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Quantitation of hydroxylated byproduct formation in a *Saccharomyces cerevisiae* Δ^9 desaturating system

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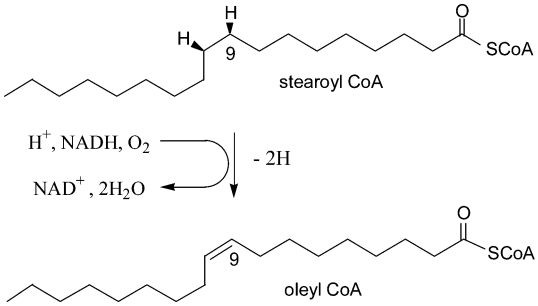
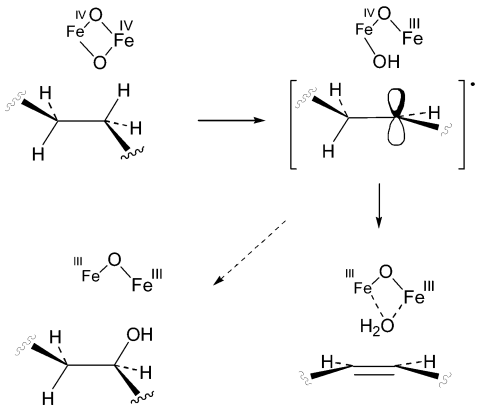
The distribution of products obtained from stearoyl CoA Δ^9 desaturase-mediated oxidation in *Saccharomyces cerevisiae* has been measured directly for the first time using an ω-fluorinated fatty acid substrate.

Desaturases catalyze the oxidation of lipidic substrates in a variety of stereochemical and regiochemical modes.[1] A prototypical reaction is shown in Scheme 1. Current interest in this ubiquitous class of enzymes is high due to their important role in a number of critical biological functions such as the response to temperature-induced stress,[2] chemical signalling[3] and lipogenesis.[4] Interestingly, in the case of membrane-bound desaturases, hydroxylated fatty acids of unknown biological function appear to be produced along with olefinic products.[5,6] A minimal mechanistic model describing the relationship between two pathways has been formulated (Scheme 2): an initial carbon-centred radical is generated by H-abstraction which collapses to either olefin (β-elimination) or alcohol (hydroxyl rebound or SH_2 reaction).[7] A more detailed mechanistic analysis is clearly warranted and the Δ^9 desaturase found in *Saccharomyces cerevisiae* (Baker's yeast) would seem to be an ideal candidate for such a study since this enzyme occurs as a single isoform in yeast, is amenable to *in vitro* study[8] and tolerates a wide range of substrate analogues.[7] In addition, this enzyme serves as a convenient microbial model for a mammalian homologue (SCD1); the latter has been targeted recently for inhibition studies in the treatment of disorders associated with the "metabolic syndrome".[9] Finally, the appropriate genetic control experiments have been conducted in yeast wherein it has been shown that hydroxylation and desaturation are mediated by the same enzyme.[5] Herein, we report on the regioselectivity of fatty acid hydroxylation in a *S. cerevisiae* strain and use of a ω-fluorinated substrate to quantitate the hydroxylation : dehydrogenation ratio directly for the first time.



Scheme 1 A prototypical dehydrogenation reaction catalyzed by stearoyl CoA Δ^9 desaturase (CoA = Coenzyme A).



Scheme 2 Mechanistic model for fatty acid-desaturase catalyzed oxidation.

A preliminary incubation experiment was performed to examine the endogenous production of hydroxy fatty acids in wild-type Baker's yeast. Cultures (200 mL) of *Saccharomyces cerevisiae S522C* were grown to stationary phase under a standard set of conditions (YPED medium,[10] 24 h, 30 °C, 150 rpm). Centrifugation (10 000 rpm) of the resultant cell suspension yielded a cell paste (~3 g, wet weight) from which the fatty acid methyl ester (FAME) fraction (~25 mg) was isolated by a hydrolysis–methylation procedure as previously described.[11] In addition, the supernatant was acidified, extracted with dichloromethane and the residue obtained upon evaporation of solvent was methylated. Aliquots of the FAME extract from the cells and from the supernatant were treated with excess bis(trimethylsilyl)acetamide–pyridine (10 min, 40 °C) to enhance the detectability of hydroxylated fatty acids by GC-MS (HP 5980 Series 2 gas chromatograph (J. & W. 30 m × 0.21 mm, DB-5 capillary column) coupled to a Kratos Concept 1H mass spectrometer). While no hydroxylated fatty acid methyl esters were detected in the supernatant extract, the total ion current (TIC) chromatogram obtained for cellular FAMEs did reveal the presence of the TMS derivatives of both methyl 9-hydroxypalmitate (hexadecanoate) **1** (0.9% ± 0.1% of total fatty acids (TFA)) and methyl 9-hydroxystearate (octadecanoate) **2** (0.3% ± 0.1% of TFA) (Fig. 1 (a), (b)).[12] **1** and **2** are thought to be byproducts of Δ^9 desaturation of palmitoyl CoA and stearoyl CoA respectively. The structural assignments of **1**-OTMS and **2**-OTMS are based on the known mass spectral fragmentation patterns of this class of compounds (**1**: *m/z* (EI) 73 ($Si(CH_3)_3$); 201 ($C_8H_{16}OSi(CH_3)_3$) and 259 ($(CH_3)_3SiOC_8H_{15}COOMe$); **2**: *m/z* (EI) 73 ($Si(CH_3)_3$); 229 ($C_{10}H_{20}OSi(CH_3)_3$) and 259 ($(CH_3)_3SiOC_8H_{15}COOMe$)).[13] The quantitation of hydroxylated analytes was calibrated by measuring the response factors for authentic reference standards

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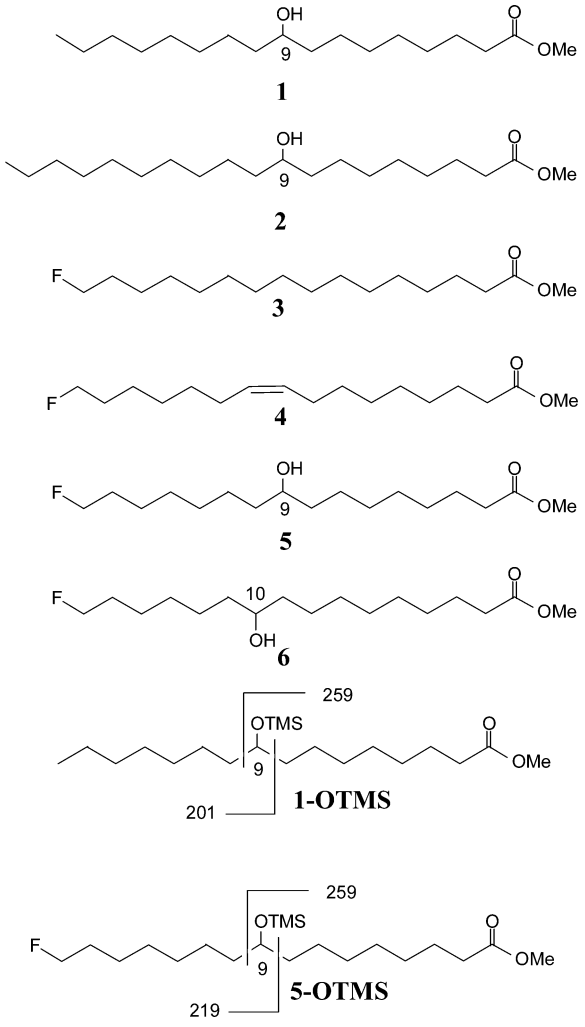
which were available from another study.[13] A careful examination of the mass spectral scans through the entire GC peak of both **1**-OTMS and **2**-OTMS indicated that these analytes were consistently accompanied by ~5% of coeluting 10-hydroxylated regioisomers (diagnostic fragment ions of the TMS derivative of 10-hydroxypalmitate: *m/z*: 187 and 273; diagnostic fragment ions of the TMS derivative of 10-hydroxystearate: *m/z*: 215 and 273). Diagnostic ions of the TMS derivatives of 8- and 11-hydroxylated fatty acids (*m/z*: 215 and 245 (C16-8-OH); 173 and 287 (C16-11-OH); 243 and 245 (C18-8-OH); 201 and 287 (C18-11-OH))) could not be detected (limits of detection: ~1% of major isomer). The observed high regioselectivity of the hydroxylation pathway is consistent with our mechanistic model (Scheme 2) and the results of KIE studies and oxo trapping studies which point to C-H abstraction at C-9 as the initiating event in yeast Δ9 desaturase-mediated oxidation.[14,15]



Having established the reproducible, endogenous production of hydroxylated fatty acids in our *in vivo* desaturating system, we sought to develop a versatile means of quantitating the hydroxylation : dehydrogenation ratio directly. This required the use of a substrate analogue with an analytical signature which 1) is distinct from those of background endogenous lipids; 2) reports the position of oxygenation accurately and 3) would be amenable for use in future *in vitro* studies. We reasoned that ω-fluorinated fatty acids such as **3** might fulfil all of these requirements. Thus, methyl 16-fluoro-palmitate **3** was prepared from the corresponding, commercially available, hydroxy acid by a methylation–fluorination sequence as previously described.[16] **3** (50 mg, in 1% Tergitol NP-40 (Sigma) solution) was incubated with actively growing cultures (200 mL) of *Saccharomyces cerevisiae S522C* under conditions similar to those used in the trial incubation (*vide supra*). Previous experiments have shown that this whole cell system efficiently incorporates fatty acyl substrates from the medium into the intracellular CoA thioester substrate pool[17] where they are processed by the endogenous Δ^9 desaturase. A portion of the FAME fraction isolated from the cells was silylated and analyzed by GC-MS. As shown in Fig. 2, excellent incorporation (62% of total fatty acids) of 16-fluoropalmitate **3** was achieved and 47% of **3** had been converted to the corresponding 16-fluoropalmitoleate **4**. Selected ion monitoring (ions at *m/z* 219 (C_8H_{15}FOTMS) and 259 (TMSOC_8H_{15}COOMe)) was used initially to locate the TMS derivative of methyl 16-fluoro-9-hydroxypalmitate **5**, which appeared as a shoulder on the peak corresponding to methyl 18-fluorostearate—a chain elongation product of **3**. The identity of the "error" product **5** was confirmed by a comparison with the retention time and mass spectral characteristics of the TMS derivatives of authentic reference standards: a 1 : 1 mixture of 16-fluoro-9-hydroxypalmitate **5** and 16-fluoro-10-hydroxypalmitate **6**. The latter was obtained by the hydration of 16-fluoropalmitoleate **4** using a well-characterized procedure ($Hg(OAc)_2$–H_2O : THF (1 : 1); $NaBH_4$ workup).[18] The ratio of **4** : **5** in the yeast FAME extract was estimated to be 98.9 ± 0.4 : 1.1 ± 0.4 *via* integration of the relevant GC-MS peaks (average of 3 analyses).[19] This product distribution is comparable to that observed for a model plant (*Arabidopsis thaliana*) Δ^{12} desaturase expressed in *S. cerevisiae* (99.4 : 0.6 for 12-ene : 12OH product ratio)[5] but is somewhat higher than that calculated for *Candida albicans* dihydroceramide Δ^4 desaturase, also expressed in *S. cerevisiae* (93 : 7 for 4-ene–4-OH product ratio).[6]

The possibility of using ^{19}F NMR, in the trace analytical mode,[20] as an alternate method of quantitating the oxidation products derived from **3** was also explored. The ^{1}H-decoupled ^{19}F NMR spectrum of a portion of the FAME extract obtained from the incubation of **3** with *S. cerevisiae* is shown in Fig. 3 and features two major peaks at δ −218.23 ppm (**3**) and −218.32 ppm (**4**). The peak assignments follow from a comparison of the ^{19}F NMR spectrum of the yeast extract with the spectrum obtained for the substrate **3** (δ −218.23 ppm) prior to incubation. The % desaturation of **3**, calculated from the NMR peak intensities (46%), is in good agreement with that obtained for the GC-MS analysis (47%, *vide supra*). However, a signal attributable to hydroxylation byproduct **5** was not detected in this spectrum. That this was in all likelihood due to the overlap of ^{19}F NMR resonances of **4** and **5** was further clarified by obtaining a ^{1}H-decoupled ^{19}F NMR spectrum of a FAME aliquot which had been treated with $Hg(OAc)_2$–H_2O (*vide supra*) to convert a portion of **4** to the regioisomeric hydration products **5** and **6** (δ −218.32 ppm and −218.36 ppm respectively (Fig. 3 (insert)). The chemical shift assignments of **5** and **6** are based on the documented tendency of mid-chain, polar substituents to shift a remote, terminal CH_2F resonance further upfield with decreasing substituent-fluorine distance.[21] This analysis suggests that it may be possible to achieve better separation between ^{19}F NMR signals of fluorine-tagged 9-ene and 9-hydroxy-products by employing substrates bearing the fluoro substituent somewhat closer to the C-9 position than is the case for **4** and **5**.

The major conclusions of this study are as follows: 1) the highly regioselective, endogenous production of 9-hydroxylated fatty acids in a wild-type strain of *S. cerevisiae* has been quantitated using GC-MS analysis. 2) It has also been shown that an ω-fluorinated substrate can be used to monitor the direct formation of olefinic and hydroxylated byproducts in *S. cerevisiae* and a product ratio of ~99 : 1 (9-ene: 9-OH) was obtained *in vivo*. 3) A combination of GC-MS and ^{1}H-decoupled ^{19}F NMR can potentially be used to assess extent of hydroxy byproduct formation. The fluorine-tag methodologies presented in this paper should greatly facilitate future *in vitro* protein engineering studies designed to pinpoint which protein residues have a critical impact on the dehydrogenation : hydroxylation ratio.

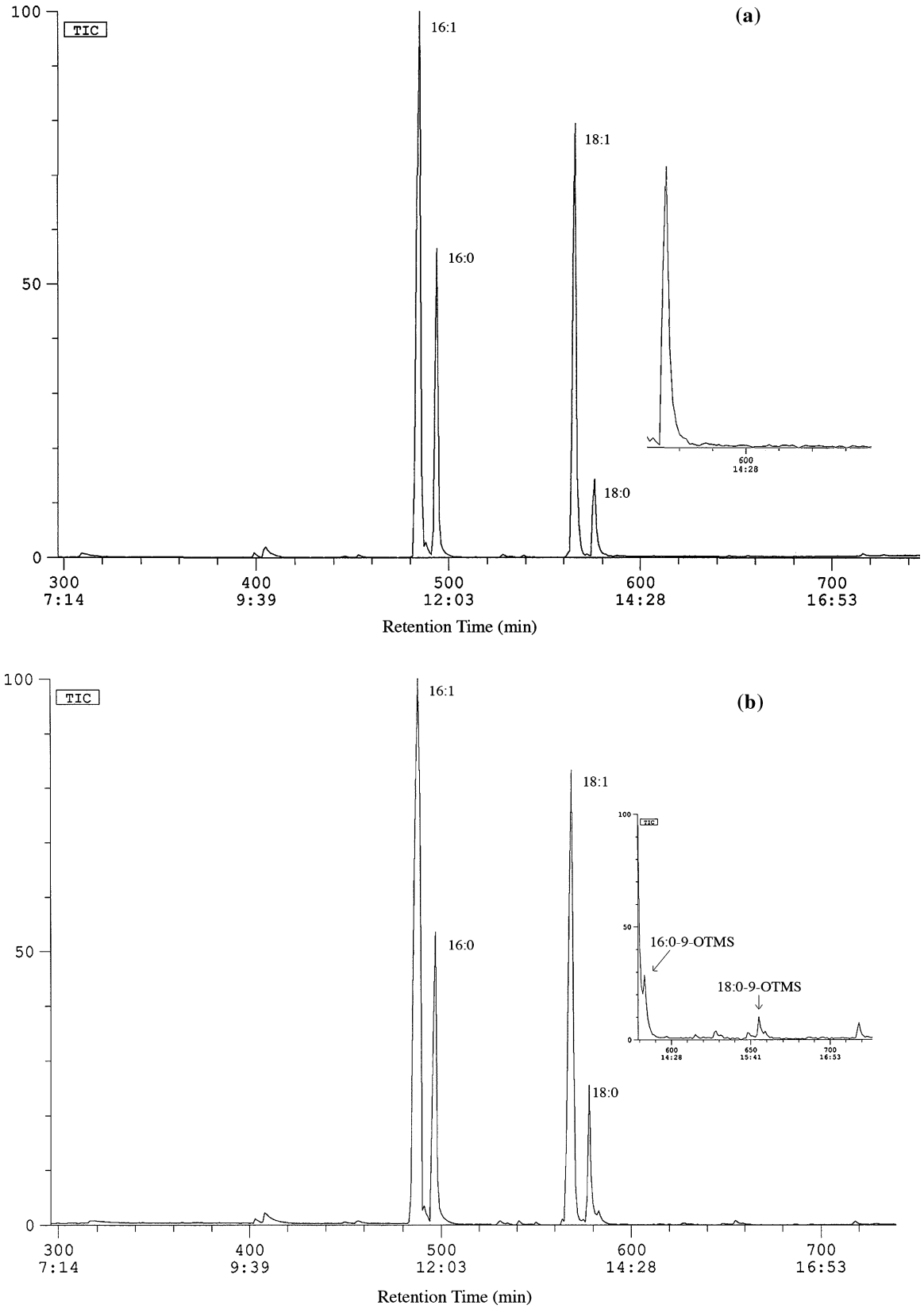


Fig. 1 GC-MS analysis of FAME (fatty acid methyl esters), total extract derived from *S. cerevisiae* cultures (a) before and (b) after silylation. The fatty acid components are indicated as 16:0 = methyl palmitate (hexadecanoate); 16:1 = methyl palmitoleate (9(*Z*)-hexadecenoate); 18:0 = methyl stearate (octadecanoate); 18:1 = methyl oleate (9(*Z*)-octadecenoate); 16:0-9-OTMS = methyl 9-hydroxypalmitate, trimethylsilyl (TMS) derivative; 18:0-9-OTMS = methyl 9-hydroxystearate, trimethylsilyl (TMS) derivative.

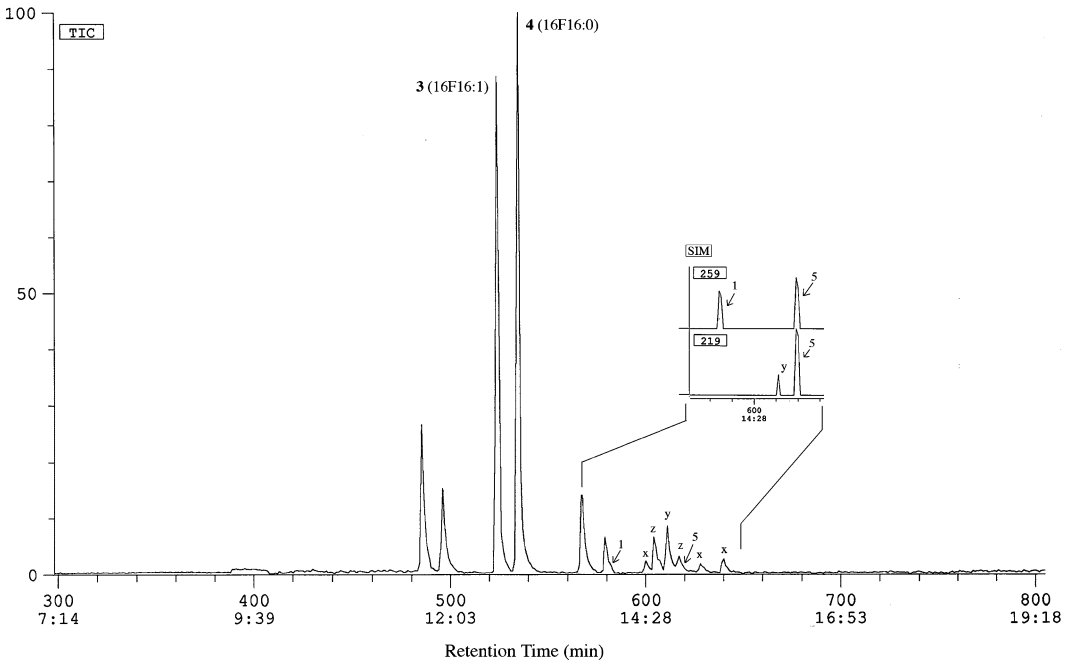


Fig. 2 GC-MS analysis of silylated FAME (fatty acid methyl esters), total extract derived from *S. cerevisiae* cultures supplied with methyl 16-fluorostearate **3**. The fluorinated fatty acid components are indicated as 16F16:0 = methyl 16-fluoropalmitate **3**; 16F16:1 = methyl 16-fluoropalmitoleate **4**; 16F16:0-9-OTMS = the TMS derivative of methyl 16-fluoro-9-hydroxypalmitate **5**. [x] Unidentified peaks of varying intensity which are believed to be artifacts of the silylation procedure. [y] Unidentified impurity accompanying substrate **3**. [z] Chain elongation products of **3** and **4**: 18F18:1 = methyl 18-fluorooleate and 18F18:0 = methyl 18-fluorostearate. ***Insert***: SIM (selected ion monitoring) chromatogram of FAME extract for ions at *m/e* 259 and *m/e* 219.

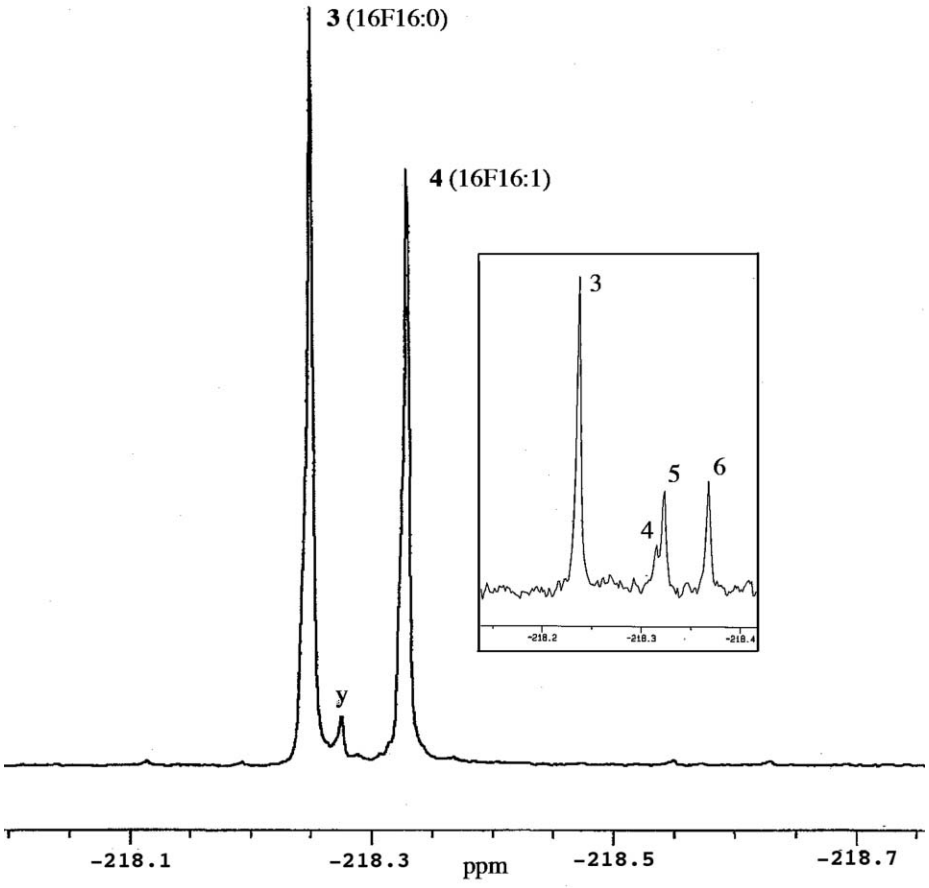


Fig. 3 [1]H-decoupled [19]F NMR spectrum of FAME (fatty acid methyl esters), total extract derived from *S. cerevisiae* cultures supplied with methyl 16-fluoropalmitate (16F16:0) **3**. [y] Unidentified impurity accompanying substrate **3**. ***Insert***: [1]H-decoupled [19]F NMR spectrum of FAME after hydration. Chemical shift assignments of **5** (methyl 16-fluoro-9-hydroxy-palmitate) and **6** (methyl 16-fluoro-10-hydroxy-palmitate) are tentative. The difference in [19]F chemical shifts assigned to **4** and **5** was found to be 0.008 ppm.

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