Synthesis and desaturation of monofluorinated fatty acids[1]

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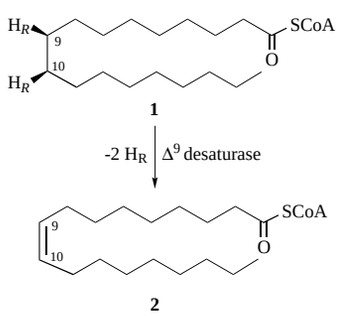
A series of monofluoro C_{16} and C_{18} fatty acids have been synthesized and used as mechanistic probes for fatty acid desaturation. Only fluoroolefinic products are obtained when these compounds are processed by an *in vivo Saccharomyces cerevisiae* Δ^9 desaturating system as determined by ^{1}H-decoupled ^{19}F NMR and GC–MS analysis. No evidence for fluorohydrin formation has been found when either methyl (*R*,*S*)-9- or 10-fluoropalmitate (stearate) 3a,b and 5a,b was incubated with the Δ^9 desaturase. On desaturation α- and β-fluorine substituent effects (k_H/k_F) of magnitude 6.2 and 2.4, respectively, have been measured by direct competition experiments between 3a and 3b and between methyl 16-fluoropalmitate 3c and 3b. These results do not support the involvement of discrete hydroxylated and carbocationic intermediates in fatty acid desaturation. Substantial apparent steric effects have been observed for monofluorostearoyl substrates 5c–f bearing a fluorine distal from the site of initial oxidation. In the case of (*R*,*S*)-methyl 12-fluorostearate 5f, we show that both enantiomers are desaturated at comparable rates.

The biological dehydrogenation (desaturation) of unactivated fatty acyl hydrocarbon chains as exemplified by the conversion of stearoyl CoA **1** into oleyl CoA **2** is an essential biochemical

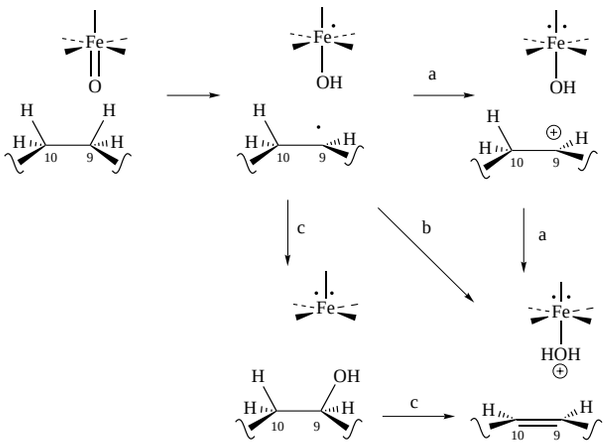


process for most aerobic lifeforms.[2a] It is also an outstanding example of enzymic regio- and stereo-selectivity and served to inspire the very early experiments in biomimetic chemistry.[2b] Recently significant progress has been made in the structural characterization of the enzymes responsible for these intriguing transformations. In two seminal papers,[3a,b] Shanklin and Fox have shown that the family of O_2-dependent, non-haem iron-containing enzymes known as desaturases can be divided into two classes: soluble plant enzymes containing a carboxylate-bridged diiron active site similar to that of methane monooxygenase (MMO) and a series of less-well characterized membrane-bound desaturases which are thought to contain a diiron active site co-ordinated to histidine groups. A recent X-ray structure of the plant Δ^9 desaturase from castor (*Ricinus communis*) reveals a tight, crescent-shaped, hydrophobic substrate binding pocket and computer modelling studies suggest that C-9 of the stearoyl substrate is near the iron atom responsible for initial oxidation.[4] No detailed mechanistic work on this enzyme has emerged due to the complexity of the substrate (a fatty acyl ACP thioester) and the lack of a suitable assay.

We have obtained a number of results using an *in vivo S. cerevisiae* (membrane-bound) Δ^9 desaturase system† which also point to initial oxidative attack at C-9 of a stearoyl substrate.[5a–d] Thus, 9-thia fatty acid analogues are more efficiently converted into the corresponding sulfoxide than are their 10-thia counterparts[5a–c] and a large primary deuterium kinetic isotope effect has been measured at C-9 whereas C–H bond cleavage at C-10 is essentially insensitive to isotopic substitution.[5d] Based on these results, the fact that hydroxylases and desaturases are linked at the structural level[3a,b,6] and the observation that dehydrogenation and hydroxylation are occasionally mediated by the same enzyme,[7a–c] we have proposed a mechanistic model for Δ^9 desaturases which involves initial rate-determining C–H bond cleavage at C-9 to form a transient carbon-centred radical followed by collapse to olefin in one of the three possible ways shown in Scheme 1 (pathways a,b,c).‡ A fascinating question remains: How do desaturases steer the putative radical intermediate to olefin? The X-ray structure[4] of the plant desaturase offers no obvious clues as to how this might be done. Newcomb *et al.* have recently presented evidence supporting the view that the closely related hydroxylation of unactivated hydrocarbons mediated by sMMO and cytochrome P_{450} proceeds by a 'nonsynchronous, concerted mechanism' and that

† We have elected to work with intact organisms because reconstituted preparations of membrane-bound Δ^9 desaturases are notoriously unstable and have very low activity. Attempts to overexpress the yeast Δ^9 desaturase have not been successful. Our decade-long experience with the *in vivo* yeast system[5e–f] has been that methyl esters of our mechanistic probes are incorporated into the cells where they are acted upon by the Δ^9 desaturase (presumably as the CoA thioesters). If the product of desaturation is sufficiently nonpolar, it accumulates in the cell whereas if desaturation is diverted to generate polar, oxygenated products they are excreted as free acids into the culture medium.

‡ For the sake of simplicity, we represent the active oxidizing species as an iron oxo complex. Although it appears that there are two irons in the Δ^9 yeast desaturase,[3b] it is interesting to note that the closely related rat liver Δ^9 desaturase was originally thought to contain one iron atom.[22] The mechanistic considerations presented in this paper are not affected by this uncertainty.



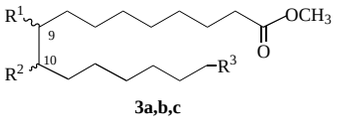
Scheme 1

the 'lifetime of the radical be shortened from that of an intermediate to that of a vibrational component in a transition state'.[8] This would imply that it would be difficult to prevent an initial hydroxylation event and that desaturases function by promoting a subsequent Lewis-acid catalyzed dehydration (pathway c).§ On the other hand, it is remarkable that biological dehydrogenation appears to be favoured when positive charge stabilizing substituents neighbour the site of initial oxidation.[7a–c] This suggests that desaturases may utilize a redox switch to force a 1 electron oxidation/deprotonation route (pathway a). We decided to explore the possibility of using the fluorine substituent as a new mechanistic probe for hydroxylated and carbocationic intermediates in desaturation. In this article, we report the synthesis of some long chain monofluorinated fatty acids and the effects of fluorine substitution on the *in vivo* Δ^9 desaturation of these substrates.

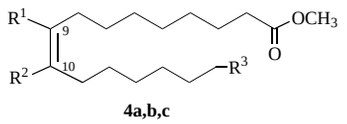
Results and discussion

We initially chose methyl (*R*,*S*)-9-fluoropalmitate **3a** and methyl (*R*,*S*)-10-fluoropalmitate **3b** as our primary test substrates since the behaviour of these compounds could conveniently be compared with that of a readily accessible, remotely fluorinated substrate—methyl 16-fluoropalmitate **3c**. Thus, **3a** and **3b** were synthesized in 8 and 7% overall yield, respectively, *via* a sequence of reactions consisting of alkyl Grignard addition to the appropriate ω-alkenal, fluorination using diethylaminosulfur trifluoride (DAST),[9] oxidative cleavage[10] and methylation[11] (Scheme 2 and Experimental section). Compound **3c** was conveniently prepared by fluorination of methyl juniperate (methyl 16-hydroxyhexadecanoate). Since the yeast Δ^9 desaturase can act on both C_{16} and C_{18} substrates, we also prepared methyl (*R*,*S*)-9-fluorostearate **5a** and methyl (*R*,*S*)-10-fluorostearate **5b** in the same manner as their C_{16} homologues. In order to check for possible steric effects of the fluorine substituent, a further three monofluorinated methyl stearates were prepared from readily available starting materials. Thus methyl (*R*,*S*)-5-fluorostearate **5c**, methyl (*R*,*S*)-7-fluorostearate **5d** and methyl (*S*)-12-fluorooctadecanoate **5e** were synthesized by DAST fluorination of the corresponding methyl hydroxystearates. All spectral and analytical data obtained for these compounds were in accord with their assigned structure.

Each fluorinated fatty acid methyl ester (25 mg) was incubated for 24 h with growing cultures (150 cm^3) of *S. cerevisiae* ATCC 12341 (Baker's yeast) as previously described.[5a] The yeast cells grew out to a normal extent in each experiment and



3a,b,c

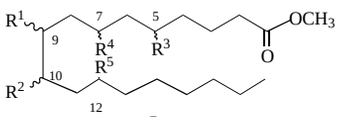


4a,b,c

a R^1 = F, R^2 = H, R^3 = H

b R^1 = H, R^2 = F, R^3 = H

c R^1 = H, R^2 = H, R^3 = F



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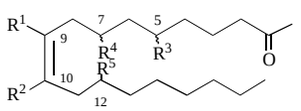


6

a R^1 = F, R^2 = H, R^3 = H, R^4 = H, R^5 = H

b R^1 = H, R^2 = F, R^3 = H, R^4 = H, R^5 = H

c R^1 = H, R^2 = H, R^3 = F, R^4 = H, R^5 = H

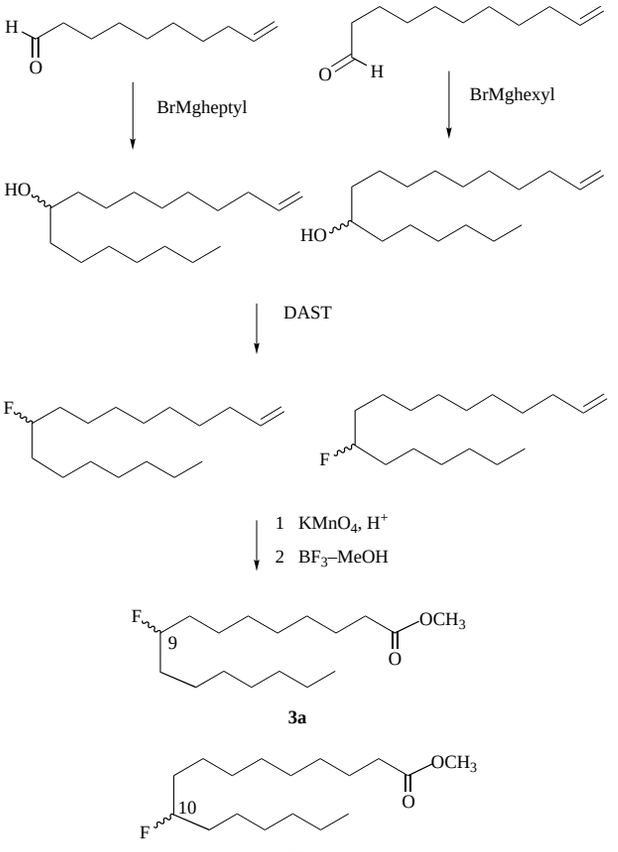
d R^1 = H, R^2 = H, R^3 = H, R^4 = F, R^5 = H

e R^1 = H, R^2 = H, R^3 = H, R^4 = H, R^5 = (*S*)-F

f R^1 = H, R^2 = H, R^3 = H, R^4 = H, R^5 = (*R*,*S*)-F

were collected by centrifugation. The cellular fatty acid fraction from each incubation experiment was isolated by a standard hydrolysis/methylation procedure[5d] and analyzed by GC–MS[5d] and ^{1}H-decoupled ^{19}F NMR.[5c] Examination of the fatty acid profiles (Table 1) revealed that uniformly high incorporation of the fluorinated substrates was achieved and with one exception (Expt. 3), no obvious effect on the % desaturation of endogenous C_{16} or C_{18} substrates was identified. (In Expt. 3, only a modest reduction in the % desaturation of endogenous fatty acids was noted—this effect was taken into account when we compared the data of the individual experiments.) Importantly, a single product (a fluoroolefin) was produced in each case. The proposed structure of the fluoroalkenes was consistent with the MS data [**4a**,**b**,**c**: *m/z* 286 (M^+); **6a**,**b**: *m/z* 314 (M^+); **6c**,**d**,**e**: *m/z* 294 ($M^+ - HF$); *m/z* 262 ($M^+ - HF, CH_3OH$)], the expected GC retention times (*ca.* 0.1–0.8 min shorter than that of the corresponding substrate) and the ^{19}F NMR chemical shifts [($CDCl_3$, $CFCl_3$ ref.) δ −105.78 **4a**; −105.57 **4b**; −218.55 **4c**; −105.78 **6a**; −105.57 **6b**; −181.22 **6c**; −179.78 **6d**; −179.57 **6e**]. The *E*-stereochemistry of the 9- and 10-fluoroolefinic products **4a**,**b** and **6a**,**b** was confirmed by comparison of the observed ^{19}F NMR chemical shifts with literature values obtained for (*E*)-7-fluorotetradec-7-ene (δ −105.6),[12] (*Z*)-7-fluorotetradec-7-ene

§ This mechanism was originally discounted by Bloch who found that 9-hydroxystearoyl CoA and 10-hydroxystearoyl CoA were not dehydrated to give olefin by the yeast Δ^9 desaturase.[23] Since it is possible that these compounds could not bind to the desaturase enzyme, this result cannot be regarded as entirely conclusive.

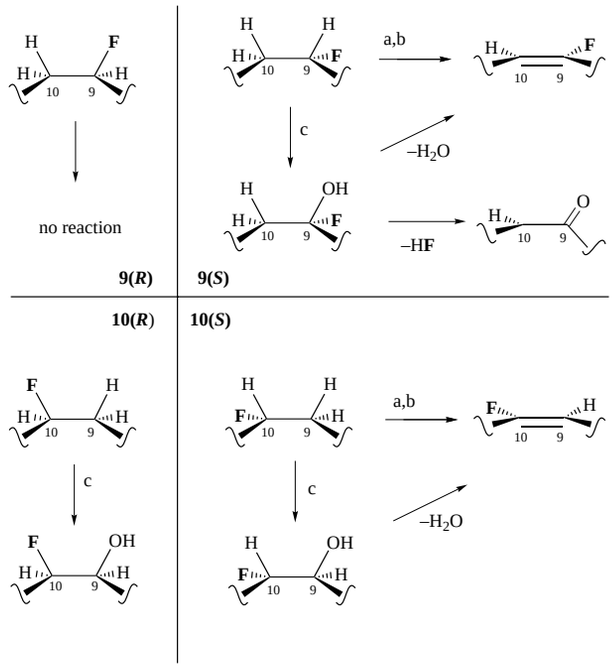


Scheme 2

(δ −110.6)[12] and (*E*)-4-fluorooct-4-ene (δ −105.0).[13] The stereochemistry of the double bond could be checked for one of the remotely fluorinated products **6e** and shown to be *Z* as expected by comparison of the observed ^{19}F NMR chemical shift of the biosynthetic product with those of authentic samples of methyl (*Z*)-12-fluorooctadec-9-enoate (δ −179.57) and methyl (*E*)-12-fluorooctadec-9-enoate (δ −179.86).¶

Examination of the methylated dichloromethane extracts of the culture medium[5a] obtained in each experiment revealed only the presence of starting material. Importantly, none (<0.1% of the total fatty acids) of the possible oxygenated products arising from diverted desaturation (Scheme 3) or their possible decomposition products/metabolites were detected by our analytical methods (GC–MS for non-fluorinated compounds or a combination of GC–MS and ^{1}H-decoupled ^{19}F NMR for fluorinated products).

We begin our discussion of these results by considering the possible fate of each enantiomer of the 9- and 10-fluoro substrates if desaturation follows a hydroxylation/dehydration pathway (Scheme 3). Since it is known that the yeast Δ^9 desaturase abstracts the *pro R* hydrogens at C-9 and C-10,[14] and that oxidation is initiated at C-9,[5a–d] the (*R*)-9-fluoro enantiomers cannot be oxidized. The corresponding (*S*)-9-fluoro enantiomer, however, would give a 9,9-fluorohydrin which should collapse spontaneously to give ketone. The α-hydroxylation of a fluorinated steroid has been shown to give a keto product.[15a] The fact that we see only fluoroolefin and no methyl 9-ketopalmitate (stearate) or their metabolites in the extracts of



Scheme 3

the **3a** or **5a** incubation militates against the involvement of an obligatory alcohol intermediate in desaturation.

This interpretation was substantiated by the results of the incubation experiments using 10-fluoro substrates **3b** and **5b.** Thus (*R*)-**3b** or (*R*)-**5b** should have given an *erythro*-1,2-fluorohydrin‖ if pathway c was involved in desaturation and failure to observe this product in our extracts also suggests that pathway c is not operative. This in turn implies that 10(*R*)-fluoro substrates should be suicide inhibitors of Δ^9 desaturases since the formation of the putative carbon-centred radical at C-9 is almost certainly irreversible. Unfortunately, our methodology relies on continuous *in vivo* production of enzyme and inactivation of the Δ^9 desaturases could go undetected under our conditions. Further experiments with purified enzyme are necessary to address this point. With respect to the fate of (*S*)-**3b** or (*S*)-**5b**, it is not surprising in light of the above discussion that these substrates were processed cleanly to fluoroolefin with no evidence of *threo*-1,2-fluorohydrin‖ formation.

Taken together, our results do not favour Lewis acid-catalyzed dehydration of a hydroxylated intermediate as a route to olefin in desaturation. This led us to consider whether the effects of fluorine substitution could be used to distinguish between the two remaining alternatives: the carbocationic and disproportionation pathways (pathway a,b, Scheme 1). Fluorine substituent effects are legitimate probes for carbocation formation. That is, a 9-fluorine substituent should favour the formation of a carbocation at C-9 by overcoming σ-inductive withdrawal with 2p-2pπ resonance donation while a 10-fluoro substituent could conceivably shut down neighbouring carbocation formation by a strong, electron-withdrawing inductive effect.[16a,b] In our case, one can only use fluorine substituent effects as evidence for or against carbocation formation if one makes the following assumptions: (1) effects of (*S*)-9-fluoro and (*S*)-10-fluoro substitution on non-catalytic steps such as substrate binding are not dramatically different and (2) fluorine substituent effects on possible carbocation formation are not completely masked by the effects of fluorine substitution on the initial hydrogen abstraction step—an event we know from our isotope effect studies[5d] to be kinetically important.

¶ These compounds were prepared by DAST fluorination of methyl (*Z*)-12-hydroxyoctadec-9-enoate and methyl (*E*)-12-hydroxyoctadec-9-enoate. These reactions do not give pure products but the desired compounds are major components which are contaminated with a number of rearrangement products due to the intermediacy of cyclopropyl carbonium ions as we have previously discussed.[24] The identity of the desired reference compounds was unambiguously determined using a combination of ^{19}F NMR and ^{13}C NMR spectroscopy.[24]

‖ The fluorohydrins were synthesized according to the method of Muelbacher and Poulter[25] (see Experimental section) and served as reference standards in our search for these intermediates. It was shown that these compounds are stable in solution under physiological conditions.

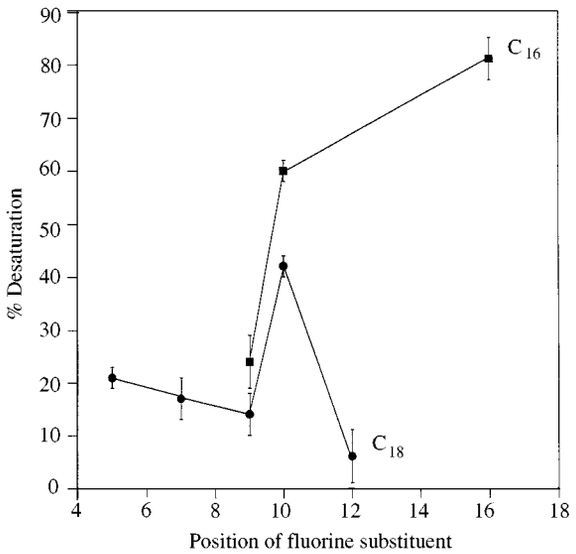


Fig. 1 Dependence of % desaturation of fluorinated substrates as a function of fluorine position (Table 1). Values for % desaturation of 9- and 10-fluorinated substrates in Table 1 have been multiplied by a factor 2 to correct for the fact that only 50% of these racemic substrates can give olefin. The value for % desaturation of methyl 16-fluoropalmitate has been corrected (×1.3) since the % desaturation of endogenous substrates in this incubation was somewhat lower than normal [83% (ave.) for C_{16} and 95% (ave.) for C_{18}].

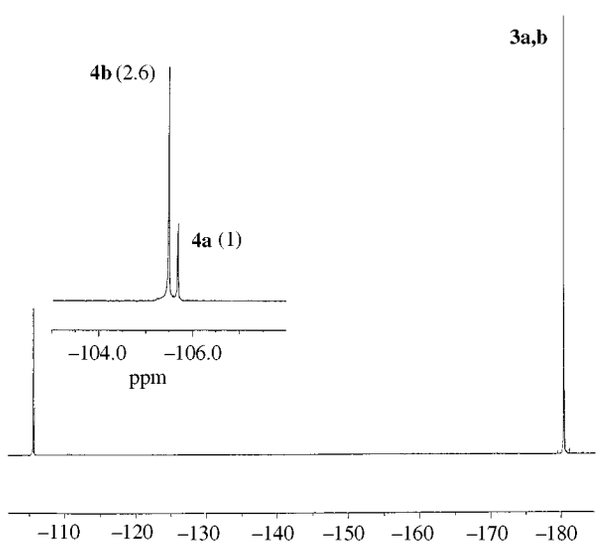


Fig. 2 ^{1}H-decoupled ^{19}F NMR (376.5 MHz) spectrum of cell extract obtained from incubation of a 1:1 mixture of methyl (*R*,*S*)-10-fluoropalmitate **3b**:methyl (*R*,*S*)-9-fluoropalmitate **3a**. ^{19}F resonances due to **3b** and **3a** as well as the products **4a** and **4b** are assigned as indicated and the relative intensities are given in parentheses.

With these caveats in mind, we examined the data of Table 1 as represented graphically in Fig. 1. The most striking feature of Fig. 1 is that 9-fluoroalkenes **4a** and **6a** are both produced *ca.* 3 times less efficiently than the corresponding 10-fluoroalkenes **4b** and **6b**. This trend was confirmed by performing a direct competition experiment in which a mixture of **3a** and **3b** (1:1) was incubated with *S. cerevisiae*. Analysis of the cell extract obtained in this experiment by ^{1}H-decoupled ^{19}F NMR spectroscopy revealed that a 2.6:1 mixture of **4b**:**4a** was obtained (Fig. 2) in good agreement with the value calculated from the data in Fig. 1 (% desaturation **3b**/% desaturation **3a** = 60/24 = 2.5). This result gives the ratio: $k_{F\beta}/k_{F\alpha} = 2.6$. In order to determine the individual α- and β-fluoro substituent effects on desaturation, we carried out a direct competition experiment between one of the fluorinated methyl palmitates **3b** and a

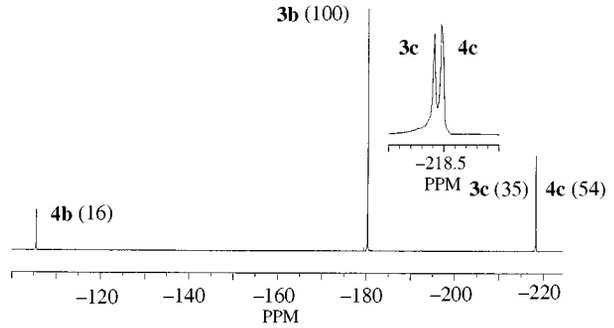


Fig. 3 ^{1}H-decoupled ^{19}F NMR (376.5 MHz) spectrum of cell extract obtained from incubation of a 1.4:1 mixture of methyl (*R*,*S*)-10-fluoropalmitate **3b**:methyl 16-fluoropalmitate **3c**. ^{19}F resonances due to **3b** and **3c** as well as the products **4b** and **4c** are assigned as indicated and the relative intensities are given in parentheses.

remotely fluorinated substrate **3c**. Thus, a mixture of **3b** and **3c** (1.4:1) was incubated with *S. cerevisiae*, the cellular fatty acids extracted and the amount of fluoroolefin formed from each substrate in the cellular extract evaluated by ^{1}H-decoupled ^{19}F NMR spectroscopy (Fig. 3). From this experiment, we can estimate a $k_H/k_{F\beta}$ = (am't **4c**/am't **4b**) ÷ [(am't **3c**)/am't (*S*)-**3b**]$_{initial}$ = (54/16) ÷ [1/(0.5)1.4] = 2.4. {This value is somewhat higher than that calculated from Fig. 1 [% desaturation **3c**/% desaturation (*S*)-**3b** = 81/60 = 1.4] but it is more accurate since it is derived from a direct competition experiment}. An α-fluorine substituent effect on desaturation can be calculated by combining the results of the two competition experiments: $k_H/k_{F\alpha} = k_H/k_{F\beta} \times k_{F\beta}/k_{F\alpha} = 2.4 \times 2.6 = 6.2$.

The significance of these values of the α- and β-fluoro substituent effects on desaturation is that they are in qualitative agreement with what is known about the effect of fluorine substitution on related biohydroxylations—rate retardations are felt most strongly α to the fluorine substituent** and decrease with increasing distance.[15a,b] Thus, our estimate of the β-fluorine effect ($k_H/k_{F\beta} = 2.4$) on desaturation can easily be accounted for by an inductive effect on the initial hydrogen abstraction step by a strongly electrophilic agent.[17] If a carbocation was being generated at C-9 during desaturation, one might have expected to see a far larger β-effect since a rate retardation of $k_H/k_{F\beta} = 79$ per β-fluorine atom on the formation of delocalized (allylic) cations has been observed[16a] and effects on non-stabilized carbocations should be even greater. Similarly, one might have expected a much reduced rate-retarding α-fluorine effect or possibly even an acceleration since a rate acceleration of $k_{F\alpha}/k_H = 71$ due to an α-fluorine effect on carbocation formation is known.[16a] Since we do not see these sorts of perturbations on the β- and α-fluorine effects, we conclude that the intermediacy of carbocations during desaturation is not supported by our data, and thus, by a process of elimination, a disproportionation mechanism (pathway b, Scheme 1) must be favoured.

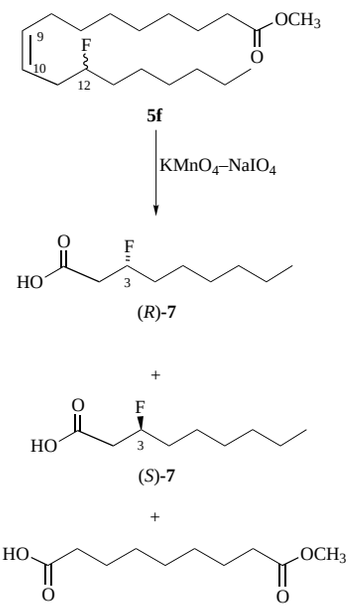
Finally, we would like to comment on the fact that the % desaturation of (*S*)-**5a** is apparently similar to the values obtained for stearoyl substrates (**5c**–**e**) bearing fluorine substituents at intermediate distances (γ, δ, ε) from the site of initial oxidation (Fig. 1). These long-range substituent effects clearly cannot be due to influences on catalytic steps but are probably due to unfavourable interactions of the fluorine substituent with the hydrophobic walls of the desaturase binding site. We thought it would be interesting to examine this

** Stoddart, Nechvatel and Tedder[17a] have shown that unlike β-fluorine effects on 'chemical' H atom abstraction which are always rate-retarding, the corresponding α-fluorine effects can be either accelerating or decelerating depending on the nature of the reagent. Thus, α-fluorine substituent effects are a potentially useful tool to evaluate chemical models of iron-oxo-mediated hydrocarbon activations.

Table 1 Incubation of fluorinated fatty acids using *S. cerevisiae*: fatty acid profiles

Expt. #	Compound incubated	Cellular fatty acid composition[a]						%(F) incorp.	%(F) desat.	%(C_{16}) desat.	%(C_{18}) desat.
		C_{16}:0	C_{16}:1	C_{18}:0	C_{18}:1	C_n:0(F)	C_n:1(F)				
Control		13	45	3	39	—	—	—	—	78	93
1	**3a**	7	35	1	24	29	4	33	12 (10)	83	96
2	**3b**	5[b]	30	1	24	28	12[b]	40	— (30)	86	96
3	**3c**	12	23	6	21	14	24	38	62 (62)	66	78
4	**5a**	6	29	1	20	41	3	44	7 (7)	83	95
5	**5b**	7	38	1	16	30	8	38	21 (20)	84	94
6	**5c**	6	29	1	21	33	9	42	21	83	95
7	**5d**	4	38	1	19	32	6	38	17 (20)	90	95
8	**5e**	8	30	1	25	34	2	36	6 (5)	80	96

[a] The relative amounts of each fatty acid component are expressed as a % of the total fatty acids; they were determined using the GC–MS Total Ion Current (TIC) chromatograms. Each experiment was carried out at least twice and each entry represents an average value (SD = 1–2%). Endogenous fatty acids include C_{16}:0 [methyl hexadecanoate], C_{16}:1 [methyl (*Z*)-hexadec-9-enoate], C_{18}:0 (methyl octadecanaoate) and C_{18}:1 [methyl (*Z*)-octadec-9-enoate]. C_n:0(F) is the saturated, fluorinated substrate, n = 16 (Expt. 1,2) and 18 (Expt. 3,4); C_n:1(F) is the unsaturated, fluorinated product, n = 16 (Expt. 1,2) or 18 (Expt. 3,4). %(F) incorp. = amount of fluorinated fatty acids as a % of the total fatty acids in extract. %(F) desat. = % desaturation of fluorinated substrate. Values in brackets were obtained by integration of the relevant peaks in the ^{1}H-decoupled ^{19}F NMR spectra of the fatty acid extracts. %(C_{16}) desat. = % desaturation of endogenous C_{16}:0 %(C_{18}) desat. = % desaturation of endogenous C_{18}:0. [b] These values are based solely on the ^{19}F NMR data due to overlap of the GC peaks.



phenomenon further by checking whether there was any enzymic discrimination between the worst substrate **5e** and its enantiomer. Thus, racemic 12-fluorooctadecanoate **5f** was prepared by fluorination of methyl (*R*,*S*)-12-hydroxyoctadecanoate (see Experimental section) and administered to *S. cerevisiae* in the usual manner. The cell extract was examined by ^{1}H-decoupled ^{19}F NMR and shown to contain a 95:5 mixture of starting material:product as expected. The extract was then submitted to von Rudloff[18] oxidation conditions and the fluorinated cleavage products **7** were analyzed by ^{1}H-decoupled ^{19}F NMR spectroscopy. With the aid of the chiral shift reagent (*S*)-(−)-1-(naphthyl)ethylamine (4 mg per 0.5 cm^3), it was shown that the ratio of (*R*)-3-fluorononanoic acid/(*S*)-3-fluorononanoic acid[19] derived from biosynthetic 12-fluorooleate was 53:47 (Fig. 4). Thus, it appears that both enantiomers of methyl 12-fluorooctadecanoate are processed at comparable rates by our desaturating system, and the rate-retarding effect of 12-fluoro substitution is not stereospecific. These phenomena clearly require more systematic study using a complete set of fluorinated fatty acids. However, our observations do serve to emphasize that while monofluorination of fatty acid hydrocarbon chains may have minimal impact on the bulk biophysical properties of condensed monolayers,[20] one cannot assume that the 'isomorphic' replacement[21] of hydrogen by fluorine will not result in unexpected effects on enzyme–substrate interactions.

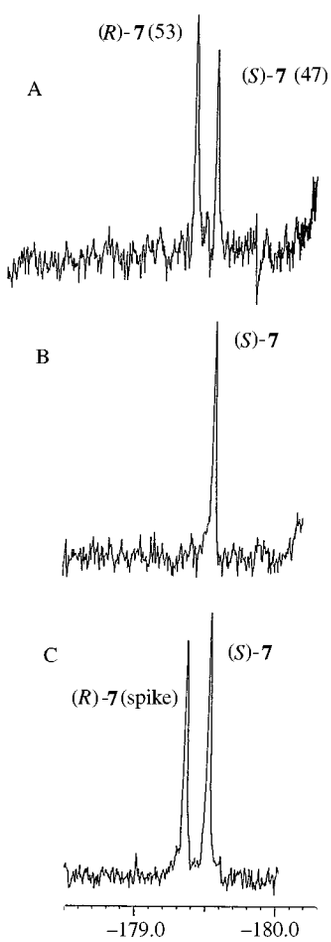


Fig. 4 Assessment of enantiomeric purity of 3-fluorononanoic acid obtained by oxidative cleavage of biosynthetic 12-fluorooleate: the effect of (*S*)-(−)-NEA on the ^{19}F (376.5 MHz) NMR resonances due to (A), (*R*)- and (*S*)-3-fluorononanoic acid (*R*,*S*)-**7** derived from desaturation of (*R*,*S*)-12-fluorooctadecanoate **5f**; (B), (*S*)-3-fluorononanoic acid (*S*)-**7** derived from desaturation of (*S*)-12-fluorooctadecanoate **5e**; (C), (*S*)-3-fluorononanoic acid (*S*)-**7** derived from desaturation of (*S*)-12-fluorooctadecanoate **5e** spiked with authentic (*R*)-3-fluorononanoic acid (*R*)-**7**

In summary, we have demonstrated that the use of fluorinated substrates shows considerable promise as a means of testing current mechanistic models for desaturation. We have provided some evidence against the intermediacy of an alcohol or carbocation in fatty acid desaturation. Additional work is

required to extend and clarify our observations using purified enzymes when these become available. It would also be interesting to carry out analogous experiments using the purified plant Δ^9 desaturase enzyme system.[3a]

Experimental

General methods

Melting points were determined on a digital Fisher-Johns melting-point apparatus and are uncorrected.

Unless otherwise noted, ^{1}H NMR spectra were obtained at 200 MHz on a Varian Gemini 200 spectrometer using dilute $CDCl_3$ solutions. Chemical shifts are expressed in ppm and are referenced to tetramethylsilane. Coupling constants (J) are reported in Hz.

All ^{19}F NMR spectra were obtained on a Bruker AM400 (9.4 T) spectrometer operating at 376.50 MHz with a dedicated 5 mm ^{19}F/^{1}H probe and ^{19}F-specific pre-amplifier. A Bruker 400 MHz band-pass filter and a 376.5 MHz band-stop filter were used in the proton decoupler channel, and 376.5 MHz band-pass filter and 400 MHz band-stop channel were used in the fluorine observe channel for all acquisitions. Proton decoupling was achieved by using low power (composite phase decoupling). Chemical shifts are reported relative to external trichlorofluoromethane ($CFCl_3$) at 0.00 ppm.

Mass spectra were obtained on a Kratos Concept IIH mass spectrometer equipped with a 70 eV EI ionization chamber. Samples were introduced using either a direct probe or a gas chromatograph (HP 5980, 30 m DB-5 capillary column) as appropriate. Single elemental analyses were performed using a Perkin-Elmer Elemental Analyzer 240C.

Flash chromatography using silica gel (230–400) mesh was used to purify substrates prior to incubation experiments. Analytical TLC was performed using Merck glass plates pre-coated with silica G/UV 254. Visualization of UV-inactive materials was accomplished by using a combination of I_2 vapour followed by a water spray.

Unless otherwise stated, all reagents and starting materials were purchased from Aldrich Chemical Company and used without purification. Chiral materials were used without improving their optical purity. All air- and moisture-sensitive reactions were performed under N_2. Unless otherwise noted, organic extracts were shaken with sat. NaCl, dried over Na_2SO_4 and evaporated on a rotary evaporator. All fluorinated methyl ester substrates were obtained as white, amorphous solids.

Synthesis of substrates

Methyl (*R*,*S*)-9-fluoropalmitate (9-fluorohexadecanoate) 3a. The title compound was prepared using a sequence of well-established synthetic procedures.

A solution of dec-9-en-1-al (prepared by Swern oxidation[26] of the commercially available dec-9-en-1-ol) (4.57 g, 29.7 mmol) in anhydrous diethyl ether (15 cm^3) was added to an ethereal solution of freshly prepared heptylmagnesium bromide (40 mmol) at −10 °C over 30 min. The reaction mixture was poured onto crushed ice (25 g), acidified with 3 M aq. H_2SO_4 and extracted with diethyl ether. The extract was washed with 3 M aq. H_2SO_4, dried and evaporated to give a yellowish solid. This material was purifed by flash chromatography with 5% EtOAc as eluent to yield (*R*,*S*)-heptadec-1-en-10-ol as a white solid (3.95 g, 52%), mp 41–43 °C; δ_H 0.89 (3H, t, *J* 7, CH_3), 1.2–1.5 (20H, br s, methylenes), 1.61 (4H, m, CH_2CHOHCH_2), 2.03 (2H, m, CH_2CH=CH_2), 3.57 (1H, br d q, C*H*OH), 4.93 (2H, m, CH=CH_2) and 5.79 (1H, m, C*H*=CH_2); *m/z* 236 ($M^+ - H_2O$).

A solution of (*R*,*S*)-heptadec-1-en-10-ol (2.3 g, 9.06 mmol) in dichloromethane (10 cm^3) was treated with diethylaminosulfur trifluoride (10.2 mmol) at −45 °C. The reaction mixture was allowed to warm to room temperature whereupon excess of reagent was carefully quenched with water. The organic layer was diluted with dichloromethane (20 cm^3), washed with sat. aq. $NaHCO_3$ and water, dried, and then evaporated to give (*R*,*S*)-10-fluoroheptadec-1-ene as a crude yellow solid (1.75 g, 51%, est.); δ_H 0.89 (3H, t, *J* 7, CH_3),1.2–1.5 (20H, br s, methylenes), 1.62 (4H, m, CH_2CHFCH_2), 2.03 (2H, m, CH_2CH=CH_2), 4.44 (1H, d of m, J_{HF} 48, C*H*F), 4.94 (2H, m, CH=CH_2) and 5.80 (1H, m, C*H*=CH_2). As judged by the integration of additional ^{1}H NMR signals at δ 5.37, this material consisted of a 3:1 mixture of fluoride:diene by-products. This intermediate was used in the next step without further purification.

A mixture of crude (*R*,*S*)-10-fluoroheptadec-1-ene (1.75 g, 75% pure, 5.1 mmol) in glacial acetic acid (12 cm^3) was oxidized at room temperature for 17 h using aqueous $KMnO_4$ (40 mmol, 60 cm^3) in the presence of hexadecyl(tributyl)phosphonium bromide (50 mg, 0.1 mmol) and with vigorous stirring. Hexane (60 cm^3) was added to the reaction mixture and excess of oxidant destroyed by the addition of sulfurous acid (prepared *ex situ*). The hexane layer was washed with water, dried and evaporated to give crude (*R*,*S*)-9-fluoropalmitic acid (800 mg) as a white crystalline product. This compound was methylated with BF_3–MeOH (pre-prepared reagent) and purifed by flash chromatography using 2% EtOAc/hexanes to give methyl (*R*,*S*)-9-fluoropalmitate, **3a** (456 mg, 31%), mp 26–28 °C (from methanol) (Found: C, 70.95; H, 11.53. $C_{17}H_{33}FO_2$ requires C, 70.78; H, 11.53%); δ_H 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (20H, br s, methylenes), 1.63 (4H, m, CH_2CHFCH_2), 2.30 (2H, t, *J* 7, CH_2CO), 3.66 (3H, s, CO_2CH_3), 4.45 (1H, d of m, J_{HF} 48, CHF); δ_F −180.50; *m/z* 268 ($M^+ - HF$) and 236 ($M^+ - HF$, CH_3OH).

The route described above was also used to synthesize the following.

Methyl (*R*,*S*)-10-fluoropalmitate (10-fluorohexadecanoate) 3b. From undec-10-en-1-al: mp 24–25 °C (Found: C, 70.71; H, 11.53. $C_{17}H_{33}FO_2$ requires C, 70.78; H, 11.53%); δ_H 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (20 H, br s, methylenes), 1.63 (4H, m, CH_2CHFCH_2), 2.30 (2H, t, *J* 7, CH_2CO), 3.66 (3H, s, CO_2CH_3) and 4.45 (1H, d of m, J_{HF} 48, CHF); δ_F −180.49; *m/z* 268 ($M^+ - HF$) and 236 ($M^+ - HF$, CH_3OH).

Methyl (*R*,*S*)-9-fluorostearate (9-fluorooctadecanoate) 5a. From dec-9-en-1-al: mp 37–39 °C (Found: C, 72.08; H, 11.79. $C_{19}H_{37}FO_2$ requires C, 72.10; H, 11.78%); δ_H 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (24H, br s, methylenes), 1.63 (4H, m, CH_2CHFCH_2), 2.30 (2H, t, *J* 7, CH_2CO), 3.66 (3H, s, CO_2CH_3) and 4.45 (1H, d of m, J_{HF} 48, CHF); δ_F −180.49; *m/z* 296 ($M^+ - HF$) and 264 ($M^+ - HF$, CH_3OH).

Methyl (*R*,*S*)-10-fluorostearate (10-fluorooctadecanoate) 5b. From undec-11-en-1-al: mp 34–36 °C (Found: C, 72.03; H, 11.96. $C_{19}H_{37}FO_2$ requires C, 72.10; H, 11.78%); δ_H 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (24 H, br s, methylenes), 1.63 (4H, m, CH_2CHFCH_2), 2.31 (2H, t, *J* 7, CH_2CO), 3.68 (3H, s, CO_2CH_3), 4.45 (1H, d of m, J_{HF} 48, CHF); δ_F −180.44; *m/z* 296 ($M^+ - HF$) and 264 ($M^+ - HF$, CH_3OH).

The following compounds were prepared by treating the corresponding alcohol with diethylaminosulfur trifluoride essentially as described above. Unless otherwise stated, purification of the crude product was achieved by flash chromatography using 2% EtOAc–hexane as eluent.

Methyl 16-fluoropalmitate (16-fluorohexadecanoate) 3c. From methyl 16-hydroxypalmitate [870 mg, 3.04 mmol; obtained by methylation[11] of 16-hydroxypalmitic acid (juniperic acid)] (450 mg, 51%), mp 32–34 °C (Found: C, 70.42; H, 11.63. $C_{17}H_{33}FO_2$ requires C, 70.78; H, 11.53%); δ_H 1.2–1.4 (24H, br s, methylenes), 1.62 (2H, m, CH_2CH$_2$F), 2.30 (2H, t, *J* 7, C$H_2CO_2CH_3$), 3.67 (3H, s, CO_2CH_3) and 4.44 (2H, d of t, J_{HF} 47, J_{HH} 7, CH_2F); δ_F −218.47; *m/z* 288 (M^+) and 257 ($M^+ - CH_3O$).

Methyl (*R*,*S*)-5-fluorostearate (5-fluorooctadecanoate) 5c. From methyl (*R*,*S*)-5-hydroxystearate (428 mg, 1.36 mmol; obtained by $NaBH_4$ reduction[27] of methyl 5-oxooctadecanoate) (134 mg, 29%), mp 39–41 °C (Found: C, 72.31; H, 11.81. $C_{19}H_{37}FO_2$ requires C, 72.10; H, 11.78%); δ_H 0.88 (3H, t, *J* 7,

CH_3), 1.2–1.5 (24H, br s, methylenes), 1.69 (4H, m, CH_2CHFCH_2), 2.34 (2H, t, *J* 7, CH_2CO), 3.67 (3H, s, CO_2CH_3) and 4.46 (1H, d of m, J_{HF} 48, CHF); δ_F −181.31; *m/z* 296 (M^+ − HF) and 264 (M^+ − HF, CH_3OH).

Methyl (*R,S*)-7-fluorostearate (7-fluorooctadecanoate) 5d. From methyl (*R,S*)-7-hydroxystearate (1.12 g, 3.5 mmol; obtained by $NaBH_4$ reduction[27] of methyl 7-oxo-octadecanoate) (350 mg, 27%), mp 30–32 °C [lit.,[28] 38.5–39.9 °C (from methanol)] (Found: C, 71.88; H, 12.01. $C_{19}H_{37}FO_2$ requires C, 72.10; H, 11.78%); δ_H 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (24H, br s, methylenes), 1.62 (4H, m, CH_2CHFCH_2), 2.30 (2H, t, *J* 7, CH_2CO), 3.66 (3H, s, CO_2CH_3), 4.45 (1H, d of m, J_{HF} 48, CHF); δ_F −180.70; *m/z* 296 (M^+ − HF) and 264 (M^+ − HF, CH_3OH).

Methyl (*S*)-12-fluorostearate (12-fluorooctadecanoate) 5e. From methyl (*R*)-12-hydroxystearate [400 mg, 1.27 mmol; obtained by BF_3-catalyzed methylation[11] of (*R*)-12-hydroxystearic (octadecanoic) acid]. Purification of the crude product was accomplished by a 3-fold recrystallization from methanol (200 mg, 50%), mp 37–39 °C (Found: C, 72.09; H, 11.77. $C_{19}H_{37}FO_2$ requires C, 72.10; H, 11.78%); δ_H 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (24H, br s, methylenes), 1.63 (4H, CH_2CHFCH_2), 2.30 (2H, t, *J* 7, CH_2CO), 3.67 (3H, s, CO_2CH_3) and 4.45 (1H, d of m, J_{HF} 48, CHF); δ_F −180.43; *m/z* 296 (M^+ − HF) and 264 (M^+ − HF, CH_3OH).

Methyl (*R,S*)-12-fluorostearate (12-fluorooctadecanoate) 5f. From methyl (*R,S*)-12-hydroxystearate (370 mg, 1.18 mmol; obtained by $NaBH_4$ reduction of 12-oxooctadecanoate). The crude fluorinated product was purified by repeated recrystallization from methanol (150 mg, 40%), mp 37–38 °C (from methanol) [lit.,[28] 39.5–40 °C (from methanol)]. Spectral data identical with those of **5e**.

Methyl *erythro*-9(10)-fluoro-10(9)-hydroxyoctadecanoate. A solution of methyl (*E*)-9,10-epoxyoctadecanoate (200 mg, 0.64 mmol) in CH_2Cl_2 was treated with pyridine polyhydrofluoride (0.2 ml) at −5 °C for 15 min. The reaction mixture was diluted with water to quench the reaction and neutralized with 5% aq. $NaHCO_3$. The organic layer was dried and evaporated to yield a yellow oil (137 mg). A portion of this material (127 mg) was purified by flash chromatography using 15% ethyl acetate–hexane as eluent to give a colourless oil (81 mg, 52%); δ_H(400 MHz, Bruker AMX 400) 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (20H, br s, methylenes), 1.51 (2H, m, CH_2CHOH), 1.62 (2H, m, CH_2CHF), 2.30 (2H, t, *J* 7, CH_2CO), 3.67 (3H, s, CO_2CH_3), 3.71 (1H, m, CHOH), 4.37 (1H, d of m, J_{HF} 49, CHF); δ_F −191.48 and −191.56; *m/z* 312 (M^+ − HF) and 294 (M^+ − HF, H_2O). This material contained a minor fluorinated contaminant of unknown structure which was detected by ^{19}F NMR and ^1H NMR spectroscopy but not by GC–MS.

Methyl *threo*-9(10)-fluoro-10(9)-hydroxyoctadecanoate. A solution of methyl (*Z*)-9,10-epoxyoctadecanoate (207 mg, 0.66 mmol) in CH_2Cl_2 was treated with pyridine polyhydrofluoride (0.2 ml) at −5 °C for 1 h. The reaction was worked up as described above to yield a crude product (184 mg). A portion of this material (159 mg) was purified by flash chromatography using 15% ethyl acetate–hexane as eluent to give a low-melting waxy solid (112 mg, 52%); δ_H(400 MHz, Bruker AMX 400) 0.88 (3H, t, *J* 7, CH_3), 1.2–1.5 (20H, br s, methylenes), 1.48 (2H, m, CH_2CHOH), 1.62 (2H, m, CH_2CHF), 2.30 (2H, t, *J* 7, CH_2CO), 3.66 (3H, s, CO_2CH_3), 3.51 (1H, m, CHOH) and 4.3 (1H, d of m, J_{HF} 49, CHF); δ_F −195.53 and −195.66; *m/z* 312 (M^+ − HF) and 294 (M^+ − HF, H_2O). This material also contained a minor fluorinated contaminant of unknown structure which was detected by ^{19}F NMR and ^1H NMR spectroscopy but not by GC–MS.

Biotransformation of fluoro fatty acids using *S. cerevisiae*

Each fluorinated substrate was incubated with growing cells of *Saccharomyces cerevisiae*, # ATTC 12341 as previously described.[5a] The desaturated fatty acid products were isolated from the cells by a standard hydrolysis–methylation procedure. Yeast cells from each incubation experiment (*ca.* 5 g, wet weight) obtained by centrifugation were treated with refluxing 1 M 50% ethanolic KOH (50 cm^3) for 3 h under N_2. Cell debris was filtered off and the resultant solution was partially evaporated *in vacuo* to remove ethanol, diluted with water (100 cm^3) acidified to pH 2 with 50% aq. H_2SO_4 and extracted with dichloromethane. This extract was washed with water and sat. brine, dried (Na_2SO_4) and evaporated to give a yellow solid. This was treated with a freshly prepared solution of ethereal diazomethane to yield fatty acid methyl esters (*ca.* 25 mg) after evaporation.

Oxidative cleavage of fatty acids

A solution of the fatty acid methyl esters (20 mg) in *tert*-butyl alcohol–water–0.002 M aq. Na_2CO_3 (20:5:5) was treated with an aqueous oxidant (10 cm^3) which was 0.0025 M in $KMnO_4$ and 0.01 M in KIO_4 with stirring at 28 °C for 48 h. Additional aliquots of oxidant were added as needed until the purple colour persisted. Excess of oxidant was destroyed by addition of $Na_2S_2O_5$ after which the solution was basified with 1 M aq. KOH (5 cm^3) and the Bu^{t}OH removed by roto-evaporation. The remaining cloudy solution was diluted with water (5 cm^3), acidified to pH 2 with 50% aq. H_2SO_4 and extracted with hexanes (4 × 5 cm^3).

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