Pergamon

Tetrahedron 55 (1999) 3595–3604

TETRAHEDRON

Studies Related to *in vivo* C-H Activation: Synthesis and Influence of 8,8- and 11,11-dimethyl oleic and 11,11-dimethyl linoleic Acids on Δ12-desaturation of *C. sorokiniana*.

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Received 3 August 1998; accepted 28 September 1998

Abstract: The synthesis of three acids was achieved using the Wittig reaction in order to study their *in vivo* influence on the Δ12-desaturase of *Chlorella sorokiniana*. It was shown that the introduction of two methyls near the double bond prevent the desaturation of these exogenous acids while they seem to be accurately incorporated. This functionality could be of interest for the design of new thiaoleic acids as probes of the different oxidation processes.

INTRODUCTION

The genesis of Gif chemistry[1] was based on the assumption that when oxygen began to be produced by photosynthesis in the earliest times on Earth, a synthetic pathway might exist between the oxidation of metallic iron and of saturated hydrocarbons. During the evolution, the carbon-hydrogen bond activation of saturated chains remained a crucial challenge for nature. Moreover, from the knowledge of the involved pathways, biomimetic oxidation studies have been extensively reported for the direct oxidation of various substrates.

Using iron as metal, the oxidative natural pathways can be divided into two groups according to the nature of the active site of the implied enzyme. For some of them, such as the well known cytochromes P450,[2,3] the iron atom was stabilized by a porphyrine moiety: the Fe(III) - protoporphyrine (IX) complex was shown to be the common prosthetic group for all the cytochrome P450 type enzymes.[4] Other metalloproteins feature dinuclear iron species multiply bridged by carboxylato residues and, in stable or resting states of the protein, by a water-derived ligand such as hydroxo or oxo.[5] Although we had identified the ribonucleotide reductase (RNR R1)[6] as the first member of the class, it appeared in fact that the methane monooxygenase (MMO) was the first true oxygenase of this class.[7] Extensive studies have been carried out due to the avaibility of pure samples of MMO, allowing, for example, spectroscopic,[8-10] crystallographic[11] and kinetic[12] investigations of their structures and of the oxidation mechanism. More recently, other enzymes have been reported to be similar to the MMO, such as toluene 2- (T2MO)[13] and 4- (T4MO)[14] monooxygenases, phenol[15] and alkane[16] hydroxylases and stearoyl desaturase.[17] This latter enzyme generates a Z-9,10 double bond into the saturated hydrocarbon

0040-4020/99/$ - see front matter
PII: S0040-4020(98)01166-1

chain of stearic acid. In this specific case, despite the requirement of O_2, no oxygen atom was found in the product of the reaction.[18] Nevertheless, the active site structure and many informations about the reaction reveal strong analogies with the other "MMO-like" enzymes.[19] The structural similarities among the diiron site of MMOH, Δ9 desaturase and RNR R2 have led to a common mechanism for the oxygen activation, which is the first step of the respective oxidation reactions catalyzed by each enzyme.[20] The Δ9 desaturase is a soluble enzyme. The other ones such as Δ12, Δ15, ... desaturases are all membranar enzymes which are not structurally characterized. Among them, the Δ12 desaturase is responsible for the biosynthesis of linoleic acid (C18:2, 9c, 12c) from oleic acid (C18:1, 9c). The reaction consists in the regio and stereospecific production of a Z,Z-bis vinyl methylene system, which cannot be obtained in one step by chemical reactions. More information about the mechanism of this biotransformation could be of interest for the design of biomimetic catalysts for this purpose. Despite their primary structural differences, the membranar Δ12 and the soluble Δ9 desaturases present significant analogies of behaviour such as the inhibition by iron complexing reagents[17,21] and the *Z*-stereospecificity of the desaturation reaction.[22,23]

Some years ago, we chose an *in vivo* approach of the Δ12 desaturase, using a green microalgae, *Chlorella sorokiniana*, as biological support for substrate-activity type experiments. Our strategy consisted first in the synthesis of modified fatty acids. Then, the evaluation of their influence on the *in vivo* desaturation of labelled [1-^{14}C] oleic acid and their metabolism by the cells are studied. Recently, we have reported the synthesis of thiaoleic acids[24] in which sulphur was used as an oxidative probe. A similar approach was reported for a whole cell *Saccharomyces cerevisiae* Δ9 desaturation system.[25,26]

$CH_3(CH_2)_nS(CH_2)_mCH{=}CH(CH_2)_7CO_2Me$

1a: m=1;n=5
1b: m=2;n=4
1c: m=3;n=3
1d: m=4;n=2
1e: m=5;n=1

During our investigations, we found that the S13-oleic acid **1b** had a dramatic effect on the activity of Δ12-desaturase. The yield of desaturation appeared to be very low in the presence of **1b** and we could observe an increase of the C18:1, 9c and a decrease of the C18:2, 9c, 12c acids in the phospholipids.[27] Moreover, the corresponding sulfoxide, **2b**, was detected in the cellular fraction by GC/MS analysis.

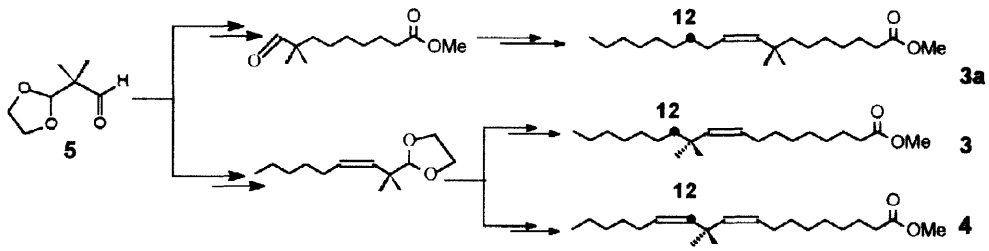
This information indicated a direct interaction between the acid and the desaturase. Actually, in the case of the Δ9-desaturase of yeast, *Saccharomyces cerevisiae*, it was shown that the presence of a sulphur atom at the 9- and 10- positions led to oxygen transfer and then to the biosynthesis of chiral 9- and 10-sulfoxides.[26] The most efficient sulfoxidation occurred at position 9 and demonstration was made that the enantioselectivity of the reaction to the R stereoisomer was very high. These results matched those of all Δ9-desaturases previously described:[28] in all cases, abstraction of pro-R hydrogens at position 9 was found. During our investigations, we have performed incubations of **1b** with whole cells of the microalgae and we followed the formation and the distribution of the sulfoxide **2b** into the different lipids classes. The sulfoxidation was found racemic except in phospholipids where a 30% ee could be measured after less than one hour incubation time (to be published). Then, the e.e. rapidly decreased suggesting a racemization process or another non stereoselective oxidation reaction of **1b**. These results led us to prepare new probes in order to evaluate the ratio between the different pathways.

Previously reported experiments[29] showed us that the *cis* double bound between C-9 and C-10 was essential for the interaction of the substrate in the active site of oleoyl desaturase. Moreover, in the case of some

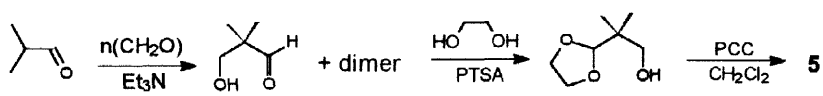
mono-methylated oleic acids,[30] the incorporation into the cells was observed but the acids seemed to have no influence on the desaturation rate. After collecting the above information, we decided to synthesise new acids with hindered double bonds ,assuming that the steric hindrance at the double bond could prevent the Δ12-desaturation. Three dimethyl acids were prepared to evaluate the effect of this kind of synthetic analogues on the algae metabolism: the two dimethyl-8,8 and -11,11-oleic acids (**3a** and **3b**) and the dimethyl-11,11-linoleic acid **4**. This paper presents the synthesis of the different substrates and the information obtained about the *in vivo* behaviour of the modified fatty acids.

RESULTS AND DISCUSSION

The three target molecules were prepared from the same precursor **5** [31] *via* two successive Wittig reactions (scheme 1):

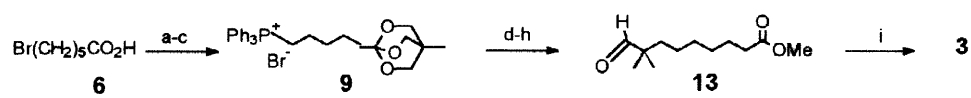


This half masked dialdehyde **5** was obtained in three steps *via* known procedures [31-34] with a 45% overall yield (scheme 2).



Synthesis of the *gem*-8,8-dimethyl acid 3a.

The *gem*-8,8 acid was synthesised *via* the key aldehyde **13** which was prepared from **5** and the commercial bromo-6-hexanoic acid **6** (scheme 3).



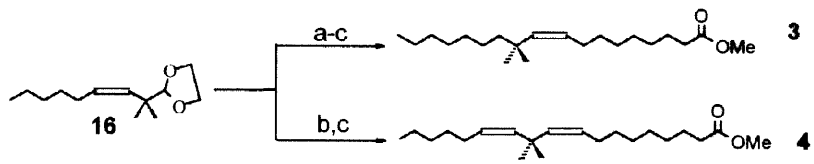
Reagents: (a) 3-hydroxymethyl-3-methyloxetane/DCC/DMAP (**7**, 85%). (b) BF_3:Et_2O (**8**, 94%). (c) Ph_3P/CH_3CN (**9**, 90%). (d) BuLi/DMPU/THF/**5** (**10**, 90%). (e) PPTS / MeOH. (f) MeOH / K_2CO_3 (**11**, 100%). (g) H_2 / Pd(C) (**12**, 100%). (h) HCl / H_2O / THF (**13**, 67%). (i) BuLi, THF/DMPU, $CH_3(CH_2)_8P^+Ph_3,Br^-$ (65%).

The acid was protected as orthoester by esterification with 3-hydroxymethyl-3-methyloxetane and dicyclohexyl carbodiimide followed by a treatment by boron trifluoride which catalyzes the rearrangement of the oxetanyl ester.[35,36] After the reaction of the protected bromide with Ph_3P was completed, the phosphonium salt **9** was isolated in a 70% overall yield from **6**. Then, the Wittig reaction between the aldehyde **5** and the phosphonium salt **9** led to the alkene **10** (90%). Acidic cleavage of the orthoester by PPTS in methanol followed by transesterification with methanol in the presence of potassium carbonate [36] gave the methyl ester **11**. Its

hydrogenation (12) and acidic deprotection led to the aldehyde **13**, which was used in a second Wittig reaction with $CH_3(CH_2)_8P^+Ph_3,Br^-$ to afford **3a** in a 65% yield. No *E* isomer was observed when n-butyllithium was added in a mixture THF/DMPU 6:1. The *Z* stereochemistry of the newly created double bond was proved by ^{1}H-NMR ($^3J(CH{=}CH)$ = 11.7 Hz).

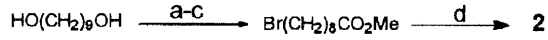
Synthesis of the *gem*-11,11-dimethyl acids 3b and 4.

They were prepared from the same protected aldehyde **16** which after deprotection was conveniently obtained from a Wittig reaction between the aldehyde **5** and the n-hexyl triphenylphosphonium bromide **15**. The second moiety of the target was introduced with a second Wittig reaction (scheme 4):



Reagents: (a) H_2 /Pd/C in MeOH (**17**, 100%). (b) HCl / H_2O / acetone. (c) BuLi / THF / DMPU / $Ph_3P^+(CH_2)_8CO_2Me,Br^-$ **20**.

The adequate phosphonium salt **20** was obtained from the commercially available nonane-1,9-diol using well known procedures (scheme 5).



Reagents: (a) HBr /C_6H_{12} (77%). (b) Jones' reagent / acetone (100%). (c) MeOH / H^+ (100%). (d) Ph_3P/CH_3CN (**20**, 88%).

As previously reported, the acids were stored as methyl esters and were saponified just before biological tests.[24]

Biological experiments

Biological experiments were carried out using the previously described procedures.[27] We first studied the influence of the hindered models (**3** and **4**) on the desaturation process. It was particularly important to know if this new kind of molecules could disrupt the algal metabolism. First, we evaluated the potential inhibitor effect of **3a**, **3b** and **4** on the *in vivo* oleoyl desaturation. Cells in the middle-logarithmic phase of growth were incubated with [1-^{14}C] oleic acid and an added exogenous free fatty acid (unlabelled oleic acid as reference, **3a**, **3b** or **4**). The Δ12-desaturase activity was evaluated from the rate of production of [1-^{14}C] linoleic acid.[24,27] Each dimethylated acid showed poor inhibitor effect on the desaturation of the labelled oleic acid (65-68% of desaturation *vs* 50% in the case of oleic acid). This behaviour does not seem to be dependant on the position (8,8 or 11,11) of the two CH_3 near the 9c double bond. The next step was the study of the incorporation and the likely metabolisation of the substrates in the cells of *Chlorella*. After a 3-hour period of incubation in standard conditions (25°C, 15000 lux, pH = 7.4 in a phosphate buffer),[27] we found that the exogenous acids were totally incorporated into the algal cells. Extraction and GLC analysis of the lipids led to a high yield recovery of the untransformed exogenous substrates: no product of desaturation was detected. Moreover, using the same culture conditions, the distribution of the modified acids into the lipids of *C. sorokiniana* was determined.[27] The three acids were mainly incorporated into neutral lipids (NL) and , to a lesser extent, into the phospholipids (PL). From these experiments, we concluded that all of them could activate the acyltransferases and then were

incorporated into lipids. The two oleic acid analogues, **3a** and **3b**, were seen to be mainly stored in the neutral lipids only. But, the 8,8-dimethylated oleic acid **3a** proved to be the less disturbing. Actually, we found that, in this case, the endogenous fatty acids distribution pattern was not modified while the 11,11-dimethyl oleic acid **3b** tended to induce a significant endogenous fatty acids decrease in the membranar lipids. No significant influence of these acids on the cells metabolism could be detected. Then, the introduction of this modification on the lipophilic chain seems to prevent the *in vivo* desaturation of the models.

CONCLUSION

We have prepared three unsaturated fatty acids with hindered double bonds. We have shown that they are incorporated into the cells of *C. sorokiniana*. Moreover, they do not induce metabolic modifications and are not desaturated to linoleic or linolenic analogues. These results lead us to design a new probe derived from the S13-oleic acid (**1b**) with two methyl groups at the 8 position.

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The aim of this new target (**21**) is to obtain a probe of oxidation to evaluate the ratio of the oxidation process related to Δ12 *vs* other *in vivo* oxidative reactions.

EXPERIMENTAL

General: ^{1}H, $^{13}C\{^{1}H\}$ and $^{31}P\{^{1}H\}$ NMR spectra and other classical technics (COSY, HMQC, DEPT, ...) were recorded on a Brucker ARX 400 spectrometer. The radioactivity incorporated was counted in Insta-Fluor scintillation liquid (Hewlett-Packard France, Les Ulis, France) with a Packard 1600 TR (correction for quenching : t-SIE/AEC) apparatus. Analytical GLC were conducted on a Carlo Erba 4130 equiped with a capillary Alltech Column (25x0.25mm; RSL-150 bonded FSOT, PDMS). For preparative chromatography, silica gel GO Merck (0.040-0.063 mm) was used and mixtures of solvents are given in volumetric ratio. Thin layer chromatography was performed on silica gel 60 F_{254} (Merck), and the spots were revealed by phosphomolybdic acid. All solvents were purified, when necessary, by standard methods.[37] Organic layers were dried over $MgSO_4$. All melting points are uncorrected. **5** was prepared according to literature procedures.[31-34] All the triphenylphosphonium salts were obtained from the corresponding alkyl bromide and triphenylphosphine in acetonitrile (reflux).

Preparation of **13**

Phosphonium salt **9**

The commercial 6-bromo-hexanoic acid was easily converted to the orthoester **8** *via* known procedures.[35,36] The corresponding phosphonium bromide **9** was obtained as a white solid (m.p. = 222°C). Yield = 90%; *Rf* = 0.73 (MeOH / CH_2Cl_2, 2:8). ^{1}H NMR ($CDCl_3$) δ: 7.85-7.65 (m, 15 H, aromatic H) ; 3.85 (s, 6 H, CH_2O) ; 3.66 (m, 2 H, CH_2P) ; 1.70-1.53 (m, 6 H, CH_2) ; 1.47-1.34 (m, 2 H, CH_2) ; 0.78 (s, 3H, CH_3). - ^{13}C NMR ($CDCl_3$) δ: 135.05 (d, J = 3 Hz, Cp) ; 133.53 (d, J = 10.3 Hz, C_o) ; 130.49 (d, J = 12.6 Hz, C_m) ; 118.08 (d, J = 85.8 Hz, C_q) ; 108.64 (s, CO_3) ; 72.44 (s, CH_2O) ; 35.78 (s, CH_2CO_3) ; 30.10 (s, CCH_3) ; 29.83

(d, J = 15.6 Hz, CH_2) ; 22.63 (d, J = 1.1 Hz, CH_2) ; 22.59 (d, J = 49.2 Hz, CH_2P) ; 22.32 (d, J = 4.2 Hz, CH_2CH_2P) ; 14.45 (s, CH_3). - ^{31}P NMR ($CDCl_3$) δ: 21.29.

General procedure of the Wittig reaction: preparation of **10**.

6.8g (12.5 mmol, 1.2 eq) of **9** were dried under vacuum (1 mbar, 3h). At -20°C, under nitrogen (N_2), to a suspension of **9** in THF (30mL), 7.9 mL of a commercial solution of BuLi (1.6M in hexanes, 12.6 mmol, 1.3 eq) were dropwise added. After 30 min at -20°C, the mixture was cooled at -78°C. DMPU (7 mL) and the semi protected bis aldehyde **5** (1.5g, 10.4 mmol) were then added. After 2h at -78°C, the mixture was allowed to warm to room temperature (RT). After extraction (diethylether (E)), the residue was chromatographed (petroleum ether (PE)) to afford **10** as a yellow oil. Yield = 90%; Rf = 0.37 (E/PE, 1:1). ^1H NMR ($CDCl_3$) δ: 5.35 (d, J = 12.2 Hz, 1 H, C*H*=CHCH$_2$) ; 5.31 (dt, J = 12.2 x 7.1 Hz, 1 H, CH=C*H*CH$_2$) ; 4.65 (s, 1 H, OC*H*O) ; 3.98-3.83 (m, 4 H, OCH_2CH_2O) ; 3.89 (s, 6 H, OCH_2C) ; 2.18-2.10 (m, 2 H, =CCH_2) ; 1.67-1.60 (m, 2 H, CH_2CO$_3$) ; 1.52-1.30 (m, 4 H, CH$_2$) ; 1.13 (s, 6 H, C(CH_3)$_2$) ; 0.80 (s, J = 6.7 Hz, CH_3). - ^{13}C NMR ($CDCl_3$) δ: 133.33 (*C*H=CHCH$_2$) ; 131.29 (CH=*C*HCH$_2$) ; 109.73 (OCO) ; 108.88 (CO$_3$) ; 72.45 (OCH$_2$C) ; 65.29 (OCH$_2$CH$_2$O) ; 40.10 (CH$_3$*C*CH=) ; 36.50 (*C*H$_2$CO$_3$) ; 30.11 (OCH$_2$*C*) ; 30.00 (CH$_2$) ; 28.75 (*C*H$_2$CH=) ; 23.25 (C(*C*H$_3$)$_2$) ; 22.96 (*C*H$_2$) ; 14.48 (*C*H$_3$).

Deprotection of the orthoester **10**

Under N_2, 2.0 g (6.1 mmol) of **10** was stirring with 1.4 eq of pyridinium p-toluene sulfonate (PPTS) in 12.5 mL of dry methanol. Three hours later, 0.25g (1.77 mmol) of K_2CO_3 was added before a new stirring period of 7 h. Then, after neutralization (oxalic acid 0.5M) and extraction (E), the residue was chromatographed (PE) to afford 1.55g of **11** as a colourless oil. Yield = 100%; Rf = 0.53 (E/PE, 1:1). ^1H NMR ($CDCl_3$) δ: 5.31 (d, J = 12.2, 1 H, (CH$_3$)$_2$CC*H*=) ; 5.25 (dt, J = 12.2 x 6.6, 1 H, =*H*CCH$_2$) ; 4.59 (s, 1 H, OC*H*O) ; 3.93-3.74 (m, 4 H, OCH_2) ; 3.59 (s, 3 H, OCH_3) ; 2.25 (t, J = 7.1, 2 H, CH_2CO$_2$) ; 2,14 (dt, J = 6.6 x 7.1, 2 H, =HCCH_2) ; 1.58 (quint, J = 7.6, 2 H, CH_2CH$_2$CO$_2$) ; 1.39-1.28 (m, 2 H, CH$_2$) ; 1.07 (s, 6 H, (CH_3)$_2$C). - ^{13}C NMR ($CDCl_3$) δ: 174.21 (C=O) ; 133.85 ((CH$_3$)$_2$*C*CH=) ; 130.89 (=H*C*CH$_2$) ; 109.83 (O*C*HO) ; 65.40 (OCH$_2$) ; 51.48 (OMe) ; 40.24 ((CH$_3$)$_2$*C*CH=) ; 33.98 (*C*H$_2$CO$_2$) ; 29.61 (*C*H$_2$) ; 28.49 (=HC*C*H$_2$) ; 24.65 (*C*H$_2$CH$_2$CO$_2$) ; 23.36 ((*C*H$_3$)$_2$C).

Catalytic hydrogenation of **11**:

Under H_2 (1180 mbar), 1.5g (5.8 mmol) of **11** in 30 ml of PE and 0.2g of palladium on carbon (10%) were stirred at RT until stoechiometric absorption. After filtration and washing (E), **12** was obtained as a colourless oil. Yield = 100%; Rf = 0.55 (E/PE, 1:1). ^1H NMR ($CDCl_3$) δ: 4.54 (s, 1 H, OC*H*O) ; 3.96-3.80 (m, 4 H, OCH$_2$) ; 3.67 (s, 3 H, CH_3) ; 2.30 (t, J = 6.7 Hz, CH_2C=O) ; 1.62 (t, J = 7.1 Hz, CH_2CH$_2$C=O) ; 1.40-1.15 (m, 8 H, CH$_2$) 0.88 (s, 6 H, C(CH_3)$_2$). - ^{13}C NMR ($CDCl_3$) δ: 174.45 (C=O) ; 110.03 (OCO) ; 65.26 (OCH$_2$) ; 51.51 (OMe) ; 37.71 (CH$_3$*C*CH$_2$) ; 37.05 (CH$_3$*C*CH$_2$) ; 34.15 (*C*H$_2$C=O) ; 30.28, 29.20 (*C*H$_2$) ; 25.01 (*C*H$_2$CH$_2$C=O) ; 23.41 (*C*H$_2$) ; 21.39 (*C*H$_3$).

Preparation of **13**:

Under N_2, at 0°C, 62 ml of aqueous HCl (5%, w/w) were slowly added to a solution of 1.5g of **12** in 62 ml of THF. After stirring overnight at RT, extraction (E) and several washings (water) afforded a crude oil which was chromatographied (PE). **13** was obtained as a colourless oil. Yield = 67%; Rf = 0.56 (E/PE, 1:1). ^1H NMR ($CDCl_3$) δ: 9.44 (s, 1 H, *H*C=O) ; 3.67 (s, 3 H, OMe) ; 2.30 (t, J = 7.6, 2 H, CH_2CO$_2$) ; 1.59 (quint, J = 7.6, 2 H, CH_2CH$_2$CO$_2$) ; 1.50-1.15 (m, 8H, CH_2) ; 1.04 (s, 6 H, CH$_3$) . - ^{13}C NMR ($CDCl_3$): δ 206.59 (*C*H=O) ;

174.31 (CO_2) ; 51.54 (OMe) ; 45.87 ($CCH{=}O$) ; 37.26 ($CH_2CCH{=}O$) ; 34.08 (CH_2CO_2) ; 29.90 and 29.01 (CH_2) ; 24.90 ($CH_2CH_2CO_2$) ; 24.17 (CH_2) ; 21.35 (CH_3).

Synthesis of the protected aldehyde **16**

As described for **10**, the reaction of **5** (1.40g, 9.7 mmol) and the hexyl triphenylphosphonium bromide (5g, 11.7 mmol) led to 2.05g of **16** as a yellow oil. Yield = 100%; *Rf* = 0.75 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$) δ: 5.40-5.34 (m, 2 H, $CH{=}$) ; 4.68 (s, 1 H, $OCHO$) ; 4.00-3.85 (m, 4 H, OCH_2) ; 2.23-2.15 (m, 2 H, $=CCH_2$) ; 1.43-1.23 (m, 6 H, CH_2) ; 1.16 (s, 6 H, $C(CH_3)_2$) ; 0.88 (t, J = 6.7 Hz, CH_3). ^{13}C NMR ($CDCl_3$) d: 133.37, 131.77 ($C{=}$) ; 109.95 (OCO) ; 65.45 (OCH_2) ; 40.25 (CH_3CCH_2) ; 31.67, 29.93 (CH_2) ; 28.96 ($CH_2CH{=}$) ; 23.37 ($C(CH_3)_2$) ; 22.65 (CH_2CH_3) ; 14.10 (CH_3).

Hydrogenation of **16**

Under the same conditions as for the preparation of **12**, **16** was reduced to afford in a quantitative yield, **17** as a colourless oil. *Rf* = 0.75 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$) d: 4.55 (s, 1 H, $OCHO$) ; 3.97-3.80 (m, 4 H, OCH_2) ; 1.36-1.17 (m, 12 H, CH_2) ; 0.90 (s, 6 H, $C(CH_3)_2$) ; 0.87 (t, J = 7.1 Hz, CH_3). - ^{13}C NMR ($CDCl_3$) d: 110.12 (OCO) ; 65.29 (OCH_2) ; 37.85 (CH_3CCH_2) ; 37.10 ($CH_3CCH_2CH_2$) ; 32.02, 30.72, 29.45, 23.63, 22.78 (CH_2) ; 21.41 ($C(CH_3)_2$) ; 14.10 (CH_3).

Deacetalisation of **16**

Using the same procedure as for **13**, **16** was deprotected to afford the aldehyde **19** as a colourless oil. Yield = 93%. *Rf* = 0.75 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$) d: 9.50 (s, 1 H, $HC{=}O$) ; 5.51 (dt, J = 11.2 x 7.6 Hz, 1 H, $=CHCH_2$) ; 5.27 (dtd, J = 11.2 x 7.6 x 2.1 Hz, 1 H, $CH{=}CHCH_2$) ; 1.38-1.22 (m, 6 H, CH_2) ; 1.29 (s, 6 H, $C(CH_3)_2$) ; 0.87 (t, J = 7.1 Hz, CH_3). - ^{13}C NMR ($CDCl_3$) d: 203.37 ($HC{=}O$) ; 134.88 ($HC{=}CHCH_2$) ; 131.37 ($=CHCH_2$) ; 47.69 (CH_3CCH_2) ; 31.49 ($CH_3CCH_2CH_2$) ; 29.45, 28.58 (CH_2) ; 23.26 ($C(CH_3)_2$) ; 28.58 (CH_2) ; 14.06 (CH_3).

Deacetalisation of **17**

Using the same procedure as for **13**, **17** was deprotected to afford the aldehyde **18** as a colourless oil. Yield = 96%. *Rf* = 0.70 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$) d: 9.45 ($HC{=}O$) ; 1.35-1.16 (m, 12 H, CH_2) ; 1.04 (s, 6 H, $C(CH_3)_2$) ; 0.88 (t, J = 7.6 Hz, CH_3). - ^{13}C NMR ($CDCl_3$) d: 206.56 ($HC{=}O$) ; 45.88 (CH_3C) ; 37.44 (CH_3CCH_2) ; 31.87 ($CH_3CCH_2CH_2$) ; 30.28, 29.23, 24.37, 22.73 (CH_2) ; 21.34 ($C(CH_3)_2$) ; 14.13 (CH_3).

Synthesis of the methyl dimethyl-8,8-oleate **3a**

In the same conditions as for **10**, a Wittig reaction was performed between **13** and the nonyltriphenyloctyl phosphonium bromide. Yield = 65%; *Rf* = 0.75 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$): d 5.19 (d, J = 11.7, 1 H, $C(CH_3)_2CH{=}$); 5.17 (dt, J = 11.7 x 7.6, 1 H, $CH_2CH{=}$); 3.67 (s, 3 H, OCH_3); 2.30 (t, J = 7.6, 2 H, $CH_2C{=}O$); 2.11 (m, 2 H, $CH_2C{=}$); 1.70-1.55 (m, 4 H, CH_2); 1.40-1.15 (m, 18 H, CH_2); 1.06 (s, 6 H, $C(CH_3)_2$); 0.87 (t, J = 7.6, 3 H, CH_3). - ^{13}C NMR ($CDCl_3$) d 174.44 ($C{=}O$); 138.48 ($C(CH_3)_2CH{=}$); 129.71 ($CH_2CH{=}$); 51.53 (OCH_3); 44.28 ($CH_2C(CH_3)_2$); 36.35 ($C(CH_3)_2$); 34.21 ($CH_2C{=}O$); 31.99 ($CH_2CH_2CH_3$); 30.40, 30.21, 29.65, 29.52, 29.41 (CH_2); 29.15 ($C(CH_3)_2$); 28.54 ($CH_2C{=}$); 25.07, 24.76 (CH_2); 22.78 (CH_2CH_3); 14.21 (CH_3). MS; *m/z* (%):324 (1.4) [M+], 293 (3.7) [M+ - OCH_3], 185 (28.7) [M+ - $CH{=}CH(CH_2)_7CH_3$], 181 (18.9) [M+ - $(CH_2)_6CO_2CH_3$]. $C_{21}H_{40}O_2$: Calcd. C 77.72, H 12.42 (M = 324.3028); Found C 77.70, H 12.45 (M = 324.3026).

Synthesis of the methyl dimethyl-11,11-linoleate **4**

In the same conditions as for **10**, a Wittig reaction was performed between **19** and the required phosphonium bromide **20**. Yield = 83%; *Rf* = 0.72 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$) d: 5.42 (d, *J* = 11.2 Hz, 1 H, $((CH_3)_2CCH=)$; 5.41 (d, *J* = 11.2 Hz, 1 H, $((CH_3)_2CCH=)$; 5.09 (dt, *J* = 11.2 x 7.1 Hz, 1 H, $CH_2CH=$) ;5.08 (dt, *J* = 11.2 x 7.1 Hz, 1 H, $CH_2CH=$) ; 3.59 (s, 3 H, OCH_3); 2.23 (t, *J* = 7.1 Hz, 2 H, $CH_2C=O$); 1.96-1.90 (m, 4 H, $CH_2C=$) ; 1.54 (q, *J* = 7.1 Hz, 2 H, $CH_2CH_2C=O$); 1.28-1.15 (m, 14 H, CH_2) ; 1.10 (s, 6 H, $C(CH_3)_2$) ; 0.81 (t, *J* = 7.1 Hz, 3 H, CH_3). ^{13}C NMR ($CDCl_3$), d 174.40 (*C*=O); 139.28, 139.16 ($C(CH_3)_2CH=$); 129.82, 129.65 ($CH_2CH=$); 51.51 (OCH_3); 36.37 ($C(CH_3)_2$); 34.18 ($CH_2C=O$); 31.81 ($CH_2CH_2CH_3$); 31.33 ($C(CH_3)_2$); 29.61, 29.38, 29.36, 29.27, 29.19 (CH_2); 25.02 ($CH_2CH_2C=O$); 22.68 (CH_2CH_3); 14.15 (CH_3). Anal. Calcd. for $C_{21}H_{38}O_2$: C, 78.20; H, 11.87. Found C 78.30, H 12.00.

Synthesis of the methyl dimethyl-11,11-oleate **3b**

In the same conditions as for **10**, a Wittig reaction was performed between **18** and the required phosphonium bromide **20**. Yield = 92%; *Rf* = 0.76 (E/PE, 1:1). ^{1}H NMR ($CDCl_3$) d : 5.14 (d, *J* = 11.7 Hz, 1 H, $C(CH_3)_2CH=$); 5.09 (dt, *J* = 11.7 x 7.2 Hz, 1 H, $CH_2CH=$); 3.67 (s, 3 H, OCH_3), 2.30 (t, *J* = 7.6 Hz, 2 H, $CH_2C=O$), 2.12 (m, 2 H, $CH_2C=$), 1.55 (quint, *J* = 7.1 Hz, 2 H, $CH_2CH_2C=O$), 1.30-1.10 (m, 20 H, CH_2), 0.99 (s, 6 H, $C(CH_3)_2$), 0.81 (t, J = 7.1 Hz, 3 H, CH_3). - ^{13}C NMR ($CDCl_3$): d = 174.44 (*C*=O), 138.48 ($C(CH_3)_2CH=$), 129.71 ($CH_2CH=$), 51.53 (OCH_3), 44.28 ($CH_2C(CH_3)_2$), 36.35 ($C(CH_3)_2$), 34.21 ($CH_2C=O$), 32.02 ($CH_2CH_2CH_3$), 30.40, 30.21, 29.65, 29.52, 29.41, 29.30 (CH_2), 29.15 ($C(CH_3)_2$), 28.54 ($CH_2C=$), 25.07, 24.76 (CH_2), 22.77 (CH_2CH_3), 14.20 (CH_3). MS; *m/z* (%):324 (0.2) [M+], 225 (14.2) [M+ - $(CH_2)_6CH_3$], 193 (13.2) [M+ - $(CH_2)_6CH_3$ - CH_3]. $C_{21}H_{40}O_2$: Calcd. C 77.72, H 12.42 (M = 324.3028); Found C 77.59, H 12.31 (M = 324.3018).

Culture conditions

Chlorella vulgaris (strain 211/8K) was purchased from CCAP (Cambridge, UK). Microalgae were maintained on nutrient agar at 20°. These cells were used to inoculate 40 ml autoclaved culture medium[38] added to glucose (5g/l), $MgSO_4$, 7 H_2O (1g/l), peptone (0.1g/l) and yeast extract (0.1g/l). Preparation of the algal biomass was performed following previously described procedure.[27]

Incubation with the labelled substrate

To 1 ml of the algal suspension previously described, 1 nmole of an EtOH solution of [1-^{14}C] oleic acid (55 mCi/mmol) was added, and ethanolic solutions of modified fatty acids were added to a final concentration of 0.20 mM. The reaction mixture was stirred for 3 hr, at 25°, and illuminated from above at 15000 lux.

Δ12 desaturase activity assays

Desaturation reaction was stopped by addition of 1 ml of 12% KOH in EtOH (w/w). After saponification at 70° for 30 min, 2 ml of 10% NaCl aqueous solution (w/w) and 10 drops of conc. H_2SO_4 were added. Free fatty acids were extracted (x 3) with 3 ml of Et_2O and treated as previously reported.[27]

Biotransformation assays

To 2 ml of a cellular suspension, an ethanolic solution of dimethyl fatty acid was added to a final concentration of 0.2 mg per ml. The reaction mixture was stirred at 25° under a 15000 lux illumination.

For kinetic studies, lipids were fixed in boiling ethanol.

Then, tubes were centrifuged at 1500 g for 10 min. Supernatants and cellular fractions were separated. Cellular fractions containing total lipids were transesterified by addition of the mixture MeOH-H_2SO_4 (2.5%). The reaction mixture was stirred during 2 hr at 70°. Fatty acid methyl esters were extracted (x 3) by 2.5 ml of PE.

Supernatants were acidified to pH=1-2 by addition of conc. H_2SO_4. Then, free fatty acids were extracted (x 3) by 2.5 ml of E. The solvent was evaporated under a stream of N_2 and endogenous and exogenous fatty acids were methylated in soft conditions[39,40] and analyzed by capillary GC.[27]

Lipid extraction

After incubation, the algal suspension was centrifuged at 1500 g during 5 min. In order to prevent hydrolysis of lipids during the procedure, the collected cells were suspended in ethanol and boiled for 5 min. Lipids were extracted by addition of 5 ml of a solution $CHCl_3$-MeOH-H_2O (1:2:1). Once tubes closed under N_2, the reaction mixture was heated at 70° during 30 min. Then, 1 ml of water and 1 ml of a 2 M solution of NaCl in potassium phosphate buffer (pH= 7.4) were added. After briskly stirring and decanting, the organic phase was collected. Total lipids were extracted (x 3) by addition of 3 ml of $CHCl_3$. Then, the solvent was evaporated under a stream of N_2.

Lipids separation

Lipids were separated by TLC. When cells were incubated with labelled substrate, the residue was resuspended in 100 ml of $CHCl_3$-MeOH (1:1). Exactly 10 ml of this lipid solution were deposited on silica gel plates which were previously activated at 60° for 1 hr. Polar and neutral lipids were separated with a first development (two-thirds of the plate) in $CHCl_3$-Me_2CO-MeOH-CH_3COOH-H_2O (50:20:10:10:5) and a second development in PE-E-CH_3COOH (70:30:1).

After developments, plates were dried under N_2 and the separated lipids were detected with I_2 vapour and identified using authentic standards. Spots, under sellotape, were cut off and dissolved into the liquid scintillation medium for radioactivity measurements.

When cells were incubated without labelled substrate, the residue was resuspended in 300 ml of $CHCl_3$-MeOH (1:1). The entire lipid solution was deposited on activated plates. After detection with I_2 vapour, the lipids strips were cut off and transesterified by addition of 3 ml of the mixture MeOH-H_2SO_4 (2.5%). The reaction mixture was stirred during 2 hr at 70°. Fatty acid methyl esters were extracted and analysed by capillary GC as previously described.

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