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Enantioselective oxidation of thiafatty acids by an algal Δ12-desaturase

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Abstract

The whole-cell *Chlorella vulgaris* (211/8 K) system allows us to use thiastearate methyl esters as a mechanistic probe of the Δ12-desaturation process. The 6-, 7-, 12- and 13-thiastearates are converted intracellularly to the corresponding Δ9-desaturated products, which can then be processed by the Δ12-desaturase. The 6- and 7-thiaoleoyl are then desaturated respectively to the 6- and 7-thialinoleoyl products; the 12- and 13-thiaoleates are oxidised to the corresponding sulfoxides with a high enantioselectivity (ee ≥ 90%), confirming the oxygenase activity of the Δ12-desaturase with appropriately modified substrates. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The carbon–hydrogen bond activation of saturated hydrocarbon chains still remains a crucial challenge for chemists while plants exhibit an extensive diversity in activating long chain fatty acids by introducing double or triple bonds, hydroxyl or epoxy groups. Interest in these highly regio- and stereoselective processes has recently intensified as a number of the metalloenzymes involved in these reactions are considered to share similar aspects of their catalytic cycle [1–5]. Important members of this class of enzymes are the non-heme, diiron-containing desaturases, which catalyse the O_2-dependent dehydrogenation of fatty acids [1,6]. Both soluble and membrane-bound enzymes have been identified: the plant acyl-ACP desaturases (including the stearoyl-ACP Δ9-desaturase from castor seeds, which is the best characterized soluble diiron-containing desaturase [7,8]) exhibit no significant sequence identity to the integral membrane desaturases (represented by the stearoyl-CoA Δ9-desaturase from yeast [9]). The latter enzymes are less well-characterized and little is known about the molecular mechanism of the biooxidations they catalyse. Like the other members of the large family of membrane non-heme iron-containing proteins (which include alkane hydroxylase, xylene monooxygenase, carotenoids oxidase, sterol methyloxidase), desaturases use a diiron cluster for catalysis and three equivalent histidine clusters, implicated in iron binding, have been shown to be essential for catalysis [6]. Considering the high de-

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gree of sequence similarity between fatty acid hydroxylases and fatty acid desaturases [3,10], some authors have considered a common intermediate in the oxidative pathway [3]. Buist and Behrouzian [11,12] have studied the cryptoregiochemistry of the $\Delta 9$- and $\Delta 12$-desaturations and determined that the site of initial oxidations were at C9 and C12, respectively.

One of our fundamental objectives has been to obtain the stereochemistry of hydrogen removal in the $\Delta 12$-desaturation process. It is known that all of the $\Delta 9$-desaturases studied to date remove pro-*R* hydrogens at C9 and C10 [13–15]. Moreover, it has been shown that the yeast stearoyl-CoA desaturase is induced to exhibit oxygenase activity with the 9- and 10-thiastearates, resulting in a highly regio- and stereoselective sulfoxidation of the thiaanalogs with the same stereochemistry as in the desaturation process [13]. We thought it would be interesting to use sulfur as a mechanistic probe of the desaturation reaction to determine whether the oleate $\Delta 12$-desaturase could also behave as a sulfoxidase. We have previously demonstrated with an in vivo *Chlorella vulgaris* $\Delta 12$-desaturase system that, among the thiaoleic acids tested, the 12- and 13-thiaoleic acids induced a significant inhibition effect on the extraplastidial oleoyl desaturation process and were oxidised to the corresponding sulfoxides in the phospholipids (PL) of the microalgae, whereas the 14-thiaoleic acid had no effect on the endogenous fatty acid composition of the PL and was not oxidised at all at sulfur [16,17]. Thus, the sulfoxides were isolated from the PL and the optical purity were determined by chiral HPLC ($ee_{max} = 30\%$) [18].

In this article, we report the new strategy we developed to target more specifically the oleoyl desaturase activity and to obtain sulfoxides with higher ee. This strategy is based on the intracellular conversion of the appropriate thiastearates to the corresponding oleoyl products.

2. Experimental

Cultivation of *C. vulgaris* and the biotransformation assays in phosphate buffer were fully described in previous papers [17,18].

2.1. Whole-cells biotransformation assays in enriched medium (glucose-SK [20] culture medium)

In the mid-logarithm phase, cells were transferred from the 2l incubator to $2 \times 1l$ Erlenmeyer flasks under sterile conditions. Ethanolic solution of methyl thiastearates (75 mg/500 ml of cellular suspension) were added and the closed Erlenmeyer flasks were stirred at 25°C, under 15,000 lx. After incubation time, the reaction mixtures were centrifuged at 1500 $\times g$ for 10 min. Total lipid-containing cellular fractions were isolated and saponified by addition of 6 ml of 12% KOH in EtOH, at 70°C for 30 min. The subsequent acidification, extraction and methylation of the endogenous and exogenous fatty acids were realized very carefully as previously described [18] in order to avoid the racemisation of the sulfoxides. The same procedure was applied to the supernatants before discarding to check that no substrates nor metabolites remained in this fraction.

2.2. Synthesis of the substrates

Methyl 6-thiastearate, methyl 7-thiastearate, methyl 12-thiastearate and methyl 13-thiastearate were previously prepared and fully characterized by Buist and Behrouzian [12] and Buist et al. [22]. Our structural data were in accordance with these ones. We prepared the methyl thiastearates according to the following general procedure.

In a first step for the synthesis of the methyl 13-thiastearate, respectively methyl 12-thiastearate, 12-bromododecanoic acid, respectively 11-bromoundecanoic acid, (3.47 mmol) was dissolved in methanol (20 ml), before H_2SO_4 (2 ml) was added. The reaction mixture was refluxed for 3 h. After cooling at room temperature, water (40 ml) was added and the aqueous phase was extracted twice with Et_2O (20 ml). The combined organic layers were washed with 10 N NaOH (10 ml), then with water (3×10 ml), dried over anhydrous $MgSO_4$ and concentrated in vacuo.

In the second step, sodium hydride (11.5 mmol) was suspended in anhydrous THF (18 ml) at room temperature and under N_2. 1-Pentanethiol, respectively 1-hexanethiol, (10.4 mmol) was added dropwise and the medium was stirred for 45 min. The

resulting colorless bromo-methyl ester obtained from the first step was added dropwise and the reaction mixture was stirred for 4 h. After extraction with Et_2O (10 ml), organics were washed with a 10% K_2CO_3 solution (10 ml) and a saturated NaCl solution (3 × 10 ml). After drying over anhydrous $MgSO_4$ and concentrated in vacuo, the crude residue was purified by chromatography on silica gel using petroleum ether as eluant.

2.3. Synthesis of the racemic methyl S-oxide thiaoleates

Methyl 12- and 13-thiaoleates (0.3 g, 0.95 mmol) were dissolved in CH_2Cl_2 (5.8 ml) and the solution was cooled to 0°C, under N_2. One equivalent of m-chloroperbenzoic acid (0.235 g) dissolved in CH_2Cl_2 (2 ml) at 0°C was added dropwise. After 3 h at 0°C, the mixture was diluted with Et_2O and successively washed with a $NaHCO_3$ solution and with a saturated NaCl solution. After drying over anhydrous $MgSO_4$ and concentrated in vacuo, the crude residue was purified by chromatography on silica gel using CH_2Cl_2 then EtOAc as eluants.

Methyl S-oxide 12-thiaoleate: colorless oil (80% yield). ^{1}H NMR (400 MHz, CDCl$_3$): δ 5.80 (1H, dt, $^3J_{H_9-H_{10}} = 10.9$ Hz, $^3J_{H_8-H_9} = 7.45$ Hz, H$_9$), 5.44 (1H, dt, $^3J_{H_9-H_{10}} = 10.9$ Hz, $^3J_{H_{10}-H_{11}} = 8.0$ Hz, H$_{10}$), 3.65 (3H, s, H$_{19}$), 3.48 (2H, dd, $^2J_{H_{11}-H_{11}} = 13.0$ Hz, $^3J_{H_{10}-H_{11}} = 8.0$ Hz, H$_{11}$), 2.65 (2H, t, $^3J = 7.49$ Hz, H$_{13}$), 2.29 (2H, t, $^3J = 7.6$ Hz, H$_2$), 2.10 (2H, m, H$_8$), 1.80–1.70 (2H, m, H$_{14}$), 1.61 (2H, m, H$_3$), 1.50–1.20 (14H, m, H$_4$–H$_7$, H$_{15}$–H$_{17}$), 0.87 (3H, t, $^3J = 7.12$ Hz, H$_{18}$). ^{13}C NMR (100 MHz, CDCl$_3$): δ 174.91 (C$_1$), 139.23 (C$_9$), 116.70 (C$_{10}$), 52.11 (C$_{19}$), 51.69 (C$_{13}$), 50.94 (C$_{11}$), 34.70, 32.00, 29.81, 29.72, 29.69, 29.59, 29.17, 22.98 (C$_2$, C$_4$–C$_7$, C$_{15}$–C$_{17}$), 28.50 (C$_8$), 22.52 (C$_3$), 23.11 (C$_{14}$), 14.57 (C$_{18}$).

Methyl S-oxide 13-thiaoleate: colorless oil (83% yield). MS (NH$_3$) 70 eV, m/z 331 [M + H]$^+$ (100), 348 [M − H$_2$O]$^+$, 365 [M − NH$_4$ − NH$_3$]$^+$. ^{1}H NMR (400 MHz, CDCl$_3$): δ 5.51–5.44 (1H, m, H$_9$), 5.40–5.30 (1H, m, H$_{10}$), 3.64 (3H, s, H$_{19}$), 2.75–2.55 (4H, m, H$_{12}$, H$_{14}$), 2.49 (2H, dt, $^3J_{H_{10}-H_{11}} = 7.62$ Hz, $^3J_{H_{11}-H_{12}} = 7.61$ Hz, H$_{11}$), 2.28 (2H, t, $^3J = 7.60$ Hz, H$_2$), 2.04 (2H, dt, $^3J_{H_7-H_8} = {}^3J_{H_8-H_9} = 6.60$ Hz,

H$_8$), 1.80–1.70 (2H, m, H$_{15}$), 1.63–1.56 (2H, m, H$_3$), 1.50–1.20 (12H, m, H$_4$–H$_7$, H$_{16}$, H$_{17}$), 0.90 (3H, t, $^3J = 7.10$ Hz, H$_{18}$). ^{13}C NMR (100 MHz, CDCl$_3$): δ 174.88 (C$_1$), 133.37 (C$_9$), 126.29 (C$_{10}$), 53.15 (C$_{14}$), 52.90 (C$_{12}$), 52.10 (C$_{19}$), 34.73 (C$_2$), 31.64, 29.90, 29.75, 29.71, 29.62, 22.95, 22.89 (C$_4$–C$_7$, C$_{15}$–C$_{17}$), 27.86 (C$_8$), 25.48 (C$_3$), 21.31 (C$_{11}$), 14.44 (C$_{18}$).

2.4. Purification of the biosynthesized products

According to previously described procedures [18], methyl S-oxide 12- and 13-thiaoleates were purified by HPLC on a Hypersil BDS C18 column (250 × 10 mm), the optical purities were determined on a Chiralcel OB column and the thiafatty acid methyl esters were analyzed by capillary GC. All chromatographic profiles were compared to the authentic substrates and metabolites standards. The structures of the obtained sulfoxides were confirmed by ^{1}H NMR and MS data.

2.5. Analysis of the methyl 6- and 7-thiastearates and their desaturated products

The 6- and 7-thiastearates and their desaturated metabolites were analysed by GC/MS using a Kratos Concept 1H mass spectrometer interface with a HP 5980 Series 2 GC equipped with a J & W 30 m × 0.21 mm DB-5 capillary column (temperature programmed from 120°C (held 2 min) to 320°C at 10°C/min).

Methyl 6-thiastearate: MS (EI, 70 eV) m/z 316 (M$^+$), 285((M−OCH$_3$)$^+$), 201 ((CH$_3$(CH$_2$)$_{11}$S)$^+$), 115 (M−(CH$_3$(CH$_2$)$_{11}$S)$^+$).

Methyl 7-thiastearate: MS (EI, 70 eV) m/z 316 (M$^+$), 285((M−OCH$_3$)$^+$), 187 ((CH$_3$(CH$_2$)$_{10}$S)$^+$), 129 (M−(CH$_3$(CH$_2$)$_{10}$S)$^+$).

Methyl 6-thiaoleate: (see Ref. [16] for chemical synthesis and full characterization); MS (EI, 70 eV) m/z 314 (M$^+$), 199 ((CH$_3$(CH$_2$)$_7$CH=CH(CH$_2$)$_2$S)$^+$), 115 (M−(CH$_3$(CH$_2$)$_7$CH=CH(CH$_2$)$_2$S)$^+$).

Methyl 7-thiaoleate: MS (EI, 70 eV) m/z 314 (M$^+$), 185 ((CH$_3$(CH$_2$)$_7$CH=CHCH$_2$S)$^+$), 129 (M−(CH$_3$(CH$_2$)$_7$CH=CHCH$_2$S)$^+$).

Methyl 6-thialinoleate: MS (EI, 70 eV) m/z 312 (M^+), 197 $((CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_2S)^+)$, 115 $(M-(CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_2S)^+)$.

Methyl 7-thialinoleate: MS (EI, 70 eV) m/z 312 (M^+), 183 $((CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2S)^+)$, 129 $(M-(CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2S)^+)$.

3. Results and discussion

We first examined the conversion of the 6- and 7-thiastearates, substrates for which the sulfur atom was not at a strategic position for the desaturations. The introduction of the first double bond in cells of *Chlorella* is assumed to be catalyzed by a chloroplastidic stearoyl-ACP $\Delta 9$-desaturase [19] and we assumed that this step should be more efficient if the photosynthetic activity were maximal. Thus, cells were incubated in a SK medium [20] enriched with glucose instead of a phosphate buffer medium. Trial incubation experiments involved addition of 75 mg of each methyl thiastearate to 500 ml of a stirred cellular suspension at mid-logarithmic phase of growth, at 25°C, under 15,000 lx. After 50 h, the cellular fraction was isolated by centrifugation, hydrolyzed and after methylation, the fatty acid methyl esters were analyzed by GC/MS. Under these conditions, the incorporation of the thiastearates appeared to be low (10%) compared with the very efficient incorporation of the thiaoleates (100%) incubated in a phosphate buffer medium [17,18]. However, the 6- and 7-thiastearates were converted to 6- and 7-thiaoleoyl products by the $\Delta 9$-desaturase and to the 6- and 7-thialinoleoyl products by the $\Delta 12$-desaturase (Fig. 1). Both 6-thiaoleate and 6-thialinoleate were obtained in 19% yields relatively to the incorporated thiastearates and we obtained, respectively, 16% and 15% of the 7-thiaoleate and the 7-thialinoleate yield. The corresponding racemic sulfoxides were obtained in less than 4% yield, pointing out the non-occurrence of any other major oxidative transformation of these thiaanalogues. As previously shown by Buist and Behrouzian [12] with a functional plant oleate $\Delta 12$-desaturase-contain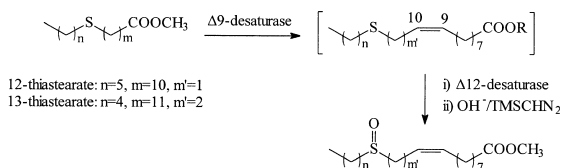ing *S. cerevisiae* strain, saturated thiafatty acids can be processed by the $\Delta 9$-desaturase before being desaturated by the $\Delta 12$-desaturase. In principle, the incubation of the 12- and 13-thiastearates should lead to highly enantioselective sulfoxidations at positions 12 and 13.

Methyl 12-thiastearate was then incubated for 50 h in the same conditions as previously described. Cellular fraction was hydrolyzed and methylated under mild conditions [18] in order to avoid racemization. Methyl 12-thiastearate and the resulting 12-thiaoleate were quantified by analysis on a capillary GC, and the corresponding biosynthetic sulfoxides were purified and analyzed by HPLC, on a preparative Hypersil BDS C18 column. The structure of the methyl 12-thiaoleate S-oxide was confirmed by ^{1}H NMR and MS data. Only 10% of the 12-thiastearate appeared to be incorporated into the cells and 30% were desaturated to 12-thiaoleoyl product. Then it was quantitatively oxidized to the corresponding 12-thiaoleate S-oxide and 5% of saturated racemic sulfoxide (likely to come from autooxidation) was also detected in the cells (Fig. 2). Unfortunately, the optical purity of the 12-thiaoleate S-oxide determined by HPLC on a ChiralCel OB column revealed a low ee of 15%. The results could be related to the existence of non-selective oxidative processes we previously showed with the incubations of 12- and 13-thiaoleates [18] or considering the allylic position of the sulfoxide, one could argue a likely racemisation process. Thus, we decided to carry out the incubations of the 12- and 13-thiastearates in a 0.2 M phosphate buffer medium as previously described [18], in order to increase the incorporation of the exogenous thiafatty acid methyl esters.

Chlorella cells in the mid-logarithmic phase were incubated in a pH = 7.4 phosphate buffer with the methyl 12-thiastearate at the concentration of 0.2 mg/ml of cellular suspension, at 25°C and under 15,000 lx. After 72 h, the cellular fraction was isolated, hydrolysed and methylated [18]. Thiafatty acids and metabolites were analyzed respectively by GC and HPLC, as described above. In these conditions, we found that the methyl 12-thiastearate was incorporated efficiently into the cells (95% of the starting material was recovered in the cell fraction) and further desaturated and oxidized to 12-thiaoleate S-oxide with an overall 6% yield. The intermediate 12-thiaoleate was not detected. The optical purity of
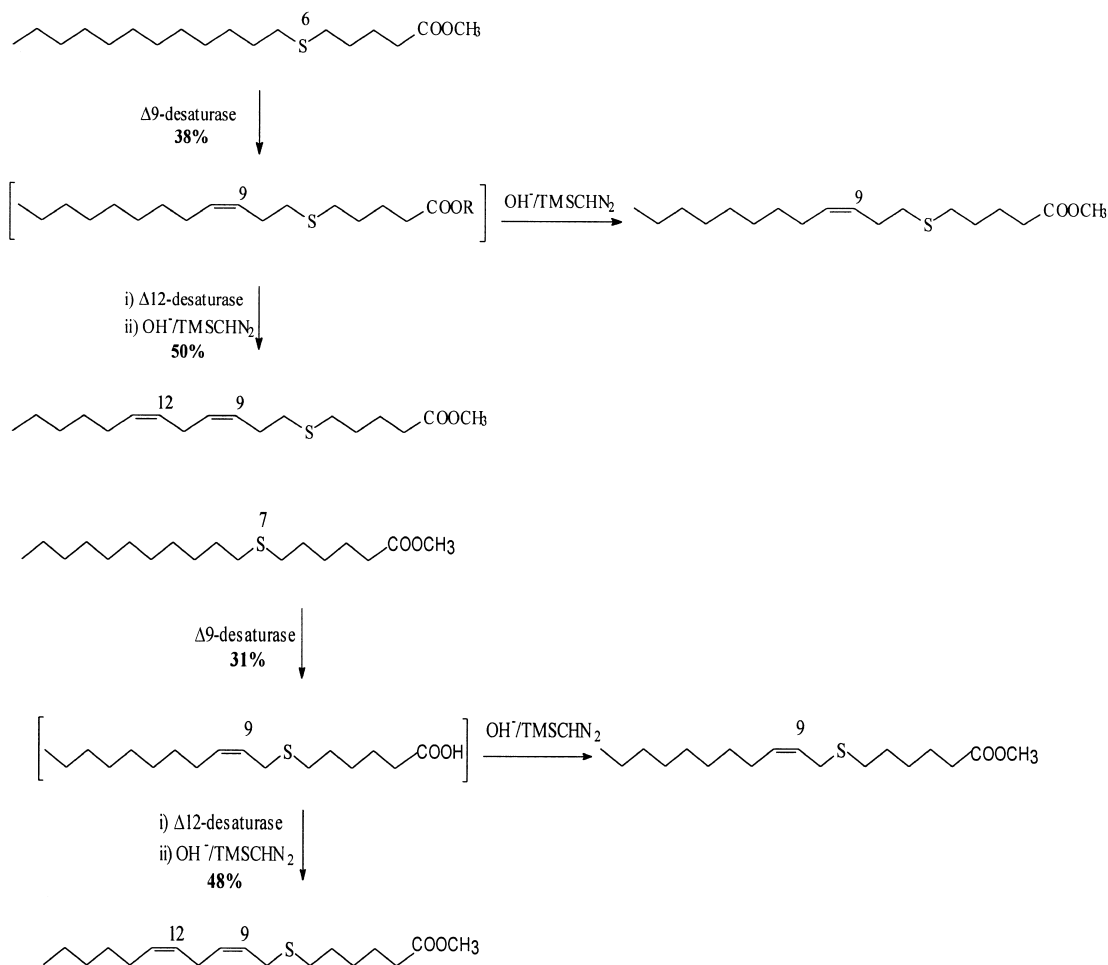
Fig. 1. Desaturation pathway of the 6- and 7-thiastearates in *C. vulgaris*.

the 12-thiaoleate S-oxide, determined by chiral HPLC, revealed a very high ee of 95%, in comparison to the racemic synthesized sulfoxide. Then, the 13-thiastearate was incubated in the same conditions for 120 h; 80% of the substrate were incorporated into the cells and 8% were oxidized to the 13-thiaoleate S-oxide. The optical purity of this sulfoxide was determined to be 90%.

In order to evaluate the chemical and/or atmospheric autooxidation of the thiastearates, control experiments were carried out either without algae or algae preheated at 100°C for 10 min. The relative amount of sulfoxides (2% yield) recovered from

12-thiastearate: n=5, m=10, m'=1
13-thiastearate: n=4, m=11, m'=2

Fig. 2. Biotransformation of the 12- and 13-thiastearates by *C. vulgaris* desaturation system.

these experiments revealed a very low level of auto-oxidation.

We have thus greatly improved the enantioselectivity of the sulfoxidation when saturated instead of monounsaturated thiafatty acid methyl esters were supplied to growing cells of *C. vulgaris*, incubated in a phosphate buffer medium. When the sulfur atom was not occupying a strategic position for the desaturation, methyl thiastearates were successively desaturated at C9–C10 and C12–C13. The 12- and 13-thiastearates were first desaturated at C9–C10 and then converted to the corresponding thiaoleate S-oxides with high enantioselectivity. So, we are able to assert a very highly selective transfer of oxygen to the sulfur of the thiaoleates by the Δ12-desaturase when the desaturation is impeded, confirming the monooxygenase behavior of this membrane-bound desaturase. In this way, we succeeded in meeting the challenge of synthesizing optically active sulfoxides with quasi-symmetrical structures. The determination of the absolute configuration of these original chiral sulfoxides would be of great importance for elucidating the mechanism of the Δ12-desaturase. In order to apply the methodology, we have developed for the stereochemical analysis of dialkylsulfoxides [21], the chemical synthesis of the models that mimic the allylic and homoallylic positions of the new thiaoleate S-oxides are currently underway in our laboratory.

Acknowledgements

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