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SYNTHESIS OF 2-AMINO-1,5-DIHYDRO-1-(METHYL-^{13}C)-4H-IMIDAZOL-4-ONE-5-^{13}C (CREATININE-$^{13}C_2$)

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SUMMARY

2-Amino-1,5-dihydro-1-(methyl-^{13}C)-4H-imidazol-4-one-5-^{13}C (creatinine-$^{13}C_2$) (8) is synthesized as follows. Glycine-2-^{13}C (1) is tosylated; the product (2) is treated with iodomethane-^{13}C; and the resultant doubly-labeled product (3) is detosylated to yield sarcosine-$^{13}C_2$ (4). Creatine-$^{13}C_2$ (7) is obtained by treating 4 with 2-methylisothiourea monohydriodide (5) or cyanamide (6). Dehydration of 7 under vacuum sublimation conditions gives 8.

Key Words: carbon-13, creatinine, creatine, sarcosine, glycine, isotope dilution-mass spectrometry

INTRODUCTION AND DISCUSSION

Isotope dilution-mass spectrometry (ID/MS) is utilized at the National Bureau of Standards for obtaining definitive concentration values for organic analytes such as cholesterol [1] and urea [2] in human serum. We have developed an ID/MS method for measuring serum creatinine [3,4] that uses creatinine-$^{13}C_2$ (8) as the internal standard.

Initially, we attempted to use 2-amino-1,5-dihydro-1-

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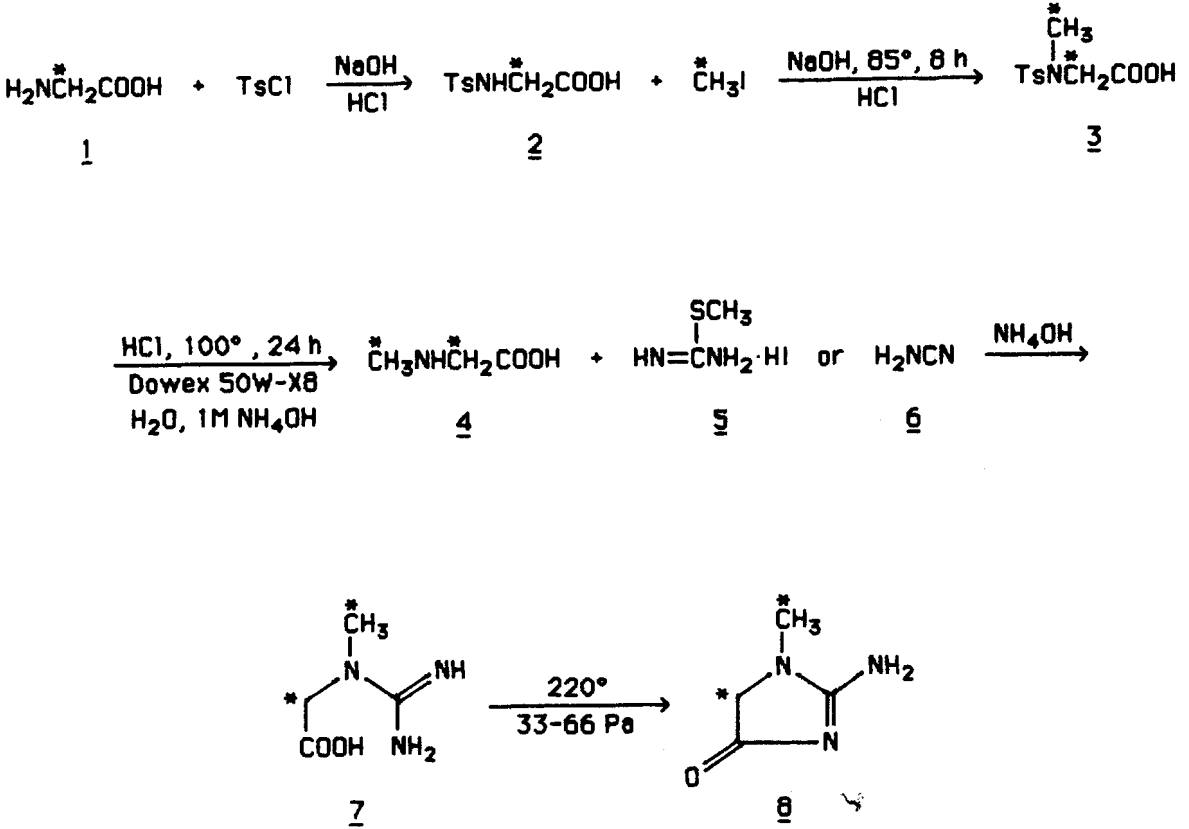
(methyl-d_3)-4H-imidazol-4-one, (creatinine-d_3) as the internal standard. Inconsistent results were obtained possibly because of an isotope effect that occurred in the derivatization of the creatinine, one of the steps in the analytical procedure. Consistent results were obtained with creatinine-$^{13}C_2$ (8) instead of creatinine-d_3. Since the procedures for synthesizing the two labeled compounds are similar, only the synthesis of 8 is described.

The synthesis of 8 is illustrated in Scheme 1. Tosylglycine-^{13}C (2) was prepared by treating 1 with 4-methylbenzenesulfonyl chloride and sodium hydroxide, following the procedure that Greenaway and Whatley [5] had used to synthesize benzenesulfonylglycine-^{15}N. We used iodomethane to convert the benzenesulfonyl- and 4-methylbenzenesulfonyl- derivatives into the corresponding sarcosine derivatives. The 4-methylbenzenesulfonylsarcosine derivative was obtained in better yield.

Fischer and Bergmann [6] had carried out the preparation of the tosylsarcosine and the hydrolysis to sarcosine in stoppered flasks or sealed tubes. We used a Teflon container with a tight-fitting, knife-edge rim [7] for the first reaction. This allowed the reaction to be run at temperatures above the boiling point of iodomethane-^{13}C and produced 3 in good yield on the small scale employed. Hydrolysis of 3 to 4 was performed with an air-cooled condenser instead of a sealed tube.

Creatine-$^{13}C_2$ (7) was prepared by reacting 4 with either 5 or 6. The use of 6 gave a side-product, the dimer of 6, which was difficult to remove. The use of 5 did not produce any detectable dimer but 5 did have to be synthesized by refluxing a solution of thiourea and iodomethane in methanol, concentrating, and recrystallizing from acetonitrile [8].

Compound 7 was usually isolated as the hydrate when prepared by the reaction of 4 with 5. This was deduced by comparing the



Scheme 1: Synthesis of 2-amino-1,5-dihydro-1-(methyl-^{13}C)-4H-imidazol-4-one-5-^{13}C

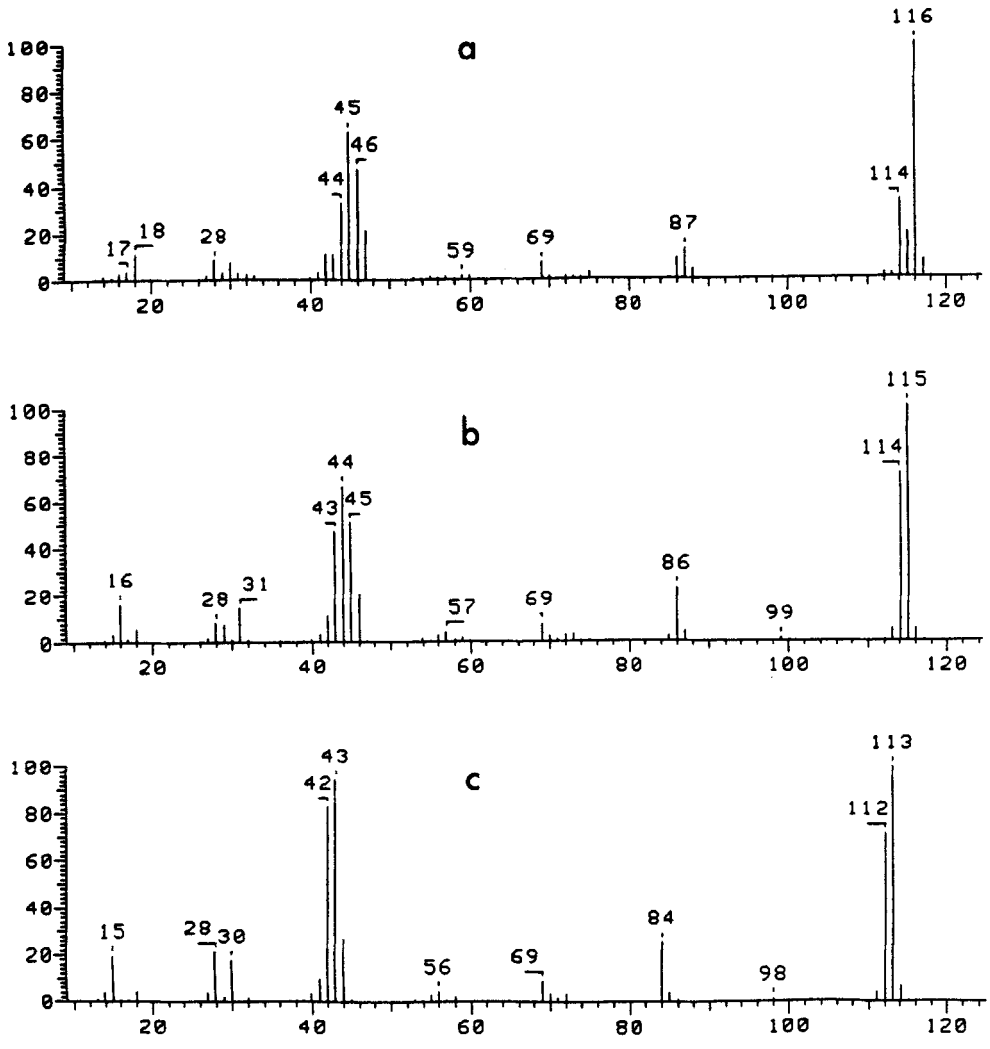


Figure 1. Electron impact mass spectra of: a. creatinine-d_3; b. creatinine-$^{13}C_2$; and c. unlabeled creatinine. Conditions: Ion source temperature, 160 °C; probe temperature, 150-165 °C; electron energy, 70 eV; resolution, 1000.

infrared spectrum of this material with that of an authentic sample of the hydrate which has a band at 1660 cm^{-1} that can be attributed to water of hydration [9]. When the sample is heated above 130 °C

under vaccuum, the 1660 cm^{-1} band and a band at 3420 cm^{-1}, which may also be attributable to water of hydration, [10] disappear and an additional band or shoulder appears at 1130 cm^{-1}. Non-hydrated 7 was isolated from the reaction of 4 with 6.

Creatinine-$^{13}C_2$ (8) was prepared by heating 7 in a sublimation apparatus at 220 °C under vacuum. Although 8 sublimes without decomposition, it cannot be melted without decomposition under vacuum.

The mass spectra of unlabeled and two labeled creatinines are shown in Figure 1. The spectrum of the unlabeled creatinine is similar to a published spectrum of the compound [11], differing only in the relative intensities of some of the ions. The fragments observed for the labeled compounds are consistent with the expected fragmentation of these compounds.

EXPERIMENTAL

Glycine-2-^{13}C and iodomethane-^{13}C (99-atom percent ^{13}C), cyanamide (99+ percent) and creatine hydrate were commercial materials. Cyanamide was stored dry and cold (-5 °C). Standard Reference Material (SRM 914) creatinine was from NBS. For high temperature pressure reactions, a Teflon container, 2.8 cm inside diameter and 3.6 cm deep, with a steel outer container was used. Before use Dowex 50W-X8 (H^+, strong cation exchange resin, 1.9 meq/mL capacity, 50-100 mesh) was treated with 1M NaOH, regenerated with 1M HCl, and washed with water until neutral and free of chloride. Thin-layer chromatography (TLC) was done with silica gel G (250-um thickness) with a fluorescent indicator. Development distance: 18 cm. Infrared spectra were recorded from KBr pellets. Mass spectra were obtained from a direct probe.

N-[(4-Methylphenyl)sulfonyl]glycine-2-^{13}C (Tosylglycine-^{13}C) (2)

4-Methylbenzenesulfonyl chloride (1.70 g, 8.89 mmol) was added to a solution of glycine-2-^{13}C (1) (0.527 g, 6.93 mmol) in 7.75 mL of 1M NaOH and the mixture stirred for one hour. Then 1.55 mL of 3M NaOH was added, and one hour later another 1.55 mL of 3M NaOH was added. After five hours, dissolution was almost complete. The stirring bar was removed, an additional 1 mL of 3M NaOH was added and the flask was tightly stoppered and shaken overnight.

The flask was cooled in an ice-water bath and 1.8 mL (22 mmol) of cold concentrated HCl was added rapidly. The mixture was kept cold for several hours, filtered, and the precipitate washed with ice cold water and dried to give 2 (1.44 g, 90%). An additional 0.19 g of crude 2 was obtained by extraction of the filtrate with ethyl ether (5 x 15 mL).

N-(Methyl-^{13}C)-N-[4-methylphenyl)sulfonyl]glycine-2-^{13}C (3) (Tosylsarcosine-$^{13}C_2$)

Ice-cold iodomethane-^{13}C (0.928g, 6.49 mmol) was added to the Teflon container which contained N-[(4-methylphenyl)sulfonyl]-glycine-2-^{13}C (2) (0.941 g, 4.09 mmol) and ice cold 3M NaOH (4.9 mL, 14.8 mmol). The container was quickly closed and inserted into a capped steel casing, and the assembly was placed in an oven, preheated to 85 °C, for eight hours, during which time the unit was briefly shaken by hand at hourly intervals. Then the assembly was allowed to cool and stored overnight at 5 °C. The container was opened and the reaction mixture was filtered. The filtrate was cooled in ice water, and 1.2 mL (14.5 mmol) cold concentrated HCl was added rapidly. After stirring several hours at 0 °C, 1.66 g (83%) of 3 was obtained. The acidic aqueous filtrate was extracted with chloroform to give 0.224 g (11%) of additional 3.

N-(Methyl-^{13}C)glycine-2-^{13}C (Sarcosine-$^{13}C_2$) (4)

N-(Methyl-^{13}C)-N-[(4-methylphenyl)sulfonyl]glycine-2-^{13}C (3) (2.13 g, 8.69 mmol) and 10 mL (121 mmol) of concentrated HCl were stirred in a 250-mL flask equipped with an air-cooled condenser. The flask was placed in an oil bath at 100 °C and, when initial foaming ceased, the flask was lowered until the oil bath level was above the level of the acid. Overnight heating resulted in a clear, pale yellow solution which was cooled and diluted with cold water. The mixture was concentrated on a rotary evaporator (water bath, 38 °C) and repeatedly diluted with water and reconcentrated to remove HCl. TLC with 95% ethyl alcohol as the developer, and detection with iodine and separately with ultraviolet light (254-nm) indicated complete hydrolysis.

The product was isolated from the reaction mixture, which consisted of product, residual HCl, and 4-methylbenzenesulfonic acid, by ion exchange chromatography [5] as follows: The mixture, as a dilute aqueous solution (87 mL) was passed at a flow rate of 0.1 bed volume/minute [12] through a column containing 18 mL (34 meq) of Dowex 50. The column was washed with water until the eluate was neutral and had a conductivity of less than 0.1 ppm as NaCl. The product was eluted with 180 mL of 1M NH_4OH, and the eluate freeze-dried to give a quantitative yield of 4.

N-(Aminoiminomethyl)-N-(methyl-^{13}C)glycine-2-^{13}C (7) (Creatine-$^{13}C_2$)

Method I: Sarcosine-$^{13}C_2$ (4) (0.0563 g, 0.618 mmol), water (2mL), cyanamide (6) (0.079 g. 1.87 mmol), and concentrated NH_4OH (0.117 mL, 1.70 mmol) were stirred together in a rubber-stoppered, 100-mL flask placed in a dish of cold water, until all of the solid material had dissolved, kept cold several hours, then left overnight. The first crop of product (0.0206 g, 25%) was collected by filtration and washed with ethanol. The second crop (0.029 g, 35%),

collected after concentration of the filtrate, was contaminated with the dimer of cyanamide. The third crop (0.030 g) was entirely dimer. The dimer was recognized by a doublet at 2198 cm^{-1} and 2157 cm^{-1} (4.55 and 4.64 μm) in its infrared spectrum.

Method II: Compound 4 (0.856 g, 9.40 mmol) was dissolved in a minimum amount of water (2 mL), compound (5) (4.10 g, 18.8 mmol) was added, and the mixture stirred to dissolution. Concentrated NH_4OH (2.14 mL, 31 mmol) was added and the flask was closed with a rubber stopper. Precipitation of 7 began within 15 minutes. After 72 hours, the product was collected and washed with ethanol. The yield of 7, as the hydrate was 0.813 g (58%). Additional 7, 0.115g (8%) was isolated from the ethanolic mother liquor. No cyanamide dimer was detected by infrared and mass spectrometric analyses.

2-Amino-1,5-dihydro-1-(methyl-^{13}C)-4H-imidazol-4-one-5-^{13}C (8) (Creatinine-$^{13}C_2$)

Compound 7 was converted to 8 by vacuum sublimation. The presence of the dimer of cyanamide (6) in 7 prepared by Method I made additional purification steps necessary.

Creatine-$^{13}C_2$ (7) from Method I (0.488 g, including cyanamide dimer impurity) was slowly heated from room temperature to 220 °C at 40 Pa (0.30 Torr) in a vacuum sublimator. The sublimate was resublimed at 180 °C and 31 Pa (0.23 Torr). The resublimed material (0.161 g) was crystallized from methanol--acetone to remove cyanamide dimer. The residue from the second sublimation (0.245 g) was again subjected to sublimation at 220 °C. The additional sublimate (0.210 g) was triturated with a small volume of acetone which was removed and the remaining solid combined with the crystallized product. Recrystallization was performed by dissolving in boiling methanol (59 mL/g), allowing the solution to cool to room temperature then refrigerating (5 °C). After maximum growth of crystals, an equal volume of acetone was slowly added, and the

mixture refrigerated (5 °C) 24 hours. The crystals were collected and washed with a minimum amount of cold methanol (precooled with dry ice-ethanol) under a gentle flow of nitrogen gas to minimize condensation of moisture. The product was powdered and dried to constant weight under vacuum at 2.7 Pa (0.02 Torr). The yield of pure 8 was 0.189 g, 36% based on sarcosine-$^{13}C_2$ (4). Isotopic purity, determined by mass spectrometry, was 95 atom percent ^{13}C. The melting point of 292 °C (dec., bath preheated to 250 °C) was identical to that of SRM 914 creatinine.

The creatinine-$^{13}C_2$ (8) was tested for traces of 7 by TLC. A sample (470 µg) of 8 dissolved in a mixture of 1:4 methanol--water was spotted on a TLC plate along with 0.9 to 1.9 µg samples of creatine (corrected for hydration) and the plate developed in 7:3 n-propanol--concentrated NH_4OH. Under these conditions, 8 has an R_F value of 0.50 and creatine has an R_F value of 0.21. Since 7 is not detected by 254-nm absorption of ultraviolet light the plate was heated for 20-40 minutes at 122 °C to convert any creatine to creatinine. In this way, the reference spots originally representing the 0.9 and 1.9 µg quantities of creatine were detectable; however compound 7 was not detectable in 8. Thus the level of 7 in 8 is less than 0.2%. Exposure of the TLC plate to iodine vapor for visualization gave similar results.

Creatine-$^{13}C_2$ (7) hydrate prepared by Method II (0.132 g, 0.873 mmol) was converted to 8 by heating from 23 °C to 220 °C under vacuum (66 Pa, 0.5 Torr); yield, 0.097 g (96%). The product was resublimed (98% recovery). Recrystallization (0.091 g) by dissolving in hot methanol and cooling, or by adding acetone to the cooled methanol solution, gave 0.085 g of 8 (93%) in several crops.

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