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**The Fluorine Substituent Effect on the Hydroxylation
of Nonane by *Pseudomonas oleovorans***

by

Tanya MacPherson, B.Sc.

A thesis
submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements
for the degree of
Master of Science

Department of Chemistry

Carleton University
Ottawa, Ontario

April 1994

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"The Fluorine Substituent Effect on the Hydroxylation
of Nonane by *Pseudomonas oleovorans*"

submitted by Tanya MacPherson, B.Sc.,
in partial fulfillment of the requirements for the degree of Master of Science

Dr. Peter H. Buist
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Abstract

The compounds 2-fluorononane, 3-fluorononane, 5-fluorononane, and 1,1-difluorononane were synthesized. These compounds plus 1-fluorononane and nonane were incubated with *Pseudomonas oleovorans* in a fluorine substituent study of the hydroxylation of nonane by this organism. Fluorine was found to retard hydroxylation at a β -carbon and a γ -carbon but to not affect hydroxylation at an ϵ -carbon. Fluorine on C-1 activated hydroxylation at this position but two fluorine substituents on C-1 deactivated hydroxylation at this carbon. These results parallel chemical studies of hydrogen atom abstractions from fluorinated alkanes in free radical reactions.

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1.0 Introduction

1.1 General

Pseudomonas oleovorans is a Gram negative bacterium which is capable of complete mineralization of aliphatic hydrocarbons.¹ The monooxygenase system induced in *Pseudomonas oleovorans* by growth on alkanes catalyzes the terminal methyl group hydroxylation of alkanes and fatty acids. This Pseudomonad was first isolated by Coon² and his students from soil by the enrichment-culture technique and was identified to be a strain of *Pseudomonas oleovorans*, a bacterium previously isolated from machine shop cutting oil by Lee and Chandler.³ The monooxygenase system is an NADH/O₂-dependent enzymatic system which contains three components: reductase, rubredoxin and hydroxylase.⁴ It is similar to another system found widely distributed in living organisms called cytochrome P-450. Cytochrome P-450 is a heme-containing enzyme which catalyzes the oxidation of many compounds including steroids, fatty acids and drugs.⁵ The stoichiometric equation of the *Pseudomonas oleovorans* monooxygenation reaction is as follows:^{6,7}

Monooxygenases are systems which catalyze dioxygen activation using two electrons and two protons, with the insertion of one oxygen atom from O₂ into a substrate and the formation of water.⁵ The reductase is a flavoprotein which transfers two electrons from NADH (DPNH) to the iron-sulphur centre of rubredoxin via its bound FAD. The rubredoxin, a red non-heme iron protein,⁴ accepts two electrons from the reduced

reductase one by one and then transfers them to the hydroxylase. ω -Hydroxylase catalyzes the reduction of O_2 to H_2O and the oxidation of an alkane to an alcohol. Electron transfer in this system is shown in Figure 1⁸ and is as follows: $NADH \rightarrow$ reductase $\rightarrow$ rubredoxin $\rightarrow$ ω -hydroxylase $\rightarrow O_2$.

Figure 1. Electron carriers in ω -hydroxylation

Both the reductase and rubredoxin have been isolated and characterized. The hydroxylase is too unstable to be purified to homogeneity but it has been characterized as a non-heme iron protein⁹ with one iron atom and one cysteine per 40,800 molecular weight polypeptide chain and contains a high concentration of phospholipid plus carbohydrate.¹⁰ The first crystal structure of a non-heme iron hydroxylase was recently obtained¹¹ from the hydroxylase protein of methane monooxygenase from *Methylococcus capsulatus*. The active site contained two iron atoms bridged by exogenous hydroxide and acetate ligands and further coordinated by four glutamate residues, two histidine residues, and a water molecule. The dinuclear iron centre lies in a hydrophobic pocket for binding methane. There is an extended canyon surrounded with many long α -helices for possible docking of the reductase and coupling proteins required for catalysis.

In 1973, Chakrabarty¹² found that the enzymes responsible for the oxidation

of *n*-octane to octanoic acid or beyond in *Pseudomonas oleovorans* are octane inducible and are coded by genes borne on a transmissible extrachromosomal element. The nonconjugative plasmid which encodes the *n*-alkane degradation pathway in *P. oleovorans* is now called the OCT plasmid. The three components or enzymes of the alkane monooxygenase system are encoded by the *alkB*, *alkG*, and *alkT* genes, located in two separate transcription units separated by at least 50 kb on this plasmid. Below is a representation of the genetic organization of the *n*-alkane pathway.¹

Scheme 1. Genetic organization of the *n*-alkane pathway

The *n*-alkane pathway is encoded by two transcription units, *alkST* and *alkBFGHJKL*, located on the OCT plasmid. The conversion of aliphatic hydrocarbons to fatty acids is catalyzed by alkane monooxygenase (*alkBGT*) and by plasmid-encoded or chromosomally encoded alcohol dehydrogenases (*alkJ* or *alcA*) and aldehyde dehydrogenases (*alkH* or *aldA*). *alkS* encodes the substrate-activated positive

regulatory of the *alkBFGHJKL* operon. No roles in the metabolism of alkanes have been assigned to the products of the *alkF*, *alkK*, and *alkL* cistrons.

The ω -hydroxylase enzyme is believed to be immersed in the lipid bilayer of the cytoplasmic membrane and also partially exposed to water.¹ Its substrate range is aliphatic hydrocarbons of six to ten carbons in length and *in vitro* larger aliphatic hydrocarbons (C_{11} - C_{18}) can also be converted into primary alcohols but these compounds do not induce the alkane pathway.¹ Hydroxylation of straight chain fatty acids and alkanes by *Pseudomonas oleovorans* always occurs at the ω -position, so the enzyme which performs this oxidation was termed ω -hydroxylase by Coon⁴ et al by 1966. However, the enzyme was found later to execute a number of other oxidations so the system is now referred to as POM¹³ (*Pseudomonas oleovorans* monooxygenase). POM readily converts terminal olefins into epoxides,¹⁴ saturated ketones into allylic alcohols,¹⁵ and sulfoxidizes methyl sulfides¹⁶ in stereo-selective reactions. As well, it catalyzes the O-demethylation of methyl ethers.¹⁶ All of these reactions were shown to be mutually competitive indicating that they occur at the same active site of the enzyme.

1.2 Mechanism of ω -hydroxylase

This enzyme is thought to operate using a free radical mechanism similar to cytochrome P-450 which also hydroxylates straight chain alkanes by the following reaction pathway:¹⁷

Scheme 2. Mechanism of ω -hydroxylase

Chi-Huey Wong¹⁶ has observed ring opening during the hydroxylation of *trans*-2-phenyl-1-methylcyclopropane by ω -hydroxylase so the oxygen insertion is not a concerted process but probably involves hydrogen atom abstraction to form a carbon-centred radical followed by hydroxyl rebound from iron to carbon similar to that implicated for heme-containing cytochrome P-450.¹⁷

Figure 2. Mechanism of oxygen insertion

The rate determining step for the oxidation reaction is probably the abstraction of a hydrogen atom from the substrate to form a radical with hydroxyl rebound occurring rather quickly.¹⁹ The mechanism of the oxygen insertion is probably a bimolecular homolytic substitution.¹⁷ Caspi found that the C-1 hydroxylation of octane proceeded with retention of configuration of the molecule, i.e. the incoming hydroxyl assumed the orientation of the displaced hydrogen atom.²⁰ Based on substrate radical stability alone, it would be expected that the $\omega - 1$ position would be the preferred position, as is observed for cytochrome P-450, since a secondary radical is thermodynamically more stable than a primary radical. Perhaps this is due to steric factors in the enzyme active site or to a fundamental difference in mechanism between heme-containing cytochrome P-450 and non-heme iron monooxygenases (POM). It is this difference in oxidation site which makes the study of the mechanism of POM interesting.

1.3 Experimental Approach

The purpose of this thesis was to measure the substituent effect of fluorine on the ω -hydroxylation of nonane as a function of distance from the terminal carbon and to compare the results obtained with the chemical work of J.M. Tedder²¹ who studied hydrogen abstraction from alkanes by various free radicals. A series of modified substrates representing different fluorine substituent effects (see Figure 3) were incubated with the organism *Pseudomonas oleovorans* in a whole cell study. They included 1-fluorononane, 2-fluorononane, 3-fluorononane, 5-fluorononane, 1,1-difluorononane and nonane itself. 1-Fluorononane, 2-fluorononane, 3-fluorononane, and 1,1-difluorononane contain two oxidizable positions which experience different effects from a fluorine substituent via an intramolecular competition. A competition

between nonane and 5-fluorononane constitutes an intermolecular competition between two different methyl hydrogens and the ratio of metabolites gives a comparison of reactivity, or the fluorine substituent effect. As well, this intermolecular competition gives an indication of the ability of a fluorinated substrate to bind to the active site of the molecule in comparison to an unsubstituted substrate.

Figure 3. Series of fluorinated substrates

The fluorine substituent effect can be broken down into the components inductive and conjugative. The inductive effect is the term given to any electron displacement due to the difference in electronegativity between the substituent and the rest of the molecule which is transmitted through σ bonds.²² Fluorine is more electronegative than carbon so the bond attaching a fluorine atom to carbon will be polar with a higher electron density at the fluorine end of the bond. The polarity will

necessarily have some effect on the adjacent bonds, both through bonds and through space. Fluorine is called an electron-withdrawing substituent by the inductive effect. In a saturated alkane derivative, the inductive effect falls off very rapidly and becomes almost negligible beyond two carbon atoms.²² The π -conjugative effect is the term given to electron displacement due to delocalization of electron density through p orbitals. There are two kinds, effects due to substituents with filled orbitals of symmetry such that they can interact with a p orbital on a neighboring carbon (donors) and effects due to substituents with low lying vacant orbitals with a symmetry which permits interaction with a p orbital (acceptors). Fluorine has lone pairs and is an electron donor by the π -conjugative effect. This type of conjugative effect is only applicable for the carbon carrying the fluorine.

In studying the substituent effect of fluorine in *n*-butyl fluoride, Tedder²¹ found that fluorine retarded hydrogen abstraction from the β -carbon atom and exerted a slight influence over the γ -carbon atom but did not affect hydrogen atom abstraction at the δ -carbon atom.

It is the inductive effect which is operative here. The α -position was slightly deactivated when $\text{Cl}\cdot$ radical was used as the hydrogen atom abstractor but it was actually activated when $\text{Br}\cdot$ radical was used as the abstractor. The higher reactivity of the α -position can be explained by resonance stabilization of the incipient alkyl radical by π -conjugation.²¹ The mesomeric effect (+M) or conjugative effect is in opposition to the inductive effect (-I). In bromination and with hydrogen abstraction

by methyl radicals²³ the resonance stabilization (+M) overcomes the inductive effect (-I), and the hydrogen atoms on the same carbon as the substituent are activated. In 1965 Fessenden and Schuler²⁴ discovered that the fluoromethane, difluoromethane, and trifluoromethane radicals each have different geometries ranging from almost planar to pyramidal in structure. Bingham and Dewar²⁵ thought that this deviation from planarity was probably due to antibonding interactions between lone pair electrons of the substituent and the MO's arising from interactions between the singly occupied carbon AO and the MO's of adjacent C-H bonds. They reasoned that a single substituent with lone pairs would be weakly bonding by the conjugative effect and result in mesomeric stabilization of the radical. Two substituents with lone pairs would probably be destabilizing since the antibonding interaction probably outweighs the bonding one. The substituents would therefore antagonize one another and this antagonism is increased the greater the resonance integral between the carbon and the substituents. The resonance integral is greatest when the radical is planar and the carbon AO is a 2p AO. This results in bending of the substituents away from planarity to form a pyramidal structure.

Based on the data presented from the literature, it would be expected that hydrogen atom abstraction would be activated at the fluoromethyl group of 1-fluorononane, retarded at the methyl group next to fluorine in 2-fluorononane, retarded slightly at the methyl group two carbons removed from the fluorine substituent, and that hydrogen atom abstraction from an ϵ -carbon would be unaffected by the fluorine substituent (5-fluorononane). Hydrogen abstraction from the difluoromethyl group in 1,1-difluorononane would be expected to be retarded as two fluorine substituents on the same carbon destabilize the incipient radical so more

energy would be required to break the C-H bond.²⁶

The transition state in hydrogen abstraction reactions must have some of the characteristics of both reactants and products. If we consider that one bond must be broken and another one formed in the transition state, the bond dissociation energies will give a measure of the ease of the reaction. The bond dissociation energy (D) is the energy required for homolytic cleavage of a bond.²⁷ The smaller D(C-H) and the larger D(FeO-H), the lower the activation energy. The breaking of the C-H bond will be greatly affected by the stability of the incipient radical, and the greater the resonance stabilization of the alkyl radical the smaller is D(C-H). D(FeO-H) is the same for all of the substrates. The breaking of the C-H bond is almost a nonpolar event, however, the forming of the FeO-H bond is a polar event. Therefore, the transition state will have some polar character.

The ease with which this structure can be formed will be hindered by electron withdrawing groups and aided by electron donating groups.²¹ Hydrogen atom abstraction will be favored at the site which can most easily accommodate a positive charge. This is the basis of the competition between the different ends of the molecule in the intramolecular competitions and the different molecules in the nonane/5-fluorononane competition. The rate of oxidation by ω -hydroxylase would then be dependent on the ability of the enzyme to break the C-H bond.

Precise measurement of the fluorine substituent effect on the ω -hydroxylation of nonane could not be realized due to time constraints, however, general comparisons between experiments with substrates fluorinated in different positions were made.

The ratio of the amounts of metabolites present at forty hours incubation time was chosen as representative of the reactivity of each methyl group.

$$\alpha \leq X \quad (1.3.1)$$

Rate of hydroxylation at nonfluorinated end
Rate of hydroxylation at fluorinated end

$\alpha = \frac{\Sigma \text{Metabolites from nonfluorinated end}}{\Sigma \text{Metabolites from fluorinated end}}$

Where X is the ratio of reactivity of the methyl group from the nonfluorinated end to the methyl group from the fluorinated end. The metabolites were analyzed by GC/MS with verification by some standards which were synthesized or obtained. Degradation of alcohols from the nonfluorinated end of the molecules would be expected to be faster than degradation of alcohols from the fluorinated end of the molecules thus all ratios can be regarded as upper limits. As not all of the metabolites were recovered, these ratios can only be regarded as very approximate. The results obtained constitute a preliminary study.

2.0 Results and Discussion

2.1 Synthesis of Substrates and Standard

The compounds utilized in this study were obtained commercially or synthesized. Nonane and 1-fluorononane were purchased from The Aldrich Chemical Company while 2-fluorononane, 3-fluorononane, 5-fluorononane and 1,1-difluorononane were synthesized from 2-nonanol, 3-nonanol, 5-nonanol and nonanal respectively. Diethylaminosulfur trifluoride (DAST) was utilized for the substitution of the alcohol group with the fluorine group by the method of Middleton.²⁸

DAST

The reaction of DAST with alcohols to replace the hydroxyl group with fluorine is a general reaction in which primary, secondary, and tertiary alcohols all react with high yields of the unrearranged fluoride usually resulting.

Two problems can occur when replacing the hydroxyl group: carbocation type rearrangements and dehydration. No carbocation rearrangements were observed, however, the alkene was present as an impurity in each synthesis. This was oxidized with KMnO_4 using a phase transfer agent^{29,30} and the fluoroalkanes were separated from the resulting carboxylic acids and any remaining starting material by the technique of flash chromatography. 1,1-Difluorononane was synthesized from nonanal also by the method of Middleton.²⁸ DAST replaces the carbonyl oxygen of

aldehydes and ketones with two fluorine atoms.

Diethylaminosulfinyl fluoride is hydrolyzed to give sulfur dioxide and diethylamine hydrofluoride.

Biological standards were necessary for the confirmation of metabolite structure by GC/MS but only a few were available. Nonanol and nonanoic acid were purchased from Aldrich, 3-fluorononanoic acid was synthesized previously in our laboratory by Reese Adeney,³¹ but 9-fluorononanoic acid was synthesized as shown in scheme 3. LiAlH_4 and DAST do not react with a double bond so they did not give side products.

Structures were assigned to fluorinated materials by ^1H NMR and ^{13}C NMR. Fluorine couples strongly with protons and with ^{13}C with characteristic coupling constants. In monofluorinated compounds J_{HF} = 47-49 Hz for geminal coupling and J_{HF} = 6-7 Hz for vicinal coupling, J_{CF} = 164-167 Hz for geminal coupling, J_{CF} = 19-23 Hz for vicinal coupling, and J_{CF} = 4-6 Hz for coupling through four bonds. In 1,1-difluorononane J_{CFF} = 239 Hz for geminal coupling, J_{CFF} = 21 Hz for vicinal coupling, and J_{CFF} = 5 Hz for coupling through four bonds. J_{HF} = 57 Hz for coupling of two fluorine substituents with a geminal proton. Protons and carbons which were not coupled to fluorine were assigned by comparison with the assignments to 1-fluorooctane.³²

Scheme 3. Synthesis of 9-fluorononanoic acid

2.2 Biological Experiments

Pseudomonas oleovorans was cultivated using octane as the sole carbon and energy source as this alkane is known to induce the monooxygenase system and if nonane had been used to grow the culture, then there may have been some metabolites present before the incubation experiments were initiated. The octane was removed from the culture by washing three times with phosphate buffer and centrifuging the cells to a pellet. There was some concern that octane or the metabolites of octane may still be present in the culture and interfere with the separation and identification of nonane metabolites. The G.C. retention times and mass spectra of authentic standards of octane, octanol, and methyl octanoate were referred to during the characterization of nonane metabolites and no such compounds were found. As well, the possibility of finding β -oxidation products with an even carbon chain length was considered during the analysis of the mass spectra and none were found.

The bacterial culture was grown up at a temperature of 28°C since above this temperature the culture turns yellow-green and the enzyme activity is reduced.¹⁰ The cells were harvested in the late exponential phase as this is the time when the culture has the largest number of growing cells. The cells were washed and pelleted by centrifugation and then resuspended in phosphate buffer. The cells were incubated at 28°C for forty hours in each experiment. The compounds were not all administered at one time so there were four different sets of experimental conditions as presented in Table 1 below:

Table 1. Experimental Conditions

Expt.	Substrate	Culture O.D.	Culture Volume	Substrate Volume
1	Nonane	1.5	30 mL	500 μ L
2	1-Fluorononane	1.0	15 mL	100 μ L
3	2-Fluorononane	1.5	26 mL	140 μ L
3	3-Fluorononane	1.5	26 mL	180 μ L
4	5-Fluorononane	1.5	30 mL	224 μ L
4	1,1-Difluoro- nonane	1.5	30 mL	220 μ L

Time did not permit the optimization of conditions. Some preliminary optimization of the experimental conditions with 500 μ L of substrate was performed, however, the purification of the synthesized compounds proved to be difficult so less was administered. The 1-fluorononane experiment had the least number of cells with an O.D. of 1.0 and a volume of 15 mL, however, it also had the smallest volume of substrate administered.

Each of the experiments was repeated at least once. A representative set of data for the incubation of each substrate was presented. The experiments which were performed with the most highly purified substrates and the experiments which showed an accumulation of the greatest variety of metabolites were chosen in each case.

After incubation with the substrates, the pH of the extracts was reduced to 2 in order to

ensure that the anticipated carboxylic acids (*vide infra*) would partition into the organic phase. The alcohols and other metabolites already preferentially partitioned into the organic phase. In order to make all products available for observation by gas chromatography/mass spectrometry, the extracts of the incubations were derivatized with diazomethane to produce the methyl ester of the carboxylic acids. The alcohols and aldehydes were volatile enough to be observed directly by gas chromatography.

In addition to the initial oxidation product expected for the ω -oxidation of a substrate, a series of metabolites was obtained for each substrate administered. Degradation led to two series of metabolites for 1-fluorononane, 2-fluorononane, 3-fluorononane, and 1,1-difluoro-nonane while one possible set of metabolites was found for each of nonane and 5-fluorononane. Nonane and 5-fluorononane each have a plane of symmetry bisecting the molecule at C-5 so metabolism at either end of the molecule would produce the same degradation series. The metabolism of both ends of a substrate concurrently to form a nonanediol or a nonanedioic acid, etc., was not observed.

Each metabolite series can be accounted for using the metabolic pathway described in Scheme 4. *Pseudomonas oleovorans* monooxygenase (POM) is the first enzyme system in the degradation of a straight chain alkane of C₆ to C₁₄ chain length³³ It oxidizes an unactivated methyl carbon to an alcohol in a mixed function oxidase reaction. The next reaction in the metabolism of the alkane is the dehydrogenation of the alcohol to form the corresponding aldehyde by alcohol dehydrogenase.³⁴ The oxidation of the aldehyde to the acid oxidation state occurs in an aldehyde dehydrogenase reaction in which the acyl-phosphate is formed from the aldehyde and inorganic phosphate. In a coupled reaction, ADP kinase removes the phosphate group to generate ATP and the conjugate base of the carboxylic acid.³⁵ Before fatty acids can be oxidized, they must be "primed" for reaction in an ATP-dependent acylation reaction to form fatty

acyl-CoA. This activation process is catalyzed by a family of at least three acyl-CoA synthetases that differ according to their chain length specificities.³⁶ Fatty acids are catabolized through the β -oxidation of fatty acyl-CoA, a process that occurs in four reactions which are shown as the last four reactions in Scheme 4 and known collectively as the β -oxidation pathway. They are formation of a *trans*- α,β double bond through dehydrogenation by the flavoenzyme acyl-CoA dehydrogenase, hydration of the double bond by enoyl-CoA hydratase to form a 3-L-hydroxyacyl-CoA, dehydrogenation of this β -hydroxyacyl-CoA by 3-L-hydroxyacyl-CoA dehydrogenase to form the corresponding β -ketoacyl-CoA, and $C_\alpha - C_\beta$ cleavage in a thiolysis reaction with CoA as catalyzed by β -ketoacyl-CoA thiolase to form acetyl-CoA and a new acyl-CoA containing two less carbon atoms than the original one.³⁷

Scheme 4. The *n*-alkane metabolic pathway

2.3 Nonane Incubation

Scheme 5 represents the expected products from the incubation of nonane with the bacterial cultures based on the metabolic pathway given in Scheme 4. Figure 4 shows the GC/MS results from the control experiment and the nonane experiment. In all of the substrate gas chromatograms presented, the peaks marked with a "c" are peaks which are intended to be ignored as they are also present in the control chromatograms. There was some variability in the control experiments as can be seen by comparing figures 1,3,7, and 15. The reason for this is unknown. Common products of almost all of the incubations were the methyl esters of four fatty acids (eluting after 17 minutes): methyl palmitoleate, methyl palmitate, methyl oleate, and methyl stearate. These are derived from fatty acids present in the phospholipid layer of the bacterial cell's outer membrane. They are marked with a "c" in the experimental chromatograms in order to prevent confusion with metabolite peaks. The other peaks present in the control chromatograms were unidentified except for the internal standard 2-nonanol which was present in the control of the nonane experiment only.

Two metabolites were identified, nonanol and methyl nonanoate. These were confirmed by comparison of the G.C. retention times and the mass spectra with those of authentic standards. No products of further metabolism were observed, although this does not mean that the metabolism of nonanoic acid did not take place.

The fragmentation pattern of methyl nonanoate is displayed in Scheme 6 for future reference to the other fluorinated metabolites which will be discussed in the thesis. The molecular ion peak at $m/z=172$ is present and the most intense peak is at $m/z=74$ which is due to the McLafferty rearrangement. A methyl ester of an aliphatic acid unbranched at the α carbon gives a strong peak at m/z 74, which, in fact, is the base peak in straight-chain methyl esters from C_6 - C_{26} .³⁸ Bond cleavage next to $C=O$ results in two ions: the resonance-stabilized acylium ion³⁹

at $m/z=141$ and the $\text{CH}_3\text{OC}=\text{O}^+$ ion³⁸ at $m/z=59$. The hydrogen rearrangement and the C-C bond cleavage two bonds removed from $\text{C}=\text{O}$ gives the second most intense peak at $m/z=87$. This is another one of McLafferty's rearrangements.⁴⁰

Scheme 5. Nonane incubation

Figure 4. Nonane chromatograms

Runname : 00499 Acquired Nonanal data (Zero baseline). 5 Jan 94 2:13
Unmarked data only:

Runname : 00503 Acquired Nonanal data (Zero baseline). 5 Jan 94 6:06
Unmarked data only:

Figure 5. Nonane mass spectra

Scheme 6. Methyl nonanoate fragmentation pathway

2.4 1-Fluorononane Incubation

Upon incubation of the bacteria with 1-fluorononane, two different series of metabolites would be expected depending on which end of the molecule was metabolized as shown in Scheme 7. Hydroxylation at the fluorinated end of the molecule would be expected to result in a spontaneous dehydrohalogenation to form the aldehyde which would then be oxidized to the acid by a dehydrogenase enzyme to form nonanoic acid. If β -oxidation also occurred, then heptanoic acid, pentanoic acid, etc., and their intermediates might also be observed. Hydroxylation at the end ω to the fluorine substituent would give 9-fluorononanol which would then be acted upon by dehydrogenases to give 9-fluorononanal and 9-fluorononanoic acid. Further metabolism by the β -oxidation pathway would give the products 7-fluoroheptanoic acid, 5-fluoropentanoic acid, etc. The products which were observed in Figure 6 were the carboxylic acids from either end of the molecule and the β -oxidation intermediates from either end of the molecule. These were 9-fluorononanoic acid, nonanoic acid, 3-hydroxynonanoic acid, and 9-fluoro-3-hydroxynonanoic acid.

9-Fluorononanoic acid was synthesized as described in Scheme 3 on page 14 and the mass spectrum of its methyl ester was used for comparison purposes (see Figure 7). The molecular ion was present at $m/z=190$ and the base peak was the McLafferty rearrangement at $m/z=74$ as was expected for a methyl ester. The second most prominent peak was the other McLafferty rearrangement at $m/z=87$. Also present were the fragment ion representing loss of the methoxy radical and the radical ion representing loss of HF. The structures of methyl 3-hydroxynonanoate and methyl 9-fluoro-3-hydroxynonanoate were inferred from their mass spectra (see Figure 8). Note that mass spectral background peaks were marked with an "x" so that they would not be confused with fragment ion peaks. This practice will be continued for the rest of the discussion. A library search indicated that an aliphatic methyl ester hydroxylated at C-2 or C-3 would give

a base peak at $m/z=103$ as did these metabolites. Such a fragment is called a 2-hydroxy-4-methoxyoxetanium ion.⁴¹ Since 3-L-hydroxyacyl-CoA is an expected intermediate in the β -oxidation cycle from the enoyl-CoA hydratase enzyme (see Scheme 4, p.19), it has been assumed that it was the 3-hydroxy metabolites which were synthesized by the bacteria. While a molecular ion was unavailable for either structure, very small peaks representing M-18 and M-31 were seen and the McLafferty rearrangement for methyl esters was observed at $m/z=74$.

The ratio for the initial hydroxylation of the fluoromethyl to the methyl group, based on the sum of the heights of the methyl nonanoate and the methyl 3-hydroxynonanoate peaks to the sum of the heights of the methyl 9-fluorononanoate and the methyl 9-fluoro-3-hydroxynonanoate peaks, was estimated to be 5:4 at forty hours incubation. However, this does not take into account possible differences in detector response for each metabolite.

An experiment to determine the detector responses of 9-fluorononanoic acid and nonanoic acid and the degradation rates of each was performed once. The GC/MS response ratio of 9-fluorononanoic acid to nonanoic acid was determined by synthesizing a mixture of the two substrates which contained 1.13 times as much nonanoic acid as 9-fluorononanoic acid by ¹H NMR. The GC/MS response ratio of nonanoic acid to 9-fluorononanoic acid was 0.88 for this mixture constitution. This is a discrepancy in response factor of 1.3 times for 9-fluorononanoic acid over nonanoic acid and shows that the peak ratios obtained for the metabolites can only be considered to be very approximate.

Table 2. 9-Fluorononanoic acid + nonanoic acid degradation results

Incubation Time (hrs)	9-Fluorononanoic acid (counts x 1,000,000)	Nonanoic acid (counts x 1,000,000)
Standard	911	799
0.2	700	491
1.0	448	112
4.0	0	0

The first set of results in Table 2 were for a standard which was analyzed by GC/MS without being incubated for the determination of response factors. The rate of degradation of nonanoic acid versus 9-fluorononanoic acid was determined by incubating 12 μ L of the substrate mixture with cultures of *Pseudomonas oleovorans* for different periods of time and comparing the integration results for each substrate. The rate of degradation was determined to be 3:2 by comparison of the slopes of the graphs for the degradation of each compound. The GC/MS response and the degradation rate for every metabolite in this thesis could not be determined as the standards were unavailable so it was assumed that the response was approximately the same for each metabolite.

There was a statistical factor of only two H atoms being available for abstraction at the fluorinated end of the molecule while there were three H atoms available at the nonfluorinated end of this molecule. If the degradation data is included with this statistical data, then the hydrogen atom abstraction at the α -carbon in 1-fluorononane was activated over that of the ω -carbon by a factor of $(5/4 \times 3/2 \times 3/2 = 45/16)$ approximately three. The fluoromethyl group was determined

to be hydroxylated at a rate three times that of the methyl group.

This result was similar to Tedder's results in his butyl fluoride experiments. Bromination at the fluoromethyl group was activated ten times over bromination at the methyl group.²¹ This result was explained by the resonance stabilization (+M) of fluorine overcoming the inductive effect (-I) and activating the hydrogen atoms on the same carbon. Bingham and Dewar approached this stabilization of a radical by a fluorine substituent in the α -position from the perspective of molecular orbital theory.²⁵ They constructed radicals from a carbon centre with a singly occupied AO and substituents with lone pairs. They reasoned that a single substituent with lone pairs would be weakly bonding by the conjugative effect and result in mesomeric stabilization of the radical due to interaction between lone-pair electrons of the substituent and the MO's arising from interactions between the singly occupied carbon AO and the MO'S of adjacent C-H bonds. This is the case of 1-fluorononane. The bond dissociation energy of fluoromethane is $D_{298}^{\circ} \text{H-CH}_2\text{F} = 101.2 \text{ kcal/mol}$ which is much less than the bond dissociation energy of methane ($D_{298}^{\circ} \text{H-CH}_3 = 104.8 \text{ kcal/mol}$). This data also supports the result that the fluorine substituent stabilizes the incipient radical.

The results presented in this thesis with respect to the ratios of ω -hydroxylation can only be regarded as approximate figures as not all of the metabolites were recovered and only one point in time was chosen as representative of the metabolite ratios over the duration of each experiment. Over a period of forty hours there have been many enzymatic cycles so the accumulation of products has probably changed over time. The GC/MS response to each compound could not be determined so it was assumed that the response was approximately the same for each metabolite in the experiments that follow and that the height of the G.C. peaks present were representative of the ratio of the amounts of metabolites formed. This also assumed that once hydroxylated, the metabolites which had been oxidized at opposite ends would then proceed through the degradation:

pathway diagrammed in scheme 4 at similar rates. However, the rate of degradation of nonanoic acid versus 9-fluorononanoic acid was already determined to be 3:2.

Scheme 7. 1-Fluorononane incubation

Figure 6. 1-Fluorononane chromatograms

Figure 7. 1-Fluorononane mass spectra

Scheme 8. Methyl 9-fluorononanoate fragmentation pathway

Figure 8. More 1-fluorononane mass spectra

8-0532 Scan 497 RT=10:29 100%-235000 mv 6 Jan 94 10:16
LSP -EI II

8-0532 Scan 474 RT=11:43 100%-191600 mv 6 Jan 94 10:16
LSP -EI II

Scheme 9. Methyl 3-hydroxynonanoate fragmentation pathway

Scheme 10. Methyl 9-fluoro-3-hydroxynonanoate fragmentation pathway

2.5 2-Fluorononane Incubation

The metabolism of 2-fluorononane occurred at both ends of the molecule until formation of the carboxylic acid but β -oxidation products were observable only for the end furthest removed from the fluorine substituent. 8-Fluorononanol, 8-fluorononanoic acid, and 2-fluorononanoic acid were all present. 2-Fluorononanol may have been present in a set of overlapping peaks at a retention time of 8:10 minutes which included a peak that was also present in the control, but no satisfactory mass spectrum was obtained. An intermediate of the β -oxidation cycle and the products of the first two complete cycles of β -oxidation were present: 8-fluoro-3-hydroxynonanoic acid, 6-fluoroheptanoic acid, and 4-fluoropentanoic acid. The retention times of the compounds listed were similar to the retention times of the corresponding unsubstituted alkanes.

The mass spectrum of methyl 2-fluorononanoate was quite distinctive with a base peak of $m/z=92$ instead of 74 which is to be expected for a McLafferty rearrangement of a methyl ester substituted in the α position by fluorine. It also gave a peak with a m/z ratio of 105 instead of 87 for the second McLafferty rearrangement, and a molecular ion at $m/z=190$. The presence of methyl 8-fluorononanoate was inferred from the fragment ions and its proximity to methyl 2-fluorononanoate in the gas chromatogram. It gave a McLafferty rearrangement with a $m/z=74$ and the second rearrangement at $m/z=87$. The molecular ion was not present but the M-20 peak representing loss of HF and the M-31 peak representing loss of the methoxy radical were present. The largest fragment ion available for 8-fluorononanol was $m/z=124$ which corresponds to the loss of $(HF + H_2O)$. The spectral fragmentation pattern which was characterized as methyl 8-fluoro-3-hydroxynonanoate gave a base peak at $m/z=103$ which is typical of the 2-hydroxy-4-methoxyoxetenium ion fragment and the two largest fragments available could be correlated to the loss of $(H_2O + HF)$ and to the loss of $(H_2O + CH_3O\cdot)$. It also had the same retention time as

the other compounds suspected of being a product of the enoyl-CoA hydratase enzyme. The presence of methyl 6-fluoroheptanoate was inferred based on an M-20 peak representing the loss of HF and an M-31 peak and a base peak of $m/z=74$ signifying a methyl ester in the mass spectrum. Dihydro-5-methyl-2(3H)-furanone was the library match for the peak at retention time 4:06 minutes. This is suggested to be 4-fluoropentanoic acid, the second product of the β -oxidation of 2-fluorononane, cyclizing back on itself.

Figure 9. Cyclization of 4-fluoropentanoic acid

The ratio of the initial hydroxylation of a methyl group in a β -position to a fluorine substituent versus a methyl group seven carbons removed from the fluorine substituent, based on the sum of the heights of all of the metabolites observed for the oxidation at either end of 2-fluorononane in an intramolecular competition was estimated to be 1:5. Tedder found that the fluorine substituent in *n*-butyl fluoride retarded hydrogen abstraction from the β -carbon atom by a factor of nine times. These results are in accord with what is expected of an electron-withdrawing substituent by the inductive effect.

Scheme 11. 2-Fluorononane incubation

Figure 10. 2-Fluorononane chromatograms

Runname : 960718 Acquired Method: dm (Zero baseline). 28 Jan 94 7:45
Uncommented data only:

Runname : 960719 Acquired Method: dm (Zero baseline). 28 Jan 94 8:10
Uncommented data only:

Figure 11. 2-Fluorononane mass spectra

Scheme 12. Methyl 2-fluorononanoate fragmentation pathway

Scheme 13. Methyl 8-fluorononanoate fragmentation pathway

Scheme 14. Methyl 6-fluoroheptanoate fragmentation pathway

Figure 12. More 2-fluorononane mass spectra

Scheme 15. Methyl 8-fluoro-3-hydroxynonanoate fragmentation pathway

Scheme 16. 8-Fluorononanol fragmentation pathway

Figure 13. Dihydro-5-methyl-2(3H)-furanone mass spectra

2.6 3-Fluorononane Incubation

The alcohol and acid products possible for the metabolism of either methyl group of 3-fluorononane are in scheme 17. 3-Fluorononanol, 7-fluorononanol, 3-fluorononanoic acid, and 7-fluorononanoic acid were all present in the gas chromatogram in Figure 11. β -oxidation was only observed for the end of the molecule furthest from the fluorine substituent. In this case, an intermediate product of the β -oxidation cycle, 7-fluoro-3-hydroxynonanoic acid, plus the first β -oxidation product, 5-fluoroheptanoate, were observed.

Methyl 3-fluorononanoate was verified by comparison of the gas chromatogram and the mass spectrum with those of the authentic standard which had been synthesized by a previous student in our laboratory. Methyl 7-fluorononanoate was inferred based on a similar fragmentation pattern and a similar polarity. It gave an M-20 fragment ion which was formed from the loss of HF. An M-31 peak from the loss of the methoxy radical and the base peak having a $m/z=74$ by the McLafferty rearrangement indicated the structure of the molecule was a methyl ester. The second McLafferty rearrangement having a $m/z=87$ peak was also indicative of a long chain methyl ester. 3-Fluorononanol and 7-fluorononanol were suggested as two of the observed G.C. peaks based on their similar polarity to nonanol and their mass spectra. No molecular ions were present for these two metabolites but the largest fragment ion present for both of them had a $m/z=124$ which is proposed to be formed from the loss of water and HF. Methyl 7-fluoro-3-hydroxynonanoate was suggested as it had a base peak of $m/z=103$ for the 2-hydroxy-4-methoxyoxatenium ion and the two largest fragments matched the mass to charge ratio expected for the loss of ($H_2O + HF$) and the loss of ($H_2O + CH_3O\cdot$). Methyl 5-fluoroheptanoate showed an M-20 peak indicating the loss of HF and a base peak of $m/z=74$ indicating a methyl ester.

The ratio of the initial hydroxylation of a methyl group in a γ -position to a fluorine substituent versus a methyl group six carbons removed from the fluorine substituent, based on the

sum of the heights of all of the metabolites observed for the oxidation at either end of 3-fluorononane in an intramolecular competition was 1:4. Tedder found that fluorine did not exert a substituent effect on bromination or chlorination of the γ -carbon atom in butyl fluoride. The results presented here show a significant retardation of hydrogen atom abstraction by *Pseudomonas oleovorans* monooxygenase at a methyl group when fluorine was a γ -substituent.

Scheme 17. 3-Fluorononane incubation

Figure 15. 3-Fluorononane mass spectra

00720 Scan 319 RT=0.32 100% = 373321 m/z 31 Jan 94 1:00
LSP -02 31 m/z 9

00720 Scan 323 RT=0.34 100% = 14946 m/z 28 Jan 94 8:37
LSP -02 3p

Figure 16. More 3-fluorononane mass spectra

8-0720 Scan 152 RT=6.26 100%-308174 ev 23 Jan 94 8:37
LAP -81 3p

8-0720 Scan 174 RT=6.13 100%-48968 ev 23 Jan 94 8:37
LAP -81 3p

Figure 17. And more 3-fluorononane mass spectra

Scheme 18. 3-Fluorononanol fragmentation pathway

2.7 5-Fluorononane + Nonane Incubation

Pseudomonas oleovorans was incubated with the two substrates 5-fluorononane and nonane in an intermolecular competition to obtain a comparison of the effect of hydrogen atom abstraction from a methyl group with an ϵ -fluorine substituent to abstraction from a methyl group in a straight chain alkane. In principle, the enzyme may be able to differentiate between the methyl groups in 5-fluorononane, even though there is a mirror plane through the molecule, if the active site binds the fluorine substituent in one particular configuration such that only one methyl group is placed in a position for hydroxylation. This occurrence would mean that 5-fluorononane is a prochiral molecule by the Ogston effect.⁴² But since the technique used for the detection and identification of the metabolites, GC/MS, could not separate or distinguish enantiomers, for the purposes of this thesis, metabolism at either end of the molecule is considered to produce the same products.

The incubation products of POM were nonanol and 5-fluorononanol and the products of alcohol and aldehyde dehydrogenase were nonanoic acid and 5-fluorononanoic acid as can be seen in figure 15. β -oxidation products were not observed. The doublet at retention time 7:05 could not be identified. Three hexadecene isomers at retention times of approximately sixteen minutes were also present.

The presence of nonanol was confirmed on the basis of the comparison of its retention time and mass spectrum to that of the authentic standard. 5-Fluorononanol was identified on the basis of its fragmentation pattern and its G.C. retention time. The largest ion in the fragmentation pattern of 5-fluorononanol was the M-20 peak for the loss of HF. The consecutive loss of water and C_2H_4 were consistent with the pattern obtained. Nonanoic acid was observed as the methyl ester and its mass spectrum matched that of the authentic standard. 5-fluorononanoic acid was also observed to be a methyl ester as it had the characteristic base peak of $m/z=74$ and the peak

M-31 representing a loss of the methoxy radical. The M-20 peak was also present indicating the loss of HF. At retention times 16:18, 16:22, and 16:31 three G.C. peaks all having the same mass spectra in the same relative abundances of fragmentation ions were observed. A library match indicated these were different isomers of hexadecene. The significance of this finding is unknown.

The result of the summation of the peak heights of the metabolites for the nonane and 5-fluorononane incubation were 5:6 for the accumulation of nonane metabolites over 5-fluorononane metabolites. Tedder found that the δ -position was unaffected by the fluorine inductive effect so hydrogen abstraction from an ϵ -carbon would be expected to be unaffected by a fluorine substituent. This data shows essentially no preference for hydroxylation of 5-fluorononane over nonane in an intermolecular competition. However, this may be misleading as the nonane metabolites may not have been accumulating while the 5-fluorononane metabolites were accumulating, as the nonane substrate had been completely utilized while there was still 5-fluorononane remaining in the extract. This is apparent from the G.C. chromatogram in which the nonane peak has disappeared while the 5-fluorononane peak remains. The fact that the nonane peak had disappeared, although equivalent amounts of substrate were added to the culture within experimental error, suggests that nonane was metabolized at a faster rate than 5-fluorononane but how much faster cannot be determined. If this is the case, then it would indicate that a fluorinated substrate was less easily bound to the active site.

Scheme 19. 5-Fluorononane + nonane incubation

Figure 18. 5-Fluorononane + nonane chromatograms

Runname: 80643 Acquired Nonanal data (Zero baseline). 19 Jan 94 7:27
Unsummed data only:

Figure 19. 5-fluorononane + nonane mass spectra

80643 Scan 235 RT=9.41 100%+63634 mv 19 Jan 94 7:27
LAP -01 26

80643 Scan 303 RT=8.33 100%+51329 mv 19 Jan 94 7:27
LAP -01 26

Scheme 20. 5-Fluorononanol fragmentation pathway

Figure 20. More 5-fluorononane + nonane mass spectra

80643 Scan 335 RT=9:10 100%-40567 m/z 19 Jan 94 7:27
LRP -82.5%

Scheme 21. Methyl 5-fluorononanoate fragmentation pathway

2.8 1,1-Difluorononane Incubation

Six metabolites accumulated upon incubation with 1,1-difluorononane (see Figure 22). A small quantity of nonanoic acid representing oxidation at the fluorinated end was observed. The rest of the metabolites were all degradation products from the methyl end of the molecule. They were 9,9-difluorononanoic acid, 9,9-difluoro-3-hydroxynonanoic acid, a β -oxidation cycle intermediate and 7,7-difluoroheptanoic acid and 5,5-difluoropentanoic acid, two β -oxidation products. Present also was an unexpected metabolite which was probably the result of a decarboxylation, 8,8-difluoro-2-octanone.

The presence of methyl 9,9-difluorononanoate was inferred from the mass spectrum which gave a molecular ion and a base peak of $m/z=74$ representing a methyl ester. Cleavage of the bond on either side of the carbonyl group also served to confirm the proposed structure. No standard was available for confirmation of the structure of methyl 9,9-difluoro-3-hydroxynonanoate, however, the spectrum showed a base peak of $m/z=103$ for the 2-hydroxy-4-methoxyoxetinium ion, an M-18 peak for the loss of water indicating the presence of the hydroxyl, an M-31 peak for the loss of a methoxy radical and a prominent $m/z=74$ fragment indicating the presence of a methyl ester. The presence of methyl 7,7-difluoroheptanoate was inferred from the mass spectrum with a base peak of 74 and an M-31 peak for the loss of the methoxy radical for a methyl ester. The cleavage of the bonds on either side of the carbonyl group supported the estimation of the molecular weight. It also had a similar retention time to methyl heptanoate. Methyl 5,5-difluoropentanoate was demonstrated by the fragmentation pattern and its position on the chromatogram. It showed the loss of HF, cleavage of the bonds on either side of the carbonyl group, however, the peak at $m/z=74$ was weak for a methyl ester. This is suggested to be a result of the shortening of the carbon chain length during the cycles of β -oxidation bringing the two fluorine substituents close to the site of hydrogen atom transfer in the McLafferty rearrangement.

The fluorine substituents strengthened the carbon-hydrogen bond making this rearrangement a less preferred fragmentation pathway.

One final product which was unexpected was characterized by mass spectrometry to be a methyl ketone, 8,8-difluorooctanone (see Scheme 23). The molecular ion was present and the loss of a methyl radical was demonstrated. Intense peaks at $m/z=43$ and $m/z=58$ are common for methyl ketones as the first represents cleavage of the bond on the other side of the carbonyl group to methyl and the second represents the McLafferty rearrangement for methyl ketones. A loss of HF was also demonstrated. It is possible that this product arises from simple thermal decarboxylation of 9,9-difluoro- β -ketononanoic acid. It may also be an enzymatic decomposition by a β -keto acid decarboxylase as whole cells were utilized in the experiments. A suggested mechanism for the enzymatic decarboxylation based on acetoacetate decarboxylase purified from *Clostridium acetobutylicum*²³ is given in Scheme 24.

The combined height of all of the G.C. peaks corresponding to compounds derived from oxidation at the methyl end of the molecule versus oxidation at the difluoro-methyl end was 71:1 at forty hours incubation time. Including consideration of the statistical factor for only one available H atom in the difluoromethyl group versus three available in the methyl group, the corrected ratio of hydrogen atom abstraction at the α -carbon in 1,1-difluorononane was 71:3 for the methyl group over the difluoromethyl group, or approximately 24:1.

This result concurs with the molecular orbital interactions described by Bingham and Dewar.²⁵ They reasoned that two substituents with lone pairs would probably be destabilizing since the anti-bonding interaction probably outweighs the bonding one. The substituents would not therefore donate electron density through π -conjugation in unison; rather, they would antagonize one another. This effect was reasoned by MO theory to be the greatest when the substituents are the same and when the relevant orbitals on them are AO's since the corresponding

resonance integral to carbon is then greater. This is the case of 1,1-difluorononane. Antagonism is also increased the greater the resonance integral between the carbon and the substituents which will be greatest when the carbon AO is a 2p AO, *i.e.* for planar methyl radicals. The resulting increase in antagonism will therefore favor a pyramidal structure (sp^3 hybridization). However, the additional substituents may lead to overall stabilization of the radical even though they destroy the symmetry of the radical centre.

The C-H bond dissociation energy for difluoromethane is $D_{298}^{\circ} \text{H-CHF}_2 = 103.2 \text{ kcal/mol}$ which is less than that for CH_4 (104.8 kcal/mol). The C-H bond dissociation energy for difluoroethane is $D_{298}^{\circ} \text{H-CF}_2\text{CH}_3 = 99.5 \text{ kcal/mol}$ which is again less than that for ethane (100.3 kcal/mol). This indicates that the pyramidal structure of the difluoroalkyl radical is indeed stabilizing overall with respect to an alkane. Based on the bond dissociation energy data for methane, the α -C-H bond in 1,1-difluorononane was much stronger than the α -C-H bond in 1-fluorononane so it would be expected that it would be much more difficult for POM to break this bond. The reason for such a low reactivity of the difluoromethyl group versus the methyl group may be that the fluorine groups are polarizing the molecule by the inductive effect and field effects thereby making the difluoromethyl group less hydrophobic so that it will prefer to stay in a water medium rather than bind to the active site.

Scheme 22. 1,1-Difluorononane incubation

Figure 22. 1,1-Difluorononane chromatograms

Runname: 80642 Acquired Nominal data (Zero baseline), 19 Jan 94 6:33
Unmatched data only:

Runname: 80646 Acquired Nominal data (Zero baseline), 19 Jan 94 8:44
Unmatched data only:

Figure 23. 1,1-Difluorononane mass spectra

Figure 24. More 1,1-difluorononane mass spectra

Scheme 23. 8,8-Difluoro-2-octanone fragmentation pathway

Scheme 24. Suggested mechanism for the decarboxylation of 9,9-difluoro- β -ketononanoic acid

2.9 Conclusions:

It was not possible in this thesis to measure the fluorine substituent effect on the ω -hydroxylation of nonane by the organism *Pseudomonas oleovorans*. However, there are some generalizations which can be made based on a comparison between experiments. Table 3 summarizes the results of all of the incubation experiments.

The reactivity of the methyl group next to or containing the fluorine substituent versus the reactivity of the methyl group further removed from the substituent is represented by the ratios.

Table 3. Ratio of reactivities of terminal groups

Substrate	F substituent end	CH ₃ end
1-Fluorononane	3	1
2-Fluorononane	1	5
3-Fluorononane	1	4
5-Fluorononane/Nonane	6	5
1,1-Difluorononane	1	24

Hydroxylation at the α -carbon of 1-fluorononane was enhanced as a result of resonance stabilization of the radical by the fluorine substituent through conjugation. Hydroxylation at the β -carbon of 2-fluorononane was significantly retarded due to the electron-withdrawing effect of the fluorine substituent and hydroxylation at the γ -carbon was retarded as well. The metabolite ratios of 5-fluorononane and nonane indicate that a methyl group in the ϵ -position was not significantly affected by a fluorine substituent although, since nonane had disappeared from the

culture while some 5-fluorononane still remained, there may have been a substituent effect. Hydroxylation of 1,1-difluorononane was significantly retarded at the fluorinated methyl group. These results parallel chemical studies of hydrogen atom abstractions from fluorinated alkanes in free radical reactions.

3.0 Experimental

3.1 General

All synthetic reagents were purchased from the Aldrich Chemical Company and used without further purification, except for oleic acid which was purchased from the Fisher Scientific Company. The salts used in the P1 medium were also purchased from the Fisher Scientific Company. Solvents were technical grade and distilled before use. A Buchi RE 111 rotavapor was utilized for the removal of solvents *in vacuo*. Flash chromatography was performed using Merck Silica Gel 60 (230-400 mesh). Thin layer chromatography was performed on Merck pre-coated Silica Gel 60 F-254 glass plates. Visualization of UV- and I_2 -inactive compounds was accomplished by spraying with water: hydrophobic spots appeared white on a grey background.

The ^1H NMR spectra (400 MHz) and the ^{13}C NMR spectra (100 MHz) were both recorded on a Bruker AMX-400 spectrometer. All chemical shifts were reported with reference to tetramethylsilane (TMS) using CDCl_3 as the solvent and were quoted in δ (ppm) values. The following abbreviations were used: s = singlet, d = doublet, t = triplet, m = multiplet, bd = broad. J values were reported in hertz.

The GC-MS data were collected by Dr. Clem Kazakoff of the University of Ottawa with a Hewlett Packard model HP 5980 gas chromatograph equipped with a 30 m DB-5 capillary column. The column temperature program was initially 80°C for 2 minutes and then ramped at 10°C per minute up to a temperature of 260°C. The mass spectrometer was a Kratos Concept model 2H equipped with a 70 eV E.I. ionization chamber and recorded on a SUN computer at a scan rate of 0.5 seconds.

3.2 Synthesis of Substrates

3.2.1 3-Nonanol

3-Nonanone (14.01g, 99%, 0.0975 mol) was dissolved in 250 mL 100% ethanol in a 1000 mL round bottomed flask in an ice bath set up with a reflux condenser. The 3-nonanone was reduced with 3.88g NaBH_4 (95%, 0.0974 mol) with stirring for 12 hours. Distilled water (500 mL) was placed in a 1000 mL separatory funnel and the reaction mixture was rinsed into it with 100% ethanol. The pH was 9-10. Glacial acetic acid was added dropwise with shaking until the pH was equal to 5 (≈ 10 ml). There was a vigorous evolution of gas due to the excess NaBH_4 . The 3-nonanol was left to separate as an oil on the surface of the ethanol/water mixture. Most of the ethanol/water layer was removed to a beaker and the oil was extracted from the remaining mixture with 3x50 mL CH_2Cl_2 . The remaining mixture was disposed of and the removed layer was reintroduced to the separatory funnel where it was also extracted with 3x50 mL CH_2Cl_2 . The organic extracts were combined and dried over

Na_2SO_4 and rotoevaporated under vacuum to constant weight. The yield was 13.82g (0.0958 mol, 98.3%) of a clear oil which was 100% pure by GC/MS.

Table 4. ^1H NMR of 3-nonanol

Carbon	C-1	C-2	C-3	C-4	C5-C8	C-9
ppm	0.94	1.5	3.52; 1.6	1.5	1.3	0.88
Mult	t	m	m bd	m	bd s	t

3.2.2 3-Fluorononane

At 0°C , under a N_2 atmosphere and fitted with a reflux condenser, DAST (diethylaminosulfur trifluoride, 6.0 mL, 0.0445 mol) was added dropwise to 3-nonanol (5.03g, 0.0349 mol) dissolved in 80 mL CH_2Cl_2 which had been dried over molecular sieves. The solution was a clear yellow color. Pyridine (5.0 mL, 0.0625 mol) dried over NaOH pellets was added dropwise with stirring to this mixture over a CaCl_2

ice/salt bath. Once the addition was complete, the solution was allowed to warm up to room temperature and stirred for four hours. The reaction was quenched in 50 mL ice and transferred to a separatory funnel. The water layer was washed with 3x25 mL CH_2Cl_2 . The combined organic layers were washed with 5% HCl (3x100 mL), saturated NaHCO_3 solution (100 mL), saturated NaCl solution (100 mL), dried over Na_2SO_4 and rotoevaporated to constant weight. The yield was 4.21g of a yellow oil. Any alkenes formed as a side reaction were removed by oxidation and column chromatography. KMnO_4 (5.0g) was dissolved in 50 mL of distilled water with 0.50g of hexadecyltributylphosphonium bromide added as a phase transfer agent and warmed gently on a hot plate. The crude 3-fluoro-nonane was added and shaken intermittently. After one hour, 6M HCl and NaHSO_3 were added alternately until the solution went from brown/purple to cloudy white in color and had a pH of 2. Saturated NaCl solution (50 mL) was added. The mixture was extracted with CH_2Cl_2 (3x50 mL). The organic layers were combined and washed with saturated NaHCO_3 solution (2x100 mL), dried over Na_2SO_4 , and rotoevaporated to constant weight. The yield was 3.26g of a yellow oil. A portion of this, 1.60g, was purified by flash chromatography in a column containing 70g silica gel with 1% CH_2Cl_2 in hexane as eluent and a pressure of 5 psi. The yield was 0.17g of a clear oil collected in the third column volume. The R_f factor was 0.25 on a silica gel tlc plate using the same solvent mixture and the b.p. was 87-88°C at 34mmHg. The compound was 99% pure by ^1H NMR and the yield was 7%.

Table 5. ^{13}C NMR of 3-fluorononane

C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
ppm	9.4	28.1	95.7	34.7	25.1	29.2	31.8	22.6	14.1
J_{CF}	5.7	21.6	167	21.0	4.4				
mult	d	d	d	d	d	s	s	s	s

Table 6. ^1H NMR of 3-fluorononane

C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
ppm	0.96		4.39						0.89
J_{HF}			49.2						
mult	t		d m						t

3.2.3 5-Nonanol

5-Nonanol was synthesized from the ketone using the same procedure described for the synthesis of 3-nonanol. 5-Nonanone (98%, 19.94g, 0.1374 mol) was reduced with NaBH_4 (95%, 10.85g, 0.2724 mol) and left to stir for 12 hours. The yield was 19.78g of a clear oil which crystallized on standing. This was a 99.8% yield which was 98.9% pure by GC/MS.

Table 7. ^1H NMR of 5-nonanol

Carbon	C-1	C-2 to C-4	C-5
Shift (ppm)	0.91	1.3 to 1.5	3.6
Multiplicity	t	m	m

3.2.4 5-Fluorononane

5-Fluorononane was synthesized using the same procedure as for 3-fluorononane. 5-

2 of/de 2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Nonanol (98.9%, 5.98g, 0.0415 mol) was reacted with DAST (6.35 mL, 0.0481 mol) and pyridine (5.8 mL, 0.0714 mol) for 4.5 hours. The product was extracted and washed to yield 4.96g of a brown oil. The oil was distilled by Kugelrohr distillation to give 2.36g (39%) of a faintly yellow oil, b.p. = 82-84°C at 29-30 mmHg. The product was found to contain alkenes so it was oxidized and purified by flash chromatography as in the 3-fluorononane procedure except that 20% CH₂Cl₂ in hexane was used as the eluent. The compound eluted in the second to third column volume with an R_f factor of 0.45 on a silica gel tlc plate with the same solvent mixture. It was rotoevaporated to give 0.10g or 1% yield of a clear oil with a b.p. of 86-88°C at 33 mmHg which was pure by ¹H and ¹³C NMR. The literature reference¹⁷ described a colorless liquid with a b.p. of 55-56°C/15 mmHg and ¹H-NMR: 4.45 (1 H, *dt*, *J* 50.0, 7.5, 4.0), 1.5 (12 H, *m*), 0.93 (6 H, *t*, *J* 7.5).

Table 8. ¹³C NMR of 5-fluorononane

Carbon	C-1	C-2	C-3	C-4	C-5
Shift (ppm)	13.9	22.5	27.3	34.8	94.5
Multiplicity	s	s	d	d	d
J _{CF} (Hz)	0	0	5	21	165

Table 9. ^1H NMR of 5-fluorononane

Carbon	C-1	C-2	C-3	C-4	C-5
Shift (ppm)	0.91	1.34	1.45	1.59	4.45
Multiplicity	t	m	m	m	d of m
$ J _{\text{HF}}$ (Hz)	7.1				49

3.2.5 2-fluorononane

DAST,
pyridine

2-Fluorononane was synthesized from the alcohol using the same procedure as the 3-fluorononane synthesis. 2-Nonanol (99%, 5.00g, 0.0347 mol) was reacted with 5.0 mL DAST and 4.8 mL pyridine for 4.5 hours. The yield was 4.31g of a yellow oil. Oxidation of the alkene side products was carried out as per the 3-fluorononane procedure yielding 3.26g of a yellow oil. This was purified by flash chromatography using 2.5% CH_2Cl_2 in hexane as eluent. 1.38g of crude product was applied to a

column containing 70g silica gel. 0.15g of a clear oil was collected in the second column volume. The R_f factor was 0.22 on a silica gel tlc plate using the same solvent mixture and the b.p. was 87-88°C at 33 mmHg. The yield was 7% of 99% pure 2-fluorononane by ^1H NMR.

Table 10. ^{13}C NMR of 2-fluorononane

C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
ppm	21.0	91.0	37.0	25.1	29.4	29.2	31.8	22.6	14.0
J_{CF}	22.9	164	20.5	5.1					
mult	d	d	d	d	s	s	s	s	s

Table 11. ^1H NMR of 2-fluorononane

C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
ppm	1.28	4.64	1.34						0.88
J_{HF}	6	48	6						
mult	d d	d m	d m						t

3.2.6 1,1-difluorononane

Dry nonanal (99%, 7.03g, 0.0495 mol) was rinsed into a 250 mL 3-necked round bottomed flask with 30 mL of dry CH_2Cl_2 . The flask had previously been set in a room temperature water bath with a condenser attached and flushed with N_2 . DAST (6.5 ml, 0.0493 mol) was added dropwise with stirring. The solution went from clear to brown. It was left to stir for 12 hours. The dark brown mixture was poured into 50 mL of ice, melted and transferred to a 250 mL separatory funnel where the dark brown organic layer was removed. The water layer was washed twice with 20 mL of CH_2Cl_2 and then the organic layers were combined. The organic layer was washed with 2x80 mL distilled water, 80 mL saturated NaHCO_3 solution, again with 80 mL distilled water, 80 mL saturated NaCl solution, dried over NaSO_4 , and rotoevaporated under vacuum to constant weight. A dark brown oil remained which was separated into its components by flash chromatography using 6% ethyl acetate in hexane as eluent. The compound eluted in the second to third column volume and the R_f factor was 0.49 on silica gel plates in the same solvent mixture. The recovered oil was yellow so it was distilled with a Kugelrohr apparatus to give a clear oil, b.p. = 84-85°C

at 32-33 mmHg. The yield was 1.83g or 23% and the purity was 96% by GC/MS and the impurity was the nonanal starting material.

Table 12. ^{13}C NMR of 1,1-difluorononane

C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
ppm	118	34.2	22.2	29.4	29.1	29.1	31.9	22.7	14.1
mult	t	t	t	s	s	s	s	s	s
J_{CF}	239	20.6	5.4						

Table 13. ^1H NMR of 1,1-difluorononane

C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
ppm	5.81	1.82	1.47						0.91
mult	tt	m	m						t
J_{HF}	57								

3.2.7 Oleyl alcohol

LiAlH_4 (4.96g, 0.131 mol) was placed in a 250 mL 3-necked round bottomed flask which was sitting in an ice bath and fitted with a reflux condenser and had been flushed with N_2 . Diethyl ether was distilled over LiAlH_4 and then 30 mL was dropped into the reaction vessel by syringe. Oleic acid (90%, 25.15g, 0.6801 mol) was diluted with 5 mL of diethyl ether and added dropwise to the vessel with stirring, using another 5 mL of ether to rinse the beaker and syringe. The mixture was stirred for 12 hours. Distilled water, 5 mL, was added by syringe and 10 mL of 6M HCl before removing the N_2 supply. CH_2Cl_2 , 20 mL, was mixed in. The water layer was washed with CH_2Cl_2 and the organic layer was washed with water and 6M HCl, to neutralize and dissolve excess LiAlH_4 alternately, until the combined organic phases were clear. The organic phase was dried over Na_2SO_4 and rotoevaporated under vacuum to constant weight. The yield was 24.59g or 103% of a faintly yellow oil, 65% pure by ^1H NMR. The major impurity was the starting material, oleic acid.

Table 14. ^1H NMR of oleyl alcohol

C	C-1	C-2	C3-C7	C-8	C9- C10	C-11	C12- C17	C18
shift (ppm)	3.64, 3.45	1.57	1.31	2.03	5.35	2.00	1.3	0.89
mult	t bd	m	m	m	m	m	bd d	t

3.2.8 Oleyl fluoride

Oleyl alcohol (9.32g, 0.0347 mol) was placed in a 500 mL 3-necked round bottomed flask fitted with a reflux condenser, flushed with N_2 , and sitting in ice. CH_2Cl_2 , 80 mL dried over molecular sieves, was stirred in and then 5.00g DAST was dropped in by syringe. Once the DAST addition was complete, the flask was placed in a CaCl_2 ice bath and 4.8 mL of dry pyridine was added dropwise. The mixture was allowed to react for 4.5 hours with stirring before being quenched by pouring into a beaker containing 50g of ice. The CH_2Cl_2 was separated from the water in a 500 mL

separatory funnel and the water layer was extracted with 3x25 mL CH_2Cl_2 . The combined organic layers were washed with 5% HCl solution (3x100 mL) and saturated NaHCO_3 solution (1x100 mL). A thick emulsion remained after the NaHCO_3 separation which was washed with 3x25 mL CH_2Cl_2 and the organic phases combined. The organic phase was then washed with saturated NaCl solution (100 mL), dried over Na_2SO_4 and rotoevaporated under vacuum to constant weight. The yield was 7.80g of a gold oil. This crude oleyl fluoride (5.18g) was purified by flash chromatography in a column containing 200g silica gel with 1% CH_2Cl_2 in hexane. The compound eluted in the second to third column volume giving a yield of 0.85g of a clear oil. The R_f factor was 0.20 on a silica gel tlc plate using the same solvent mixture and the yield was 14%.

Table 15. ^{13}C NMR of oleyl fluoride

C	C-1	C-2	C-3	C-9 ⁴⁴	C-10	C-16	C-17	C-18	C4-C8 11-15
ppm	84.2	30.4	25.1	129.8	130.	31.9	22.7	14.1	27-29
J_{CP}	164	19.3	5.6						
mult	d	d	d	s	s	s	s	s	m

Table 16. ^1H NMR of oleyl fluoride

C	C-1	C-2	C-3	C4- C7	C-8	C9- C10	C11	C12- C17	C18
ppm	4.45	1.71	1.4	1.33	2.05	5.4	2.03	1.29	0.91
mult	d t	d m	m	m	m	m	m	m	t
J	47.4	24.8							

3.2.9 9-Fluorononanoic acid

9-Fluorononanoic acid was synthesized as a mixture of 9-fluorononanoic and nonanoic acid. KMnO_4 (1.52g) and 0.26g of hexadecyltributylphosphonium bromide were dissolved in 25 mL distilled water in a 125 mL erlenmeyer flask on a hot plate set at low. Oleyl fluoride, 0.85g, was added dropwise and the mixture heated gently and shaken intermittently for one hour. The flask was removed from the heat and 10 mL 6M HCl was shaken into it. NaHSO_3 and acid were added alternately until the solution cleared and the pH was 2. Saturated NaCl solution was poured into the

mixture (25 mL) and a yellow oil was floating on top. The mixture was extracted with 3x25 mL CH_2Cl_2 and the combined organic phases dried over Na_2SO_4 and rotoevaporated to constant weight. The yield was 1.15g of a yellow oil. The oil was purified by flash chromatography in a column containing 70g of silica gel beginning with 10% ethyl acetate in hexane to remove the starting material and less polar impurities (3 column volumes). Elution was continued with 40% ethyl acetate in hexane and then graded up to 100% ethyl acetate. The product eluted in the third to sixth column volumes of this series. The yield was 0.77g of a slightly yellow oil with 22% impurities by GC/MS.

Table 17. ^{13}C NMR of 9-fluorononanoic acid

C	C-1	C-2	C-3	C4-C6	C-7	C-8	C-9
ppm	180.4	34.1	24.6	29.0- 29.2	25.1	30.4	84.1
$ J _{\text{CF}}$					5.5	19	164
mult	s	s	s	m	d	d	d

Table 18. ^1H NMR of 9-fluorononanoic acid

C	C-1	C-2	C-3	C4-C7	C-8	C-9
ppm	11.4	2.35	1.64	1.2-1.45	1.69	4.43
$ J _{\text{HF}}$						47.4
mult	bd	t	m	m	m	d t

3.3 Biological Methods

Pseudomonas oleovorans was purchased from American Type Culture Collection (ATCC 29347). The culture was revived by transferring a small piece of freeze-dried material aseptically to 10 mL of autoclaved P1 medium (see p.92) in a baffled and teflon-capped 50 mL erlenmeyer flask which contained 3-4 drops of filter-sterilized n-octane, 99%. The bacteria were incubated at 28°C with shaking at 150 rpm for three days. The population was increased by aseptically inoculating with 10 mL of broth a 2-L baffled and teflon-capped erlenmeyer flask containing 200 mL of autoclaved P1 medium and 20 drops of filter sterilized n-octane as sole carbon source and incubating at 28°C with shaking at 150 rpm for 36 hours. Growth was increased once more by transferring 30 mL of this culture into each of six baffled 2-L flasks with 20 drops of octane and 200 mL of medium and incubating under the same conditions for 10 to 16 hours (until the late exponential phase). The culture was a cloudy white suspension. The cells were harvested at the late exponential phase by centrifugation in a Du Pont Instruments Sorvall RC-5B refrigerated superspeed centrifuge with a GSA rotor for 15 minutes at 8000 rpm and room temperature. The resulting pellet was pink and was washed and centrifuged under the same conditions three times with phosphate buffer to remove the octane. The cells were then resuspended in phosphate buffer and the optical density was recorded with a Beckman spectronic 21 spectrophotometer at 660 nm.⁴³ Once the cells had been prepared, the culture was divided into 125 mL erlenmeyer flasks and Triton X-100 was added to each flask in 0.5% concentration. The appropriate amount of substrate was administered and the flasks were stoppered with a rubber septum to prevent evaporation. The flasks were placed in a New Brunswick Scientific Gyrotory Water Bath Shaker, model G76, and shaken at 28°C

and 250 rpm for 40 hours.

After forty hours, the flasks were removed from the shaking water bath and 6M HCl was added to reduce the pH to 2. The contents of each flask was poured into a 125 mL separatory funnel and the empty flask rinsed into the funnel with 20 mL of CH_2Cl_2 . The culture was extracted with the CH_2Cl_2 rinsings and the emulsion was broken by centrifugation. The water layer was poured off the top and any remaining emulsion was broken by the addition of Na_2SO_4 . The organic layer was transferred to a 50 mL erlenmeyer flask where it was dried over Na_2SO_4 . It was reduced to a 2 mL volume by rotoevaporation in a 50 mL rbf and then 2 mL of ether saturated diazomethane was mixed in which turned the solution yellow. Diazomethane reacts almost immediately and in high yield so the remaining yellow color indicated sufficient diazomethane to esterify the sample. The sample was rotoevaporated to dryness to remove all of the diazomethane and diluted in 2 mL of CH_2Cl_2 . The cloudy suspension was allowed to settle and 1.5 ml was pasteur pipetted off the top into G.C. vials for analysis.

P1 medium used for bacterial growth^{45,46} (pH = 7.4)

$(\text{NH}_4)_2\text{HPO}_4$		10.0 g
K_2HPO_4		5.0 g
Na_2SO_4		0.5 g
CaCl_2	(50 g/l)	1.0 mL
Salt B		10.0 mL
microelements		1.0 mL
Distilled water to		1.0 L

Salt B:

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	40.0 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	2.0 g

MnSO ₄ ·H ₂ O	1.6 g
NaCl	2.0 g
Distilled water to	1.0 L

Microelements:

H ₃ BO ₃	0.50 g
CuSO ₄ ·5H ₂ O	0.04 g
Na ₂ MoO ₄ ·2H ₂ O	0.20 g
ZnSO ₄ ·7H ₂ O	8.00 g
CoCl ₂ ·6H ₂ O	0.20 g
Distilled water to	1.00 L

Phosphate Buffer (pH = 7.4):

Na ₂ HPO ₄	4.26 g
KH ₂ PO ₄	1.22 g
Distilled water to	1.00 L

3.3.1 Derivatization of Fatty acids with Diazomethane

Diazomethane was prepared for the esterification of fatty acids by the following method. A 50% aqueous solution of KOH, 6 mL, was placed on ice in a 40 mL test tube and 20 mL of diethyl ether was poured on top. Nitrosomethylurea,⁸⁷ 1 g, was added gradually, waiting until it had reacted in between additions. Diazomethane gas bubbled into the ether layer and turned it yellow in color. Diazomethane is unstable so it could not be stored.

3.3.2 Degradation of 9-Fluorononanoic acid versus Nonanoic acid

9-Fluorononanoic acid was synthesized as a mixture (see pp. 92-94). This mixture was incubated with cultures of *Pseudomonas oleovorans* for various lengths of time and then extracted and analyzed by GC/MS as described previously (see pp. 95-97). The mixture was added by syringe (12 µL) to a bacterial culture (26 mL, O.D.

= 1.5 at 660 nm) to which two drops of Triton X-100 had been added.

4.0 Appendix

ppm

100

¹H NMR of 3-nonanol

3.553
3.535
3.523
3.516
3.505
3.494

2.069

1.529
1.523
1.520
1.517
1.505
1.498
1.486
1.479
1.467
1.460
1.442
1.423
1.415
1.407
1.359
1.371
1.292
0.959
0.941
0.922
0.902
0.886
0.879
0.869
0.858

^{13}C NMR of 3-fluorononane

¹H NMR of 3-fluorononane

¹NMR of δ -nonanol

^{13}C NMR of 5-fluorononane

MS-400 Carleton University
Tanya 27-07-83

¹H NMR of 5-fluorononane

^{13}C NMR of 2-fluorononane

max-400 carleton university

¹H NMR of 2-fluorononane

^{13}C NMR of 1,1-difluorononane

gem-400 carleton university
sample : Tm10

^1H NMR of 1,1-difluorononane

¹H NMR of oleyl fluoride

ark-400 carleton university

^{13}C NMR of 9-fluorononanoic acid +
nonanoic acid

¹H NMR of 9-fluorononanoic acid +

- 11.431 ppm nonanoic acid

5.0 References

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