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Project Synthesis of N Acetyl L Histidine Ethyl Amide and Standard Operating Procedure for Fractional Collector

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PROJECT SYNTHESIS OF N ACETYL L HISTIDINE ETHYL AMIDE AND STANDARD OPERATING PROCEDURE FOR FRACTIONAL COLLECTOR

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ABSTRACT

Photo oxidation of proteins leads to the formation of cross links of the amino acids in the proteins and leads to induction of cataracts, photo aging of the skin and in the Photodynamic therapy of tumors.

Oxidation of proteins involves significant modification of particular amino acids residues with consequent photodynamic damage. Studies show that tryptophan, tyrosine and histidine side chains gives rise to semi stable peroxides on exposure to singlet oxygen these oxidized structures are studied with low temperature NMR. These peroxides or species derived from them have been postulated as key intermediates in subsequent reactions such as cross linking of oxidized amino acids with unmodified residues. It is difficult to identify modified amino acids in photo dynamically treated proteins and to determine their positions/locations .It is tedious to isolate the cross linked amino acid moieties from proteins, hence low molecular weight histidine and imidazole derivatives have been used to study the detailed reaction mechanisms.

photo oxidation of N acetyl histidine ethyl amide derivative of histidine in which the amine group and the carboxylic acid group have been converted to amide bonds .this conversion makes this amino acid a peptide resembling amino acid so that its photo oxidation mimics the photo oxidation of histidine moiety in proteins.

Use of N-Benzoyl-L histidine as a model compound elucidates the chemical structure and mechanisms of the formation of His-His cross links .Benzoyl group protects the alpha amino group of histidine participation in the cross link reactions .Benzoyl absorbs strongly in UV hence permits the spectroscopic identification

Rose Bengal is used as a photo sensitizer which sensitizes primarily by the singlet oxygen mechanism in the aqueous solution It causes extensive damage to the imidazole ring of Histidine by forming semi stable peroxides which results in the formation of Hydrated Imidazolone with the second molecule of N acetyl histidine

ethyl amide. The formation of this dimer shows the strong possibility of cross linking in the histidine residues in proteins.

Lab synthesized N acetyl L histidine ethyl amide was analyzed by TLC, Melting point and NMR and declared that it was impure.

Purification process was done through re-crystallization and Running the analyte down through silica column by gradient elution using three different solvents Hexanes, Ethyl acetate and Methanol.

Synthesis of N acetyl L histidine ethyl amide meant to synthesize in lab

Lab synthesis is done in two steps:

Step1. Synthesizing N acetyl L histidine ethyl ester from L Histidine

Step2. Synthesizing N acetyl L Histidine ethyl amide from N acetyl L histidine ethyl ester

N acetyl L histidine ethyl ester is synthesized in lab on proceeding upon second step reconversion of N acetyl L histidine ethyl ester to L histidine was observed.

EXPERIMENTAL SECTION:

This involves the following steps

Step1: Preliminary examination of lab synthesized N acetyl L Histidine ethyl amide

Step2: Lab synthesis of N acetyl L Histidine ethyl ester.

Step3: Lab synthesis of N acetyl L Histidine ethyl amide (not proceeded)

Experimental section of Step1 Preliminary examination of lab synthesized N acetyl L Histidine ethyl amide.

Aim: Preliminary analysis of previously synthesized product and Synthesis of N-acetyl L Histidine

Reagents used:

Ethyl amine

Water

L Histidine mono hydrate

Methanol

Ethanol

Dowex Ion exchange resin

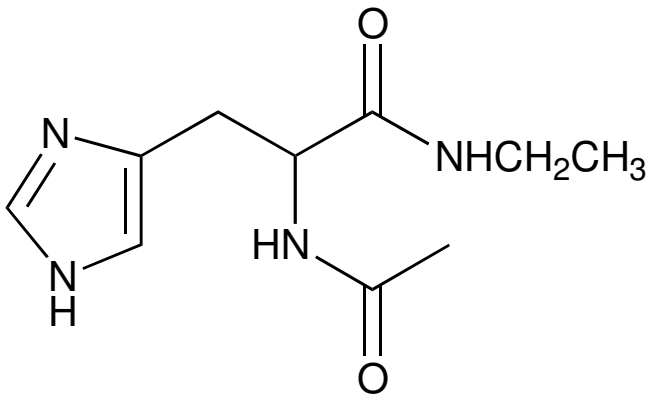
Chloroform

Silica

Concentrated sulfuric acid

Experiment and results:

TLC, melting point, IR analysis and NMR analysis of lab synthesised N acetyl L Histidine ethyl amide is done



N-Acetylhistidine N-ethylamide

Synthesized in our lab earlier

Determination of melting point:

Melting points of 3 different samples are determined

The long range of melting point proves that the sample contains impurity so to confirm the presence of impurity we ran the samples in TLC plate the distance travelled by the samples in TLC plate should be compared with that of the Manufactured N acetyl Histidine ethyl amide so if there is differences in the Rf values of Manufactured and Lab synthesized products we can say that the compound is impure and we have to re-synthesize the compound again

Melting point Observations are recorded in the table shown up below:

Sample Number	**Start (^{0}C)**	**End(^{0}C)**
1	140	221
2	145	169
3	145	170

The melting point range is very high compared to the literature value which was 219 ^{0}C to 220 ^{0}C

TLC analysis of Lab synthesized sample:

The sample was dissolved in ethanol and with capillary tube it was spotted on the TLC plates the following observations are recorded.

Silica plates with U.V absorbance are used and the mobile phases used are ethanol and ethyl acetate.

Sample	Spots	Distance travelled Ethanol/EA(10:90)	Distance travelled Ethanol/EA (50:50)	Distance travelled in Ethanol/EA (90:10)
Sample 1	Spot1	4.9	5.0	5.5
	Spot2	5.0		5.5
Sample2	Spot1	5.0	5.0	5.5
	Spot2			5.5
Sample3	Spot1	5.0	5.0	5.5
	Spot2	5.0	5.0	5.5

IR analysis of Lab synthesized sample:

We have run the sample in the Fourier transform Infrared spectroscopy and determined the characteristic peaks of our compound.

The characteristic peaks are shown in the table below

S.NO	FUNCTIONAL GROUP	CHARACTERISTIC ABSORPTION	PEAKS ABSERVED
1	Amide(Carboxyl acid derivative)	1650	1659.4
2	CH_3-CH_2 Alkyl C-H stretch	1470 2950 – 2850	1473.4 2750
3	CH=CH Alkenyl C-H stretch	1640-1680 3010-3100	1659 & 1684 3035.2, 3137.9
4	Amide C=O stretch	1690 – 1630	1627.3
5	Amide N-H stretch	3500- 3700	--
6	Amine N-H	3300 – 3500	3035.2, 3137.9 , 3230.8, 3298.4,3334.3

NMR analysis of Lab synthesized Sample:

We had analyzed the N-acetyl histidine N ethyl amide which was synthesized in our lab earlier on Proton NMR with DMSO as solvent.

The Assignment of peaks for our sample is shown in the table below:

Peak #	Chemical Shift, ppm	Area (Relative # of H)	Splitting	Assignment
1	0.9	3H	-	A
2	1.3	3H	Singlet	J
3	2.1	H	-	ABMX
4	2.5	2H	Doublet of doublet	B
5	3.80	1H		D
6	6.10	1H		G
7	7.2	1H	Singlet	H

9	7.9	1H	Doublet	I

Results regarding Preliminary studies of Pre synthesized product:

From previous analysis we have found that the sample (N-acetyl Histidine ethyl amide) synthesized previously by Jagadeesh is impure as the melting points are very broad. Hence purification of the product is necessary.

So we have done Recrystallization

Recrystallization is a process for purifying the compound.

Re-crystallization Procedure:

To recrystallize the sample about 1.008g of sample is dissolved in 35 ml of ethyl acetate

And this is refluxed under a very low heat and about 83.5 ml of ethanol is added in 0.5 ml intervals until the product is dissolved

99% ethanol is used; this is kept in refrigerator for overnight until the crystals are formed.

Analysis of re-crystallized product: (Discuss about recovered product)

Melting point determination:

The Melting point is determined to be 163^{0C} – 175^{0}C

Recovered mass after re-crystallization is 0.91 g

From this we have concluded that N-acetyl Histidine ethyl amide synthesised by previous batch is impure or may be the reaction does not preceded hence it needs to be resynthesized.

Sep2: Synthesis of N acetyl L Histidine Ethyl Ester:

Experimental section for Synthesis of N acetyl L Histidine ethyl amide:

Step 2 Synthesis of N acetyl L histidine ethyl ester:

Take 1.02 g of N acetyl L Histidine and added 50ml of methanol and 1 ml of Sulfuric acid for 4 hours.

After 4 hours of reflux we tested it on TLC plates for confirming that the reaction is done at different concentrations of Ethyl acetate and ethanol.

The starting material is N acetyl L Histidine and final product is N acetyl L Histidine ethyl ester.

TLC analysis of L Histidine and L Histidine ethyl ester is done

These samples are dissolved in methanol

Ethyl acetate concentration	Ethanol concentration
50%	50%
90%	10%
60%	40%
80%	20%
30%	70%

On TLC analysis the first spot is starting material and the second spot is the final product.

There is a drag in the second spot is observed.

It means not all the L histidine is converted to N acetyl L Histidine ethyl ester.

Hence the reaction is kept for some more hours by refluxing it adding 0.5 ml of sulfuric acid.

This time we have refluxed it for 6 hours.

Rotovap: This is done for removing the ethanol and a liquid film is observed after rotovapping.

Separation of Ester through DOWEX column:

Add 100 ml of water to it after adding water the ester is dissolved in water.

Building a DOWEX column:

30g of Dowex Ion exchange resin is taken and 70ml of water is added to it and this slurry is prepared.

At the bottom of the column a cotton plug is inserted which acts as a slit.

This slurry is added to the column.

The sample solution is added to the column with a pasteurized pipette.

Collect all 100 ml of water with sample in the Erlenmeyer flask.

Add 50 ml of water to the column collect it in an Erlenmeyer flask

Add 25 ml of water to the column collect it in an Erlenmeyer flask

All this solution is rotovapped a liquid oily film is obtained.

Next add 50ml of water again to the resin column and Rotovap it. this is to ensure that everything sample in the column came outside.

This 50ml is also rotovapped.

IR Analysis:

IR analysis will determine the functional group as N acetyl L histidine is carboxylic acid whereas the N acetyl L Histidine ethyl ester is an Ester.

On doing IR analysis we have not seen the carboxylic acid peak but we have observed the OH peak may be while rotovapping there is a little bit of water is left in the flask and this was showing up in the IR.

From the IR data we have confirmed that the reaction was proceeded and the product ester was formed.

Results for IR peaks observed after Reaction with methanol with L Histidine:

Observed values	**Allotted functional groups**
3300- 3500 cm^{-1}	Broad alcohol peak
1754 cm^{-1}	Ester peak

Synthesis of N acetyl L Histidine ethyl Amide:

To the sample solution rotovapped from the previous step, an oily layer is obtained.

25 ml of 70% ethyl amine solution is added to the oily layer and kept for stirring over night (10 hours).

The solution after the overnight stirring, it is rotovapped, after rotovapping an oily yellow liquid film is observed.

Addition of chloroform:

To the yellow liquid film 55 ml of chloroform is added. Yellow layer floating on the chloroform is observed. This is kept on a low heat and refluxed for 40 mins.

An oily creamy white precipitate is observed.

This is rotovapped to remove chloroform, till a level of 15 ml of chloroform should be present in the flask.

After rotovapping vacuum filtration is done to remove the chloroform and saved the filtrate as it contains the unreacted ester in it.

Chloroform dissolves the unreacted ester.

Rotovapping the filtrate from the vacuum pump is done and I observed precipitate.

I have emptied the flask by adding chloroform second time and I have observed no precipitate in the flask after vacuum filtration. After rotovapping it I have NOT observed precipitate in it.

Solubility test:

This product formed have showed up Insolubility in ethyl acetate and slightly soluble in ethanol

Recrystallization:

I have recrystallized the First crop with 125 ml of ethanol added to it and kept overnight for recrystallization.

The recrystallized product is recovered and the weights are as follows

Weight of the empty vial: 2.0565 g

Weight of the vial and product: 2.1680 g

Recovered weight: 0.1115 g

IR analysis:

IR to the recrystallized product is done. We have observed the broad Carboxylic acid peak. Further analysis is needed and re synthesis should be done. Here reconversion of the product back to the acid is observed.

IR results for Peaks observed after reaction of N acetyl L histidine methyl ester with Ethyl amine

Observed values	**Standard values**	**Assigned functional group**
2624-2966 cm^{-1}(Cow peak)	2500-3300 cm^{-1}	Carboxylic acid O-H stretch
1639.5 cm^{-1}	1640- 1700 cm^{-1}	C=O Amide stretch
1566.8-1639.5 cm^{-1}	1550-1640 cm^{-1}	N-H amide bend

Step3: Purification of the Pre-synthesized N acetyl L histidine ethyl amide:

Experimental section for purification:

Solubility Test for Jagdeesh sample 1:

Ethanol: Sparingly soluble

Methanol: soluble

Ethyl acetate: insoluble

Hexanes: Insoluble

Total weight of compound 1: 0.1053g

Building up a silicon column:

First the column was cleaned with acetone and next washed with Hexanes.

Hexanes solvent is filled in the column by continuous addition of silica and tapping the column making sure that there are no air bubbles in it.

About 50 grams of silica is used for building the column.

The mobile phases used are Hexanes, Ethyl acetate and Methanol.5%, 10%, 20%, 30%, 50% Hexanes, Ethyl acetate and Ethyl acetate and Methanol increasing concentrations.

Concentration	Hexanes/Ethyl Acetate(25ml)	Ethyl acetate/Methanol(25 ml)
5%	23.75/1.25	23.75/1.25
10%	22.5/2.5	22.5/2.5
20%	20/5	20/5
30%	17.5/7.5	17.5/7.5
50%	12.5/12.5	12.5/12.5

The column is placed on a Fraction collector and the program in the fraction collector is set for collection for about 200 seconds.

Fractions are collected from fraction collector From Tubes 38 to79 and 85, 86, 189.

Before running the sample on column I have tested the Sample on TLC with different concentrations

5%, 10%, 15% methanol and Ethyl acetate concentrations for L histidine and Compound 2

And found out that at all the concentrations the starting material and the final product have same RF values.

And we have changed the concentration to be 5 % Hexanes, 4 drops of Methanol and 94% ethyl acetate we have got two different spots.

Hence at this concentration we have run all the fractions TLC (5% Hexane, 4 drops of methanol, 94% Ethyl acetate).

Standard operating procedure for Fraction collector:

1. **OBJECTIVE:**

   1.1 To describe a procedure operating Spectra/Chrom CF-1 fraction collector

2. **ACCOUNTABILITY:**

   2.1 Prof. Dr. Kumar

   2.2 Prof. Kent

3. **RESPONSIBILITY:**

   3.1 Graduate assistant/Research student operating it

4. SCOPE AND APPLICABILITY:

4.1 This procedure illustrates the operation of Fraction collector for collection of the analyte that separates from the LC columns. Collection of the analyte can be done based on time, Drop counts and fraction volume.

5. SUMMARY OF THE METHOD:

5.1 First the instrument is setup on a clean bench top and the separation column is aligned straight above the collection tubes and the method in the fraction collector is setup to collect the analytes separated through drop counts.

We can operate the instrument in two ways with peak separator and without peak separator.

6. TIPS OPERATING THE INSTRUMENT:

6.1 Make sure that the collection tubes are of sizes between 10-28 mm. for better results use 12 or 13 mm tubes

6.2 Ensure the collection tubes are properly placed in the allotted rack

6.3 Ensure that the bench top on which the instrument is placed on clean surface

6.4 Make sure that all the collection tubes racks are properly labeled with designated numbers.

6.5 For organic solvents or mobile phases better use glass collection tubes.

6.6 While operating the instrument for collection of fractions through drop counts the drop counter should be used.

6.7 Ensure that the drop collector is plugged at the back of the fraction collector

6.8 Ensure that tube counter is placed high enough because of two reasons

- It can scan the drops falling through the column
- The test tubes able to pass through the drop counter

7. PROCEDURE FOR OPERATING THE FRACTION COLLECTOR WITH OUT PEAK SEPARATOR:

7.1 Turning off the peak separator

7.1.1 Turn POWER button ON by clicking it

7.1.2 Press C key to set the time format

7.1.3 Press B key to select time format in minutes and seconds

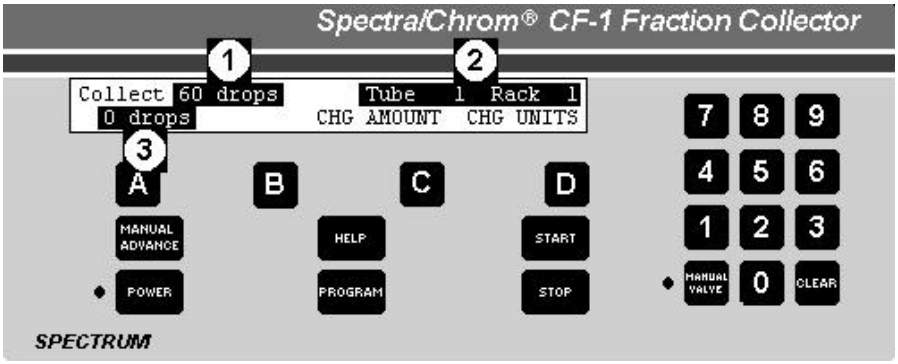
7.2 Selection of valve

7.2.1 Press A key to select not using the valve (We have to select the valve on when using peak separator ON)

7.2.2 The final question if we opt to use Peak separator. Press A key to select NO

7.3 Normal Operation for fraction collection

7.3.1 Here the display will be like fig following figure



7.3.2 Press D key and change the units the display says fraction collection size through Drop count.

7.3.3 To select the drop count Press B key.

7.3.4 Enter the desired amount to be collected in the test tube.

7.3.5 220 drops is approximately 10 ml.

7.4 Stopping the fraction collection:

7.4.1 Press D key to have the fraction collector stop when the red rack is reached.

7.4.2 Press START to resume the run with the specifications given.

7.5 Storing the new method and retrieving the saved program:

7.5.1 Press STOP

7.5.2 Press PROGRAM

7.5.3 Press D key once then the second program menu will be shown

7.5.4 Press C key to select memory options

7.5.5 Press A to save the method or Press B to recall already saved programs

7.5.6 After pressing A select letters A or B or C or D in which slot you wanted to save the program.

7.5.7 After pressing B select letters A or B or C or D which program you want to select, which was saved already

7.6 Preview the edited or selected program

7.6.1 Press STOP

7.6.2 Press PROGRAM

7.6.3 Press D key twice until the third program menu is shown

7.6.4 Press the B key to select the preview.

7.6.5 Press A key enable the preview

7.6.6 Press D to display the method which we have developed or wanted to recall already saved method.

7.6.7 Press START to begin the fraction collection

OBSERVATIONS:

And observed spots at 55, 56.

Combined the fractions 39-49, 50-59, 60-69, 70-79, (189, 85, 86).

And Roto-vaporated them

We have got a light yellow color oily layer from the fraction of 50-59.

No Oily liquid film is observed at 39-49, 60-69, 70-79 and 85 -86.

On preliminary examination we have found out that the compound was impure so we have purified the compound through re-crystallization and by building a silica column and running the sample with different concentrations of mobile phase Hexanes, Ethyl acetate and Methanol.

DISCUSSION:

Discussion on Melting point results of Pre synthesized sample:

As the melting points of pre- synthesized sample reveals broader trend which means the product N acetyl L histidine ethyl amide synthesized is impure.

Discussion on IR results of Pre synthesized sample:

The IR results of pre synthesized sample reveals that the presence of amide peak, absence of Ester peak and absence of carboxylic acid peak which means that all the N acetyl L Histidine monohydrate (carboxylic acid) is converted to N acetyl L histidine ethyl amide.

But the broader melting points reveal that the product is impure. Hence purification procedures like Re-crystallization is performed.

Discussion of IR results taken after reaction of the L Histidine with methanol and Sulfuric acid:

Here in this reaction concentrated sulfuric acid acts as a catalyst. The IR data reveals the absence of Carboxylic acid cow peak which will be present in L Histidine is not seen. The presence of ester peak at 1754 cm^{-1} explains the conversion of carboxylic L Histidine is completely converted to N acetyl L Histidine ethyl ester.

The presence of Broad Alcohol OH peak at 330-3500cm^{-1} is due to the presence of methanol which is remained in the round bottomed flask even after rotovapping.

Discussion of IR results taken after the reaction of N acetyl L Histidine ethyl ester with ethyl amine:

Here in this IR data three different peaks are observed

The carboxylic acid C=O cow peak is observed

The ester peak at 1754 cm^{-1} is not observed which illustrates that all the ester is reacted.

The amide C=O stretch is also observed and Amide N-H bend is observed in the IR data. We can say that the reaction of amine and ester produced two products one amide is formed and the other is converted back to Carboxylic acid L Histidine.

Discussion on Building up the silica and Ion exchange resin columns:

While building up the silica column N Hexanes is used as a solvent.

Care is taken to prevent the air bubbles in the column through continuous tapping of the column while filling up the column. The presence of air bubbles in the column causes improper separation of the product.

Ion exchange resin column is used for purifying the ester product.

Discussion on Standard operating procedure:

The fractional collector can be collected in two ways one by collecting just the fractions and by collecting the peaks along with the fractions. This can be done by connecting the UV detector to the fractional collector. Here I have just collected the fractions separated through the Silicon column.

We can collect the fractions based on Time, Drop count and Volumes.

Collection of fractions based on Time can be selected if the flow is consistent.

Collection of Fractions based on Drop count can be done by placing the Drop counter on the Collector unit.

Drop counter have two diodes placed on either side and when the light between the diodes is interrupted by falling drop then it counts the drop.

Collection of fractions based on volumes this can be also selected but we need special cables and pumps to collect through volumes.

Hence by considering all these factors I have selected collection of fractions through Drop counts.

The Drops to count is selected as 220 drops.

CONCLUSION:

Preliminary examination of pre synthesized lab sample (N acetyl L histidine ethyl amide) demonstrates that the sample is impure may be not all the Ester is converted to Amide.

The synthesis 1 (synthesis of N acetyl L Histidine ethyl ester) is done as we can confirm from the IR taken after the synthesis we have not observed any carboxylic acid peak.

The synthesis 2 (Synthesis of N acetyl L Histidine ethyl amide) is not done may be further reaction by the addition of Ethyl amine is necessary.

About 0.1115g of the product is recovered may be the unreacted ester is present in the Crop 1 Vacuum filtrate flask, which should be reacted again with the ethyl amine again.

Standard operating procedure for Fraction collector is written.

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