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Note

In vitro enzymatic oxidation of a fluorine-tagged sulfido substrate analogue: a ^{19}F NMR investigation

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^{1}H-decoupled ^{19}F NMR has been used to monitor the highly regioselective oxidation of a fluorine-tagged thia-fatty acid derivative by castor stearoyl-ACP Δ^9 desaturase. The major enzymatic product, after reductive work-up, was identified as 9-fluoro-1-nonanol. This compound could be easily distinguished from substrate and a 9-sulfoxy by-product on the basis of its ^{19}F NMR chemical shift and spiking experiments using authentic standards. Structural assignment of the cleavage product was confirmed by GC-MS analysis of the enzymatic products. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{19}F substituent effects; oxidation; desaturase

INTRODUCTION

^{19}F NMR is a useful technique for monitoring the biotransformation of fluorine-tagged substrates at the trace analytical level.[1] We have recently developed a methodology that features the use of ω-fluorinated fatty acyl thia-analogues such as **1-ACP** to probe for oxo-transfer reactions carried out by an important class of enzymes known as *desaturases* (Scheme 1).[2] The latter enzymes are critical to lipid biosynthesis in virtually all aerobic life forms[3,4] but are relatively poorly understood from a mechanistic point of view. Our fluorine-based approach was tested recently using an *in vivo* membrane-bound yeast Δ^9 desaturating system[5] and a purified *in vitro* soluble plant Δ^9 desaturase enzyme.[6] In both cases, regioselective sulfoxidation could be easily monitored by ^{19}F NMR owing to long-range substituent effects on a remote fluorine reporter group. Interestingly, in the case of the plant Δ^9 desaturase, we were able to detect an unknown fluorinated product (Compound **U**) produced from **1-ACP** in addition to the sulfoxide product.[6] We proposed that this compound was a chain-cleavage product[6,7] and in this paper we confirm this hypothesis.

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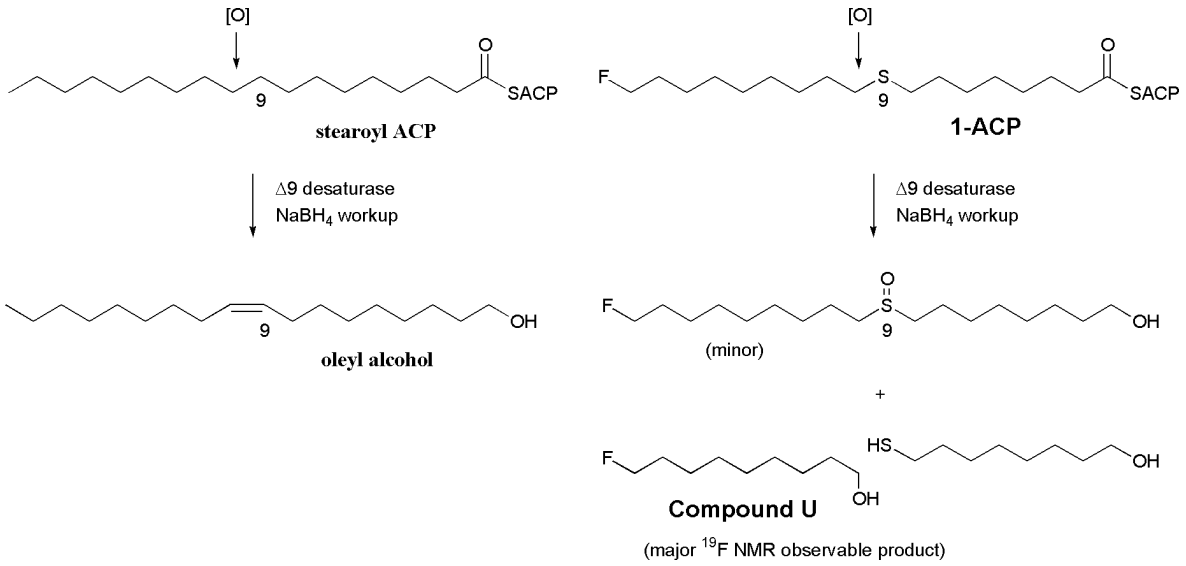
RESULTS AND DISCUSSION

The ACP thioester derivative of 18-fluoro-9-thiaoctadecanoic acid (**1-ACP**, 160 nmol) was incubated for 40 min with freshly prepared Δ^9 desaturase (25 nmol) and cofactors essentially as previously reported.[7] The enzymatic reaction was quenched by the addition of THF/$NaBH_4$ and the resultant terminal alcohols were extracted with dichloromethane; previous experiments have demonstrated that ^{19}F NMR signals of related compounds in aqueous solutions are substantially broadened. An aliquot of the organic extract was allowed to evaporate and the residue so obtained examined by ^{1}H-decoupled ^{19}F NMR after the addition of $CDCl_3$ (570 µl). The ^{19}F NMR spectrum of the product mixture was similar to that obtained previously[6] and is presented in Fig. 1(A). As alluded to in the Introduction, previous experiments[6,7] had suggested that the major fluorinated analyte (**U**) observed at −218.23 ppm might be derived from chain cleavage of the substrate (Scheme 1). Accordingly, 9-fluorononanol was prepared from commercially available 9-bromononanol (See Experimental Section) and an appropriate amount of this material (7 nmol) was added to the enzymatic product mixture (Fig. 1(B)). A single, unbroadened signal of enhanced intensity relative to the minor peaks was obtained at the expected chemical shift (−218.23 ppm). The identification of compound **U** as 9-fluoro-1-nonanol was confirmed by GC-MS analysis of a portion of the enzymatic extract after silylation (See Experimental Section for GC conditions). A GC peak corresponding to the TMS derivative of 9-fluoro-1-nonanol was observed at the anticipated retention time (Fig. 2(A)). The mass spectrum of this analyte also matched that of the reference standard (Fig. 2(B)).



Scheme 1

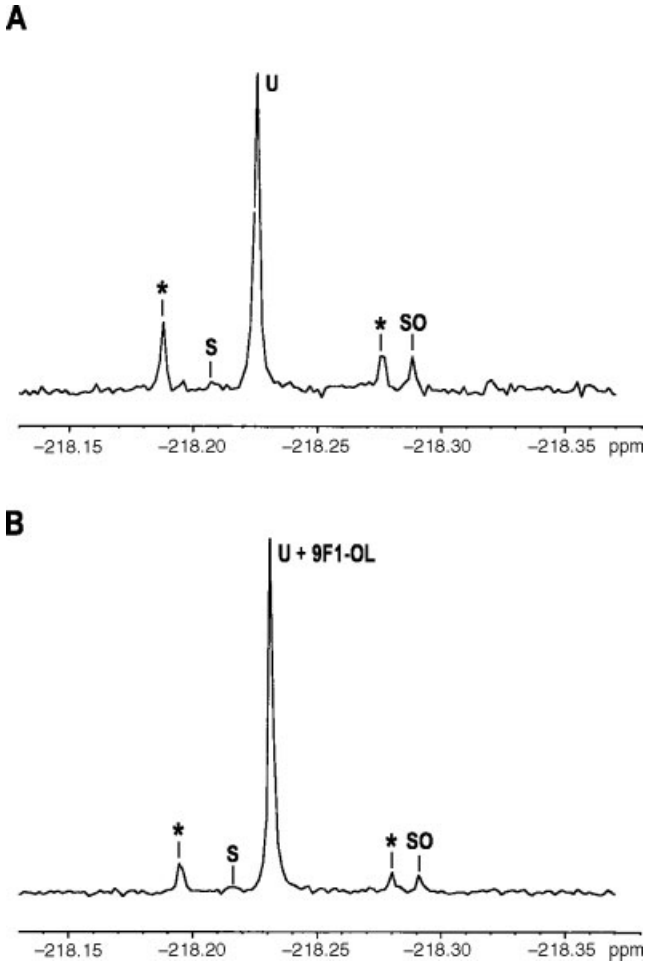


Figure 1. ^{1}H-decoupled ^{19}F NMR (376.5 MHz) spectra of the products ($CDCl_3$ solution) obtained from Δ^9 desaturase-mediated oxidation of 18-fluoro-9-thiaoctadecanoyl ACP before (A) and after (B) addition of 9-fluorononanol reference standard. Analytes were obtained by reductive work-up of the enzymatic reaction using $NaBH_4$, followed by CH_2Cl_2 extraction. *Unidentified resonances. S = Substrate; SO = 9-sulfoxide product; U = Previously unidentified resonance; 9F1-OL = 9-fluorononanol.

Two of the minor peaks in the ^{19}F NMR spectrum of the enzymatic product mixture were identified as those corresponding to the residual substrate (S, −218.22 ppm) and sulfoxide product (SO, −218.29) via spiking experiments with authentic reference standards (data not shown). Two minor signals (−218.19, −218.28 ppm) remain unidentified; these were not present in the ^{19}F NMR spectrum of the starting material (S) (detection limit: 0.05% of major analyte), and the GC-MS analysis of the extract did not reveal appreciable amounts of any analyte in addition to the TMS derivative of 9-fluorononanol (the sulfoxide by-product is thermally labile and is not GC-observable). It should be noted that owing to the enhanced volatility of 9-fluoro-1-nonanol relative to longer chain compounds, the intensity of these minor peaks in the ^{19}F NMR spectrum has, in all likelihood, been artificially enhanced upon evaporative concentration of the extract.

The identification of compound **U** substantiates our previous prediction that **1-ACP** undergoes desaturase-mediated oxidation primarily α to sulfur owing to a strict enzyme-imposed regiochemical imperative.[6,7] Early work using thia-analogues as mechanistic probes[8] also led to the hypothesis that when the enzyme oxidant (probably a hypervalent diirondioxo complex)[3] is not optimally aligned with the sulfur atom of a thia-substrate, a chain-cleavage process can occur. Our data correlate well with the results obtained recently by Fox and coworkers who used mass spectrometry to analyze enzymatic products from Δ^9 desaturase-mediated oxidation of the nonfluorinated analogue of **1-ACP**.[9] One possible pathway to compound **U** is illustrated in Scheme 2 and involves hydroxylation at C-10 followed by spontaneous collapse of the intermediate hemithioketal to aldehyde and thiol. $NaBH_4$ reductive work-up generates 9-fluorononanol (**U**) from the intermediate aldehyde.

CONCLUSIONS

A novel reaction product of a desaturase-mediated reaction was detected for the first time by ^{1}H-decoupled ^{19}F NMR[6]

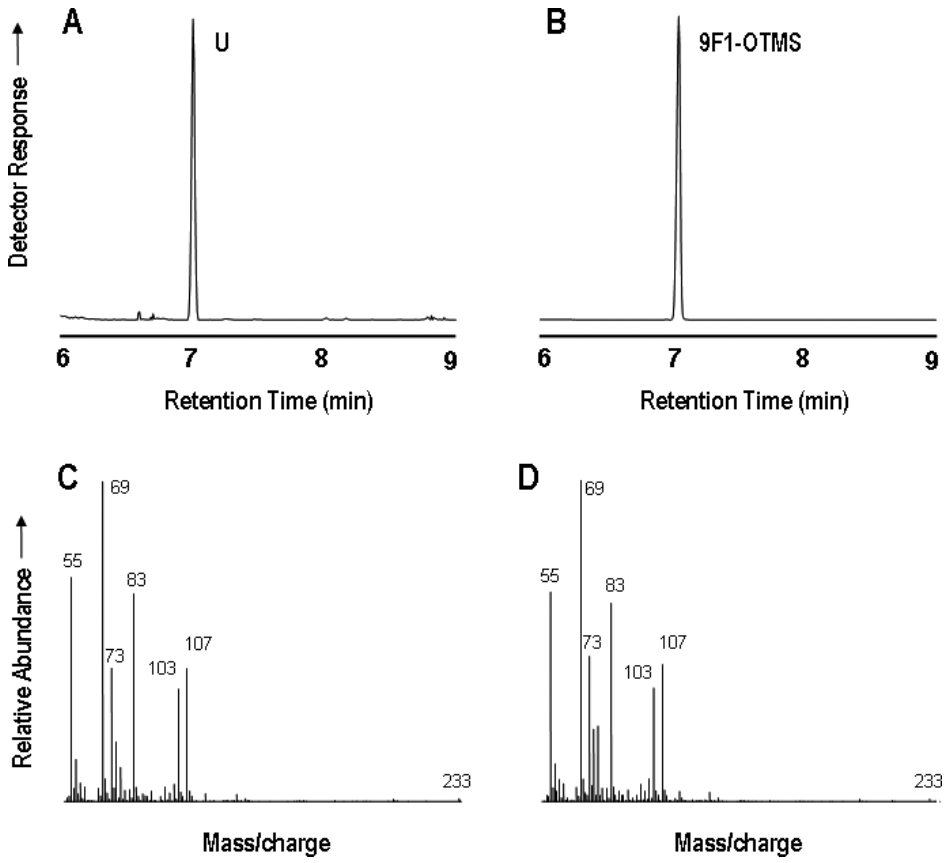
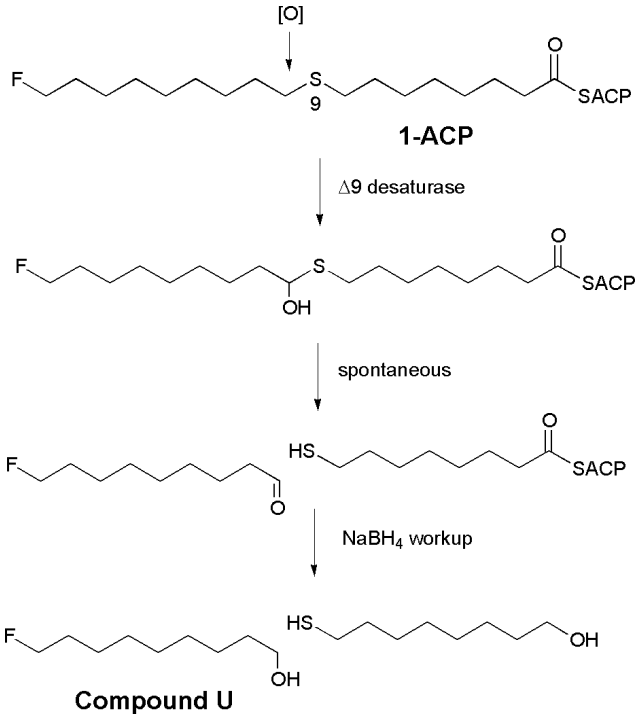


Figure 2. Partial GC-MS TIC chromatogram of products (TMS derivatives) obtained from Δ^9 desaturase-mediated oxidation of 18-fluoro-9-thiaoctadecanoyl ACP (A) and TMS derivative of reference standard, 9-fluorononanol (B). Mass spectral analysis of major enzymatic product **U** (C) and TMS derivative of reference standard, 9-fluorononanol (D). U = Previously unidentified enzymatic product; 9F1-OTMS = TMS derivative of 9-fluorononanol.



Scheme 2

and in the present work, the predicted structure for this compound has now been independently confirmed by ^{19}F NMR and GC-MS. This result, in combination with other mechanistic information,[2] suggests that the putative diiron-dioxo oxidant initiates the parent dehydrogenation reaction at C-10 (Scheme 1). The lack of background interferences, high sensitivity and wide chemical shift range of ^{19}F NMR greatly enhance the utility of a remote fluorine substrate tag in probing enzymatic reactions. We are currently exploring other applications of ^{19}F NMR in the trace analytical mode that will contribute to the bioorganic investigation of enzyme mechanisms.

EXPERIMENTAL

Materials

18-fluoro-9-thiaoctadecanoic acid and the corresponding sulfoxide were available from a previous study.[6] 9-Fluoro-1-nonanol was synthesized by fluorination of the corresponding commercially available 9-bromo-1-nonanol following a standard literature procedure:[10] A solution of tetrabutylammonium fluoride TBAF (5.86 g, 22 mmol) in THF (45 ml) was stirred over 3A molecular sieves under N_2 for 1 h. 9-bromo-1-nonanol (1.02 g, 4.5 mmol) in THF (10 ml) was transferred to the TBAF solution and stirred under N_2 at r.t. for 3 h. After THF was removed *in vacuo*, H_2O (20 ml) was added to the resultant syrup and the aqueous layer extracted with hexanes (4 × 20 ml). The organic layer was washed with sat. NaCl (30 ml) and dried (Na_2SO_4). The crude

product (456 mg) was purified using flash chromatography (Hex/EtOAc 1 : 20) to remove residual starting material. A sample of 9-fluoro-1-nonanol was obtained as a colorless liquid[11] (55 mg, >99% pure by GC-MS). The analytical data (MS, ^{1}H, ^{13}C, ^{19}F NMR) of this material was in accord with expectations: TLC (SiO_2 hexane/EtOAc 70 : 30): R_f 0.21. IR (film): γ_{max} 3336, 2929, 2856, 1465, 1391, 1058, 723 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$): 4.44 (*dt*, $^2J_{HF}$ = 47.4, 2 H); 3.64 (*t*, $^3J_{HH}$ = 6.6, 2 H); 1.69 (*dm*, $^3J_{HF}$ = 25.0, 2 H); 1.57 (*tt*, $^3J_{HH}$ = 7.2, 2 H); 1.45 (*bd s*, 1 H); 1.28–1.45 (*m*, 10 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 84.23 (*d*, $^1J_{CF}$ = 164.0); 63.02; 32.76; 30.39 (*d*, $^2J_{CF}$ = 19.4); 29.45; 29.31; 29.16; 25.70; 25.13 (*d*, $^3J_{CF}$ = 5.5). ^{19}F NMR (376.5 MHz, $CDCl_3$): −218.23. EI-MS: *m/z* 144 (0.4, $[M-18]^+$), 116 (19), 88 (32), 69 (66), 55 (100), 41 (79). EI-MS (TMS derivative): *m/z* 233 (<1, $[M-H]^+$); 214 (<1, $[M-HF]^+$); 199 (<1, $[M-HF, CH_3]^+$), 107 (52), 103(49), 83(65), 69(100), 73(55), 55(88).

Incubations

The purification of castor stearoyl-ACP Δ^9 desaturase, required cofactors and the synthesis of substrate ACP derivatives has been previously described.[12,13] Incubations of acyl-ACP derivatives (10 × 16 nmol batches) with freshly prepared Δ^9 desaturase (2.5 nmol) were carried out at room temperature. The reaction was initiated by the addition of NADPH (1.0 mg, 1.2 μmol) in 100 mM Tricine, pH 8.0 buffer (50 μl) and allowed to continue for 30 min. The reaction was terminated with the addition of THF (100 μl), and the thioester linkage of the ACP derivatives was reduced to the corresponding alcohol with $NaBH_4$ at 37 °C for 15 min. The residue was diluted with H_2O (1 ml) and extracted with CH_2Cl_2 (2 × 2 ml). The phases were separated by centrifugation; the organic layer was collected and concentrated by passive evaporation for 15 h at 20 °C. Owing to the volatility of the major analyte (9-fluorononanol), evaporation of the extract under a N_2 stream was avoided.

The product mixture was analyzed directly by ^{19}F NMR and by GC/MS (60 m Supelco 2340 poly(biscyanopropyl siloxane) 0.25 mm ID capillary column with 1.5 ml/min flow rate operated in the splitless mode with an inlet temperature of 250 °C and a gradient of 100–140 °C at 5 °C/min) after silylation. Using the latter technique, it could be demonstrated that the enzymatic reaction had consumed all but traces of the substrate. This observation was confirmed by subsequent ^{19}F NMR analysis.

NMR measurements

^{19}F NMR spectra were recorded at 300 K on a Bruker AVANCE 400 (9.4T) spectrometer operating at 376.50 MHz with a dedicated 5 mm ^{19}F/^{1}H probe and a ^{19}F-specific preamplifier. A Bruker ^{1}H band-pass/^{19}F band-stop filter was used in the proton channel and a ^{19}F band-pass/^{1}H band-stop filter was used in the fluorine observe channel for all acquisitions. WALTZ-16 was used for proton decoupling. Standard microprograms from Bruker software were employed. All spectra were run using 128 K data points with a spectral width of 37 650 Hz, which gave a final spectral resolution of 0.287 Hz. Exponential multiplication with a line broadening of 0.28 Hz was applied. The spectra were acquired using the 90 degree pulse, a delay time of 1.0 s, an acquisition time of 1.74 s, and 20 000 scans. All chemical shifts are referenced to neat external trichlorofluoromethane ($CFCl_3$) at 0.00 ppm. Samples were dissolved in 0.57 ml $CDCl_3$, which had been previously filtered through neutral alumina.

Acknowledgements

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