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SYNTHESIS AND CHARACTERIZATION OF A TRYPSIN INHIBITOR

A THESIS

The Honors Program

College of St. Benedict/ St. John's University

In Partial Fulfillment

of the Requirements for the Distinction "All College Honors"

and the Degree Bachelor of Arts

In the Department of Chemistry

by

Eric Schneider

April 25, 1998

SYNTHESIS AND CHARACTERIZATION OF A TRYPSIN INHIBITOR

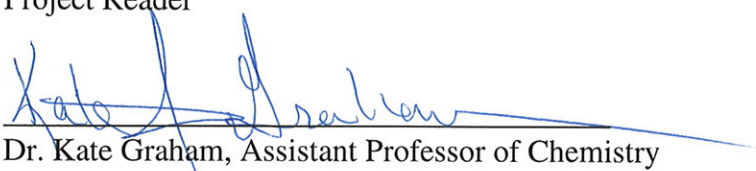
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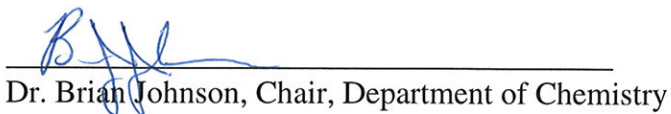
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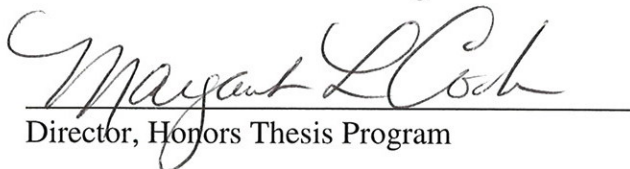
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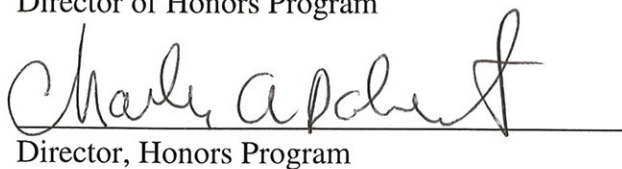
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Director of Honors Program



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Abstract

Inhibitors of biological enzymes are frequently produced by mimicking the molecular structure of the enzyme's natural substrate. Because of the extensive studies that have already been performed on the trypsin enzyme, its natural substrates and its mode of interaction with them are well understood.¹ By finding an easily synthesized molecule to inhibit the trypsin enzyme, college level laboratory experiments could be designed and integrated into organic chemistry and biochemistry courses. In this project, a possible trypsin inhibitor molecule, 4-fluorobenzylamine, was chosen based on its similarity to the natural trypsin substrates and because it has been predicted through computational studies to be a potential trypsin inhibitor. 4-fluorobenzylamine was synthesized and tested for inhibition using Michaelis-Menten kinetics. The inhibition constant, K_i , determined experimentally for a standard 4-Fluorobenzylamine was found to be 0.65 mM, which compared closely to the K_i calculated by Kurinov and Harrison.² However, the K_i determined experimentally for the synthesized 4-Fluorobenzylamine was found to be 3.2 mM.

Introduction

The trypsin enzyme has been one of the most extensively studied enzymes to date.¹ Because of this the structure and function of trypsin have become well understood. The trypsin enzyme is a proteolytic enzyme and is found in the pancreatic juices of the digestive tract.³ Its main function is to break down other proteins during digestion.

Trypsin has been found to be a very specific enzyme. It mainly hydrolyzes amide bonds on the carboxyl side of arginine and lysine amino acids.⁴ This specificity is due to the structure

of the enzyme, especially the active site (Figure 1). Inside the active site of trypsin lies an aspartate residue, Asp 189, which contains a negatively charged carboxylic acid group. This negative charge is a large factor in the active site's preference for arginine and lysine residues.

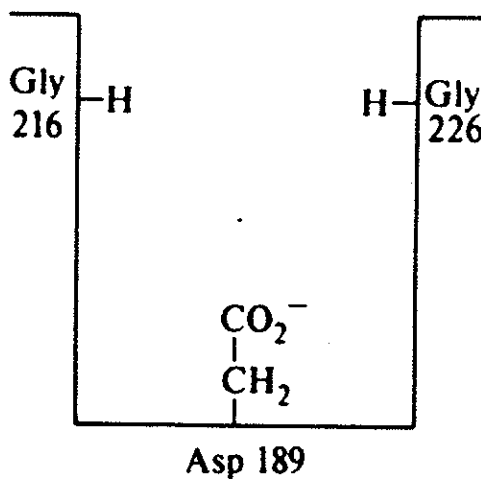


Figure 1. A model of the trypsin active site.⁵

The positively charged amine groups on the end of the lysine and arginine side chains are attracted to the negatively charged Asp 189 (Figure 2).⁵

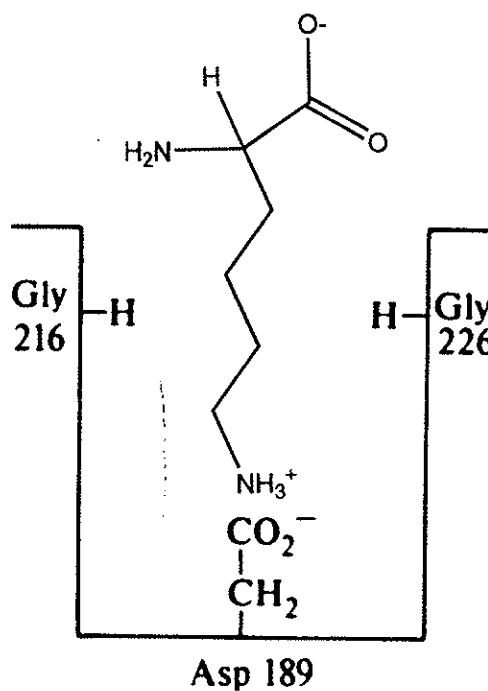


Figure 2. The binding of lysine in the trypsin active site.

This ionic interaction allows the enzyme to bind the substrate tightly as well as align it correctly within the active site. The specific binding increases the substrate hydrolysis by 10^5 times over trypsin enzymes without the Asp 189, a fact that was determined through studies involving mutagenesis of Asp 189.¹ The remaining area of the active site is comprised mainly of hydrophobic amino acids, such as glycine, which help to further stabilize the arginine and lysine side chains.

Once the arginine or lysine has been stabilized within the active site, a catalytic triad of amino acids begins to hydrolyze the amide bond on the carboxyl side of the target amino acid. The catalytic triad is composed of serine 195, histidine 57, and aspartate 102.¹ Binding of the substrate with the active site aligns the substrate in such a way that a nucleophilic attack by serine 195 on the arginine/lysine carbonyl carbon is possible. Histidine 57 is in position to accept a proton from serine 195 as aspartate 102 stabilizes the protonated histidine 57 (Figure 3I). The amide bond is then broken as histidine 57 gives up its proton to the nitrogen in the amide bond (Figure 3II). Then peptide bond is broken and the peptide chain that was located on the carbonyl side of the target amino acid is released while the half containing the arginine/lysine amino acid remains bound to the active site (Figure 3III). A water molecule then acts as a nucleophile and begins the deacylation reaction to release the second half of the protein chain (Figures 3IV-VI).⁶

The well understood mechanism and active site of the trypsin enzyme allow for good inhibitors to be found and designed. This is desirable because trypsin is able to provide a good model for the examination by college undergraduate students. One way to design an inhibitor of an enzyme is to build a molecule that mimics the structure of the enzyme's target molecule.

Hypothetically the molecule should bind with the enzyme's active site in a way that is similar to the natural substrate's interaction with the enzyme. Depending on how well the molecule fits into the active site and how tight the enzyme-molecule binding is, the molecule may prove to be an excellent inhibitor.¹

Following this theory, a possible inhibitor of the trypsin enzyme would be structurally similar to the lysine and arginine amino acid side chains. Two requirements of the inhibitor molecule are that it contain a positive charge on one end and the rest of the chain would be relatively compact and hydrophobic. In addition, the molecule should be easy to synthesize. The molecule 4-fluorobenzylamine (4-FBA) was chosen for this research because it fit these criteria. The choice of this molecule also derived from a paper containing computational analysis on possible trypsin inhibitors which gave a predicted K_i value to use for comparison.² Under neutral to acidic conditions, 4-FBA contains a positively charged amine on one end of a compact, non-polar carbon chained molecule, similar to lysine (Figure 4) and it can readily be purchased from Acros Chemical company. A final reason for choosing 4-FBA is because it can hypothetically be synthesized through a reductive amination of 4-fluorobenzaldehyde (Figure 5). This is important because the ability to easily synthesize the inhibitor would allow this experiment to be integrated into various chemistry course syllabi. An organic chemistry course could focus on the synthesis of the molecule while a later biochemistry course would return to that molecule and look at its inhibition characteristics. Experiments like this would allow students to view their chemistry courses as complementary instead of separate.

By the nature of the designed inhibitor's structure and intended function, it is expected to act as a competitive inhibitor of the trypsin enzyme. In other words, it is expected to bind to the

same active site on the enzyme as the substrate. Because the inhibitor is bound to the active site, the substrate is blocked out of the active site and is unable to bind with the enzyme (Figure 6b). This reduces trypsin's ability to catalyze the hydrolysis of its substrate and is the reason for the inhibition.⁸

However, just because an inhibitor is similar in structure to an enzyme's substrate does not guarantee that it functions as a competitive inhibitor.⁹ There is also the possibility that it functions as a noncompetitive inhibitor or an uncompetitive inhibitor. As a noncompetitive inhibitor, the molecule binds to either the enzyme or the enzyme-substrate at a site other than the substrate active site. Upon binding, the inhibitor alters the shape of the enzyme, including the active site. The altered shape of the active site continues to allow binding of the substrate but no longer allows for proper catalysis (Figure 6c).

As an uncompetitive inhibitor, a molecule is able to bind only to the enzyme-substrate complex. When the substrate binds to the enzyme, a conformational change of the enzyme takes place and a binding site for the inhibitor molecule is created. Binding of the inhibitor to this site prevents the enzyme-substrate complex from forming product (Figure 6d).

Because it is impossible to predict with any certainty which type of inhibition a molecule causes, kinetics experiments are used to determine inhibition types. The trypsin enzyme has been shown to display Michaelis-Menten kinetics, which can be used to experimentally determine the type of inhibition a molecule displays along with the molecules's inhibition constant, K_i .

Using the most basic model of enzyme catalysis, the reaction can be written as



where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex and P is the product. The rate constant k_1 refers to the formation of ES from E + S, k_2 refers to the formation of E and S from the dissociation of ES, and k_3 refers to the formation of E and P from ES. Under the method of initial rates, the formation of ES from E and P can be ignored due to the small concentration of P.

From this reaction equation, the rate of catalysis, V, can be written as ¹⁰

$$V = \frac{dP}{dT} = k_3 * [ES]$$

along with the rates of formation of ES (V_f) and consumption of ES (V_c).

$$V_f = k_1[E][S]$$

$$V_c = k_2[ES] + k_3[ES] = (k_2 + k_3)[ES]$$

If the steady state approximation is now applied, then $V_c = V_f$ and

$$k_1[E][S] = (k_2 + k_3)[ES]$$

which can be rearranged to form

$$[ES] = \frac{k_1[E][S]}{k_2 + k_3} = \frac{[E][S]}{K_M}$$

where $(k_2 + k_3)/k_1 = K_M$, the Michaelis constant. Also, the maximum velocity (V_{max}) of the reaction can be assumed to occur when all of the enzyme in the reaction, E_T , is complexed with the substrate. An equation for this can be written as

$$V_{max} = k_3 * [E_T]$$

Because all of the enzyme in the reaction is found in either the free form, E, or the complexed form, ES, equation 4 can also be written as

$$V_{max} = k_3 * ([E] + [ES])$$

Upon division of equation 4 by equation 5 and substituting equation 3 in for [ES], an expression for V can be written as

$$V = \frac{V_{max} * [S]}{[S] + K_M}$$

which is well known as the Michaelis-Menten equation. The reciprocal of this equation is

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} * \frac{1}{[S]}$$

which can be plotted as $1/V$ versus $1/[S]$. The result is a straight line where the y-intercept equals $1/V_{max}$, the slope equals K_M/V_{max} , and the x-intercept equals $-1/K_M$ (Figure 7).

The Michaelis-Menten equation and the Lineweaver-Burke plot are very helpful in determining the type of inhibition an inhibitor-enzyme complex exhibits. During competitive inhibition the total enzyme, E_T , must be accounted for by including $[EI]$, the enzyme-inhibitor complex. This reveals the equation for a Lineweaver-Burke plot

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_M}{V_{\max}} \left(1 + \frac{[I]}{K_{is}}\right) \left(\frac{1}{[S]}\right)$$

with K_{is} equal to the dissociation constant for the enzyme-inhibitor complex. The equation, and its plot, reveal that the y-intercept is still equal to V_{\max} but the apparent K_M , from the x-intercept, changes along with the slope, which changes by a factor of $(1 + ([I]/K_{is}))$ (Figure 8).

During uncompetitive inhibition the reciprocal expression turns out to be

$$\frac{1}{V} = \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_{ii}}\right) + \frac{K_M}{V_{\max}} \left(\frac{1}{[S]}\right)$$

where K_{ii} is equal to the enzyme/substrate-inhibitor complex. When the expression is plotted at varying substrate concentrations and increasing inhibitor concentrations it reveals an unchanged slope. However, both the V_{\max} and apparent K_M change with a change in the inhibitor concentration (Figure 9).

Noncompetitive inhibition reveals the reciprocal plot

$$\frac{1}{V} = \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_{ii}}\right) + K_M \left(1 + \frac{[I]}{K_{is}}\right) \left(\frac{1}{[S]}\right)$$

with K_{ii} = dissociation constant for E and I and K_{is} = dissociation constant for ES and I. This equation, along with the plot of varying [S] and [I] reveals an unchanged K_M . However, the slope and V_{max} change with a change in inhibitor concentration (Figure 10).

Because each type of inhibition reveals a different change in V_{max} , K_M , and slope of the Lineweaver-Burke plot, these characteristics determine which inhibition model an enzyme-inhibitor complex follows. By plotting $1/V$ versus $1/[S]$ at increasing [S] for different constant [I] the inhibition model can easily be determined because it will reveal a pattern for the changes in slope, V_{max} , and K_M that resembles one of the patterns listed above for competitive, noncompetitive and uncompetitive inhibition.¹⁵

Results/Discussion

An important part of the experiment was to show that 4-FBA has a solubility high enough to effectively inhibit the trypsin enzyme. Because no literature was found on the solubility of 4-FBA, the solubility was first tested visually. Visual examination of the solutions of 4-FBA in varying pH buffered solutions revealed that the maximum solubility was between 1.1g 4-FBA/ 1L buffer (8.8 mM) and 0.55g 4-FBA/ 1L buffer (4.4 mM) at a neutral to acidic pH (below pH 6). The solubility in an acidic solution is due to the protonated form of the amine (the structurally similar benzylamine molecule has a pK_a of 9.35⁽¹²⁾ indicating that the amine would be protonated at a pH less than 9.35). Protonation of the amine allows for a greater solubility in a water solvent.

The use of spectral analysis allowed for a more accurate prediction of the molecule's exact solubility in an aqueous solution. As more 4-FBA goes into solution, the absorbance of the

solution should increase linearly. The graph of absorbance versus concentration of 4-FBA (Figure 11) reveals a linear correlation for the values from 0.11 g/L (0.88 mM) to 0.77 g/L (6.1 mM) of 0.996 with slope of 0.912. The values from 0.88 g/L (7.0 mM) to 1.75 g/L (13.9 mM) have a linear correlation of 0.956 with a slope of 0.592. This evidence suggests that the maximum solubility of 4-FBA in pH 7.5 buffer is about 0.77 g/L (6.1 mM).

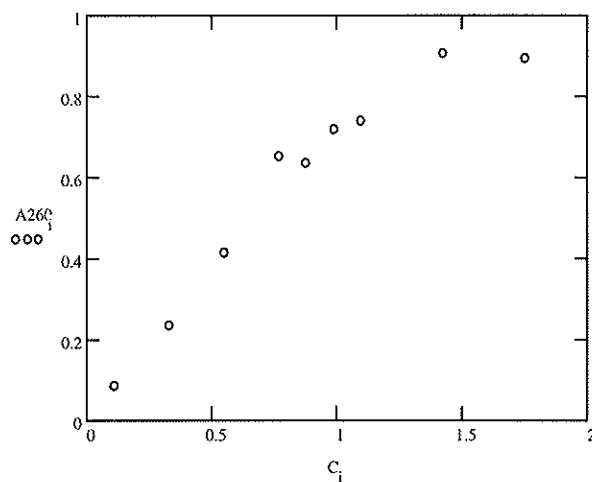


Figure 11. Absorbance at 260nm vs. concentration of 4-FBA in TBS solution

Upon comparison with the K_i of 4-FBA predicted by Kurinov and Harrison², 0.43mM, the maximum solubility of 0.77g/L (6.1 mM) is high enough to produce reliable kinetic assays. Although it is only slightly soluble in water, the 4-FBA has a solubility high enough to allow for the necessary inhibitor-substrate interactions to occur.

Because the 4-FBA was determined to be soluble in an aqueous solution, kinetic assays could be performed with the inhibitor. The kinetic assays were run using Chromozym TH (tosyl-gly-pro-arg-p-nitroanilide) as the substrate. This substrate allowed for easy determination of the

reaction's progress by spectroscopically following the formation of yellow colored p-nitroanaline product (absorbance at 405nm) upon its cleavage by the trypsin enzyme.¹³ However a few problems were encountered. First, after running assays with no inhibitor, 0.5mM inhibitor, and 1mM inhibitor with different substrate concentrations the absorbance values were found to be similar between each assay at the same substrate concentrations. This implied that there was no inhibition occurring with the addition of 4-FBA. However, it was determined that the substrate was being cleaved through base catalyzed hydrolysis when 6M NaOH was added to quench the enzyme-substrate reaction. The hydroxide ion not only denatured the enzyme but also increased hydrolysis of the substrate.

Instead of quenching the reaction by denaturing the enzyme, the absorbance at 450nm was taken while the enzyme catalyzed reaction was in progress. When this was done, the absorbance values for each assay were found to be lower, indicating that the hydroxide ion did hydrolyze the substrate at a very significant rate.

However, the assays still did not indicate any inhibition by the 4-FBA. The absorbance at 405nm every minute of an assay containing 80mM CZTH and 1mM trypsin revealed that the substrate consumption was linear only through 10 minutes (Figure 12). After 10 minutes the rate of substrate consumption continued to drop due to a significant change in the substrate concentration.

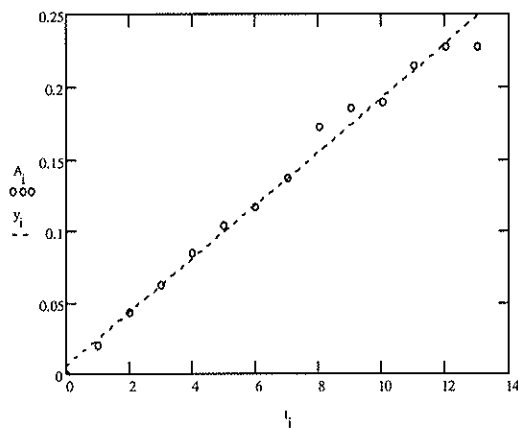


Figure 12. Absorbance at 450nm versus time (minutes)

For this reason, the assays could not be run for 15 minutes. This length of time violated the steady state assumption that $[S_o]=[S_f]$ and allowed for any effect of the inhibitor to be overcome by time.

In order to also verify that the initial rate was linear with respect to the enzyme concentration, another necessity for valid kinetic experiments, the product absorbance at 3 minutes for varying trypsin concentration in 80mM CZTH solutions was determined. A graph of the absorbance versus trypsin concentration revealed a linear graph with a correlation of 0.997 (Figure 13).

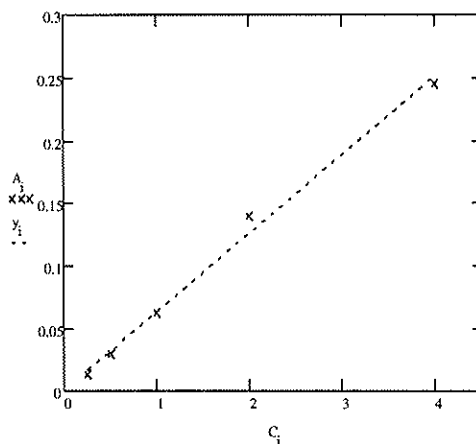


Figure 13. Absorbance (450nm) at 3 minutes vs. trypsin concentration

This graph validates the assumption that the product formation has a linear dependence on the enzyme concentration.

Taking absorbance readings at 7 minutes, a definite effect from the inhibitor was noticed. After entering the kinetic data into the Cleland program, the results indicated that the 4-FBA displayed competitive inhibition, due to the high probability the program gave the competitive fit. (Table 1). A high probability was also given for uncompetitive competition, slightly lower than competitive inhibition. An exact determination between the two models is difficult due to the similar probabilities. The K_{ij} value calculated by the Cleland program for competitive inhibition was $0.65100 \text{ mM} \pm 0.14059 \text{ mM}$ which compares favorably with the calculated value of 0.43 mM . This gives a little stronger indication for the competitive model. A Lineweaver-Burke plot of the experimental and the calculated best fit data also show a good fit for competitive inhibition (Figure 14).

	K_{is}	K_{ij}	weight
Competitive	$0.65100 \pm 0.14059 \text{ mM}$	***	50.593
Noncompetitive	$1.1838 \pm 0.55493 \text{ mM}$	$2.2693 \pm 1.3262 \text{ mM}$	0.56855, 3.2573
Uncompetitive	***	$0.92347 \pm 0.21768 \text{ mM}$	21.104

Table 1. Cleland results for pure 4-FBA

Because the 4-FBA does exhibit inhibition of the trypsin enzyme, it is necessary to attempt the synthesis of the molecule in order for a curriculum addition to be developed.

Theoretically the synthesis of 4-fluorobenzylamine can easily be achieved through a reductive

amination of 4-fluorobenzaldehyde.

Using anhydrous starting reagents, running the reaction under inert atmosphere, and adding 3Å sieves to absorb the water produced by the reaction itself, the reaction ran smoothly. When the reaction was complete the mixture was a yellow color, similar to 4-FBA. After extracting with anhydrous ether, the remaining product was a white crystal. An IR and a melting point were performed on the crystals. The IR compared well to an IR for the 4-fluorobenzylamine hydrochloride salt found in the Aldrich Catalog of IR spectra.¹⁴ The melting point of the crystals was sharp at 295° C (literature value 274-275°C)¹⁵ which may support the hypothesis that the crystals were the impure hydrochloride salt .

The problem in the procedure was determined to be in the extractions. After acidifying the reaction mixture, a white insoluble solid formed and collected on the interface of the ether-aqueous layers. This solid had been taken up with the ether extractions in the previous experiment. However, if this was the hydrochloride salt then it should have been soluble in the aqueous layer. The later addition of KOH base would have returned it to the deprotonated form and allowed it to be extracted with the ether. This is exactly what happened. Upon adding KOH to the solution with crystals, the crystals disappeared. In the end, the extracts from the basic solution produced a yellow colored liquid, exactly where the 4-FBA product was expected to appear.

The IR analysis of the product contained many of the peaks expected for a 4-fluorobenzylamine product. The important IR peaks are summarized in Table 2.

Absorption (cm ⁻¹)	Corresponding Vibration	Absorption (cm ⁻¹)	Corresponding Vibration
3500-3400	N-H stretch, 1° amine	1150	C-F stretch
3050	sp ² C-H stretch	900-650	N-H oop bend
2900-2800	sp ³ C-H stretch	810	1,4 disubstituted benzene
2000-1600	1,4-disubstituted benzene	****	****

Table 2. Important IR peaks and their related vibration for synthesized 4-FBA

The crucial primary amine peaks at 3500 and 3400 appeared very weak (Figure 15) when compared to the IR spectra of pure 4-FBA (Figure 16). This could possibly be due to a less concentrated salt plate when the IR was taken or dilution due to impurities in the product. The absence of a peak around 1700 indicated that none of the initial aldehyde starting reactant remained. This, along with other IR peaks, gives good evidence that the product synthesized was 4-FBA.

The NMR spectra also gave evidence that the desired 4-FBA molecule had been synthesized. The peaks are summarized in Table 3.

Chemical shift (ppm)	Integration	Coupling	Corresponding Fraction
1.65	3.5	Singlet	-NH ₂
3.78	4.0	Singlet	benzene -CH ₂ - amine
7.05	5.0	Multiplet	disubstituted benzene
7.30	5.0	Multiplet	disubstituted benzene
1.2	1.0	Multiplet	contaminant
2.0	1.0	Triplet	contaminant
3.5	1.5	Singlet	contaminant

Table 3. Summarization of NMR peaks for synthesized 4-FBA

The NMR data show that there is no aldehyde starting reagent remaining in the reaction product. Again this is evidence that a reaction has occurred and the rest of the NMR data (Figure 17) correspond well with the NMR data from the pure 4-FBA (Figure 18) and the NMR spectra found in the Aldrich Library of ^{14}C and ^1H FTNMR spectra.¹⁶ The integration of the peaks for 4-FBA should theoretically be 1:1:1:1 because there are two amine hydrogens, two carbon hydrogens, and two aromatic hydrogens on each half of the disubstituted ring. The ratio from the NMR data close to 1:1:1:1, providing more evidence that 4-FBA is present in the product. Contamination is obvious from the extra, small peaks present in the NMR data. The peaks at 1.2 ppm and 3.5 ppm may correspond to some remaining ether solvent because the ether was removed by using only a flow of nitrogen gas.

Because the 4-FBA appeared to have been synthesized, the next step was to test its inhibition of trypsin. Following the same procedure developed for the pure 4-FBA, assays with the synthesized 4-FBA were run. When the data was interpreted by the Cleland program, the results indicated that the synthesized 4-FBA acted as either a competitive or an uncompetitive inhibitor (Table 4).

	K_{is}	K_{ii}	weight
Competitive	3.1795 ± 1.7079	***	0.34284
Noncompetitive	2.1708 ± 0.70974	-7.1190 ± 6.8032	$1.9852, 2.1606 \times 10^{-2}$
Uncompetitive	***	2.8443 ± 0.69643	2.0618

Table 4. Cleland results for synthesized 4-FBA

The weights given to both the competitive and uncompetitive inhibition constants are very close

which makes a determination between the two hard. Because the pure 4-FBA exhibited competitive inhibition, the Lineweaver-Burke plot corresponding to competitive inhibition was plotted (Figure 19). As the Cleland program indicates, the experimental data does not fit this plot as well as the pure 4-FBA data.

A possible reason for this discrepancy is the contaminants in the synthesized 4-FBA. Because there were contaminants in the synthesized 4-FBA the expected 4-FBA concentration in the kinetic assays would be lower than the concentration of inhibitor believed to have been present in the assays. The lower inhibitor concentration would be expected to result in less inhibition of the enzyme and therefore a result in the calculation of a higher inhibition constant, just like the data that is observed with the contaminated inhibitor. Another possibility is that the contaminants may have interfered in some way with the mechanism of the enzyme.

Conclusion

The data for the pure 4-FBA shows that the molecule can function as an inhibitor of the trypsin enzyme. It even compared well with the inhibition constant predicted by Kurinov and Harrison.² However, the synthesis of the inhibitor proved to be difficult. The spectral data shows that if the reaction is run under strict anhydrous conditions 4-fluorobenzylamine can be made from 4-fluorobenzaldehyde. However, the experimental procedure used in this project results in a contaminated 4-FBA product. Future attempts at the synthesis of 4-FBA need to include a purification step. Recrystallization of the product in the form of the hydrochloride salt is one possibility for purification. The liquid form could easily be recovered by placing the recrystallized chloride salt into a basic aqueous solution and extracting it out with ether.

Future work on this project should also include repetitions of the kinetic assays. Due to time constraints the kinetic assays were only run one or two times in this project. More kinetic assays would lead to better support for the inhibition constants determined in this experiment.

A possibility for extension of this work would include the designing of other easily synthesized trypsin inhibitors. Inhibitors with higher solubilities in an aqueous phase could be designed by replacing the fluorine atom with a more polar group, for example a hydroxyl group, to decrease the insolubility factor. Inhibitors could also be designed to bind more tightly with the substrate. These inhibitors may be designed to have covalent or ionic interactions with one of the nearby residues in the catalytic triad (the 4-hydroxybenzylamine molecule may work here also).

Upon completion of this work, the possibility of adding this experiment to the curriculum of different chemistry courses is very good. The organic synthesis, once perfected, should be easy enough for an introductory organic course. It would also be very simple for the inhibition of trypsin to be integrated into the curriculum of a biochemistry lab. These possibilities hold great promise for a more integrated college chemistry curriculum.

Experimental Procedure

General procedure. 4-Fluorobenzylamine (Acros Organics), 4-Fluorobenzaldehyde (Aldrich Chemical Company), trypsin (Sigma Chemical Company), sodium cyanoborohydride (Aldrich Chemical Company), and Chromozym TH (Sigma Chemical Company) were used as obtained. The 3Å sieves were dried under vacuum while in a 160°C oil bath for 24 hours before use. The ammonium acetate was dried in a vacuum desiccator in the presence of P₂O₅ for 24 hours before use.

All kinetic assays were followed spectroscopically by measuring the absorbance of p-nitroaniline, the product upon cleavage of Chromozym TH (tosyl-gly-pro-arg-p-nitroanilide), at 405nm minus a reference spectrum at 650nm. The solutions of Chromozym TH (CZTH) were prepared from a 1mM CZTH solution in 10 mM HCL. The Tris Buffer solutions (TBS) were 20 mM Tris, 0.1 M NaCl, and 1mM CaCl₂. All Trypsin solutions were prepared by diluting a standard solution of 10 μM trypsin and 0.1% Bovine serum albumin in TBS. The Molecular Devices Vmax microplate reader was used to read the absorbance of the p-nitroaniline. The spectroscopic measurements on the solubility of 4-FBA were performed using a Hewlett Packard 8452A Diode Array spectrophotometer with a 1 cm quartz cuvette. A Mattson Galaxy Series FTIR 5000 was used for all IR measurements and a Varian Gemini 2000 300MHz NMR was used for all NMR measurements.

The kinetic data was evaluated using the Cleland program.¹⁷ All graphical data was plotted using the Mathcad 6.0 program.¹⁸

Visual determination of 4-fluorobenzylamine solubility. The solubility of 4-Fluorobenzylamine (4-FBA) was tested by visual examination. Aqueous buffers ranging from pH 5 to pH 10 were prepared. Four 1 mL samples of each buffer were placed in small vials along with 10 μL, 50 μL, 100 μL, and 200 μL of 4-FBA. The vials were shaken and allowed to sit for 24 hours. After equilibration, the vials containing 5 μL or more 4-FBA had noticeable droplets of insoluble 4-FBA at the bottom of the vials. Only the vial containing 1 μL 4-FBA appeared to be completely soluble in the aqueous solution. The acidic to neutral pH solutions also appeared to allow a higher solubility (ie. less insoluble 4-FBA) than the basic solutions.

For pH 5 through pH 7 cosolvents were used to help increase solubility. Solutions of

buffer/ 1%, 5%, or 10% methanol, and buffer/ 5% DMSO were tested in a similar manner to the pure buffer solutions. Visual examination did not determine an increase in solubility upon addition of the cosolvents.

Larger scale solutions were prepared using a pH 7.5 Tris buffer solution (TBS) (20 mM Tris, 0.1 M NaCl, and 10 mM CaCl₂). To 1 L of this buffer solution, 1 mL of 4-FBA was added, shaken and allowed to sit for 24 hours. To 250 mL of the TBS, 0.125 mL 4-FBA was added shaken and allowed to equilibrate for 24 hours. This produced an equivalent 0.55 g 4-FBA/ 1 L buffer solution (4.4 mM). The larger scale was prepared to aid with more precise visual determination of the solubility. The determination of solubility was made by observing if any insoluble 4-FBA remained on the bottom of the flask. Beads of 4-FBA remained in the 1.1 g/1 L solution (8.8 mM). In the 0.5 g/1 L (4.4 mM) solution there appeared to be no insoluble 4-FBA.

Spectroscopic determination of 4-FBA solubility. Solutions of 13.9 mM, 11.4 mM, 8.8 mM, 7.9 mM, 7.0 mM, 6.1 mM, 4.4 mM, 2.6 mM, and 0.87 mM 4-Fluorobenzylamine in TBS were prepared and allowed to equilibrate for up to 48 hours. A 1 mL portion of each solution was diluted 10 fold with TBS. Using TBS as a blank, the absorbance of each of the 9 samples was measured. The maximum absorption was found to be 206 nm; the absorbance at 206 nm versus the 4-FBA concentration (Beer's law plot) was plotted.

The plot revealed a linear increase in absorbance through a concentration of 0.77 g/ 1L (6.1 mM). At concentrations above 0.77 g/ 1L the absorbance no longer remained linear, revealing a maximum solubility at approximately 0.77 g/ 1L.

Validation of kinetic assays. Using a 96 well micro titer plate, in wells A1-A15 175 μ L of TBS and 50 μ L of 400 μ M CZTH were placed. At time 0 minutes 25 μ L of 10nM trypsin was

added to the well A1, producing a final volume of 250 μL and final concentrations of 1nM trypsin and 80 μM CZTH. At times 1 minute, 2 minutes, 3 minutes, etc. 25 μL of 10 nM trypsin was added to wells A2, A3, A4, etc. respectively. At 14 minutes an absorbance reading at 405-650nm was taken using an 80 μL solution of CZTH as a blank. No trypsin was added to well A15. The absorbance measurements versus time were graphed. The graph revealed a linear relation between substrate cleavage and time through ten minutes. The rate after ten minutes began to decrease.

In order to also show that the initial velocity is a linear function of the trypsin concentration, the following procedure was followed. Assays with final trypsin concentrations of 4nM, 2nM 0.5nM and 0.25nM were used. For each of these four assays, the procedure above (using a 1nM trypsin final concentration) was followed TBS/ 10nM trypsin volumes added to 100 μL /100 μL , 150 μL /150 μL , 187.5 μL /12.5 μL , and 193.75 μL /6.25 μL to produce the desired trypsin concentrations in 250 μL total solution. The absorbance measurements versus time was plotted for each of these assays. Also, a plot of the absorbance at 3 minutes versus the trypsin concentration was graphed. The graph revealed a linear correlation between the substrate cleavage at 3 minutes and the trypsin concentration.

Kinetic assays with pure 4-fluorobenzylamine inhibitor. In a total volume of 250 μL , eight different substrate (Chromozym TH) concentrations were used: 80 μM , 40 μM , 20 μM , 10 μM , 5 μM , 2.5 μM , 1.25 μM , 0.625 μM . The absorbance values at 405 nm minus 650 nm for the eight concentrations were measured. These values were used to account for the absorbance of the unreacted Chromozym TH in the kinetic assays. Kinetic measurements were made using

reactions containing zero inhibitor, 0.5 mM 4-FBA inhibitor, and 1mM 4-FBA inhibitor with each of the eight substrate concentrations and 1 nM trypsin. All reactions were run for 7 minutes at which time the absorbance at 405nm minus 650 nm was measured. After subtracting the Chromozym TH absorbance values the data was interpreted by the Cleland program. The data from Cleland was then plotted. The results from the Cleland program indicated competitive inhibition with a K_{is} value of 0.65100 ± 0.14059 mM.

Kinetic assays with synthesized 4-Fluorobenzylamine inhibitor. Assays with the synthesized 4-FBA were run using the same procedure for the pure 4-FBA. The results from the Cleland program indicated competitive inhibition with a K_{is} value of 3.1795 ± 1.7079 mM.

*Synthesis of 4-Fluorobenzylamine.*¹⁹ Using completely anhydrous conditions, a solution of 10 mL anhydrous methanol, 2.5 mmol sodium cyanoborohydride, 33.6 mmol ammonium acetate, and 0.2472 g 3Å sieves was mixed in a 25 mL round bottom flask. A magnetic stirbar was added and the flask was capped with a septum. Using a hypodermic needle as an escape hole, the air in the flask was evacuated with a flow of nitrogen gas. Through the septum, 3.8 mmoles 4-fluorobenzaldehyde was injected into the reaction mixture. The reaction was run for 48 hours under N_2 gas using the apparatus shown in Figure 20.

The reaction mixture was worked up using an acid-base technique. Using suction filtration, the sieves were removed from the reaction mixture and washed with 2x1 mL methanol, which was added back to the reaction mixture. The solution was then made acidic ($pH < 2$) by addition of approximately 10 mL of 6M HCl and tested using pH paper with pH 0-14 range. This caused the solution to lose its yellow color and become colorless. The reaction mixture was concentrated at low pressure by rotoevaporation. The remaining solution and white solid was

taken up in 25 mL deionized water, causing some of the white solid to go into solution, but not all of it. The solution was checked to still have a pH < 2 with pH paper (addition of more HCl is necessary if the pH is no longer < 2). The organics were extracted with 3x5 mL portions of anhydrous ether. During the extractions, a layer of white crystals collected at the solvent interface and was left in the aqueous fraction. The extractions were set aside and solid KOH pellets were slowly added to the remaining aqueous solution until the pH > 10 by pH paper. Upon addition of KOH the white crystals left in the aqueous phase began to disappear. The aqueous solution was saturated with NaCl and the organics were extracted with 3x5 mL ether. The collected organics were dried with anhydrous MgSO₄ and filtered to remove the drying agent. The ether was evaporated with a stream of N₂ gas to give the 4-Fluorobenzylamine product: ¹H NMR (CDCl₃, ppm) δ 1.2 (m, 0.5H), 1.65 (s, 2H, FC₆H₄CH₂NH₂), 2.0 (t, 0.5H), 3.5 (s, 1H), 3.78 (s, 2H, FC₆H₄CH₂NH₂), 7.05 (m, 2.5H, FC₆H₄CH₂NH₂), 7.30 (m, 2.5H, FC₆H₄CH₂NH₂); IR (NaCl, cm⁻¹) 3400-3500, 3050, 2800-2900, 1900, 1750, 1150, 810.

Later attempts at the synthesis of 4-Fluorobenzylamine should include evaporation of the ether solvent from the product extractions under low pressure, for example rotoevaporation. An increase in the volume of starting material may also be necessary in order to recover enough product for multiple kinetics experiments.

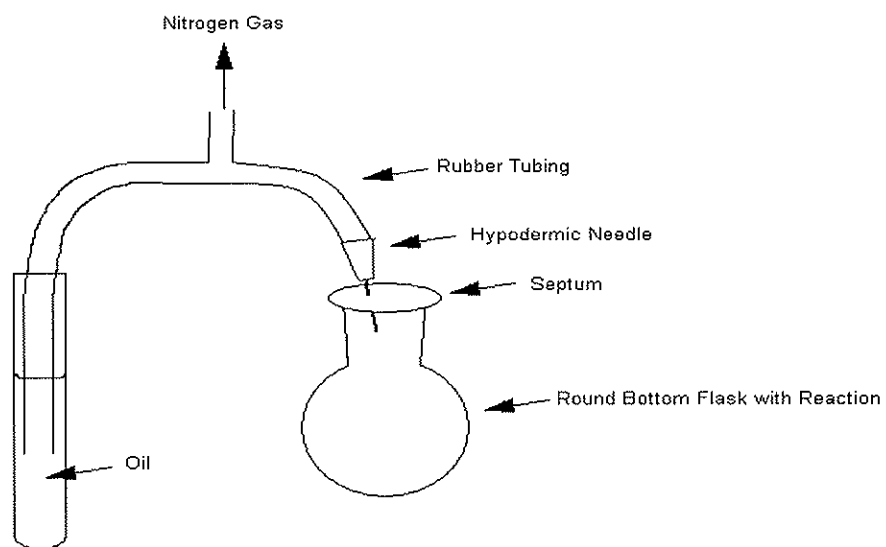


Figure 20. Apparatus for synthesis of 4-Fluorobenzylamine

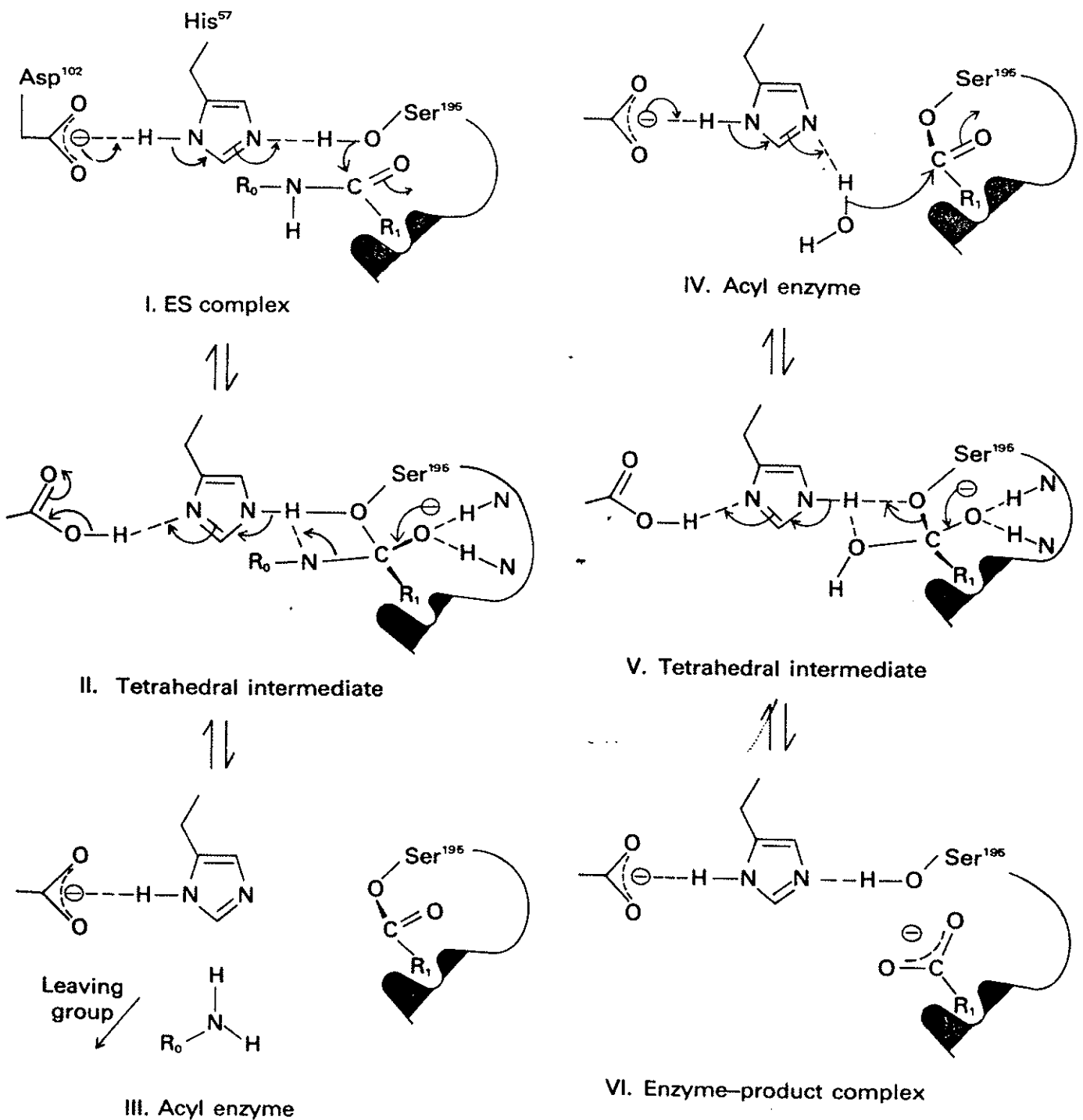
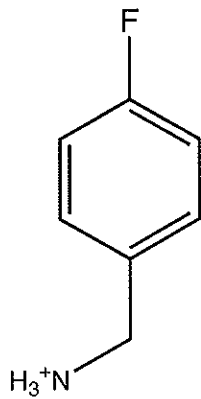
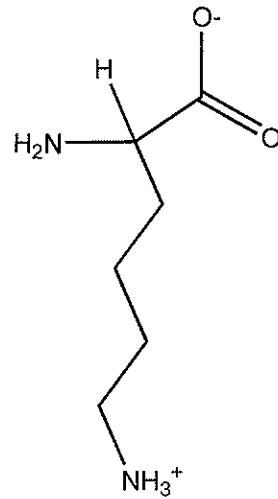


Figure 3. Mechanism of of the trypsin enzyme.⁶

Figure 4. **Structure of 4-fluorobenzylamine compared to lysine**



4-fluorobenzylamine



Lysine

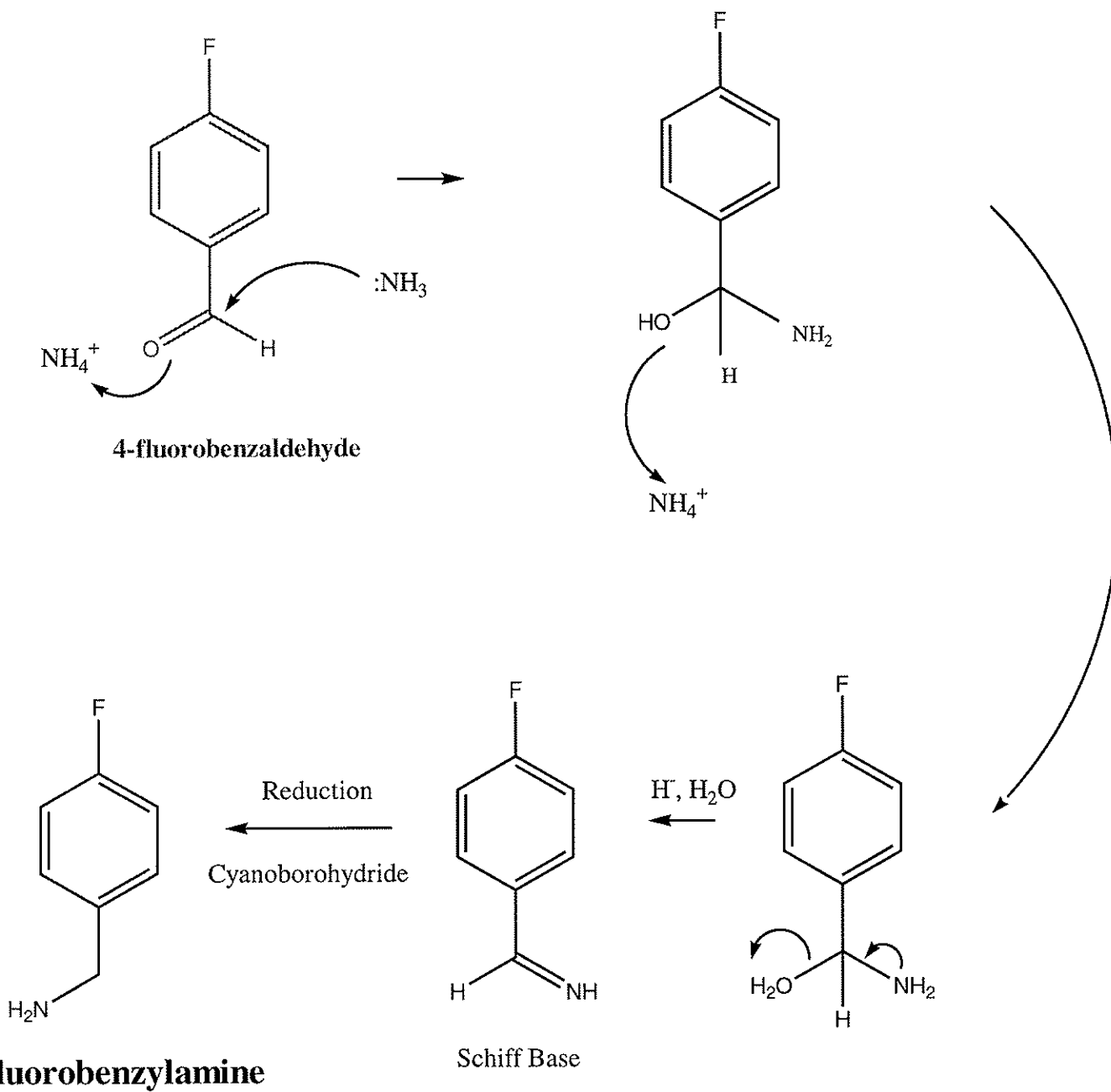


Figure 5. Mechanism for reductive amination of 4-fluorobenzaldehyde

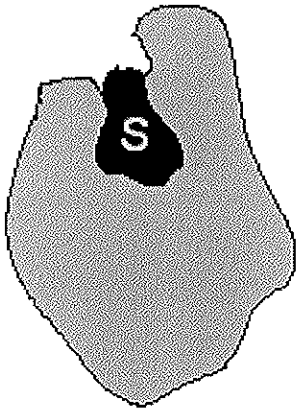


Figure 6a. Enzyme-substrate complex

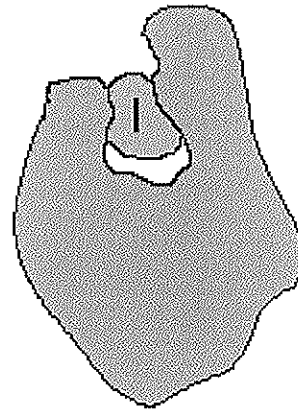


Figure 6b. Competitive Inhibition

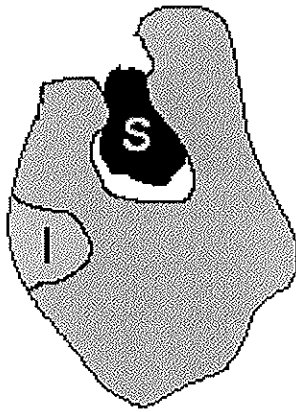


Figure 6c. Noncompetitive Inhibition

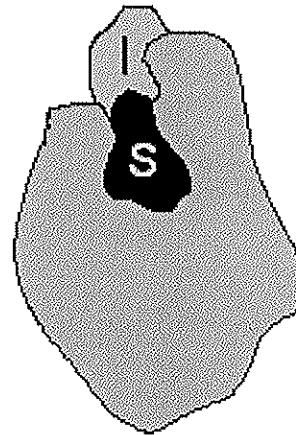


Figure 6d. Uncompetitive Inhibition

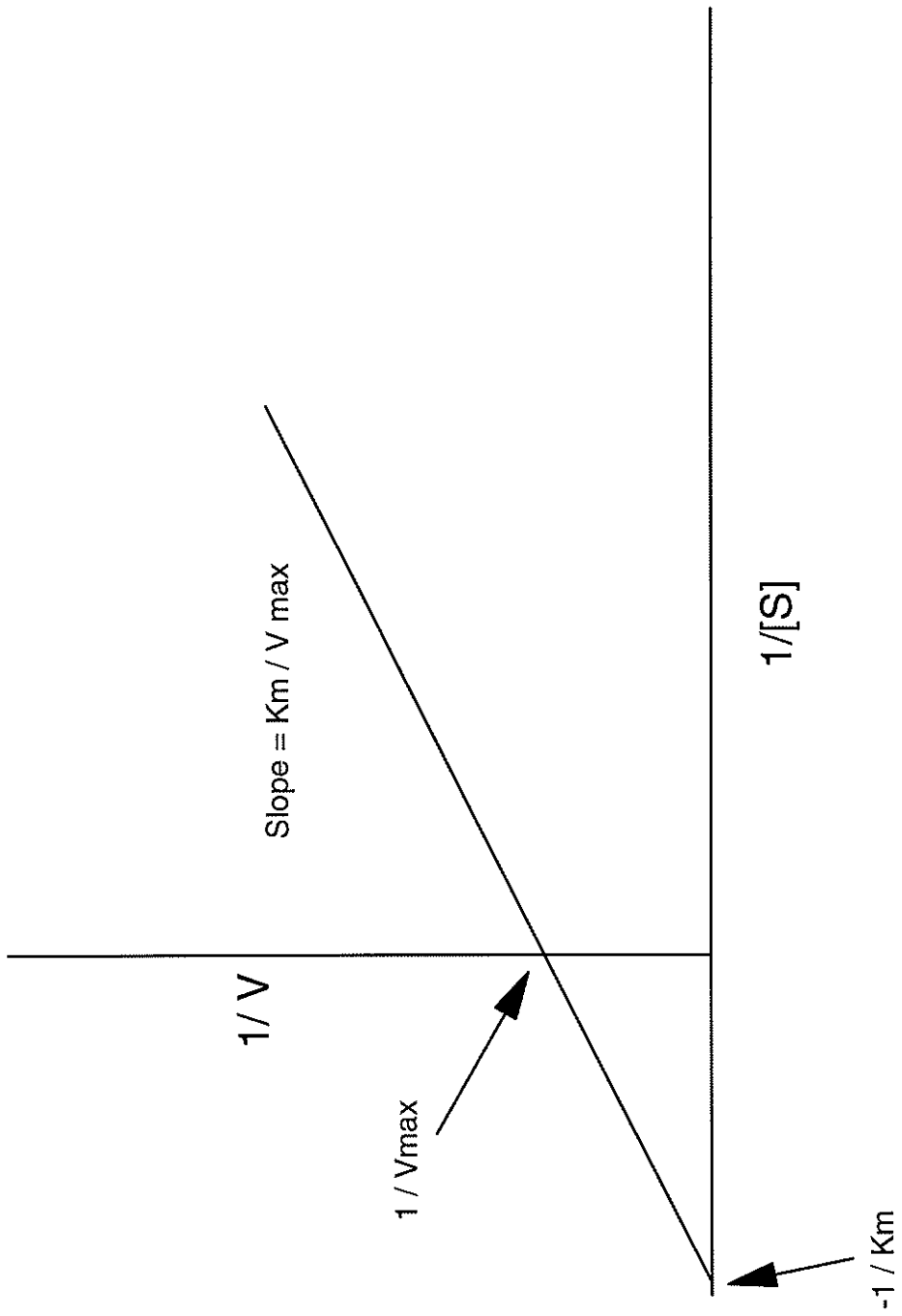


Figure 7. Lineweaver-Burke Plot

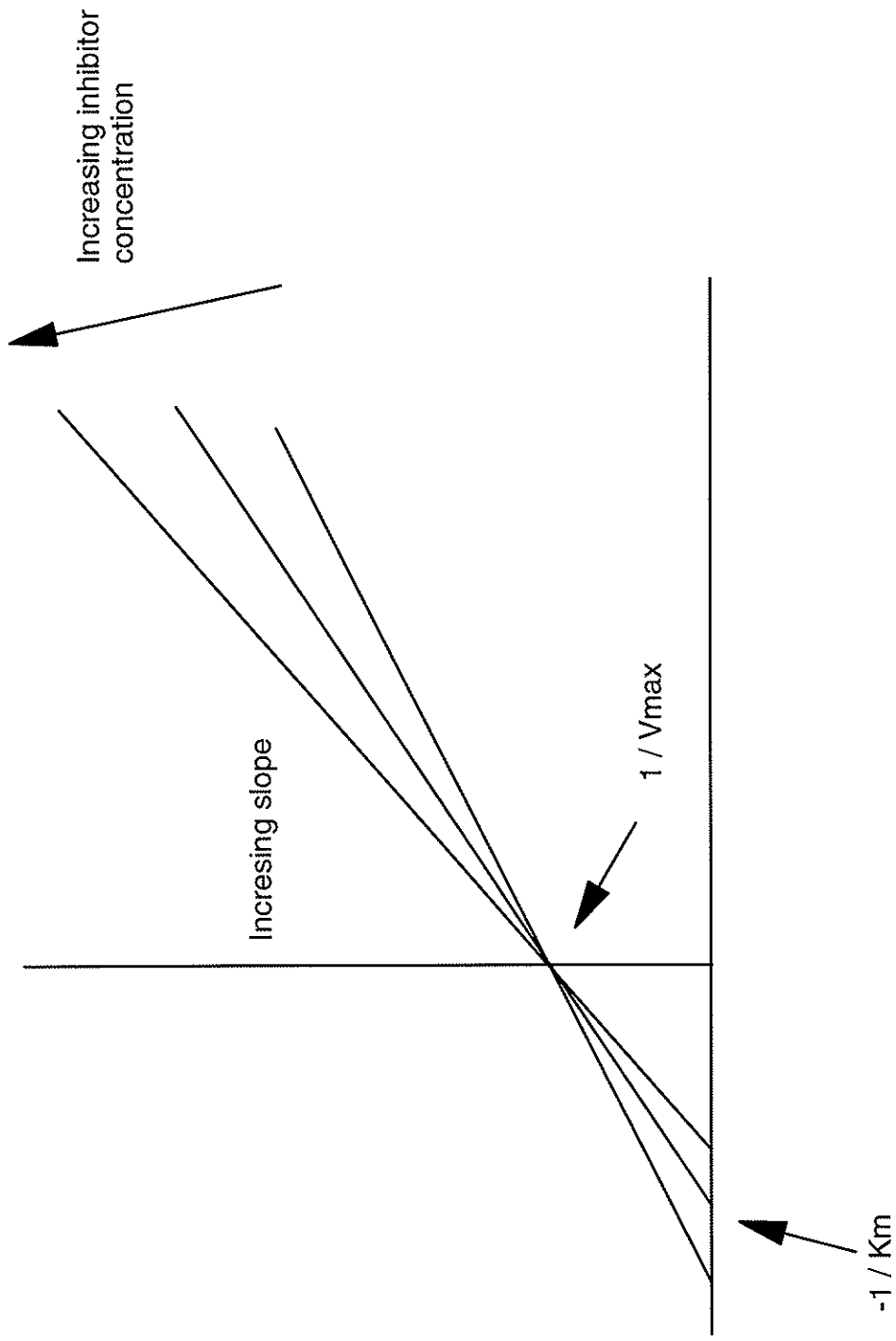


Figure 8. Competitive Inhibition

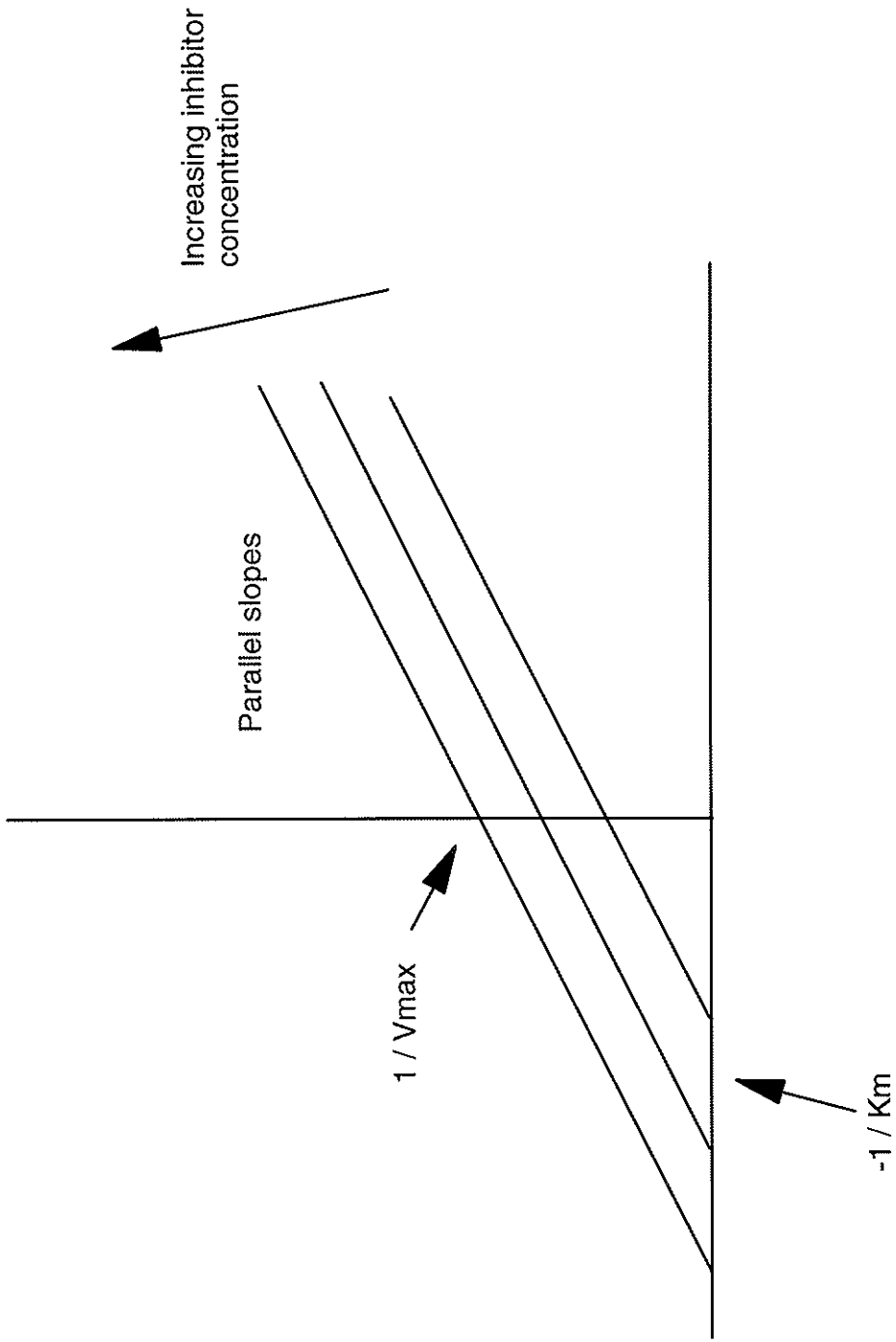


Figure 9. Uncompetitive Inhibition

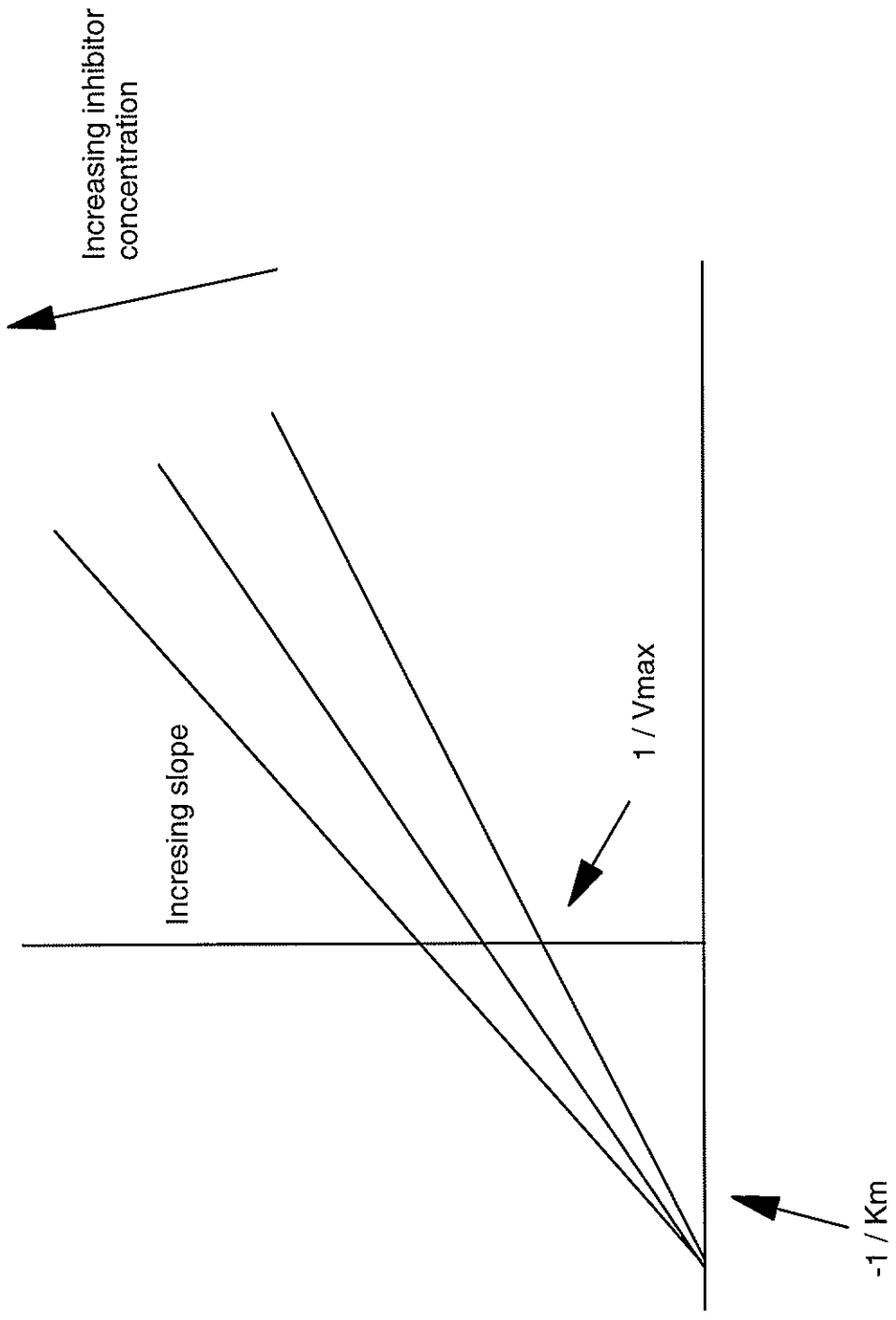


Figure 10. Noncompetitive Inhibition

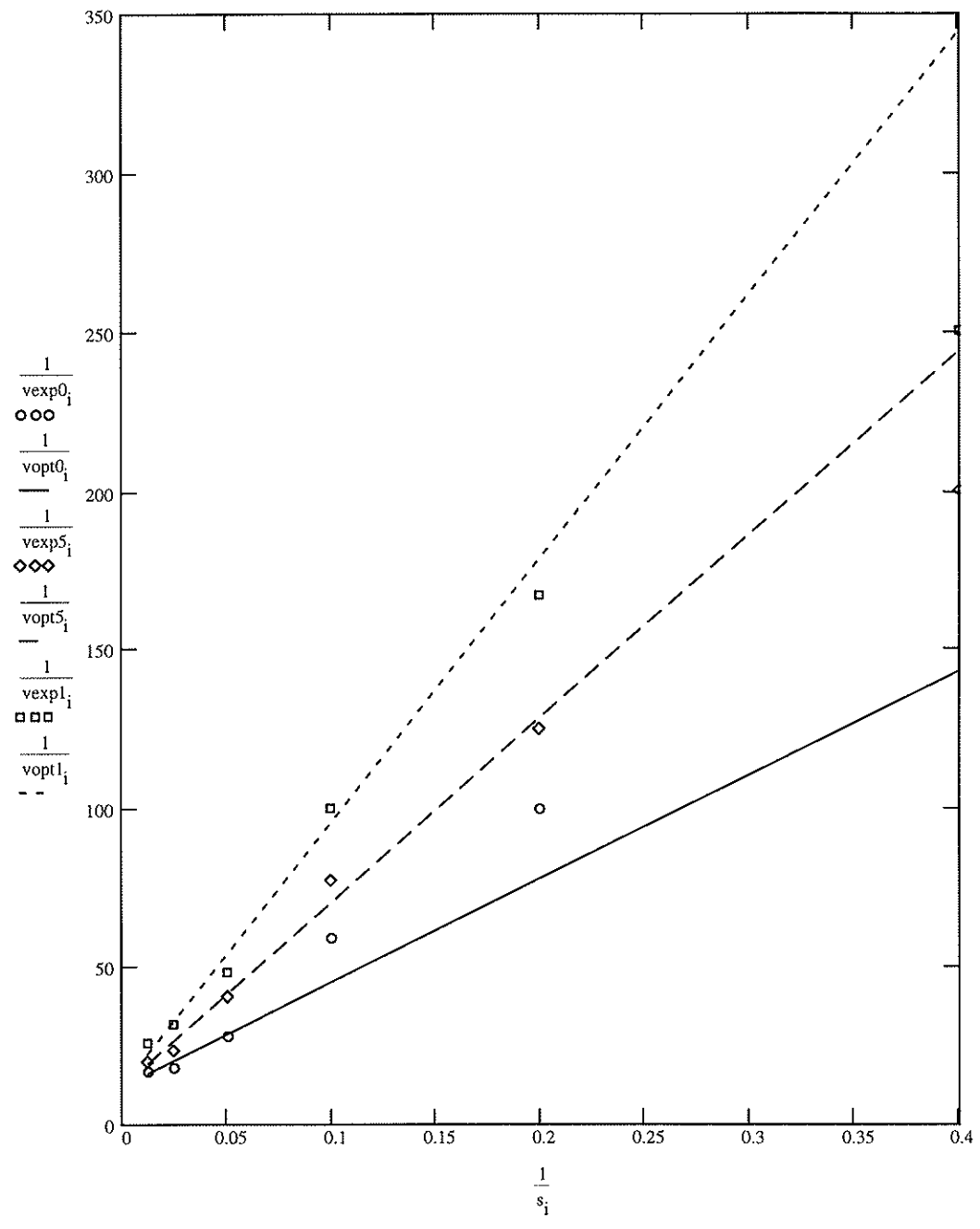


Figure 14. Lineweaver-Burke plot for inhibition with pure 4-FBA

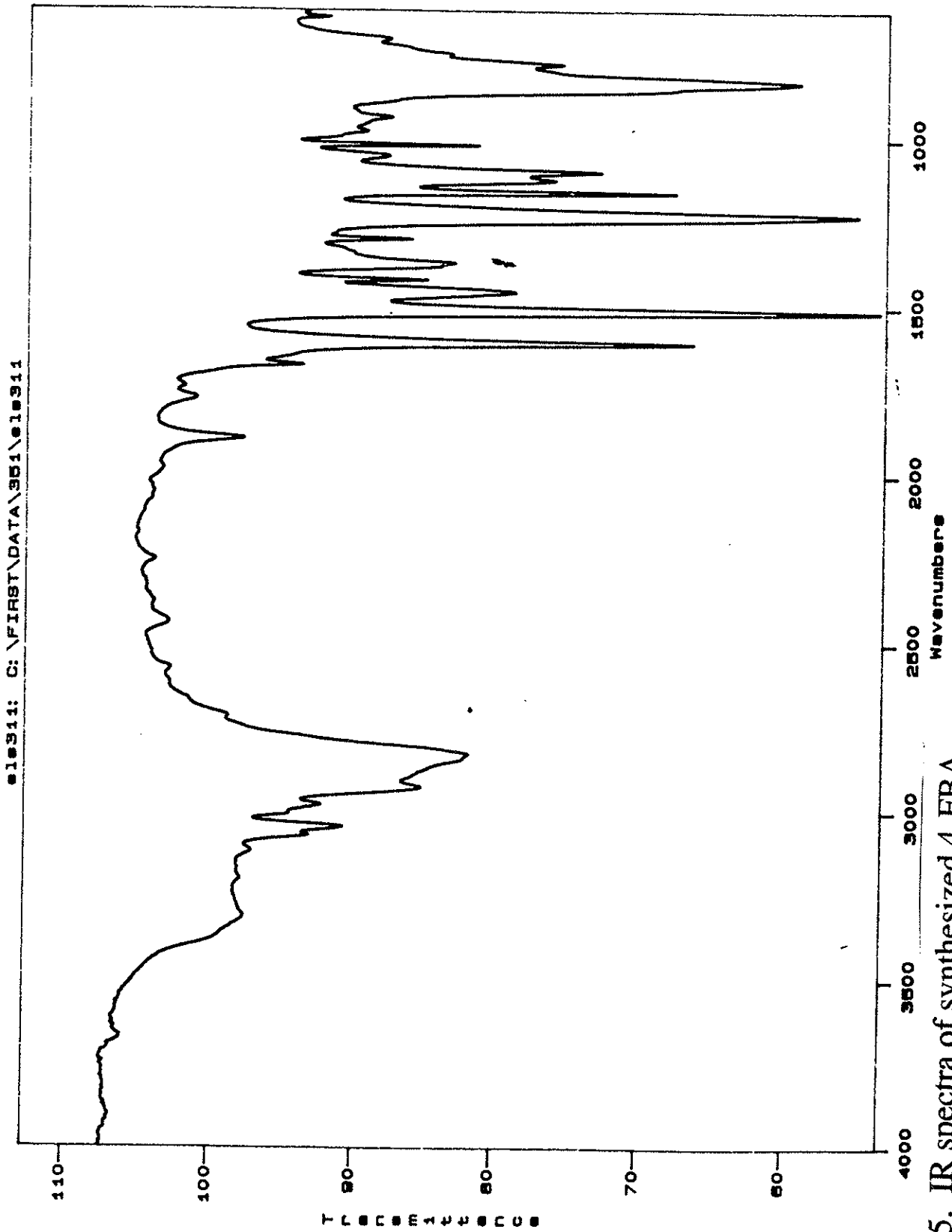


Figure 15. IR spectra of synthesized 4-FBA.

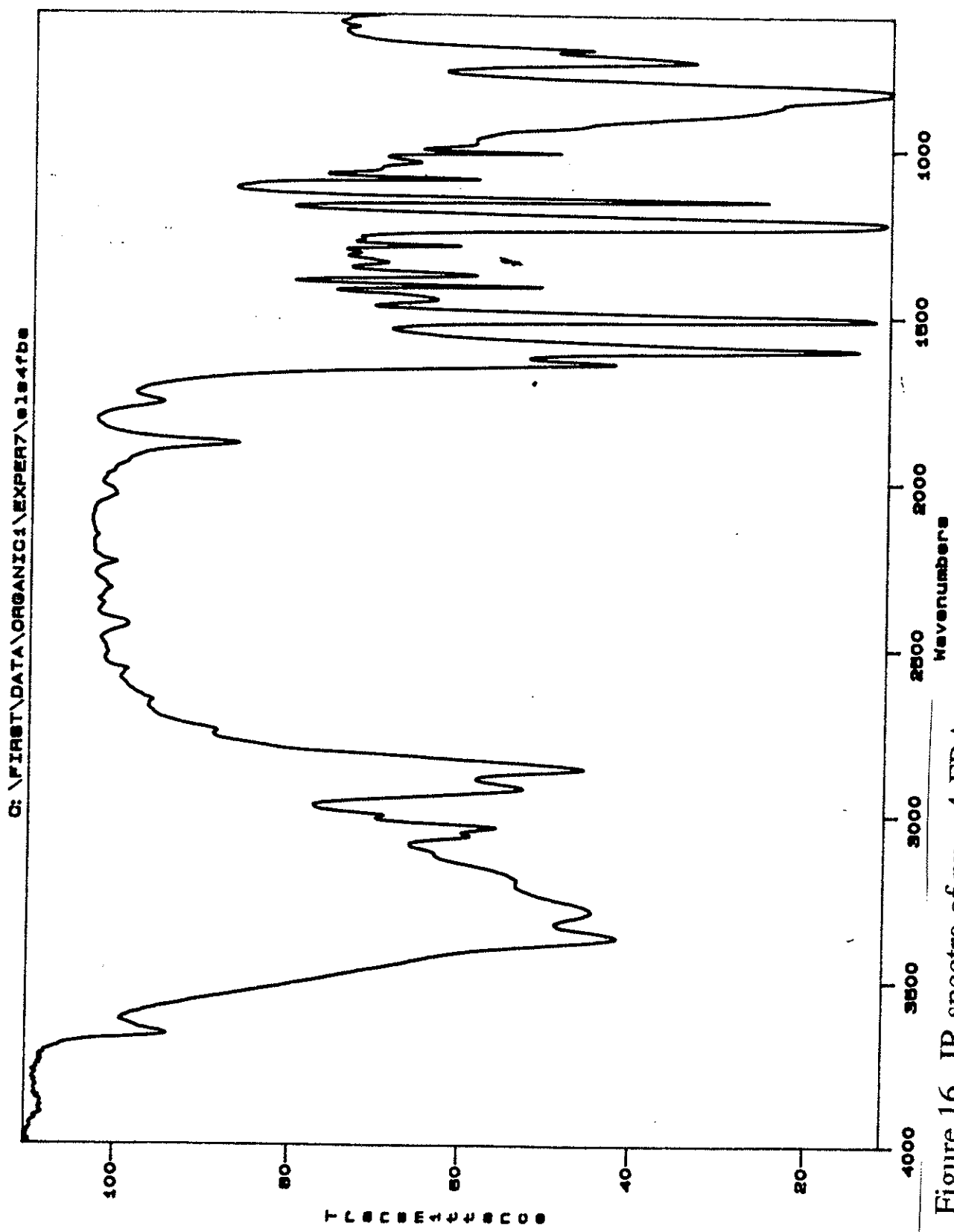


Figure 16. IR spectra of pure 4-FBA.

STANDARD 1H OBSERVE

Solvent: CDCl3
Ambient temperature
File: e1s312
INOVA-500 "kekule"
PULSE SEQUENCE
Relax. delay arrayed
1st pulse arrayed
2nd pulse 14589.5 degrees
Acq. time 1.998 sec
Width 4500.5 Hz
Arrayed repetitions
OBSERVE H1 0.0000000 MHz
DATA PROCESSING
F1 size 32768
Total time 1 minute

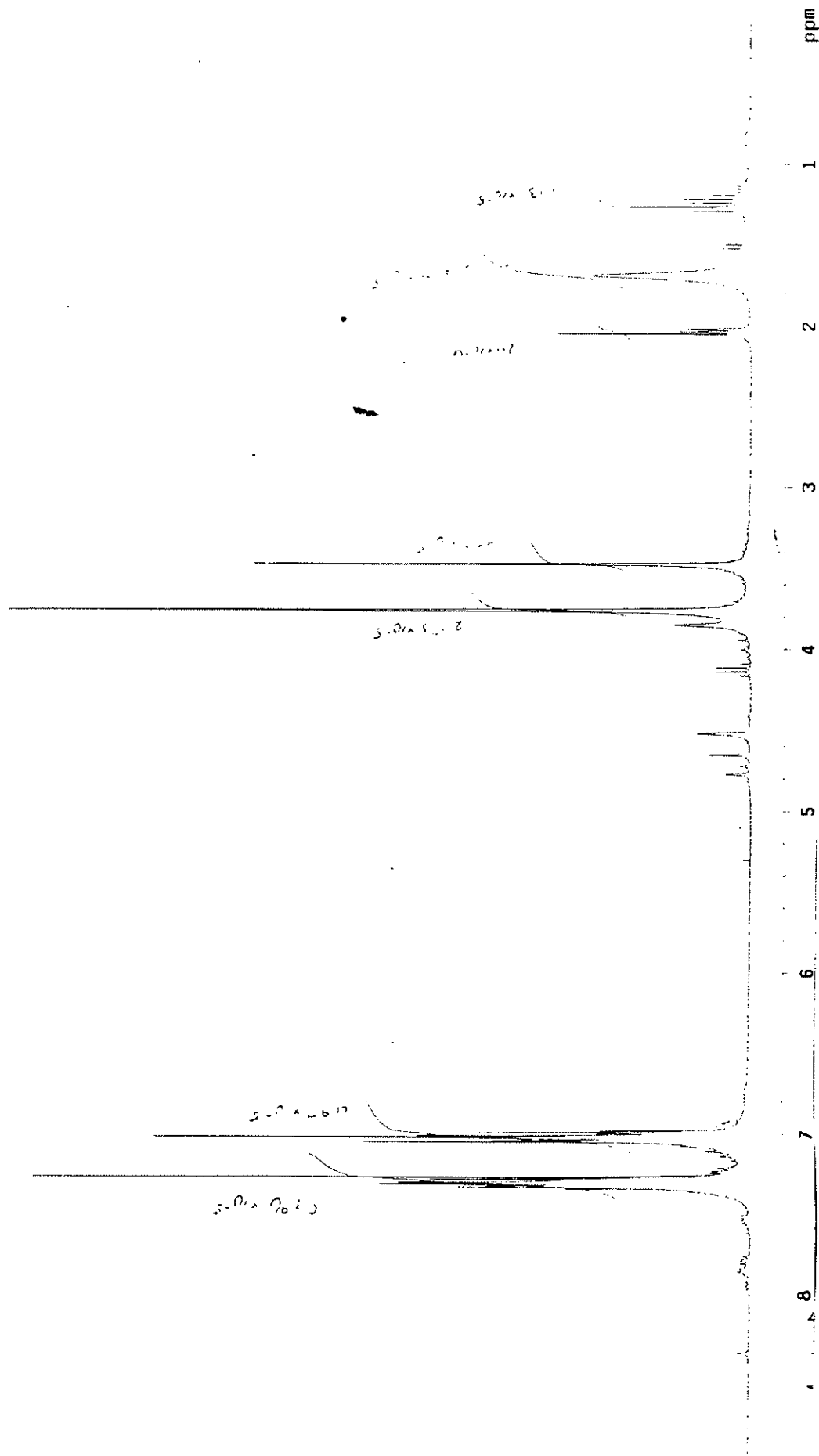


Figure 17. NMR spectra of synthesized 4-FBA.

4-FBA

STANDARD 1H OBSERVE
Solvent: CDCl3
Ambient temperature
File: e1s
INDVA-500 "kekule"
PULSE SEQUENCE
Relax. delay arrayed
1st pulse arrayed
2nd pulse 14589.5 degrees
Acq. time 1.998 sec
Width 4500.5 Hz
Arrayed repetitions
OBSERVE H1 0.0000000 MHz
DATA PROCESSING
FT size 32768
total time 1 minute



H₂O

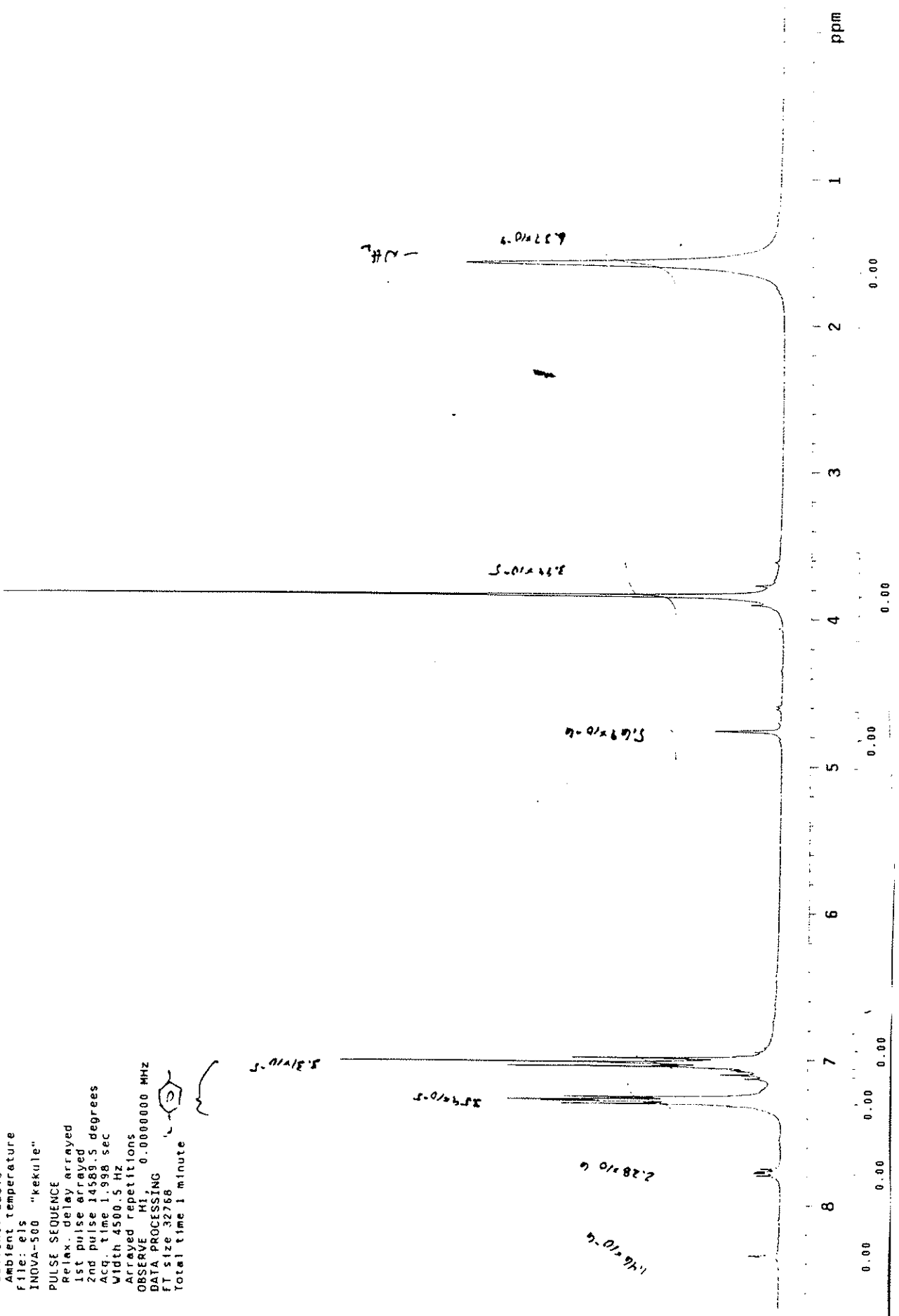


Figure 18. NMR spectra of pure 4-FBA.

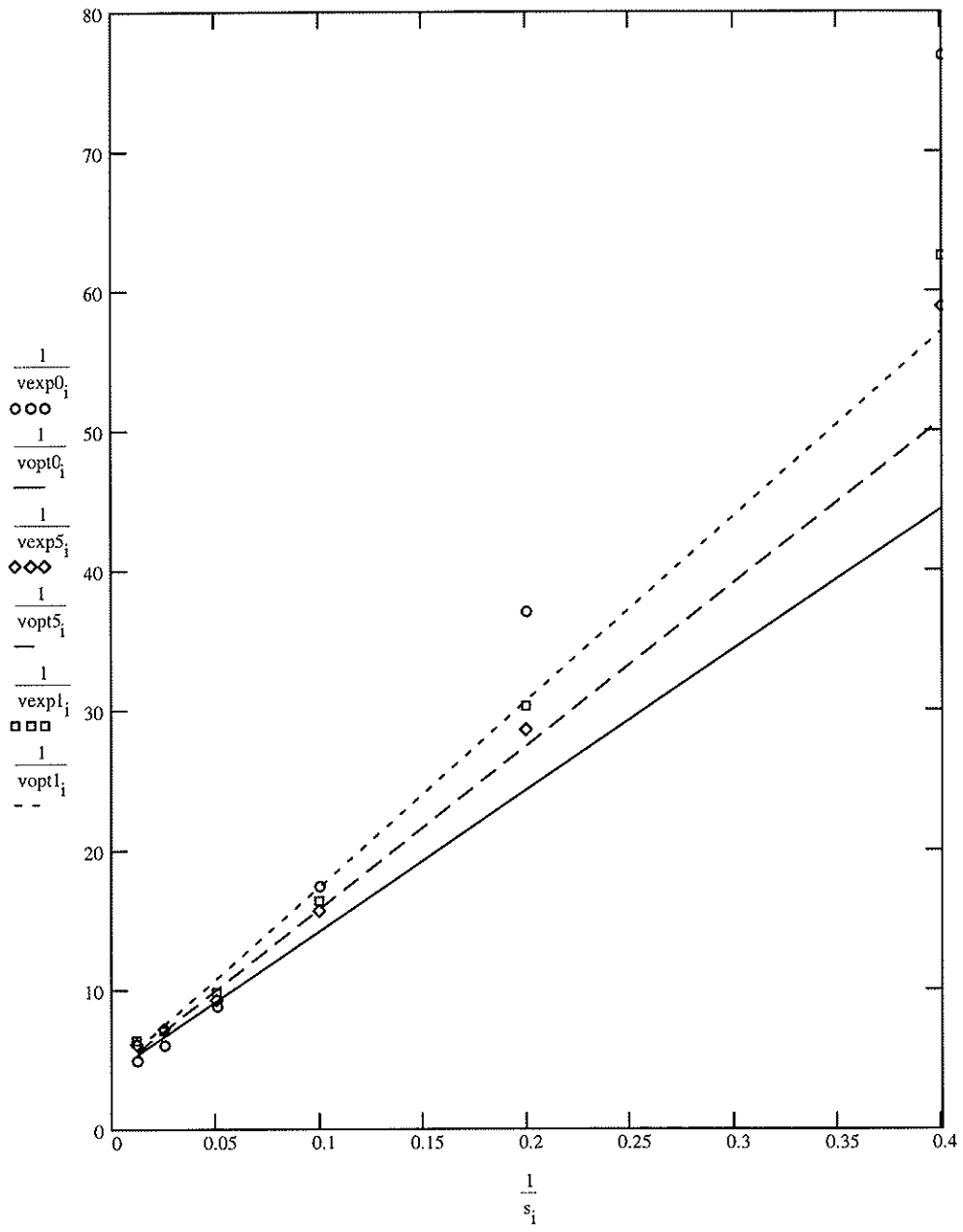


Figure 19. Lineweaver-Burke plot for inhibition with synthesized 4-FBA

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