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Quantitative ^2H NMR analysis of deuterium distribution in petroselinic acid isolated from parsley seed

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

We have previously demonstrated that ^2H distribution in fatty acids is non-statistical and can be related to isotopic discrimination during chain extension and desaturation. Petroselinic acid (C18:1Δ^6), a fatty acid characteristic of the seeds of the Apiaceae, has been shown to be biosynthesised from palmitoyl-ACP (C16:0) by two steps, catalysed by a dedicated Δ^4-desaturase and an elongase. We have now demonstrated that the isotopic profile resulting from this pathway is similar to that of the classical plant fatty acid pathway but that the isotopic fingerprint from both the desaturase and elongase steps show important differences relative to oleic and linoleic acid biosynthesis.

Keywords: *Petroselinum crispum*; A.W. Hill; Apiaceae; Dehydrogenation; ^2H NMR; ^2H Distribution; Fatty acids; Isotope effects; Petroselinic acid

1. Introduction

Petroselinic acid (C18:1Δ^6) is a fatty acid characteristic of the seeds of the Apiaceae, where it typically accumulates as the major (up to 80%) storage FA in the seed (Kleinman and Spencer, 1982). While there is no reason not to assume the Δ^6-desaturation to be introduced by a Δ^6-desaturase equivalent to the Δ^9-desaturase responsible for the formation of oleic acid (C18:1Δ^9), this has recently been proved not to be the case. Rather, petroselinic acid is formed by a unique pathway, in which five specific enzymes have been identified. Critically, the biosynthesis differs from that of oleic acid in that the desaturation is introduced prior to elongation. Thus, a specific Δ^4-desaturase acts on palmitoyl-ACP to produce Δ^4-hexadecenoyl-ACP (C16:1Δ^4) (Cahoon et al., 1994), itself the substrate for a dedicated elongase that produces petroselinoyl-ACP.

In the elongation, specificity appears related to the properties of the 3-ketoacyl-ACP synthase (KAS) (Mekhedov et al., 2001). This step is followed by two steps of reduction, comparable to the fatty acid synthase (FAS) responsible for the C2 to C16 chain elongation (Harwood, 1997; Ohlrogge and Browse, 1995). In either case, elongation involves the incorporation of hydrogen atoms into the chain, either from malonyl-CoA or from $NAD(P)H + H^+$.

It has previously been shown that the hydrogen atoms in different positions of plant long-chain fatty acids contain different levels of ^2H and that the distribution of ^2H is non-statistical (Royer et al., 1999; Quémerais et al., 1995; Billault et al., 2001; Duan et al., 2002). Furthermore, this distribution can largely be explained in terms of the origin of individual hydrogen atoms and the kinetic isotope effects (KIEs) intrinsic to the pertinent enzymes (Billault et al., 2001; Duan et al., 2002; Robins et al., 2003). Notably, during the 3-ketoacyl-reductase and enoyl reductase steps, two hydrogen atoms are incorporated from $NAD(P)H + H^+$ at each uneven position of the chain and one at each odd position. The other odd-position hydrogen is derived from malonyl-CoA. Thus, positional variation in the $(D/H)_i$

Abbreviations: ACP, acyl carrier protein; FA, fatty acid; FAME, fatty acid methyl ester; FAS, fatty acid synthase complex; KAS, 3-ketoacyl-ACP synthase; KIE, kinetic isotope effect; LAME, linoleic acid methyl ester; OAME, oleic acid methyl ester; PAME, petroselinic acid methyl ester.

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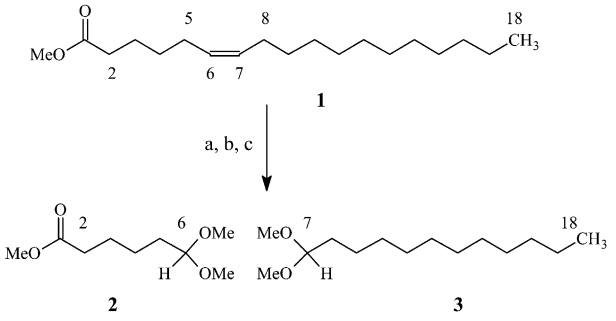
ratios essentially will reflect the $(D/H)_m$ ratio of the water of the environment plus the KIEs of FAS enzymes. In addition, desaturases acting on acyl-ACP (Billault et al., 2001; Duan et al., 2002) and on acyl-PC (Billault et al., 2001; Duan et al., 2002; Behrousian and Buist, 2003) show marked sensitivity to ^2H.

In this paper, we report an analysis by quantitative ^2H NMR of the natural abundance distribution of ^2H in PAME extracted from seeds of *Petroselinum crispum* (Mill.) A.W. Hill. It is found that, while the ^2H distribution in PAME shows certain similarities to that in OAME and LAME, differences are observed that may be related to the specialised nature of the petroselinic acid biosynthetic machinery.

2. Results

The ^2H distribution in methyl petroselinate **1** (Scheme 1) was determined for two preparations independently obtained from the same batch of *Petroselinum crispum* (Mill.) A.W. Hill seeds.



Scheme 1. The chemical modification of methyl petroselinate **1**: (a) OsO_4, *N*-Methylmorpholine oxide, $CHCl_3/H_2O$; (b) $NaIO_4$, MeOH; (c) TsOH, molecular sieves (3 Å).

2.1. Sample preparation

Soxhlet extraction of seeds (20 g) of *P. crispum* gave about 3.5 g of oil (17.5% yield, based on two extractions) and transesterification gave a mixed preparation of FAMEs composed of methyl palmitate (3.7%), methyl hexadecenoate (0.4%), methyl stearate (1.3%), methyl oleate and methyl petroselinate (85.0%), and methyl linoleate (9.7%). This compositional analysis is in good agreement with literature values (Kleinman and Spencer, 1982).

The C18:1 fatty acid methyl esters (PAME + OAME) were isolated by argentation chromatography (Duan, 2002; van Beek and Subrtova, 1995). Typically, 2.0 g of FAME mixture gave 1.58 g (yield 95%) of C18:1 (PAME and OAME). This was composed of 96.8±0.6% of PAME and 3.2% of OAME as determined using ^{13}C NMR spectroscopy (Mallet et al., 1990).

2.2. Quantitative ^2H NMR spectrometry

This sample of C18:1 (PAME + OAME) was submitted to quantitative ^2H NMR spectrometry. To verify that the values obtained are characteristic of PAME, the ^2H distribution in a commercial PAME purchased from Sigma-Aldrich was also determined. The site specific isotopic ratios $(D/H)_i$ measured using quantitative ^2H NMR spectrometry (Martin and Martin, 1990) and expressed in parts per millions (ppm), are given in Table 1. Because of the low quantity of OAME present (3.2%), its contribution to the data can be considered negligible as it falls within the standard deviation range. The standard deviations are between 0.7 and 5.2 ppm, indicating good reproducibility for the work-up procedure.

Table 1
$(D/H)_i$ (in ppm) of C18:1 fraction of *Petroselinum cripum* (97% methyl petroselinate)

Sample no.[a]	Carbon atom no.								*D*/*H* total
	2	3	4[b]	5[c]	6, 7	8[c]	9–17[b]	18	
RMN **1a**	145.3	85.2	123.5	134.6	114.9	134.6	123.5	126.9	123.1
S.D.	(2.4)	(0.7)	(2.1)	(0.9)	(3.1)	(0.9)	(2.1)	(0.9)	(1.5)
RMN **1b**	154.3	86.3	126.3	139.1	122.2	139.1	126.3	128.2	126.7
S.D.	(1.4)	(2.0)	(1.1)	(0.9)	(2.8)	(0.9)	(1.1)	(0.8)	(3.0)
Mean	149.8	85.8	124.9	136.9	118.5	136.9	124.9	127.5	124.9
S.D.	(5.2)	(1.5)	(2.1)	(2.6)	(4.8)	(2.6)	(2.1)	(1.0)	(2.1)
Commercial PAME[d]	158.7	98.9	125.9	148.6	114.1	148.6	125.9	134.3	128
S.D.	(2.5)	(2.6)	(1.5)	(0.7)	(2.3)	(0.7)	(1.5)	(2.4)	(2.2)

[a] $(D/H)_i$ values obtained independently on two C18:1 fractions (a and b) corresponding to the two separations and transesterifications are designated RMN 1a and RMN 1b.
[b] Carbons **4** and **9–17** resonate at the same frequency.
[c] Sites **5** and **8** resonate at the same frequency.
[d] Sigma-Aldrich mixed lots no. 65h1193, 44h0368, 128h0964.

2.3. Chemical modification

As is evident from Table 1, numerous hydrogen atoms have co-incident resonance positions. To reveal isotopic data thus masked in the ^{2}H NMR spectrum of PAME, the partial chemical degradation technique developed on OAME (Billault et al., 2001) has been adapted for PAME. Essentially, the molecule is cleaved with minimal oxidation at the site of the double bond, the resulting aldehydes are protected and the protected products are isolated by chromatography.

Each C18:1 FAME preparation was independently submitted to partial chemical degradation (Scheme 1). The products derived from **1**, methyl 6,6-dimethoxyhexanoate (63% yield) **2** and 1,1-dimethoxydodecane (59% yield) **3** were separated and purified by chromatography. The residues of OAME cleavage were largely eliminated by this procedure.

The products **2** and **3** were submitted independently to quantitative ^{2}H NMR spectral analysis (Table 2). The overall standard deviations are acceptable, (1.1–6.7 ppm), except for carbon position 2 (S.D. = 9.3). However, position 2 is not considered useful, as this position risks exchanging hydrogen with the medium during transesterification (Duan et al., 2002).

In order to verify that chemical modification has not introduced isotopic fractionation, the values obtained for **2** and **3** may be compared with those obtained from the intact molecule **1**. Table 3 shows the difference observed between $(D/H)_i$ of **1** and derivatives **2** and **3**. A direct comparison of the $(D/H)_i$ values determined at sites C2 and C18 for starting material **1** and their products **2** and **3** shows no significant fractionation had occurred. Similarly, the mean value of 120.4 ppm calculated from $(D/H)_6$ and $(D/H)_7$ of **2** and **3**, respectively, compares favourably with the $(D/H)_{6,7} = 118.5$ ppm measured for **1**. Again, the calculated mean value $(D/H)_3 + (D/H)_{5,8} = 118.1$ ppm from **1** can be compared favourably with the calculated mean value of the $(D/H)_{3,5} + (D/H)_8 = 121.5$ ppm from **2** and **3**.

A small difference was observed between $(D/H)_{4,9-17} = 124.9$ ppm measured from **1** and $(D/H)_{9-17} + (D/H)_4 = 132.5$ ppm from **2** and **3**. This difference can be explained by the difficulty of the quantitative calculation of the ^{2}H content when the cluster[1] $(D/H)_x$ is composed of several 'near-coincident' $(D/H)_i$ values. Even so, this difference is not very large compared with the standard deviations (S.D. of $(D/H)_4 = 3.9$ ppm and S.D. of $(D/H)_{9-17} = 3.5$ ppm).

On the bases of these internal checks, it can be concluded that chemical modification has not introduced aberrant values for $(D/H)_i$ of the sites in **2** and **3**.

From the data in Table 2, the $(D/H)_i$ values for a number of positions can be accessed, either by direct measurement or by difference. Thus, the $(D/H)_6$ and $(D/H)_7$, coincident in **1**, are measured directly in **2** and **3**. $(D/H)_4$ and $(D/H)_8$ are also obtained directly in **2** and **3**. $(D/H)_5$ may be obtained by calculation: although in **1** the C5 and C8 resonances are coincident, the value measured directly for C8 in **3** may be used to calculate a value of 122 ppm for $(D/H)_5$. The various measured and calculated values for PAME are summarised in Table 4. Where more than one direct measurement has been made, a mean value is given.

Table 2
$(D/H)_i$ (in ppm) of methyl-9,9′-bis(methoxy)nonaoate **2** and 1.1′-bis(-methoxy)dodecane **3**

Sample no.[b]	Carbon atom no.[a]					*D*/*H* total
	2	3[c]	4	5[c]	6	
RMN **2a**	155.8	107.5	138.1	107.5	139.3	144.4
S.D.	(6.5)	(1.1)	(4.2)	(1.1)	(1.4)	(2.5)
RMN *2b*	140.8	102.5	133.5	102.5	135.2	136.5
S.D.	(2.0)	(4.0)	(6.2)	(4.0)	(2.0)	(3.6)
Mean	148.3	104.9	135.8	104.9	137.2	140.5
S.D.	(9.3)	(3.9)	(4.9)	(3.9)	(2.7)	(3.4)
	7	8	9–17	18	*D*/*H* total	
RMN **3a**	103.0	151.7	129.4	130.5	129.2	
S.D.	(5.5)	(0.8)	(1.6)	(1.3)	(1.8)	
RMN **3b**	103.9	157.9	134.8	131.8	133.9	
S.D.	(8.4)	(7.3)	(2.5)	(0.3)	(3.2)	
Mean	103.5	154.8	132.1	131.1	131.5	
S.D.	(6.7)	(5.7)	(3.5)	(1.1)	(2.4)	

[a] Carbon atom numbering is given as in PAME **1**.

[b] $(D/H)_i$ values obtained independently on products **2** and **3** obtained by chemical modification of two C18:1 fractions (a and b) corresponding to the two separations and transesterifications are designated RMN **2a**, **3a** and RMN **2b**, **3b**, respectively.

[c] Carbons 3 and 5 resonate at the same frequency.

3. Discussion

The procedure described here has, for the first time, given access to $(D/H)_i$ values for PAME **1** extracted from plant seed oil. The data confirm that the distribution of ^{2}H along the chain is non-statistical, as reported

Table 3
Comparison of the $(D/H)_i$ ratios measured for the initial PAME **1** with those measured for the derivatives **2** and **3**

Sample	Carbon atom no.[a]				
	2	3, 5, 8	6, 7	4, 9–17	18
1	149.8	118.1	118.5	124.9	127.5
2 or **3**	148.3	121.5	120.4	132.5	131.1
Δ (**Der**−**1**)	−1.5	3.4	1.8	7.6	3.6

[a] Carbon atom numbering is given as in PAME.

[1] In order to distinguish between $(D/H)_i$ values for clusters and for individual carbon positions, those for clusters are not expressed in italics.

Table 4
Summary of mean $(D/H)_i$ values (in ppm) of methyl petroselinate obtained from **1**, **2** and **3**

	Carbon atom no.[a]									D/H total
	2	3	4	5	6	7	8	9–17	18	
Mean value[b]	149	86	136	*122*	137	104	155	129	129	133

[a] $(D/H)_i$ values obtained by direct measurement are given in standard text: those calculated in italics.
[b] Where more than one direct measurement was obtained (for example position 3 is measured in both **1** and **2**), the mean value is given.

for OAME and LAME (Billault et al., 2001; Duan et al., 2002). As previously discussed, this can be considered due to three principal causes: the origin of the hydrogen atoms present, the KIEs of the enzymes in the FAS complex and the KIEs of the desaturase and elongase enzymes implied in the biosynthesis.

The PAME was extracted from mature *P. crispum* seeds in which the biosynthesis of fatty acids had ceased. According to the literature (see Introduction), the key precursors of petroselinate are palmitate and Δ^4-hexadecenoate. In the extract studied, only 3.4% is methyl palmitate and 0.4% is methyl hexadecenoate (presumably Δ^4), indicating that these fatty acids have essentially been completely desaturated (palmitate) and elongated (hexadecenoate) to petroselinate during maturation. Therefore, the measured levels of enrichment or impoverishment in ^2H at different sites in PAME should reflect the overall influence of the various isotopic effects that have taken place.

It is apparent that the methylenic sites in PAME, which are derived from the even (C2) position of acetate, possess $(D/H)_i$ values greater than those at equivalent odd-numbered (C1-derived) sites, as observed previously (Billault et al., 2001; Duan et al., 2002). This tendency can be seen in Fig. 1, in which C3, C5 and C7 are all impoverished relative to C4, C6 and C8. For the positions C9 to C17, only a mean value can be obtained as these sites resonate at the same frequency even in **3**. By calculation using the values of $(D/H)_4$ and $(D/H)_5$, a value of 128 ppm is obtained for positions C9–C17, the same as the measured value (128 ppm). Hence, by direct

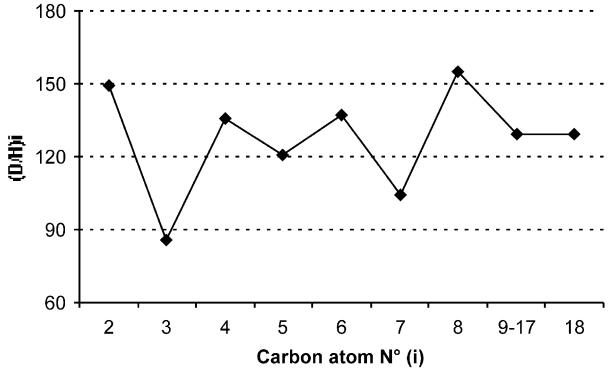


Fig. 1. The variation in $(D/H)_i$ values between different positions in methyl petroselinate.

and indirect means, it is reasonable to conclude that petroselinate, like oleate and linoleate, shows an alternating pattern of $(D/H)_i$ values, which may be explained in terms of the origin of the hydrogen atoms and the KIEs of certain enzymes of the FAS complex (Billault et al., 2001; Duan et al., 2002).

Of particular interest are the relative values of $(D/H)_i$ for the C6 and C7 ethylenic hydrogens. Both the commercial PAME sample (extracted from *Coriandrum sativum* seed) and the PAME extracted from *P. crispum* seed show depletion in deuterium at the sites of desaturation. While this is rather less than seen in a number of other desaturated fatty acids (Royer et al. 1999; Quémerais et al., 1995; Billault et al., 2001; Duan et al., 2002), it follows the generally established pattern. Furthermore, following separate analysis of the C6 and C7 positions in **2** and **3**, it is clear that the C7 position (100 ppm) is significantly depleted compared to the C6 position (135 ppm). This difference of 35 ppm is more than double that observed between the C4 and C5 positions (14 ppm), which show a difference similar to that previously determined in LAME (Billault et al., 2001; Duan et al., 2002). Furthermore, it should be noted that, while C6 has a $(D/H)_i$ value equivalent to C4, the C7 $(D/H)_i$ is 18 ppm lower than the C5.

The question therefore arises as to what is the origin of these differences in isotopic values at the sites of desaturation. Two explanations can be put forward. One possibility is that the pro-(*R*) and pro-(*S*) positions at each methylene group differ in their ^2H content. At the even positions, these hydrogens are derived respectively from acetate (with the allowance for post-malonate exchange with water) and water, while at the odd positions these are both derived from NAD(P)H via hydride transfer at the steps of 3-ketoactylreductase and enoyl reductase.

If no KIE exists, then the hydrogen removed by the desaturase from the C6 position can be predicted to be close to 155 ppm on the basis of the mean value (146 ppm) for the C4 and C8 positions. As the hydrogens at these even positions have two different origins, it would be reasonable to find a slight difference (predicted as ca. 20 ppm) between the pro-(*R*) and pro-(*S*) at this position.

Similarly, if there is no KIE at the C7 position, the hydrogen removed by the action of the desaturase can be predicted to have a value of ca. 140 ppm by com-

parison with the C5 position (122 ppm). It seems improbable, however, that the two hydrogens at the odd positions will differ by ca. 40 ppm as they are both introduced from the NADPH pool. While no isotopic data is known for the individual reactions in the FAS, data from comparable soluble aldehyde- and keto-reductases (Barski et al., 1995; Bohren et al., 1987; Nidetzky et al., 2001; Zheng and Blanchard, 2000) and from enoyl reductase (Ikeda et al., 1985; Parikh et al., 1999) show that these hydride transfers have KIEs of similar strength (1.5–3). Hence, there is no evidence to suggest that the hydrides transferred by these activities are likely to differ greatly in D/H values. A similar argument was previously made for OAME and LAME (Billault et al., 2001).

The alternative explanation is that the soluble Δ^4-desaturase involved in petroselinate synthesis (Cahoon and Ohlrogge, 1994) shows a secondary KIE at the C5 position of (C16:0). A similar but stronger depletion was observed in the D/H value at the C9 of methyl oleate, indicating that the soluble Δ^9-desaturase shows similar properties but at the site closer to the acyl group. In contrast, no KIE was observed for the soluble Δ^9-desaturase when examined by classical high-enrichment methods—when any effect is apparently masked by other rate-determining steps in the catalytic process (Behrouzian et al., 2001)—even though the membranous (insoluble) Δ^9-desaturase showed a high KIE by these techniques (Buist and Behrouzian, 1996).

Other 'low-position' desaturases for which kinetic isotopic data are available all apparently show initial oxidation at the position closer to the acyl head-group (Behrouzian and Buist, 2003). In contrast, the depleted position of all desaturations in fatty acids so far examined is the uneven carbon C7, C9 and C13 respectively for PAME (this study) OAME and LAME (Billault et al., 2001; Duan et al., 2002). This is irrespective of whether the activity is soluble or membranous. While it cannot be ruled out that this is related to differences in the pro-(R) and pro-(S) positions at each methylene groups of the substrate, it is, in view of the argument outlined above, more likely to be due to a KIE(II) at these positions that is not observed by the enrichment approach. Further work is required to examine these two possibilities.

The C3 position of PAME shows a very strong impoverishment compared with all other positions, including the ethylenic carbons (Table 1, Fig. 1). In this, it differs from previous observations of stearate, oleate and linoleate (Royer et al., 1999; Billault et al., 2001; Duan et al., 2002), in which the C3 value is similar to that at other uneven positions. Crucially, PAME differs from OAME in that the elongation of the C16 chain to the C18 chain occurs following rather than preceding desaturation. The highly specific Δ^4-hexadecenoyl-ACP elongase identified in coriander (Mekhedov et al., 2001) shows the properties of a KAS-I type enzyme, rather than those of the KAS-II type activity normally associated with C16:0-ACP elongation. Hence, it can be tentatively suggested that the low $(D/H)_3$ might reflect KIEs occurring during the 3-ketoacylreductase and enoyl reductase steps that differ from those found in the equivalent steps of KAS-II. Other KAS-I products need to be examined to test this hypothesis.

4. Concluding remarks

Petroselinic acid has already been shown to be the product of an unusual pathway, with at least five specialised activities: acyl-ACP desaturase (Cahoon et al., 1994), ACP (Suh et al., 1999), KAS (Mekhedov et al., 2001), ferredoxin (Schultz et al., 2000) and acyl-ACP thioesterase (Dörmann et al., 1994). It is apparent that certain of these activities possess sensitivity to ^2H that differs significantly from those of the better-described enzymes of the palmitoyl-ACP→stearoyl-ACP→oleoyl-ACP pathway. Whether there are consistent relationships between desaturase and elongase types and the associated isotopic fingerprints will require further analyses.

5. Experimental

5.1. Chemicals

Methyl petroselinate extracted from coriander seed (*Coriandrum sativum L.*) was purchased from Sigma-Aldrich Chemical Co. Solvents were distilled and dried before use.

5.2. Preparation and purification of FAMEs from seed oil

Parsley [*Petroselinum crispum* (Mill.) A.W. Hill] seed (20 g) was ground to a powder with an electric mill. The powder was soxhlet-extracted with MeOH (2 h), then cyclohexane (8 h). The cyclohexane was dried ($MgSO_4$) and removed in vacuo to give the seed oil (3.5 g).

FAMEs were prepared from seed oil with boron trifluoride (Duan et al., 2002).

The separation of FAMEs was performed by modified argentation CC on silica (Duan et al., 2002). For separation of 2 g of FAME, the column was prepared as follows: an aqueous $AgNO_3$ soln. (150 ml, 45% w/v) was added with stirring to silica (110 g, Sigma, 70–230 mesh). The silica was then dried in an oven at 130 °C (20 h).

The column (375×30 mm) was filled with $AgNO_3$-impregnated silica suspended in *n*-hexane (130 ml). FAME (2 g) was applied and developed as follows: 0.5 l

n-hexane; 1.0 l cyclohexane/toluene (9:1); 1.0 l cyclohexane/toluene (8:2); 1.0 l cyclohexane/toluene (7:3); 1.0 l cyclohexane/toluene (6:4). Fractions of 60 ml were collected and the presence of FAMEs checked by GC. After pooling of the fractions containing the C18:1 FAMEs and removal of solvent in vacuo, the purity of the C18:1 fraction as determined by GC as > 99%.

Methyl petroselinate (**1**): ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 5.38 (2H, m, H-6 and H-7), 3.68 (3H, s, OCH_3), 2.30 (2H, *t*, $J = 7.5$ Hz, H-2 and H-2′), 2.02 (4H, *m*, H-5, H-5′, H-8 and H-8′), 1.63 (2H, *m*, H-3 and H-3′), 1.40–1.17 (20H, *m*, CH_2), 0.88 (3H, *t*, $J = 6.7$ Hz, Me).

5.3. Gas chromatography

The FAME mixture was analysed by GC on a Supelco SPB-PUFA capillary column (30.0 m×0.25 mm×0.20 μm). Conditions of analysis: carrier gas, He at 1.0 ml min^{-1}; split injection 1:40; injection volume, 1.0 μl; FID temperature, 260 °C; injector temperature, 250 °C; thermal gradient, 140 °C for 2 min/8 °C min^{-1} to 210 °C/210 °C for 40 min.

5.4. Compositional analysis of C18:1 mixture

^{13}C-NMR spectra were recorded on a Bruker DRX500 spectrometer operating at 125 MHz. Samples were prepared in a 5 mm o.d. tube by mixing the FAMEs with $CDCl_3$ (1:4 v/v). TMS was added as internal standard. Acquisition conditions were: spectral width, 6000 Hz; pulse delay, 4.5 μs; acquisition time, 1.4 s; number of data points, 16 k; number of scans, 2000. The relative composition of PAME and OAME was calculated from peak intensity ratios of the ethylenic carbons as described previously (Mallet et al., 1990).

5.5. ^{2}H-NMR spectrometry

The samples were previously characterised by their ^{1}H and ^{13}C NMR spectra. The ^{2}H-NMR spectra were recorded on a Bruker DPX400 spectrometer operating at 61.4 MHz and fitted with a ^{19}F field-frequency locking device as described previously (Billault et al., 2001). Three spectra were recorded for each sample and an average $(D/H)_i$ was calculated from the area under the peak from each measurement. The $(D/H)_i$ ratio of samples were calculated from Eq. (1)

$$\left(\frac{D}{H}\right)i = \frac{P_{\text{ref}} \times m_{\text{ref}} \times M_s \times S_{\text{si}}}{P_i \times m_s \times M_{\text{ref}} \times S_{\text{ref}}} \times \left(\frac{D}{H}\right)\text{ref} \quad (1)$$

where P_i and P_{ref} are the stoichiometric numbers of hydrogens in site *i* and in the reference, S_{si} and S_{ref} are the area of the signal, and M_s, m_s and M_{ref}, m_{ref} are the molecular weight and mass of the samples and the reference used, respectively.

The internal reference was pyridine. The isotopic ratio of pyridine, $(D/H)_{\text{pyr}}$ was calibrated relative to *N*,*N*,*N*′,*N*′-tetramethylurea (TMU) and $(D/H)_{\text{TMU}}$ was calibrated on the V.SMOW scale (Gonfiantini et al., 1995). Areas under the peaks were calculated by least squares analysis (Martin, 1994) using the Interliss software (Eurofins Analytical, Nantes, France).

5.6. Chemical modification

Chemical modification were carried out as previously described (Billault et al., 2001). As the chemical modification were performed on 1 g of C18:1 fraction, two separate chemical modifications were carried out. Modification of C18:1 fraction gave derivatives **2** and **3** (Scheme 1).

Methyl-9,9′-bis(methoxy)hexanoate (**2**): ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 4.30 (1H, *t*, $J = 5.5$ Hz, H-6), 3.61 (3H, s, MeOCO), 3.26 (6H, s, 2 OMe), 2.26 (2H, *t*, $J = 7.5$ Hz, H-2 and H-2′), 1.58 (4H, *m*, H-3, H-3′, H-5 and H-5′), 1.33 (2H, m, H-4 and H-4′).

1.1′-Bis(methoxy)dodecane (**3**): ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 4.31 (1H, *t*, $J = 5.5$ Hz, H-1), 3.25 (6H, *s*, 2 OMe), 1.53 (2H, *m*, H-2 and H-2′), 1.30–1.17 (18H, m, 9 CH_2), 0.82 (3H, *t*, $J = 6.5$ Hz, Me).

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