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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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ENTITLED INCORPORATION OF FLUORINATED AROMATIC AMINO ACIDS

INTO CYTOCHROME P-450_{cam} AND PUTIDAREDOXIN

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF BACHELOR OF SCIENCE IN BIOCHEMISTRY

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FLUORINATED AROMATIC AMINO ACIDS
INTO
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BY

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THESIS

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INTRODUCTION

In vivo incorporation of fluorinated aromatic amino acids into proteins allows for the study of their structure and function via nuclear magnetic resonance (NMR) (1,2,3,4,5). Having a window into the active site of cytochrome P-450_{cam} or putidaredoxin would help to determine the conformation of the cytochrome P-450_{cam}-putidaredoxin complex (6). Although the crystalline structure of cytochrome P-450_{cam} is known (7), putidaredoxin has not been crystallized. Therefore, NMR spectra of this fluorine labelled protein could help in the elucidation of its structure. The COOH-terminus (tryptophan residue) of putidaredoxin has been shown to play a role in cytochrome P-450_{cam} hydroxylation reactions (8). Replacement of putidaredoxin's only tryptophan residue could further these studies. Therefore, the motivation behind this work is to show that fluorinated aromatic amino acids can be incorporated into the proteins, cytochrome P-450_{cam} and putidaredoxin.

MATERIALS AND METHODS

Bacterial Strains. E. coli (pUS200) amp^R contains the CamC gene encoding the protein, cytochrome P-450_{cam}. As determined from the known sequence, this protein has 18 phenylalanine residues (14-UUC and 4-UUU codons), five tryptophan residues (5-UGG codons), and nine tyrosine residues (7-UAC and 2-UAU codons) (9).

E. coli (pKM356) amp^R contains the CamB gene encoding the protein, putidaredoxin. As determined from the known sequence, this protein has one phenylalanine residue (1-UUC codon), one tryptophan residue (1-UGG codon), and three tyrosine residues (1-UAC and 2-UAU codons) (Koga 1986).

Chemicals. The fluorinated amino acids, DL-4-fluorophenylalanine, DL-6-fluorotryptophan and DL-3-fluorotyrosine were purchased from Aldrich Chemical Co. Other amino acids were purchased from Sigma Chemical Co.

Equipment. All O.D.₆₆₀ assays for bacterial growth curves were made on a Varian Cary 219 Spectrophotometer. Photospectra and extinction coefficients for determining

*Abbreviations: amp is ampicillin (200 g/L), fphe is 1 mM DL-4-fluorophenylalanine, ftrp is 1 mM DL-6-fluorotryptophan

protein concentration were made on a Hewlett Packard 8450A UV/VIS Spectrophotometer. Nuclear Magnetic Resonance (NMR) spectra were obtained from a NT-360 with a fluorine probe.

Growth Curves. Overnights of E. coli (pUS200) and E. coli (pKM356) were grown in two-5 ml LB w/amp at 37°C. They were used to inoculate two-50 ml LB w/amp controls and six-50 ml LB w/amp and each supplemented with a fluorinated amino acid, 1mM final concentration. The eight-50 ml cultures were incubated at 37°C and O.D.₆₆₀ readings were taken every hour until well into stationary phase. Samples of each were taken at mid exponential phase and frozen at -70°C in glycerol for later use.

Growth of Bacteria for ¹⁹F-labelled Proteins. E. coli (pUS200) and E. coli (pKM356) were each grown in 5 L LB w/amp at 37°C with aeration by shaking for 16 hours. E. coli (pUS200) was supplemented with DL-4-fluorophenyl-alanine and E. coli (pKM356) with DL-6-fluorotryptophan, both 1 mM final concentration. Each of the cell cultures were centrifuged and stored at -70°C as cell pastes.

Preparation of cell free extract.¹ The cell pastes, 1.3 g E. coli (pUS200) w/fphe and 5.0 g E. coli

¹Abbreviation: O. D.₆₆₀ is optical density at 660 nm.

(pKM356) w/ftsp, were suspended separately in 50 ml Lysis Buffer (50 mM Tris pH7.4, 5.0% glycerol, 0.2 mM sat. camphor, 20 mM B-mercaptoethanol). A Lysis Mix (2 mM TSC, 0.58 units/ml RNase A, 16.0 units/ml DNase I, 0.5 mg/ml lysozyme) was then added and stirred at 4°C for 14 hours. The suspension was centrifuged at 10,000 g for 70 minutes. The supernatant was saved and the pellet resuspended in 25 ml lysis buffer and lysis mix. This second suspension was stirred at 4°C for 4 hours and then sonicated. The suspension was centrifuged at 10,000 g for 20 minutes. The supernatant was added to the first.

Protein Purification.¹ A 72 ml volume DE-52 ion-exchange column was previously equilibrated with a running buffer (same as lysis buffer). The cell free extract was loaded on the column and washed with 400 ml running buffer. The protein was eluted from the column using a 50 mM to 500 mM linear potassium chloride gradient (same as lysis buffer). All fractions showing the characteristic absorbance spectra for the particular protein was pooled due to the possibility of a low yield of fluorinated protein. The pooled fractions underwent dialysis for 24 hours. The protein was concentrated using

¹Procedures based on those provided by David Jollie.

In succession: a 50 ml and 10 ml Amicon with N₂ gas, and then centrifuged in a Centicon at 5,000 g until a volume of less than 0.4 ml. Absorbance spectra was taken to determine protein concentration. The proteins, cytochrome P-450_{cam} w/fphe and putidaredoxin w/ftpr, were stored in liquid N₂.

NMR Spectroscopy.² The frozen proteins were thawed on ice at 4°C. D₂O was added to each to a final volume of 10%. Standards of 100mM fphe and 100mM ftrp, both in D₂O, were prepared. An external sample of CF₃COOH was used to relatively determine the fluorine chemical shifts found in the proteins. All spectra was taken with sample at 20°C.

RESULTS AND DISCUSSION

A fair amount of study has been associated with the incorporation of fluorinated aromatic amino acids in proteins. An inherent problem with this type of research is obtaining the relatively high yields of active protein necessary for NMR spectra. Fluorinated aromatic amino acids have an inhibitory effect on cell division (3, 10, 11, 12

²The ¹⁹F NMR spectra was obtained by Dr. Tom Pochapsky.

and 13). This suggests that the addition of the aromatic amino acid analog to a growth medium at time equals zero would not allow for a sufficient cell density. Researchers have found that, in the case of isomers of fluorotryptophan, 4-fluorotryptophan allowed two doublings of optical density, while 5- and 6-fluorotryptophan allowed less than two doublings (3, 5).

Although 6-fluorotryptophan was used in these studies, the growth curves in Fig. 2 may reflect those findings stated above. Cells grown in the presence of a fluorine analog show a decrease in optical density during lag phase relative to the control. The sharp recovery of the cells grown with the analog may represent those cells which could select against the analog by reducing permease activity.

A second problem for the incorporation of fluorinated aromatic amino acids may exist with the transfer of the amino acid versus its analog to tRNA. It has been suggested that fluorophenylalanine can only be incorporated into the protein at a UUU-codon on the mRNA while phenylalanine can be incorporated at either a UUU- or a UUC codon (13, 14). As stated earlier, cytochrome P-450_{cam} contains only four UUU codons versus 14 UUC codons and putidaredoxin contains one UUC codon. Therefore, if both the amino acid and its analog are present for tRNA binding, the event of incorporating the analog into the protein are greatly

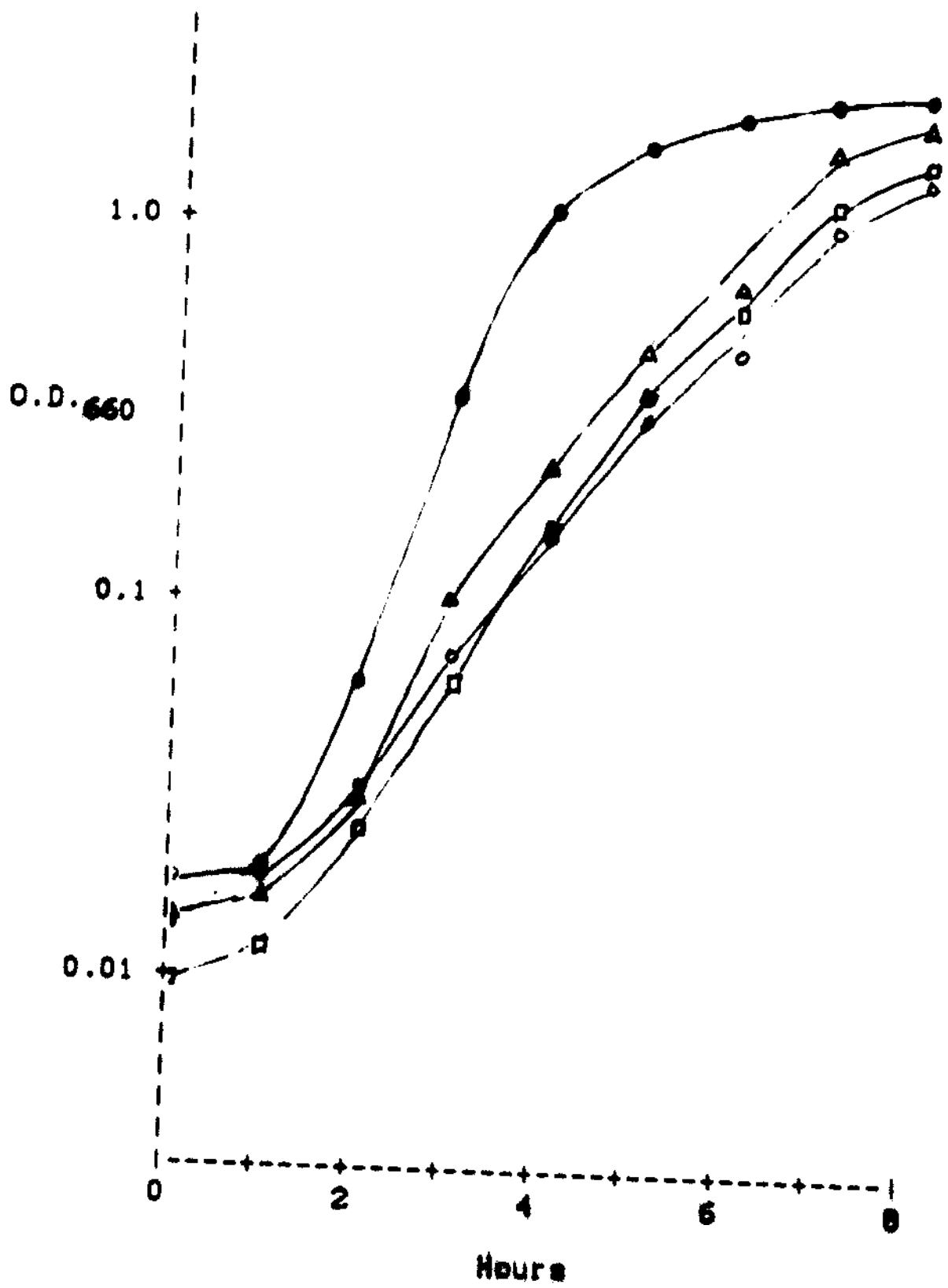


Figure 1: Growth curve for *E. coli* (pUS200). Optical density 660 was determined as a function of time for the control (solid dots), 1 mM DL-4-fluorophenylalanine (open triangles), 1 mM DL-6-fluorotryptophan (open squares), and 1 mM DL-3-fluorotyrosine (open circles).

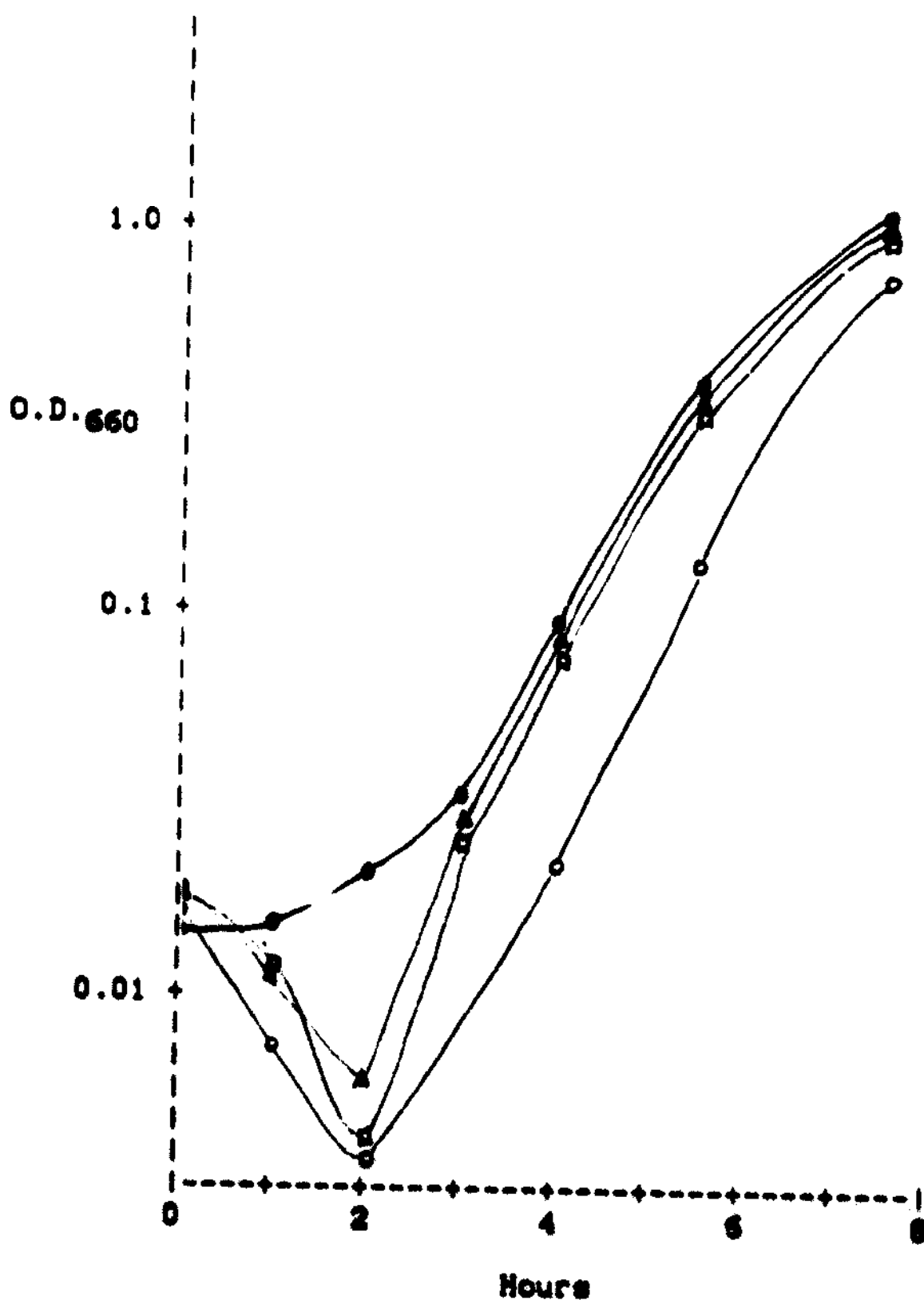


Figure 2: Growth curve for *E. coli* (pKN356). Optical density 660 was determined as a function of time for the control (solid dots), 1 mM DL-4-fluorophenylalanine (open triangles), 1 mM DL-6-fluorotryptophan (open squares), and 1 mM DL-3-fluorotyrosine (open circles).

reduced. This specificity in tRNA binding may also exist for the other aromatic amino acids (15).

Some researchers have developed ways to use an auxotroph having the appropriate biosynthetic pathway mutation for the incorporation of fluorinated aromatic amino acids in proteins (3, 4, 5). This approach requires the exhaustion of the amino acid that is to be replaced followed by the addition of the analog, because of possible competition for permease and tRNA enzymes. Determination of exhaustion may be difficult. On the other hand, one could pellet the cells by centrifugation, wash and resuspend cells in medium containing the analog. But, this would be difficult considering the large quantity of culture needed to grow enough cells to produce sufficient protein for NMR studies (2).

Another approach involves feedback inhibition and repression of the biosynthetic pathway of the aromatic amino acid to be replaced (13, 16 and 17). Researchers have determined that the DAHP isoenzymes can be repressed by feedback inhibition by the presence of its respective amino acid or its analog in the growth medium (18, 19, 20, 21, 22 and 23). The cells can, therefore, be grown in a rich medium supplemented with two aromatic amino acids and grown until late-log phase shutting down the biosynthetic pathway within the cell and exhausting the supply of the third that

is to be replaced by the analog. This method was tried in 5 L LB w/amp supplemented with 1 mM phenylalanine and 1 mM tyrosine at time zero. At time six hours, 1 mM DL-6-fluorotryptophan was added and growth continued until time 16 hours. The cells (15.0 g) were collected and the protein purified as described in Materials and Methods with the exception of a 40% ammonium sulfate cut prior to the loading of the ion-exchange column. The cell growth yield was three-fold higher than described in the Materials and Methods. But, the absorbance at 325, 415 and 455 nm was 0.204, 0.225 and 0.185 respectively. This gave a molar concentration of 0.02 mM for putidaredoxin w/ftrp. The NMR spectra for this small yield indicated no fluorine incorporation.

The growth conditions described in the Materials and Methods in this manuscript, although provided a smaller yield of cell growth, the absorbance values were 10-fold greater for putidaredoxin. The molar concentrations were as follows: 0.03 mM cytochrome P-450_{cam} w/fphe and 0.11 mM putidaredoxin w/ftrp. The NMR spectra shown in figures 4 and 6 were obtained from these proteins. Although the yield of protein was low, the splitting due to the coupling between the fluorine and the hydrogens of the indole ring structure is evident and compares favorably to that of the standards.

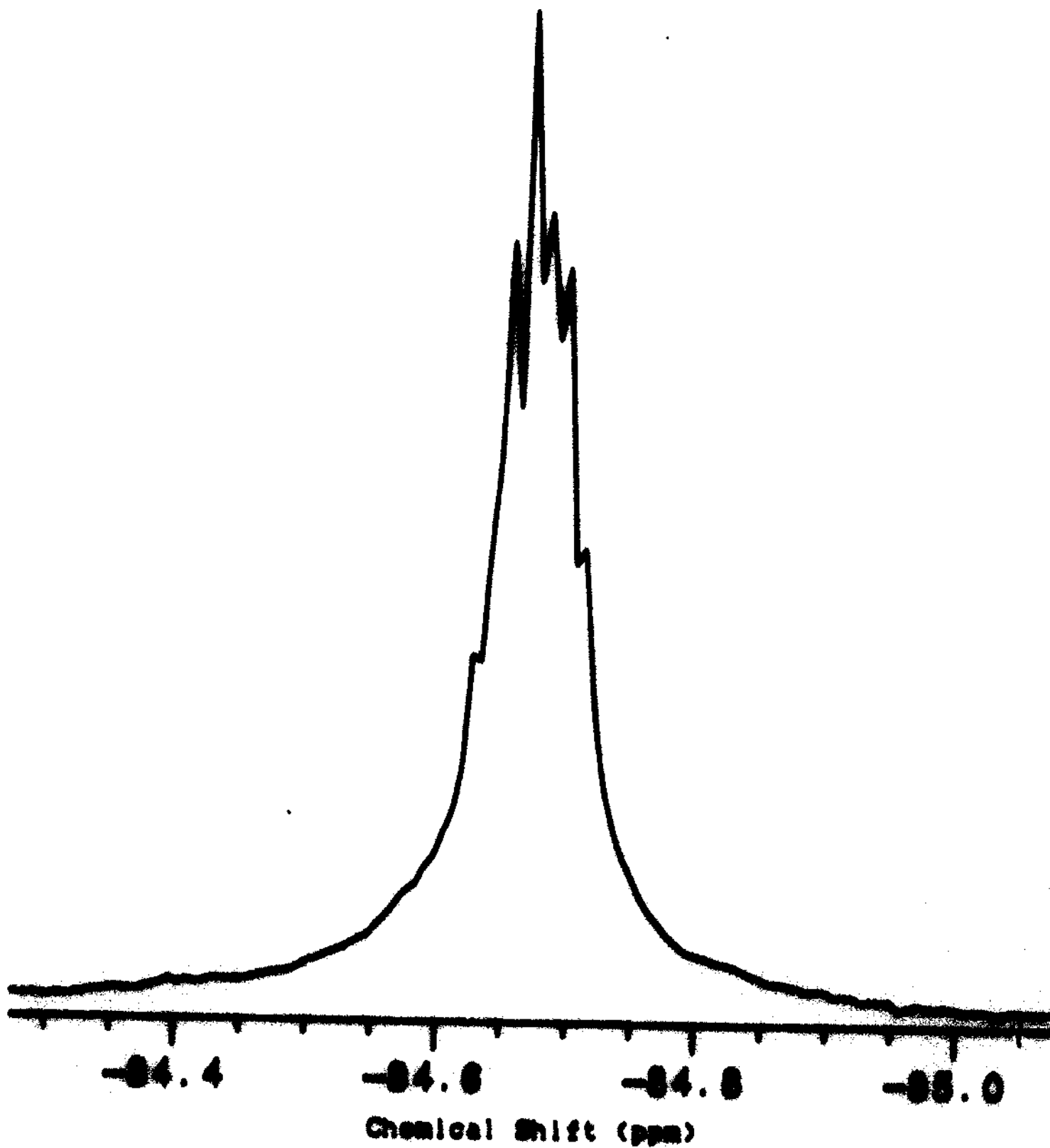


Figure 3: ^{19}F NMR spectrum of DL-4-fluorophenylalanine in D_2O . The chemical shift is measured in parts per million. The external standard is trifluoroacetic acid.

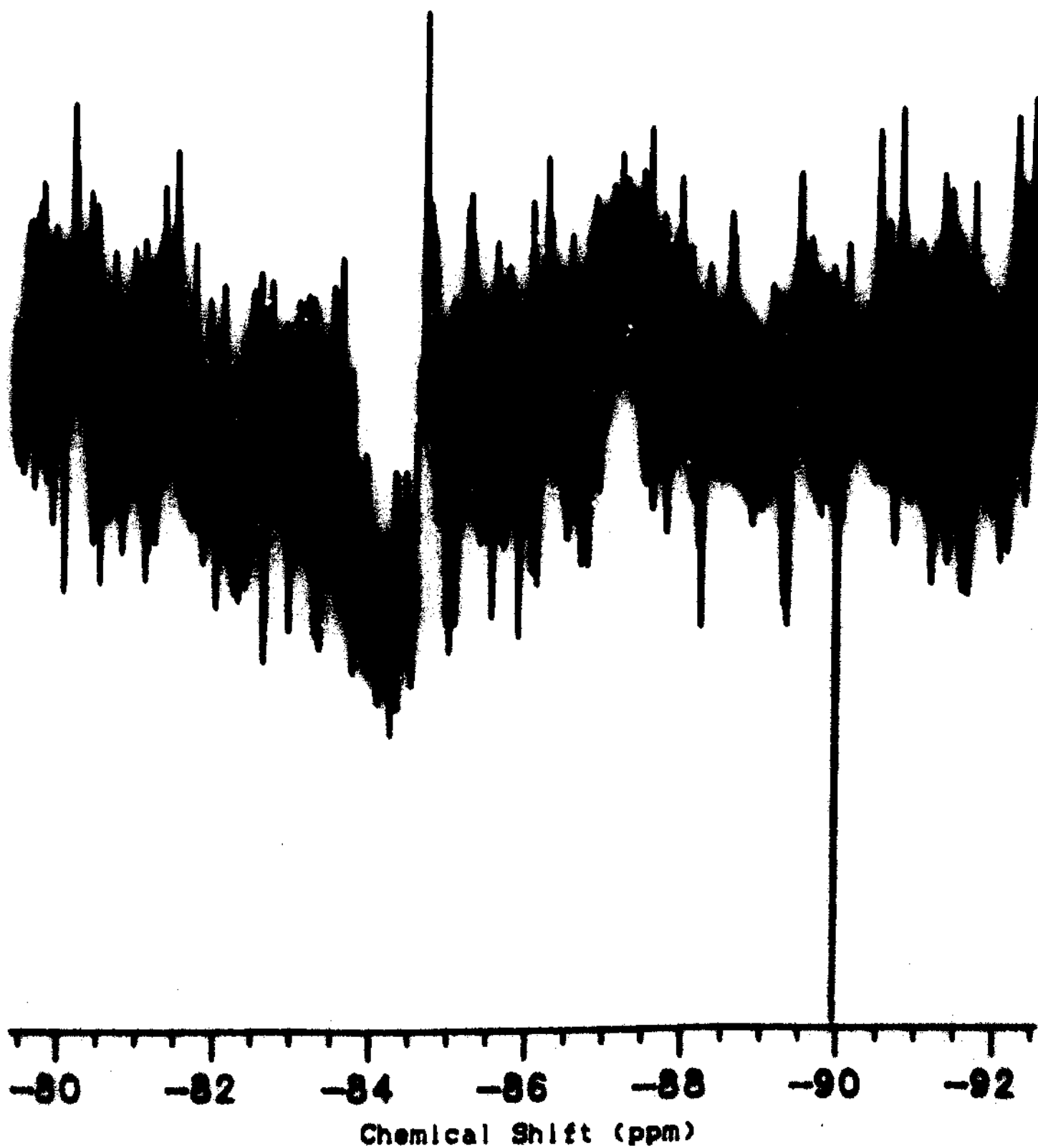


Figure 4: ^{19}F NMR spectrum of 4-fluorophenylalanine cytochrome P-450_{cam} in D_2O . The chemical shift is measured in parts per million. The external standard is trifluoroacetic acid.

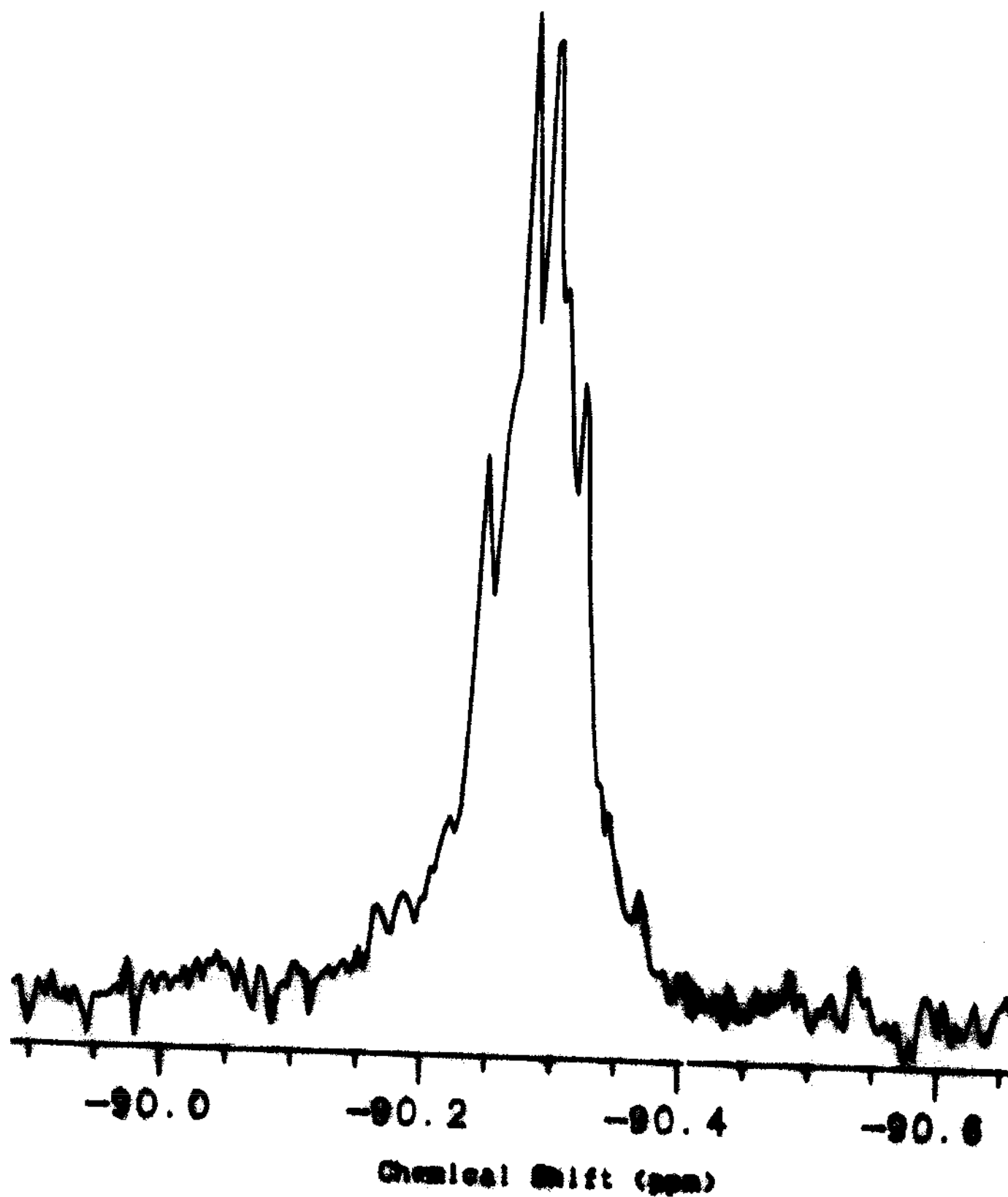


Figure 5: 19F NMR spectrum of DL-6-fluorotryptophan in D2O. The chemical shift is measured in parts per million. The internal standard is trifluoroacetic acid.

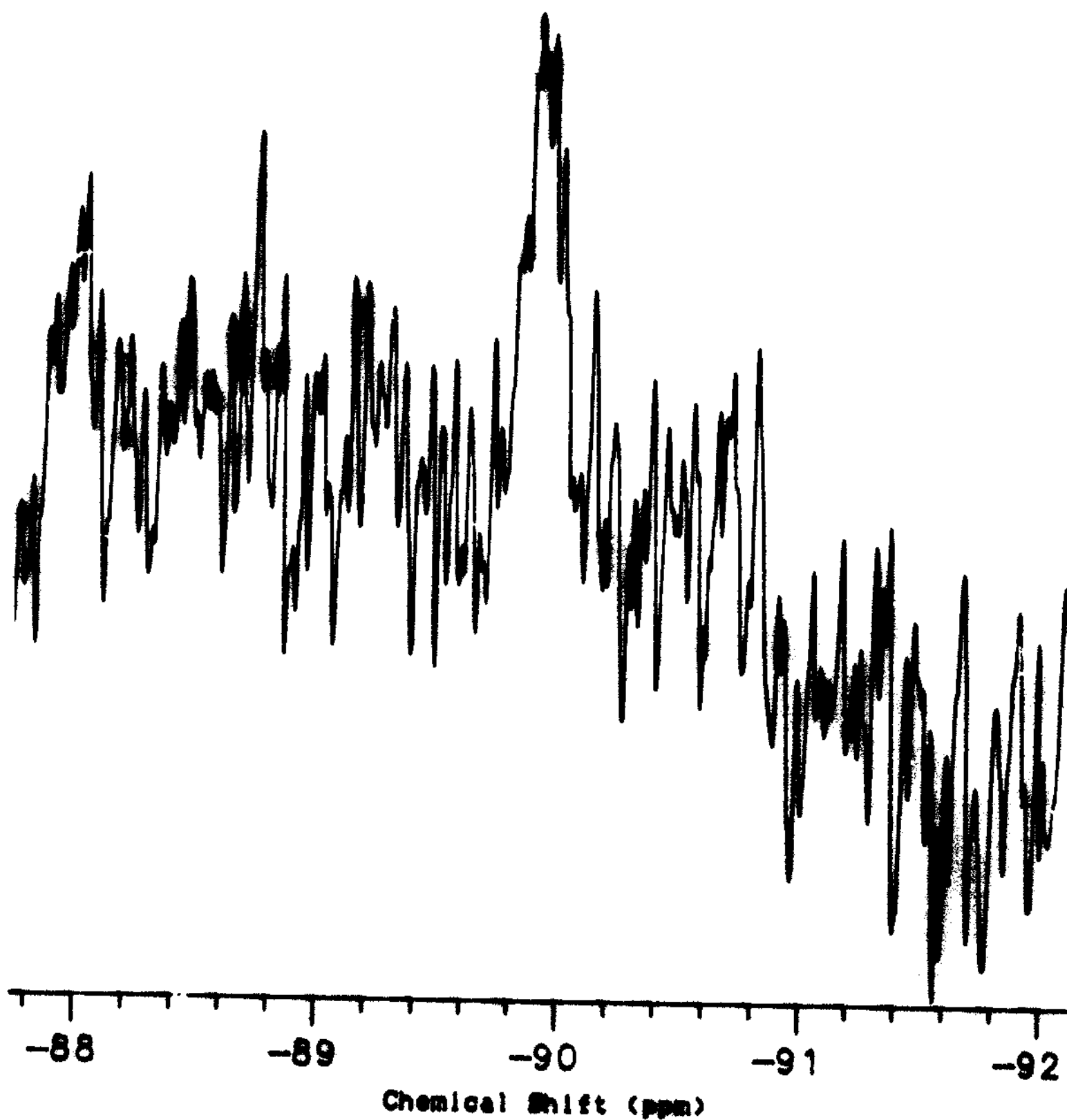


Figure 6: ^{19}F NMR spectrum of 6-fluorotryptophan putidaredoxin in D_2O . The chemical shift is measured in parts per million. The external standard is trifluoroacetic acid.

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