THE HYDROGENATION OF HISTIDINE METHYL ESTER

by

Walter T. Kurek

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Chemistry

Program

Warren Lubkin February 14, 1977

Advisor Date

Dean Land February 19, 1977

Dean of Graduate School Date

YOUNGSTOWN STATE UNIVERSITY

March, 1977

ABSTRACT

HYDROGENATION OF HISTIDINE METHYL ESTER

Walter T. Kurek

Master of Science

Youngstown State University, 1977

This investigation undertakes the synthesis of an N-acetylated imidazoline derivative of histidine methyl ester. The synthetic method involved hydrogenating a sample of histidine methyl ester using an acetic anhydride solvent and a platinum oxide catalyst. The reaction was carried out at room temperature and at a pressure of 60 psi. Four reaction products were found using thin layer chromatography developed with five per cent methanol in chloroform.

From previous work with the hydrogenation products of imidazole, three products were expected for the reduction of histidine methyl ester, namely, the unreduced N-acetylhistidine methyl ester, the partially reduced imidazoline derivative and the completely reduced imidazolidine derivative. The fourth product was suspected of being an alcohol or acid (COOH) imidazoline derivative.

A number of chromatographic methods were investigated for purifying the imidazoline derivative, the desired product. Silica gel G proved suitable for the separation

of N-acetylhistidine methyl ester, a major contaminant. Under the conditions used, two successive column runs were necessary to remove N-acetylhistidine methyl ester.

Hydrazinolysis of the residue obtained after removal of the substituted histidine ester resulted in a white solid having a melting point of 92-94°C. The hydrazide, while impure, appeared to contain as a major component the hydrazide derived from an N-acetylated imidazoline derivative of histidine methyl ester. This was supported by the fact that the hydrazide showed a strong UV absorbance at approximately 280 nm corresponding to the N,N-diacetylimidazoline ring.

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Marvin Lukin, first for his help and guidance over the past two years and secondly for an interesting and stimulating project. I would also like to thank all faculty members, especially Dr. Leonard Spiegel, for his suggestion to use silica gel G, and the graduate students for their assistance during the course of this work. Finally, I would like to thank my wife, Karen, for her time and patience in typing this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

Introduction

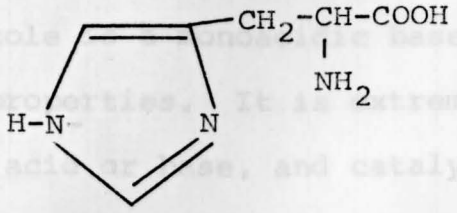
Scientists are now able to identify the amino acid sequence of biologically important polypeptides among them secretin, glucagon, cholecystokinin and angiotensin. By substitution of a single amino acid in biologically active peptides they have been able to create analogs of a number of peptides and as a result been able to identify the active groups. They have also been able to produce specific antagonists (compounds that have the same affinity for the receptor as the agonist have, but are devoid of intrinsic activity).[1]

The biological importance of histidine is well known. The amino acid histidine is essential in young children and has been found to be present at the active site of a number of enzymes like chymotrypsin, trypsin, and pancreatic ribonuclease. Histidine has also been found in a number of small peptides present in the body. In particular angiotensin II, an octapeptide, contains a histidine residue at position six. This peptide has been suspected of playing a major role in some forms of hypertension. Experimentation has reached the point where more than one hundred analogs of angiotensin have been prepared and results indicate that out of the total eight amino acids

present in the active polypeptide five (amino acids four through eight) are the most important for activity.[2] Few of the analogs prepared have had significant and prolonged inhibitory activity on angiotensin II[3] and scientists seem to have extensively covered a large number of possible replacements and sequence changes in the peptide. This then suggests another area of research. That involves the synthesis of new amino acids or modifications of naturally occurring amino acids and substitution of these in biologically active peptides. This could be important not only in the synthesis of new peptides, but also in research dealing with the role of an individual amino acid in biological systems. For example, a histidine analog could possibly be incorporated in the growth media of bacteria which need histidine in the synthesis of proteins. This could cause inhibition of protein synthesis by the organism or the modification of the activity of those proteins synthesized. It might also be interesting to see if this analog caused a decrease in the synthesis of histidine itself by affecting the enzymes necessary for synthesis through feedback inhibition. Today, a synthetic analog of a single amino acid could be used in a wide range of chemical research.

This investigation deals with the initial steps in the synthesis of a new amino acid analog of histidine.

Histidine (α-amino-4(or 5)-imidazolepropionic acid) has the following structure:

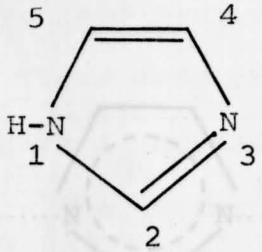


Histidine

I

It is a non-essential amino acid in adult humans[4] but an essential amino acid in children. It was isolated separately by Kossel[5] and Hedin[6] in 1896.

The portion of the molecule which is of major concern to us is the imidazole nucleus. Imidazole is a five-membered heterocyclic ring which contains two nitrogens. The nitrogen which is first in the numbering sequence is an imino nitrogen, the other nitrogen which is third in the numbering sequence is a tertiary nitrogen:



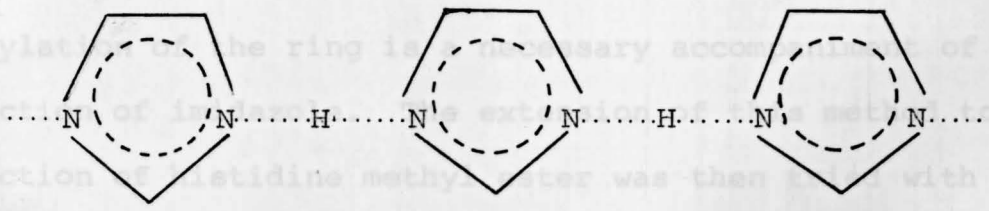
Imidazole

II

The solubility of imidazole is high in polar and low in non-polar solvents.[7] The simple imidazoles fail to exhibit selective absorption in the ultra-violet region.[8,9]

However, selective absorption is observed in imidazoles in which the imidazole ring is conjugated with a carbonyl group, such as in imidazole carboxaldehyde and imidazolecarboxylic acids.[7] Imidazole is a monoacidic base and also exhibits weakly acidic properties. It is extremely stable to drastic treatment with acid or base, and catalytic hydrogenation leaves the imidazole ring unaffected. Since imidazole obeys Huckel's rule for aromaticity it should show aromatic character and it has been shown that imidazole undergoes typical aromatic substitutions.

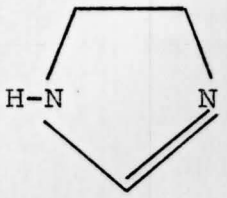
Examination of the physical properties of imidazole shows that its boiling point (256°C at 760 mm Hg) is much higher than that anticipated because of extensive hydrogen bonding. Due to the absence of this factor, N-alkyl derivatives (but not C-alkyl substituents) have much lower boiling points. "In fact, close study has demonstrated that in solution imidazole exists as aggregates of twenty or more molecules."[10]



III

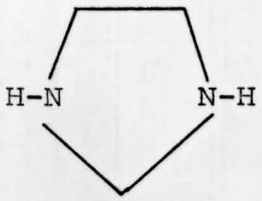
It is this imidazole ring and its chemical characteristics that give histidine its activity in biological systems and it is this same ring that will be altered in this work.

Substituted imidazolines and imidazolidines are known and the parent rings have the following structures:



2-Imidazoline

IV



Imidazolidine

V

Until 1961 substituted imidazolines and imidazolidines were not prepared by catalytic hydrogenation of imidazoles.

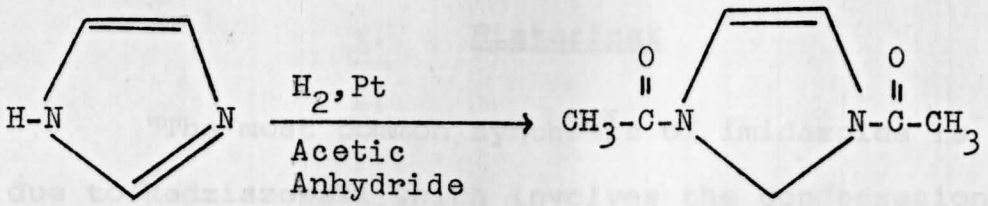
In this work the previously reported synthesis of N,N-Diacetyl-4-imidazoline was studied.[11] This synthesis was performed by catalytic hydrogenation of imidazole using a platinum oxide catalyst and acetic anhydride as the solvent (Reaction 1). Their results indicate that N-acylation of the ring is a necessary accompaniment of the reduction of imidazole. The extension of this method to the reduction of histidine methyl ester was then tried with the subsequent attempt at the isolation, purification and identification of the products (Reaction 2).

Future work would involve removal of the three acetyl groups, conversion of the ester to the free carboxyl and possible substitution of the imidazoline analog into a

biologically active polypeptide normally containing a histidine residue.

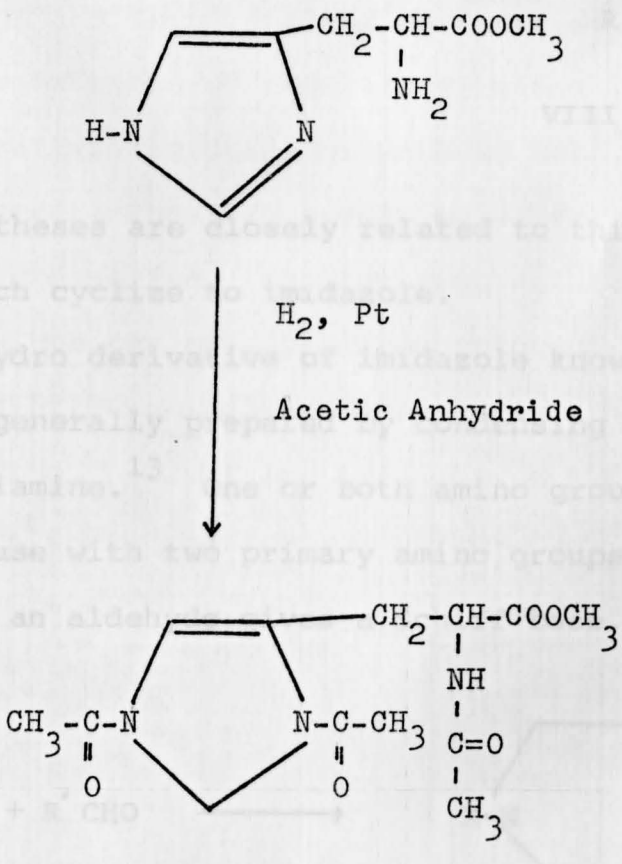
VI

VII



(1)

VI



(2)

VII

CHAPTER II

Historical

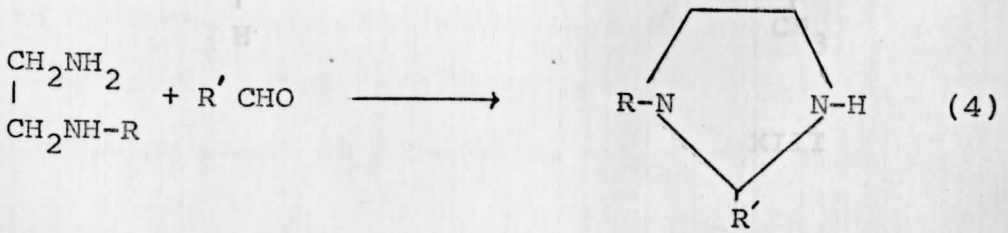
"The most common synthesis of imidazoles is that due to Radziszewski which involves the condensation of a dicarbonyl compound with ammonia and an aldehyde."[12]

(3)

VIII

Several other syntheses are closely related to this and give intermediates which cyclize to imidazole.

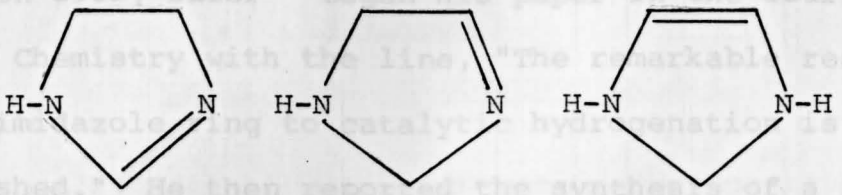
The tetrahydro derivative of imidazole known as imidazolidine is generally prepared by condensing an aldehyde with an ethylenediamine.[13] One or both amino groups must be secondary because with two primary amino groups, condensation with an aldehyde gives a Schiff base.



(4)

IX

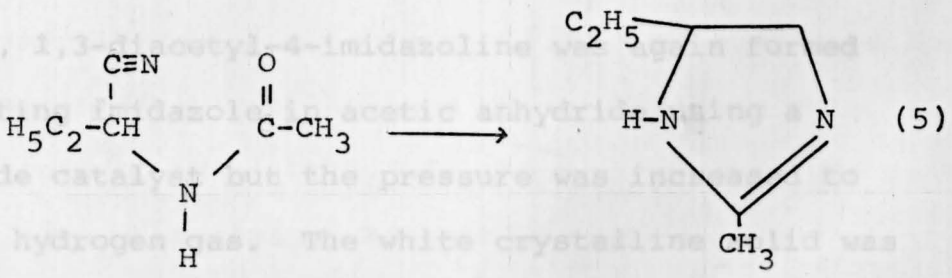
The dihydro derivative of imidazole gives the possibility of three isomeric imidazolines, depending on the position of the double bond:



2-Imidazoline 3-Imidazoline 4-Imidazoline

X XI XII

Several 3-imidazolines have been prepared[14] but 2-imidazolines are the most common and have received most attention. 2-Imidazolines can be prepared from 1,2 diamines by reaction with a mono-or dicarboxylic acid, or with an acid derivative. Reaction of ethylenediamine with acetic acid, for example, gives 2-methyl-2-imidazoline. Another synthesis of 2-imidazoline involves the catalytic reduction of the mono acetyl derivatives of α-aminonitriles.[15]



(5)

XIII

It was thought that the di-and tetrahydro derivatives had to be prepared by special synthetic methods and no method used imidazole as the starting material.

In 1961, Bauer[16] began his paper in the Journal of Organic Chemistry with the line, "The remarkable resistance of the imidazole ring to catalytic hydrogenation is well established." He then reported the synthesis of a previously unknown compound, namely, 1,3-diacetylimidazolidine. This reduction of imidazole was done by catalytic hydrogenation. The hydrogenation proceeded smoothly at room temperature and atmospheric pressure in an acetic anhydride solvent using platinum oxide as the catalyst. Hygroscopic white crystals were obtained after recrystallization from ethanol-ether in eighty per cent yield and having a melting point of 95°C. The diacetylimidazolidine was found to be very soluble in water, ethanol, acetone and in hot benzene. It was sparingly soluble in ether.

In 1966, Vail, Barker and Moran[11] reported the synthesis of another related new compound, an intermediate in the synthesis of diacetylimidazolidine but not reported by Bauer. The compound, 1,3-diacetyl-4-imidazoline was again formed by hydrogenating imidazole in acetic anhydride using a platinum oxide catalyst but the pressure was increased to 500 psi with hydrogen gas. The white crystalline solid was recrystallized from ethanol resulting in a fifty per cent yield and a melting point of 239-240°C.

CHAPTER III

Experimental

Preparation of Platinum Oxide

The procedure for this preparation of platinum oxide, Adams catalyst, is a modification of that given by Vogel in his "Textbook of Practical Organic Chemistry."[17]

A sample of platinum wire weighing 6.17 g was dissolved in aqua regia. A solution was achieved by heating a mixture of finely cut platinum wire and aqua regia on a steambath for at least eight hours. The solution was then evaporated to dryness, the residue dissolved in concentrated hydrochloric acid and the solvent again evaporated. Dissolving in concentrated hydrochloric acid followed by evaporation was repeated twice. The result on cooling to room temperature was brown, rod-like crystals of chloroplatinic acid. These crystals were dissolved in a minimum amount of water, followed by addition of a saturated solution of ammonium chloride to give 13.50 g of bright yellow crystals of ammonium chloroplatinate which were then dried at 100°C. The yield of $(NH_4)_2PtCl_6$ was about ninety six per cent.

To 35.72 g of potassium nitrate was added 3.04 g of ammonium chloroplatinate. The mixture was powdered, thoroughly mixed and then slowly heated in an evaporating dish over a Fisher burner until the evolution of gas slackened

and the solids melted to a brown solution. The heating was continued until the temperature rose to about 300°C when the solution was placed in a muffle furnace at 500-530°C for thirty minutes.

After cooling, the solid mass was broken up to aid the solution of nitrates in warm water. The dark brown platinum oxide was washed twice by decanting and then using Whatmann #541 (hardened, purified) filter paper until the filtrate was clear and the catalyst was free of nitrates (care must be taken that the precipitate does not become collodial from excess washing). There was obtained 1.42 g of catalyst which was then dried in a desiccator. A yield of eighty seven per cent was the best obtained in several runs.

It must be remembered that the catalyst thus obtained ($PtO_2 \cdot H_2O$) must be converted to the active form of finely divided platinum black which is the actual catalyst. This was done by hydrogenating the catalyst in an appropiate solvent under a hydrogen atmosphere at a pressure of about 60 psi. The theoretical uptake can be calculated from the chemical equation:

$$PtO_2 + 2H_2 \longrightarrow Pt + 2H_2O \qquad (6)$$

In the case where the platinum oxide was hydrogenated in an anhydride solvent the actual hydrogen uptake was somewhat higher than that calculated. This was also reported by Bauer[12] and has not yet been explained. Bauer did report,

however, that acetic anhydride consumed a small amount of hydrogen with the formation of aldehyde and a little ethyl acetate.

Calibration of Hydrogenation Apparatus

A 0.1 g sample of platinum oxide was suspended in 150 ml of ninety five per cent ethanol and the mixture was then hydrogenated for thirty minutes in a Parr hydrogenator to activate the catalyst. Next, 11.6 g (0.1 mole) of maleic acid was added and a total uptake of 7.0 psi was observed after approximately seventy minutes. Hydrogenation for another hour resulted in no further uptake of hydrogen. The suspension was then filtered and the filtrate evaporated on a rotary evaporator to give white crystals with a melting point, after recrystallization from water, of 187-9°C (literature melting point of succinic acid 188°C).[18]

A 7.0 psi drop has been shown to correspond to the reduction of one double bond for 0.1 mole of any compound containing one or more double bonds.

Preparation of N,N-Diacetylimidazoline From Imidazole

To 150 ml of acetic anhydride was added 1.00 g of platinum oxide. This suspension was placed on the hydrogenator, the reaction vessel was evacuated using a water aspirator and hydrogen introduced. The evacuation

and introduction of hydrogen was repeated three times as in all subsequent hydrogenation experiments. After the third time the hydrogen pressure was adjusted to 60 psi and the suspension shaken vigorously. The amount of hydrogen taken up by the catalyst-solvent mixture and the time necessary to reach the maximum uptake varied somewhat in different runs with the same amount of catalyst but in every case the hydrogen uptake was greater than that calculated. The calculated pressure drop for 1.00 g of platinum oxide is 0.62 psi.

When no further hydrogen was taken up by the mixture, 2.04 g (0.03 mole) of imidazole was dissolved in acetic anhydride, the suspension placed on the hydrogenator, evacuated several times as stated above and finally reduced at a starting pressure of 60 psi (the maximum limit for our hydrogenator). After approximately twenty four hours the pressure dropped 3.0 psi (0.9 psi more than the theoretical uptake). At this point the reaction mixture was removed from the hydrogenator and poured over 300 g of crushed ice to convert excess acetic anhydride to acetic acid. The suspension was stirred until only one liquid layer was present, filtered, and the filtrate evaporated under vacuum on a rotary evaporator at a temperature never exceeding 50°C.

White crystals weighing 4.86 g were left after evaporation. This is greater than the 4.61 g calculated as the theoretical yield and is due to the presence of

some residual acetic acid, detectable by its odor.

The diacetylimidazoline was recrystallized twice from ninety five per cent ethanol to a constant melting point of 238-39°C (reported melting point of diacetylimidazoline by Vail, Barker and Moran[11] 239-40°C). There was recovered 2.69 g of recrystallized diacetylimidazoline, a yield of fifty eight per cent.

Preparation of Diacetylimidazoline From 1-Acetylimidazole

The identical procedure used for the preparation of diacetylimidazole from imidazole was also followed for 1-acetylimidazole. The total pressure drop for 3.31 g (0.03 mole) of 1-acetylimidazole was 2.6 psi (0.5 psi greater than the theoretical uptake); the total time elapsed, twenty five hours. The product recrystallized once from ninety five per cent ethanol had a melting point of 234-37°C and weighed 1.30 g, a yield of twenty eight per cent.

Attempted Reduction of Histidine

The hydrogenation procedure described above was used with histidine. A 4.65 g (0.03 mole) sample of histidine free base was shaken with hydrogen using Adam's catalyst and acetic anhydride as the solvent. After hydrogenating for over twenty four hours the pressure drop amounted to only 0.4 psi, much less than the expected 2.1 psi. The evaporation product was a dark brown viscous oil. An

attempt was made to form crystals by recrystallization from ninety five per cent ethanol, but the result was the original brown oil.

Preparation of Anhydrous Methanol

Approximately 1200 ml of methanol and 15.0 g of magnesium turnings were placed in a 2 l round bottom flask equipped with a reflux condenser. The system was protected from atmospheric moisture with a drying tube containing Drierite (anhydrous calcium sulfate). After about one minute, hydrogen evolution began. Later, it was necessary to slow the reaction by cooling the reaction flask in an ice bath. When all magnesium had reacted, the suspension which was cloudy, was refluxed for five hours. The methanol was then distilled, the first and last 100 ml fractions being discarded. Material boiling at 64°C was collected and used in the preparation of histidine methyl ester dihydrochloride.

Preparation of Histidine Methyl Ester Dihydrochloride[19]

An apparatus was set up that would allow hydrogen chloride gas to be passed through a solution without allowing any trace amounts of water into the system. The apparatus consisted of three separate chambers connected in series. The first chamber was a drying tower filled with calcium chloride (mesh 4) that removed water vapor from

the hydrogen chloride gas. The next chamber was a trap to prevent any solution from backing up into the drying tower. The third chamber was a gas bubbling apparatus in which the reaction was carried out.

In the reaction chamber was placed 460 ml of dry methanol to which 30.33 g of L-histidine free base was added. As hydrogen chloride gas was slowly bubbled into the mixture all but a very small amount of the histidine dissolved in the methnaol. As the solution became saturated with hydrogen chloride, fine white crystals of histidine methyl ester dihydrochloride precipitated. The suspension was filtered and the solid was washed with anhydrous ether. There were obtained 45.36 g of crystals, a yield of ninety six per cent. These crystals were recrystallized from methanol.

Preparation of Sodium Methoxide

A reflux apparatus was set up and protected from moisture with a drying tube containing Drierite. To 200 ml of dry methanol was added 10.0 g of sodium metal spheres. The sodium was added over a period of about fifteen minutes to control the reaction. When the reaction was complete, a 25 ml aliquot of the sodium methoxide solution was diluted to 250 ml with distilled water in a volumetric flask. This solution was then standardized against potassium hydrogen phthalate (.005 mole) and the concentration of the original sodium methoxide was calculated

to be 0.218 M.

Preparation of Histidine Methyl Ester Free Base

To 150 ml of dry methanol was added 10.0 g (0.041 mole) of histidine methyl ester dihydrochloride. This suspension was then heated on a hot plate to aid in the solution. Next, 37.88 ml of sodium methoxide in methanol (0.218 M) was added dropwise from a buret. The resulting suspension was heated to insure that all histidine methyl ester dihydrochloride had dissolved and reacted. The suspension was cooled in an ice bath for approximately one hour for maximum precipitation of the sodium chloride. The suspension was filtered using a small conical Buchner funnel and the filtrate evaporated on a rotary evaporator under vacuum at a temperature never exceeding 50°C. The residue was a mixture of sodium chloride and an oil. To this mixture approximately 30 ml of chloroform was added and the mixture filtered. The residue (NaCl) was washed with chloroform and the filtrate evaporated to give a clear oil of histidine methyl ester. The ester was dissolved in methanol and evaporated on a rotary evaporator, first with the aid of a water aspirator then for approximately thirty minutes with a vacuum pump. The weights of 6.98 g for the histidine methyl ester free base and 4.82 g for sodium chloride are very close to the theoretical yield (98-99 per cent).

Reduction of Histidine Methyl Ester Free Base

The hydrogenation procedure described previously was used for the histidine methyl ester free base. To 150 ml of acetic anhydride was added 2.00 g of platinum oxide. The suspension was hydrogenated until no further uptake of hydrogen occurred to reduce the platinum oxide.

A 5.07 g (0.03 mole) sample of histidine methyl ester free base was dissolved in 15 ml of acetic anhydride with cooling in an ice bath. This solution and another 5 ml acetic anhydride wash of the flask were added to the hydrogenation bottle. This entire suspension was then hydrogenated for forty three hours until the total pressure drop amounted to 3.5 psi (1.4 psi over the calculated drop).

The synthesis of the desired product was followed during the course of the hydrogenation. This was done by stopping the hydrogenation, taking a 0.5 ml aliquot and diluting it to 5 ml in a volumetric flask. The suspension was filtered and the filtrate diluted 1:625. The absorbance of these diluted aliquots was then measured at 280 nm. The point in the course of the reduction at which the highest absorbance is obtained corresponds to the maximum formation of the desired product (the diacetylimidazoline derivative). This was expected from theoretical as well as experimental considerations.

Theoretically it is known that selective absorption was expected since the imidazole ring is conjugated with a carbonyl group (see introduction). The desired compound contains two carbonyl groups one bound to each nitrogen of the imidazole ring. Previous experimental results indicate that imidazole and N,N-deacetylimidazolidine have very little ultra-violet absorbance whereas N,N-diacetylimidazoline shows strong ultra-violet absorbance around 280 nm.[20]

The hydrogenation mixture was poured over 300 g of crushed ice. Upon melting of the ice, the two layers were stirred until the acetic anhydride had hydrolyzed. The suspension was filtered and the filtrate evaporated to dryness under vacuum. The result was 10.23 g of a clear viscous oil. The theoretical yield was 8.91 g. Thus, the oil contained some residual acetic acid.

A graph of the kinetic data for this hydrogenation is given on the following page (Figure 1).

Preparation of Pauly Reagent[21]

The Pauly reagent must be prepared immediately prior to its use. Three separate solutions are necessary: (1) sulfanilic acid - to 90 ml of concentrated hydrochloric acid was added 0.907 g of sulfanilic acid. This was then diluted by the addition of 90 ml of water, (2) five per cent sodium nitrite, (3) ten per cent sodium carbonate.

To prepare the reagent, 20 ml of cold sulfanilic

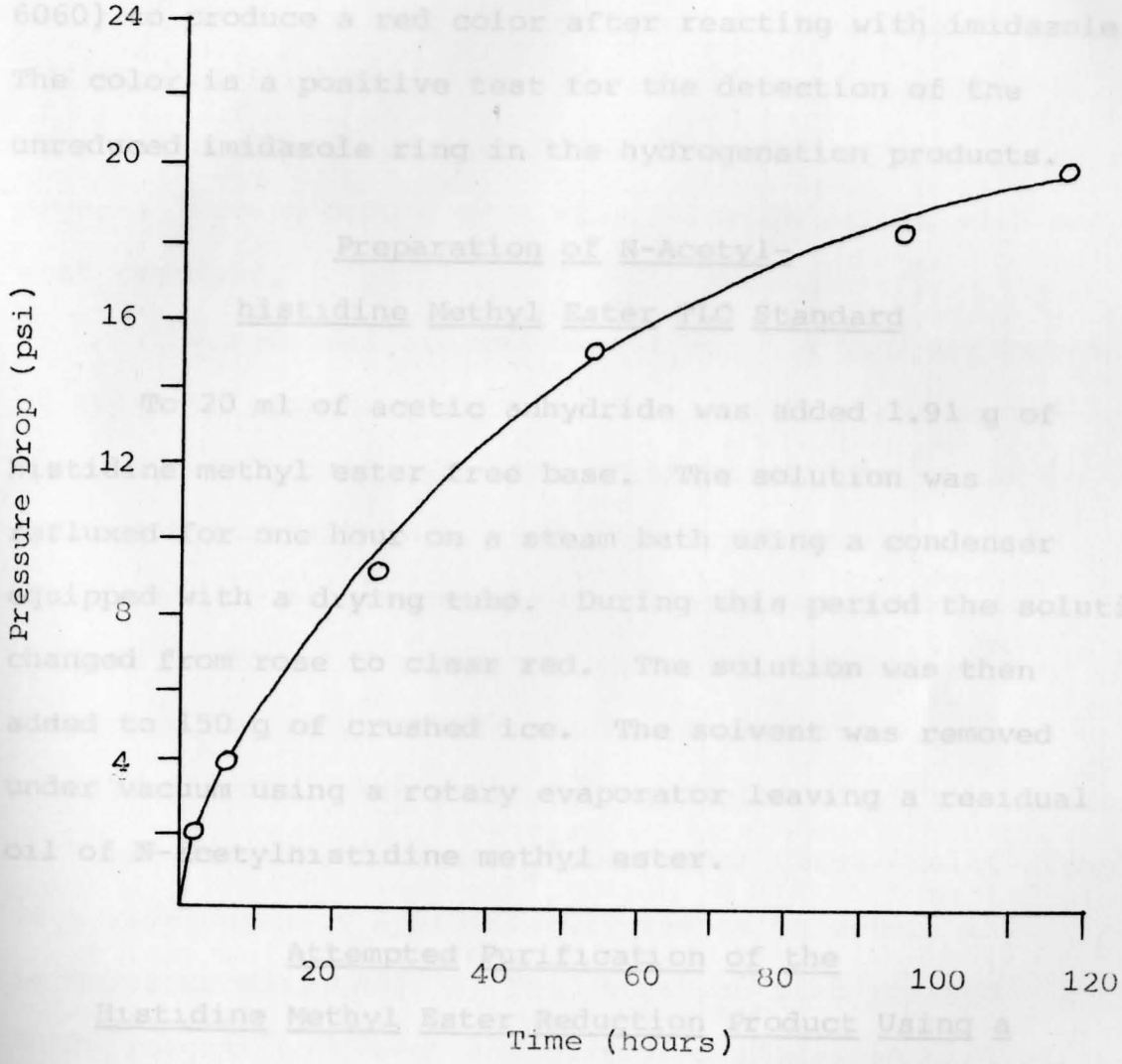


Figure 1. Rate of Hydrogenation of Histidine Methyl Ester.

acid and 20 ml of cold sodium nitrite were added together and allowed to stand below 20°C for five minutes. Then 40 ml of sodium carbonate was added.

The reagent was used by spraying a fine mist of the solution on a thin layer chromatography plate (Eastman no. 6060) to produce a red color after reacting with imidazole. The color is a positive test for the detection of the unreduced imidazole ring in the hydrogenation products.

Preparation of N-Acetyl-histidine Methyl Ester TLC Standard

To 20 ml of acetic anhydride was added 1.91 g of histidine methyl ester free base. The solution was refluxed for one hour on a steam bath using a condenser equipped with a drying tube. During this period the solution changed from rose to clear red. The solution was then added to 150 g of crushed ice. The solvent was removed under vacuum using a rotary evaporator leaving a residual oil of N-acetylhistidine methyl ester.

Attempted Purification of the Histidine Methyl Ester Reduction Product Using a Cation Exchange Batch Method

Dowex 50W X 4 cation exchange resin (1.15 g) was washed with 50 ml of deionized water by stirring for two hours. The resin was filtered with suction and the resin resuspended in another 150 ml of deionized water.

The resin suspension in water was stirred mechanically while cooled in an ice bath. A 1.00 g sample of the reduced histidine methyl ester product was dissolved in a minimum amount of water and also cooled in an ice bath. The solution was then added dropwise to the suspension with stirring, and stirring was continued for two hours after the addition with cooling in an ice bath. The suspension was then filtered with suction. The filtrate was evaporated to dryness leaving 0.50 g of a clear viscous oil, a 43.5 per cent recovery.

The residual oil was dissolved in a minimum amount of solvent (approximately 5 to 10 ml of five per cent methanol in chloroform). The solution was spotted at different concentrations on a thin layer chromatography sheet made of silica gel with a fifteen per cent calcium sulfate backing. The coating contained a fluorescent indicator for easy identification of any ultra-violet absorbing spots. The sheet was developed with five per cent methanol in chloroform and viewed under ultra-violet light at a wavelength of approximately 254 nm to detect the imidazoline compound(s). The sheet was also sprayed with Pauly reagent to detect any unreduced imidazole derivative. An iodine chamber was used for the detection of all spots separated after development of the TLC plate and ninhydrin reagent was sprayed on the plate to detect free amino groups.

The above identification methods were all done by cutting the TLC plate into strips containing three spots per

strip. A particular visualization technique was then used on each strip. The ultra-violet absorbances were performed using an ultra-violet viewer and could be done without cutting the sheet since this method did not alter the compounds on the sheet.

Preparation of Silica Gel Columns

A glass wool plug was placed in the bottom of a glass column filled with solvent (five per cent methanol in chloroform). Sand was added to cover the glass wool and a slurry of silica gel and solvent was poured into the column and the gel allowed to settle. The solvent level was then lowered to the top of the silica gel, the compound dissolved in a minimum amount of solvent was added, adsorbed onto the column and the reservoir filled with solvent. A Buchler Fractomat automatic fraction collector was used to collect 5 ml fractions. Ultra-violet absorbance values were then determined for selected fractions.

Since the amount of silica gel used varied with the quantity of compound to be separated it is difficult to give exact amounts here. But, in general, for 1.0 g of reduced histidine methyl ester product to be separated, 65 g of silica gel for column chromatography (particle size 0.2 to 0.5 mm) was used.

In later work silica gel G for thin layer chromatography acc. to stahl (particle size 10-40μ) was used. This silica gel afforded a better separation but,

due to the much smaller particle size, the flow rate was drastically reduced and it was necessary to decrease the amount of silica gel in the column to increase the flow rate. To separate a 1.20 g sample of reduced histidine methyl ester, 25 g of silica gel G was used. A 4.13 g sample was separated on a column containing 50 to 55 g of silica gel G. This amount (55-60 g) of silica gel approaches the upper limit for this column (60 X 2.5 cm). Larger amounts would result in too slow a flow rate. The above separations were done on a 600 mm column with a 25 mm internal diameter. It was found subsequently that a narrower column gave a better separation. Also, the flow rate for this large column (50-55 g silica gel G) was about 0.5 to 0.75 ml per minute, slower than was desired.

The ultra-violet absorbance data was collected by determining the absorbance of every other fraction at a wavelength of 280 nm using a Beckman DB Spectrophotometer. Then from a plot of absorbance vs. fraction number, one could determine the ideal fractions to spot on the thin layer chromatography plate. The Pauly positive material was determined from thin layer chromatography of these selected fractions. The fractions from both columns that contained the least amount of Pauly positive material, due to the unreduced imidazole ring, and contained the largest amount of ultra-violet absorbing material, due to the presence of the imidazoline ring, were combined and evaporated. Evaporation of these fractions under vacuum

yielded 2.06 g of residual oil.

The residual oil was dissolved in a minimum amount of five per cent methanol in chloroform and added to another silica gel G column containing 25 g of gel. The column was 600 mm long and had an internal diameter of 15 mm. A flow rate of about 1.0 ml per minute was achieved by making the total volume of the solvent in the reservoir about 800 ml. As the solvent in the reservoir was depleted the flow rate decreased. The ultra-violet absorbances were determined for every other fraction at a wavelength of 280 nm as stated above. Thin layer chromatography was performed on selected fractions and those fractions containing the least amount of Pauly positive material and those showing the greatest ultra-violet absorbance were combined and evaporated.

An ester test was also done on selected fractions. This test was performed for the first time on these fractions and will be described in more detail in the following pages.

Experimentally it was found that a 600 mm column with an internal diameter of 15 mm gave the best separation. Although a constant flow rate was difficult to achieve, one varying from 0.75 (fifteen drops) to 1.00 ml (twenty drops) per minute gave a good separation with little diffusion. The columns used were obtained from Ace Glass Incorporated and were equipped with either a glass stopcock or a teflon screw, which had a hole in the center to allow solvent to pass through.

Preparation of Cation Exchange Columns

The same method used for the preparation of the silica gel columns was used for the cation exchange columns. The column was equipped with a glass wool plug covered with sand and the resin added to the column as a slurry. A 300 mm column was used with an internal diameter of 11mm. The flow rate was regulated with the stopcock to a constant flow rate of 1 ml per minute. The reduced histidine methyl ester product was dissolved in a minimum amount of deionized water, and adsorbed onto the column. Solvent (deionized water) was added to the reservoir and eluted through the column. Fractions of 5 ml were collected on the Fractomat automatic fraction collector and checked for ultra-violet absorbance at 280 nm. Thin layer chromatography was performed on selected fractions by spotting a 10 ml sample of each fraction and developing the plate with five per cent methanol in chloroform. The fractions were then combined and evaporated.

Preparation of Anion Exchange Column

An anion exchange column was prepared in an attempt to convert histidine methyl ester dihydrochloride to the free base. Bio-Rad analytical grade anion exchange resin AG 1 X 8 was used. The resin, to begin with, was in the chloride form and it was necessary to convert it to the hydroxide form.

To 300 ml of 2 M sodium hydroxide was added 125 g of the anion exchange resin. The suspension was stirred for twenty three hours, and poured into a column 600 mm long and having an internal diameter of 25 mm. The reservoir was filled with 2 M sodium hydroxide and the flow rate adjusted to .5 ml per minute. The eluent was checked for chloride by collecting ten drops from the column, acidifing with nitric acid and adding ten drops of .1 M silver nitrate to give a white precipitate of silver chloride as a positive test. After 12 l of 2 M sodium hydroxide had passed through the column the eluent showed a negative test for chloride. Next, 4 l of deionized water was passed through the column until the pH of the eluent was neutral. The resin at this point was in the hydroxide form and was ready for use.

To 15 ml of deionized water was added 9.68 g of histidine methyl ester dihydrochloride. This solution was then added to the top of the column. Upon addition of the dihydrochloride, heat was liberated and bubbles appeared at the top of the column. After approximately three minutes the resin had resettled. The eluent was then checked periodically for the imidazole ring of histidine using the Pauly reagent described previously. After passing 3.2 l of deionized water through the column, all tests for histidine were negative.

The resin was then removed from the column, added to 500 ml of 2 M ammonium hydroxide and stirred for one

hour. The suspension was then decanted and the process repeated with another 500 ml of ammonium hydroxide. Pauly tests of the ammonium hydroxide washes were both negative.

The 9.68 g of histidine methyl ester dihydrochloride was never recovered. A positive test observed when the resin was sprayed with Pauly reagent indicates that the compound was still bound to the resin. No further work was done to recover the histidine methyl ester. The failure of the desired compound to be eluted may be due to hydrolysis of the ester by the resin, a strong base. The carboxyl group so liberated would be strongly bound.

Determination of An Ester Using Hydroxylamine[22]

This test was used semi-quantitatively to determine the fractions containing the maximum amount of an ester. The 5 ml fractions were collected from a column chromatography separation of a reduced histidine methyl ester mixture using silica gel G as the stationary phase.

The following four reagents were used to detect the presence of ester: (1) 2 M hydroxylamine hydrochloride (this solution should be stored in the cold), (2) 3.5 N sodium hydroxide, (3) concentrated hydrochloric acid (specific gravity 1.18) diluted with two parts by volume of distilled water, (4) 0.37 M ferric chloride.

The alkaline hydroxylamine was prepared by adding equal volumes of hydroxylamine hydrochloride and sodium hydroxide. This solution keeps for approximately three

hours at room temperature. To a 0.1 ml aliquot, taken from each fraction to be tested, was added 2.0 ml of alkaline hydroxylamine. After reacting for at least one minute, 1.0 ml of acid was added followed by 1.0 ml of ferric chloride. The absorbance of the purple-brown colored solution was determined immediately at a wavelength of 540 nm using a Bausch and Lomb Spectronic 20. The formation of gas bubbles was avoided by shaking the solution after the addition of each reagent. Since our test solutions were in five per cent methanol in chloroform, it was necessary to shake the mixture to ensure that the reduced histidine methyl ester had been extracted from the chloroform and reacted with the hydroxylamine.

Hydrazinolysis of Reduction Product of Histidine Methyl Ester

The following procedure was used to convert the ester to the corresponding hydrazide. A 1.61 g sample of reduction product from which Pauly positive material was removed by column chromatography, was dissolved in 48 ml of absolute methanol. A 30 ml aliquot of this solution was placed in a 50 ml round bottom flask. To this solution, 1.5 ml of an eighty five per cent solution of hydrazine hydrate was added. The resulting solution was refluxed for three hours using a condenser equipped with a drying tube. The reaction mixture was then evaporated to dryness, followed by the addition of methanol and a second evaporation

using a water aspirator to achieve the desired pressure. Residual solvent and hydrazine was then evaporated overnight using a vacuum pump. The result was a white hygroscopic solid weighing 2.23 g, having a melting point between 45°C and 75°C. The solid was then suspended in 45 ml.of ethyl acetate and the solvent was boiled with the result that part of the solid dissolved. The hot suspension was filtered and the solid extracted with an additional 50 ml of boiling ethyl acetate. After being allowed to stand at 5°C overnight there was obtained from the first filtrate a small amount of a white solid, melting point 92-94°C.

Reagents and Materials

The following is a list of the reagents and equipment used in the experimental section of this work. Note that the manufacturer and other pertinent information is also given.

1. Acetic anhydride - Fisher Scientific Company.
2. 1-Acetyl imidazole - Aldrich Chemical Company.
3. Ammonium chloride - 'Baker Analyzed' Reagent.
4. Ammonium hydroxide - 'Baker Analyzed' Reagent.
5. Anion exchange resin - Bio-Rad Analytical Grade AG 1 X 8, 100-200 mesh, chloride form.
6. Beckmann DB Spectrophotometer - Fisher Scientific.
7. Calcium chloride - 'Baker Analyzed' Reagent, mesh 4.
8. Cary 14 Spectrophotometer - Cary Instruments.
9. Cation exchange resin - Dowex 50W X 4, 50-100 mesh, hydrogen form, polystyrene type, sulfonic acid.
10. Chloroform - 'Baker Analyzed' Reagent.
11. Drierite - anhydrous calcium sulfate, Hammond.
12. 95 per cent Ethanol - I.S.I. Gold Shield.
13. Ethyl acetate - 'Baker Analyzed' Reagent.

14. Ferric (III) chloride hexahydrate - 'Baker Analyzed' Reagent.
15. Fraction collector - Fractomat Automatic Fraction Collector, Buchler Instruments.
16. Glass columns - Ace Glass Incorporated.
17. Glass wool - A.H. Thomas.
18. Hydrazine hydrate - 85 per cent solution, Fisher Scientific Company.
19. L-Histidine (free base) - Nutritional Biochemicals Corp.
20. Hydrochloric acid (conc.) - Analytical Reagent 37 per cent.
21. Hydrogenator - Parr Instruments.
22. Hydrogen chloride (gas) - Matheson.
23. Hydroxylamine hydrochloride - Fisher Scientific Company.
24. Imidazole - Eastman.
25. Magnesium turnings - Mallinckrodt.
26. Maleic acid - Eastman.
27. Methanol - Fisher Scientific Company.
28. Nitric acid - DuPont Reagent 70-71 per cent.
29. Platinum wire - Engelhart Industries.
30. Potassium nitrate - 'Baker Analyzed' Reagent.
31. Rotary evaporator - Buchler Instruments.
32. Silica Gel - 0.2-0.5 mm for chromatography, Merck AG Darmstadt (Germany).
33. Silica Gel G for thin layer chromatography acc. to Stahl, 10-40 μ, Merck AG Darmstadt (Germany).
34. Silver nitrate - 'Baker Analyzed'Reagent.
35. Sodium carbonate - 'Baker Analyzed' Reagent.
36. Sodium hydroxide - J.T. Baker, U.S.P.
37. Sodium metal (spheres) - Matheson, Colman, Bell, Analyzed Reagent.
38. Sodium nitrite - 'Baker Analyzed'Reagent.
39. Sprayers for thin layer chromatography (TLC) - Nutritional Biochemicals Corp., contains dichlorodifluoromethane.
40. Silica Gel G TLC sheets - Eastman, with and without fluorescent indicator.
41. Spectronic 20 Spectrophotometer - Bausch and Lomb.
42. Sulfanilic acid - Fisher Scientific Company.
43. TLC chromagram chamber plate set - Eastman.

CHAPTER IV

Results and Discussion

The work of Bauer,[16] together with the later investigations of Vail, Barker and Moran,[11] showed that the hydrogenation of imidazole with platinum oxide catalyst in acetic anhydride can yield either N,N-diacetylimidazoline or N,N-diacetylimidazolidine, depending on how far the reaction is allowed to proceed.

The ultra-violet absorbance spectrum of a 0.000500 per cent solution (3.25×10^{-5} M) of N,N-diacetylimidazoline in water was run on a Cary 14 Spectrometer (Figure 2). This solution showed a maximum absorbance at 277 nm with a molar absorptivity of 1.93×10^{4}.

Diacetylimidazoline is a conjugated system and would therefore be expected to show ultra-violet absorbance. Diacetylimidazolidine, on the other hand, does not exhibit significant absorbance in the region of 280 nm.[20]

The ultra-violet absorbance of diacetylimidazoline derivatives was an important tool in locating and identifying the analogous reaction product of histidine methyl ester. All identification techniques used in this early work were essential in later experimentation. The UV absorbance was determined for fractions from column chromatography to locate and estimate the desired compound. The absorbance

was also used as an easy visualization technique in fluorescent thin layer chromatography (TLC). The diacetylimidazoline derivatives appeared in the UV viewer as a blue spot against an orange background. The fact that imidazole couples with diazotized sulfanilic acid to form highly colored azo compounds was used as a test for any unreduced imidazoles. The Pauly reagent used in this test was very sensitive and convenient. It was used as a visualization spray on TLC plates or added to solutions to give a qualitative determination of imidazole derivatives. Iodine vapor gave a dark spot with all products separated by TLC. Finally, a semi-quantitative test for esters was performed on fractions from column chromatography. The ester test was also used as a crude visualization spray for TLC. The techniques described here were all used later to investigate the reduction of histidine methyl ester.

The hydrogenation of histidine (I) was attempted as an extension of previous work with imidazole. Histidine was dissolved in acetic anhydride and hydrogenated with a platinum oxide catalyst. The result after hydrolysis of excess acetic anhydride, removal of platinum black and evaporation of solvent was a dark brown viscous oil. Since there was no hydrogen uptake during the reaction it was concluded that the imidazole ring was left unaffected. Thin layer chromatography of the product developed with 1-butanol (80 ml), glacial acetic acid (20 ml) and distilled water (20 ml) showed only one spot. The spot gave a positive

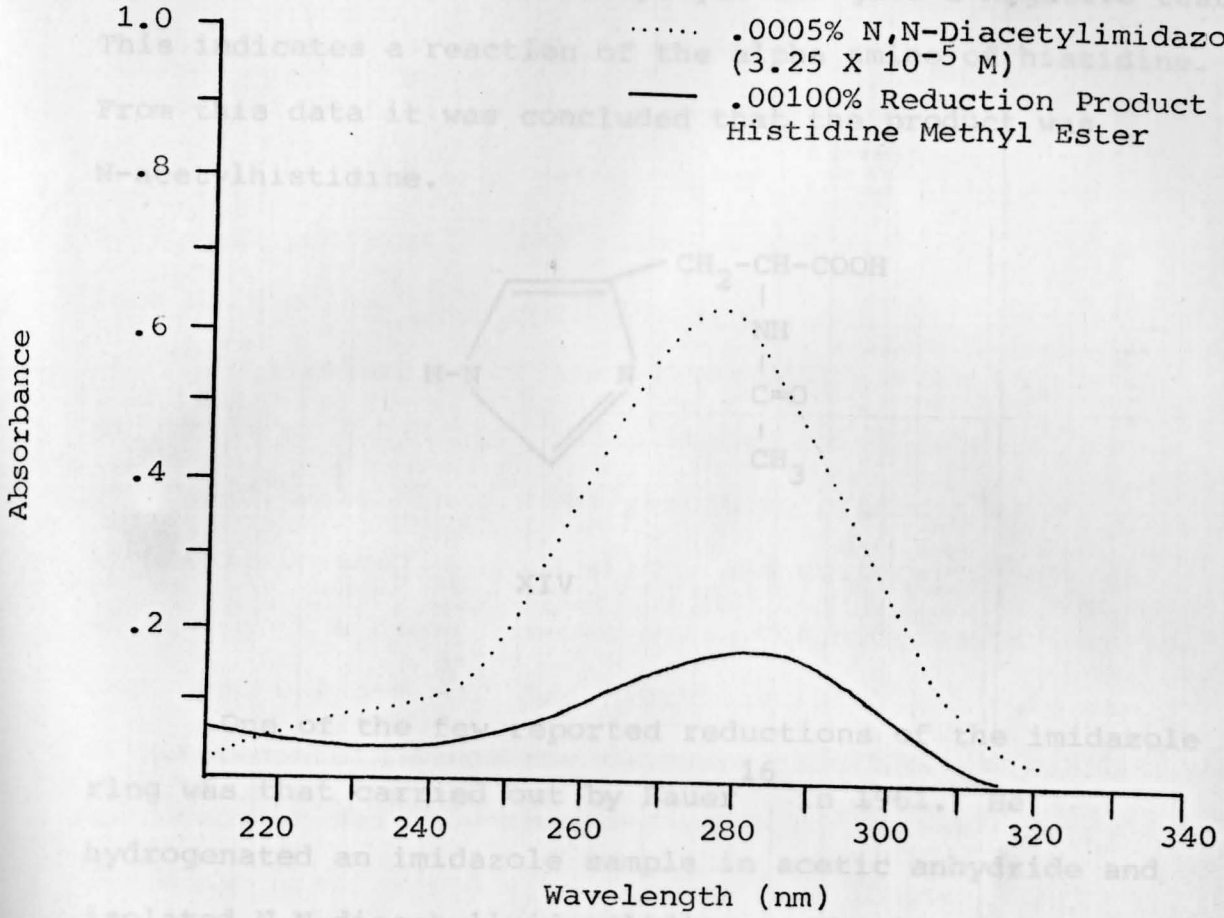
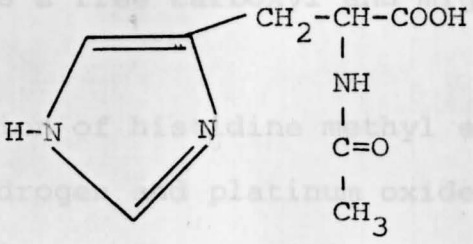


Figure 2. UV Absorption Spectrum of N,N-Diacetylimidazoline and Reduction Product of Histidine Methyl Ester.

Pauly test indicating an intact imidazole ring and showed no UV absorbance. Ninhydrin, used to identify free amino groups in amino acids, was sprayed and gave a negative test. This indicates a reaction of the alpha amino of histidine. From this data it was concluded that the product was N-acetylhistidine.



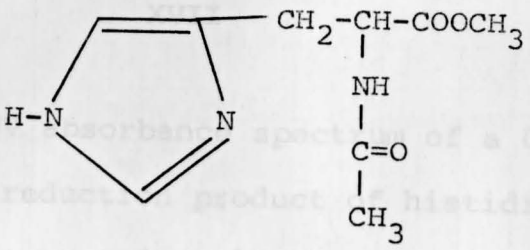
XIV

One of the few reported reductions of the imidazole ring was that carried out by Bauer[16] in 1961. He hydrogenated an imidazole sample in acetic anhydride and isolated N,N-diacetylimidazolidine as the final product. Their results indicate that N-acylation of the ring is a necessary accompaniment of the reduction of imidazole. Other workers[23] using derivatives of histidine reported that under ordinary conditions of acylation, introduction of an acyl group into the imidazole portion of histidine residues, possessing a free carboxyl group, was not possible. This then suggests a possible explanation of the fact that the catalytic hydrogenation of histidine was unsuccessful. Without N-acylation of the imidazole nucleus of histidine reduction of the ring would be impossible. However, in

the case of methyl esters of α-N-acylhistidines, a second acyl group can be readily introduced into the imidazole ring. Thus, histidine methyl ester meets the condition for the hydrogenation of the imidazole ring, whereas histidine, itself, does not. The next step then was the synthesis of histidine methyl ester (VII). This compound no longer contains a free carboxyl and might be reduced with less difficulty.

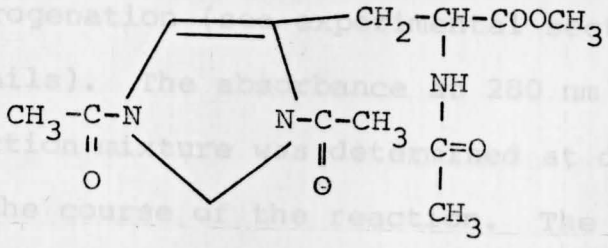
The reduction of histidine methyl ester in acetic anhydride with hydrogen and platinum oxide catalyst was performed three times. The resulting products were identical in each hydrogenation, and evaporation of the solvent left a clear viscous oil. The hydrogen uptake in each case was greater than that calculated but this was of minor concern since the pressure gauge on the Parr hydrogenator was only accurate to about $\pm$0.2-0.3 psi. The scale also varied with fluctuations in room temperature.

Since the hydrogenation of imidazole gave two products, three components were suspected in the product of the hydrogenation of histidine methyl ester. These were:



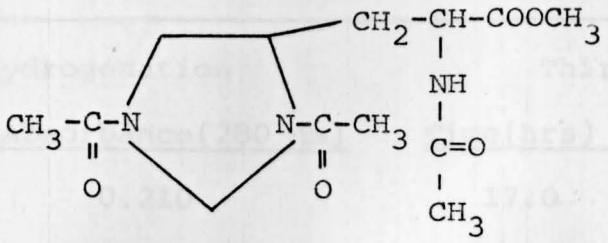
N-Acetylhistidine Methyl Ester

XV



Imidazoline Derivative

XVI



Imidazolidine Derivative

XVII

The UV absorbance spectrum of a 0.001 per cent solution of reduction product of histidine methyl ester in water was run on a Cary 14 Spectrometer (Figure 2). The solution showed a maximum absorbance at 280 nm. This absorbance was ascribed to the diacetylimidazoline ring.

Since a diacetylimidazoline derivative shows UV absorption, this could be used to follow the progress of the hydrogenation (see experimental section, Chapter III for details). The absorbance at 280 nm of an aliquot of the reaction mixture was determined at different times during the course of the reaction. The absorbance should increase with increased concentration of the imidazoline derivative, the maximum absorbance corresponding to maximum formation of the desired compound. The absorbance should then decrease as the imidazoline derivative is converted to the imidazolidine derivative. The rate of the reduction was followed for the last two hydrogenations. The results were similar and are given below.

Table I

Hydrogenation of Histidine Methyl Ester

Second Hydrogenation		Third Hydrogenation	
Time(hrs)	Absorbance(280 nm)	Time(hrs)	Absorbance(280 nm)
19.5	0.210	17.0	0.215
23.5	0.235	19.3	0.235
29.0	0.260	22.0	0.225
43.0	0.225	24.5	0.170

The total hydrogen uptake for the second hydrogenation was 1.4 psi over that calculated for the reduction of one double bond. The total hydrogen uptake for the third hydrogenation was only 0.4 psi over the theoretical uptake. Obviously, the second reduction was run for too long. The difference in the rate of formation of the imidazoline derivative could be attributed to the activity of the catalyst since the hydrogenations were done with different catalyst preparations. The point to be made is that the formation of the desired product could be followed by UV absorbance as was suspected.

After the proper solvent had been found a thin layer chromatographic separation of the reduction products of histidine methyl ester was achieved. The chromatogram developed with five per cent methanol in chloroform showed three separate spots. The following table summarizes the TLC data collected to this point.

Table II

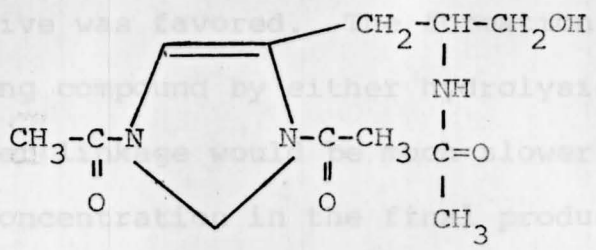
Thin Layer Chromatography

Sample	UV Viewer (254 nm)	Pauly	Ninhydrin
1 Imidazole	-	+	-
2 Diacetylimidazoline	+	-	-
3 Histidine	-	+	+
4 Reduction Product of Histidine	-	+	-
Reduction Products of Histidine Methyl Ester			
5a Highest R_f Spot	+	-	-
5b Middle R_f Spot	+	-	-
5c Lowest R_f Spot	-	+	-

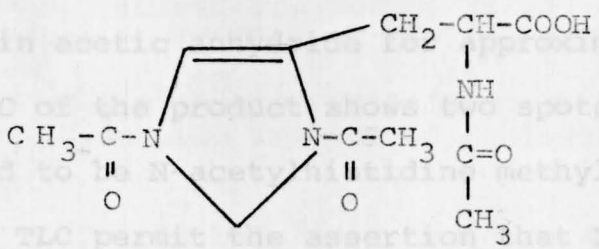
The R_f values for these compounds are not given because of their variability. The sequencing of the spots and their orientation with respect to each other was very reproducible.

The TLC data for the hydrogenation mixture for histidine methyl ester varied slightly from what was expected. The Pauly positive and non-UV absorbing spot was ascribed to N-acetylhistidine methyl ester for reasons to be given later. The Pauly negative but UV absorbing spot was suspected of being the imidazoline derivative. The second UV spot was totally unexpected and did not appear to be an imidazolidine derivative since a completely reduced ring compound would not be expected to

absorb UV light. The second UV spot would then have to be an imidazoline derivative since the latter shows UV absorbance and is also Pauly negative. These results suggest a side reaction most likely involving the ester linkage. Two possible reactions are: (1) reduction of the ester to give a primary alcohol, or (2) hydrolysis of the ester producing a free carboxyl. The structure of the corresponding compounds are as follows:



XVIII

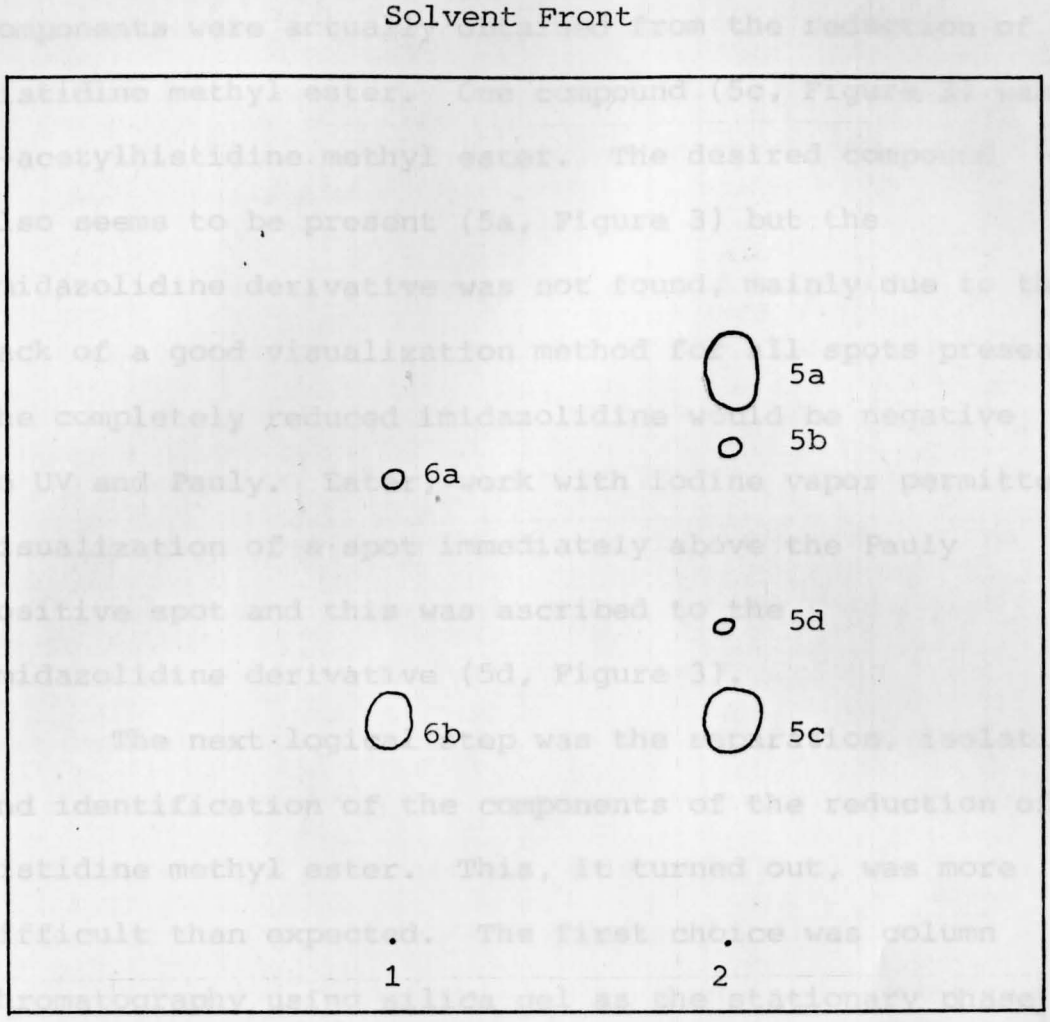


XIX

The following (Figure 3) is a replication of a typical TLC of the reduction products of histidine methyl ester. The chromatogram was developed with five per cent methanol in chloroform and identification of the spots is given. The spot on the left is a separation of N-acetyl-histidine methyl ester prepared in the lab.

The highest R_f spot (5a, Figure 3) for the reduction of histidine methyl ester was thought to be the desired imidazoline derivative. This is the larger UV spot and it was expected that the formation of this imidazoline derivative was favored. The formation of the second UV absorbing compound by either hydrolysis or reduction of the ester linkage would be much slower, resulting in a lower total concentration in the final product. This UV absorbing compound would then be seen as the smaller UV spot, on a TLC chromatogram.

The N-acetylhistidine methyl ester seen spotted in Figure 3 (6b) was prepared by refluxing histidine methyl ester in acetic anhydride for approximately three hours. The TLC of the product shows two spots, the larger one was assumed to be N-acetylhistidine methyl ester. The results of the TLC permit the assertion that N-acetylhistidine methyl ester is present in the reduction product of histidine methyl ester. The much smaller second spot (6a, Figure 3) may be unreacted histidine methyl ester. This is supported by the fact that the spot is Pauly positive and shows no UV absorption.



1. N-Acetylhistidine Methyl Ester
2. Reduction Product of Histidine Methyl Ester

Figure 3. TLC of N-acetylhistidine Methyl Ester and Reduction Product of Histidine Methyl Ester.

Spot 5a was Pauly negative and UV positive, spot 5b was also Pauly negative and UV positive and spot 5c showed no UV absorbance and was Pauly positive. Spot 5d was only iodine positive. Spot 6a was only Pauly positive, and spot 6b was also only Pauly positive. All spots were iodine positive.

The results to this point indicate that three components were actually obtained from the reduction of histidine methyl ester. One compound (5c, Figure 3) was N-acetylhistidine methyl ester. The desired compound also seems to be present (5a, Figure 3) but the imidazolidine derivative was not found, mainly due to the lack of a good visualization method for all spots present. The completely reduced imidazolidine would be negative to UV and Pauly. Later, work with iodine vapor permitted visualization of a spot immediately above the Pauly positive spot and this was ascribed to the imidazolidine derivative (5d, Figure 3).

The next logical step was the separation, isolation and identification of the components of the reduction of histidine methyl ester. This, it turned out, was more difficult than expected. The first choice was column chromatography using silica gel as the stationary phase and a methanol in chloroform solvent since this system separated the reaction mixture on a TLC sheet. The silica gel used had a particle size of 0.2-0.5 mm.

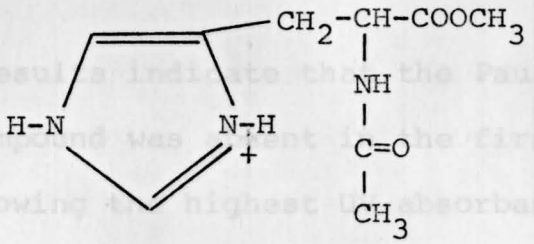
A 1.0 g sample of the reduction product was dissolved in a minimum amount of chloroform and adsorbed onto the column. The first forty five tubes were eluted with two per cent methanol in chloroform then the remaining tubes with five per cent methanol in chloroform. A total of 280 fractions, containing 5 ml of eluent each, were collected. The results indicate that a partial separation

did occur, the last fractions containing only Pauly positive material, but the fractions containing the most UV absorbing material (fractions 100-140) also contained Pauly positive material. Evaporation of the latter fractions yielded only 70 mg of a brown oil.

Two methods were used to locate the compounds eluted from the column. First, the UV absorbance was determined of selected fractions using a Beckmann DB Spectrophotometer at a wavelength of 280 nm. Having applied the same technique to follow the formation of the desired product during the hydrogenation, one could also locate the imidazoline derivative in the fractions from column chromatography rapidly. The strongest UV absorbing fractions should contain the highest concentration of the desired imidazoline derivative. Selected fractions were then spotted on a TLC sheet and developed with five per cent methanol in chloroform. The spots were identified with a UV viewer at 254 nm and by spraying the entire sheet with Pauly reagent.

The drawback to this separation was that too many tubes were collected. This proved tedious when measuring the UV absorbances and the first tubes evaporated slightly before the remaining tubes were collected. The two per cent methanol in chloroform solvent was abandoned in favor of a five per cent methanol in chloroform solution. This concentration should reduce the number of fractions required.

The ease of separation found using thin layer chromatography was not transferable to column chromatography as was expected. A different method to remove the N-acetyl-histidine methyl ester impurity was attempted. It was believed that the unreduced compound (since it contained the basic imidazole ring) would be positively charged in the acidic environment of a strong cation exchange resin, like Dowex 50W. The positively charged compound:



XX

would be held by the resin allowing the uncharged products to pass through the column.

The first use of this method employed very small quantities of resin and sample. A 0.070 g partially purified sample from the silica gel column mentioned previously (fractions 100-140) was adsorbed onto a column containing 0.5 g of cation exchange resin. Fractions of 5 ml were collected and the UV absorbance determined. A spot of each fraction was made on a TLC sheet and sprayed with Pauly reagent. The results are given below.

Table III

Ion Exchange Chromatography

Fraction	Absorbance(280 nm)	Pauly	UV Viewer(254 nm)
1	0.70	-	+
2	0.315	+	-
3	0.120	+	-
4	0.085	+	-
5	0.070	+	-

The results indicate that the Pauly positive unreduced compound was absent in the first fraction. This fraction, showing the highest UV absorbance, should contain the largest amount of the desired imidazoline derivative. Evaporation of the first fraction yielded 0.013 g of a brown oil. Since this method was successful on such a small scale it was decided to increase the amounts of sample and resin to 2.00 g and 10.0 g respectively.

The resin was added as a slurry to a column 300 mm long and having an internal diameter of 11 mm. A 2.06 g sample of reduction product was dissolved in a minimum amount of deionized water and adsorbed onto the column. Fractions of 5 ml were collected and the UV absorbance determined at 280 nm, the fractions were also checked for unreduced imidazoles with Pauly reagent. This was done in an ice bath by mixing 0.1 ml of sulfanilic acid with 0.2

ml of test solution and then 0.1 ml of five per cent sodium nitrite was added and left to stand for three minutes. Finally, the solution was made alkaline with addition of 0.3 ml of ten per cent sodium carbonate. A red color is a positive test for unreduced imidazole.

Results after one column chromatography separation indicated that all Pauly positive material had not been removed. The fractions containing the most UV absorbing material were combined and evaporated. The residual oil was chromatographed on a second column using 10.0 g of cation exchange resin. Again the fractions containing the most UV absorbing material and the least amount of Pauly positive material were combined and evaporated. TLC of this residue developed with five per cent methanol in chloroform showed streaking rather than discrete spots with iodine and Pauly reagent. This was attributed to either impurities present from the resin, hydrolysis of the compounds by cation exchange resin, or possible hydration of the double bond in the imidazoline ring.

To minimize possible hydrolysis of the desired compound, a different method to remove the Pauly positive material was attempted using Dowex 50W X 4 cation exchange resin. This time the reaction mixture was simply stirred with the resin rather than being passed through a column. The resin was first washed by stirring a suspension in deionized water for approximately two hours. The filtered resin was then stirred again with deionized water while

being cooled in an ice bath. A cooled solution of reduced histidine methyl ester dissolved in a minimum amount of deionized water was added dropwise to the suspension. After all the sample had been added, the suspension was stirred for an additional two hours. Next, the suspension was filtered and the filtrate evaporated to dryness. TLC of this first residue showed a small amount of Pauly positive material. The above procedure was repeated using the first residue but TLC of this second residue showed the same type of streaking found with the eluent collected from column chromatography using cation exchange resin. Dowex 50W X 4 cation exchange resin was then abandoned as a possible method for removal of the Pauly positive material. Two batch methods were enough to remove the unreacted imidazole derivative but apparent side reactions caused by the resin made this method undesirable. Possible a batch method using a weaker cation exchange resin would prove to be more favorable.

Since the cation exchange method could not be used, silica gel for column chromatography was again tried as a method of separation. Three separate columns were prepared containing 45 g, 25 g and 15 g of silica gel. The solvents used were ten per cent, seven per cent and five per cent methanol in chloroform, respectively. A sample of reduced histidine methyl ester weighing 1.1 g was added to the first column, the fractions that had the highest UV absorbance and the least amount of Pauly positive material

were combined, evaporated and the residue added to the second column. Fractions from the second column were combined and evaporated as above and added to the third column. The final residue from the third column (fractions 9-27) weighing 0.18 g contained no Pauly positive material and TLC of fractions showed two UV absorbing spots, one of these being much more prominent. The data for the above separation can be found in the following pages.

The data from these separations showed that the Pauly positive material could be removed by three separate column chromatography runs. The only drawback of this method was poor recovery of UV absorbing material. A different silica gel adsorbent labeled for thin layer chromatography was tried in another column, namely silica gel G. This silica gel G had a particle size of 10-40μ and smaller columns were prepared to achieve a desirable flow rate of about 0.5-0.75 ml per minute.

The first two separations of the reduction product of histidine methyl ester using silica gel G were carried out using samples of 1.19 g and 4.13 g. The solvent for each column was five per cent methanol in chloroform. The column used was 600 mm long and had an internal diameter of 25 mm. Silica gel G weighing 25 g and 50 g respectively were used for each column. The fractions were combined and evaporated as described previously. The results indicate that a larger amount of Pauly positive material

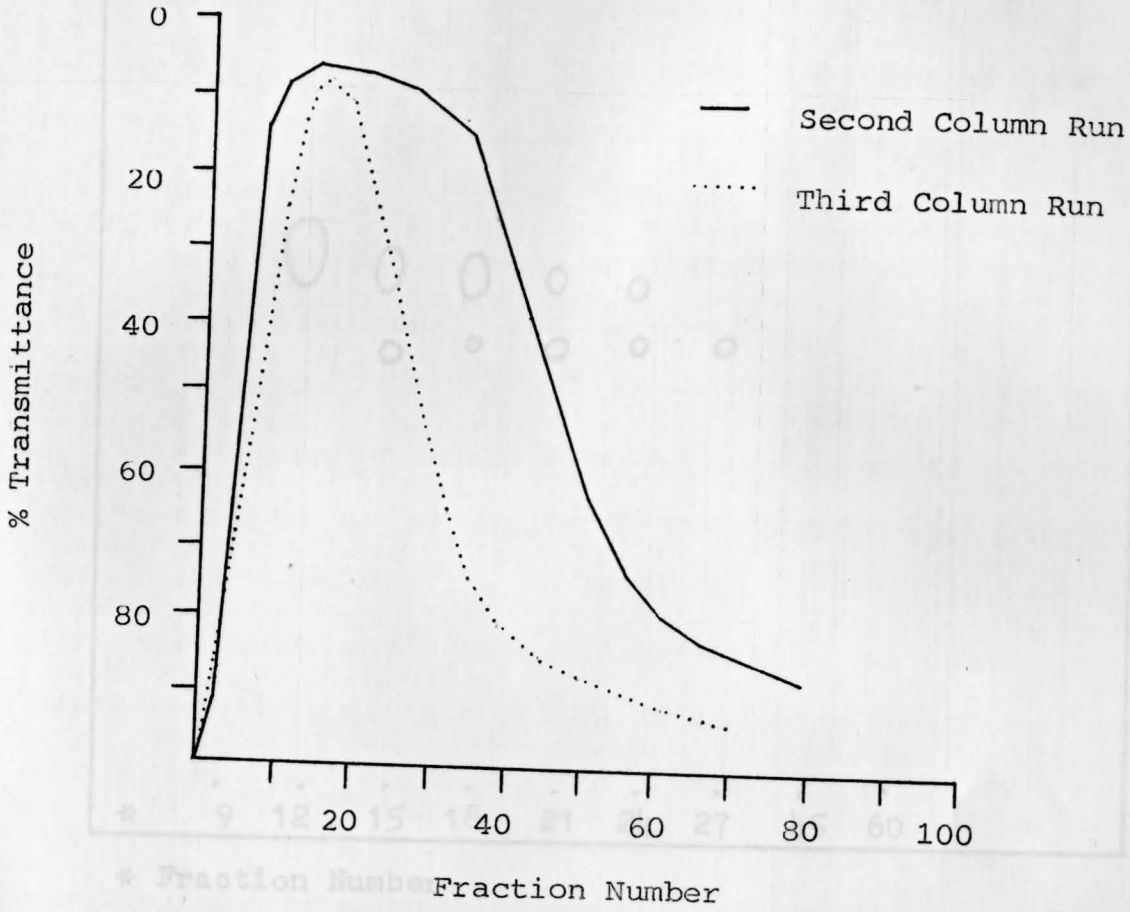
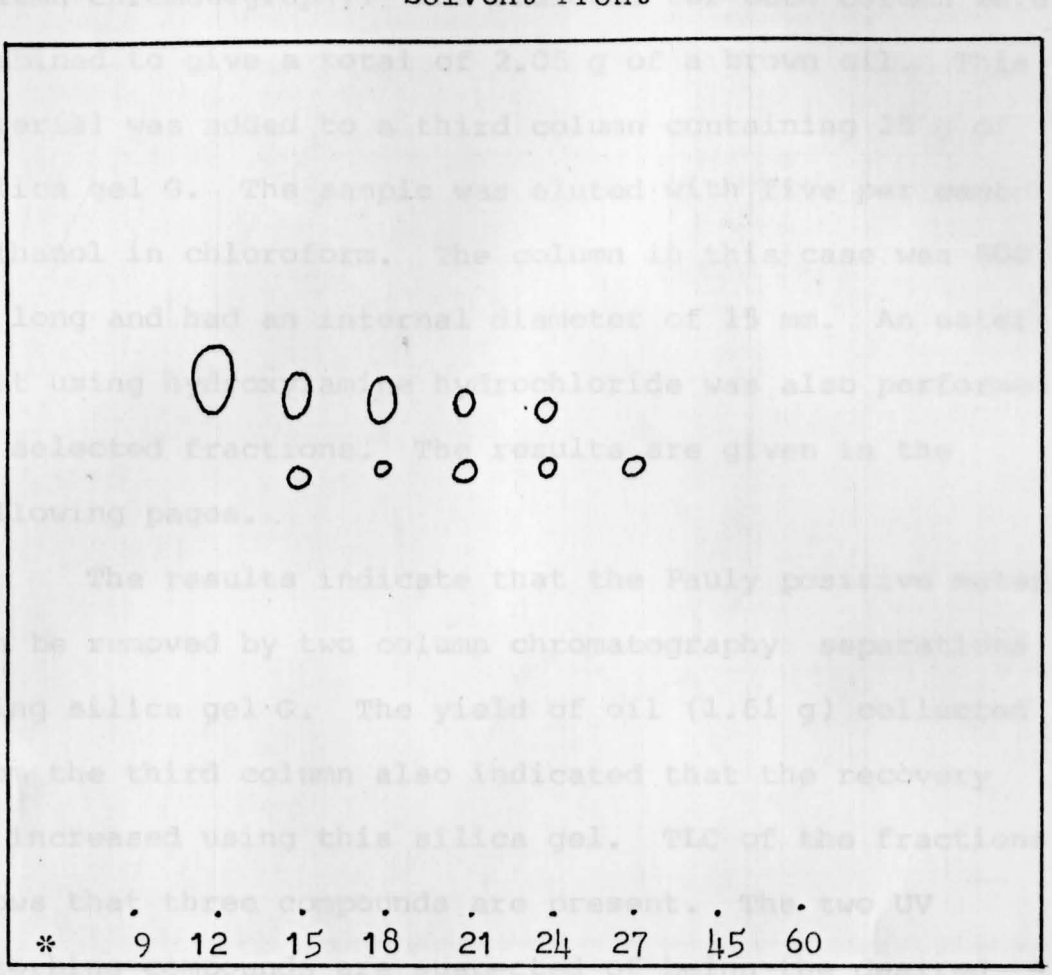


Figure 4. The Second and Third Column Chromatography Runs of the Reduced Histidine Methyl Ester.

The resin used was silica gel for column chromatography (particle size 0.2-0.5 mm). The column dimensions were 600 X 15 mm.



* Fraction Number

Figure 5. TLC of Fractions Collected from Third Column Chromatography Run on Previous Page.

The solvent was five per cent methanol in chloroform. All spots identified by UV absorbance, no spots were Pauly positive.

was removed using silica gel G than for silica gel for column chromatography. The residues for each column were combined to give a total of 2.06 g of a brown oil. This material was added to a third column containing 25 g of silica gel G. The sample was eluted with five per cent methanol in chloroform. The column in this case was 600 mm long and had an internal diameter of 15 mm. An ester test using hydroxylamine hydrochloride was also performed on selected fractions. The results are given in the following pages.

The results indicate that the Pauly positive material can be removed by two column chromatography separations using silica gel G. The yield of oil (1.61 g) collected from the third column also indicated that the recovery is increased using this silica gel. TLC of the fractions shows that three compounds are present. The two UV absorbing compounds are suspected of being the desired compound and an alcohol or acid (COOH) derivative of an imidazoline. The UV negative but iodine positive spot was suspected of being the completely reduced imidazolidine. The data collected from the ester test proves that the UV absorbing material is an ester, strong evidence that the desired imidazoline derivative was synthesized. Spots F,G,H, and I (page 56) were identified with iodine only but are probably only the partially reduced imidazoline and completely reduced imidazolidine compounds, the imidazoline spot was not seen under the UV viewer because

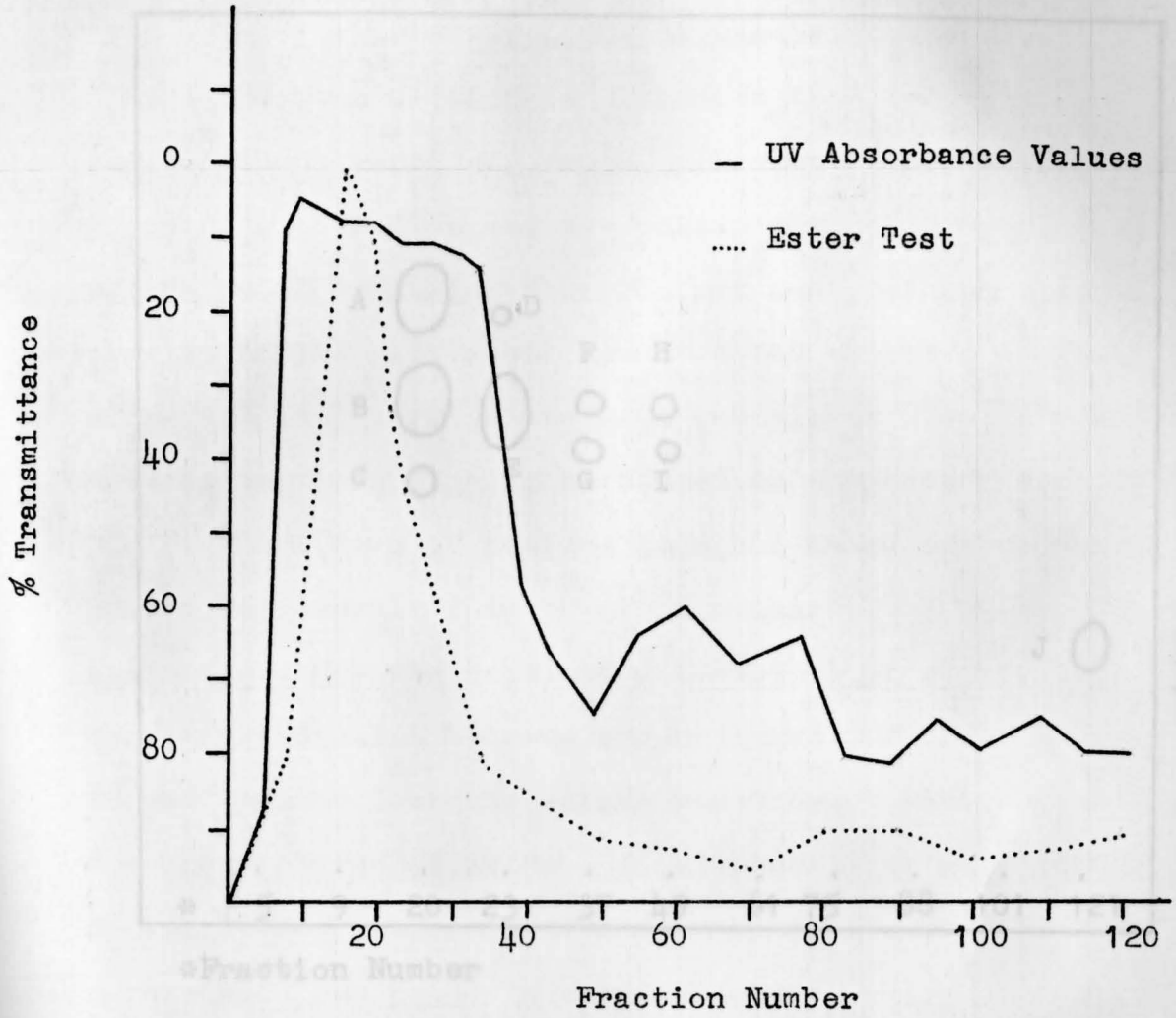


Figure 6. The Third Column Chromatography Run of Reduced Histidine Methyl Ester Using Silica Gel G.

The data from the ester test is also given. The sample was eluted with five per cent methanol in chloroform and the column dimensions were 600 X 15 mm.

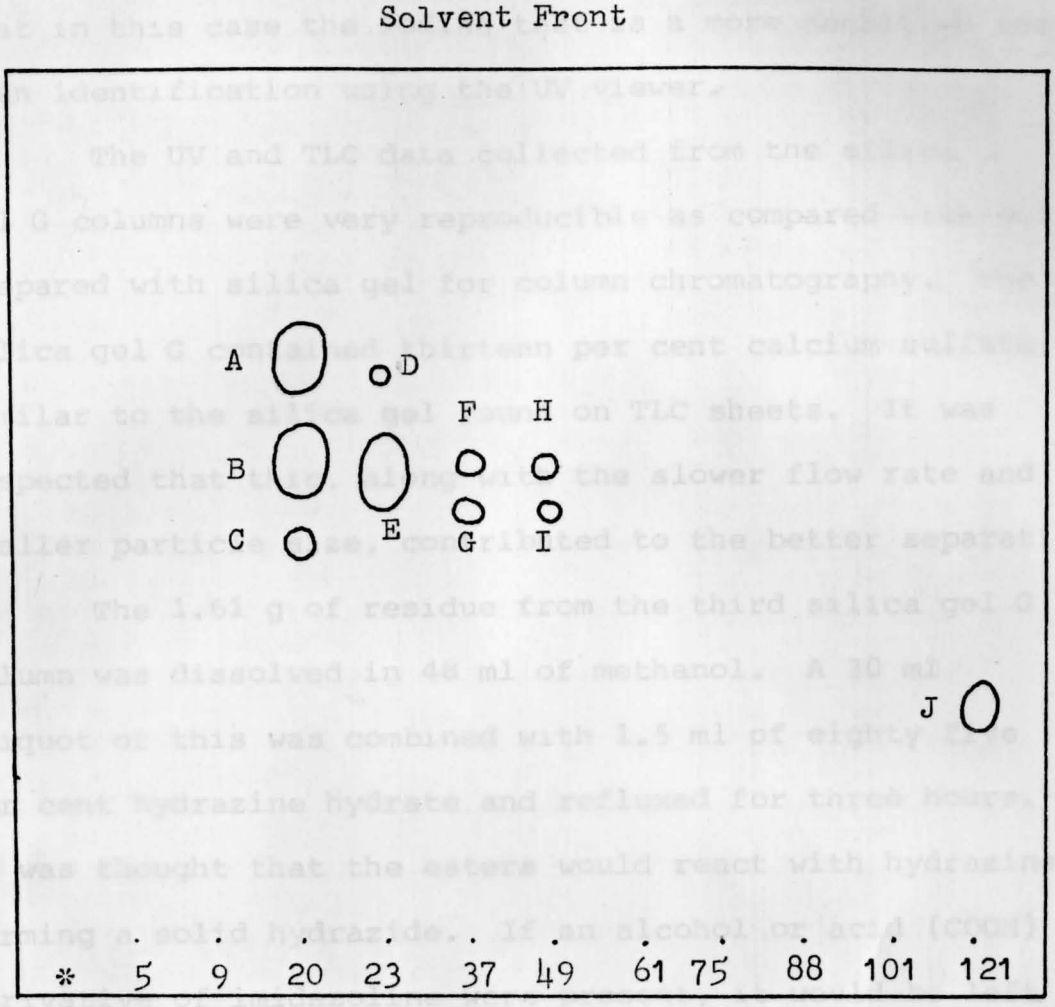


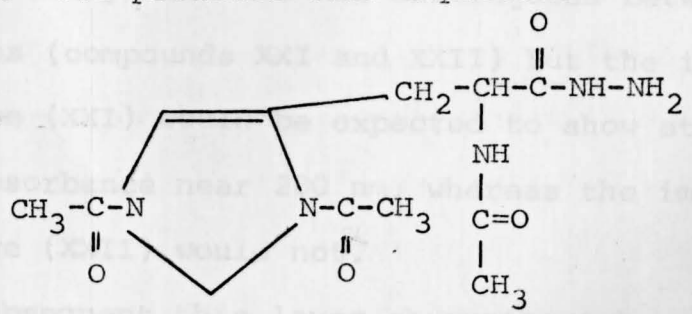
Figure 7. TLC of Fractions Collected from Third Column Chromatography Run of Reduced Histidine Methyl Ester Using Silica Gel G (see previous page).

The developing solvent was five per cent methanol in chloroform. Only spot J was Pauly positive, spots A, B, D and E were UV positive. Spots C, F, G, H and I were only iodine positive. All other spots also gave a positive iodine spot.

the spot was not concentrated enough. This only indicates that in this case the iodine test is a more sensitive test than identification using the UV viewer.

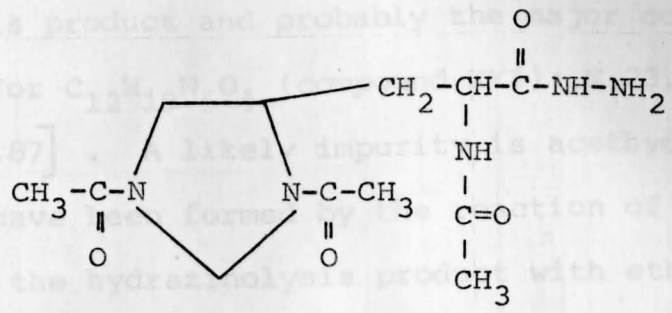
The UV and TLC data collected from the silica gel G columns were very reproducible as compared with columns prepared with silica gel for column chromatography. The silica gel G contained thirteen per cent calcium sulfate, similar to the silica gel found on TLC sheets. It was suspected that this, along with the slower flow rate and smaller particle size, contributed to the better separation.

The 1.61 g of residue from the third silica gel G column was dissolved in 48 ml of methanol. A 30 ml aliquot of this was combined with 1.5 ml of eighty five per cent hydrazine hydrate and refluxed for three hours. It was thought that the esters would react with hydrazine forming a solid hydrazide. If an alcohol or acid (COOH) derivative of imidazoline were present, it would be left unreacted. Two products were expected:



Imidazoline Hydrazide

XXI



Imidazolidine Hydrazide

XXII

Evaporation of the reaction mixture first with a water aspirator to achieve the desired pressure and finally with a vacuum pump overnight resulted in approximately 0.8 g of an off white solid. By recrystallization from hot ethyl acetate, there was obtained approximately 30 mg of white solid, melting point 92-94°C (Fisher Johns stage). The slightly hygroscopic solid was dried with the use of an Abderhalden dryer and submitted for elemental analysis. Elemental analysis would not distinguish between the two hydrazides (compounds XXI and XXII) but the imidazoline derivative (XXI) would be expected to show strong ultra-violet absorbance near 280 nm, whereas the imidazolidine derivative (XXII) would not.

Subsequent thin layer chromatography showed that the compound submitted for analysis was impure. However, the nitrogen analysis was sufficiently close to the calculated value that, when coupled to other data cited below, the inference seems justified that the imidazoline hydrazide

derivative (compound XXI) was a component of the hydrazinolysis product and probably the major component [calculated for $C_{12}H_{19}N_5O_4$ (compound XXI): N,23.56; found N, 24.60, 24.87] . A likely impurity is acethydrazide which could have been formed by the reaction of residual hydrazine in the hydrazinolysis product with ethyl acetate, the recrystallization solvent.

The ultra-violet spectrum of a 0.0016 per cent solution of the recrystallized hydrazinolysis product (i.e. of the same sample which was submitted for analysis) was determined using a Cary 14 Spectrophotometer (Figure 9). Strong UV absorbance with a peak near 280 nm was observed. As mentioned earlier, diacetylimidazoline shows an absorption maximum at 280 nm. Thus, the UV spectrum supplies evidence that the hydrazinolysis product contains as a major product a compound with the diacetylimidazoline ring intact.

That the diacetylimidazolinyl segment of compound XXI is unaffected by the conditions of the hydrazinolysis was further supported by an experiment in which diacetylimidazoline, itself, was refluxed with hydrazine in absolute methanol. A mixture of 1.00 g of diacetylimidazoline, 60 ml of absolute methanol and 3.0 ml of hydrazine hydrate was refluxed for three hours. After evaporation of solvent and excess hydrazine hydrate under vacuum, the melting point of the white solid residue was the same as that of the starting material (melting

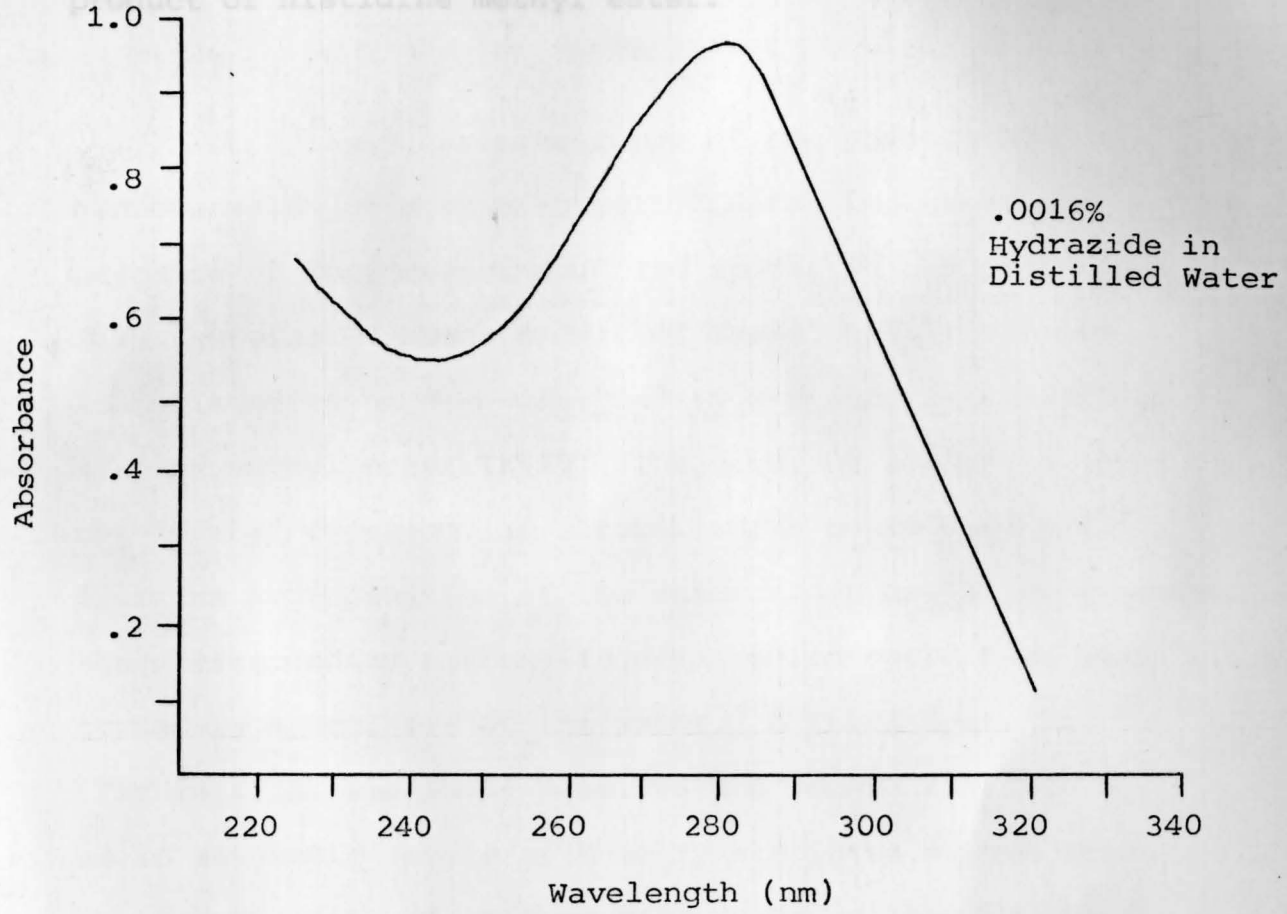


Figure 8. UV Apsorption Spectrum of Hydrazide Formed From Hydrazinolysis of Partially Purified Reduction Product of Histidine Methyl Ester.

point 238-240°C). Thus, diacetylimidazoline survives treatment with hydrazine under the conditions employed in the hydrazinolysis of the partially purified reduction product of histidine methyl ester.

CHAPTER V

Summary

Thin layer chromatography of the product of hydrogenation of histidine methyl ester indicates the presence of four compounds. Two spots, 5a and 5b (Figure III), showing UV absorbance. Of these, the larger one is believed to be α-N-acetyl-β-(N,N-diacetyl-4-imidazolinyl) alanine methyl ester (XVI). The other UV absorbing spot may be the corresponding alcohol which could have arisen from the hydrogenation of the ester group or, alternatively, the corresponding carboxylic acid, which could have been formed by hydrolysis of the ester. A third spot, 5c (Figure III), was Pauly positive and showed the same R_f as an authentic sample of N-acetylhistidine methyl ester. The fourth spot, 5d (Figure III), gave neither UV absorbance nor a Pauly test but appeared upon exposure to iodine vapor. In accord with expectations on the basis of the reported hydrogenation of imidazole in acetic anhydride[16], spot 5d is ascribed to α-N-acetyl-β-(N,N-diacetyl-4-imidazolidinyl) alanine methyl ester (XVII).

Dowex 50W X 4 cation ion exchange resin was found effective in removing the unreduced Pauly positive material. However, the resin caused some destruction of the desired imidazoline derivative and was not used in later experimentation.

Silica gel G for thin layer chromatography (particle size 10-40 μ) was found to give the best separation of reduced histidine methyl ester. These were no apparent side reactions and two column runs were necessary to remove the Pauly positive material.

A color test for identifying esters, involving the use of alkaline hydroxylamine and ferric chloride, was applied to fractions obtained by column chromatography with silica gel G. The spectrophotometric peak for the ferric ion-hydroxamate complex (the product of the ester test) corresponded to the ultra-violet absorption peak for these fractions (Figure 6). This is strong evidence supporting the synthesis of an N-acylated imidazoline derivative (XVI).

The partially purified product obtained after column chromatography with silica gel G, was treated with hydrazine to form the corresponding hydrazide. Although the hydrazide of the desired ester was impure from TLC results and elemental analysis, the ultra-violet absorbance at 280 nm is again strong evidence for the formation of α-N-acetyl-β-(N,N-diacetyl-4-imidazolinyl) alanine methyl ester (XVI by the hydrogenation of histidine methyl ester in acetic anhydride with platinum oxide catalyst.

REFERENCES

1. E.J. Ariens, Molecular Pharmacology, Academic Press, New York, N.Y., 1964.

2. F.M. Bumpus, R.R. Smeby, and P.A. Klairaliah, Peptides: Chemistry and Biochemistry, Dekker Inc., New York, N.Y., 1970, p 128.

3. Ibid., p 148.

4. S. Tatsuoka, and M. Honjo, J. Pharm. Soc. Japan, 73, 355 (1953).

5. A. Kossel, Z. Physiol. Chem., 22, 176 (1896).

6. S. Hedin, Z. Physiol. Chem., 22, 191 (1896).

7. K. Hofman, Chemistry of Heterocyclic Compounds - Imidazole and Derivatives, Part 1, Interscience Publishers, New York, N.Y., 1953, p 7.

8. G. Hunter, Biochemistry J., 30, 1183 (1936).

9. L.F. Cavalieri, A. Bendich, J.F. Tinker and G.B. Brown, J. Am. Chem. Soc., 70, 3875 (1948).

10. L.A. Paquette, Modern Heterocyclic Chemistry, Benjamin Inc., New York, N.Y., 1968, p 186.

11. S.L. Vail, R.H. Barker and C.M. Moran, J. Org. Chem., 31, 1643 (1966).

12. G.M. Badger, The Chemistry of Heterocyclic Compounds, Academic Press, New York, N.Y., 1961, p 188.

13. J.L. Riebsomer, J. Org. Chem., 15, 237 (1950).

14. G. Kirchner, Ann., 98, 104 (1959).

15. W.L. Hawkins and B.S. Biggs, J. Am. Chem. Soc., 71, 2530 (1949).

16. H. Bauer, J. Org. Chem., 26, 1649 (1961).

17. A.A. Vogel, The Textbook of Practical Organic Chemistry, Longman, Green and Co., London, England, 1956 p 470.

18. R.C. West, Handbook of Chemistry and Physics, CRC Press, Cleveland, Ohio, 1975, p C-495.

19. H. Heath, A. Lawson and C. Rimington, J. Chem. Soc., 2215 (1951).

20. M. Lukin: Personal Communication.

21. J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, Freeman and Co., San Francisco, Calif., 1969, p 57.

22. S. Hestrin, J. Biol. Chem., 180, 249 (1949).

23. K. Hofman, Chemistry of Heterocyclic Compounds, Imidazole and Derivatives, Part I, Interscience Publishers, New York, N.Y., 1953, p 201.