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N-Methylamidinoglycine의 합성 및 동정 Creatine의 이성질체

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Synthesis and Characterization of N-Methylamidinoglycine: an Isomer of Creatine

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요 약. 시험관내에서 효소에 의해 생성될 수 있을 것으로 생각되는 N-methylamidinoglycine (isocreatine)을 glycine과 N, S-dimethylthiopseudouronium iodide로 부터 약 60% 수득율로 합성하였고, isocreatine의 산성수용액을 가열하여, creatine이 creatinine으로 탈수 고리화되는 것처럼, 고리화된 isocreatinine도 얻었다. 한편 이들 화합물에 대해 원소분석, nmr 스펙트럼, 박층 크로마토그라피(Rf) 및 아미노산분석기에서의 elution rate도 점토하였으며, 등전점을 측정하기 위해서 ^{14}C-creatine, ^{14}C-creatinine, ^{14}C-isocreatine 및 ^{14}C-isocreatinine도 합성하였다.

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, ^{14}C-labeled creatine, creatinine, isocreatine and 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[1,2], their own biosynthesis[3], biosynthesis of actin and myosin heavy chains[4], fusion of muscle cells[5], rate of heart beat[5], and intracellular transport of high energy phosphate from mitochondrial inner

membrane to contractile fibers[6,7]. 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[8].

McDermott[9] earlier examined the arginine: glycine amidinotransferase activity [EC 2.1.4.1] in various rabbit tissues with either L-arginine or N^G-monomethyl-L-arginine as a substrate, and observed that N^G-monomethyl-L-arginine was utilized at substantial rates (approximately 10~20% of that of L-arginine) in both liver and kidney.

This observation strongly suggests formation of N-methylamidinoglycine (or N^G-methylguanidinoacetic acid), an isomer of creatine: N^G-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^G-monomethyl-L-arginine is present in relative abundance in all mammalian tissues[10] and some creatine analogues such as cyclocreatine were shown to increase brain energy stores[11], 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 glycocyamines[12].

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}C] Methylamine hydrochloride (60 mCi/mmol), [^{14}C] thiourea (60mCi/mmol) and [2-^{14}C] glycine (49.54 mCi/mmol) were purchased from Amersham. Methyl iodide and α-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}C], S-methylthiopseudouronium iodide was synthesized according to the method previously published[13].

Synthesis of N-methylamidinoglycine flavianate [I] (isocreatine)

$CH_3NHCSNH_2$ + CH_3I ⟶

Methylthiourea　　Methyl iodide

$CH_3NHC(SCH_3)NH.HI$

N, S-Dimethylthiopseudouronium iodide

NH_2CH_2COOH

⟶ $CH_3NHC(=NH)NHCH_2COOH$

Glycine

[I]

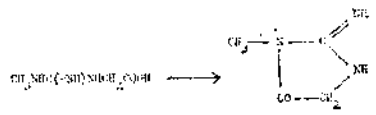
Methyl iodide (1 m*l*; 0.016 mol) was added to a 100m*l* round-bottom flask containing N-methylthiourea (0.9g; 0.01 mol) and acetone (10 m*l*), which was surrounded in an ice-water bath. The mixture was then refluxed on a water-bath at around 60~70°C for 10 min, and was evaporated *in vacuo*.

The white crystalline residue was dissolved in 10m*l* of aqueous ammonia [water:NH_4OH= 1 : 1] and this was mixed with 10m*l* of a solution of glycine (0.75g; 0.01 mol) in aqueous ammonia [1 : 1]. The mixture was stirred at room temperature for 48h with a magnetic stirrer, evaporated *in vacuo* to dryness and the residue washed twice with 5 m*l* of anhydrous

alcohol. The white solid formed was dissolved in 5 m*l* of water and mixed with 10 m*l* 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 $C_{14}H_{15}N_5O_{10}S$ (445.4) as N-methylamidinoglycine flavianate: C, 37.75; H, 3.40; 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) (II)

[I] (2.23g; 0.005 mol) in 10 m*l* water was mixed with 5m*l* 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 10m*l* of water. The colorless clear filtrate was evaporated to dryness *in vacuo*, the residue was dissolved in 20m*l* of 4N HCl and heated on a boiling water-bath for 2h. The solution was evaporatde to dryness *in vacuo* and residue was dissolved in 5m*l* of water and treated with 2m*l* of Dowex-2 suspension in order to remove the HCl. The mixture was filtered and the resin was washed twice with 2.5m*l* of water.

The colorless soluton was mixed with 5m*l* of flavianiciacid 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).

Anal. Calculated from $C_{14}H_{13}N_5O_9S$ (427.4) as cyclized N-methylamidinoglycine (isocreatinine) flavianate: C, 39.35; H, 3.07; N, 16.39; S, 7.50. Found: C, 39.18; H, 3.18; N, 16.28; S, 7.23.

N-Methylamidlno [2-^{14}C] glycine flavianate [III]. N, S-Dimethylthiopseudouronium iodide, which is used for coupling with [2-^{14}C] 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, 20m*l* of acetone and 2m*l* of methyl iodide. The formed white crystalline residue was dissolved in 10 m*l* of an aqueous ammonia, and was mixed with a solution of glycine (0.75g; 0.01mol) and [2-^{14}C] glycine (50 μCi) in 10 m*l* of an aqueous ammonia. The remainder of the experimental procedure was the same as described above for the synthesis of non-labeled N-methylamidinoglycine 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-^{14}C] glycine flavianate.

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

[III] [IV]

[IV] was prepared by the same procedure employed for the synthesis of [II].

{Guanidino-^{14}C}creatine flavianate(V).

$$NH_2\overset{*}{C}SNH_2 + CH_3I \longrightarrow$$

[^{14}C] Thiourea Methyl iodide

$$NH_2\overset{*}{C}(SCH_3)NH.HI \xrightarrow[\text{Sarcosine}]{CH_3NHCH_2COOH}$$

S-Methylisothiourea iodide

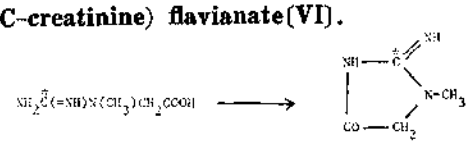
$$NH_2\overset{*}{C}(=NH)N(CH_3)CH_2COOH$$

(V)

Thiourea(0.46g; 6mmol), [^{14}C] thiourea (12.5 μmol; 0.75mCi) and acetone(10m*l*) were placed in a 100m*l* round-bottom flask surrounded in an ice-water bath. After addition of methyl iodide (1 m*l*; 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 m*l* of water and mixed with a 20 m*l* of solution of sarcosine (0.53g; 6 mmol) in aqueous ammonia (1 : 1). The mixture was stirred at room temperature for 48h with magnetic stirrer, and concentrated to dryness *in vacuo*. The residue was washed twice with 10 m*l* of acetone, and dissolved in 5m*l* of water. Addition of 10m*l* of an aqueous solution of flavianic acid (3.14g; 0.01mol) and storage at 4°C overnight gave yellow crystals which were recrystallized from water to yield 1.92g 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}C] creatine flavianate.

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



[V] [VI]

The compound[V] (0.45g; 0.001mol) was treated as described for the synthesis of [II] from [I]. Yield was 0.3g(53%). 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 flavianic 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 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[a]

Compounds	CH_3	CH_2	NH
Creatine	Singlet, 3.27ppm 3H	Singlet, 4.40 ppm 2H	Broad singlet 6.1~6.6 ppm, 4H
N-Methylamidinoglycine flavianate[b] (Isocreatine)	Doublet[c], 3.12 ppm J=4.5 Hz, 3H	Doublet[c], 4.42 ppm J=6 Hz, 2H	Complex envelope, 6.2~6.9ppm, 4H
Creatinine	Singlet, 3.57 ppm, 3H	Singlet, 4.57 ppm, 2H	Broad singlet, 7.6~8.1 ppm, 2H[d]
Cyclized N-methylamidinoglycine flavianate (Isocreatinine)[b]	Singlet, 3.46ppm, 3H	Singlet[e], 4.53ppm, 2H	Broad singlet, 7.9ppm, 2H Broad singlet, 8.40 ppm, 1H

[a] 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. [b] The signals for the aromatic protons of flavianic acid appeared between 8.4 and 9.6 ppm. [c] Singlet in trifluoroacetic acid-d. [d] The proton on a ring nitrogen is presumed to be in fast exchange with the solvent. [e] 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

Compounds	With reagents			
	FCNP[a]	alkaline picrate	Nessler's reagent	α-naphthol diacetyl
Creatine(A)	red[b]	—	—[c]	red
Creatinine (B)	yellow	red	black	yellow
Isocreatine (C)	red	—	—	—
Isocreatinine(D)	yellow	—	black	—
Ratio of color yield: (A):(B):(C):(D)	100:13:36:9(140min)[d]		0:100:0:100 (immediately)	100:4:5:0 (40min)

[a] FCNP: Potassium ferricyanide(0.5g) and sodium nitroprusside (0.5g) in 50ml of 0.5N NaOH[14]. Alkaline picrate: a saturated solution of picric acid in 5% sdioum carbonate. α-Naphthlo-diacetyl: Solution A contains 25mg of α-naphthol and 8g of anhydrous sodium carbonate in 50ml of 1.5N NaOH, and solution B 0.025ml of diacetyl in 50ml of H_2O. After solution A was sprayed on the filter paper and dried, followed by solution B spray. [b] Spot test on filter paper whatman #1. [c] Symbol indicates colorless. [d] The intensity of color was time-dependent.

the reagents than creatine and creatinine.

The reagent FCNP (a mixture of $K_3Fe(CN)_6$ and $Na_2Fe(CN)_5NO$) 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 isocreatine 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

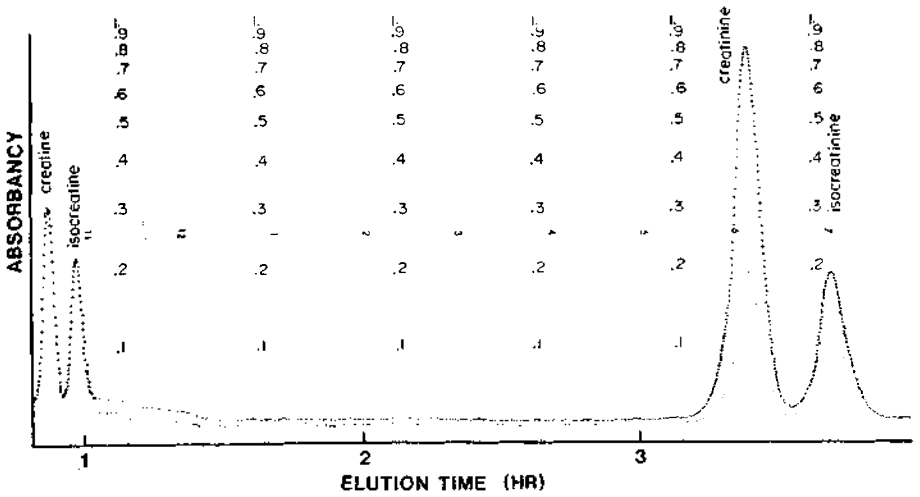


Fig. 1. Analysis of isocreatine and isocreatinine on automatic amino acid analyzer. Amino acid analysis was carried out with a Perkin-Elmer KLA-3B automatic amino acid analyzer with a column of Aminex A-5 resin (0.9×43cm; particle size 13±2μ). The column was eluted with 0.38 (Na^+) sodium citrate buffer, pH 5.84, at 24°C. The flow rate was 45m*l*/h. 7 μmol of creatine, 2 μmol of creatinine, 20 μmol of isocreatine and 40μmol of isocreatinine were applied.

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

Compounds	Rf values×100 in solvents				
	A[a]	B	C	D	E
Creatine	58	63	24	17	29
Creatinine	70	67	55	37	49
Isocreatine	61	65	36	25	37
Isocreatinine	69	70	67	35	49

[a] Composition of the solvent. A: *n*-Butanol: acetic acid: H_2O (50 : 25 : 25). B: Ethyl acetate: formic acid: H_2O (70 : 20 : 10). C: Pyridine: acetone: 3N NH_4OH(50 : 30 : 20). D: *n*-Butanol: ethyl alcohol: H_2O(40 : 25 : 35). E: *n*-Propanol: H_2O(3 : 1)
Plate; precoated with Avicel F(250 μm thickness; product of Analtech, Inc., Newark, Delaware). For identifying the spots, FCNP solution [0.5g of potassium ferricyanide and0.5g of sodium nitroprusside in 50m*l* of 0.5N NaOH] was sprayed.

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

Compound	pI values
Creatine	9.37 (9.35; 9.38)[a]
Creatinine	7.35 (7.23; 7.46)
Isocreatine	8.77 (8.74; 8.80)
Isocreatinine	8.57 (8.57; 8.56)

[a] The numbers in parentheses indicate the values of two independent experiments. In order to facilitate the identification of the peak position during isoelectrofocusing, ^{14}C-labeled compounds were used. Isoelectric focusing was carried out according to the published method[15].

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 N^G-monomethyl-L-arginine, glycine and rabbit tissue homogenate[9]. If isocreatine is indeed the product and serve as a substrate for

creatine 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[16], formation of isocreatine will spare S-adenosyl-L-methionine *in vivo* by exploiting the methyl group already incorporated into N^G-monomethyl-L-arginine: N^G-Monomethyl-L-arginine arises *in vivo* from the hydrolysis of N^G-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][17].

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