that ω3-desaturation in *M. subterraneus* occurs in both the chloroplast and the cytoplasma (Fig. 2). Chloroplastic desaturation which involves the prokaryotic molecular species of MGDG, 20:4ω6/16:0 and 20:4ω6/14:0, is inhibited by SAN 9785, while the cytoplasmic desaturation, which utilizes phospholipid-bound fatty acids as substrates is not affected by this herbicide. Presumably, EPA produced in the latter pathway, can be exported through the chloroplast membrane as the 20:5/20:5 diacylglycerol moiety and converted to the respective MGDG derivative. In the presence of the inhibitor, chloroplastic ω3-desaturation is inhibited and the fatty acid flux in the cytoplasmic ω6 pathway is diverted into the direction of the ω3-cytoplasmic pathway, which can serve as a by-pass. Similarly, Norman and St John [9] have shown that a mutant of *Arabidopsis thaliana* defective in the prokaryotic pathway was able to compensate by enhanced production of eukaryotic molecular species. Supporting evidence to the possible existence of an ω3-cytoplasmic pathway to EPA was obtained when linoleic acid-fed *P. cruentum* produced 18:3ω3, 18:4ω3 and 20:4ω3 in PC (Cohen *et al.*, unpublished). Such a pathway may be operative also in the presence of SAN 9785 and may explain the increase, in the presence of the inhibitor, of 20:5/16:0 in *P. cruentum* (Table 4).

Plants are divided into 18:3 and 16:3 species [15]. In leaves of pea, an 18:3 plant, 18:3ω3 in the chloroplast is imported from the cytoplasm. In 16:3

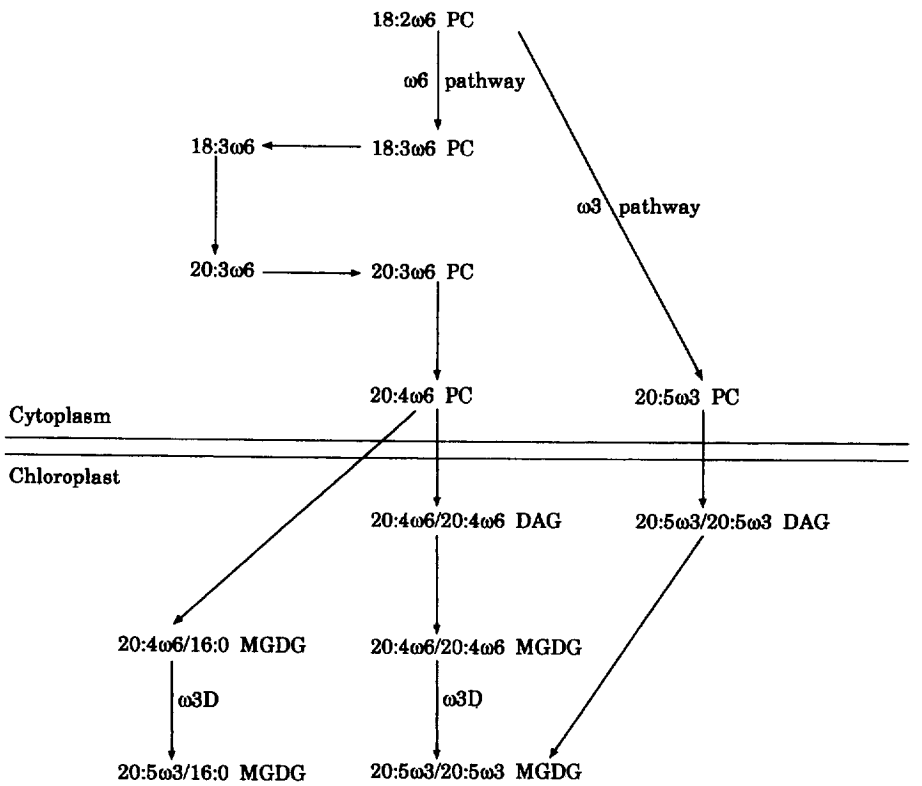

Fig. 2. Suggested scheme for EPA biosynthesis in *Monodus subterraneus*.

plants, such as spinach, ω3-desaturation would be mainly chloroplastic [4]. We suggest that a similar distinction can be also made among EPA-producing algae. Thus, ω3 desaturation in *P. cruentum* is predominantly chloroplastic, while in *N. oculata* only the cytoplasmic ω3-desaturation is operative. *M. subterraneus*, however, apparently has chloroplastic ω3-desaturase activity, as in *P. cruentum*, and a cytoplasmic activity, as in *N. oculata* and *P. tricornutum*. The content of 20:4ω6 in MGDG in a given alga may thus be an indicator of the type of biosynthetic pathways existing in the alga. Those with a high abundance of 20:4ω6 may resemble *P. cruentum*.

EXPERIMENTAL

Organism and culture conditions. *M. subterraneus* UTEX 151 was obtained from the University of Texas Culture Collection and cultivated on BG-11 medium as described in ref. [16]. *P. cruentum* strain 1380 1d was obtained from the Göettingen Algal Culture collection (Göettingen, Germany) and was grown on Jones' medium as previously described [14]. Cultures were grown in Erlenmeyer flasks, placed in a shaking incubator and illuminated from above at a light intensity of 115 μE m^{-2} s^{-1}, under an air–CO_2 (99:1) atmosphere at 25°. Cultures were grown exponentially (with proper diln) under the appropriate conditions for at least 4 days prior to each expt. Specific growth rates were estimated by measurement of chlorophyll concn. Solns of SAN 9785 in DMSO were added to exponentially growing cultures to a final concn of 0.16 mM. The final concn of DMSO in treated and control cultures did not exceed 1%.

Lipid fractionation. Freeze-dried samples of biomass were extracted by sand-grinding with $CHCl_3$–MeOH–H_2O (2:1:0.8) according to ref. [17]. Separation into neutral lipid, galactolipid and phospholipid frs was performed using a silica gel cartridge (Sep-pak); individual frs were eluted successively with $CHCl_3$, Me_2CO and MeOH, respectively. Galactolipid frs were further separated by TLC on silica gel developed with $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1).

Separation, identification and quantitation of molecular species of MGDG. This was achieved by HPLC on Lichrocart 250-4 (RP-18, 5μ) cartridges (Merck) and elution with 93% MeOH in H_2O at a flow rate of 2 ml min^{-1}. Peaks were detected at 205 nm, isolated and transmethylated as described below. The fatty acid composition was determined by GC analysis. For quantitation of HPLC chromatograms, an evaporative light-scattering detector was used; the settings were: tube temperature 130°, N_2 flow 40 ml min^{-1}.

Fatty acid analysis. Freeze-dried cells or lipid extracts were *trans*-methylated with MeOH–AcCl and analysed by GC as previously described [11]. The data shown represent mean values with a range of less than 5% for major (over 10% of fatty acids) peaks and 15% for minor peaks, of at least two independent samples, each analysed in duplicate.

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