Iso-Orotate Decarboxylase:

Attempts at Purification by Mechanism Based Affinity Chromatography

by

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Attempts at Purification by Mechanism Based Affinity Chromatography

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Thesis Abstract

Iso-orotate decarboxylase (IDCase) has been partially purified by affinity chromatography. The protein lysates used in this purification were from *Neurospora crassa*. Affinity chromatography was carried out by synthesizing *N*-3-(3-carboxypropyl) 5-nitrouracil (N3 conjugated 5NU) as the ligand. Partial purification was successful yielding a 7-fold purification scheme.

Kinetic and inhibition data was collected for IDCase from *Rhodotorula glutinis*. The K_m, Michaelis constant, of IDCase was determined to be 64.45 ± 0.05 μM. IDCase from *N. crassa* had a K_m of 35 ± 9 μM, meaning that IDCase from *R. glutinis* has less affinity for the substrate, iso-orotate (IOA), than the enzyme from *N. crassa*. The K_i for 5NU, a potent inhibitor of IDCase, was found to be ~25 nM while the inhibitor had a K_i of ~0.5 nM from *N. crassa* IDCase. This indicated that *R. glutinis* IDCase has a lower affinity for 5NU than *N. crassa* IDCase. The K_i for N3 conjugated 5NU for IDCase from *R. glutinis* was established as ~7.9 μM, which suggests that IDCase binds to this inhibitor with more affinity than the substrate. Since IDCase binds to N3 conjugated 5NU with high affinity, the compound could be used as a ligand in affinity chromatography.

The proposed mechanism of IDCase involves a covalent attachment to C6 of IOA. This was somewhat demonstrated by an experiment in which labeled inhibitor-was added to IDCase and the protein was trapped on a membrane. The results show that inhibitor alone does not bind to the membrane while IDCase with the inhibitor does.

Acknowledgements

I would first like to acknowledge Dr. Jeffrey Smiley for all of his patience and understanding. I am extremely grateful for his motivation and commitment towards me and this project. I would also like to thank the Department of Chemistry and Graduate School here at Youngstown State University for giving me the opportunity to continue my education. Thanks to all the professors and graduate students for making me feel welcome for the time that I've been here. I would also like to thank Sue Viglione and Dorothy Untch for keeping me in line. Special thanks go to Dr. Michael Serra and Dr. Peter Norris for taking time to review this thesis.

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Table of Contents

List of Tables

List of Figures

List of Symbols and Abbreviations

β	beta
D	dextrorotatory
UV	ultraviolet
g	gram
mmol	millimole
mL	milliliter
v/v	volume by volume
μL	microliter
°C	degrees Centigrade
NMR	nuclear magnetic resonance
ppm	parts per million
nm	nanometer
mg	milligram
nM	nanomolar
μM	micromolar
M	molarity
cm	centimeter
v/w	volume by weight
μg	microgram
L	liter
rpm	revolutions per minute
min	minute

List of Symbols and Abbreviations

mm	millimeter
mM	millimolar
MHz	megahertz

Chapter 1: Introduction

In a living organism, information is passed from generation to generation in the form of deoxyribonucleic acid (DNA). The DNA is transcribed to ribonucleic acid (RNA), which is then translated to proteins. DNA and RNA are made up of sugar phosphates with a nitrogenous base attached. DNA is a polymer of nucleotides linked to one another by phosphate groups at the 5′ and 3′ positions of the sugars (Figure 1). The sugar in DNA is 2-deoxy-β-D-ribofuranose (deoxyribose), while the sugar in RNA is β-D-ribofuranose (ribose). In each of these nucleic acids there are unique bases. The four major bases of RNA are adenine, guanine, cytosine, and uracil; the major bases of DNA are adenine, guanine, cytosine, and thymine. Thymine, a DNA base, is a derivative of uracil, a RNA base, and is also called 5-methyluracil. There are also minor bases which occur in very minute amounts depending on organism and species. The larger purines, adenine and guanine, are found in both DNA and RNA. The pyrimidines, uracil and cytosine, are found in RNA, while thymine and cytosine are found in DNA. Oftentimes, the metabolism of these nucleotides is the target of drugs by synthesized inhibitors or substrate analogs. While both purines and pyrimidines are equally important, this thesis is focused on pyrimidine metabolism, especially the enzymes involved in the thymidine salvage pathway.

The purines, adenine and guanine, are bicyclic molecules comprised of two fused heterocycles—pyrimidine and imidazole rings (Figure 2, (**1**)). The differences in structure between adenine and guanine involve substitutions at positions 2 and 6 (Figure 2, (**2**) and (**3**)). The synthesis of purines occurs via a ten-step pathway known as the *de*

novo biosynthesis of inosine 5′-monophosphate (IMP) (Figure 2, (**4**)). Although the nucleotide is IMP, the purine is hypoxanthine (6-oxopurine) (Figure 2, (**5**)). IMP is the precursor purine monophosphate from which both adenosine 5′-monophosphate (AMP) and guanosine 5′-monophosphate (GMP) are synthesized (Figure 3).

Uracil, cytosine, and thymine (Figure 4) are the small molecules which form hydrogen bonds with purines in DNA (Figure 1) and RNA. Pyrimidine metabolism is similar to purine biosynthesis since in both cases amino acids are used in building the rings. But, besides the differences in enzymes and steps involved in each of the pathways, the product of the *de novo* pyrimidine synthesis (Figure 5) is uridine 5′-monophosphate (UMP), a nucleotide that can be directly used after phosphorylation in RNA synthesis.

The first step of pyrimidine biosynthesis in prokaryotes involves the enzyme carbamoyl phosphate synthetase with the formation of carbamoyl phosphate from glutamine, bicarbonate, and the hydrolysis of two molecules of adenosine triphosphate (ATP). The enzyme aspartate transcarbamoylase (ATCase) catalyzes the substitution of aspartate for inorganic phosphate forming carbamoyl aspartate. In the next step, dihydroorotase catalyzes ring closure forming dihydroorotate as water is released. Dihydroorotate is oxidized with the reduction of a quinone by dihydroorotate dehydrogenase in the formation of orotate. The formation of orotidine 5′-monophosphate (OMP), catalyzed by orotate phosphoribosyltransferase, results from the substitution of the pyrophosphate group at the 1 position of 5-phosphoribosyl-1-pyrophosphate (PRPP) and the coupling of the pyrimidine and the ribose phosphate. The final step in the *de novo* pathway for pyrimidine synthesis is the decarboxylation reaction which forms the

product uridine 5′-monophosphate (UMP) catalyzed by OMP decarboxylase.[1]

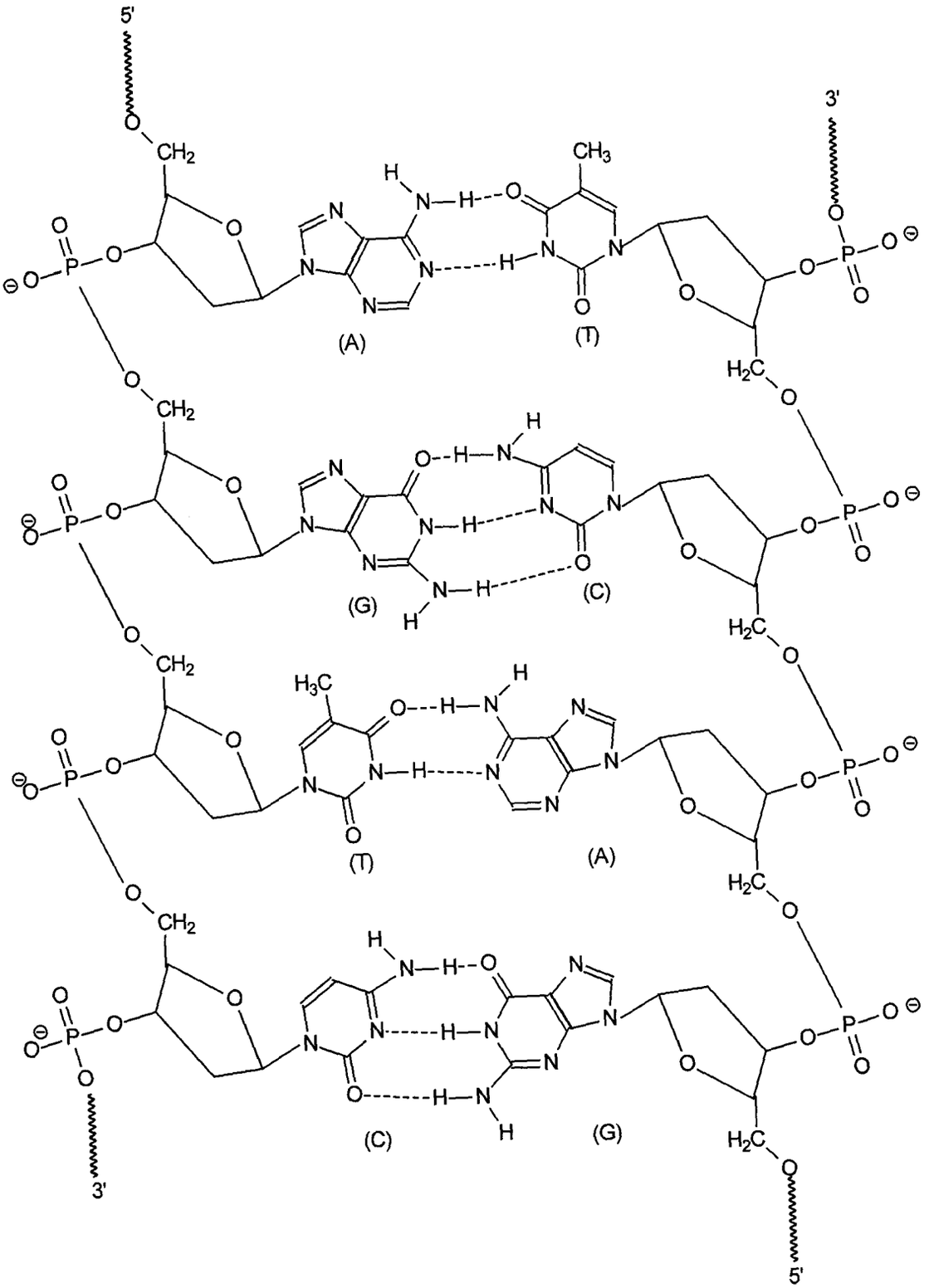
Uridine triphosphate (UTP) and deoxyuridylate (dUMP) are the precursor molecules for the synthesis of cytidine triphosphate (CTP) and thymidylate (Figure 6), respectively. While both uracil and cytosine are a part of the total pyrimidine pool, thymine is not since it is only a part of the DNA pyrimidine pool (Figure 7). The thymidine salvage pathway (Figure 8), which is not present in most organisms, recycles thymidine (thymine) as a part of the total pyrimidine pool. The pathway begins with the oxidation of thymidine, a deoxyribonucleoside, to thymine ribonucleoside. The second step involves hydrolytic cleavage of the C1 and N1 bond between ribose and thymine, respectively. In the next three steps, thymine is converted to 5-hydroxymethyl uracil and 5-formyl uracil before further oxidation to uracil-5-carboxylate (iso-orotate, IOA). All three of these reactions are catalyzed by thymine hydroxylase. The final step of the pathway involves the decarboxylation of IOA by iso-orotate decarboxylase (IDCase). The enzymes found in the thymidine salvage pathway have only been isolated from a few organisms such as *Neurospora crassa* and *Rhodotorula glutinis*.[2,3]

Iso-orotate decarboxylase (IDCase), the major focus of this study, is an important enzyme that catalyzes the final step in the metabolic pathway from thymidine to uracil. The mechanism of IDCase is not completely understood, but it is thought to proceed similarly to thymidylate synthase, the enzyme responsible for the conversion of dUMP to TMP. Both of these enzymes involve a group transfer from the 5 position of uracil. In thymidylate synthase the methyl group is transferred from 5,10-methylenetetrahydrofolate to the 5-position of the pyrimidine, whereas in IDCase the carboxylate group at the 5-position is removed to form uracil.

Very few IDCase studies have been reported.[2,4] IDCase has been assayed by using ^{14}C labeled substrate to detect the activity of the enzyme and substrate specificity. Another method for assaying IDCase has been the use of UV spectroscopy.[4] The kinetics and mechanism of the enzyme as well as inhibitors of IDCase have not been completely reported since the enzyme has not yet been purified. Different purification techniques have been postulated, including the use of 5-nitrouracil (5NU) bound to a column support with elution of an IDCase preparation.

The synthesis of a conjugated 5NU for affinity chromatography has been chosen as an option for purifying IDCase. This synthetic scheme was used to bind conjugated 5-nitrouracil to a column for purification. After IDCase is purified, it may be possible to isolate the gene to allow protein over-production and more easily characterize the mechanism of this unique enzyme.

Figure 1: Double stranded DNA showing hydrogen bonding between base pairs. Adenine (A) hydrogen bonds to thymine (T) while guanine (G) forms hydrogen bonds with cytosine (C)


5'
3'
(A)
(T)
(G)
(C)
(T)
(A)
(C)
(G)
3'
5'

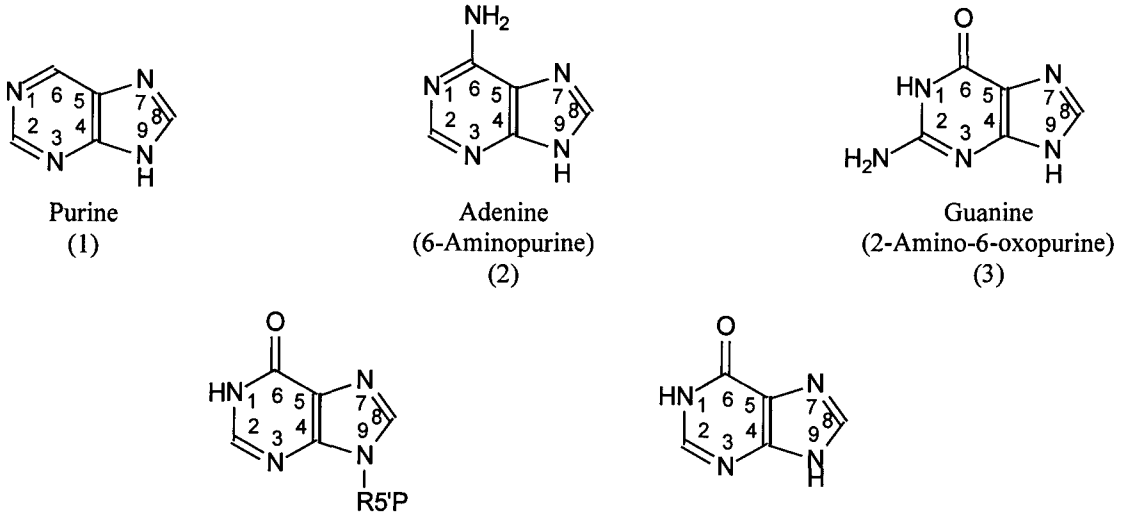
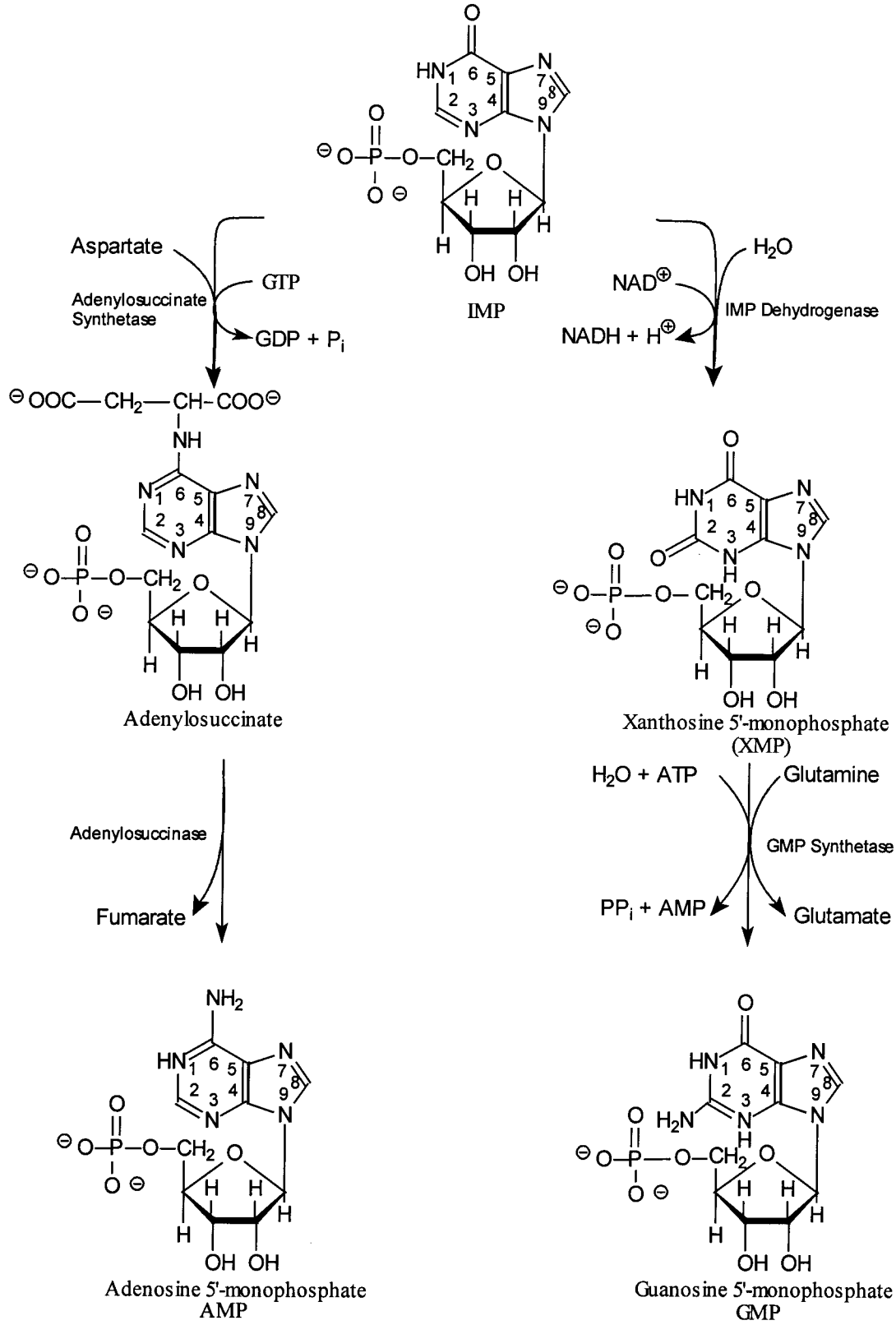
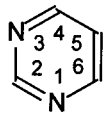


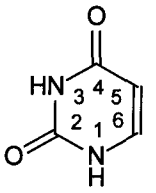
Figure 2: The planar purine ring (1) is made up of an imidazole ring (atoms 4, 5, 7, 8, and 9) and a pyrimidine ring (atoms 1, 2, 3, 4, 5, and 6). The major purines adenine (6-aminopurine) (2) and guanine (2-amino-6-oxopurine) (3), both synthesized from inosine 5′-monophosphate (IMP) (4). The purine of IMP is hypoxanthine (6-oxopurine) (5). The current numbering system is shown for all of the molecules.

Figure 3: The synthesis of adenosine 5′-monophosphate (AMP) and guanosine 5′-monophosphate (GMP) from IMP

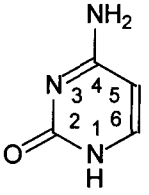

IMP
Aspartate
GTP
Adenylosuccinate Synthetase
GDP + Pi
Adenylosuccinate
Adenylosuccinase
Fumarate
Adenosine 5'-monophosphate
AMP
NAD⊕
H2O
IMP Dehydrogenase
NADH + H⊕
Xanthosine 5'-monophosphate
(XMP)
H2O + ATP
Glutamine
GMP Synthetase
PPi + AMP
Glutamate
Guanosine 5'-monophosphate
GMP



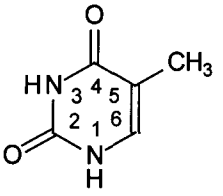
Pyrimidine
(6)



Uracil
(2,4-Dioxopyrimidine)
(7)



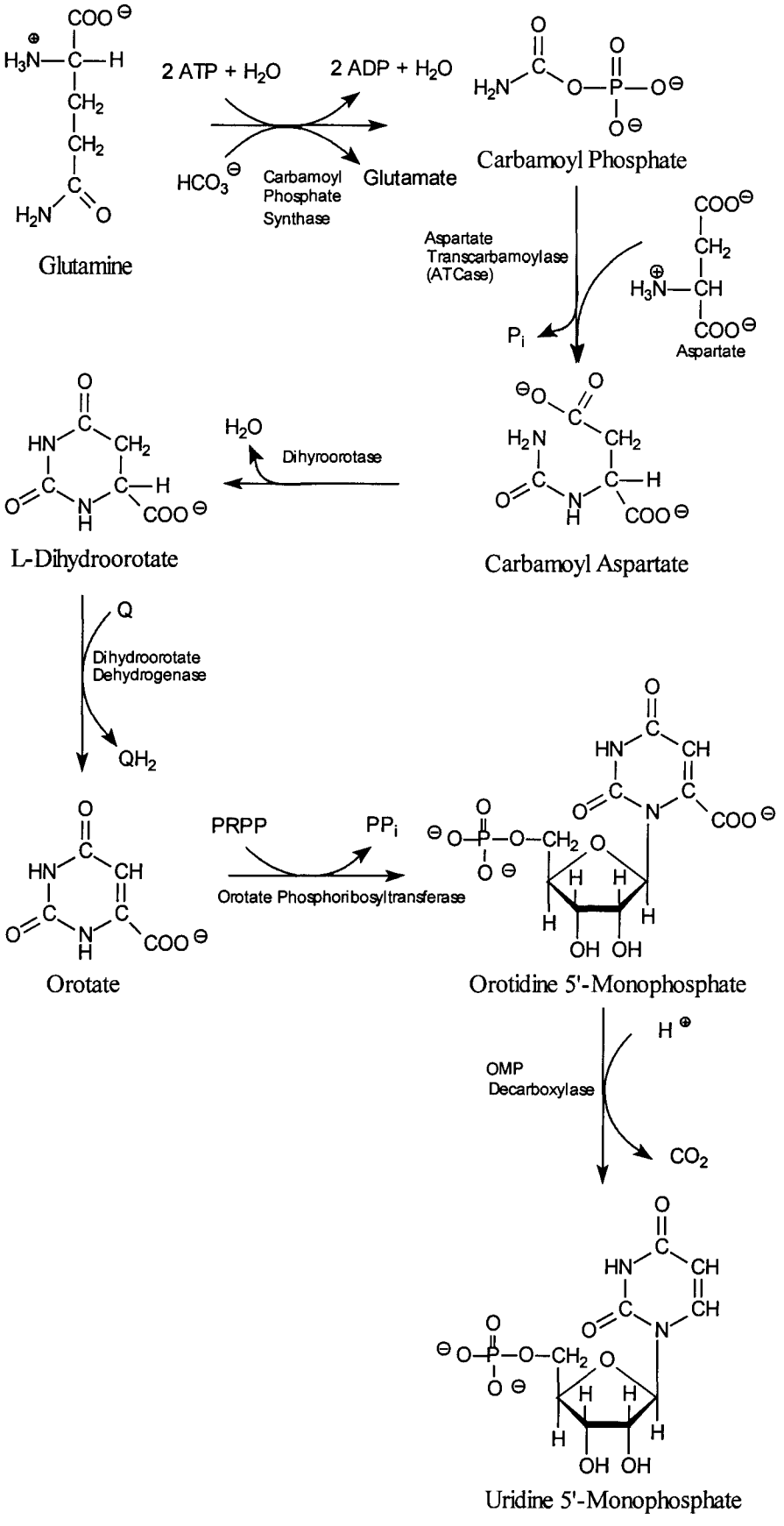
Cytosine
(2-Oxo-4-aminopyrimidine)
(8)



Thymine
(5-Methyluracil)
(9)

Figure 4: The heterocyclic pyrimidine ring (6) is a benzene derivative with nitrogen at the 1- and 3-positions. Uracil (2,4-dioxopyrimidine) (7) is a RNA pyrimidine base and thymine (5-methyluracil) (9) is a DNA pyrimidine. Cytosine (4) is found in both RNA and DNA. The current numbering system is shown for all of the molecules.

Figure 5: The *de novo* pathway for pyrimidine biosynthesis results in the synthesis of uridine 5′-monophosphate. The last step of this pathway is catalyzed by an enzyme noted for its high proficiency, OMP decarboxylase.


Glutamine
2 ATP + H2O
2 ADP + H2O
HCO3⊖
Carbamoyl Phosphate Synthase
Glutamate
Carbamoyl Phosphate
Aspartate Transcarbamoylase (ATCase)
Pi
Aspartate
Carbamoyl Aspartate
H2O
Dihyroorotase
L-Dihydroorotate
Q
Dihydroorotate Dehydrogenase
QH2
Orotate
PRPP
PPi
Orotate Phosphoribosyltransferase
Orotidine 5'-Monophosphate
H⊕
OMP Decarboxylase
CO2
Uridine 5'-Monophosphate

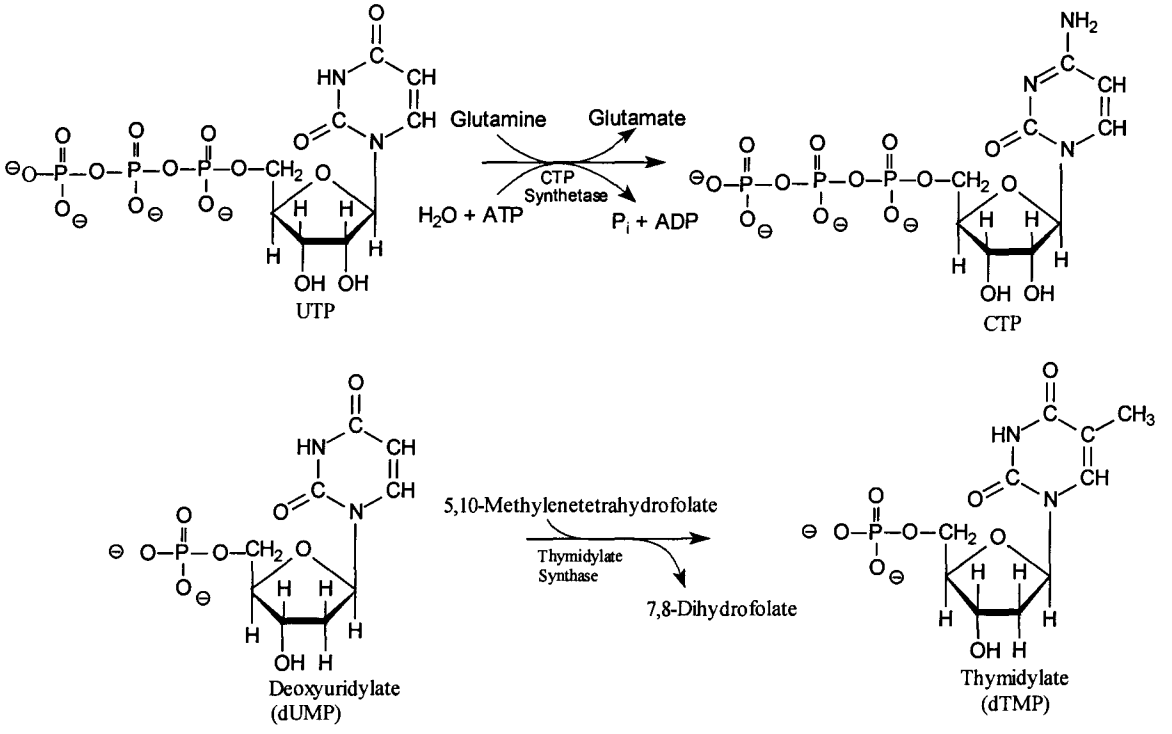


Figure 6: The synthesis of pyrimidine nucleotides, cytidine triphosphate (CTP) and thymidylate (dTMP), from two different forms of uridine nucleotides. In the synthesis of thymidylate, the 2′ hydroxyl is removed from UMP. Thymidylate synthase uses the cofactor, 5,10-methylenetetrahydrofolate, from which a methyl group transfer occurs to the 5-position of dUMP.

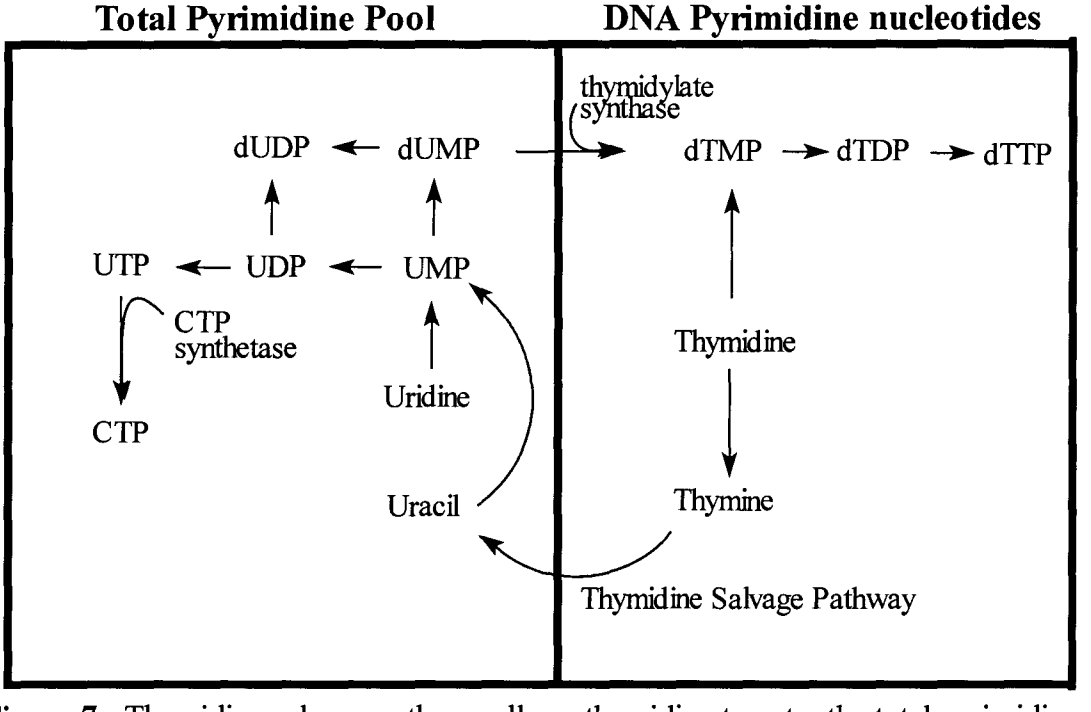
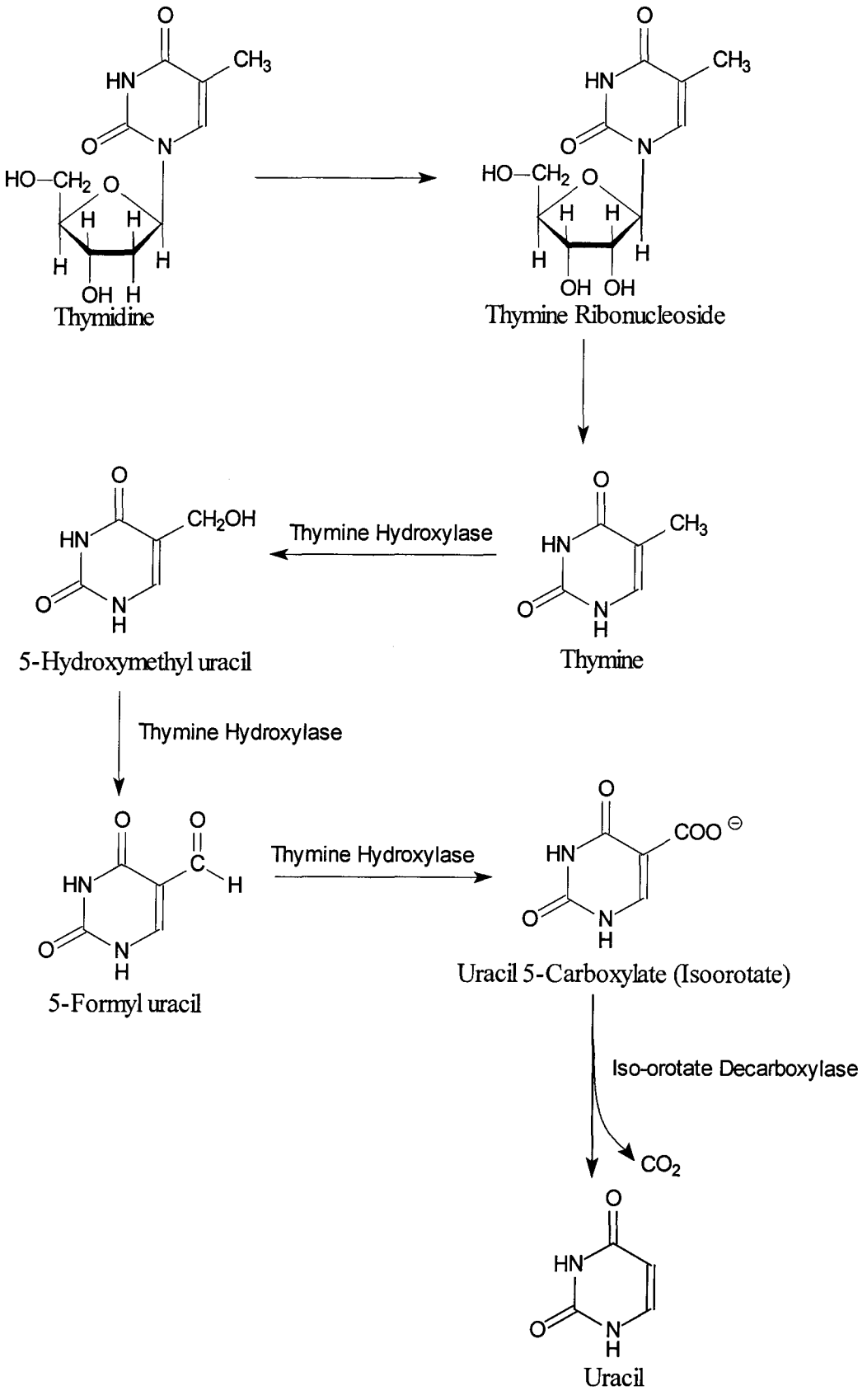


Figure 7: Thymidine salvage pathway allows thymidine to enter the total pyrimidine pool.

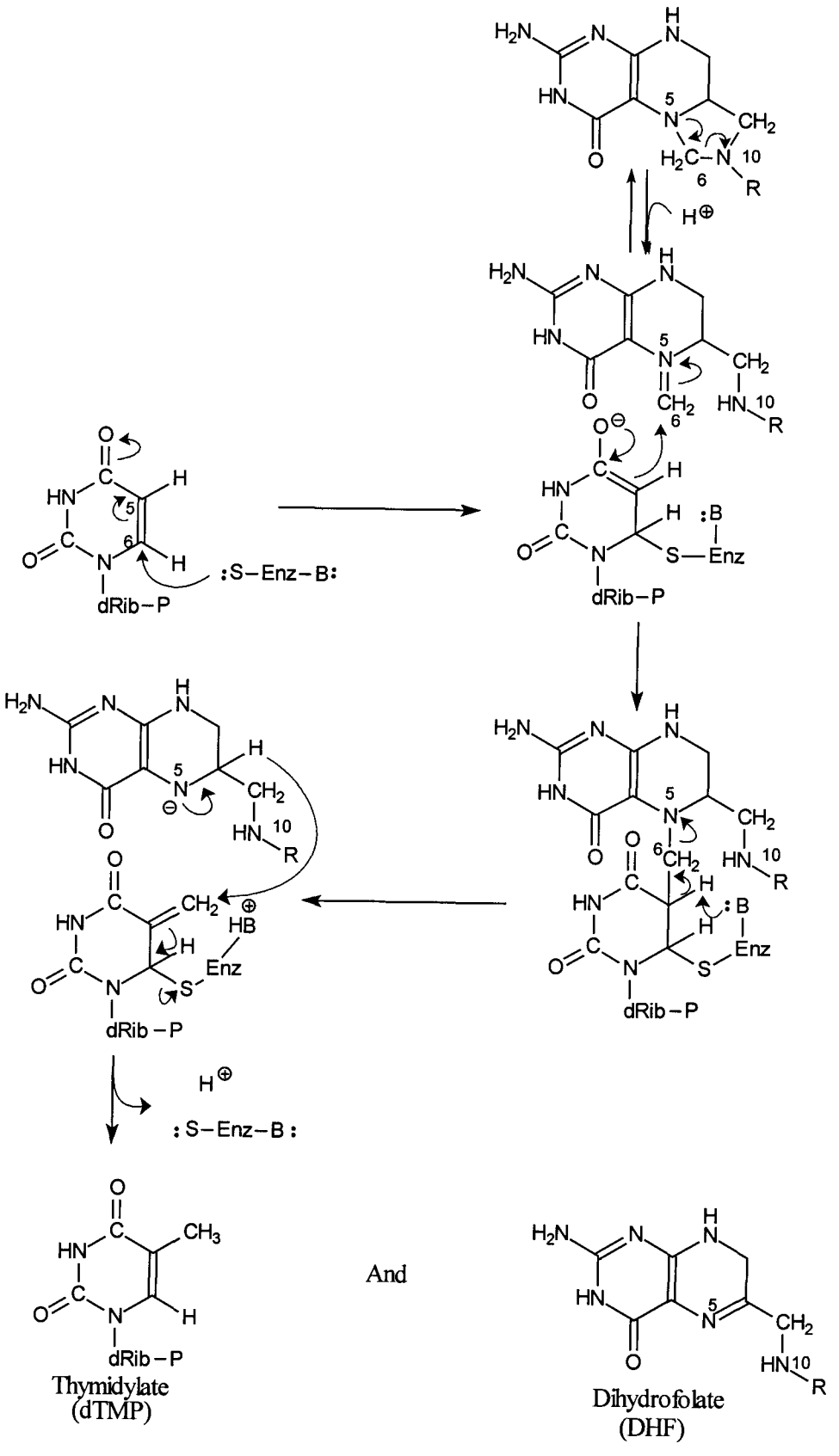
Figure 8: The thymidine salvage pathway is an unusual way for some organisms to recycle thymidine into the total pyrimidine pool. The final step in the pathway is catalyzed by iso-orotate decarboxylase.

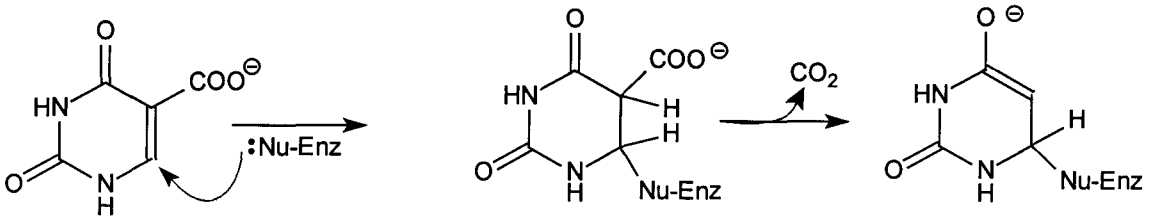

Thymidine
Thymine Ribonucleoside
Thymine Hydroxylase
5-Hydroxymethyl uracil
Thymine
Thymine Hydroxylase
Thymine Hydroxylase
5-Formyl uracil
Uracil 5-Carboxylate (Isoorotate)
Iso-orotate Decarboxylase
CO_2
Uracil

Thymidylate synthase (TS) catalyzes the methylation of dUMP to dTMP by using 5,10-methylenetetrahydrofolate (THF) as the methyl donor. The mechanism of TS (Figure 9) occurs via a covalent attachment between enzyme and substrate at the C6 position of the pyrimidine ring. This activates the pyrimidine ring by making the C5 position more electron rich. Formation of a ternary complex then occurs involving TS, dUMP, and THF. The electron rich C5 attacks the methylene group at N5 of THF. The cofactor, THF, is then freed from the complex by abstraction of an acidic proton at C6 of the pyrimidine substrate. Thymidylate is formed after a hydride ion attacks the C5 methylene group and dihydrofolate is a by-product.

The binding studies between TS and dUMP were performed based on mechanism-based inhibition studies.[5] The inhibitor 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) is similar to the actual substrate, dUMP, and the enzyme binds to the C6 position of FdUMP. But, catalysis cannot occur since fluorine is attached at the 5-position consequently inactivating the enzyme. Inhibition studies have always been very important in mechanistic studies of enzymes. Because of the strong inhibition of IDCase by 5NU, which is electrophilic at C6, it is hypothesized that nucleophilic attack occurs at the 6-position of IOA (Figure 10) before decarboxylation can occur.

Figure 9: The mechanism of the enzyme thymidylate synthase involves nucleophilic attack at C-6 of the pyrimidine ring by the sulfhydryl group of Cys 198. This is followed by methylation by the cofactor 5,10-methylenetetrahydrofolate at C-5.


:S–Enz–B:
dRib–P
Enz
HB
H⊕
:S–Enz–B:
dRib–P
Thymidylate
(dTMP)
And
Dihydrofolate
(DHF)



IOA | Enzyme-Substrate (ES) Intermediate | Enzyme + Product (E + P)

Figure 10: The proposed mechanism of catalysis by iso-orotate decarboxylase.

Enzyme inhibitors prevent either the formation of the enzyme-substrate (ES) complex or the breakdown of enzyme and product (E + P) by binding to the enzyme. The inhibition constant, K_i (Figure 11), is the dissociation constant of inhibitor from the enzyme-inhibitor (EI) complex for competitive inhibition. Enzymatic mechanisms and

$$K_i = \frac{[E][I]}{[EI]}$$

Figure 11: The equation for the inhibition constant, K_i

metabolic pathways can be determined from inhibitor studies. Some natural inhibitors are metabolic regulators and some natural and synthetic inhibitors are medicinal drugs. There are two major types of inhibitors: reversible and irreversible. In the case of IDCase studies, two inhibitors, 5-nitrouracil (5NU) and 5-nitrocytosine (5NC), were studied.[6]

Reversible inhibitors bind to enzymes noncovalently and are removed easily by gel filtration (chromatography based on size) or dialysis. The majority of reversible inhibitors can be classified as competitive, uncompetitive, or noncompetitive inhibitors. Competitive inhibition (Figure 12) is the most common type and has been widely

studied.[7] As in the case of TS mechanistic studies, FdUMP, the inhibitor, binds to the active site of free enzyme preventing the binding of substrate. Competitive inhibition

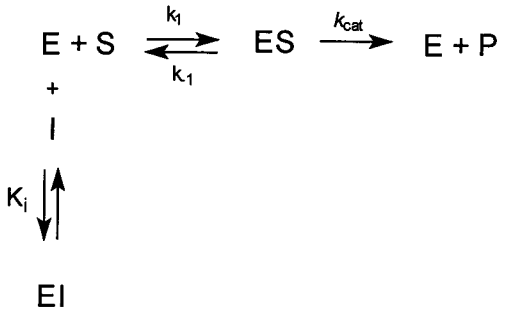


Figure 12: The kinetic scheme for competitive inhibition

occurs when both substrate and inhibitor compete for binding to the enzyme. As the concentration of a competitive inhibitor increases, the V_{max}, maximum velocity, remains constant while the K_m, Michaelis constant, increases. Uncompetitive inhibition (Figure 13) occurs when both substrate and inhibitor binds to ES and not to free enzyme, E.

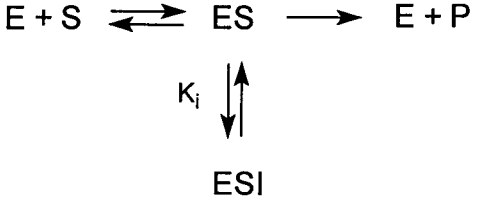


Figure 13: The kinetic scheme for uncompetitive inhibition

When uncompetitive inhibitor concentrations are increased, both K_m and V_{max} decrease. Only multi-substrate reactions are usually susceptible to uncompetitive inhibition. Noncompetitive inhibition (Figure 14) is characterized by a decrease in V_{max} and no change in K_m. This type of inhibitor binds to both E and ES, forming inactive EI and ESI

complexes. Although noncompetitive inhibition is rare, some allosteric enzymes are regulated by these inhibitors. Another type of noncompetitive inhibition is termed mixed

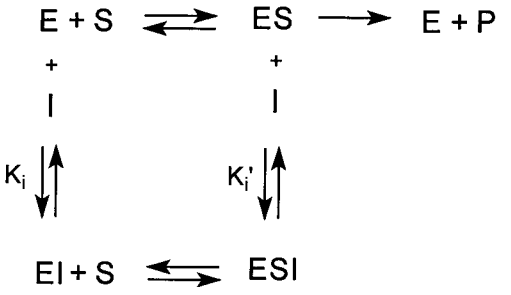


Figure 14: The kinetic scheme for noncompetitive inhibition.

noncompetitive inhibition. Mixed noncompetitive inhibition occurs when an inhibitor has unequal affinity for E and ES. These cases are described by a decrease in V_{max}, and K_m either decreases or increases depending on whether K_i is higher or lower, respectively, than K_i'.

The effects of two inhibitors, 5-nitrouracil and 5-nitrocytosine, have been studied for IDCase. 5-Nitrouracil has been previously reported as a strong, competitive inhibitor of IDCase.[4] 5NU is a substrate analog with an electron-withdrawing nitro group substituting for the carboxylate group at C5. In both substrate and inhibitor, the C6 position is electrophilic allowing for nucleophilic attack by the enzyme. After a covalent attachment is made at C6 of 5NU, the nitro group cannot be removed like the carboxylate in IOA. Previous studies have shown that IDCase from *N. crassa* has a K_i value in the nM range for 5NU.[4] 5-nitrocytosine (5NC) is similar to 5NU with an amino group substitution at C4. Studies indicate that 5NC is an adequate inhibitor with a K_i of ~26 μM for IDCase from *N. crassa.*[6]

The ability of IDCase to bind 5-nitropyrimidines can be potentially utilized in protein purification. Affinity chromatography is a selective type of column chromatography which involves the binding between a protein and a ligand. The ligand, which is covalently attached to the stationary matrix, is a molecule, group, or atom that binds to another molecule. As a protein solution is passed across the column, nonspecific proteins pass while specific proteins bind to the column. After several washings of the column to ensure complete removal of nonspecific proteins, the target protein can be removed from the matrix by washing the column with high concentrations of the free ligand, a concentrated salt solution, or changing the pH of the wash buffer. These different conditions cause changes in protein-ligand interactions and allow for protein elution.

In some cases custom affinity chromatography is necessary for the purification of proteins. This requires the synthesis of a ligand which is a substrate analog or inhibitor. Then the target enzyme can bind to the ligand while nonspecific proteins are removed. Custom affinity chromatography provides very specific purification of the desired protein based on the specific interactions between ligand and protein.

In a similar purification of OMP decarboxylase, a column was synthesized by attaching the ethylenediamine amide of 5-(2-carboxyethyl)-6-azauridine 5′-phosphate to carboxymethyl agarose.[8] This scheme is similar to one proposed for the purification of IDCase using similar column material with a synthesized conjugate of 5-nitrouracil. The purification of OMP decarboxylase afforded a 4,000-fold purification. Additional types of commercial chromatography, such as a carboxymethylcellulose column and a Sephadex G-100 column, gave a 6700-fold purification. Multiple step purifications are

necessary if the protein is not overexpressed and is present at a normal concentration of perhaps 1/10,000 of total cell protein. Overexpressed systems provide enough of the desired proteins so that the protein of interest is present as conceivably 10 % of the total cell protein, and only 10-fold purification is necessary. Previous attempts at purification with commercial affinity chromatography resin resulted in unsuccessful purification of IDCase.

The most sensitive method for assaying IDCase activity was determined to be $^{14}CO_2$ radioassay (Table 1) involving ^{14}C-labeled iso-orotate.[4] This assay was based on the original experiment which first detected the presence of this enzyme.[2] The amount of product produced over a given amount of time is calculated by the detection of the amount of radioactivity in counts per minute (cpm), which is directly related to the amount of product. The radioactivty assay allows measurement of the kinetics and any inhibition involved in the enzymatic reaction.

Table 1: ^{14}C Assay Terms

Term	**Definition of Term**	**Operation**
cpm	Amount of $^{14}CO_2$ detected per minute	Radioactivity counts per minute
min	Time point and length of each assay (10 minutes)	
cpm/nmol	Conversion factor used to obtain the nmol (~200 cpm/nmol)	cpm/ (cpm/nmol) = nmol
nmol/min/mg	The specific activity of the enzyme	nmol/ min/ mg of protein

This project is an attempted purification of the enzyme iso-orotate decarboxylase using custom affinity chromatography. Included in this study is the Michaelis constant,

K_m, determination for IDCase in the organism *Rhodotorula glutinis*, the K_i determination for the synthesized inhibitor conjugate, and some evidence supporting a nucleophilic attachment model for the mechanism of this enzyme.

Chapter 2: The Synthesis of Conjugated inhibitors

The IDCase inhibitors 5-nitrocytosine and 5-nitrouracil were considered for possible construction of a conjugated substrate analog. The amino group at the C4 position of 5NC was an appealing site for an acylation reaction. By acylating the amino group an amide bond would be used to tether a linker for the attachment to a stationary matrix, such as agarose. With a linker arm of about four atoms long, the enzyme could bind the substrate analog, making possible a method to purify the enzyme by chromatography. After acylation the conjugated 5NC should retain the properties of inhibiting the enzyme in order to perform adequately in purification.

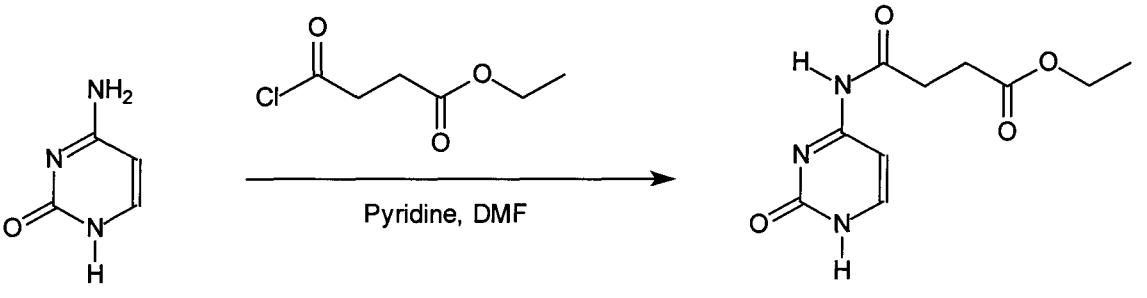
In the case of 5-nitrouracil, alkylation is possible at N1 or N3, and different reaction conditions can favor one or the other. Previously, *N*-1-(3-carboxypropyl) 5-nitrouracil was synthesized which showed very little inhibition of IDCase indicating no binding. This indicated that the enzyme might have an active site requiring no substitution at the N1 position of the substrate or inhibitor. Alkylation could also be performed at the N3 position of 5NU. If inhibition of IDCase activity still took place then the N3 conjugated 5NU could be an option for purification.

All the organic synthesis involved in this research has been previously performed on similar molecules.[9,10] Synthesis of a conjugated 5NC was unsuccessful, but there was some success in synthesizing a conjugated 5NU. The two compounds, N_4-ethyl succinyl-cytosine and *N*-3-(3-carboxypropyl)-5-nitrouracil, were synthesized and analyzed by thin layer chromatography (TLC), ^{1}H NMR (Varian Gemini 2000, 400 MHz spectrometer) and UV spectroscopy (Hewlett Packard 8452A diode array spectrophotometer).

Materials and Methods

Synthesis of N_4-ethyl succinyl-cytosine (compound 11)

All reagents in this synthesis were purchased from Sigma. The synthesis of N_4-ethyl succinyl-cytosine (11) was accomplished (Figure 15) by combining 1.004 g of cytosine (9 mmol), 1.5 mL of ethyl succinyl chloride (10.8 mmol), 2.1 mL of dry pyridine (27 mmol) and 20 mL of dry dimethylformamide (DMF) in a pre-dried 50-mL round-bottomed flask at room temperature. After 15.5 hours, the reaction was quenched



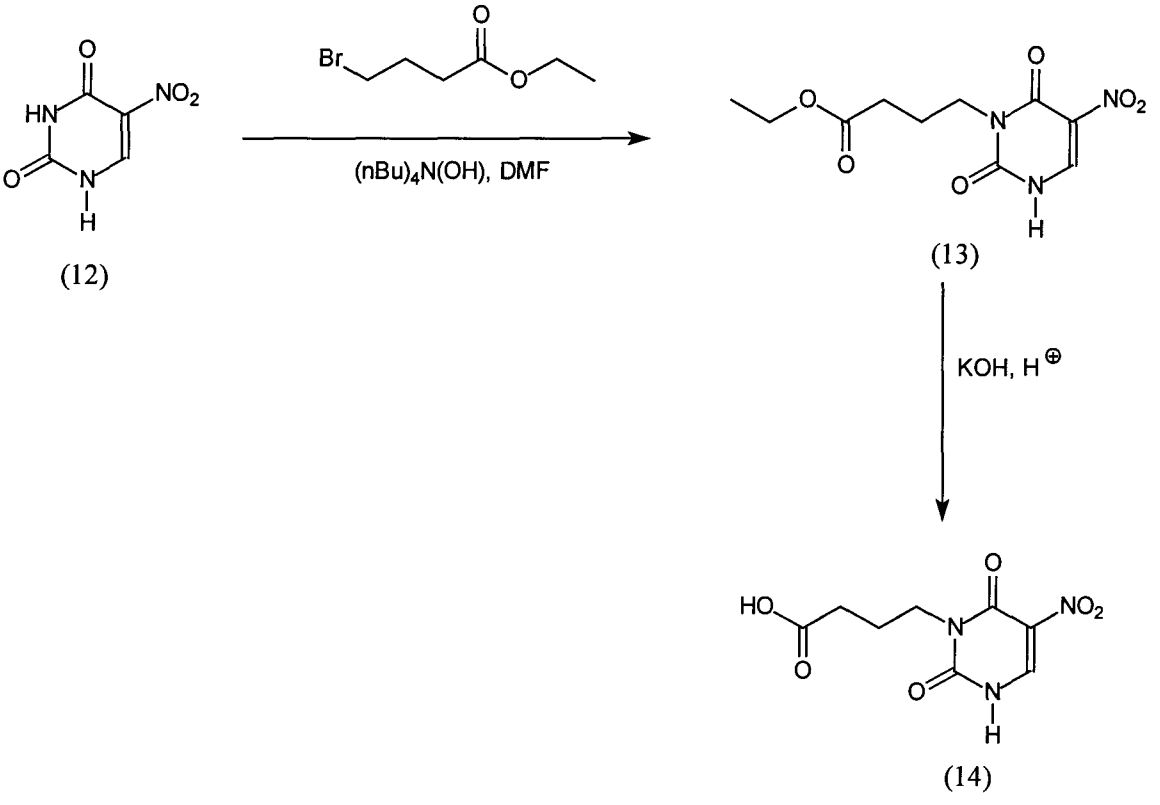
(10) (11)

Figure 15: The synthesis of compound 11 from cytosine (10) and ethyl succinyl chloride.

by adding 5 mL of H_2O. The white crystalline product was recovered by suction filtration and was dried overnight under vacuum. Analysis by thin layer chromatography (TLC) in 9:1 CH_2Cl_2: CH_3OH (v/v) revealed that the product was different from cytosine and uracil, a possible by-product. ^1H NMR (Figure 17) of the product in deuterated dimethylsulfoxide (d_6-DMSO) indicated shifts that are consistent with the desired product. UV spectrum analysis was performed by dissolving compound 11 in H_2O.

Synthesis of *N*-3-(3-carboxypropyl)-5-nitrouracil (compound 14)

All reagents were purchased from Sigma. The synthesis of N3 alkylated 5NU was performed (Figure 16) according to a general procedure for the synthesis of 3-alkyl-5-nitrouracils.[10] 5-nitrouracil (0.5 g, 3.2 mmol), 25% tetrabutyl ammonium hydroxide in methanol (6.4 mL, 6.4 mmol), and DMF (5.0 mL) in a 50-mL round bottomed flask and



(12) (13) (14)

Figure 16: The synthesis of compound 14 via ester intermediate (13) from 5-nitrouracil (12) and ethyl-4-bromobutyrate.

evaporating these compounds to a syrup. The syrup was redissolved in DMF (5.0 mL) and ethyl-4-bromobutyrate (458 μL, 0.24 g, 3.2 mmol) was added at room temperature. After 24 hours, the reaction was quenched with acetic acid (4.0 mL). An aqueous workup was performed to extract the product from solution with water (30 mL) and ethyl acetate (20 mL x 3). The organic layer was dried with anhydrous $MgSO_4$, gravity filtered, and evaporated. The product was separated from starting material and by-

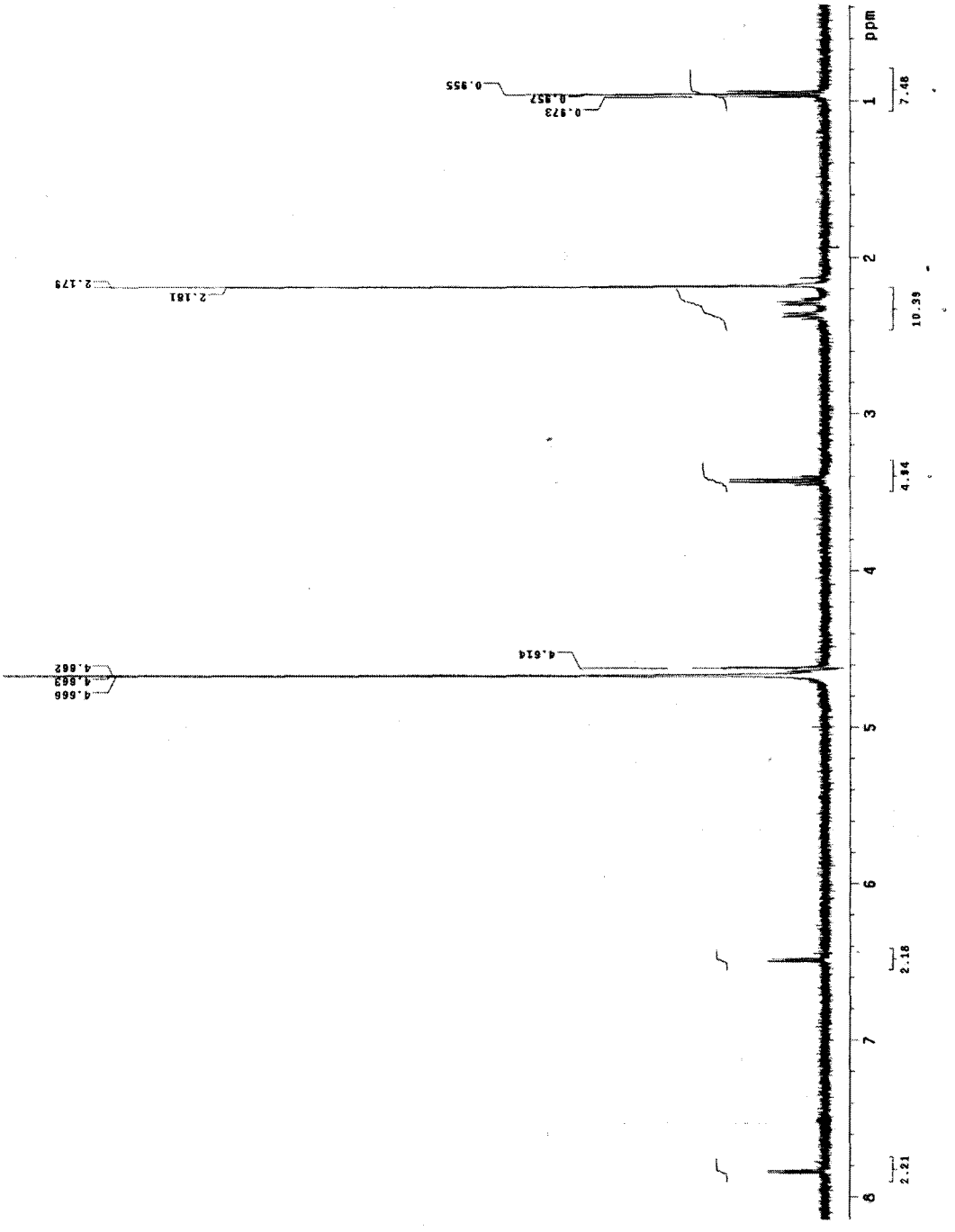
products by flash chromatography using silica gel and with 85:15 CH_2Cl_2: CH_3OH. The product was analyzed by TLC (85:15 CH_2Cl_2: CH_3OH, (v/v)). The second step of the reaction was the hydrolysis of the ester and was completed by adding potassium hydroxide (KOH, 0.3 M) to the ester. The reaction was refluxed with gentle heating (40 °C) for 4 hours. At the completion of hydrolysis, the reaction mixture was evaporated. To exchange K^+ ions for H^+, the mixture was applied to Dowex 50W-X8 (20-50 mesh) cation exchange resin which had been previously washed with HCl and H_2O. The product was evaporated and dried under vacuum yielding yellow-brown crystals. Analysis was performed by ^1H NMR in D_2O (Figure 18) and UV spectrophotometry.

Results and Discussion

Compound 11 was synthesized with a yield of 44.7%. The ^1H NMR of the product (Figure 17) showed six separate signals, which had shifts of 0.96 ppm (3 H, triplet), 2.3 ppm (2 H, triplet), 2.5ppm (2 H, triplet), 3.5 ppm (2 H, quartet), 6.5 ppm (1 H, doublet), and 7.8 ppm (1 H, doublet). TLC analysis in three different solvents (19:1 CH_2Cl_2: CH_3OH, 9:1 CH_2Cl_2: CH_3OH, and 5:2:3 butanol: acetic acid: H_2O (v/v/v)) showed that the product migrated further than both uracil and cytosine. The λ_{max}, wavelength of maximal absorbance, was found to be 244 nm at pH 7 as opposed to cytosine, 267 nm at pH 7, and uracil, 260 nm at pH 7.

Nitration at the C5 position of N_4-ethyl succinyl-cytosine proved unsuccessful because the amide bond formed in the reaction is hydrolyzed by the basic conditions

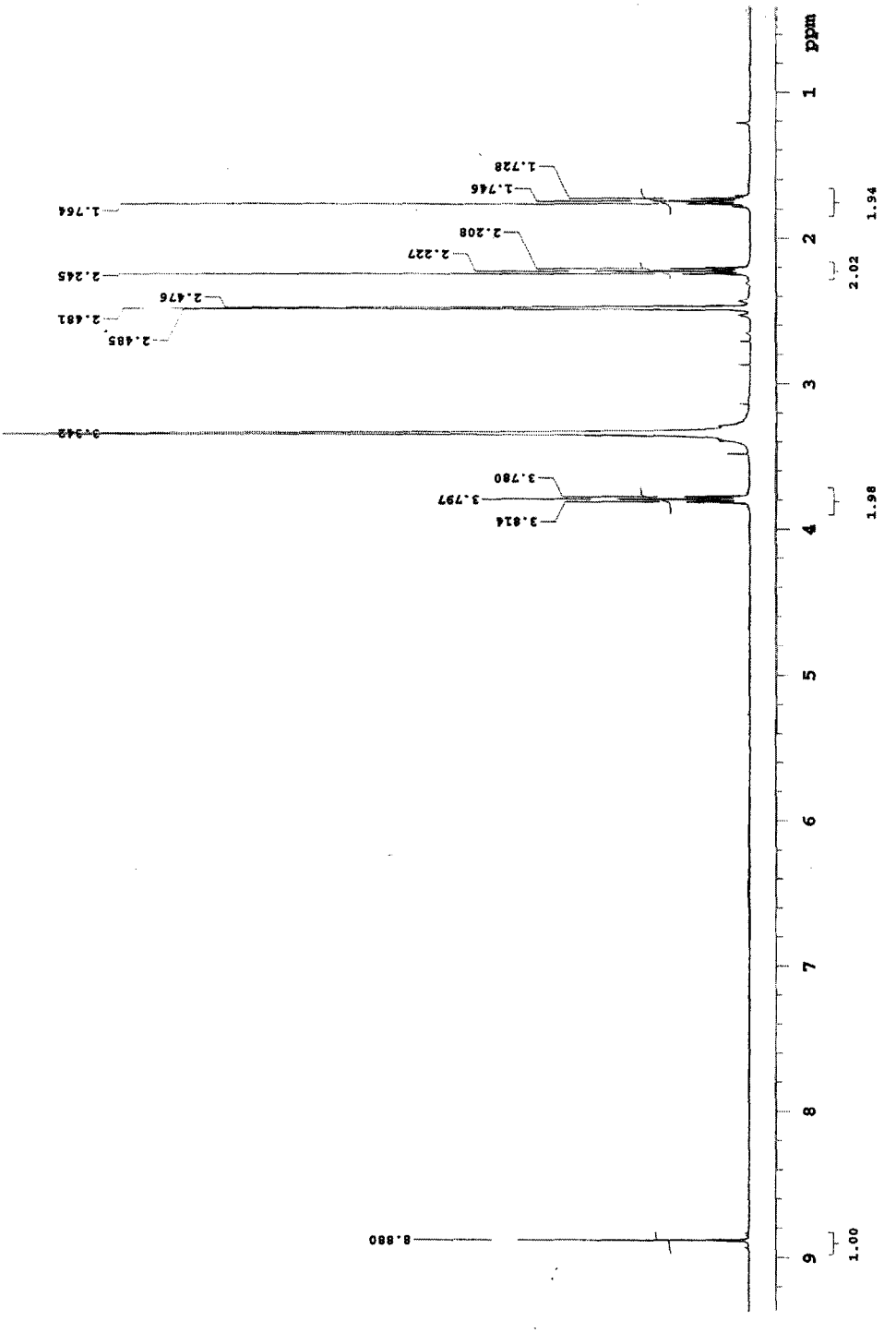
Figure 17: ^{1}H NMR of compound 11.


ppm
0.955
0.857
0.873
2.179
2.181
4.614
4.666
4.863
4.882
7.46
10.39
4.84
2.16
2.21

present in the ester hydrolysis reaction. This compound was ineffective as an inhibitor and could not be used to construct a custom affinity column.

Compound 14 was successfully synthesized in two steps and was analyzed by ^{1}H NMR, TLC, and UV spectroscopy. After the first step in the synthesis, the N3 alkylated 5-nitrouracil was separated from the N1 alkylated 5-nitrouracil. This required multiple separations by flash chromatography until enough of the desired pure product remained. The first reaction had an 81% yield of the ester product while the yield in the second step was 73%. The overall yield for the complete synthesis was 59%. The ^{1}H NMR (Figure 18) had four signals, which had shifts of 1.75 ppm (2 H, triplet), 2.23 ppm (2 H, triplet), 3.80 ppm (2H, triplet) and 8.80 ppm (1 H, singlet). TLC evidence established the reaction yielded three separate products. Only the desired product was characterized, but it is thought that the other two products might be the N1 alkylated 5NU and the dialkylated 5NU. The λ_{max} found for the N3 alkylated 5-nitrouracil was 302 nm at pH 4, which correlated with the value for 3-methyl 5-nitrouracil, 298 nm at pH 4. The λ_{max} of 5-nitrouracil is 342 nm at pH 8.[11] The synthesis of compound 14 permitted the synthesis of a custom affinity column.

Figure 18: ^{1}H NMR of compound 14.


8.880
3.814
3.797
3.780
3.342
2.485
2.481
2.476
2.245
2.227
2.208
1.764
1.746
1.728
ppm
1
2
3
4
5
6
7
8
9
1.00
1.98
2.02
1.94

Chapter 3: Preparation of Iso-orotate Decarboxylase

Neurospora Crassa: Source of IDCase

N. crassa is a filamentous fungus belonging to the group ascomycetes, which are also referred to as sac fungi. The strain used in this study was the mutant strain, FGSC #2203. Strain #2203 contains two critical mutations: one in the gene uc2, that encodes for thymine 2′ hydroxylase, which is responsible for the conversion of thymidine to thymine ribonucleoside (Figure 8) and deoxyuridine to uridine; and the gene pyr-4, which encodes for OMP decarboxylase, the last step in the *de novo* pathway for the synthesis of pyrimidines. Because of the lack of pyrimidine synthesis, a pyrimidine source such as uracil must be added to the growth media of this strain.

N. crassa was grown on solid Horowitz complete media, which included agar and uracil as a pyrimidine source. Conidia from growing cultures were then transferred to sterile liquid Westergaard's media. More IDCase activity was detected from cultures that were grown from liquid Westergaard's media.[12]

Materials and Methods

Protease Inhibitors

Solutions of protease inhibitors, leupeptin, phenylmethylsulfonylfluoride (PMSF), pepstatin A, and ethylenediamine tetraacetic acid (EDTA, sodium salt) (Sigma), were prepared with the following concentrations. Leupeptin was prepared with a concentration of 0.6 mM by dissolving 25 mg in 90 mL of H_2O. A solution of 100 mM PMSF was prepared by dissolving 0.1742 g in 10 mL of absolute ethanol. 2 mM pepstatin A

solution was prepared by dissolving 25 mg in 18.2 mL of absolute ethanol. EDTA solution with the concentration of 100 mM was prepared by dissolving 37.22 g in 1 L of H_2O and dissolving with the addition of NaOH. PMSF, leupeptin, and pepstatin A were stored at -20°C until they were used.

N. crassa Growth

Horowitz complete media (one liter) was prepared (Table 2) and sterilized by autoclaving (Yamato SM 32) 50 mL portions in 250-mL Erlenmeyer flasks. In order to solidify the Horowitz complete media, agar (Fisher) was added at a concentration of 1.5% (w/v) and each of the flasks were covered with aluminum foil before sterilization. Upon cooling to room temperature, 100 μL of 20 mg/mL filtered sterilized uracil was added to the Horowitz complete media and the flasks were inoculated with a flame sterilized loop of *N. crassa* strain #2203. These flasks were allowed to incubate at room temperature and adequate light for 3-5 days until the orange, filamentous fungi had grown.

Westergaard's media (one liter)[12] was prepared (Table 3). 50 mL of Westergaard's media was placed in 250-mL Erlenmeyer flasks and also covered with aluminum foil for sterilization similarly to the Horowitz media. After growth of the fungi had appeared on Horowitz solid media, 10 mL of Westergaard's liquid media was transferred to the flasks with the growth using sterile pipets. The conida that were suspended in the liquid media were removed and transferred back to the original flask with Westergaard's liquid media. 800μL of filter-sterilized uracil (20 mg/mL) was also added as a pyrimidine source. The flask was then allowed to incubate with shaking at 30°C for 18 hours, which showed the highest activity.

Table 2: Horowitz Complete Medium

Compound	Amount
potassium tartrate (Sigma)	5.0 g
sodium nitrate (Sigma)	4.0 g
potassium dihydrogen phosphate (Sigma)	1.0 g
magnesium sulfate (7 H_2O) (Sigma)	0.5 g
sodium chloride (Fisher)	0.1 g
calcium chloride (Sigma)	0.1 g
glycerol (Amresco)	16 mL
hydrolyzed casein (Fisher)	0.25 g
yeast extract (Fisher)	5.0 g
malt extract (Sigma)	5.0 g
dissolved in 950 mL of H_2O and diluted to 1 L	1.0 L
agar (Fisher)	15.0 g

Table 3: Westergaard's Medium

Compound	Amount
potassium nitrate (Sigma)	1.0 g
potassium dihydrogen phosphate (Sigma)	1.0 g
magnesium sulfate (7 H_2O) (Sigma)	0.5 g
sodium chloride (Fisher)	0.1 g
calcium chloride (Sigma)	0.1 g
trace elements (50X)[a]	0.2 mL
biotin (50X)[b]	0.2 mL
dissolved in 950 mL of H_2O and diluted to 1 L	1.0 L
sucrose	20.0 g

[a] See Table 4 for preparation

[b] dissolve 5.0 mg of biotin in 50 mL of 50% ethanol

Table 4: Trace Elements Solution

Compound (Sigma)	Amount
citric acid·1H_2O	5.0 g
$ZnSO_4$·7H_2O	5.0 g
$Fe(NH_4)(SO_4)_2$·6H_2O	1.0 g
$CuSO_4$·5H_2O	0.25 g
$MnSO_4$·1H_2O	0.25 g
H_3BO_3 anhydrous	0.05 g
Na_2MoO_4·2H_2O	0.05 g
dissolved in 80 mL of H_2O and diluted to 100 mL	100 mL
$CHCl_3$ [a]	1.0 mL

[a] added after all components were dissolved in H2O to suppress contaminating microbial growth

Harvest and Tissue Preparation

After 18 hours of growth, the tissue was harvested by vacuum filtration using a side arm flask, Büchner funnel, filter vac, and a #1 Whatman filter disk. The tissue was then weighed and placed in a 50-mL conical tube (Fisher). The usual amount of tissue from a harvest was between ~0.5-2.0 g. A 2:1 ratio (v/w) of GDH buffer (10 mM EDTA, 33 mM Tris (pH 8.0) and 0.007% β-mercaptoethanol) to tissue was added to the conical tube along with protease inhibitors, PMSF (0.4 mM), leupeptin (1.2 μM), and pepstatin A (4 μM). The tissue was disrupted on ice by 30 second bursts at the maximum setting of 30,000 rpm using a tissue homogenizer (Biospec model 985-370). The cell suspension was cooled on ice at 30 second intervals between each burst. This was repeated five times to ensure that all the tissue had been disrupted. The lysates were then transferred to polystyrene tubes (Beckman) and centrifuged (Beckman L7 Ultracentrifuge, rotor type

50.2 TI) at 25,000 rpm for 20 minutes and 4 °C. The supernatant was transferred to a 15-mL conical tube (Fisher) and the cell debris was discarded.

Ammonium Sulfate Precipitation

In the case of the *N. crassa* IDCase protein, a 60% and 90% fractionation was performed.[4] After determining the volume of the tissue lysate, ammonium sulfate, $(NH_4)_2SO_4$, (Amresco) (Table 5), was slowly added over the course of ~10 minutes with constant stirring at 4 °C. The mixture was allowed to stir for one hour to precipitate the

Table 5: Ammonium Sulfate Fractionation[13] at 60% and 90 %[a] and 45% and 65%[b]

Final Percent Saturation of $(NH_4)_2SO_4$	**40**	**60**	**65**	**90**
Initial % $(NH_4)_2SO_4$	**Solid $(NH_4)_2SO_4$ (g) added to 100 mL of Solution**			
0	***22.6***[b]	***36.1***[a]	39.8	60.3
30	5.6	18.1	21.4	40.2
40	0	12.0	***15.3***[b]	18.7
60	-	0	3.1	***20.1***[a]
65	-	-	0	16.8

[a] IDCase from *N. crassa*

[b] IDCase from *R. glutinis*

first of two $(NH_4)_2SO_4$ fractionations. After the mixture had stirred for one hour, it was centrifuged at 25,000 rpm for 20 minutes at 4 °C. Then the volume of the supernatant was measured and $(NH_4)_2SO_4$ was slowly added to a saturation of 90% at 4 °C. The suspension was allowed to stir for an hour and then was again centrifuged at 25,000 rpm for 20 minutes at 4 °C. When centrifugation was completed, the pellet was resuspended

in 2 mL of lysis buffer (50 mM K_2HPO_4, pH 7.4; 10% glycerol; 0.035% β-mercaptoethanol; 2 mM PMSF; 6 μM leupeptin; 20 μM pepstatin A; and 2 mM EDTA) and the supernatant was discarded. The protein was transferred to a dialysis bag and was dialyzed overnight (~15 hours) in dialysis buffer (50 mM Tris-HCl, pH 7.4; 10% glycerol; and 0.035% β-mercaptoethanol). IDCase remains in the supernatant in the first 60% cut, while it is precipitated in the pellet in the 90% fraction.

R. Glutinis: Source of IDCase

R. glutinis is a type of yeast which has not been completely studied. *Rhodotorula* species have been known to produce pigments that are red or pink in color. These carotenoids are composed mainly of two oxygenated compounds, torulene and torularhodin. The pigments of *R. glutinis* can be affected by the environmental conditions; when grown at 25°C a particular strain is pink in color, but at 5°C it is a dark yellow. *R. glutinis* has also been noted for accumulating large amounts of lipids when grown in a medium with a limiting nitrogen supply.[14] These organisms have recently been found to have IDCase activity.

The original idea of *R. glutinis* having IDCase activity came from the assays of thymine 7-hydroxylase, the enzyme preceding IDCase in the thymidine salvage pathway. The literature has shown that thymine 7-hydroxylase has been purified from cultures of *R. glutinis* grown with thymine as the only nitrogen source. Since thymine is the only nitrogen and pyrimidine source, the organism can use thymine 7-hydroxylase to eventually convert thymine to iso-orotate. This would lead to an accumulation of IOA, a potentially lethal incident, if IDCase was not present.

Unfamiliarity with *R. glutinis* led to some questions. Because this organism was not used in previous studies of IDCase, conditions for growth, cell lysis, and protein preparation were unknown. In the literature, *R. glutinis* was grown to obtain a similar enzyme so similar conditions were used to obtain IDCase from these cultures with slight modifications. The protocol in the literature called for a large *R. glutinis* preparation which unfeasible for first IDCase assays. The scale for production was decreased to accommodate the research and the cells were grown to optimize IDCase activity.[3]

Materials and Methods

Media Preparations

YM agar plates were prepared by dissolving all the components (Table 6) and autoclaving. 20 mL of the sterilized medium was transferred by sterile pipets to Petri plates. The medium was allowed to solidify at room temperature. Unused plates were stored at 4 °C and discarded if left for longer than three weeks.

11.7 g of the powdered yeast carbon base medium (YCB, Table 7) was added to 1 L of H_2O. Thymine (1g/L) was added as the nitrogen source and 100 mL of the medium was transferred to 250-mL Erlenmeyer flasks. The flasks were covered with aluminum foil and autoclaved.

R. glutinis Growth

R. glutinis (ATCC 2527) cells were first grown on YM agar plates. After at least three days growth, the cells were scraped off the plates and transferred to a yeast carbon base (YCB) media (Table 6) with thymine (1 g/L) at a concentration of about 9×10^6 cells/mL. This value was obtained by calculating the optical density (OD_{600}) of the cell

suspension and using the conversion factor for *Saccharomyces cerevisiae* where an OD_{600} of 2 is equal to 4 x 10^7 cells/mL.[15] The cells were incubated between 24-28°C for 20-22 hours.

Table 6: YM Medium Components

Components	Amount
yeast extract (Fisher)	3.0 g
malt extract (Sigma)	3.0 g
peptone (Fisher)	5.0 g
dextrose (Sigma)	10.0 g
dissolved in 700 mL of H_2O, the pH was adjusted to 4.5 with acid, and the mixture was diluted to 1 L	1.0 L
agar (Fisher)	20.0 g

Harvest and Cell Preparation

After ~21 hours of growth, cells were transferred to a 50-mL conical tube (Fisher) and harvested by centrifugation (Beckman GPR Centrifuge) at 2,500 rpm for 5 minutes at room temperature. The supernatant was decanted off, and the remaining cells were weighed. The approximate wet cell weight varied between 0.5-1.0 g. The cells were resuspended in 2 mL of lysis buffer with PMSF (2 mM), leupeptin (6 μM), pepstatin A (20 μM), and EDTA (2 mM). The cells were lysed by first placing the suspension in a 2.5-mL screw cap tube with ~0.5 mL of 0.1 mm diameter beads. Then the tube was placed in a mini beadbeater (Biospec Products Mini Beadbeater) at 5,000 rpm for 30 seconds with intermittent cooling. To ensure complete cell lysis, the agitation was repeated at least seven times. The cellular debris was removed by centrifugation (Fisher Scientific MicroV) at 4,000 rpm for 1.5 minutes at room temperature. The supernatant

was decanted and the pelleted debris was discarded. This method resulted in ~1.0 mL of cell lysate which was placed on ice.

Ammonium Sulfate Precipitation

The ammonium sulfate precipitation conditions for IDCase from *R. glutinis* were determined to be different than IDCase from *N. crassa*. These values were determined by precipitation at different $(NH_4)_2SO_4$ concentrations and assaying the supernatant from each (Table 8). There were some difficulties as the protein seemed less stable in *R. glutinis* after salting it out. The volume of the cell lysate was measured and was brought to 40 % $(NH_4)_2SO_4$ saturation by slowly adding the salt to the lysate at 4 °C over the course of 10 minutes. After one hour of vigorous stirring, the suspension was transferred to a polystyrene tube (Beckman) and was centrifuged at 25,000 rpm for 20 minutes at 4 °C. The volume of the supernatant was measured to determine the amount of $(NH_4)_2SO_4$ needed to bring the solution to 60% saturation. $(NH_4)_2SO_4$ was added slowly to the suspension over 10 minutes at 4 °C. After one hour of stirring, the mixture was centrifuged at 25,000 rpm for 20 minutes at 4 °C. The supernatant was removed and discarded while the pellet was redissolved in 2.0 mL of lysis buffer with PMSF (2 mM), leupeptin (6 μM), pepstatin A (20 μM), and EDTA (2 mM). The resuspension was transferred to a dialysis bag and placed in dialysis buffer (~15 hours) to remove salts.

Determination of Protein Concentrations

The protein concentration was determined for the lysates and resuspended $(NH_4)_2SO_4$ pellets by Bradford assay. A standard curve was generated by using 0, 5, 10, 15, and 20 μL of 0.5 mg/mL BSA (2 mg/mL stock in 0.9% NaCl with NaN_3, Pierce).

Table 7: Yeast Carbon Base Medium[14]

Constituent (Difco)	Amount
D-glucose	10.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2 \cdot 2H_2O$	0.1 g
L-histidine·HCl·H_2O	1.0 mg
DL-methionine	2.0 mg
DL-tryptophan	2.0 mg
H_3BO_3	500 μg
$CuSO_4 \cdot 5H_2O$	40 μg
KI	100 μg
$FeCl_3 \cdot 6H_2O$	200 μg
$MnSO_4 \cdot H_2O$	400 μg
$NaMoO_4 \cdot 2H_2O$	2 μg
$ZnSO_4 \cdot 7H_2O$	400 μg
biotin	2 μg
calcium pantothenate	400 μg
folic acid	2 μg
inositol	2000 μg
niacin	400 μg
para-aminobenzoic acid	200 μg
pyridoxine·HCl	400 μg
riboflavin	200 μg
thiamine·HCl	400 μg

Each of the five amounts of BSA was placed in a separate Eppendorf tube and the volume was brought up to 100 μL with 0.15 M NaCl. The standards were duplicated to decrease error. To each of the tubes, 900 μL of Coomassie Blue dye (Pierce) was added and the tubes were vortexed for ~30 seconds. The protein sample was prepared similarly to the standards with the concentration of the protein solution falling in the range of the standard curve. After about 5 minutes, the absorbance at 596 nm (A_{596}) was measured on a Hewlett Packard 8452A diode array spectrophotometer using the standards with no BSA as the blank. Once all the A_{595} measurements were made, the standards were used to construct a standard curve. The protein concentration (μg/μL) was then determined by using the equation of the linear standard curve.

Table 8: Determination of Percent $(NH_4)_2SO_4$ Fractionation for *R. glutinis*

supernatant from percent $(NH_4)_2SO_4$	cpm
20 %	779
30 %	982
40 %	827
50 %	166
60 %	164

Chapter 4: Kinetic Parameters of IDCase from *R. glutinis*

Determination of Kinetic Constants for IDCase

Early kinetic studies by Michaelis and Menten resulted in evidence for enzyme (E) binding to the substrate with the formation of an enzyme-substrate complex (ES) before product (P) formation.[7] Specificity between enzyme and substrate is the basis of the lock and key theory and the concept of an active site in an enzyme evolved subsequently. Kinetic experiments provide a good description of general properties of enzymes, such as the efficiency of an enzyme and conditions for optimal and maximal activity. Kinetic data also provides a relationship between the amount of product formed and the time that it takes to form. The basis for enzyme kinetic measurements is the observation that the velocity varies directly with the concentration of substrate or enzyme.

The kinetics of IDCase from *N. crassa* have been previously determined from ^{14}C radioassays.[4] However, no kinetic information has been available for the enzyme from *R. glutinis*. The kinetic constant for this enzyme was determined in assays performed with varying amounts of substrate. Lineweaver-Burk plots derived from the Lineweaver-Burk equation (Equation 1) were used in the K_m determination. Inhibition data was

$$\frac{1}{v_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \qquad \text{Equation 1}$$

gathered for 5NU and the synthesized conjugated 5NU. All kinetic data were obtained by assaying IDCase from *R. glutinis* protein preparations using the ^{14}C radioassay.

The proposed mechanism of catalysis by IDCase involves the enzyme making a covalent attachment to C6 of the substrate. An experiment for determining whether this attachment is realistic was performed on IDCase from both *R. glutinis* and *N. crassa* using ^{14}C 5NU. Polyvinylidene difluoride (PVDF), a membrane used in Western and dot blotting, was used to bind the protein. IDCase with ^{14}C 5NU can be bound to PVDF and the amount of inhibitor bound to the enzyme can be detected by scintillation counting. The experiment was performed by adding the labeled inhibitor and IDCase to a well on a dot blot apparatus. The mixture can then be pulled across the membrane by vacuum. Since only proteins bind to PVDF, unbound ^{14}C 5NU would not be detected. This experiment was performed with protein and ^{14}C 5NU, heat-killed protein (HK) and ^{14}C 5NU. Two controls for this experiment were protein with 5NU and ^{14}C 5NU, and ^{14}C 5NU alone. Included in this chapter is the K_m determination, K_i determination, and a proposal of a nucleophilic attack that is presumed to occur at C6.

Materials and Methods

^{14}C Radioassay

The assay mixture (0.5 mL) contained substrate ([7-^{14}C] IOA) (Moravek Biochemicals), water, buffer (tris(hydroxymethyl)aminomethane-hydrochloric acid [Tris-HCl], pH 7.4), and protein. Any liberated $^{14}CO_2$ adsorbs to a 2 M sodium hydroxide saturated wick (Whatman 3 MM filter paper) positioned in a reservoir suspended from a septum fitted over the reaction vessel. After 10 minutes, the reaction was quenched by the addition of 2 M hydrochloric acid by syringe and allowed to incubate at

Table 9: ^{14}C Radioassay Components

^{14}C IOA (μL)	Tris-HCl (μL)	Protein (μL)	H_2O(μL)	Total Volume (μL)
50[a]	25	100	325[a,b]	500[c]

[a] when the volume of substrate was changed, the volume of H_2O was changed to keep the total volume constant

[b] in the presence of inhibitor, the volume of H_2O was changed to keep the total volume constant

[c] constant

room temperature for one hour. The wicks were dried in a vacuum oven for approximately 30 minutes and then they were placed in scintillation fluid (ScintiSafe™ Econo 1, Fisher) filled vials. $^{14}CO_2$ displacement was measured by scintillation counting (Packard Tri-Carb 1900CA Liquid Scintillation Analyzer) and the cpm correlated with the amount of enzymatic activity (Table 1)

Preparation of Solutions for Kinetic Determinations

A 5NU solution was prepared with a concentration of 200 nM from a stock solution of 200 μM 5NU. 50 μL of 200 μM 5NU was diluted to 50 mL with H_2O and the concentration was measured by UV spectroscopy using Beer's Law, $A = \varepsilon \times b \times c$, where A is the absorbance, ε is the extinction coefficient ($M^{-1} \cdot cm^{-1}$), b is the path length of the quartz cell (cm), and c is the concentration (M). The λ_{max} of 5NU is 342 nm and the extinction coefficient (ε) is 13,500 $M^{-1} \cdot cm^{-1}$.[11]

A 190 μM solution of 3-(3-carboxypropyl)-5-nitrouracil was prepared from a 11.67 mM stock solution of the compound. 815 μL of the stock solution was diluted to 50 mL with H_2O. The λ_{max} of 3-(3-carboxypropyl)-5-nitrouracil was determined to be

302 nm and the extinction coefficient of 3-methyl 5-nitrouracil, 9440 $M^{-1} \cdot cm^{-1}$,[16] was used to determine the concentration.

Determination of the Michaelis constant, K_m

Protein from *R. glutinis* was prepared as described in chapter 3. Precipitation by ammonium sulfate was unnecessary because of the high levels of activity. The K_m was determined using two separate ^{14}C radioactivity assays with varying ^{14}C IOA concentrations (assay 1: [S] = 35.8, 17.9, 11.8, 7.2 , 5.0, and 3.6 μM, 100 μL of protein; assay 2: [S] = 80, 40, 27, and 20 μM, 150 μL of protein).

Determination of Inhibition Constants, K_i, for 5-nitrouracil and 3-(3-carboxypropyl)-5-nitrouracil

The K_i for 3-(3-carboxypropyl)-5-nitrouracil was determined by assaying IDCase in the presence of varying substrate and inhibitor concentrations ([I] = 0, 5, and 30 μM; [S] = 12.5, 25, 37.5, and 50 μM). The ^{14}C radioassay was used to detect the effects of the inhibitor on IDCase. An estimated K_i for 5NU was determined by conducting an assay where the K_m equaled the substrate concentration ([I] = 200 nM; [S] = 65 μM).

Solutions for Covalent Attachment Experiment

A solution of 500 μM 5NU was prepared from an 18 mM stock solution of 5NU. The stock solution was prepared by dissolving 0.1414 g of 5NU in 50 mL of pH 8 H_2O-NaOH; then 1.38 mL of the stock solution was diluted to 50 mL with pH 7.4 Tris-HCl buffer.

^{14}C-labeled 5NU was synthesized by Dr. J. A. Smiley (Department of Chemistry, Youngstown State University) from uracil (Moravek Biochemicals) which was ^{14}C labeled at positions 2, 4, 5, and 6. The final concentration was 5 μM (2200 cpm/μL).

Covalent Attachment Experiment

Protein was collected from both *R. glutinis* and *N. crassa*. Both of these lysates were subjected to a single 90% ammonium sulfate fractionation. The pellet was resuspended in 2.0 mL of lysis buffer and it was dialyzed for two hours. After the first hour of dialysis, the dialysis buffer was exchanged for fresh buffer. The activity and protein concentration were calculated for both of the lysates by ^{14}C radioassay and Bradford assay, respectively, to ensure that IDCase was present.

Before beginning the experiment, PVDF was saturated in methanol for two hours and washed with H_2O prior to use. It was then placed on a dot blot apparatus. 200 μL of protein was aliquoted into an eppendorf tube along with 10 μL of ^{14}C 5NU (5 μM). The reaction was allowed to proceed at 25 °C for two minutes. The next sample was prepared by adding 10 μL of ^{14}C 5NU to 200 μL of protein which was subjected to heat-killing at 95 °C for five minutes. 10 μL of 5NU (500 μM) was added to 200 μL of protein for the first control in the experiment. This reaction proceeded for two minutes at 25 °C and then 10 μL of ^{14}C 5NU (5 μM) was added for an additional two minutes at 25 °C. The final sample used in this experiment was 10μL of ^{14}C 5NU (5 μM). All the samples were applied to separate wells in the dot blot apparatus and the samples were pulled across the membrane by vacuum for 30 minutes. Then each well was washed with 200 μL of lysis

buffer which was drawn through the membrane by vacuum for 30 minutes. Duplicate assays were performed for all of these reaction samples.

The PVDF membrane was removed from the dot blot apparatus and each sample was carefully cut out. The pieces of membrane were dried in a vacuum oven for ten minutes and then placed in scintillation fluid-filled vials for radioactivity counting.

Results and Discussion

The K_m of IDCase from *R. glutinis* was determined to be 64.45 ± 0.05 μM, which differed from *N. crassa* IDCase (K_m = 35 ± 9 μM).[4] This value was obtained from the kinetic plots of two assays (Figure 19 and 20). The Lineweaver-Burk plot, a linear transformation of the Michaelis-Menten equation, is the graph of $1/v_0$ vs. 1/[S], where v_0 is the rate (nmol/min/mg). The lower K_m value observed in IDCase from *N. crassa* indicates that the enzyme from *R. glutinis* has a lower affinity for the substrate than IDCase from *N. crassa*.

The K_i for 3-(3-carboxypropyl)-5-nitrouracil was determined to be ~7.9 μM. This value was obtained from data that was collected from an assay which had varying substrate and inhibitor concentrations. A Lineweaver-Burk kinetic plot (Figure 21) was constructed from the rate and substrate concentrations. The slopes from each of the inhibition graphs of the kinetic plot were used in a replot versus inhibitor concentrations (Figure 22) to arrive at the K_i value, which is the negative abscissa intercept.[17]

The K_i value of 5NU was estimated by using the Segal Plot[18] (Figure 23) because it could not be directly measured. The Segal Plot is implemented by assaying an enzyme with an inhibitor, where the substrate concentration is equal to the K_m of the enzyme. At

200 nM 5NU, 20% of the activity was retained. The $[I]/K_i$ was extrapolated to ~8 and the K_i was determined to be 25 nM. IDCase from *N. crassa* was estimated to have a K_i for 5NU of ~0.4 nM using the same approximation.[4] This indicates that IDCase from *R. glutinis* has a lower affinity for 5NU than IDCase from *N. crassa*.

The covalent attachment experiment showed some encouraging results (Table 10). The cpm for IDCase from both organisms were higher than both of the controls. During this experiment, there was difficulty in drawing off the mixtures from the wells by vacuum, which may have led to the high cpm in the wells with only ^{14}C 5NU. The indication that this experiment may be successful was from the data obtained for the other control. The other control, protein with both labeled and unlabeled 5NU, showed relatively few cpm indicating competition for binding by the two inhibitors. The concentration of unlabeled 5NU was intentionally 100 times that of ^{14}C 5NU to determine whether the cpm were from actual enzyme-inhibitor interactions or inhibitor-membrane interactions. This experiment might be the first of many to prove the covalent attachment mechanism.

Table 10: Results of Covalent Attachment Experiment

Sample	cpm
IDCase (*N. crassa*) and ^{14}C 5NU	819
HK IDCase (*N. crassa*) and ^{14}C 5NU	1446
IDCase (*N. crassa*), ^{14}C 5NU, and 5NU	464
IDCase (*R. glutinis*) and ^{14}C 5NU	924
HK IDCase (*R. glutinis*) and ^{14}C 5NU	1107
IDCase (*R. glutinis*), ^{14}C 5NU, and 5NU	388
^{14}C 5NU	554

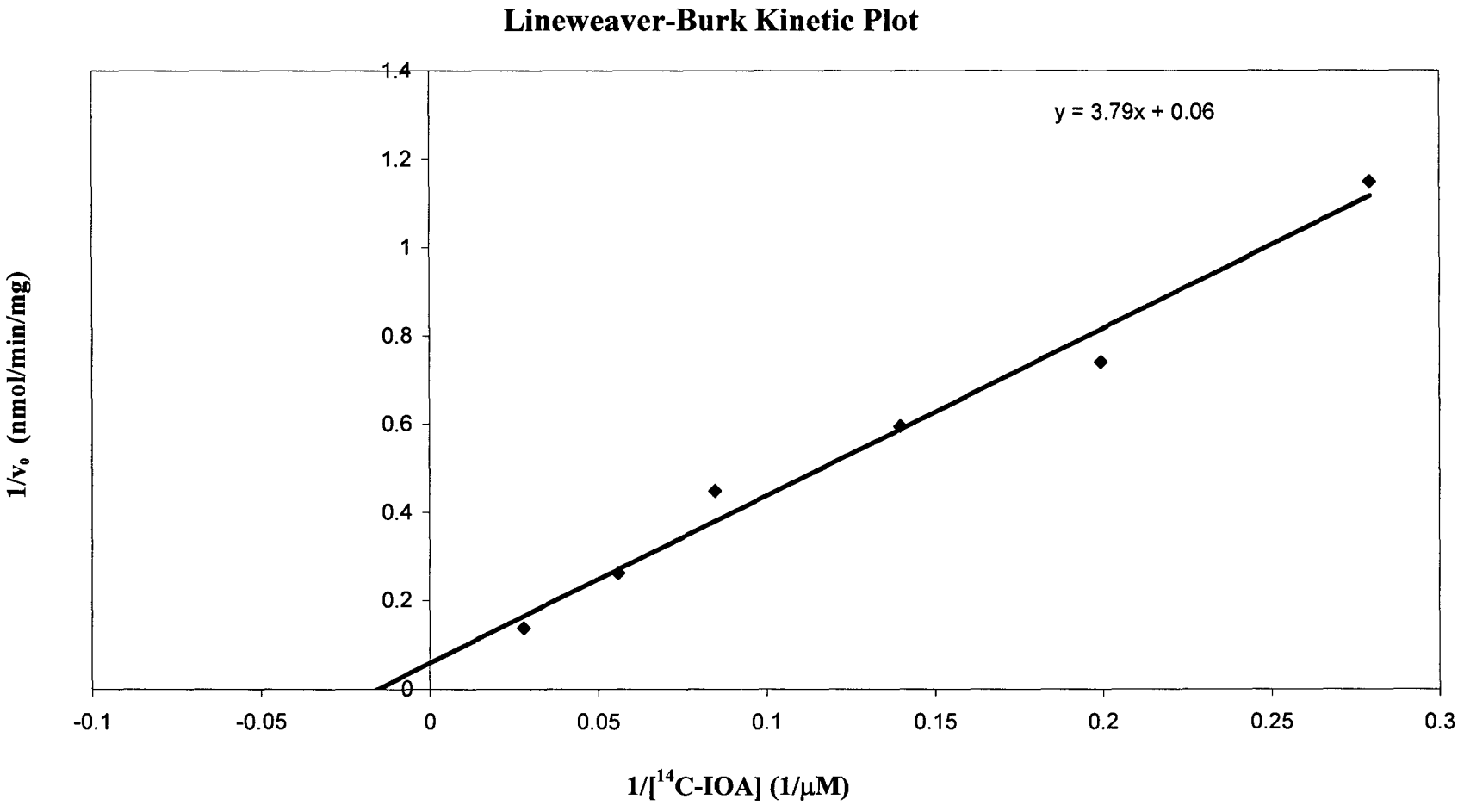


Figure 20: Km = ~64.4 μM, average Km = 64.45 ± 0.05 μM

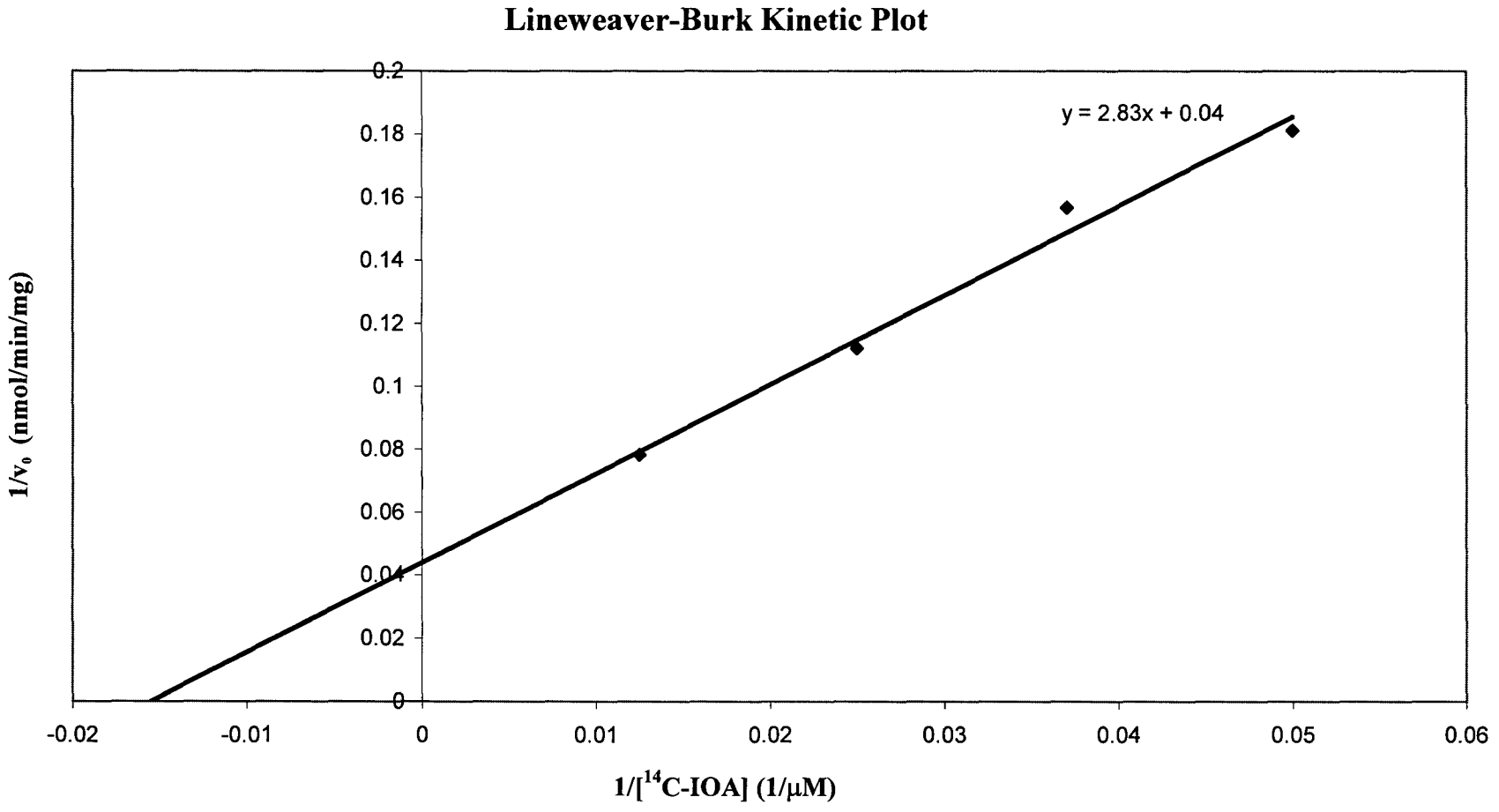


Figure 21: K_m = ~65.5 μM, average K_m = 64.45 ± 0.05 μM

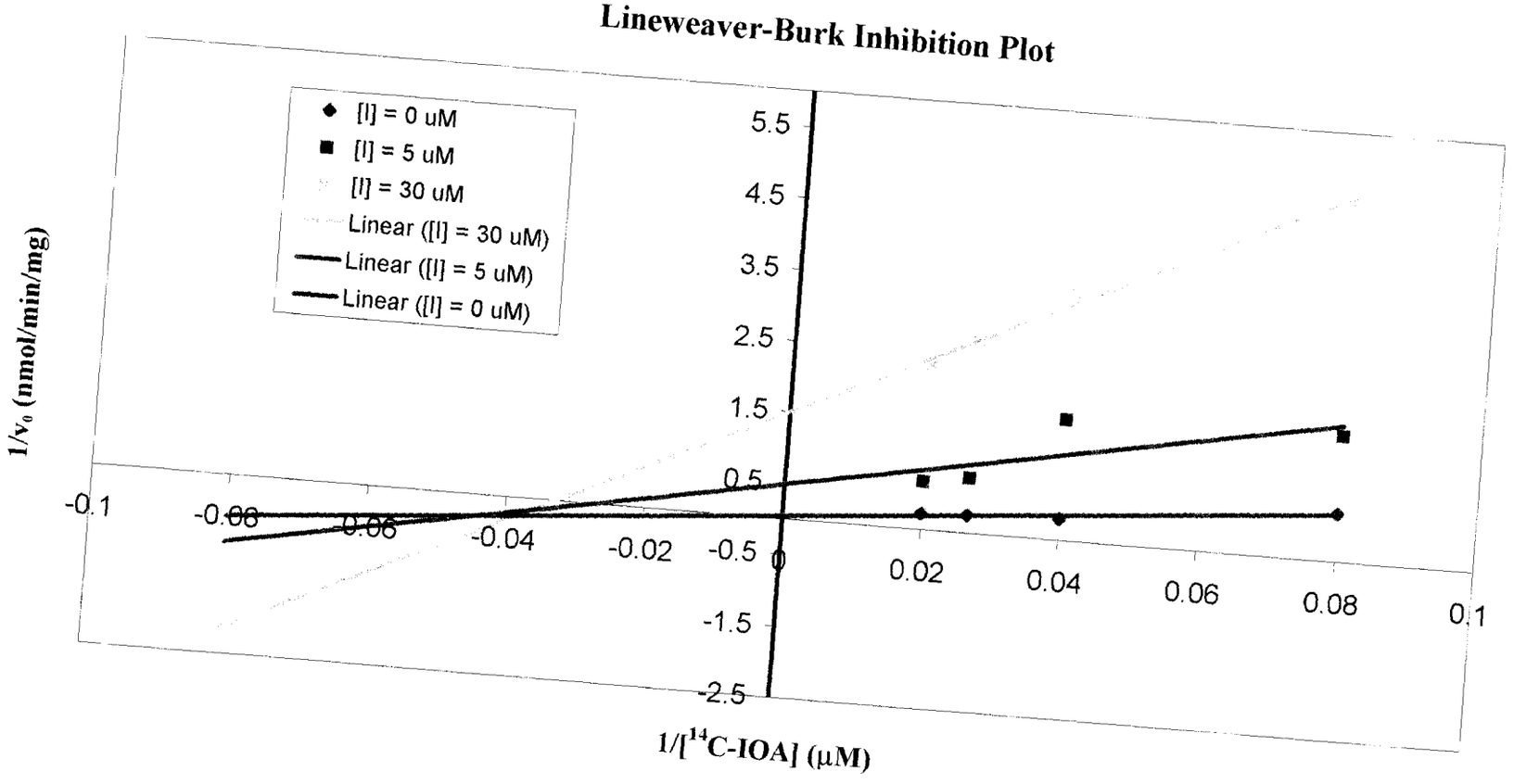


Figure 22: K_i determinations at various conjugated 5NU concentrations

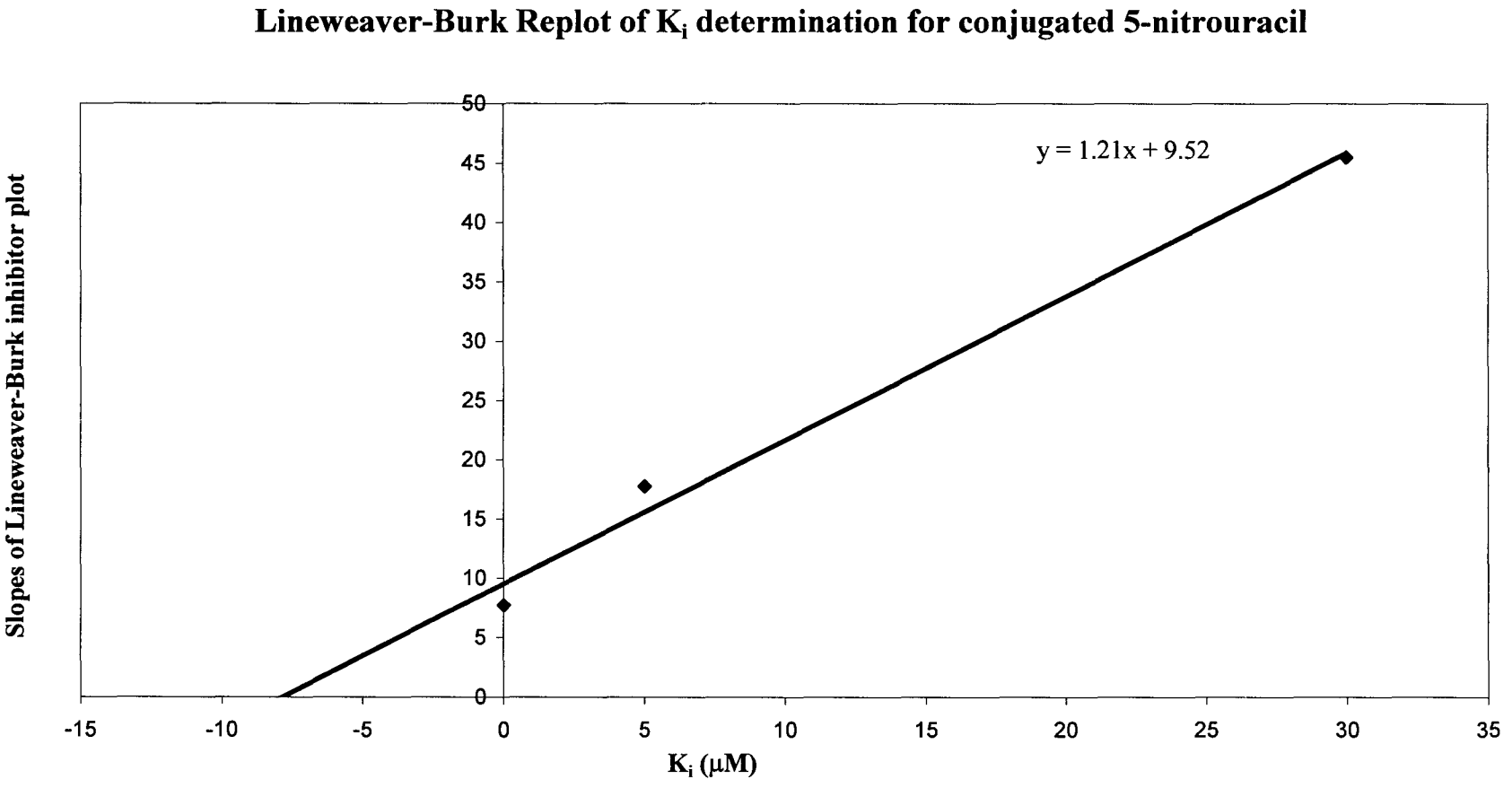


Figure 23: K_i = ~7.9 μM

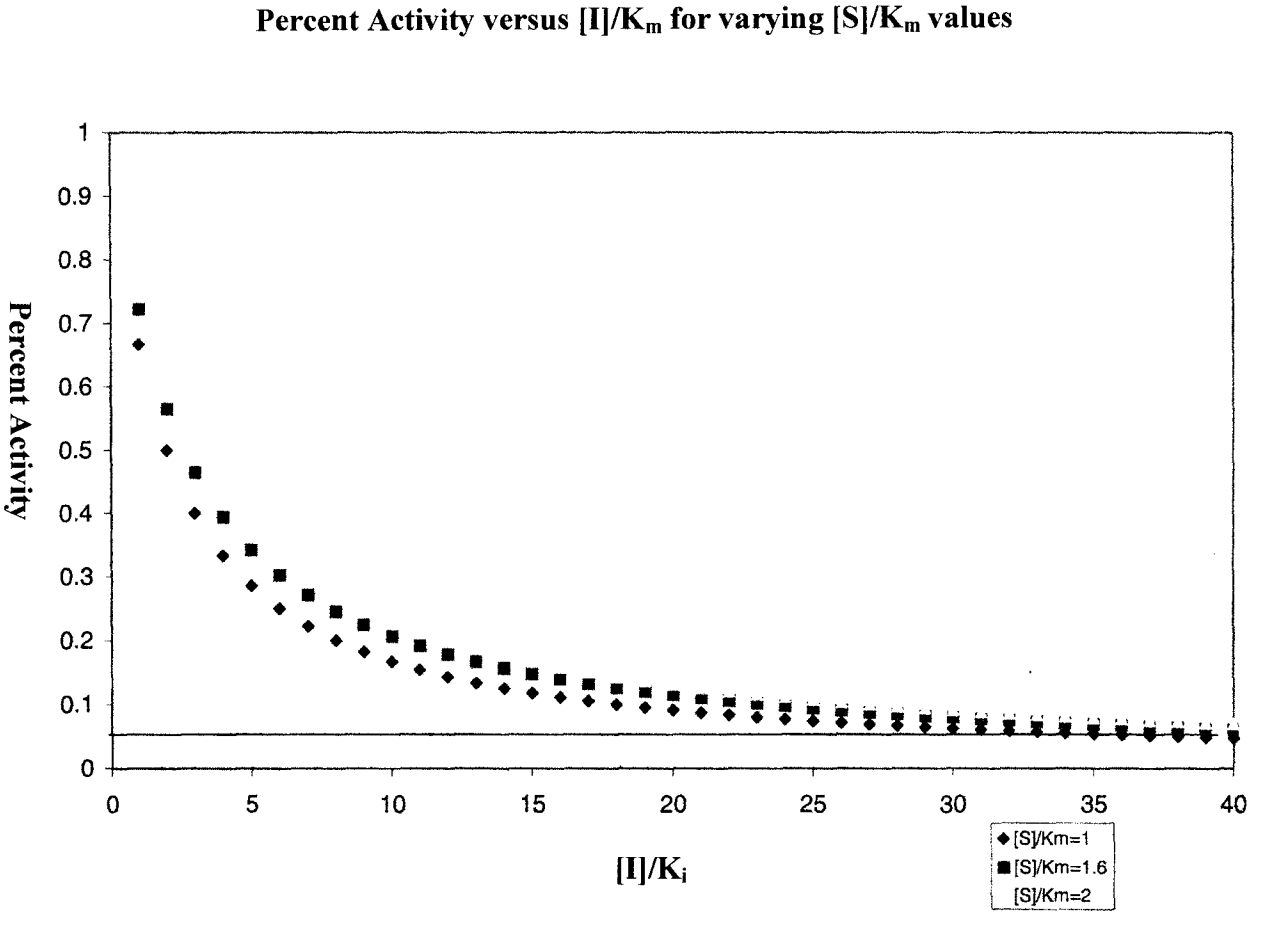


Figure 23: Segal Plot[18] used in determining the K_i for 5NU

Chapter 5: Synthesis of Custom Affinity Column and Partial Purification of IDCase

Custom Affinity Column Construction

In order to construct a custom affinity column, a ligand capable of binding with the desired enzyme must be synthesized. There are important factors that are required for success in affinity chromatography. The matrix, which should present the largest surface area for a ligand to bind, must be inert and stable. A porous material, such as agarose, is best suited for this purpose. The most appropriate ligand, which only binds to a single component in a mixture, must be selected for the desired interactions present in the active site of the enzyme. Stability of the ligand is also necessary to ensure that experimental conditions do not cause degradation of the ligand. The ligand must also form a bond with the matrix that does not involve any functional groups necessary for binding the desired enzyme resulting in the minimal loss of affinity for the enzyme. Due to steric hindrance between large molecules, such as enzymes, and the matrix, a linker or spacer arm is necessary to separate the ligand from the stationary phase. The protocol for 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/ diaminodipropylamine immobilization kit from Pierce was followed for the immobilization.

After the construction of the custom affinity column, many variables still exist in the purification attempt. It was not known if the column could handle large amounts of protein, how these proteins may be removed from the column without disturbing either enzyme-ligand interactions or ligand-matrix interactions, or whether the column would bind to IDCase. Purification attempts had been previously done with commercially available ligands, but with very little success.[6]

Materials and Methods

Column Immobilization

All reagents were purchased from Pierce unless noted otherwise. A column was constructed using the previously synthesized *N*-3-(3-carboxypropyl) 5-nitrouracil. 2 mL of diaminodipropylamine (agarose slurry), the column material, was placed in a 10-mL syringe with a cotton plug. The column was washed five times with 2 mL of H_2O and then five times with conjugation buffer (0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma), 0.9% NaCl, pH 4.7). The column was not allowed to go dry and stoppered when the liquid level descended to the top of the gel bed. Approximately 50 mg of 3-(3-carboxypropyl) 5-nitrouracil (~200 μmol) was dissolved in 4 mL of conjugation buffer and the OD_{302} was determined. Then this solution was applied to the column and after placing a cap on the syringe, it was placed on a shaker. 0.5 mL of conjugation buffer was added to 60 mg of EDC. The EDC solution was then added to the slurry. The reaction proceeded for 24 hours with gentle shaking. The following day, the solution within the syringe was drained and the volume was measured. The column was washed with 1mL of wash buffer (1 M NaCl) ten times. Each of the 1 mL fractions were collected and along with the reaction mixture that was first drained, the OD_{302} was measured. The amount of ligand bound to the stationary phase at the end of the reaction was determined from the amount originally applied, minus the amount recovered upon washing the column after the reaction.

Partial Purification of IDCase

IDCase from *N. crassa* was prepared as previously described. An ammonium sulfate precipitation was performed on the protein and it was dialyzed for 12 hours. 1 mL

of this protein lysate was applied to the column. The column was washed with 1.0 mL of lysis buffer for each fraction and increasing amounts of NaCl. The salt concentrations were increased from 0 to 800 mM. After all of the fractions were collected, the amount of protein in each of the fractions was determined along with the IDCase activity. The activity and protein concentration were also determined for the protein before applying it to the column.

Results and Discussion

In the construction of the column, the optical density units (ODU's) of the solution applied to the column was 585. The fractions in the wash buffer totaled 292.3 ODU's. The actual amount of ligand attached to the column was calculated to be ~24 μmol.

The protein purification was partially successful by using varying salt concentrations to elute nonspecific binding proteins and separating them from IDCase. The results show that most of the total protein eluted from the column after washing with 200 mM NaCl in lysis buffer. Table 11 shows each fraction with the amount of protein (μg/μL), concentration of NaCl, and cpm from ^{14}C radioassays which are directly related to IDCase activity. Figure 25 is a graphical representation of this data, showing that most of the protein ran off the column before the activity. This only led to a 7-fold purification, but future results may improve upon these. Protein from *R. glutinis* was also prepared for purification, but results were not as promising.

Table 11: Partial Purification of IDCase from *N. crassa*

Fraction	[NaCl] in lysis buffer	[protein] (μg/μL)	cpm
1	0	0.071	29
2	0	0.051	41
3	200 mM	0.165	39
4	200 mM	0.118	50
5	400 mM	1.242	61
6	400 mM	0.291	241
7	600 mM	0.205	622
8	600 mM	0.179	138
9	800 mM	0.122	45
10	800 mM	0.105	29

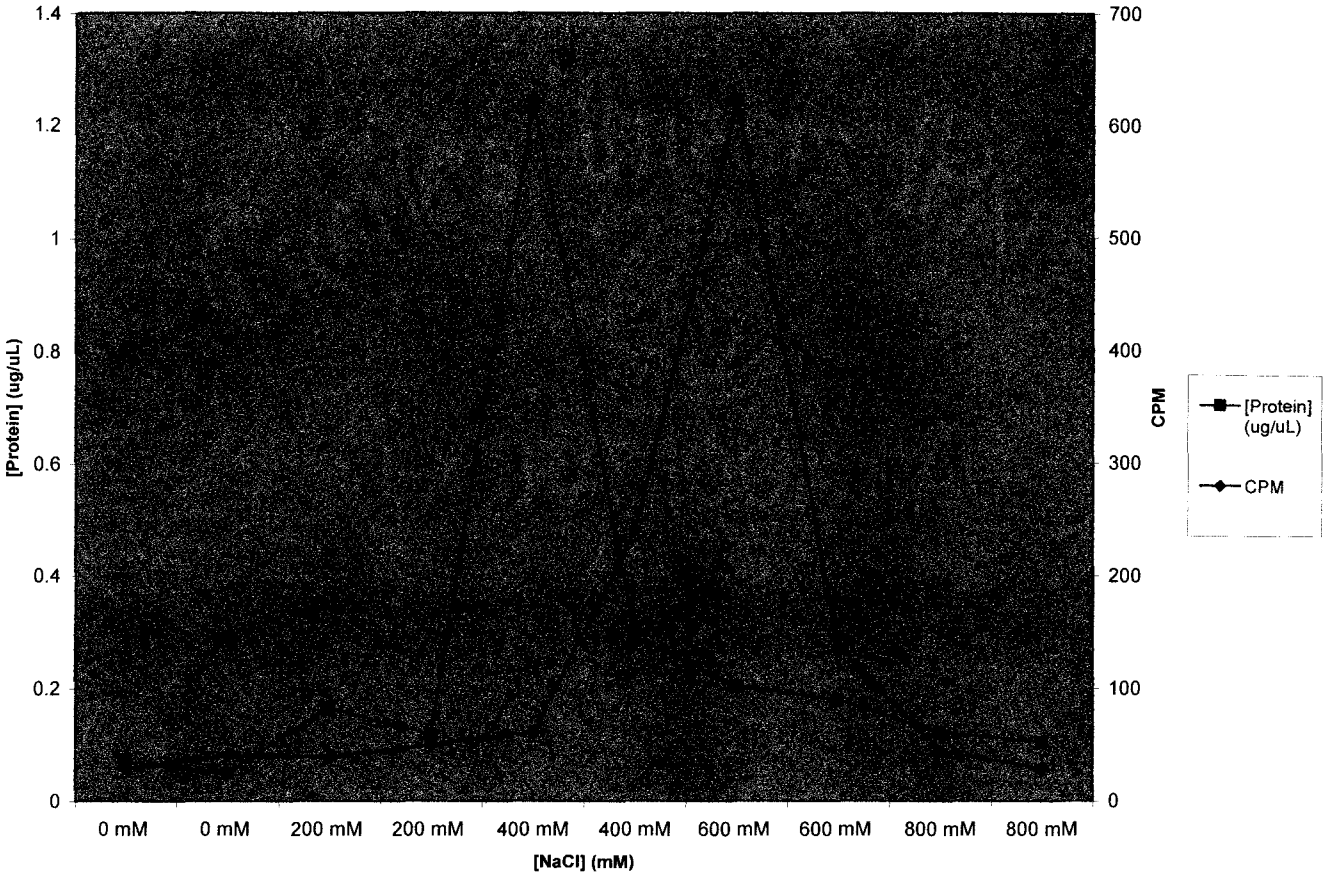


Figure 25: Graphical analysis of the data which shows partial purification of IDCase. After most of the protein is eluted from the column, the activity emerges.

Conclusion

The use of IDCase from R. glutinis has proven to result in high levels of activity. Measurements of kinetics have shown that IDCase from R. glutinis is different than IDCase from N. crassa.

Purification of IDCase by affinity chromatography was not as successful as other similar purifications, but the results are an indication that this purification scheme is feasible.

The covalent attachment experiment could be a factor in determining the mechanism of IDCase. Other information about IDCase, such as molecular weight, may be determined by similar experiments.

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