Iso-Orotate Decarboxylase:

Progress Toward Protein Isolation, Gene Screening, and the Non-Enzymatic Decarboxylation Mechanism

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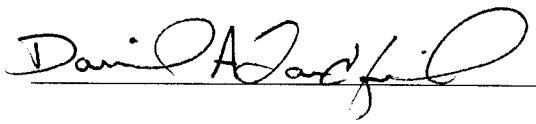

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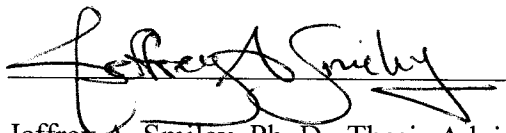

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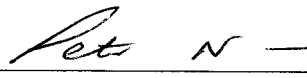

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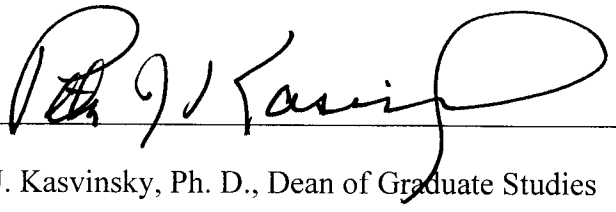

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Thesis Abstract

Iso-orotate decarboxylase (IDCase) is the final enzyme in the thymine salvage pathway, which is possessed by few organisms. A possible mechanism for this enzymatic decarboxylation is similar to a β-keto acid decarboxylation, but multiple mechanism schemes are possible for the non-enzymatic decarboxylation reaction. Protein taken from *Rhodotorula glutinis* cells was assayed for IDCase by decarboxylation of the labeled [*carboxy*-^{14}C] iso-orotate to $^{14}CO_2$. Attachment of a known inhibitor to an agarose column allowed for a five-fold purification of IDCase, with a seven percent recovery. A cDNA library of *R. glutinis* was constructed and converted to a plasmid library to screen for the gene responsible for the IDCase protein. Isolation of the gene responsible for IDCase could help increase effectiveness of IDCase isolation. However, colonies of *E. coli* grown on restrictive media, designed to select for the presence of IDCase, did not contain active IDCase, according to assay data. For the non-enzymatic decarboxylation, spontaneous decarboxylation of iso-orotic acid does not occur at a measurable rate, but the Bu_4Am^+-iso-orotate salt is decarboxylated at a rate of 2.45×10^{-6} sec^{-1}. This rate is much slower than the non-published data of iso-orotic acid in water.

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List of Symbols and Abbreviations

α	alpha
β	beta
g	gram
L	liter
LCMS	liquid chromatography mass spectrometry
M	molarity
μ	micro-
MHz	megahertz
min	minute
mol	mole
nm	nanometer
NMR	nuclear magnetic resonance
°C	degrees Centigrade
ppm	parts per million
rpm	revolutions per minute
TLC	thin layer chromatography
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume

Chapter I: Introduction

Nucleotides are involved in almost every type of biochemical process and exist in two distinct forms: purines and pyrimidines. Adenine and guanine are classified as purines, whereas cytosine, thymine and uracil are classified as pyrimidines. In both DNA and RNA, cytosine consistently pairs with guanine. However, this consistency does not apply to the pairing of adenine in DNA and RNA (Figure 1, Appendix). Specifically, in DNA adenine pairs with thymine, while in RNA adenine pairs with uracil. The purines and pyrimidines adenine, guanine, cytosine, thymine and uracil are the monomeric units of nucleic acids. Nucleotide triphosphates (NTPs), most commonly ATP, store energy via phosphate bonds, the release of which is used to propel thermodynamically unfavorable reactions. NTPs regulate many degradative and biosynthetic metabolic pathways. Finally, nucleotides are found as parts of coenzymes, such as NAD^+, FAD, and others.

The *de novo* pyrimidine biosynthetic pathway, through which pyrimidine ribonucleotide biosynthesis occurs, was discovered in the 1950's and 1960's in *Neuorospora crassa,* as reviewed by Jones.[1] It was determined that *N. crassa*, which normally requires cytosine and uracil, grew typically with orotic acid as the sole pyrimidine source. The *de novo* pyrimidine nucleotide biosynthesis is a six step process (Figure 2, Appendix). In higher organisms, this is a channeled pathway, meaning intermediates are not released into the medium, but instead moved from one enzyme to the next.[2] It starts with the cytosolic enzyme carbamoyl phosphate synthase II combining glutamine, water, and carbonate anion to produce carbamoyl phosphate. Two molecules of ATP are required for this reaction, one to provide a phosphate group, and the other to

energize the reaction. Aspartate transcarbamoylase (ATCase) then catalyzes the addition of aspartate to the carbamoyl group, with loss of a phosphate. Next is a ring formation by the enzyme dihydroorotase, producing dihydroorotate, and loss of water. Oxidation by dihydroorotate dehydrogenase gives orotate, with quinone being reduced. Orotate phosphoribosyltransferase adds a ribose monophosphate group in the β configuration to form orotidine-5'-monophosphate (OMP). The final step is a decarboxylation by orotidine-5'-monophosphate decarboxylase (ODCase) to produce uridine-5'-monophosphate (UMP).

Regulation of the *de novo* pyrimidine biosynthetic pathway in bacteria normally occurs at ATCase with activation by ATP and inhibition by CTP or UTP. In animals, regulation occurs by activation of carbamoyl phosphate synthase II with ATP or PRPP, and inhibition with UDP or UTP.

Once UMP has been synthesized, it is converted to UTP. From there, it is converted to dNTP by a ribonucleotide reductase. There are three classes of ribonucleotide reductases, dependent on the type of organism. Types I and II are found in prokaryotic organisms. All eukaryotic organisms utilize the type I ribonucleotide reductase. Type III is found in anaerobic organisms living in O_2 depleted environments. All three classes follow the same basic mechanism - a free radical replacement of OH at the 2' position by an H atom.

For incorporation into DNA, uracil nucleotides must be converted to dTTP (Figure 3).

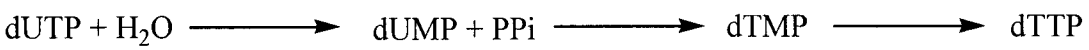


Figure 3. Conversion of deoxyuridine triphosphate to deoxythymidine triphosphate.

The first reaction is present in most organisms to diminish the concentration of dUTP in cells. This seemingly wasteful process occurs so that uracil is not incorporated into DNA. Methylation of dUMP to dTMP is catalyzed by thymidylate synthase, with N^5, N^{10} methylenetetrahydrofolate as a methylene donor (Figure 4, Appendix). The reaction starts with an enzymatic nucleophilic attack at C6 of dUMP, followed by C5 attack of the methylene group. Loss of the proton at C5, followed by hydride transfer to the methylene group, displaces the enzymatic nucleophile, thereby concluding the reaction.

The pyrimidine metabolic pathway is normally unidirectional. Thymidine ribonucleotides and ribonucleosides cannot be reused in most organisms. However, a pathway discovered by Palmatier *et al.*, named the thymidine salvage pathway, which converts thymidine to uracil in a four step process.[3] The uracil can then be converted to UMP and reincorporated into the total pyrimidine pool. Surprisingly, Palmatier and co-workers found that, when ring-labeled thymidine was taken into the system, the label which normally is incorporation into only DNA, had also been incorporated in RNA.

The thymidine salvage pathway is found in only a few organisms, among them *N. crassa, Aspergillus nidulans,* and *Rhodotorula glutinis*. The pathway begins with an oxidation of thymidine to thymine ribonucleoside[4] (Figure 5, Appendix). Next, the cleavage of the ribonucleotide produces thymine and ribose. Oxidation of the thymine to give 5-carboxyuracil (iso-orotate, IOA) occurs next, followed by a decarboxylation at the 5 position to produce uracil.

Iso-orotate decarboxylase (IDCase) completes the conversion of thymidine to uracil in the unusual thymidine salvage pathway identified to date only in certain fungi.[5] This enzyme has been studied very little in the past, and therefore little is known about

it.[3,4] A possible mechanism for the enzymatic decarboxylation of iso-orotate has been offered by Smiley *et al.*[4] (Figure 6). The proposed mechanism involves an attack by a nucleophilic residue in the enzyme at the 6 position of the iso-orotate, followed by the loss of carboxylate, leaving the enolate of uracil bound to the active site residue. Finally, the enzyme is removed from the substrate, leaving uracil.

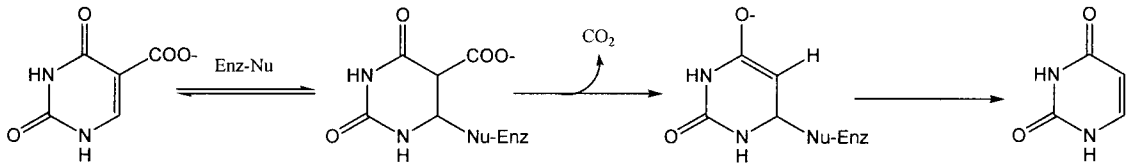


Figure 6. Proposed IDCase mechanism. Nucleophilic attack by the enzyme (Enz-Nu) initiates the reaction.

There are four main types of decarboxylation reactions in enzymatic mechanisms: α-keto acid, β-keto acid, amino acids, and oxidative decarboxylations.[6] α-Keto acid decarboxylation occurs in the presence of a metal ion. One example is pyruvate decarboxylase (Figure 7, Appendix). This reaction requires both thiamin pyrophosphate (TPP) and a divalent metal ion. Anion formation occurs by addition to C2 of pyruvate, forming a covalent adduct, then decarboxylation occurs, where the TPP is protonated. The keystone of this reaction is the heterocyclic compound acting as an electron sink, to provide intermediate stabilization.

As with α-keto acid decarboxylases, β-keto acid decarboxylation reactions require a metal ion for catalysis. However, in some cases, no other cofactors are needed. An α carboxyl and keto carbonyl are formed, with the metal stabilizing the enolate formed upon decarboxylation. Related to this reaction is oxidative decarboxylation. Two distinct steps occur in oxidative decarboxylation: oxidation to the ketone, followed by

the decarboxylation of the β CO_2. Schiff base dependent β-keto acid decarboxylations rely on nitrogen stabilization of the negative charge formed after loss of CO_2 (Figure 8, Appendix). The reaction requires no metals or other cofactors. It begins by covalent attachment of an amine group, then loss of water to form an imine. Loss of CO_2 occurs, then hydrolysis and loss of the amine to form the carbonyl product.

Pyridoxyl-5'-phosphate is used in the decarboxylation of amino acids. (Figure 9, Appendix). As with β-keto acid decarboxylations, the aldehyde group of PLP forms a Schiff base with the amino acid. The reaction begins with PLP tightly bound to the enzyme. After Schiff base formation between PLP and the substrate, loss of CO_2 and charge neutralization by the pyridinium nitrogen occurs to form a stabile quinonoid intermediate. Reprotonation occurs, and the amine product is released.

Though it is not one of the types of decarboxylases discussed above, a possible mechanism for IDCase resembles a β-keto acid decarboxylation. Several pyrimidine-metabolizing enzymes, such as thymidylate synthase, make this type of nucleophilic addition (Figure 10). Therefore, this provides a unique and interesting enzyme to study.

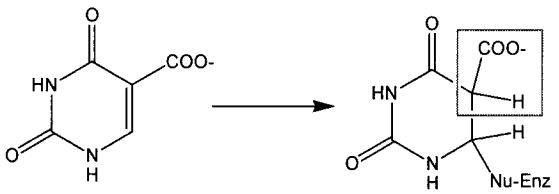


Figure 10. β-keto acid like mechanism of IDCase.

Abbott and co-workers published the first research of the IDCase enzyme.[3] They state that mammals are capable of producing IOA, but lack the capacity to decarboxylate it to form uracil. The presence of the thymidine salvage pathway was shown by [^{14}C]-

labeled thymidine being incorporated into RNA, but not DNA. This led them to believe that thymidine, normally a precursor of DNA thymine nucleotides, undergoes a demethylation to uracil, a precursor to RNA nucleotides. They also described the procedures they used to show that IDCase is present in the cells, and determined from the results that an enzyme or pathway which converts thymidine to RNA pyrimidines is present in *N. crassa.*

In research published by Smiley *et al.*, two different assaying methods are described to determine the amount of IDCase activity, as well as proposed a mechanism for the thymidine salvage pathway.[4] One technique discussed involves the use of radioactive labeled [*carboxy*-^{14}C]IOA to assay for IDCase activity. The other technique spectrophotometrically records the enzymatic conversion of 2-thioIOA to 2-thiouracil.

The final research to be mentioned, done by Brody and Westheimer, deals with orotidine-5'-monophosphate decarboxylase (ODCase).[7] Their interest resided in the isolation of pure, intact, and active enzyme. To achieve their goal, they used a custom affinity chromatography column to purify the ODCase enzyme. To construct a custom affinity chromatography column, a spacer arm terminated by an amino group was attached to 6-azaUMP, a known inhibitor of the ODCase enzyme. This amino group could then form an amide bond with carboxymethyl agarose, thus creating an agarose chromatography resin decorated with covalently bound 6-azaUMP. The total protein slurry collected from yeast cells was allowed to pass over the column, which bound only ODCase. All other proteins flowed through the column unhindered. Utilization of this method afforded the authors a 3200-fold purification after one pass over the column, and multiple columns yielded up to a 6700-fold purification of ODCase.

We will try an approach similar to that of Brody and Westheimer's for the purification of IDCase. 5-Nitrouracil (5NU) is known to inhibit IDCase[8], and may be a mechanism based inhibitor. We will attach 5NU to activated agarose via a spacer arm, and a protein slurry containing IDCase will be passed over the agarose chromatography resin in an effort to separate the IDCase from unwanted proteins.

Another way to purify IDCase is to isolate the gene responsible for encoding the enzyme. To effectively isolate the gene, a DNA library of an organism which contains IDCase will be transformed into an organism which does not contain the enzyme. Subsequent screening of the transformed cells on a limiting media plate may produced individual colonies that can be isolated and tested for insertion of the gene.

This screening process was earlier performed by Theisen, Kelln, and Neuhard.[9] Their goal was to isolate and characterize the *pyrF* gene, which encodes the ODCase of *Salmonella typhimurium*. By transforming an ODCase deficient bacterium with plasmid containing the *pyrF* gene and plating the transformed bacteria on a selective media, only those bacteria that contained the *pyrF* gene were able to grow. The *pyrF* gene in plasmid form to the enzyme deficient cells yielded a 15-20 fold increase in ODCase activity. Analysis of the plasmid harvested from the deficient bacteria grown on the selective media confirmed the plasmid contained the *pyrF* gene.

We intend to isolate the IDCase gene from a cDNA library derived from *R. glutinis*. The library will then be converted to the plasmid form, then transformed into SØ1259, a bacterial strain which is deficient in both IDCase (as are all *Escherchia coli*) and ODCase. Allowing the transformed cells to incubate on minimal media, with IOA as the sole pyrimidine source, only the bacteria which incorporate the plasmid containing

the gene responsible for IDCase should be able to grow. We will then be able to isolate and sequence the plasmid. We will also be able to use the plasmid to overexpress IDCase, so that the protein can be isolated and further studied.

Chapter II: Cell Growth, Protein Isolation and Assay for IDCase Activity

Introduction

For our research, IDCase was obtained from *R. glutinis* yeast cells. *Rhodotorula glutinis* has no telemorphic stage, has been studied little in the past, and many questions are still unanswered concerning the yeast. It is known, however, that it is a non-fermenting yeast, which is red-pink in color when grown at between 25-30°C. It has been previously isolated in a variety of conditions and areas worldwide.[10-12] Because thymine-7-hydroxylase has previously been isolated from *R. glutinis*, it was believed that it could also be used to isolate the IDCase enzyme.[13]

Two types of media were utilized in the growth of *R. glutinis*: YM, a rich medium, and a restrictive medium containing thymine. YM medium allows for the best growth of the *R. glutinis* cells. In the restrictive media, thymine is the only source of nitrogen, therefore presumably IDCase must be present for the cells to grow. The rich media was used to obtain viable cells, which were inoculated into the restrictive media, and the protein isolated from these cells.

Materials/Methods

Cell Growth

10mL of autoclaved YM media (Table 1) was inoculated with yeast freezer stock, and the inoculated media was shaken for 24 hours in a 28 °C incubator. A fresh, 200mL flask of autoclaved YM media was inoculated with the entire 10mL culture and

incubated, with shaking, for 24-36 hours at 28 °C. The yeast cells were removed from the YM media by centrifugation, and the resultant pellet was used to inoculate 1L thymine media (Table 2) and was shaken in a 28 °C incubator for 18-24 hours.

Components	Amount
Yeast Extract (Amresco)	3.0g
Malt Extract (BD)	3.0g
Peptone (Amresco)	5.0g
Glucose (Amresco)	10g
Agar, Bacteriological (if necessary) (Amresco)	20g
Dissolve in 950mL dH_2O, adjust to pH 6.5, and dilute to 1L	1.0L

Table 1. YM Media.

Components	Amount
Yeast Carbon Base (Difco)	11.7g
Thymine (Sigma)	1.0g
Dissolve in 950mL dH_2O, adjust to pH 6.5, and dilute to 1L	1.0L

Table 2. Thymine Media.

Protein Isolation

Yeast cells were separated from media by centrifugation for 15 minutes at 10,000 rpm. The cell pellet was resuspended in a small volume (about 2.0mL per 1.0g wet cell mass) of lysis buffer (10% v/v glycerol, 50μM β-mercaptoethanol, 20μL pH 7.4 Tris buffer). Also added to the lysis buffer were protease inhibitors: phenylmethyl-sulfonylfluoride (PMSF, 0.4mM), leupeptin (1.2μM), and pepstatin-A (4μM). This

solution was transferred to a screwcapped vessel (50mL) containing 1.0mm glass beads (2.5cm, Biospec). The mixture was then blended using a Biospec Bead Beater six times for one-minute intervals, with two minutes in-between blendings to keep the cell lysate cool. The liquid was then removed from the glass beads, and centrifuged at 10,000 rpm to separate the cell material from the soluble proteins.

$AmSO_4$ Fractionation

50-70% $AmSO_4$, at 5% increments, was added to total soluble protein over a 10 minute interval (Table 3). Protein/$AmSO_4$ solution was then mixed by magnetic stirring at 4 °C for 30 minutes. The solution was then transferred to a 30mL glass centrifuge tube (Corex) and centrifuged at 12,000 rpm for 20 minutes at 4 °C. The supernatant was decanted and saved for later quantification by ^{14}C assay.

	Final Concentraction				
Initial Concentration	**50**	**55**	**60**	**65**	**70**
0	29.1	32.6	36.1	39.8	43.6
50					12.5
55					9.3
60					6.2
65					3.1

Table 3. $AmSO_4$ fractionation (in grams, per 100mL)

To the total soluble protein taken from broken cells, 50% $AmSO_4$ was added over a 10 minute interval, while mixing by magnetic stirring at 4 °C. The resultant mixture was stirred for 30 minutes, then centrifuged at 12,000 rpm for 20 minutes. The supernatant was collected, and $AmSO_4$ was added over 10 minutes, to bring the final

$AmSO_4$ concentration to 70%. Again, this was mixed for 30 minutes, then centrifuged at 12000 rpm for 20 minutes. The supernatant was decanted, and the pellet kept.

The pellet was dissolved in a minimal (about 1.0mL/g) volume of buffer, and pipetted into a selective membrane dialysis bag, and placed into about 1L buffer, slowly stirred at 4 °C, and dialyzed for about 8-12 hours.

^{14}C Assay

^{14}C assays were performed by adding [carboxy-^{14}C] IOA to the protein solution. If present, the IDCase removes the carboxyl group from the IOA, leaving $^{14}CO_2$ and uracil. In an assay vial, 25μL pH 7.4 Tris buffer, 50μL [carboxy-^{14}C] IOA (5000 cpm/assay), and 375μL dH_2O were combined. Wicks made of filter paper (Whatman) were moistened with 2M KOH to collect the CO_2 driven off by the reaction. To the assay vial, 50μL of protein solution was added, then the reaction capped with a rubber septum and basket containing the KOH dampened wick. The reaction was run for 5 minutes in a 30 °C shaking water bath, then quenched with 0.1mL 2M HCl. The quenched reaction was allowed to sit for 1 hour, allowing time for the wick to absorb any $^{14}CO_2$ released. The wicks were then removed and placed in a 70 °C oven for about twenty minutes, or until dry but not to the point of discoloration. Once dry, each wick was individually inserted into a pre-counted vial containing scintillation fluid (ScintiSafe Econo 1) and the amount of ^{14}C present was quantitated with a scintillation counter (Packard 1900CA Liquid Scintillation Analyzer).

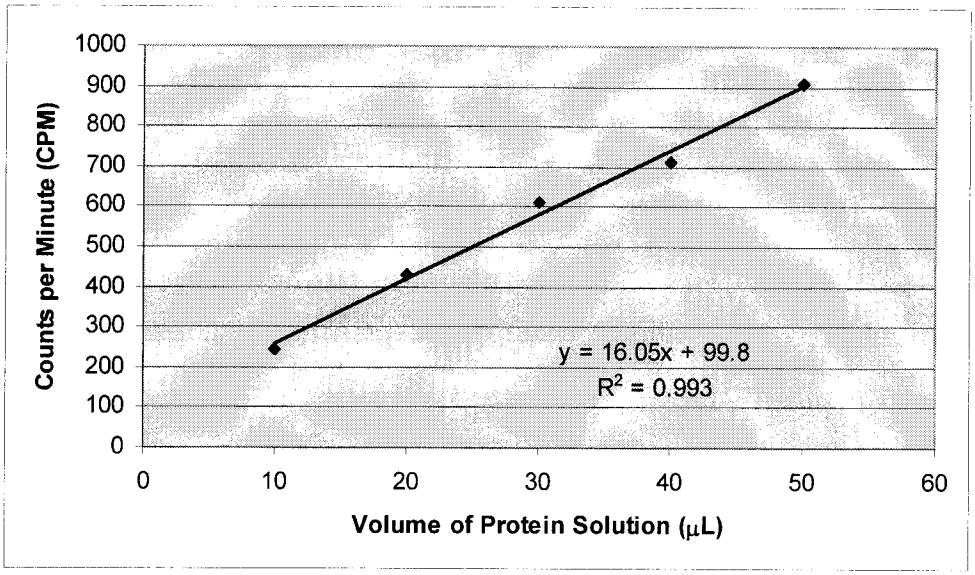
Bradford Assay

In duplicate, five different amounts of 0.5mg/mL BSA are added to 0.9mL of Bradford Assay Reagent (Pierce), and diluted to 1mL with 0.15M NaCl. For the standard, amounts of 0, 5, 10, 15, and 20 μL of BSA were added.[14] An amount of sample protein (normally about 5μL) was added to 0.9mL Bradford Assay reagent, and diluted to 1mL with 0.15M NaCl. All samples were then measured for absorbance (HP 8452 diode array spectrophotometer, λ_{595}).

The intent is to use an appropriate amount of sample protein so that the absorbance is between the values from the standard. A graph of the μg protein vs. absorbance was plotted, and from this graph, μg sample protein can be determined. Specific activity of the protein can be calculated once μg protein and cpm of protein have been determined.

Results/Discussion

Protein solution isolated from broken cell material was assayed by ^{14}C assay (Graph 1). A linear relationship between volume of protein solution and cpm was seen, and all volumes were below 50% of total ^{14}C counts. Therefore, a volume of up to 50μL protein solution could be used to obtain a high amount of cpm without extending beyond the linear range of the assay.



Graph 1. Comparison of volume of protein solution vs. CPM.

Protein slurry was divided into 6 equal aliquots, containing 50, 55, 60, 65, and 70 percent $AmSO_4$ respectively. No $AmSO_4$ was added to the 6th aliquot. It was determined that IDCase from *R. glutinis* precipitates out of solution between 50 and 55 percent $AmSO_4$ (Table 4), and that only a minimal amount of IDCase protein was lost when a 50-70% ramp of $AmSO_4$ was utilized (Table 5a). It was also determined that a majority of activity is retained with either a 50-70% or 70% $AmSO_4$ pellet (Table 5b). Therefore, 50% $AmSO_4$ was added to total protein, with the supernatant kept, and the $AmSO_4$ concentration increased to 70%. The pellet could then be kept and dialyzed to remove the $AmSO_4$.

	cpm	% conversion
Direct Spot	4042	
Total Protein – No $AmSO_4$	2328	57.6
50% supernatant	1704	42.1
55% supernatant	282	7.0
60% supernatant	192	4.8
65% supernatant	76	1.9
70% supernatant	195	4.8
No Protein	70	1.7

Table 4. ^{14}C counts of supernatant from various percentages of $AmSO_4$ added to protein slurry containing IDCase.

a)

	cpm	% conversion
Direct Spot	1901	-
Total Protein – No $AmSO_4$	1936	100
50-70% supernatant	112	5.9
No Protein	52	-

b)

	cpm	% conversion
Direct Spot	3766	
50% pellet	762	20.2
50-70% pellet	1311	34.8
70% pellet	2660	70.6
No Protein	111	-

Table 5. a) ^{14}C counts of total protein and 50-70% $AmSO_4$, indicating that only 5.9% of IDCase is lost in $AmSO_4$ fractionation.
b) ^{14}C counts of pellets from various percentages of $AmSO_4$ added to protein slurry containing IDCase.

Chapter III: Inhibitor Synthesis

Introduction

Use of an inhibitor attached to a column to isolate a specific protein has been done previously,[7,8] and it is believed that 5-nitrouracil (5NU) can be used in an effort to isolate IDCase. Previous inhibition studies of N1 alkylated 5NU by Smiley (unpublished) showed little to no inhibition of IDCase. However, inhibition was retained when the N3 alkylated 5NU was used. Therefore, the protein still binds to the N3 linked 5NU. This product can be conjugated to an activated agarose column to produce the custom affinity resin for chromatography and separation of the IDCase.

Chains of 5 and 8 atoms in length will be attached to the 5NU. Similar molecules have been previously synthesized.[8] Analysis of products were obtained by thin layer chromatography (TLC), ^{1}H NMR (Varian Gemini 2000, 400MHz spectrometer), UV spectroscopy (HP 8452 diode array spectrophotometer), and mass spectrometry (Bruker DataAnalysis Esquire LC).

Methods/Materials

The following synthesis was originally described by Yun.[8] In a clean, dry, 50mL roundbottom flask, 0.5g 5-nitrouracil (3.2 mmol), 6.4mL 25% tetrabutylammonium hydroxide in methanol (6.4 mmol), and 5.0mL *N,N*-dimethylformamide (DMF) were mixed at room temperature, until all the 5-nitrouracil was dissolved. The resultant mixture was evaporated until a syrupy solution was obtained. This syrup was then redissolved in 458μL ethyl-4-bromobutyrate (3.2 mmol) and 5.0mL DMF. This solution

was mixed at room temperature for 24 hours, then quenched with 4.0mL glacial acetic acid. The product was extracted with 30mL water and 3x 20mL ethyl acetate. The organic layer was dried over $MgSO_4$, then evaporated to dryness. Starting material and unwanted side products were removed from the desired product by flash chromatography, using a 19:1 methylene chloride:methanol solvent system. Column fractions were checked by thin layer chromatography (TLC) using the same CH_2Cl_2:MeOH solvent system. Those fractions containing only the desired product were pooled and evaporated to dryness (Figure 11).

Product 1 (800mg, 2.95mmol) was dissolved in 2M KOH (5mL) in a clean, dry roundbottom flask, and gently heated at 65 °C for 4 hours. The resultant mixture was checked by TLC.

Product 1 (800mg, 2.95 mmol) and 3.0mL ethylenediamine were mixed in a clean, dry, roundbottom flask, and allowed to mix under reflux for four days. The resultant mixture was separated by flash chromatography, using a 4:1 isopropanol:ammonium hydroxide solvent system.

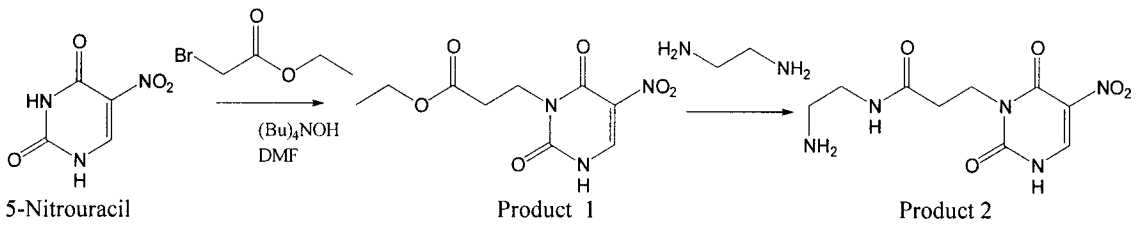


Figure 11. Synthesis of inhibitors, Product 1 and Product 2.

Analysis of products were done by 50μL injection into a HPLC on a 25 minute run at 4mL/min with a 100% H_2O to 100% of 80% sodium bicarbonate ramp. Analysis was also done by mass spectrometry using electrospray ionization (ESI) in both positive and negative modes. Finally, nuclear magnetic resonance (NMR, Bruker 400MHz) was utilized to check the products.

Results and Discussion

Product 1 was synthesized with a 26.4% yield. The HPLC chromatogram provided 3 peaks, minor peaks at 7.25 and 7.40 minutes, and a major peak at 10.09 min (Figure 12, Appendix). The successful conversion of Product 1 to the hydrolyzed form was complete, as shown by TLC (data not shown). The sample was considered clean enough to be used to synthesize Product 2, which was synthesized with a 35% yield. The HPLC of compound 2 after 5 days showed new peaks compared to the Product 1 chromatogram, including peaks at 4.04, 6.68, 7.69, 8.59 minutes (Figure 13, Appendix). Flash chromatography of the Product 2 mixture yielded two distinguishable spots when checked by TLC, each of which were isolated and checked by mass spectrometry. The spot with an R_f of 0.4 (data not shown) the base peak in positive mode was 251.30, with no peak present at the molecular weight of the desired product. In positive mode MS for the spot with R_f of 0.35, the base peak was 242.65, consistent with tetrabutylammonium hydroxide (Figure 14, Appendix). The next largest peak was at 286.25, which corresponds to the molecular weight of the desired product plus a proton. Negative mode showed a base peak of 284.20, consistent with the desired product minus a proton (Figure

15, Appendix). ^{1}H NMR of the sample produced peaks of: 1.77(m, C), 2.10(t, D), 3.23(t, F), 3.46(t, E), 3.73(t, B), and 8.79(s, A) (Figure 16, Appendix; and Figure 17).

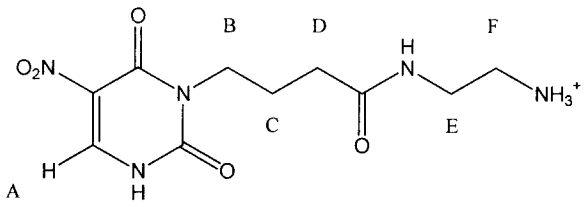


Figure 17. Product 2. Letters represent peaks of ^{1}H signals on NMR.

Chapter IV: Custom Affinity Column Chromatography

Introduction

One potential way to purify IDCase is the use of custom affinity chromatography. As stated previously, Brody and Westheimer noted a 3200 fold purification of ODCase after one pass over their column of agarose attached to the ODCase inhibitor, 6-azaUMP.[7]

For an affinity column to work, an active-site binding inhibitor is needed. This inhibitor must have a linker arm attached, one which will not interfere with binding to the protein. The linker arm must have an end which can selectively bind to the matrix. The matrix must be inert as not to interfere with the protein, nor interact with the column buffer. The matrix must also be porous, as to allow free flow of buffer and unwanted proteins.

Custom affinity chromatography is a selective technique to purify IDCase. Many factors can affect its effectiveness however. While it is possible to determine how many molecules of inhibitor are attached to the column, it is impossible to know how many molecules are attached to an agarose bead, or the position of the inhibitor in relation to another molecule of inhibitor or another agarose bead. If the linker arm is not long enough, the protein will not be able to bind. If the linker arms are bound too closely together, there will not be enough room for the protein to bind.

Product 2 was attached to an AminoLink agarose resin, and hydrolyzed Product 1 was attached to a diaminodipropylamine agarose resin, both using the protocol described by the manufacturer (Pierce).[15]

Materials/Methods

Product 2 Attachment

Amino-Link Coupling Gel (Pierce Chemical Co.) contains aldehyde groups, to which amino groups on ligands such as Product 2 can form Schiff Bases, and subsequently reduced with $NaCNBH_3$ to form stable secondary amines. Gel slurry was washed with 5 x 2mL water then 5 x 2mL conjugation buffer (0.1M MES, 0.9% NaCl, pH 4.7). Product 2 (20mg, 70mmol) was dissolved in 2mL conjugation buffer and OD at 282nm was determined. Buffer plus Product 2 mixture was added to gel and shaken. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 60mg) was dissolved in 0.5mL conjugation buffer, then added to the column gel. Mixture was gently shaken at room temperature for three hours. The solution was drained to gel bed top and the volume recorded. Slurry was transferred to a new tube and 1.0mL wash buffer (1.0M NaCl) was added and allowed to drain until the liquid was to the gel bed top. A polyethylene disk, supplied by Pierce, was inserted 1mm above bed, and solution drained. Column was transferred to fresh tube and 12 x 1mL wash buffer was added, drained, and collected. OD of fractions was determined by UV spectroscopy.

10mL of agarose resin was drained by vacuum filtration in a Büchner funnel, then washed 3x with 0.1M NaH_2PO_4 (pH 7.0). In a clean, dry screw-cap tube, the gel was mixed with an equivalent volume of inhibitor. In a hood, 0.5mL of 1M $NaCNBH_3$ per mL of slurry was mixed in. Solution was capped and mixed for about 2 hours by gentle side by side shaking. Solution was allowed to sit for four hours, then drained by vacuum filtration and washed twice with 1M Tris·HCl (pH 7.4). The resultant cake was returned

to the screw-cap tube and an washed twice with an equivalent volume of 1M Tris·HCl. 1M $NaCNBH_3$ (0.05mL of per mL of slurry) was added and mixed for 30 minutes at room temperature. The gel was drained by vacuum filtration and washed with 10 volumes of 1M NaCl, then 10 volumes of 0.05% NaN_3.

Hydrolyzed Product 1 Attachment

Hydrolyzed Product 1 was attached to the diaminodipropylamine agarose resin by activation with EDC. Agarose slurry (20mL) is placed in a 50mL syringe, drained, and washed 5x with 20mL (0.1M MES, 0.9% NaCl, pH 4.7). Hydrolyzed Product 1 (20mg) was dissolved in 2mL conjugation buffer and OD at 282nm was determined. Buffer plus hydrolyzed Product 1 mixture was added to gel and shaken. EDC (60mg) was dissolved in 0.5mL conjugation buffer, then added to the column gel. Mixture was gently shaken at room temperature overnight. The solution was drained to gel bed top and the volume recorded. Slurry was transferred to a new tube and 12 x 1.0mL wash buffer (1M NaCl) was added and allowed to drain until the liquid was to the gel bed top. OD of fractions was determined by UV spectroscopy.

Chromatography Method

The column was washed with buffer (10% glycerol, 50mM pH 7.4 Tris, 50μM β–ME) to ensure any salt, protein, or unattached inhibitor from previous use was removed. Crude protein, having been $AmSO_4$ precipitated and dialyzed, was placed on top of the column bed. Column buffer was run over the column at 4mL/min. Fractions were tested by 900μL Bradford reagent and 100μL fractionate. Once no color change

was visible, 50mL of 0.2M NaCl in buffer was run over the column, followed by equivalent volume washes of 0.4, 0.6, and 0.8M concentrations. All fractions were tested for IDCase activity by ^{14}C scintillation, and total protein amount was determined by Bradford Assay.

Results/Discussion

No increase in activity was seen with use of the column with Product 2 attached. However, a modest increase in specific activity was seen after using the custom affinity column with hydrolyzed Product 1. Use of UV spectroscopy determined that 440 μM of the hydrolyzed Product 1 inhibitor remained on the column. About 10g of *R. glutinis* cells were obtained from 1L of thymine media, and total protein was isolated as previously described. Table 4 shows the total and specific activities of crude (total) protein isolated from the *R. glutinis* cells, protein after $AmSO_4$ precipitation and dialysis, an early column fraction, and fraction after 0.8M NaCl was applied to column. A 5-fold increase in specific activity of the IDCase protein, with a 7.3% recovery of protein was determined after the one pass over the column.

Purification Step	Volume (mL)	Total Activity (nmol/min)	Total Protein (mg)	Specific Activity (nmol min^{-1} mg^{-1})	Percent Recovery %
1. Crude Protein	32.5	8391.6	171	49.1	100
2. 50-70% $AmSO_4$	7.5	4062.0	70.1	58.0	48.4
3. Fraction 8	7.5	2429.9	30.0	81.1	28.9
4. Fraction 20	7.5	613.38	2.53	242	7.31

Table 6. Stepwise table of purification of IDCase. 1) unaltered protein, 2) protein after precipitation and dialysis, 3) protein not binding to column, 4) protein after high salt elution.

As seen in Table 6, fraction 8 represents the amount of IDCase which passed through the column unhindered along with undesired proteins. For this reason, we are unable to determine the total amount of IDCase present compared to total protein in *R. glutinis*.

Chapter V: Non-Enzymatic Decarboxylation of Iso-Orotate

Introduction

To fully understand the enzymatic decarboxylation of IDCase, it is also important to understand the non-enzymatic decarboxylyation of iso-orotate to uracil. It is believed that the non-enzymatic decarboxylation is a pH dependent mechanism (Figure 18, Appendix). In acidic conditions, the carboxylate is protonated, therefore no reaction occurs. In neutral conditions there is possibly a slow reaction. The carboxylate is lost, followed by protonation at the C5 position. In basic conditions, nucleophilic attack by hydroxide on C6 could begin the reaction. This would give a C4-C5 enolate. Protonation at C5 would occur next, followed by loss of CO_2 and nucleophile. In very basic conditions, there would likely be no reaction due to the negative charges at N1 and the carboxylate.

To test the proposed mechanisms described above, a variety of experiments have been designed. Because the non-enzymatic rate of reaction is very slow, all reactions were run at either 100 °C or 130 °C. Rate of the IOA decarboxylation reaction was tested at neutral pH in a non-aqueous solvent to ensure absence of any nucleophile. Also in a non-aqueous solvent, the rate of a salt form of iso-orotate was tested. Finally, the rate of reaction for IOA was tested in the presence of nucleophile.

The extent of reaction was checked by ^{1}H NMR. A singlet at 7.8 ppm corresponds to the single hydrogen on C6, a doublet at 7.3 ppm corresponds to the single hydrogen on C5 of the product uracil, and is split by the coupling with the hydrogen on C6. By comparing the area of the IOA peak versus the area of the uracil peaks, a

percentage of conversion can be obtained. From there, the negative slope of the natural log of IOA concentration versus time in seconds gives the rate constant of a reaction.

Materials/Methods

Decarboxylation of Iso-Orotic Acid in DMSO

IOA (0.078g, 0.5mmol) was dissolved in 10mL DMSO. Into each of two pressure tubes (Ace Glass), 1-2mL solution were placed, and heated in an oil bath at 100°C for 24 and 48 hours, respectively. Another pressure tube containing 1-2mL solution was heated for 24 hours at 130 °C.

Decarboxylation of Tetrabutylammonium Salt of Iso-Orotate in DMSO

IOA (0.195g, 1.25mmol) was dissolved in 55% tetrabutylammonium hydroxide ($Bu_4Am^+OH^-$, 305μL, 1.25mmol) and 10mL dH_2O. The mixture was gently warmed to about 50 °C to dissolve all the IOA. Once a homogeneous solution was obtained, it was dried by evaporation and the resultant dry $Bu_4Am^+IO^-$ (0.1985g, 0.5mmol) was redissolved in 10mL DMSO. 1-2mL were placed into 2 tubes. One was heated for 24 hours at 100 °C, and the other for 72 hours at 130 °C, with samples pulled at various intervals up to 72 hours.

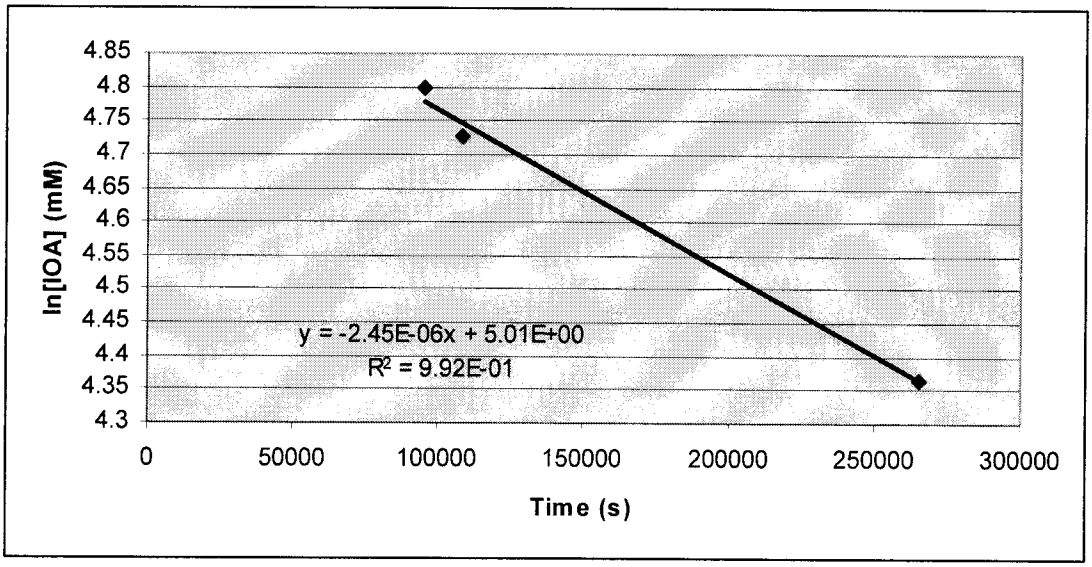
Decarboxylation of Iso-Orotate in Water with Sulfide as Potential Catalyst

IOA (0.078, 0.5mmol) and $Na_2S{\cdot}9H_2O$ (0.120g, 0.5mmol) were dissolved in 0.25M KH_2PO_4 (10mL, 1mmol), adjusted to pH 7.2, and diluted to 50mL. 1-2mL solution was placed in a pressure tube and heated 18 hours at 100 °C. IOA (0.078, 0.5mmol) and $Na_2S{\cdot}9H_2O$ (0.048g, 0.2mmol) were dissolved in 0.25M KH_2PO_4 (10mL,

1mmol), adjusted to pH 7.2, and diluted to 50mL. 1-2mL solution was placed in a pressure tube and heated 18 hours at 100 °C.

Results/Discussion

No decarboxylation of 50mM IOA in DMSO after heating at 100 °C or 130 °C was detected. There was also no decarboxylation detected of $Bu_4Am^+IO^-$ after heating at 100 °C. After 26.5 hours of heating at 130 °C, 15% of the 143mM $Bu_4Am^+IO^-$ in DMSO was converted to uracil (Figures 19, 20, Appendix). 30 hours of heating at 130°C yielded a 21% conversion to uracil (Figures 21, 22, Appendix), and 45% conversion was noted after 74 hours of heating (Figures 23, 24, Appendix). From this data, it was determined that the rate constant of 143mM $Bu_4Am^+IO^-$ in DMSO is $2.45x10^{-6}$ sec^{-1} (Graph 2). The reaction occurs, but is much slower than the reaction rate in water with OH^- as the proposed nucleophile (C. R. Smith - unpublished).



Graph 2. Rate of reaction for tetrabutylammonium salt of iso-orotate.

A conversion of 32% of IOA in the presence of 0.4mM Na_2S and dissolved in H_2O occurred in 20.75 hours at 100 °C (Figures 25,26, Appendix). Increasing the sodium sulfide concentration to 1mM, with a heating time of 19.75 hours at 100 °C, increased the conversion of IOA to 47% (Figures 25, 26, Appendix). In unpublished work by Smith, it was determined that a 50% conversion of 50mM IOA at pH 7.2 occurs after 18 hours of heating at 100 °C. It is possible that an unknown complex is being formed between the IOA and the SH^-, though further studies will need to be performed to determine what is happening. Using another sulfide, such as $NaSCH_3$, could provide more information into how a nucleophile affects rate of reaction.

Chapter VI: Library Screening

Introduction

Another way to isolate IDCase is to clone the gene responsible for encoding the enzyme. It is possible to effectively isolate the responsible gene by a technique known as gene screening. By transforming a gene library of an organism which contains IDCase into an organism which does not contain the enzyme, and plating the transformed cells onto a restrictive medium, only those cells which acquire the plasmid containing the desired gene will grow.

A good example of the screening process was performed by Theisen, Kelln, and Neuhard.[9] Their goal was to isolate and characterize the *pyrF* gene, which encodes the ODCase of *Salmonella typhimurium*. Transformation of an ODCase deficient *E. coli* with *S. typhimurium* plasmid library allowed for growth on restrictive media only of cells which acquired plasmid containing the *pyrF* gene. Analysis and characterization of the isolated plasmid confirmed that the plasmid contained the *pyrF* gene. Incorporation of the plasmid yielded a 15 – 20 fold increase in ODCase activity.

The cDNA library from *R. glutinis* was derived by Clontech, Inc. The cDNA library was then converted to plasmid form, then transformed into SØ1259, a bacterial strain which does not contain IDCase, and is deficient in ODCase. Allowing the transformed cells to grow on minimal media, with iso-orotate as the sole pyrimidine source, only the cells which incorporated plasmid containing the IDCase gene were able to grow. Isolation of this plasmid would allow for the gene sequence to be determined, and overexpression of IDCase would facilitate easier isolation of the protein.

Materials/Methods

R. glutinis was grown according to previously stated procedures. Wet cell mass was collected in 50mL screw-cap tubes (Falcon) by centrifugation at 5000rpm for 5min. Tubes containing cells were then frozen in dry ice. The cells were sent to BD Biosciences, Clontech Laboratories, Inc., who extracted the mRNA and converted it to λTriplEx cDNA library.

In accordance with the protocol from the manufacturer, a fresh, autoclaved 10mL culture tube of LB was inoculated with freezer stock of BM25.8 (Table 4), and incubated with shaking at 31 °C overnight.[16] A new 10mL LB culture was inoculated with 0.5mL of the overnight cells, and incubated with shaking at 31 °C for 3 hours. After this time, 1M $MgCl_2$ (100μL, 0.1mmol) was added to the culture. A 200μL aliquot was placed in a 15mL screw-cap tube (Falcon), and to it was added $1x10^5$ pfu of the unamplified phage library, and mixed by gentle pipetting. The mixture was incubated for 1 hour without shaking at 31 °C. LB (500μL) was added to the mixture, and incubated at 31 °C with shaking at 190rpm for 1 hour. The resultant mixture was equally divided and spread on five 150mm plates containing LB + ampicillin media. The plates were incubated at 37 °C overnight (no longer than 14 hours).

Each plate, covered with thousands of colonies, was washed with 10mL LB media to remove all the colonies. The LB was pooled and the plasmid isolated and purified (QIAGEN HiSpeed Plasmid Midi Kit). Presence of plasmid was determined by 0.8% agarose gel, and quantity was determined by UV/VIS (λ_{260}) spectroscopy.

Conversion of Unmethylated Plasmid Library to Methylated Plasmid Library by Passing Through *E. coli* Strain XL-1 Blue

A 5mL culture of LB was inoculated with XL-1 blue freezer stock (Table 4), and incubated at 37 °C overnight with shaking. From the overnight culture, 0.5mL cell solution was transferred to 50mL fresh LB media, and incubated at 37 °C for three hours with shaking. The cells were centrifuged for 15 minutes at 5000 rpm. The pellet was resuspended in 25mL cold 50mM $CaCl_2$ – 10mM Tris pH 8.0 solution. This mixture was centrifuged at 5000rpm for 15 minutes. The resultant pellet was then resuspended in 3.0mL of the $CaCl_2$-Tris solution, and kept on ice until use, but for no longer than 2 days.

Transformation

Plasmid library (1μg) was added to 200μL competent XL-1 blue cells in a 14mL capped roundbottom tube (Falcon), and heat pulsed at 42 °C for 2 minutes. LB (1.0mL) media was added to the cells, and incubated with shaking for 1 hour at 37 °C. Next, 100 μL aliquots were then spread on five 150mm plates containing LB + agar media, and incubated overnight at 37 °C. 10mL LB media was used to wash each plate of cells, and total washings were pooled and purified by plasmid midi prep.

Treatment to make competent and transformation of strain SØ1259 *E. coli* (Table 7) was performed according to previously stated procedures, where plasmid collected from XL-1 blue was used. Next, 100 μL aliquots were then spread on twenty 100mm restrictive media plates (Table 8), and incubated at 30 °C for 4-5 days. Colonies which appeared were picked, and separately inoculated in 5mL LB culture tubes. After an hour

of incubation at 37 °C, 50μg/mL ampicillin was added, and the culture was incubated overnight at 37 °C. Finally, 0.8mL of culture was mixed with 0.2mL glycerol, and stored at -70 °C for future use.

Plasmid mini-prep

1.5mL of grown culture was placed in a microfuge tube and centrifuged at maximum speed in an Eppendorf microcentrifuge for 30-60 seconds. The supernatant was decanted and the cell pellet was resuspended by pipetting in 200μL Solution A (50mM glucose, 25mM Tris, pH 8.0, 10mM EDTA). To this solution was added 300μL Solution B (0.2M NaOH, 1% sodium dodecyl sulfate), and inverted 4-6 times to mix. Then 300μL cold Solution C (3M potassium acetate, 2M acetic acid, adjusted to pH 4.8) was then added and let sit on ice for 5 minutes. The tubes were then centrifuged for 5 minutes at maximum speed. 750μL of the supernatant was transferred to a new tube, and 450μL of isopropanol was added, and held at room temperature for 5 minutes. The solution was then centrifuged for 5 minutes at maximum speed, and the supernatant removed. The pellet was resuspended with 50μL 1x TE. Presence of plasmid was tested by 0.8% agarose gel electrophoresis.

Plasmids collected were transformed into uninfected competent SØ1259, and spread on LB + ampicillin, restrictive media with iso-orotate, and restrictive media without iso-orotate. Plates were incubated for 4-5 days at 37 °C to determine presence of IDCase, ODCase, or neither.

IDCase Assays of *E. coli* Cultures with Suspected IDCase Gene

5mL LB + ampicillin liquid media was inoculated with 100μL of 5mL liquid culture from above, and incubated at 30 °C overnight. Restrictive media (4mL) minus iso-orotate was inoculated with 1mL overnight culture, and incubated for 3 hours at 30 °C. Cell growth (300μL) was added to 100μL fresh restrictive media minus iso-orotate and 100μL ^{14}C-IOA assay mix. Vials were capped with rubber septa holding KOH damp wicks. After 3 hours of shaking at 28 °C, 0.1mL 2M HCl was added to quench the reaction, and allowed to sit overnight. The wicks were dried and cpms determined with the scintillation counter.

Strain	**Genotype**	**Source**
XL-1 Blue[17]	*endA1, gyrA96, hsdR17, lac$^-$, recA1, relA1, supE44, thi-1, F` lacI^{q}Z ΔM15, proAB, Tn 10*	Clontech Laboratories, Inc
BM25.8[18]	*supE44, thi Δ(lac-proAB) [F` traD36, proAB$^+$, lacI^{q}Z ΔM15] λimm434 (kan^R)P1 (cam^R) hsdR (r_{k12}-m_{k12}-)*	Clontech Laboratories, Inc
SØ1259[9]	MC4100, *araD139, lacU169, rpsL, thi, pyrF*	K. F. Jensen

Table 7. Bacteriological strain information.

Components	Amount
10x M9 Salts (Table 9)	10.0mL
Agar (bacteriological)	1.5g
20% Glycerol	10.0mL
1M $MgSO_4$	100µL
0.1M $CaCl_2$	100µL
20mg/mL Thiamine	100µL
50mg/mL Ampicillin	100µL
40mg/mL Iso-orotate	100µL
Dissolved in 79mL dH_2O and diluted to 100mL	100.0mL

Table 8. Restrictive media.

Components	Amount
Na_2HPO_4	60.0g
KH_2PO_4	30.0g
NH_4Cl	10.0g
NaCl	5.0g
Dissolve in 950mL dH_2O and dilute to 1L	1.0L

Table 9. 10xM9 Salts.

Results/Discussion

After attempts to obtain *R. glutinis* cDNA library in the lab, it was determined that it would be more time and cost effective to send out for the construction of the cDNA library (data not shown). Conversion of the λTriplEx cDNA library to plasmid library proceeded successfully, with a final concentration of 0.083 µg/µL. Transformation into competent SØ1259 did not proceed successfully however. It was later realized that BM25.8 cells are methylation minus, while the SØ1259 cells are methylation plus.

Because the plasmids were not methylated, they could not be incorporated into the SØ1259 competent cells.

Transformation into competent XL-1 Blue cells occurred successfully, with a 0.2 μg/μL concentration. Then 0.7% agarose gel electrophoresis was performed to ensure that a variety of plasmids were produced, and a streak between 0.5-5 Kbp was visible (data not shown). Next, 5μL of methylated plasmid library was successfully transformed in competent SØ1259.

After 4 days of growth on restrictive medium at 30 °C, visible colonies were picked and restarted in 5mL LB media + ampicillin, and incubated at 37°C overnight. Of the 35 colonies pulled off restrictive plates, sixteen grew in the LB + ampicillin media. Freezer stock solutions with 20% glycerol were made with cultures that grew. Cells were cultured for plasmid mini preps, and the plasmid was run on a gel. Results showed bands indicative of both supercoiled and circular plasmid DNA, along with RNA fragments. For the representative plasmid for each colony which grew, 5μL of plasmid was transformed and plated on LB + ampicillin, restrictive media plus iso-orotate, and restrictive media minus iso-orotate. After a week, growth appeared on all LB + ampicillin plates. Very small colonies appeared on the restrictive media plus iso-orotate plates, and colonies also appeared on the restrictive media minus iso-orotate plates. From this screen, it is impossible to determine whether the cells incorporated the IDCase gene from plasmid, the ODCase gene from plasmid, or because the SØ1259 strain is ODCase deficient, but reverts back to ODCase active easily.

Scintillation counts showed no more than background counts on any of the cells. This does not mean that IDCase was not present in any of the cultures, but could mean

that the iso-orotate is not imported easily by the cells. Instead of adding the cells directly to the ^{14}C-IOA assay mix, perhaps the cells should be broken as described earlier, then assayed. This would be a more definitive determination of whether IDCase was present in any of the cultures.

Conclusion

^{14}C assaying of protein from *R. glutinis* cells is an effective way to determine the amount of active IDCase in a total protein solution. Synthesis and attachment of Product 2 to an agarose column was achieved effectively, and one pass through the custom affinity column afforded a five-fold purification. It is possible that by increasing the size of the column, a larger increase in purification could be attained. If hindrance between the protein and agarose is a problem, a longer linker arm to attach to the column could give the protein more room to bind the inhibitor.

When dissolved in a non-aqueous solvent, iso-orotic acid does not have a detectable non-enzymatic decarboxylation rate. The tetrabutylammonium salt form of iso-orotate in non-aqueous solvent has a reaction rate of 2.45×10^{-6} sec^{-1}, which is much slower than iso-orotate dissolved in H_2O. This data is consistent with the thought that two different non-enzymatic reaction mechanisms are present, with the nucleophilic reaction having a much higher reaction rate. The rate of reaction with addition of a sulfide, Na_2S, to iso-orotate dissolved in water did not change significantly. Use of a different sulfide might work as a better nucleophile.

Conversion of the *R. glutinis* cDNA library to plasmid library, then subsequent conversion to methylated plasmid library were both successful. Screening of SØ1259 cells transformed with methylated plasmid library on restrictive media produced colonies containing plasmid. However, no activity was shown with ^{14}C assays. It is possible that the SØ1259 cells could have reverted back to being ODCase active. Addition of an ODCase inhibitor, such as 5-fluoroorotate, could allow only cells which acquire the plasmid containing the gene responsible for IDCase to grow.

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Appendix

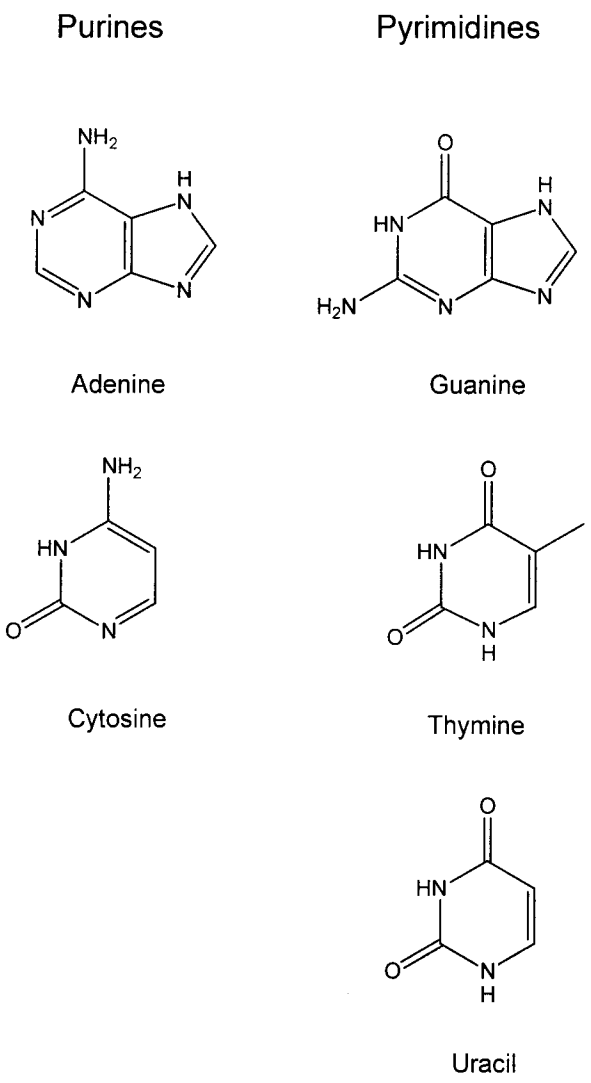

Figure 1. Common Nucleic Acids. Adenine pairs with guanine, cytosine with thymine in DNA, and cytosine with uracil in RNA.

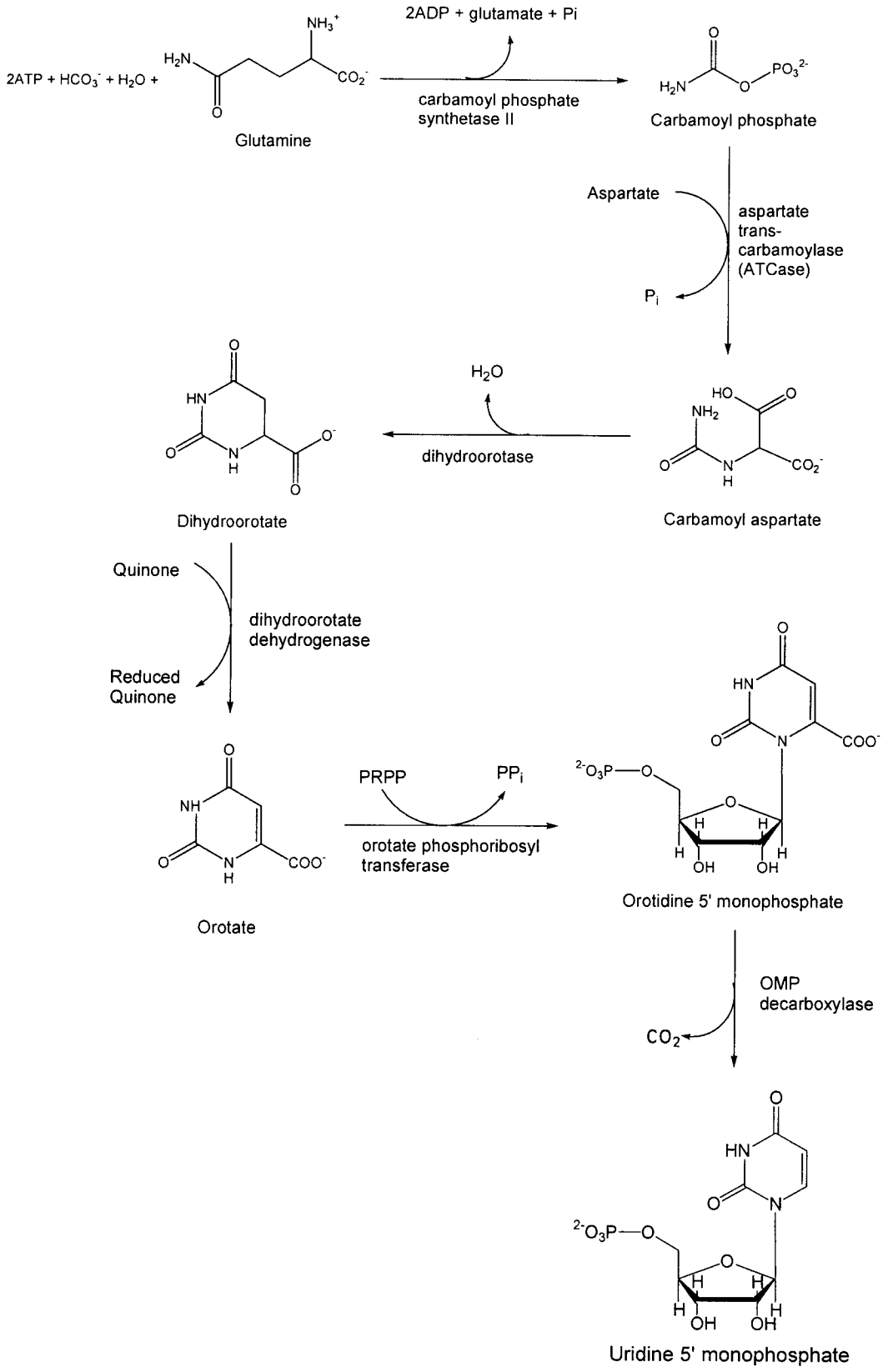

Figure 2. The *de novo* pyrimidine biosynthetic pathway.

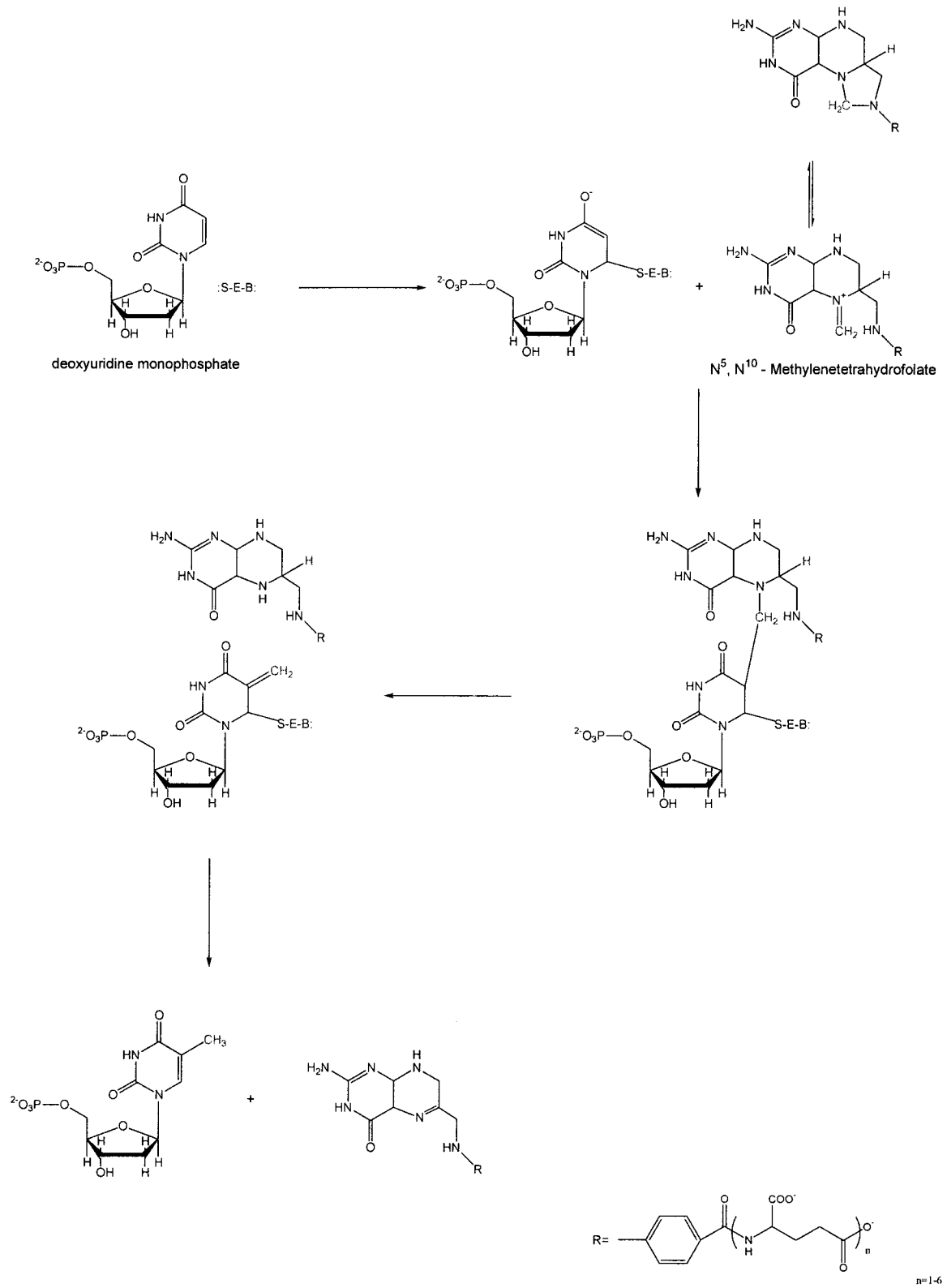

Figure 4. The thymidylate synthase pathway.

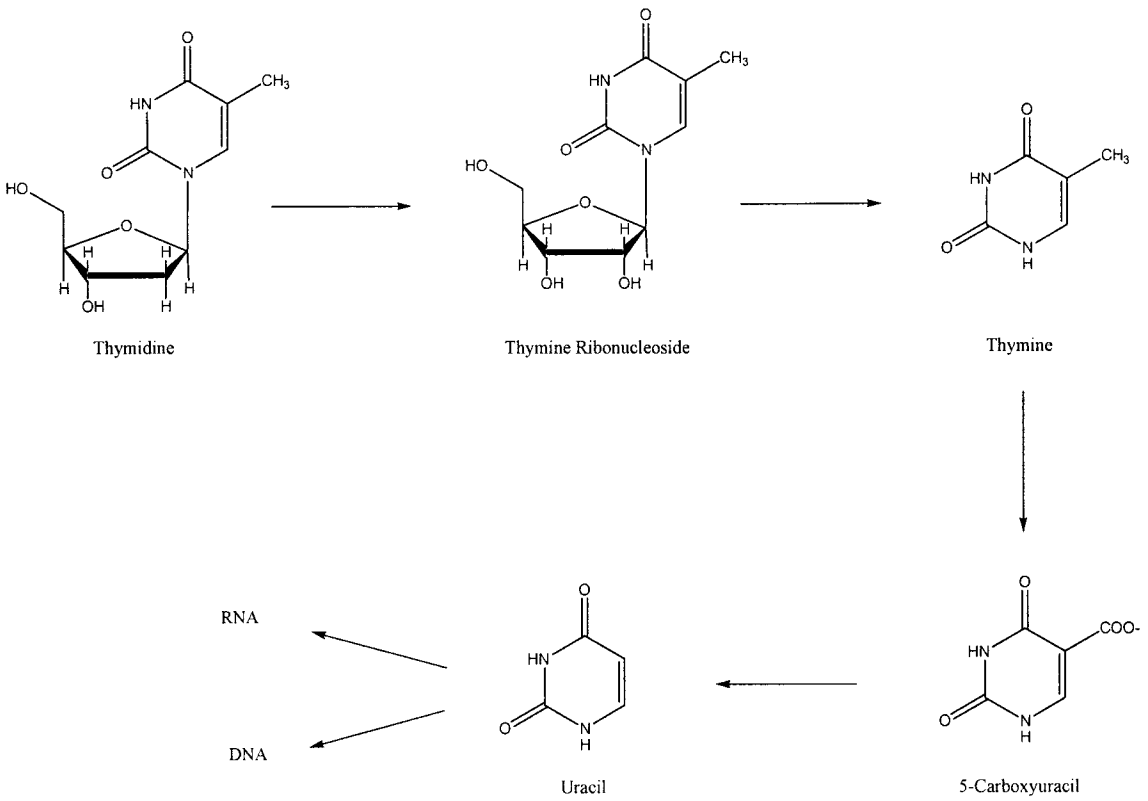

Figure 5. The thymine salvage pathway.

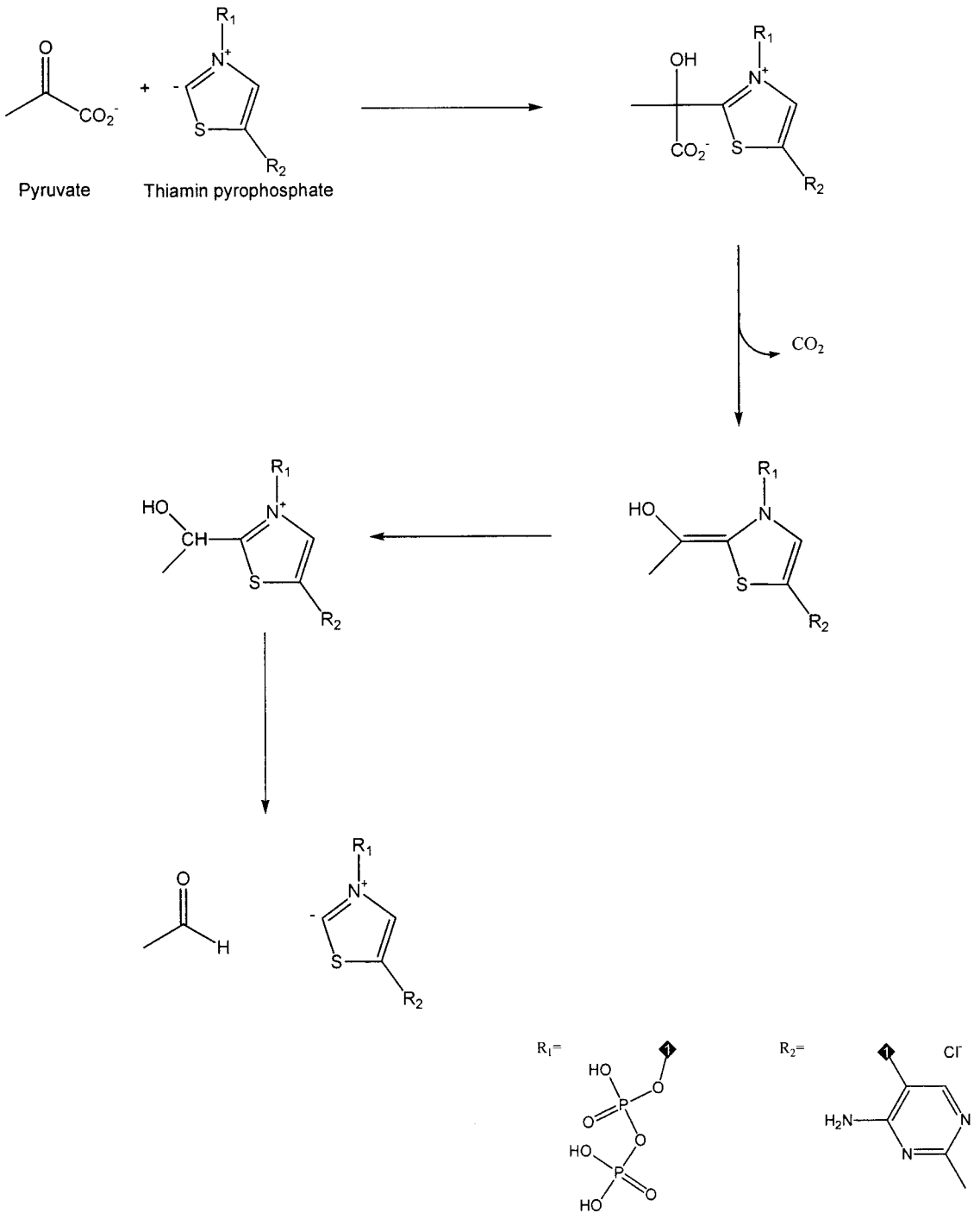

Figure 7. Pyruvate decarboxylase. An example of an α-keto acid decarboxylase mechanism.

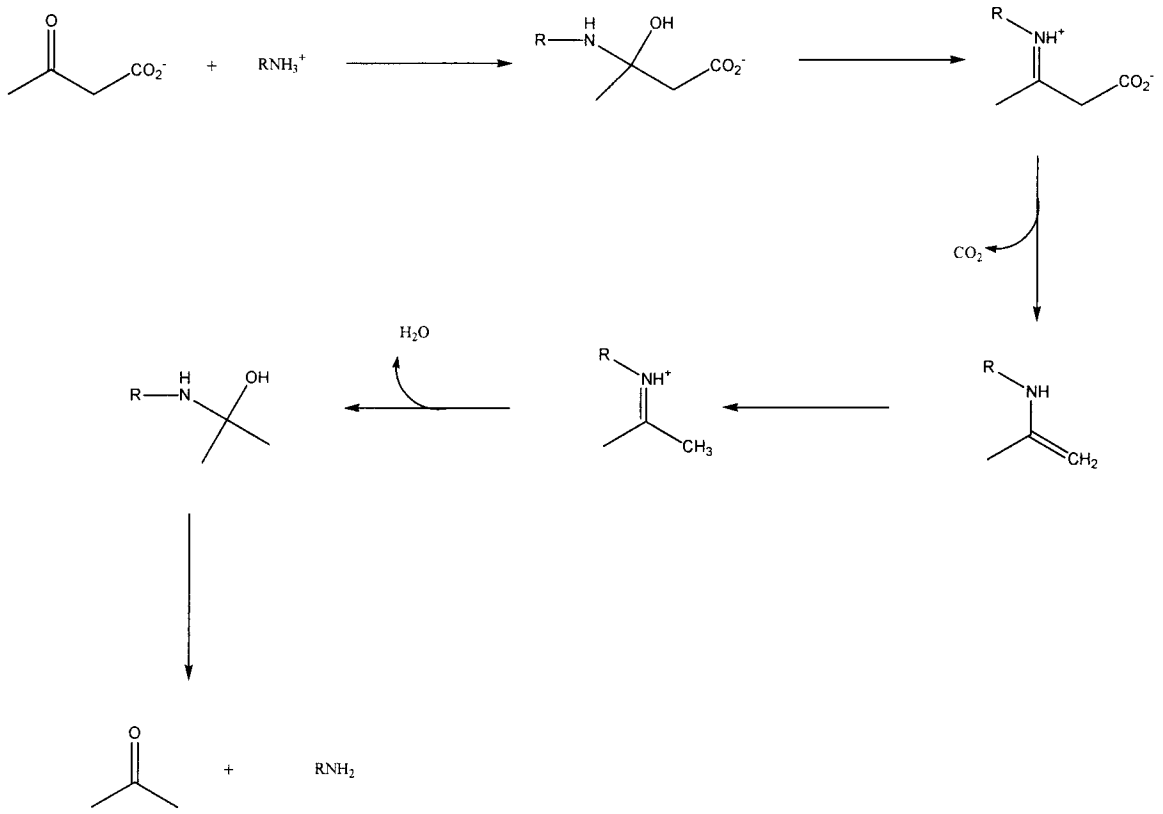

Figure 8. β-acid decarboxylase mechanism.

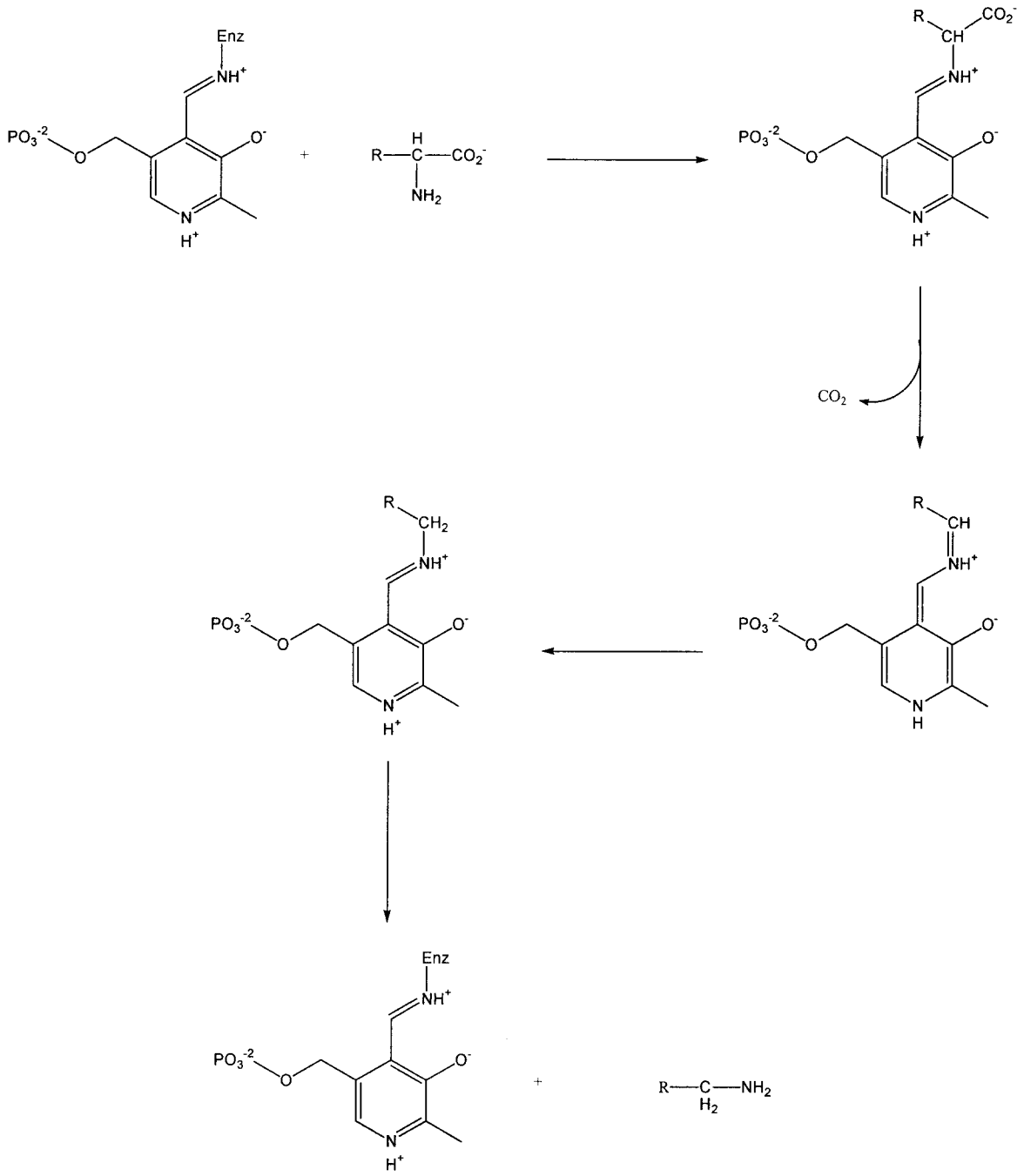

Figure 9. Amino acid decarboxylase mechanism.

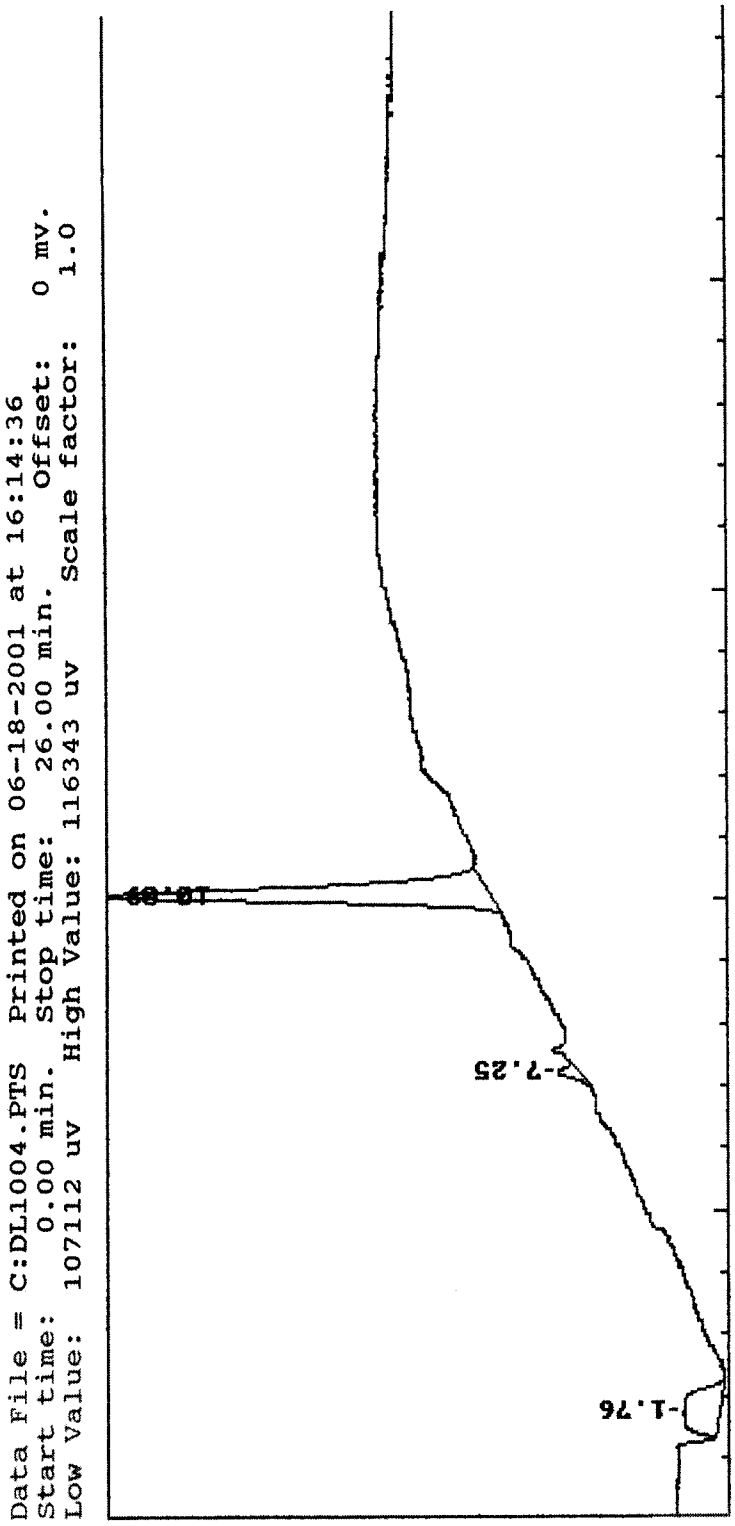

Figure 12. HPLC of Product 1.

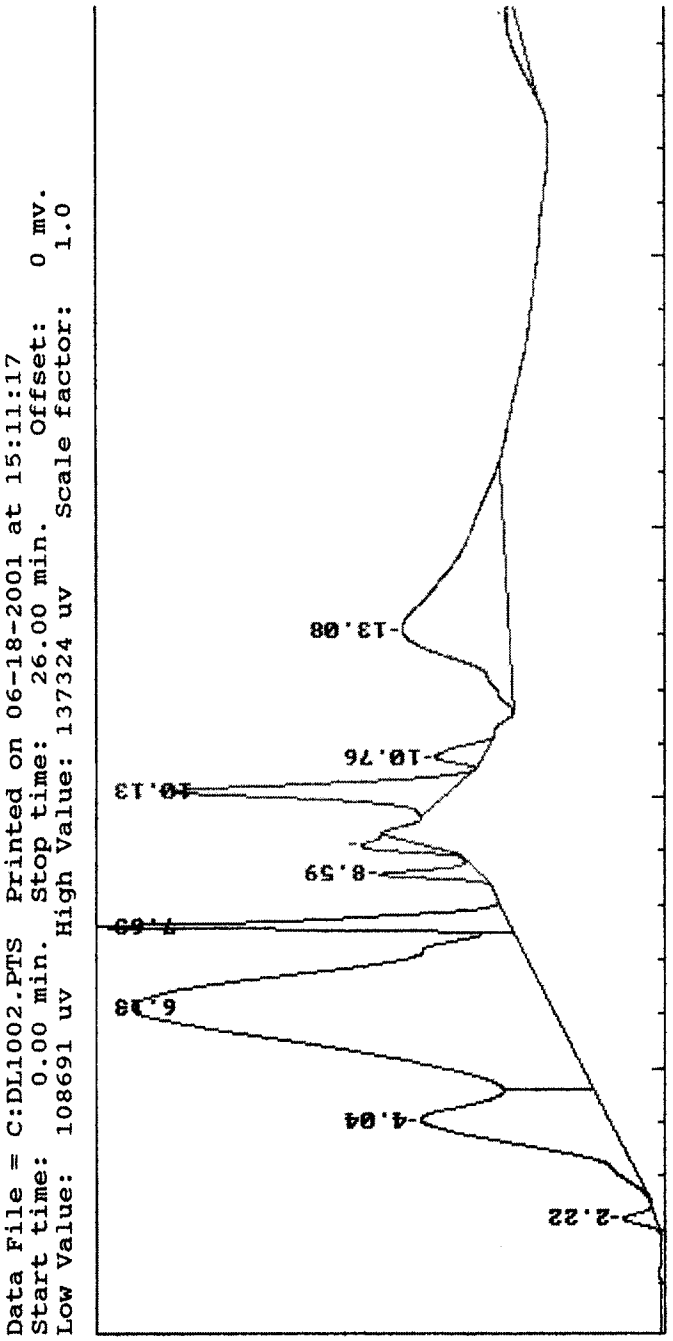

Figure 13. HPLC of unpurified Product 2.

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Instrument: EsquireLC_00135
Task :
Method :

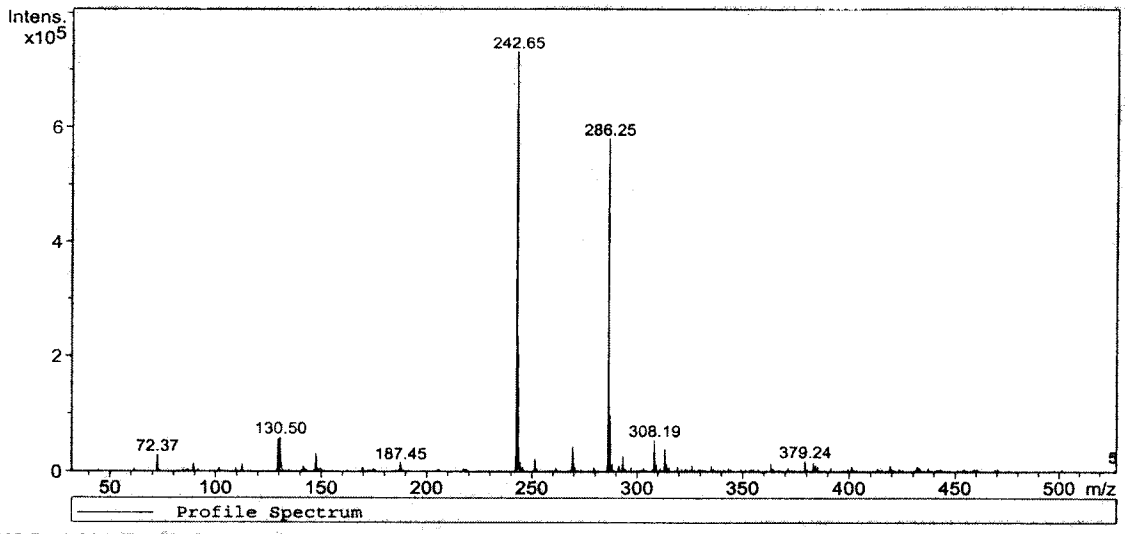
Printed: Tue Apr 15 17:11:04 2003
Operator : Administrator
Sample : Product 2

Acquisition Parameter:

Source : ESI
Mode : Std/Normal
CapExit : 92.7 Volt
Scan Range: 15.00 - 2200.00 m/z
Accum.time: 13242 µs
MS/MS :

Polarity : Positive
Skim 1 : 22.0 Volt
Trap Drive: 35
Summation : 10 Spectra

Profile Spectrum, No.: 1, Time: 0 min



MS Peak List (Profile Spectrum):

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89.47	13415	0.40	251.41	21916	0.40	314.23	11575	0.30
112.51	11878	0.50	269.33	44171	0.30	326.25	10208	0.30
129.58	57757	0.60	286.25	580532	0.30	363.33	13301	0.30
130.50	59366	0.50	287.06	98267	0.30	379.24	18639	0.30
147.52	30406	0.60	288.19	13051	0.30	383.28	14446	0.20
187.45	15500	0.30	293.24	26366	0.30	384.28	10583	0.40
242.65	732238	0.30	308.19	54840	0.40	536.11	10483	0.30
243.29	223509	0.30	309.20	11183	0.40	570.97	21084	0.90

End of report

Figure 14. Positive mode ESI MS of Product 2.

Profile MS Report

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Instrument: EsquireLC_00135
Task :
Method :

Printed: Tue Jul 31 14:23:11 2001
Operator Administrator
Sample : Product 2

Acquisition Parameter:

Source : ESI
Mode : Std/Normal
CapExit : -98.7 Volt
Scan Range: 15.00 - 2200.00 m/z
Accum.time: 20000 µs
MS/MS :

Polarity : Negative
Skim 1 : -28.0 Volt
Trap Drive: 45
Summation : 10 Spectra

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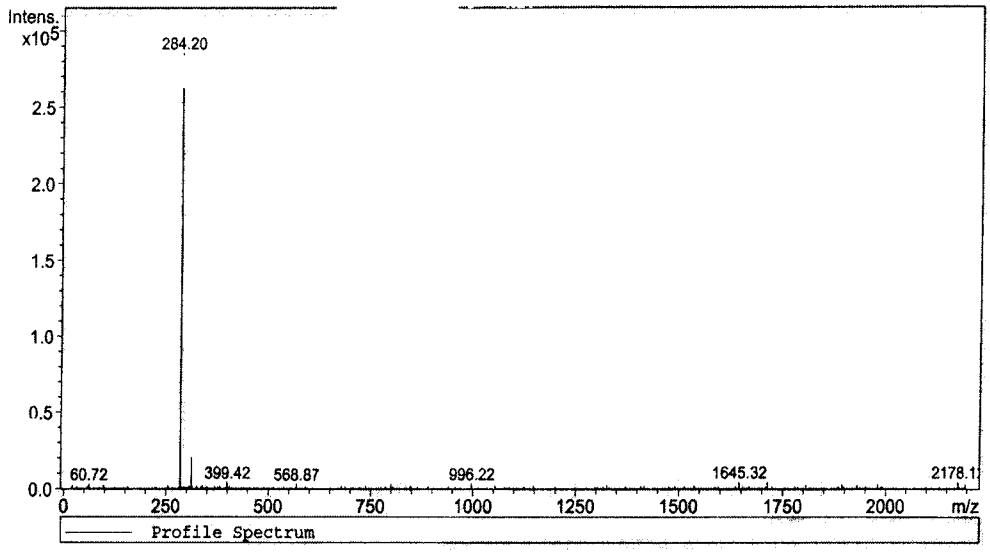

MS Peak List (Profile Spectrum):

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60.72	3295	0.20	799.26	3149	0.20	1806.62	2371	0.20
96.83	2326	0.20	848.06	2113	0.20	1893.24	2757	0.20
284.20	285760	0.20	996.22	3561	0.20	1930.12	2365	0.20
285.90	4819	0.40	1053.85	2229	0.20	1979.62	3102	0.20
312.09	20573	0.30	1326.72	2549	0.20	2178.12	4644	0.20
313.07	3542	0.30	1537.43	2119	0.20	2195.65	2893	0.30
399.42	4404	0.20	1645.32	4854	0.20			

End of report

Figure 15. Negative mode ESI MS of Product 2.

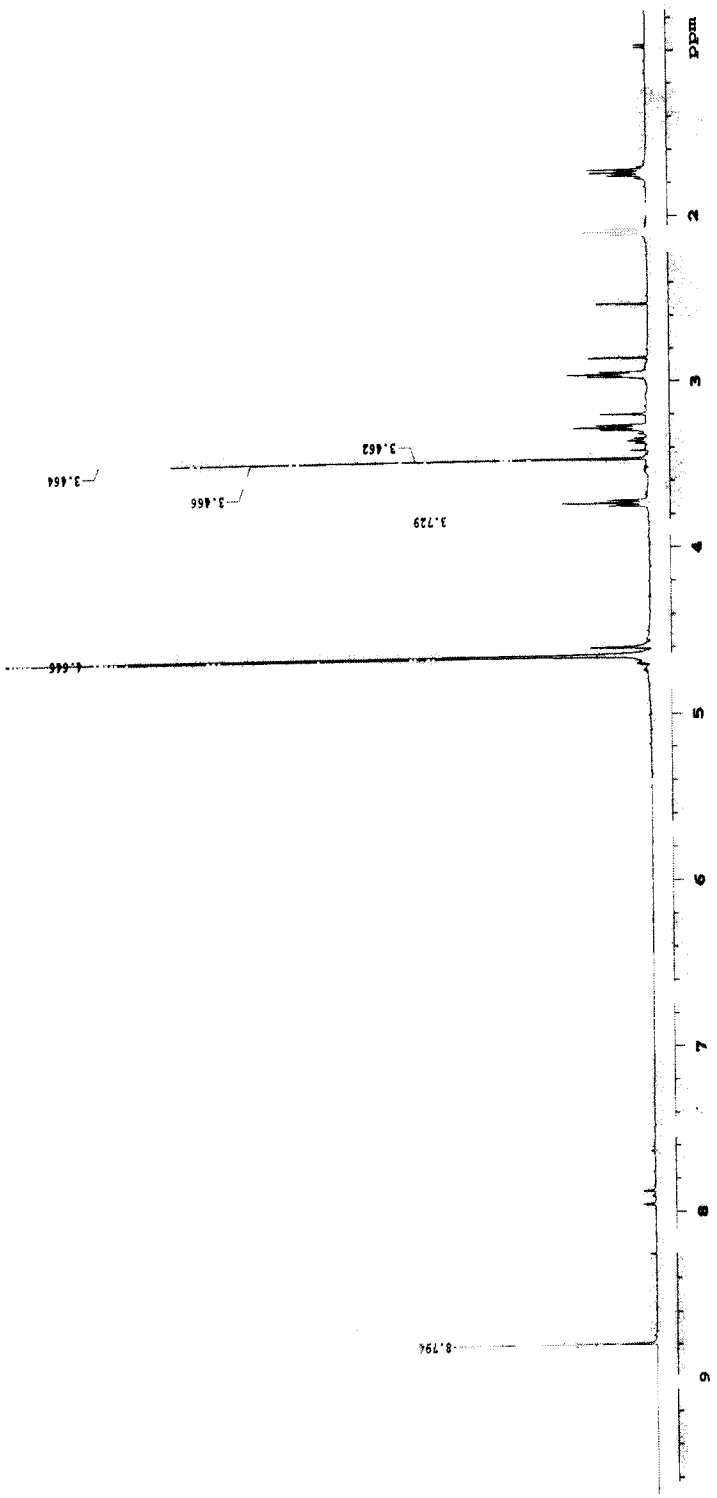

Figure 16. Product 2 ^{1}H NMR.

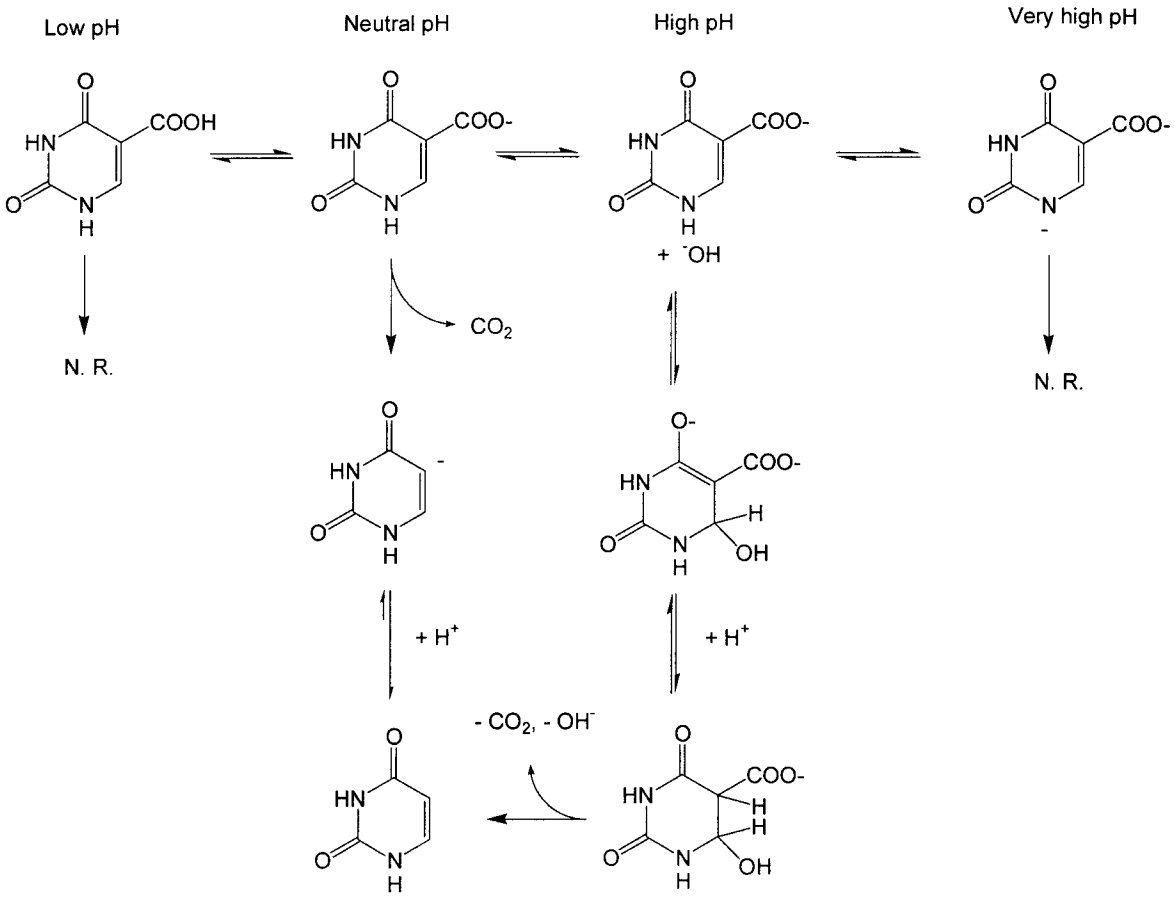

Figure 18. Non-enzymatic decarboxylation mechanism diagram of iso-orotate.

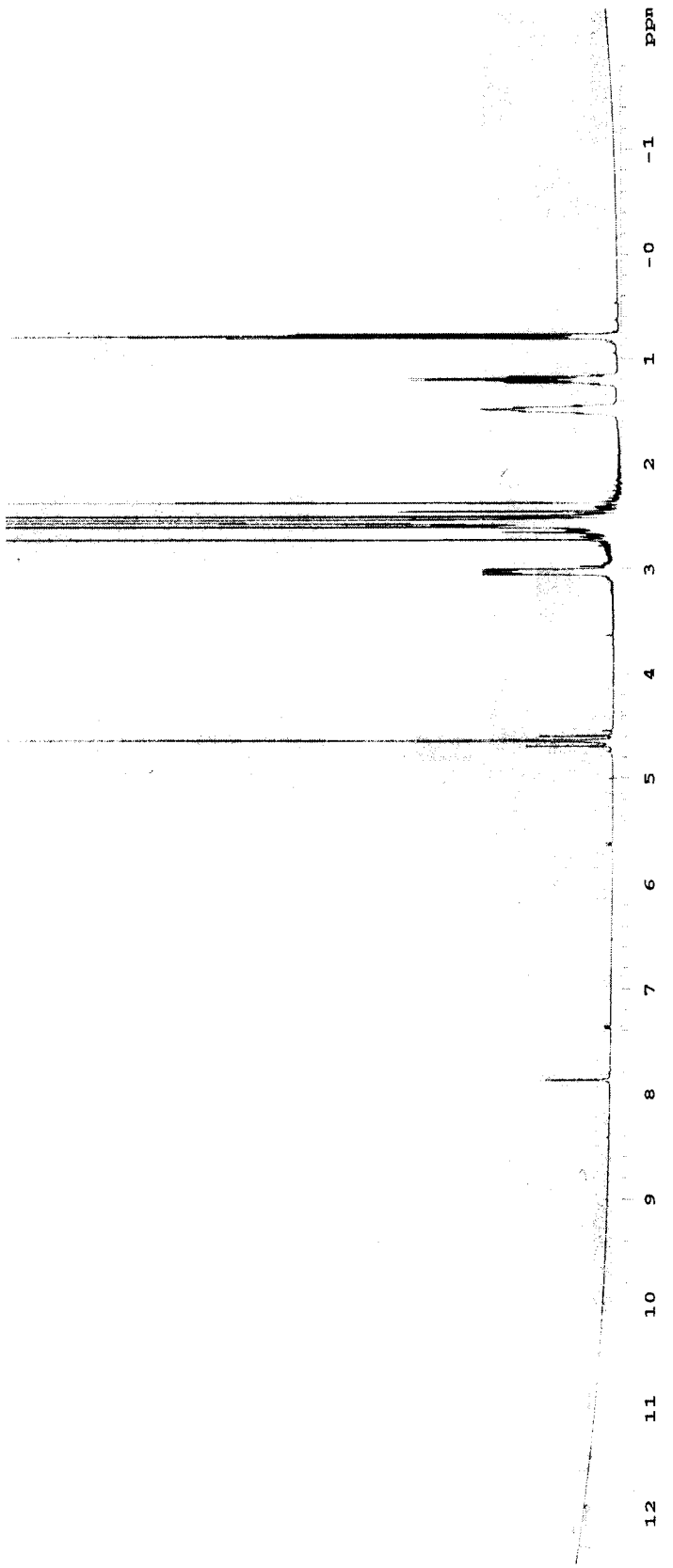

Figure 19. 143mM IOA in DMSO after 130°C heating for 24 hours.

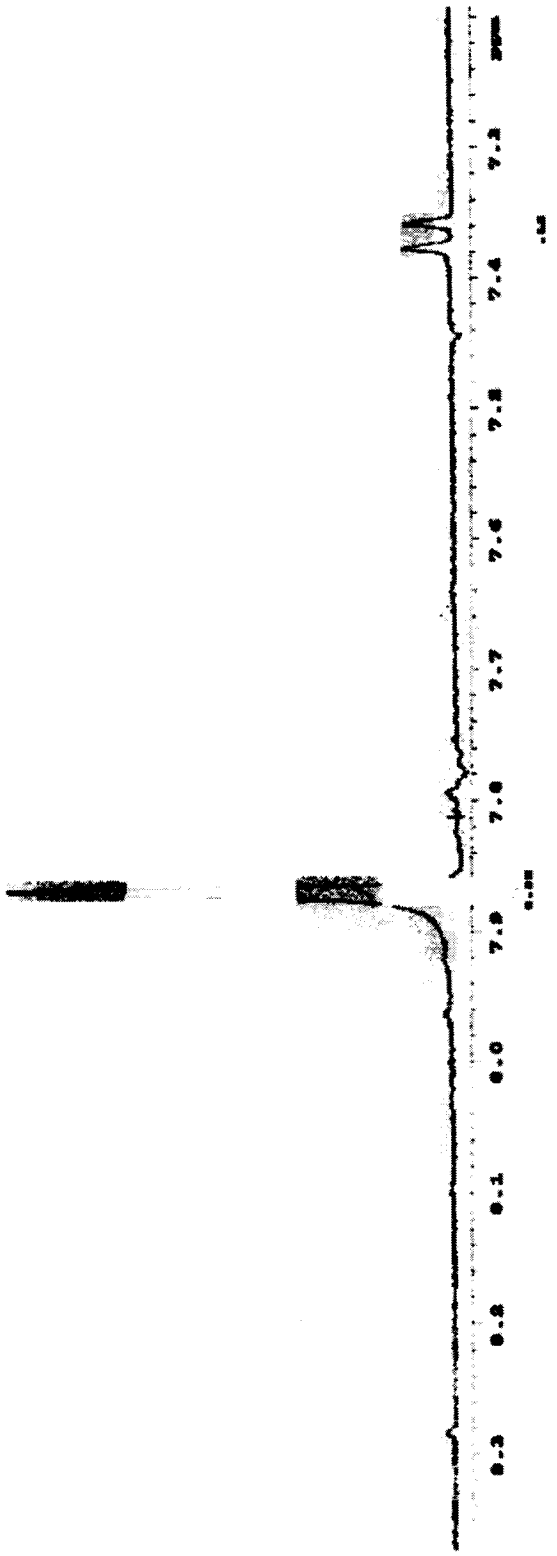

Figure 20. IOA and uracil peaks of 143mM IOA in DMSO after 130°C heating for 24 hours.

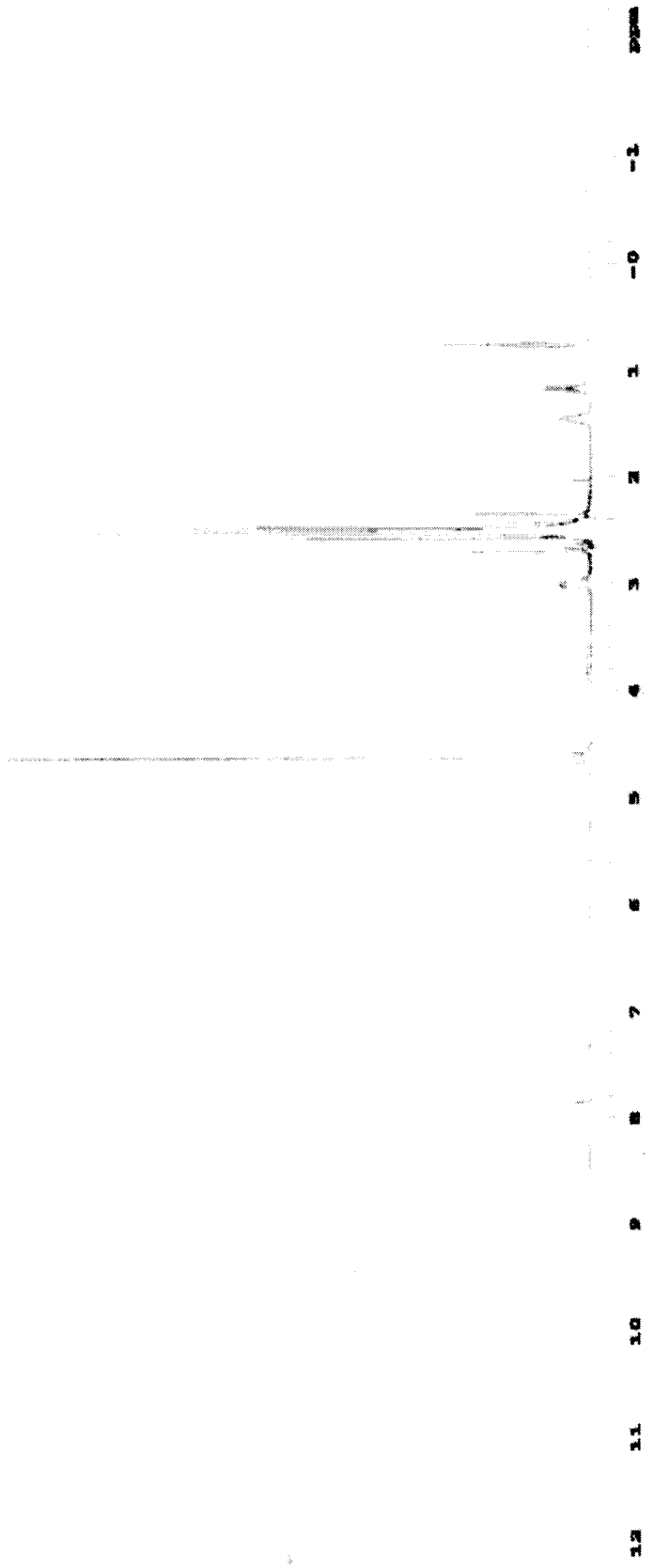

Figure 21. 143mM IOA in DMSO after 130°C heating for 30 hours.

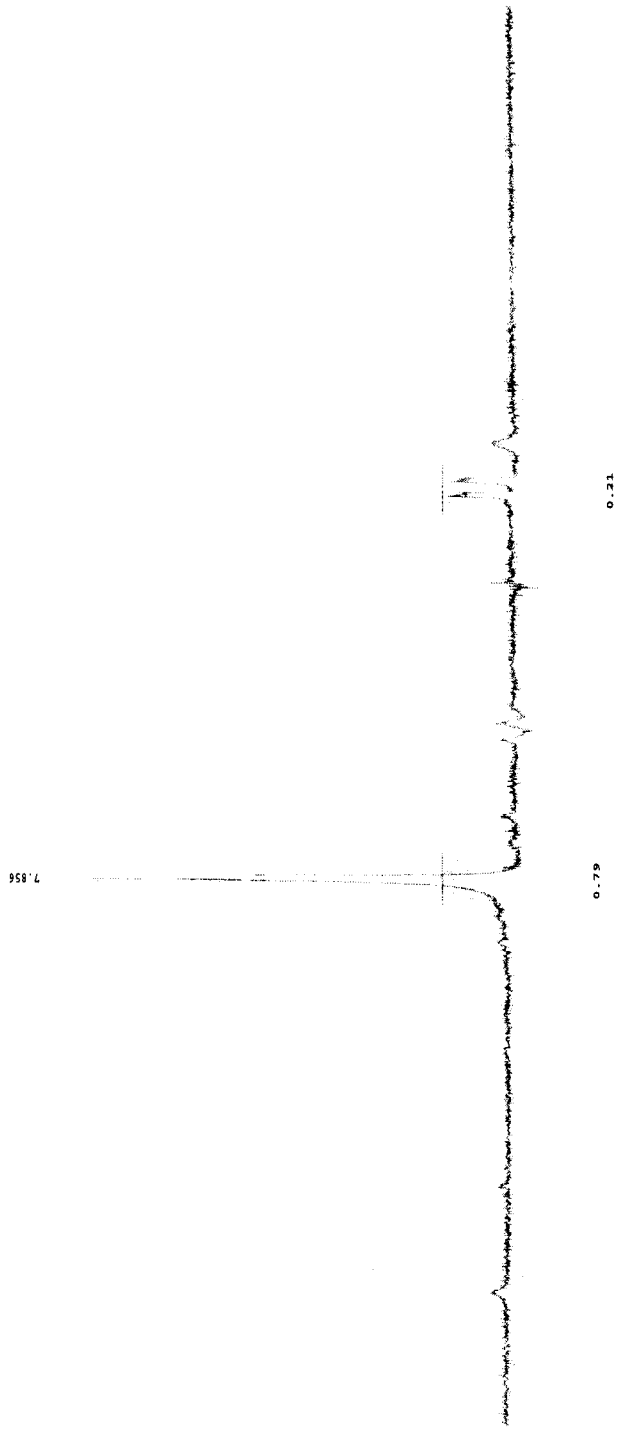

Figure 22. IOA and uracil peaks of 143mM IOA in DMSO after 130°C heating for 30 hours.

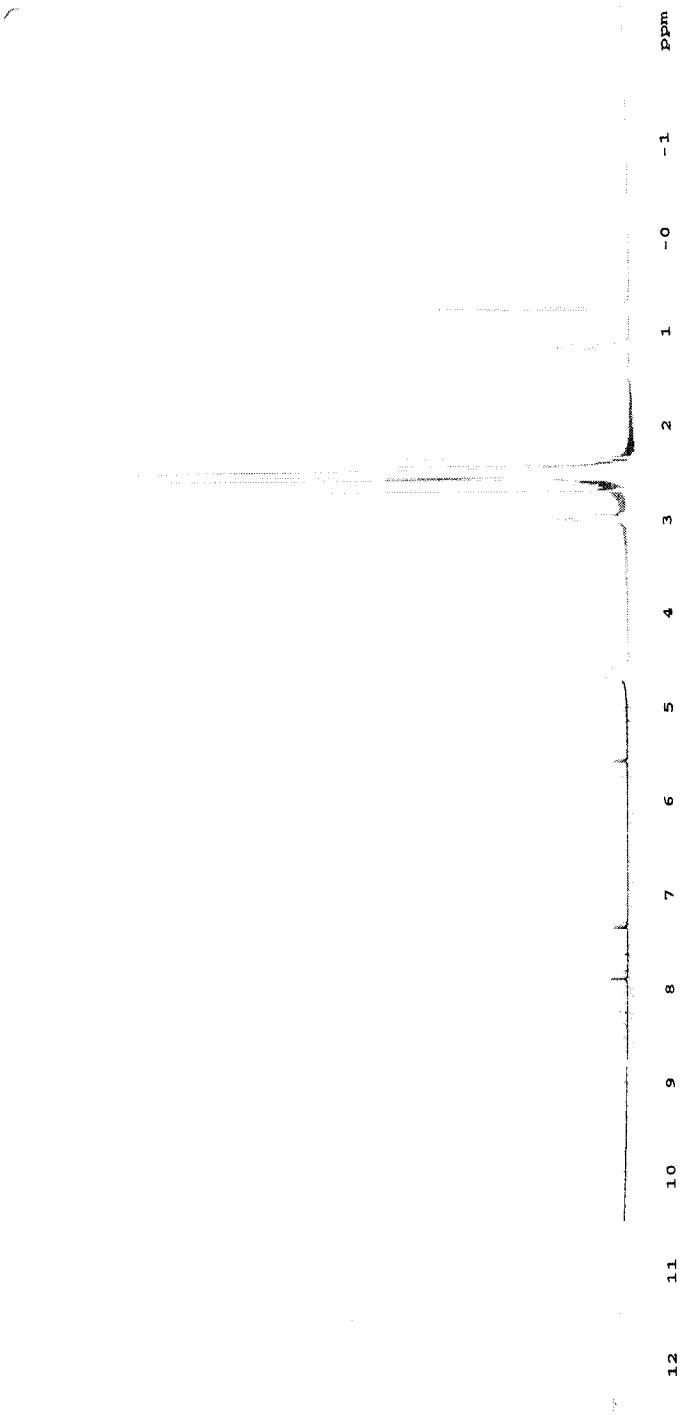

Figure 23. 143mM IOA in DMSO after 130°C heating for 74 hours.

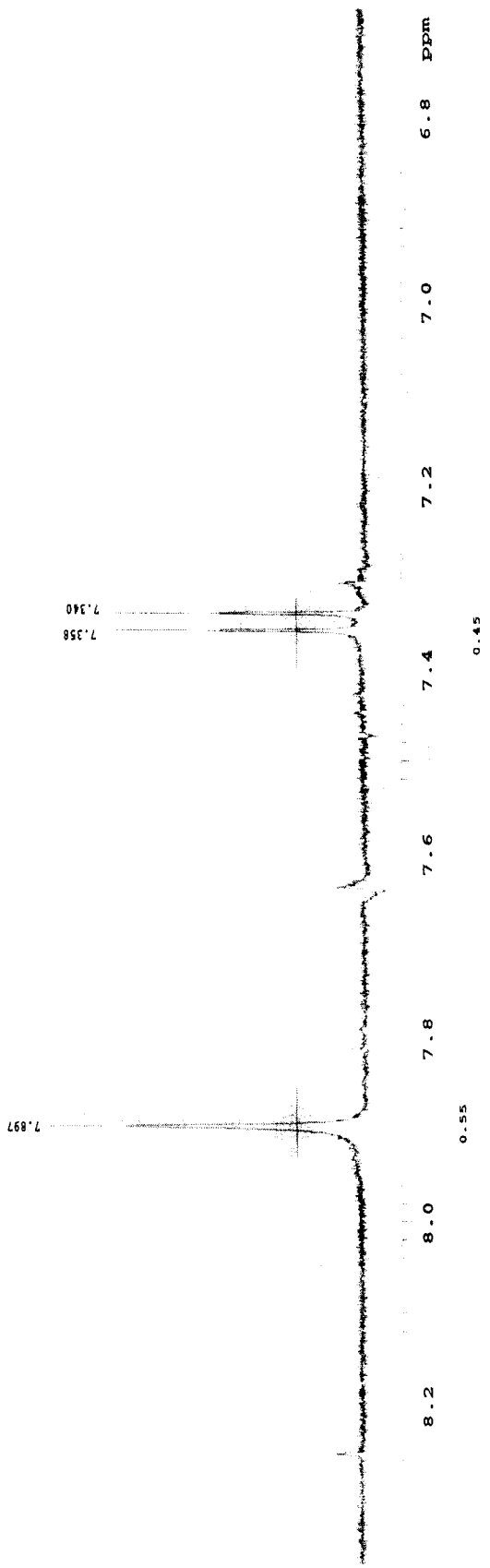

Figure 24. IOA and uracil peaks of 143mM IOA in DMSO after 130°C heating for 74 hours.

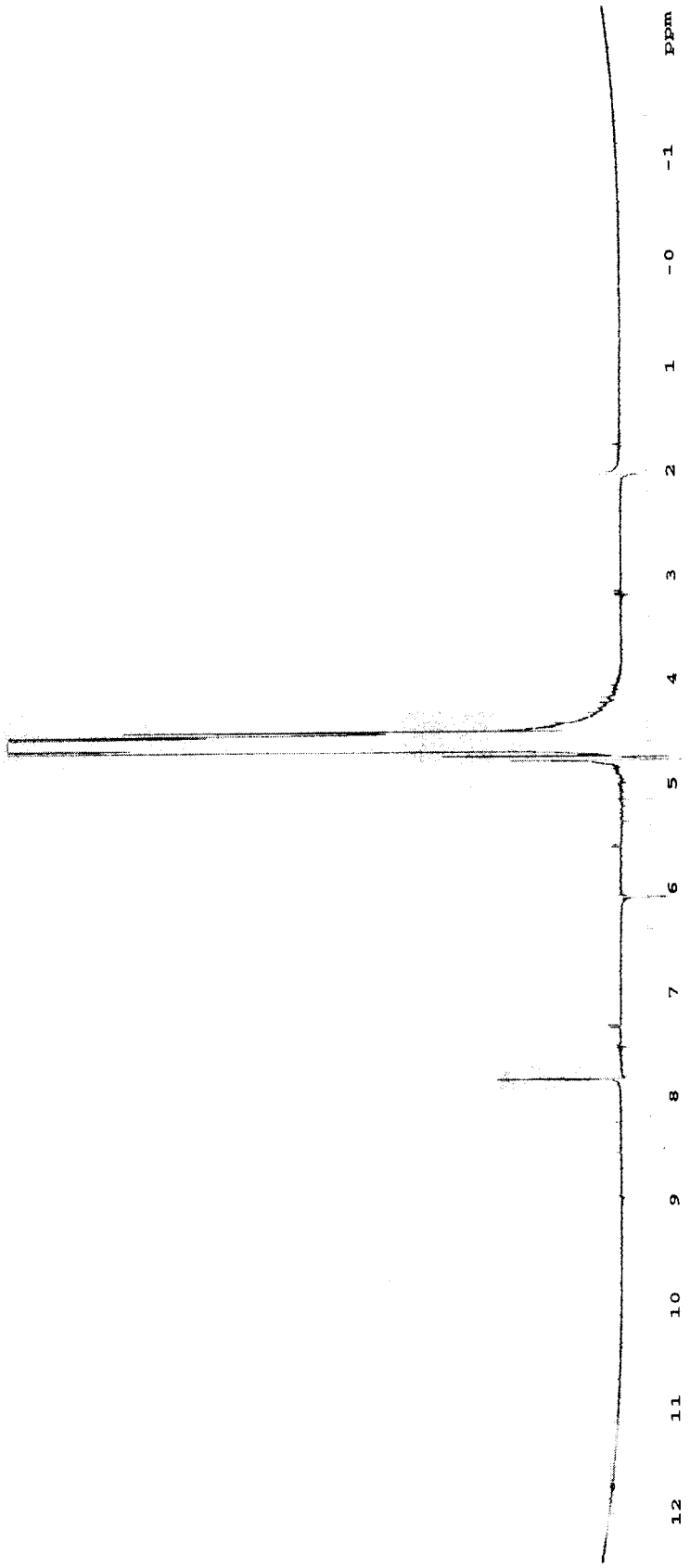

Figure 25. IOA in H_2O with 0.4mM Na_2S.

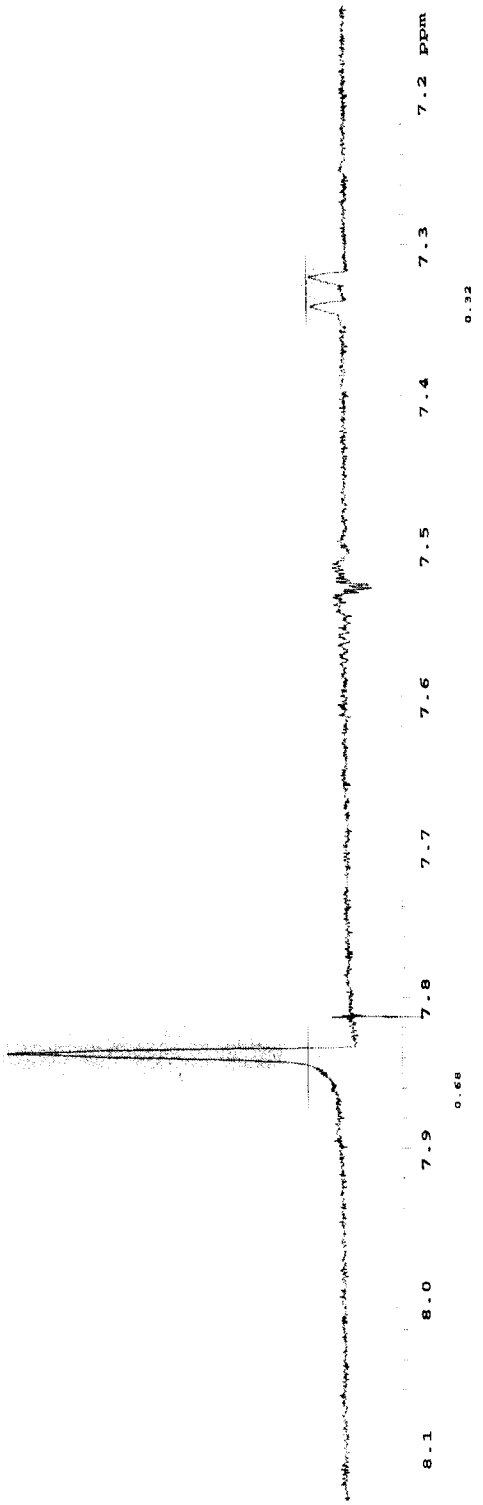

Figure 26. Uracil and IOA peaks of IOA in H_2O with 0.4mM Na_2S.

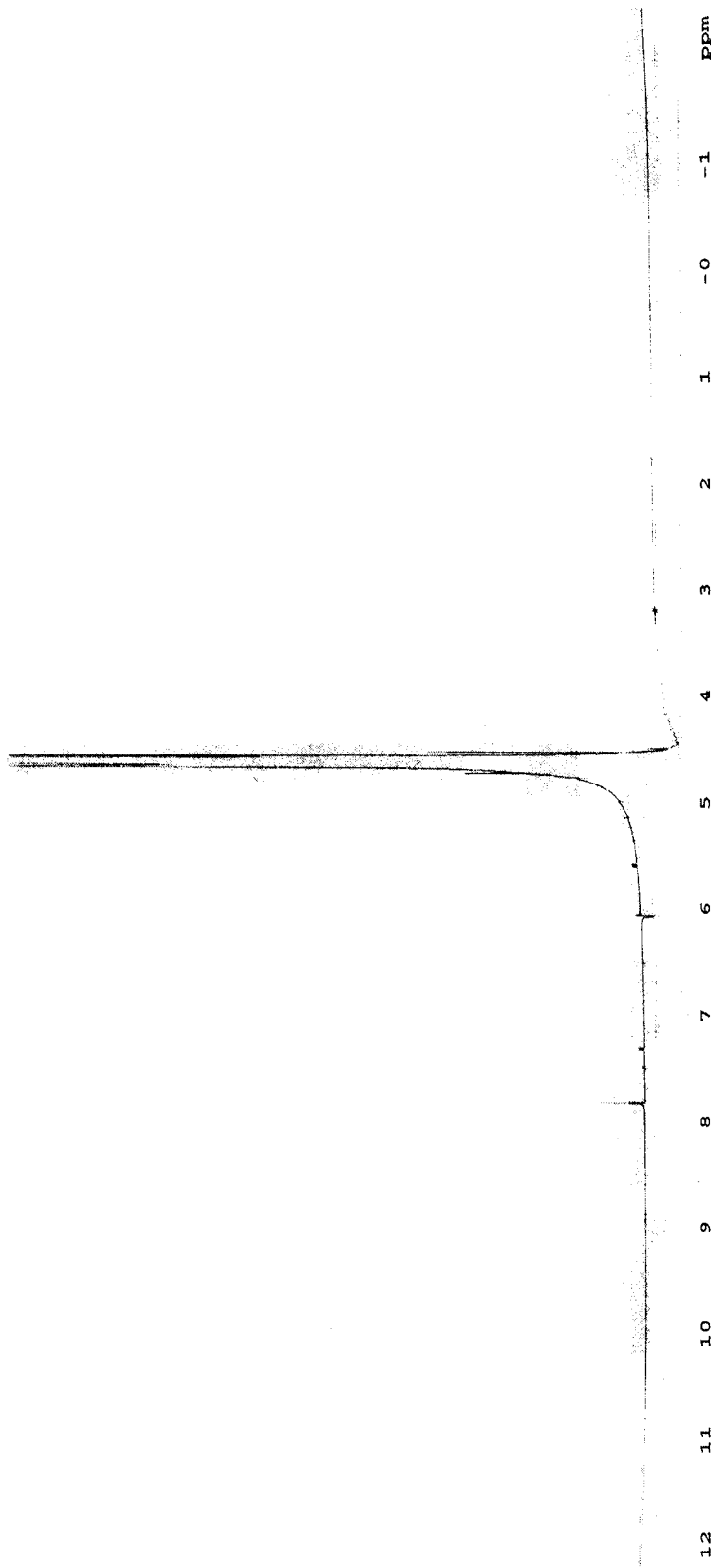

Figure 27. IOA in H_2O with 1.0mM Na_2S.

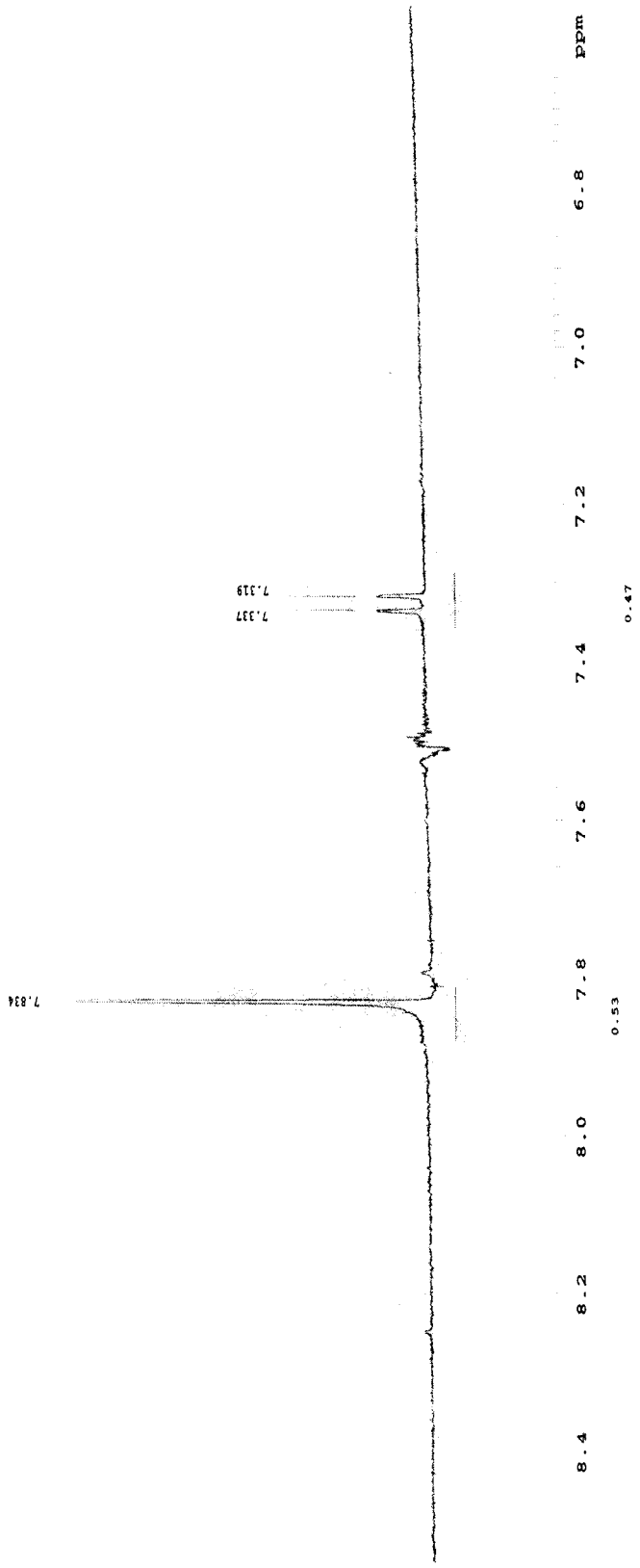

Figure 28. Uracil and IOA peaks of IOA in H_2O with 1.0mM Na_2S.