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Reaction of Glycylglycine with Formaldehyde in the Presence of Cupric Ion[1]

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The reaction mixture of glycylglycine with formaldehyde in an alkaline aqueous solution containing cupric ion at 100°C gave seven ninhydrin positive components which were identified as follows; α-hydroxymethylserine, serine, glycine, 3-carboxymethyl-4-imidazolidone, α-hydroxymethylseryl-glycine, serylglycine and glycylglycine. The yield of serylglycine increased in proportion to the amount of cupric ion, whereas the yield of 3-carboxymethyl-4-imidazolidone was maximum when no cupric ion was added. A single-step synthesis in preparative scale of serylglycine or 3-carboxymethyl-4-imidazolidone under the best condition was achieved in a good yield.

Sato, Okawa and Akabori[3] reported the synthesis of a diastereomeric mixture of threonine and allothreonine by the reaction of an aqueous alkaline solution of copper glycinate with acetaldehyde. Benoiton *et al.*[4] described a general preparation of β-hydroxy-α-amino acid by the condensation of copper glycinate with various aldehydes, ketones and α-keto acids under comparable conditions. Akabori *et al.*[5] observed that the reaction of glycine with formaldehyde in the presence of cupric ion caused formation of serine and an unidentified ninhydrin reactive substance. Later Otani and Winitz characterized the unidentified substance as α-hydroxymethylserine, and they indicated that this amino acid could be derived from the interaction of serine and formaldehyde with cupric ion.[6]

Regarding the reaction of glycyl peptide with aldehyde, Akabori *et al.*[7] attempted to introduce side-chains into polyglycine, which had been dispersed on a clay, with formaldehyde or acetaldehyde in an alkaline medium; they observed that 0.4—3.1% or 1.4—1.5% of glycine residues were converted to serine or threonine residues. Furuyama *et al.*[8] treated polyglycine with acetaldehyde in

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3) M. Sato, K. Okawa and S. Akabori, This Bulletin, **30**, 937 (1957).

4) L. Benoiton, M. Winitz, R. F. Colman, S. M. Birnbaum and J. P. Greenstein, *J. Amer. Chem. Soc.*, **81**, 1726 (1959).

5) S. Akabori, T. T. Otani, R. Marshall, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **83**, 1 (1959).

6) T. T. Otani and M. Winitz, *ibid.*, **90**, 254 (1960).

7) S. Akabori, K. Okawa and M. Sato, This Bulletin, **29**, 608 (1956).

8) T. Furuyama, F. Sakiyama and K. Narita, *ibid.*, **36**, 903 (1963).

liquid ammonia in the presence of metallic sodium; 7—8% of glycine residues were shown to be converted to threonines.

This paper will describe the isolation and characterization of several components from a reaction mixture of glycylglycine with formaldehyde in the presence of cupric ion. Convenient single-step synthesis of serylglycine, which was conceived as a consequence of these studies, will also be described.

We assumed the formation of either serylglycine or glycylserine as a major product when glycylglycine is subjected to the reaction with formaldehyde in the presence of cupric ion. The methylene group in copper glycylglycinate might be more active than that in free glycylglycine and the amino group in copper salt might be protected from unfavorable side reactions. This supposition suggested the possibility for such a reaction sequence shown in Fig. 1.

$$NH_2CH_2CO\text{-}NHCH_2COOH \xrightarrow[OH^-,\ Cu^{2+}]{HCHO}$$

$$NH_2\underset{\displaystyle CH_2OH}{\underset{|}{C}}HCO\text{-}NHCH_2COOH$$

or $$NH_2CH_2CO\text{-}NH\underset{\displaystyle CH_2OH}{\underset{|}{C}}HCOOH$$

Fig. 1. Assumed reaction sequence.

In this investigation, several dipeptides such as serylglycine, glycylserine and α-hydroxymethylserylglycine were prepared in a conventional manner as the first step of the experiment. These dipeptides were to be used as authentic samples. For example, α-hydroxymethylserylglycine was synthesized as follows.[9]

$$\text{Z-HMSer} + \text{Gly-OBzl} \xrightarrow{DCC}$$

$$\text{Z-HMSer-Gly-OBzl} \xrightarrow{H_2/Pd} \text{HMSer-Gly}$$

Fig. 2. Synthesis of a dipeptide.

We observed that a reaction mixture of glycylglycine and formaldehyde with cupric ion in an alkaline medium gave seven ninhydrin positive components which were identified by the use of an amino acid analyzer (Fig. 4-B). We could easily identify six components to be α-hydroxymethylserine, serine, glycine, α-hydroxymethylserylglycine, serylglycine and glycylglycine by means of paper chromatography and an amino acid analyzer. However, one component which emerged at the fourth peak in the chromatogram by the amino acid analyzer (Fig. 4-B) could not be characterized at this stage. Therefore, the reaction mixture was prepared on a large scale, and was fractionated with a column (1.8×110 cm) of Dowex 50 (NH_4^+ form) using 0.2M ammonium acetate buffer as a solvent.[10] Thus, the unidentified component emerging at the fourth peak could be isolated as crystals, and finally its structure was characterized as 3-carboxymethyl-4-imidazolidone (Cm-Iz) shown in Fig. 3, by means of elemental analysis, molecular weight determination and IR and NMR

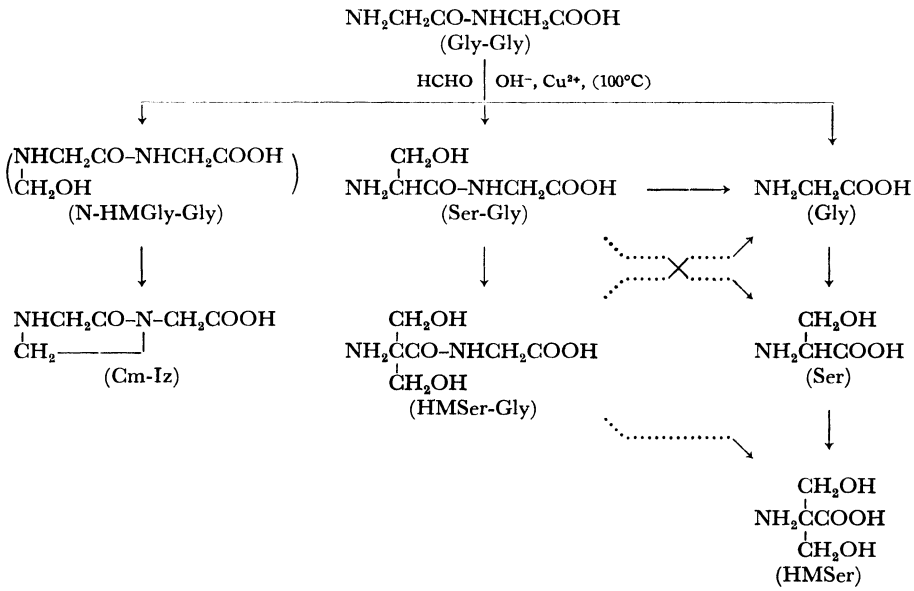

Fig. 3. Summary of the reaction of Gly-Gly and formaldehyde with cupric ion.

9) Abbreviations: Z-, benzyloxycarbonyl; -OBzl, benzyl ester; HMSer, α-hydroxymethyl-DL-serine; Cm-Iz, 3-carboxymethyl-4-imidazolidone. Amino acid symbols except Gly denote the DL-configuration.

10) K. Noda, H. Okai, T. Kato and N. Izumiya, This Bulletin, **41**, 401 (1968).

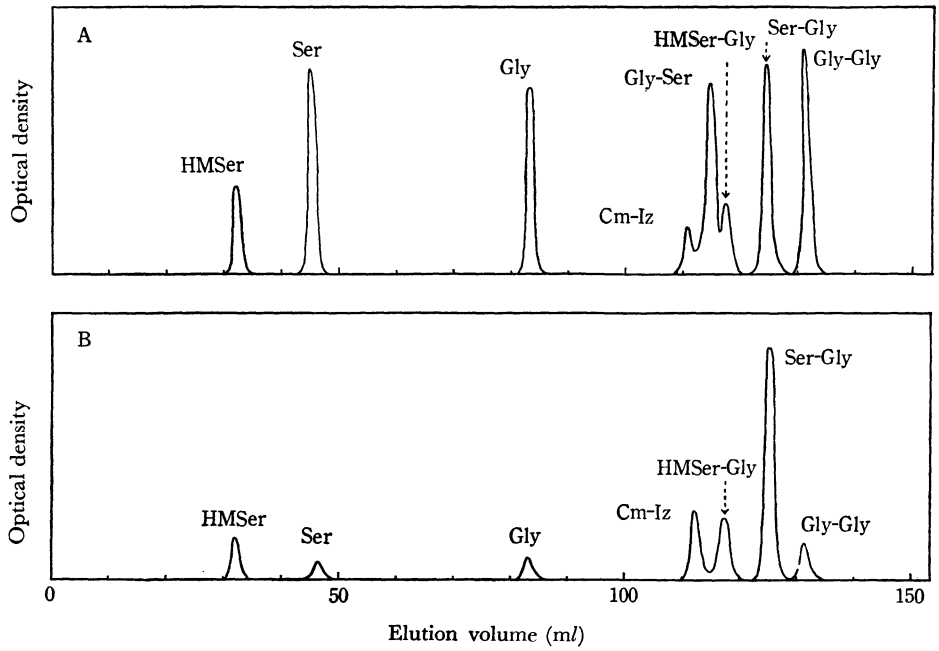

Fig. 4. Elution pattern of a model mixture (A) and a reaction mixture (B) by amino acid analyzer.

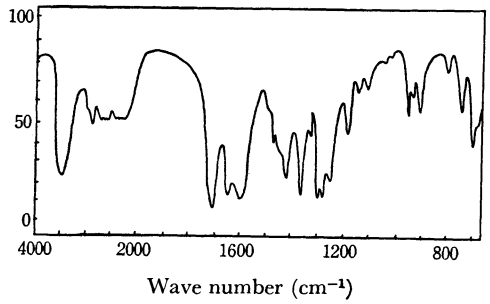

Fig. 5. Infrared spectrum of 3-carboxymethyl-4-imidazolidone in KBr disk.

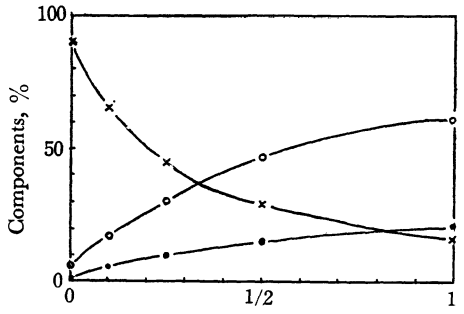

Fig. 6. Effects of cupric ion concentration.
—○—, Ser-Gly; —×—, Cm-Iz; —●—, HMSer-Gly

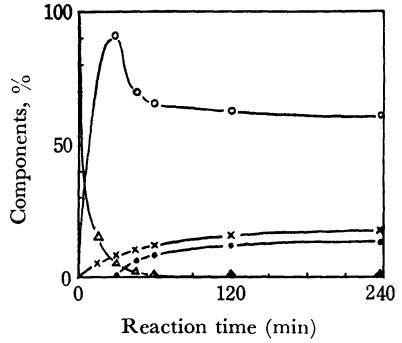

Fig. 7. Effects of reaction time.
—○—, Ser-Gly; —×—, Cm-Iz; —●—, HMSer-Gly; —△—, Gly-Gly

spectra. Later it was found that this substance, Cm-Iz, was isolated easily in a good yield from the reaction mixture of glycylglycine and formaldehyde without cupric ion (see Fig. 6).

Figure 3 indicates the reaction sequence of glycylglycine and formaldehyde with cupric ion. Kim and Martell[11] have reported the structure of copper glycylglycinate in aqueous solution in detail. They have indicated that the amino group in copper salt in sodium carbonate is protected by cupric ion. It is therefore considered that the methylene group is more active than that of free glycylglycine. We have already described the formation of serylglycine and α-hydroxymethylserylglycine. However, it should be mentioned that not even a trace of glycylserine could be found, contrary to our ex-

11) M. K. Kim and A. E. Martell, *Biochemistry*, **3**, 1169 (1964).

pectation indicated in Fig. 1. When the amount of cupric ion is decreased, the amino group in free glycylglycine is apt to react with formaldehyde to produce 3-carboxymethyl-4-imidazolidone (Cm-Iz); Cm-Iz may be produced *via* a possible intermediate, *N*-HMGly-Gly (Fig. 3). It is considered that the components of amino acid were derived by an alkaline hydrolysis at peptide bond, and some amino acid such as glycine was converted further to serine and hydroxymethylserine as Otani and Winitz reported.[6]

The effects of variations of concentration of cupric ion and reaction time were studied by using the amino acid analyzer in order to find optimal condition for the serylglycine formation. The experiments revealed that the formation of serylglycine was favored when the ratio of copper to glycylglycine increased, and no serylglycine was formed in the absence of cupric ion (Fig. 6). Within the conditions we studied, the maximum formation of serylglycine was observed when the molar ratio of copper to glycylglycine was 1 : 1 and the reaction mixture was heated at 100°C for 30 min (Fig. 7).

As an experiment on a preparative scale, we tried to prepare serylglycine under the best conditions described above. Thus, a practicable one-step synthesis of serylglycine has been achieved in a good yield of 70%. The method of synthesis of serylglycine developed in these studies has merits of simplicity, convenience and inexpensive starting materials.

Experimental

The melting points were uncorrected. Paper chromatography was run on Toyo Roshi No. 52 in 80% pyridine. The infrared spectra were measured on a Hitachi EPI-S2 spectrophotometer. The NMR spectra were recorded on a Varian Associates A-60 model, using tetramethylsilane as the internal standard and acetic acid as solvent.

Gly-Ser. This peptide was prepared by the hydrogenolysis of Z-Gly-Ser-OBzl;[12] 85%, mp 197—199°C (decomp.), R_f 0.36.

Ser-Gly. This was also prepared from Z-Ser-Gly-OBzl;[12] 82%, mp 204—206°C (decomp.); R_f 0.45.

Z-HMSer. To a solution of α-hydroxymethylserine[6] (1.62 g, 12 mmol) and sodium bicarbonate (2.52 g) in water (50 m*l*) was added benzyloxycarbonyl chloride (1.82 m*l*, 14.4 mmol) portionwise under stirring at room temperature. Stirring was continued for 4 hr and the solution was extracted with ether. After the aqueous layer was acidified with hydrochloric acid, it was extracted with ethyl acetate (4 times, each 50 m*l*). The combined extracts were dried over sodium sulfate and evaporated *in vacuo*. The residual syrup was solidified by the addition of petroleum ether. It was recrystallized from ethyl acetate-petroleum ether; yield, 1.64 g (51%); mp 109—110°C.

Found: C, 53.50; H, 5.78; N, 5.19%. Calcd for $C_{12}H_{15}O_6N$: C, 53.53; H, 5.62; N, 5.20%.

Z-HMSer-Gly-OBzl. To a solution of Z-HMSer (0.7 g, 3 mmol) and glycine benzyl ester *p*-toluenesulfonate (1.01 g, 3 mmol) in chloroform (15 m*l*) with triethylamine (0.42 m*l*) was added DCC (0.62 g, 3 mmol) at room temperature. The mixture was left to stand overnight, and evaporated *in vacuo*. To the residue was added ethyl acetate, and dicyclohexylurea was filtered off. The filtrate was washed with a 4% sodium bicarbonate solution, 2% hydrochloric acid and water, and dried over sodium sulfate. The filtered solution was evaporated and the oily residue was solidified by the addition of petroleum ether. It was recrystallized from ethyl acetate-petroleum ether; yield, 0.75 g (61%); mp 91—93°C.

Found: C, 60.48; H, 5.89; N, 6.70%. Calcd for $C_{21}H_{24}O_7N_2$: C, 60.56; H, 5.81; N, 6.73%.

HMSer-Gly. A solution of Z-HMSer-Gly-OBzl (0.42 g, 1 mmol) in a mixture of acetic acid (5 m*l*), *t*-butanol (3 m*l*) and water (1 m*l*) was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* to dryness. The residual crystals were recrystallized from water-ethanol; yield, 0.154 g (80%); mp 190—192°C (decomp.); R_f 0.56.

Found: C, 37.26; H, 6.38; N, 14.70%. Calcd for $C_6H_{12}O_5N_2$: C, 37.50; H, 6.29; N, 14.58%.

3-Carboxymethyl-4-imidazolidone (Cm-Iz). A solution of Gly-Gly (1.32 g, 10 mmol), sodium carbonate (4.24 g, 40 mmol) and 37% aqueous formaldehyde (6 m*l*, 80 mmol) in water (200 m*l*) was kept at 100°C for 1 hr, and evaporated to a small volume (about 10 m*l*) *in vacuo*. It was put on a column of Dowex 50×8 (1.8×30 cm, 100—200 mesh, H^+ form). After the column was washed with water, it was treated with 2N ammonium hydroxide. The eluate was evaporated *in vacuo* and the residual crystals were recrystallized from water-ethanol; yield, 1.18 g (82%); mp 200°C (decomp.); R_f 0.42.

Calcd for $C_5H_8O_3N_2$: C, 41.66; H, 5.59; N, 19.44%. Found: C, 41.64; H, 5.67; N, 19.43%.

A definite amount of the product was hydrolyzed with 6N hydrochloric acid at 110°C for 12 hr; the hydrolysate indicated the value of $Gly_{2.03}$ by the amino acid analysis and the presence of formaldehyde qualitatively by a reagent of chromotropic acid.[13] The following observations confirm the structure of the product to be Cm-Iz. The molecular weight was determined on a Hitachi Osmometer, type 115, using methanol as a solvent. Calcd, 141.1; Found, 141.5. Infrared spectrum was shown in Fig. 5: ν_{max} 3400 (>N–H); 2750—2350 (this series is not seen in amino acid and acyldipeptide acid); 1718 (C=O in δ-lactam); 1647 (–CO–N<), 1590, 1314, 1302, 1012 (C–N–C) cm^{-1}. The NMR spectra were in accord with that assumed for Cm-Iz: δ=4.10 (singlet, methylene protons, 2H); 4.23 (singlet, methylene protons, 2H); 4.98 (singlet, methylene protons, 2H).

Chromatography of Model Mixture by Amino Acid Analyzer. We tried to obtain sufficient separation of each component of an artifical mixture by an amino acid analyzer, and found the following conditions to satisfy this purpose. A mixture of each one μmole of eight compounds listed in Table 1 was treated with a Hitachi amino acid analyzer, model KLA-3B, using

12) G. Fölsch, *Acta Chem. Scand.*, **12**, 561 (1958).

13) E. Eegriwe, *Z. Anal. Chem.*, **110**, 22 (1937).

Table 1. Values of compounds in a model mixture

Compound	Effluent volume (ml)	Color constant[a]
HMSer	45	3.74
Ser	57	11.90
Gly	86	11.50
Cm-Iz	114	3.07
Gly-Ser	117	11.63
HMSer-Gly	119	2.71
Ser-Gly	129	11.00
Gly-Gly	135	12.15

a) Observed constants for the integration of peaks obtained from each 1 μmol of peptides and amino acids (see D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958)).

a 0.9×50 cm column with spherical resin under the conditions of flow rate 60 ml/hr and jacket temperature 55°C. The 0.2M sodium citrate buffer at pH 3.25 was applied until 70 ml, and then the system was changed to the pH 4.25 buffer. The effluent volumes and color yields of the compounds are summarized in Table 1, and the pattern of elution is shown in Fig. 4-A.

Isolation of Components in Reaction Mixture of Gly-Gly and Formaldehyde with Cupric Ion. *a) Preparation of Reaction Mixture.* A solution of Gly-Gly (1.32 g, 10 mmol), sodium carbonate (4.24 g, 40 mmol), cupric sulfate pentahydrate (1.25 g, 5 mmol) and 37% formaldehyde (6 ml, 80 mmol) in water (200 ml), was kept 100°C for 1 hr. The mixture was cooled with tap water and insoluble material in a small amount was filtered off. After the filtrate was acidified with acetic acid, the solution was evaporated *in vacuo*. The residue was dissolved in water, then the solution was treated with a column of Dowex 50×8 (1.8×30 cm, H^+ form). The column was washed with water and eluted with 2N ammonium hydroxide. The eluate was evaporated *in vacuo*, the residue was dissolved in water (1 ml), and the solution (about 1 ml) was used for the separation experiment as described in the following section. Approximately 1/2000 part of the solution was analyzed with the amino acid analyzer under the conditions described before, the pattern indicating the presence of seven components (Fig. 4-B).

b) Isolation and Characterization of Components. After several runs under various conditions, we found the following conditions to be satisfactory for separating the reaction mixture into each component. The solution (about 1 ml) was put on a column (1.8×110 cm) of Dowex 50×8 (200—400 mesh, NH_4^+ form), and the column was eluted with 0.2M ammonium acetate buffer of pH 4.0 with a flow rate of 30 ml/hr. An aliquot of each fraction (3 ml) was tested on a paper strip with ninhydrin, and the strip demonstrated that the reaction mixture was also separated into seven portions. The fractions containing each component were combined and evaporated to dryness *in vacuo* for removal of most of the ammonium acetate. The residue was dissolved in water and treated with a column of Dowex 50×8 (H^+ form) to remove the remaining acetate ion. After washing with water, the column was eluted with 2N ammonium hydroxide and the eluate was evaporated *in vacuo*. A residual solid was recrystallized from water-ethanol. The amount obtained is shown in Table 2 together with methods of characterization of the product isolated.

Table 2. Isolation and characterization of components in a reaction mixture[a]

Component	Numbers of test tubes treated (tube, 3 ml)	Amount isolated and yield mg (%)	Method of characterization
HMSer	55—65	71(5)	PPC
Ser	67—80	5(0.5)	PPC
Gly	95—115	8(1)	PPC
Cm-Iz	117—145	353(25)	PPC, EA, AAA
HMSer-Gly	155—170	195(10)	PPC, EA, AAA
Ser-Gly	180—250	605(37)	PPC, EA, AAA
Gly-Gly	250—270	12(1)	PPC, AAA

a) The reaction mixture was prepared from Gly-Gly (1.32 g), formaldehyde (6 ml), sodium carbonate (4.24 g) and cupric sulfate (1.25 g) (for details, see Experimental).
b) PPC, paper chromatography compared with authentic sample. EA, elemental analysis (C, H and N). AAA, analysis by amino acid analyzer on hydrolysate derived from the component with 6N hydrochloric acid at 110°C for 12 hr.

As an example, a procedure for identification of Cm-Iz is described below. The fractions from tube number 117 to 145 were treated as described above, and the crude product obtained was recrystallized from water-ethanol; yield, 353 mg (25%); mp 200°C (decomp.); R_f 0.42; amino acid analysis, $Gly_{2.05}$.

Calcd for $C_5H_8O_3N_2$: C, 41.66; H, 5.59; N, 19.44%. Found: C, 41.59; H, 5.71; N, 19.33%.

Effect of Cupric Ion and Reaction Time on Formation of Dipeptides. *a) Effect of Cupric Ion.* Effect of variation in concentration of cupric ion was examined. A solution of Gly-Gly (132 mg, 1 mmol), sodium carbonate (424 mg, 4 mmol), 37% formaldehyde (0.6 ml, 8 mmol) and an appropriate amount of cupric sulfate (0 to 1 mmol) in water (20 ml) was heated at 100° for 1 hr, and treated as described before. The residue was dissolved in 0.2M citrate buffer (20 ml) of pH 2.2, and 0.1 ml of the solution was applied to the amino acid analyzer. The results obtained were shown in Fig. 6.

b) Effect of Reaction Time. Effect of variation in reaction time was examined. A solution of Gly-Gly (1 mmol), sodium carbonate (4 mmol), cupric sulfate (1 mmol) and 37% formaldehyde (8 mmol) in water (20 ml), was heated at 100°C. After various time intervals a portion of the solution was treated as described before. Each solution in citrate buffer at pH 2.2 was applied to the amino acid analyzer, the results being shown in Fig. 7.

Preparation of Ser-Gly from Gly-Gly. A solution of Gly-Gly (5.28 g, 0.04 mol), sodium carbonate (17 g, 0.1 mol), cupric sulfate (1.0 g, 0.04 mol) and 37% formaldehyde (24 ml) in water (800 ml) was heated at 100°C for 30 min. The reaction mixture was treated with a Dowex 50×8 column (2.5×60 cm, 100—200 mesh, H^+ form). It was washed with water and eluted

with 2N ammonium hydroxide. The eluate was evaporated *in vacuo*, and the residual crystals were recrystallized from water-ethanol; yield, 4.42 g (70%); mp 203—205° (decomp.); R_f 0.45.

Found: C, 37.15; H, 6.11; N, 17.29%. Calcd for $C_5H_{10}O_4N_2$: C, 37.03; H, 6.62; N, 17.28%.

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