N-Methylamidinoglycine의 합성 및 동정 Creatine의 이성질체

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ABSTRACT. N-Methylamidinoglycine, an isomer of creatine which was postulated to be formed enzymatically in vitro, has been synthesized by coupling glycine with N,S-dimethylthiopseudouronium iodide in a yield of approximately 60%. On heating in acidic solution, it was converted to a cyclized form (isocreatinine) in analogy with the conversion of creatine to creatinine (anhydrous form). Structures were confirmed by an elemental analysis and proton NMR spectroscopy. Further studies on their characteristics were compared with those of creatine and creatinine in regard to isoelectric points (pI), retardation coefficients (Rf) on thin layer chromatography, and elution profiles on amino acid analyzer. In order to facilitate the comparison, 14C-labeled creatine, creatinine, isocreatine, 14C-isocreatine, and 14C-isocreatinine were also synthesized.

Phosphocreatine plays a unique role as a temporary storage form of high energy phosphate groups in muscle and other excitable tissues, such as brain and nerve. In addition, creatine and creatine phosphate have been postulated to play regulatory roles in glycolysis\textsuperscript{1,2}, their own biosynthesis\textsuperscript{3}, biosynthesis of actin and myosin heavy chains\textsuperscript{4}, fusion of muscle cells\textsuperscript{5}, rate of heart beat\textsuperscript{6}, and intracellular transport of high energy phosphate from mitochondrial inner...
membrane to contractile fibers. Mammals can use dietary creatine per se, but most of the body’s large stores of creatine are synthesized primarily in the liver from L-arginine; L-arginine donates its guanidino group to glycine to form guanidinoacetic acid which is subsequently methylated to creatine in an S-adenosyl-L-methionine dependent reaction.

McDermott earlier examined the arginine:glycine amidinotransferase activity [EC 2.1.4.1] in various rabbit tissues with either L-arginine or N\textsuperscript{δ}-monomethyl-L-arginine as a substrate, and observed that N\textsuperscript{δ}-monomethyl-L-arginine was utilized at substantial rates (approximately 10\textendash20% of that of L-arginine) in both liver and kidney.

This observation strongly suggests formation of N-methylamidinoglycine (or N\textsuperscript{δ}-methylguanidinoacetic acid), an isomer of creatine: N\textsuperscript{δ}-Monomethyl-L-arginine possibly donates its N-methylguanidino group to glycine to form N-methylamidinoglycine. This reaction bypasses the transmethylation step when L-arginine is used as a substrate.

Since N\textsuperscript{δ}-monomethyl-L-arginine is present in relative abundance in all mammalian tissues and some creatine analogues such as cyclocreatine were shown to increase brain energy stores, we felt it worthwhile to synthesize this isomer of creatine by coupling glycine with N,S-dimethylthiopseudouronium iodide.

Rowley et al. described earlier a general synthetic procedure for substituted glycocya-mines.

However, detailed experimental conditions as well as a description on the characteristics of N-methylamidinoglycine was lacking entirely.

Therefore, in the present communication we report on the detailed synthetic condition and have studied in detail the characteristics of this compound.

**EXPERIMENTAL SECTION**

**Materials.** \(^{14}\text{C}\)Methylamine hydrochloride (60 mCi/mmol), \(^{35}\text{S}\) thiourea (60 mCi/mmol) and \((2-^{14}\text{C})\) glycine (49.54 mCi/mmol) were purchased from Amersham. Methyl iodide and \(\alpha\)-naphthol were obtained from Fisher Chemical Co., diacetyl, flavianic acid, glycine, creatine and creatinine from Sigma Chemical Co., and N-methylthiourea from Aldrich Chemical Co. The remaining chemicals were purchased from various commercial sources and were of the highest grade. N-(Methyl-\(^{14}\text{C}\)), S-methylthiopseudouronium iodide was synthesized according to the method previously published.

**Synthesis of N-methylamidinoglycine flavianate (I) (isocreatine)**

\[
\text{CH}_3\text{NHCSNH}_2 + \text{CH}_3\text{I} \rightarrow \text{Methylthiourea} \quad \text{Methyl iodide}
\]

\[
\text{CH}_3\text{NHCSCH}_3\text{NH}_2\text{HI} \quad \text{N},\text{S-Dimethylthiopseudouronium iodide}
\]

\[
\text{NH}_2\text{CH}_3\text{COOH} \rightarrow \text{CH}_3\text{NHC} (=\text{NH})\text{NHCH}_2\text{COOH} \\
\text{Glycine (I)}
\]

Methyl iodide (1 ml; 0.016 mol) was added to a 100 ml round-bottom flask containing N-methylthiourea (0.9 g; 0.01 mol) and acetone (10 ml), which was surrounded in an ice–water bath. The mixture was then refluxed on a water-bath at around 60\textendash70°C for 10 min, and was evaporated in vacuo.

The white crystalline residue was dissolved in 10 ml of aqueous ammonia (water:NH\textsubscript{4}OH = 1:1) and this was mixed with 10 ml of a solution of glycine (0.75 g; 0.01 mol) in aqueous ammonia (1:1). The mixture was stirred at room temperature for 48 h with a magnetic stirrer, evaporated in vacuo to dryness and the residue washed twice with 5 ml of anhydrous...
alcohol. The white solid formed was dissolved in 5 ml of water and mixed with 10 ml of an aqueous solution of flavianic acid (3.14g; 0.01 mol). The yellowish crystals appeared on storage at 4°C. The crystals were filtered, washed with a small amount of cold water, and recrystallized from water to yield 2.8g of flavianate salt(I). Yield, based on glycine, 63%. m.p., 212~214°C (with decomposition).

Anal. Calculated from C_{14}H_{15}N_{5}O_{10}S (445.4) as N-methylamidinoglycine flavianate: C, 37.75; H, 3.49; N, 15.73; S, 7.20. Found: C, 37.89; H, 3.35; N, 15.69; S, 7.26.

**Cyclized form of N-methylamidinoglycine flavianate (II) (Isocreatinine) (2-Imino-3-methyl-4-imidazolidinone).**

(I) (2.23g; 0.005 mol) in 10 ml water was mixed with 5ml of suspension of Dowex-2 resin (OH⁻ form; 200~400 mesh), and the mixture was stirred with magnetic stirrer until the crystals dissolved. The resin was removed by filtration, and washed twice with 10ml of water. The colorless clear filtrate was evaporated to dryness in vacuo, the residue was dissolved in 20ml of 4N HCl and heated on a boiling water-bath for 2h. The solution was evaporated to dryness in vacuo and residue was dissolved in 5ml of water and treated with 2ml of Dowex-2 suspension in order to remove the HCl. The mixture was filtered and the resin was washed twice with 2.5ml of water.

The colorless solution was mixed with 5ml of flavianic acid solution (1.5g; 0.005 mol) and the mixture was stored at 4°C overnight. The yellowish crystals formed were filtered and the compound(II) was recrystallized from water to yield 1.7g (84% yield, based on (I)). m.p., 251~253°C (with decomposition).

**N-Methylamidino[2-¹⁴C] glycine flavianate (III).** N,S-Dimethylthiopseudouronium iodide, which is used for coupling with [2-¹⁴C] glycine, was synthesized according to the method described for the synthesis of [I], described above, except that twice the amount of each component was used; 1.8g of N-methylthiourea, 20ml of acetone and 2ml of methyl iodide. The formed white crystalline residue was dissolved in 10 ml of an aqueous ammonia, and was mixed with a solution of glycine(0.75g; 0.01mol) and [2-¹⁴C] glycine (50 μCi) in 10 ml of an aqueous ammonia. The remainder of the experimental procedure was the same as described above for the synthesis of non-labeled N-methylamidoglycine flavianate. After recrystallization from water, 2.5g of the product was obtained (56% yield, based on glycine). m.p., 212~214°C (with decomposition).

**Specific activity.** 9,200 dpm/μmol of N-methylamidino[2-¹⁴C] glycine flavianate.

**Cyclization of N-methylamidino[2-¹⁴C] glycine flavianate (IV) (¹⁴C-isocreatinine).**

(IV) was prepared by the same procedure employed for the synthesis of (II).
\[ \text{(Guanidino-}^{14}\text{C)} \text{ creatine flavianate} (\text{V}). \]
\[ \text{NH}_2^\text{S} \text{CSNH}_2 + \text{CH}_3\text{I} \rightarrow \]
\[ ^{14}\text{C} \text{ Thiourea Methyl iodide} \]
\[ \text{NH}_2^\text{S} \text{(SCH)} \text{NH}_2 \text{HI} \ Qt\text{NHCH}_2\text{COOH} \rightarrow \text{Sarcosine} \]
\[ \text{S-Methylisothiourea iodide} \]
\[ \text{NH}_2^\text{S}(=\text{NH})\text{N(CH}_3\text{CH}_2\text{COOH} \]
\[ \text{(V)} \]

Thiourea (0.46 g, 6 mmol), \(^{14}\text{C} \) thiourea (12.5 mmol, 0.75 mCi) and acetone (10 ml) were placed in a 100 ml round-bottom flask surrounded in an ice-water bath. After addition of methyl iodide (1 ml, 0.016 mol), the mixture was stirred magnetically for 30 min and then refluxed for 10 min on a water bath at around 60~70°C. Concentrating to dryness in vacuo gave a white residue which was dissolved in 3 ml of water and mixed with a 20 ml of solution of sarcosine (0.53 g, 6 mmol) in aqueous ammonia [1:1]. The mixture was stirred at room temperature for 48 h with magnetic stirrer, and concentrated to dryness in vacuo. The residue was washed twice with 10 ml of acetone, and dissolved in 5 ml of water. Addition of 10 ml of an aqueous solution of flavianic acid (3.14 g, 0.01 mol) and storage at 4°C overnight gave yellow crystals which were recrystallized from water to yield 1.92 g of the compound [V] (72% yield, based on sarcosine). m.p. 253~256°C (with decomposition).

Specific activity, 57,000 dpm/umol of (guanidino-\(^{14}\text{C} \) creatine flavianate.

\[ ^{14}\text{C} \text{2-Imino-1-methyl-4-imidazolidinone} \]
\[ ^{14}\text{C-creatinine} \text{ flavianate(VI).} \]

The compound [V] (0.45 g, 0.001 mol) was treated as described for the synthesis of (I) from (I). Yield was 0.3 g (33%). m.p. 253~255°C (with decomposition).

RESULTS AND DISCUSSION

Synthesis. Isocreatine was prepared satisfactorily and simply by coupling glycine with unpurified N,S-dimethylthiopseudouronium iodide derived from methyl thiourea and methyl iodide at room temperature.

The purification of the compound in the presence of unreacted glycine is very difficult, since both are freely soluble in water. However, using flavanic acid (as applied in our study) which selectively precipitates isocreatine, it is very easy to purify the compound.

Proton NMR spectroscopy. Proton NMR spectroscopy of the N-methylamidinoglycine(isocreatine) and its anhydrous derivative (isocreatinine) was carried out in trifluoroacetic acid (Table 1).

In contrast to creatine, which in this solvent shows both the methyl and methylene protons as singlets, isocreatine displays the proton on carbon as two doublets split by vicinal proton on nitrogen.

Both creatine and isocreatine in trifluoroacetic acid solution undergo cyclization in the imidazolidinone derivatives. Interestingly, isocreatine cyclizes much more slowly than creatine\(^{18}\); as might be expected, isocreatine gives a mixture of products, with the methyl group on either a ring or an exocyclic nitrogen.

When the cyclization was carried out in aqueous hydrochloric acid, a single product with the methyl group on a ring nitrogen was isolated.

Color reactions. Some reagents used to develop colors with creatine have been examined with isocreatine and isocreatinine (Table 2). These two compounds are generally sensitive towards
Table 1. Proton NMR spectra

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CH₃</th>
<th>CH₂</th>
<th>NH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine</td>
<td>Singlet, 3.27 ppm 3H</td>
<td>Singlet, 4.40 ppm 2H</td>
<td>Broad singlet 6.1~6.6 ppm, 4H</td>
</tr>
<tr>
<td>N-Methylamidinoglycine flavianate (Isocreatinine)</td>
<td>Doublet, 3.12 ppm (J=4.5) Hz, 3H</td>
<td>Doublet, 4.42 ppm (J=6) Hz, 2H</td>
<td>Complex envelope. 6.2~6.9ppm, 4H</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Singlet, 3.57 ppm 3H</td>
<td>Singlet, 4.57 ppm 2H</td>
<td>Broad singlet. 7.6~8.1 ppm, 2H²</td>
</tr>
<tr>
<td>Cyclized N-methylamidinoglycine flavianate (Isocreatinine)</td>
<td>Singlet, 3.46 ppm, 3H</td>
<td>Singlet, 4.53 ppm, 2H</td>
<td>Broad singlet, 7.9ppm, 2H Broad singlet, 8.40 ppm, 1H</td>
</tr>
</tbody>
</table>

* All spectra were run in trifluoroacetic acid. Chemical shifts are reported in ppm from internal TMS.
Coupling constants may not be accurate because of partial coalescence of multiplets by exchange of protons on nitrogen with the solvent. * The signals for the aromatic protons of flavianic acid appeared between 8.4 and 9.6 ppm. * Singlet in trifluoroacetic acid-d. * The proton on a ring nitrogen is presumed to be in fast exchange with the solvent. * The absence of apparent coupling to the vicinal proton on nitrogen may be ascribed to an unfavorable dihedral relationship.

Table 2. Colors and their yield of isocreatine and isocreatinine with various reagents

<table>
<thead>
<tr>
<th>Compounds</th>
<th>With reagents</th>
<th>FCNP*</th>
<th>Nessler's reagent</th>
<th>α-naphthol picrate</th>
<th>α-naphthol diacetyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine(A)</td>
<td>red</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (B)</td>
<td>yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocreatinine (C)</td>
<td>red</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocreatinine (D)</td>
<td>yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of color yield:</td>
<td>100:13:36:100 (40min)²</td>
<td>0:100:0:100 (immediately)</td>
<td>100:4:5:0 (40min)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* FCNP: Potassium ferricyanide (0.5g) and sodium nitroprusside (0.5g) in 50ml of 0.5 N NaOH. Alkaline picrate: a saturated solution of picric acid in 5% sodium carbonate. α-Naphthol-diacetyl: Solution A contains 25mg of α-naphthol and 8g of anhydrous sodium carbonate in 50ml of 1.5 N NaOH, and solution B 0.025ml of diacetyl in 50ml of H₂O. After solution A was sprayed on the filter paper and dried, followed by solution B spray. * Spot test on filter paper whatman #1. * Symbol indicates colorless. * The intensity of color was time-dependent.

the reagents than creatine and creatinine.

The reagent FCNP (a mixture of K₃Fe(CN)₆ and Na₃Fe(CN)₆NO) appears to be most useful for identification and quantitation. The compounds also react with ninhydrin (Fig. 1), and can be resolved on an automatic amino acid analyzer from creatine and creatinine.

However, even the most sensitive compound isocreatinine is still approximately 10 times less sensitive than the ordinary amino acids.

Thin-layer chromatography. As shown in Table 3, the Rf values of both isocreatine and isocreatinine are similar to those of creatine and creatinine. However, the former pair appears to move slightly faster than the latter.

PI values. Table 4 lists the PI values (isoelectric point) determined by isoelectrofocusing technique. While dehydration and cyclization of creatine decreased the PI value quite significantly, cyclization of isocreatine does not change to any significant extent.

In summary, isocreatine is shown to be less basic, but more stable than the physiologically occurring structural isomer, creatine. Since creatine phosphate serves as a readily available source of high energy phosphate in muscle and
Fig. 1. Analysis of isocreatine and isocreatinine on automatic amino acid analyzer. Amino acid analysis was carried out with a Perkin-Elmer KLA-3B automatic amino acid analyzer with a column of Aminex A-5 resin (0.9 x 43 cm; particle size 13 ± 2 μm). The column was eluted with 0.38 (Na+) sodium citrate buffer, pH 5.84, at 24°C. The flow rate was 45 ml/hr. 7 μmol of creatine, 2 μmol of creatinine, 20 μmol of isocreatine and 40 μmol of isocreatinine were applied.

Table 3. Rf values of isocreatine and isocreatinine on thin layer chromatography

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RF values x 100 in solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Creatine</td>
<td>58</td>
</tr>
<tr>
<td>Creatinine</td>
<td>79</td>
</tr>
<tr>
<td>Isocreatine</td>
<td>61</td>
</tr>
<tr>
<td>Isocreatinine</td>
<td>69</td>
</tr>
</tbody>
</table>

Plate, precoated with Avicel F (250 μm thickness; product of Analtech, Inc., Newark, Delaware). For identifying the spots, FCNP solution (0.5 g of potassium ferricyanide and 0.5 g of sodium nitroprusside in 50 ml of 0.5N NaOH) was sprayed.

Table 4. Isoelectric point (pl) of isocreatine and isocreatinine

<table>
<thead>
<tr>
<th>Compound</th>
<th>pI values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine</td>
<td>9.37 (9.35; 9.39)*</td>
</tr>
<tr>
<td>Creatinine</td>
<td>7.35 (7.33; 7.46)</td>
</tr>
<tr>
<td>Isocreatine</td>
<td>8.77 (8.74; 8.80)</td>
</tr>
<tr>
<td>Isocreatinine</td>
<td>8.57 (8.57; 8.58)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses indicate the values of two independent experiments. In order to facilitate the identification of the peak position during isoelectrofocusing, ¹³C-labeled compounds were used. Isoelectrofocusing was carried out according to the published method.

brain, and has been postulated to play a role in its biosynthesis, in glycolysis, and in biosynthesis of muscle proteins and heart function, it might be worthwhile to examine the possible effect of isocreatine in these mentioned functions.

The successful synthesis of N-methylamidinoglycine (isocreatine) described herein should enable us to pursue the identification of the reaction product arising in the incubation mixture of NG-monomethyl-L-arginine, glycine and rabbit tissue homogenate. If isocreatine is indeed the product and serve as a substrate for
creatinine kinase [EC 2.7.3.2], this observation will possibly have an important biological significance. Since a large proportion of tissue S-adenosyl-L-methionine (over 80%) is utilized for the synthesis of creatine from guanidinoacetic acid\textsuperscript{16}, formation of isocreatine will spare S-adenosyl-L-methionine \textit{in vivo} by exploiting the methyl group already incorporated into N\textsuperscript{\text{\textbeta}}-monomethyl-L-arginine: N\textsuperscript{\textbeta}-Monomethyl-L-arginine arises \textit{in vivo} from the hydrolysis of N\textsuperscript{\textbeta}-methylated protein which had been synthesized by the action of protein methylase I [S-Adenosyl-L-methionine: protein-arginine N-methyltransferase; EC 2.1.1.23]\textsuperscript{17}.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{REFERENCE}

18. About 10\% of guanidinoacetic acid in trifluoroacetic acid cyclizes in 20h at 60\(^\circ\)C, whereas under such conditions \textbeta-guanidinopropionic acid does not cyclize at all (unpublished data).