Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXVI, No. 10

A Reinvestigation of the Synthesis of [$^{15}N_2$]Hydroxymethylimidazole: Useful in an Improved Synthesis of (D,L)-[τ,π-$^{15}N_2$]Histidine

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SUMMARY

We have reinvestigated the synthesis of [$^{15}N_2$]hydroxymethyl imidazole from dihydroxyacetone, formaldehyde, and labelled ammonia in an attempt to optimize the yield from the isotope. Conversion of the hydroxymethyl imidazole to histidine was accomplished in good yield without the need for picric acid as an ion pairing agent.

Keywords: [$^{15}N_2$]Hydroxylmethylimidazole, (D,L)-[τ,π-$^{15}N_2$]Histidine.

INTRODUCTION

The amino acid histidine plays an important role in many biological functions. It is well known from structural and mechanistic studies that serine proteases, such as chymotrypsin[1] and ribonuclease[2], have one or more essential histidine residues at their active sites. Respiratory enzymes, such as the Rieske type, have also been shown to possess two histidines which are coordinated to the [2Fe-2S] cluster[3]. In addition, the enzyme nitrous oxide reductase possesses a histidine metal cluster[4]. Moreover, histidine is an essential component in a wide variety of other reaction types and protein functions. A very elegant example of the use of ^{15}N labeled histidine has led to the clear confirmation of the Blow charge-relay[5] for the catalytic triad found at the active sites of serine proteases[6]. The utility of histidine in enzyme structure and function is related to its relatively high nucleophilicity and subsequent formation of unstable intermediates, its ability to serve as the donor in a hydrogen bond (thereby acting as a general acid catalyst)

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CCC 0362–4803/95/100947–05

Received 13 February 1995
Revised 24 April 1995

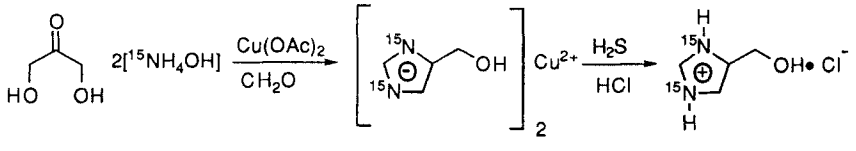
and the ability of the neutral imidazole ring to serve as the acceptor in a hydrogen bond (thereby acting as a general base). Essential to all such studies is the ability to provide specifically labelled histidine to the appropriately engineered organisms that are producing the enzyme or protein of interest.

Histidine was discovered in both 1896 in salmon sperm and a hydrolysate of casein. A number of syntheses of this amino acid have been described, beginning in 1930 with the synthesis of hydroxymethylimidazole (HMI) from fructose, NH_3, HCHO, and copper carbonate salts with a claimed yield of 50 - 70 %[7]. Subsequently, it was found that a mixture of dihydroxyacetone, NH_3, HCHO, and copper carbonate salts would give HMI in ~70 % yield (based on dihydroxyacetone)[8]. Several procedures have been described for obtaining isotopically labelled histidine[9]. Because the above synthesis begins with simple synthons, the formation of HMI from dihydroxyacetone, NH_3, HCHO, and cupric salts is appealing. We have revisited this procedure for the construction of labelled HMI and have optimized its conversion to labelled histidine.

RESULTS AND DISCUSSION

The construction of HMI is illustrated in Scheme 1. Starting from the dihydroxyacetone dimer, aqueous [^{15}N]ammonia, and an aqueous solution of formaldehyde, formation of the HMI is rapid, occurring in less than 30 minutes. It is well known that HMI forms complexes with copper salts which are insoluble in aqueous solution[10]. This process serves to remove the product from the reaction medium. The brown-green precipitate is collected by filtration and then resuspended in water. After extensive TLC analysis, we have concluded that the copper salt precipitation occurs in a relatively pure manner. Ligand exchange was affected with hydrogen sulfide and the copper sulfide rapidly precipitated from the solution. The solution was cooled and then filtered. The solution was then treated with Norite, filtered and acidified with HCl (pH =

Scheme 1



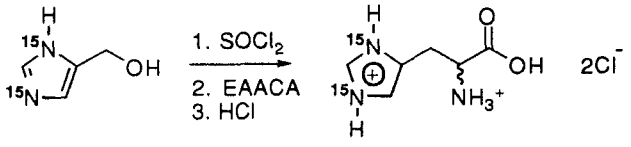
0.9). Based on these results we feel that ion-pairing of the HMI with picric acid, as described with previous syntheses[7,8], is no longer a necessary process step. Picric acid (2,4,6-trinitrophenol) is not only toxic, but is highly shock sensitive, undergoing spontaneous ignition when in a slightly desiccated form. Furthermore, this hazardous material requires unique disposal efforts. We have found that simply removing the volatiles in the acidification step gives the HMI HCl salt, which is identical to the commercial grade material.

With the goal of optimizing the reaction, we have performed a set of experiments which used varying amounts of ammonium hydroxide. The results of these experiments indicate that a 1.55 molar excess of ammonium hydroxide with respect to the dihydroxy acetone gives consistent results. The use of stoichiometric amounts resulted in drastically diminished yields. Generally, the yield of the one-pot reaction affords the HMI HCl in

59% based on dihydroxyacetone, and 37% on $^{15}NH_4OH$. Considering the complexity of this reaction the fact that it occurs in 37% yield is noteworthy.

Conversion of the HMI into DL-histidine is straightforward, as illustrated in Scheme 2. Conversion of the HMI to the chloromethyl derivative is effected with freshly distilled boiling thionyl chloride[11]. The excess thionyl chloride was removed, followed by the addition of the sodium anion of ethyl acetamidecyanoacetate (EAACA) in freshly distilled and dried ethanol (from Mg^0). The protecting groups were then cleaved under acidic conditions to give the crude histidine. Purification was performed using BioRad Dowex 50W-X8 resin and using HCl as the eluent to give histidine in 80% yield. We have performed this reaction to give the histidine racemate in 8 gram quantities. In addition, this series of experiments can be performed in a period of 3-4 days.

Scheme 2



CONCLUSION

This 2-step synthesis of labeled histidine from inexpensive precursors is simple and can be conveniently scaled up to provide 10 gram quantities. Moreover, the synthesis of other isotopomers, such as [2-^{13}C] or [2-^{13}C, $^{15}N_2$]HMI, can be realized by the use of [^{13}C]formalin. We are currently investigating routes using the labeled 4-chloromethyl-[$^{15}N_2$]imidazole with a chiral auxiliary-promoted synthesis of (L)-histidine isotopomers[12].

Chemicals--[^{15}N]Ammonia (99.2% ^{15}N) was prepared at Los Alamos National Laboratory. Dihydoxyacetone dimer, thionyl chloride, ethyl acetamidecyanoacetate (EAACA), formaldehyde (aqueous), and copper acetate were purchased from Aldrich Chemical Co. and used without purification.

NMR Methods--Proton, The ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded as DMSO-d6, CD_3OD, or D_2O solutions on an Bruker AM-200, AC-250, WM-300, AMX-500 NMR spectrometers. ^{1}H chemical shifts are expressed in parts per million with respect to tetramethylsilane at 0.0 ppm; ^{13}C chemical shifts are referenced with respect to internal $CDCl_3$ (δ = 77.0 ppm with respect to tetramethylsilane at 0.0 ppm), DMSO (39.5 ppm), CD_3OD (49.0 ppm), or D_2O (externally referenced to a D_2O/methanol mixture); ^{15}N NMR chemical shifts are referenced with respect to 2.5 M solution of potassium [^{15}N]nitrate. Analytical thin-layer chromatography (TLC) was carried out on glass plates (silica gel 60 Å 250 μm thickness) obtained from EM science. TLC visualization was accomplished with a UV lamp, I_2 staining, and an ethanolic solution of phosphomolybdic acid (PMA). Moisture-sensitive reactions were performed in flame-dried glassware under a positive pressure of nitrogen or argon.

[$^{15}N_2$]Imidazole-4-methanol--To a 2-neck, 1 L-flask containing a magnetic stir bar was placed 150 mL of deionized, distilled water. To this, with stirring at ambient temperature, was added 20.0 g (0.11 mol) of coppper (II) acetate. The flask was placed in a 95°C water bath and stirred until the solution was at 75-80°C. To this was added 7.75 M $^{15}NH_3$ (5.58 g; 0.31 mol) in water. The solution clouded momentarily then turned a deep blue. To the blue solution, at 85°C, was added 9.0 g (0.05 mol) of the dihydroxyacetone dimer in 30 mL of water, and 20 mL (0.27 mol) of a formaldehyde solution at a rate of 6 mL per minute until the addition was completed. After one-half of the solution was added the reaction mixture became a brown/olive green suspension. All of the dihydroxyacetone solution was added after 10 minutes. The reaction was heated for an additional 1.5 h at 90-96°C and then chilled to 4°C. The resulting copper complex was then filtered to give 19 g of a brown solid. The complex was then suspended in 250 mL of water, heated to 45°C, and H_2S was bubbled through for 20 min. The copper sulfide sludge was filtered and the clear reddish brown liquid was collected. Norite was added and the mixture was stirred overnight. The solution was filtered through washed Celite and the solution of pH = 8.0 was acidified with 7 N HCl to pH = 0.9 with stirring. Removal of the volatiles gave 8.11 g (0.059 mol) of a white to slightly tan HMI hydrochloride. ^1H (D_2O) δ 4.68 (s, 2H, C*H*$_2$OH), 7.45 (t_{app}, J = 4.2 Hz, 1H, H_5), 8.85 (t, J = 5.6 Hz, 1H, H_2); ^{13}C (D_2O) δ 54.4 (s, CH_2OH), 117.6 (d;^{13}C-^{15}N), 133.5 (d;^{13}C-^{15}N), 134.9 (t;^{13}C-^{15}N, C_2); ^{15}N (D_2O) δ -204.5 (br s), -205.4 (br s).

4-Chloromethyl-[$^{15}N_2$]imidazole--The dried HMI HCl solid in 20 mL of thionyl chloride was refluxed for 3 h. The dark solution was reduced *in vacuo* and the resulting brown solid was dried under a vacuum. This crude material was used directly without purification. ^1H (DMSO-d6) δ 4.83 (s, 2H, C*H*$_2$Cl), 7.32 (d, J = 84 Hz, 1H, ^{15}N-H), 7.67 (t, 1H, H_5), 9.1 (t, 1H, H_2); ^1H (CD_3OD) δ 4.83 (d, 2H, 5.8 Hz, C*H*$_2$Cl), 5.8 (br s, HDO), 7.67 (t, J = 3 Hz, 1H, H_5), 8.97 (t, J = 4 Hz, 1H, H_2); ^{13}C (CD_3OD) δ 34.6 (s, CH_2Cl), 119.2 (d, $J_{^{13}C\text{-}^{15}N}$ = 9.1 Hz), 136.0 (d), 136.3 (t, $J_{^{13}C\text{-}^{15}N}$ = 15 Hz, C_2).

(D,L)-[τ,π-$^{15}N_2$]Histidine-dihydrochloride--Chloromethyl imidazole (4.3 g; 0.062 mol) was dissolved in dry freshly distilled ethanol (distilled from Mg^o), in a dried round bottom flask, under argon. The solution was then chilled to -5°C and was added to a -5°C ethanol (60 mL) solution of the sodium salt of EAACA [8.9 g; 0.05 mol of EAACA with 0.13 mol of NaOEt). The mixture was then stirred at ambient temperature for 14 h under argon. The solvents were removed *in vacuo* to give the crude material. The resulting solids were then dissolved in 50 mL of 6 N HCl and refluxed for 16 h. The mixture was concentrated and the crude histidine was taken up in 100 mL of water and stirred with Norite overnight. The mixture was then filtered through Celite, concentrated, and applied to a Dowex 50W-X8 (500 g) column. The column was prepared as follows: The resin was washed with 6.0 L of water, then filtered and stirred with 1 N HCl. The resin was washed with water and filtered. The resin was washed with in 1 N NaOH solution, stirred, filtered, washed with water, then filtered. Water was applied to the resin until the pH = 7.0. The resin was then washed with 2.5 L of 1.5 N HCl. The crude histidine was dissolved in 200 mL of water and poured onto the column and allowed to

elute. Water (6 L) was run through the loaded column, then 2 L of 1.45 N HCl followed by 4 N HCl. The histidine eluted with the 4 N HCl. The fractions containing the histidine were concentrated to dryness. A total of 5 g of a yellow solid (80%) was isolated. The histidine was recrystallized by dissolving the material in a minimal amount of methanol. The methanolic solution was then added dropwise to a 1:100 methanol: 2-propanol solution with stirring. The resulting white suspension was then reduced to a volume of ~25 mL and the solids were filtered give the purified material. ^1H (D_2O) δ 2.98 (m, 2H, CH_2), 3.96 (t, J = 8 Hz, 1H, CH), 6.96 (t_{app}, $J_{^1H-^{15}N}$ = 3 Hz, 1H, H_5), 8.2 (t, $J_{^1H-^{15}N}$ = 8 Hz, 1H, H_2); ^{13}C (CD_3OD) δ 26.5 (C_3), 53.0 (C_2), 119.7, 128.5 (d, $J_{^{13}C-^{15}N}$ = 10 Hz), 135.6 [t, $J_{^{13}C-^{15}N}$ = 14 Hz, $C_{2(imid)}$], 170.1 (C_1); ^{15}N (D_2O) δ -208.4 (t_{app}, $J_{^{15}N-^1H}$ = 4 Hz, N_τ), -210.6 (m, N_π).

Acknowledgment. This work was supported by the National Stable Isotopes Resource, NIH Division of Research Resources (RR 02231).

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