BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 1205—1208 (1967)

A New Method of Reducing Nitroarginine-peptide into Arginine-peptide, with Reference to the Synthesis of Poly-L-arginine Hydrochloride*[1]

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(Received May 12, 1966)

With stannous chloride in formic acid, nitroarginine can be reduced to L-arginine hydrochloride quantitatively. This new method for the removal of the nitro group was applied successfully in the synthesis of poly-L-arginine hydrochloride from poly-L-nitroarginine, which had been prepared by the polymerization of *N*-carbothiophenyl-L-nitroarginine in dimethylsulfoxide at 120°C for 14 hr. The infrared absorption spectra of these polymers showed absorptions at 1708 cm^{-1} and 1766 cm^{-1}, these absorptions might correspond to the C=O group in the hydantoin of the *N*-terminal. The poly-L-arginine hydrochloride showed positive Biuret and Sakaguchi reactions.

The guanido group in arginine is generally protected for peptide synthesis by nitration, and it is then coupled with other amino acids. The nitroarginine residue was converted back to the parent amino acid residue, with the removal of the nitro group by catalytic[1] or electrolytic[2] reduction. In some cases, however, these methods failed in the removal of the nitro group because of the limited solubility of peptide or because of other factors.

This paper will present a convenient and efficient method of preparing L-arginine hydrochloride from L-nitroarginine with stannous chloride in formic acid without any detectable loss in optical activity. It will also deal with the application of this method to the synthesis to poly-L-arginine hydrochloride from poly-L-nitroarginine.

The indirect synthesis of poly-arginine by the guanidization of poly-ornithine was described by Katchalski,[3] Ariely,[4] and Debarov,[5] but it is very difficult to guanidize quantitatively the ornithyl residues of poly-ornithine into poly-arginine. Poly-L-nitroarginine failed to be converted into poly-L-arginine by palladium reduction, as has been reported,[6] but the present method produces good results in the conversion from poly-L-nitroarginine into poly-L-arginine without any cleavage of the peptide bond. Moreover, the method seems applicable to other nitroarginyl peptides.

To a solution of L-nitroarginine in 60% formic acid, 8 mol/mol of stannous chloride against L-nitroarginine was added; the solution was then heated in a boiling water bath for 3 hr. As a result the L-nitroarginine was completely converted into L-arginine.

In order to remove the nitro group of the nitroarginyl residue in peptides under mild conditions without any cleavage of the peptide bond, the reductive conditions were tested at a set temperature of 50°C. When 4.4—6.0 mol/mol of stannous chloride against L-nitroarginine in 60% formic acid is used, more time is required to complete the reduction; a trace of nitroarginine remains even after 40 hr or more in 50% formic acid, as is shown in Fig. 1. Various concentrations of formic acid, 50, 60, 70 and 85%, were tested at 8 mol/mol stannous chloride against L-nitroarginine, as is shown in Fig. 2.

L-Nitroarginine was quantitatively reduced to L-arginine by treatment for 40 hr with 50%, for 25 hr with 60%, for 20 hr with 70%, and for 12 hr with 85% of formic acid respectively. From these results, the most suitable conditions for reduction were finally determined, one part of L-nitroarginine was dissolved in 30 parts of 60% formic acid; 8 mol/mol of stannous chloride against L-nitroarginine was added to it, and then the mixture was kept for 25 hr at 50°C.

The carbobenzyloxy group of ε-*N*-carbobenzyloxy-lysine or *S*-carbobenzyloxy-cysteine could not be split off into lysine or cysteine by this reduction. Hence, only the nitro group of the arginine residue in the peptide was selectively removed. This procedure was also applied with success in the synthesis of poly-L-arginine hydrochloride from poly-L-nitroarginine. The process of synthesizing

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*1 Presented in part at the 19th Annual Meeting of the Chemical Society of Japan, Yokohama, April, 1966.

1) M. Bergmann, L. Zervas and H. Rinke, *Z. Physiol. Chem.*, **224**, 40 (1934); K. Hofmann, W. D. Peckham and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).

2) P. M. Scopes, K. B. Walshaw, M. Welford and G. T. Young, *J. Chem. Soc.*, **1965**, 782.

3) E. Katchalski and P. Spitnik, *J. Am. Chem. Soc.*, **73**, 3992 (1951).

4) S. Ariely, M. Wilchek and A. Patchornik, *Biopolymers* **4**, 91 (1966).

5) V. G. Debabov and V. D. Davydov, *Izv. Akad. Nauk. SSSR, Ser. Khim.*, **1965**, 203.

6) T. Hayakawa, T. Mizuno and J. Noguchi, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **81**, 618 (1960).

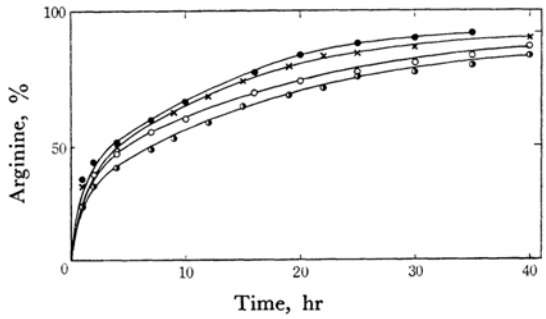

Fig. 1. Reduction to L-arginine from L-nitroarginine.
-●- with 6.0 mol/mol stannous chloride, 60% formic acid
-×- with 4.4 mol/mol stannous chloride, 60% formic acid
-○- with 6.0 mol/mol stannous chloride, 50% formic acid
-◑- with 4.4 mol/mol stannous chloride, 50% formic acid

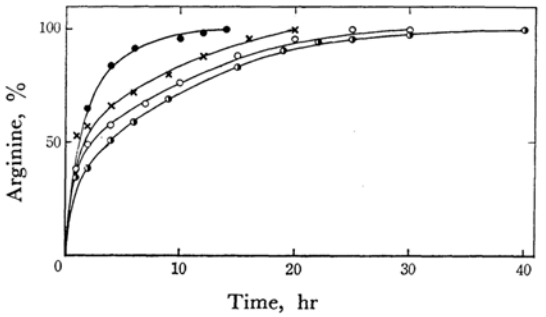

Fig. 2. Reduction to L-arginine from L-nitroarginine with 8.0 mol/mol stannous chloride.
-●- in 85% formic acid
-×- in 70% formic acid
-○- in 60% formic acid
-◑- in 50% formic acid

poly-L-nitroarginine, which was prepared by the polymerization of *N*-carbothiophenyl-L-nitroarginine in a previous paper, was improved with respect to the condition of polymerization in dimethylsulfoxide at 120°C for 14 hr, but the degree of polymerization was as low as before because of the termination with the formation of the hydantoin group. The degree of polymerization (DP) of this polymer was about 10.

These polymers showed infrared absorptions, at 3300 cm^{-1}(νNH), 1650 cm^{-1}(Amide I) and 1530 cm^{-1} (Amide II), which were related to the specific absorption of polypeptide; they also showed absorptions, at 1708 cm^{-1} and 1766 cm^{-1}, which might correspond to the C=O group in the hydantoin group, as is shown in Fig. 3. The elementary analysis of the polymers is in good agreement with the calculated values as hydantoin derivatives.

The degree of polymerization of poly-L-arginine hydrochloride was determined to be 9—10 with

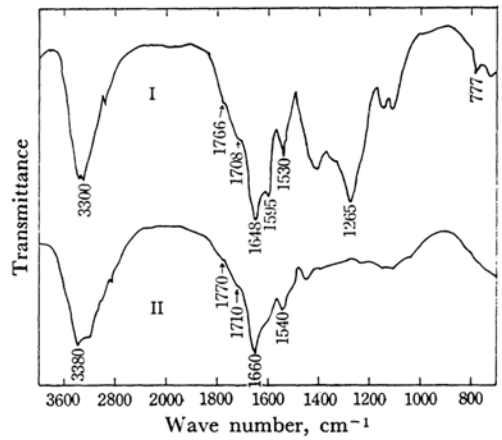

Fig. 3. Infared absorption spectra of poly-L-nitroarginine (I) and poly-L-arginine hydrochloride (II).

a vapor-pressure osmometer; this is in good agreement with the value obtained from the titration of the carboxyl end group of poly-L-nitroarginine. The acid hydrolyzate of poly-L-arginine gave only one spot of arginine by paper chromatography and showed no racemization of the arginine produced. This polymer was soluble in water, 85% formic acid, dimethylsulfoxide, and dichloroacetic acid, but it was insoluble in ether, methanol, ethanol, acetone, glacial acetic acid, dimethylformamide, and dimethylacetamide. It shows positive Biuret and Sakaguchi reactions.

L-Nitroarginine was prepared in an 86% yield under mild conditions from L-arginine hydrochloride by treatment with ammonium nitrate in concentrated sulfuric acid. This is applicable to the preparation of L-nitroarginine on a large scale and is more convenient than Bergmann's method.[1]

Experimental

Preparation of L-Nitroarginine. L-Arginine hydrochloride (100 g) was slowly stirred into 200 m*l* of concentrated sulfuric acid at room temperature. The hydrogen chloride thus produced was removed by a water pump until the gas bubbles disappeared. To a clear solution of the reaction mixture, powdery ammonium nitrate (50 g) was added slowly under stirring, and then, after 15 min, the gas bubbles were removed again, *in vacuo*. The mixture was poured onto cracked ice with stirring. The resulting solution was brought to pH 6.8 with concentrated ammonium hydroxide under cooling, and finally allowed to stand at 0°C for several hours. The precipitate of L-nitroarginine was filtered over suction, washed with cold water, recrystallized from hot water, washed with water, ethanol, and ether, and dried. As there is little difference in the solubility of nitroarginine in cold or hot water, recrystallization from water was partially carried out using repeatedly the mother liquor which was used prior to the recrystallization. Yield, 90 g

(86.5%); mp 255°C (decomp.); $[\alpha]_D^{30}$ +24.1° (*c* 1.93, 2 N HCl).

Found: C, 32.68; H, 5.97; N, 31.91%. Calcd for $C_6H_{13}O_4N_5$: C, 32.87; H, 5.98; N, 31.95%.

Preparation of L-Arginine Hydrochloride by the Reduction of L-Nitroarginine. *A) Reduction in Boiling Water.* L-Nitroarginine (1.0 g) was dissolved in 30 m*l* of 60% formic acid; stannous chloride (8.3 g) was added to it, and then the solution was heated for 3 hr in a boiling water bath. The precipitate was filtered, the filtrate was diluted with 30 m*l* of water, and then hydrogen sulfide was added to it until the precipitation of stannous sulfide ceased. The sulfide was then filtered and extracted with hot water. The combined solution of the filtrate and the extract was treated with active carbon. The clear solution was concentrated to dryness, and the residue was dried in a vacuum desiccator. To the syrup dissolved in absolute ethanol (30 m*l*), triethylamine (0.63 m*l*) was added; then the mixture was allowed to stand for 3 hr in a cold room. The crystalline formed was filtered, washed with ethanol, and dried. The crude product was 0.90 g (93.7%). This was recrystallized from water and ethanol. Yield, 0.67 g (70.0%); mp 215°C (decomp.). The mixed melting point of the crystalline and the standard sample of L-arginine hydrochloride did not show any depression. The paper chromatogram, ascending with a *n*-butanol-glacial acetic acid - water mixture (4 : 1 : 5), gave only one spot of R_f=0.13 (standard sample R_f=0.13), which corresponded to L-arginine; there was no evidence of the existence of nitroarginine. The optical rotation was $[\alpha]_D^{25}$ +22.3° (*c* 2.00, 6 N HCl), which was in good agreement with that of the standard sample.

Found: C, 33.85; H, 7.18; N, 26.60; Cl, 16.35%. Calcd for $C_6H_{15}O_2N_4Cl$: 34.21; H, 7.18; N, 26.68; Cl, 16.83%.

B) Reduction in Warm Water (50°C). To a solution of L-nitroarginine (1.0 g) in 30 m*l* of 60% formic acid, stannous chloride (8.3 g) was added; then the mixture was warmed at 50°C for 25 hr. The reaction mixture was treated in the way as described in A). The crude product was 0.90 g (93.7%). This was recrystallized from water and ethanol.

Yield, 0.65 g (67.8%). The melting point, the R_f value, the optical rotation, and the results of elementary analysis were in good agreement with those of the standard sample. $[\alpha]_D^{25}$ +22.3° (*c* 1.98, 6 N HCl).

Found: C, 34.13; H, 7.24; N, 26.57; Cl, 16.49%. Calcd for $C_6H_{15}O_2N_4Cl$: C, 34.21; H, 7.18; N, 26.68; Cl, 16.83%.

***N*-Carbothiophenyl-L-nitroarginine.** Methyl-*N*-carbothiophenyl-L-nitroargininate was prepared from carbothiophenyl chloride (8.7 g) and methyl-L-nitroargininate hydrochloride (13.7 g) in a way similar to that used for ethyl-*N*-carbothiophenyl-L-nitroargininate in a previous paper. This product was recrystallized from methanol. Yield, 16.2 g (88%); mp 143°C, $[\alpha]_D^{23}$ −10.2° (*c*, 1.85, acetone).

Found: C, 45.65; H, 5.18; N, 18.98%. Calcd for $C_{14}H_{19}O_5N_5S$: C, 45.52; H, 5.19; N, 18.97%.

The above ester was hydrolyzed to *N*-carbothiophenyl-L-nitroarginine by the method used in a previous paper.[6] It was recrystallized from methanol and ether. mp 152°C, $[\alpha]_D^{15}$ +14.2° (*c* 1.71, ethanol).

Found: C, 43.78; H, 4.75; N, 19.76%. Calcd for $C_{13}H_{17}O_5N_5S$: C, 43.98; H, 4.82; N, 19.71%.

Poly-L-nitroarginine. To *N*-Carbothiophenyl-L-nitroarginine (10 g), 5.0 m*l* of dimethyl sulfoxide was added, the mixture was heated at 115—120°C for 14 hr in a sealed tube with a nitrogen gas atmosphere, and then kept at the same temperature for 2 hr *in vacuo*. The polymerized product was taken out with acetone after cooling, centrifuged, and washed with acetone. The polymer was dissolved in 10 m*l* of dimethylformamide, reprecipitated with ethanol, and washed with ethanol and ether. Yield, 5.6 g (99%); $[\alpha]_D^{30}$ −16.6° (*c* 2.01, dimethylformamide). It showed a positive Biuret reaction. Equiv. mol wt≒2020, DP≒10 (by the titration of the carboxyl end with N/50 CH_3ONa in methanol). It has no free amino end from the perchloric titration in glacial acetic acid.

Found: C, 35.75; H, 5.76; N, 33.55%. Calcd for $C_{61}H_{110}O_{32}N_{56}$: C, 35.63; H, 5.39; N, 34.07%.

Poly-L-arginine Hydrochloride. To a solution of poly-L-nitroarginine (5.0 g) in 100 m*l* of 60% formic acid, stannous chloride (45.0 g) was added; the mixture was then allowed to stand for 25 hr at 50°C. The precipitate was filtered and washed with water. The filtrate and the washed water were collected and concentrated *in vacuo* to one-half volume within 35°C. This was diluted by adding 100 m*l* of water and saturated with hydrogen sulfide. The precipitation of stannous sulfide was filtered off and washed three times with 150 m*l* of hot water. The filtrate and the washed water were combined and concentrated *in vacuo* to approximately 100 m*l* at a temperature below 35°C. The concentrated solution was adjusted to pH 5 with triethylamine on cooling and evaporated to dryness *in vacuo*. The residue was dissolved in 400 m*l* of absolute ethanol and reprecipitated with 600 m*l* of acetone, and the precipitate was centrifuged and dried. Yield, 3.0 g (62.7%). This polymer was redissolved in a small amount of water and reprecipitated with ethanol. The precipitate in a syrup was separated by decantation, treated with absolute ethanol until a powder is formed, and then centrifuged, washed with ethanol and ether, and dried. Yield, 2.5 g (52.3%); $[\alpha]_D^{20}$ −21.3° (*c* 2.35, water). Even though we have assume no racemization, the optical rotation of this polymer was low compared with the value reported by Ariely.[4] This may be due to the low molecular weight of the polymer, which has a hydantoin group at about the tenth mole of the component as the *N*-terminal. This shows positive Biuret and Sakaguchi reactions, mol wt≒1750, DP≒9—10 (by the measurement of the molecular weight with a vapor-pressure osmometer, Model 301A, Mechro Lab., Inc.).

Found: C, 37.30; H, 6.87; N, 28.01; Cl, 17.97%. Calcd for $C_{61}H_{130}O_{12}N_{40}Cl_{10}$: C, 37.23; H, 6.65; N, 28.43; Cl, 17.99%.

Hydrolysis of Poly-L-arginine Hydrochloride. A portion of this polymer was hydrolyzed with 6 N hydrochloric acid. The hydrolyzate gave one spot of arginine with ninhydrin by paper chromatography; this spot corresponded to the quantitative amount of arginine from the polymer as determined by measurement with a densitometer. The hydrolyzate has $[\alpha]_D^{25}$ +22.2 (*c* 0.27, 6 N HCl), which agrees with the $[\alpha]_D^{25}$ value of L-arginine.

Infrared Absorption Spectra of Poly-L-nitroarginine and Poly-L-arginine Hydrochloride. The

infrared absorption spectra of poly-L-nitroarginine and poly-L-arginine hydrochloride were measured by the KBr pellet method (Fig. 3). Both spectra showed absorption bands at 3300 cm^{-1} (ν NH), 1650 cm^{-1} (Amide I), and 1550 cm^{-1} (Amide II) which are the specific absorption spectra of polypeptides; each also has absorption shoulders at 1708 cm^{-1} and 1766 cm^{-1}. The latter spectra may be due to the C=O group in hydantoin derivatives. Poly-L-arginine hydrochloride failed to show any of the absorption bands of the nitroamine group, 1595, 1265, and 777 cm^{-1}.

The authors are very much indebted to the Fuji Film Co., Ltd., and to the Institute of Fiber Research for their financial support. This work was supported in part by a grant from the Ministry of Education.