Kitakanto Med.J.
47 (3) : 133~139, 1997

N^{ω}-HYDROXY-L-ARGININE, AN INTERMEDIATE IN NITRIC OXIDE SYNTHESIS, IS MORE POTENT THAN L-ARGININE FOR ENDOTHELIUM-DEPENDENT VASCULAR RELAXATION.

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Abstract : L-Arginine (Arg) and related amino acids are involved in the pathophysiology of cardiovascular disease. Endothelium-derived relaxing factor, nitric oxide (NO), is generated by vascular endothelial cells from Arg. In this study, high purity N^{ω}-hydroxy-L-arginine (OHArg), an intermediate in the pathway generating NO, was synthesized, and the effects of Arg and OHArg on endothelium-dependent vascular relaxation were examined.

OHArg caused a significant relaxation of endothelium-intact rat aortic rings that had been contracted by norepinephrine. OHArg was more potent than Arg at aortic relaxation ($OHArg_{ED80}$= $10^{-7.4}$M versus Arg_{ED80}= $10^{-5.5}$M). Neither OHArg nor Arg relaxed the endothelium-denuded or NO synthase-inhibited aortic rings. These results suggest that OHArg is more potent than Arg at inducing vascular relaxation that is endothelium-dependent and NO synthase-dependent. OHArg may be useful in the treatment of hypertension and in the prevention of atherosclerosis.

Key words : Arginine, Hydroxyarginine, Nitric Oxide, Vascular relaxation
(Kitakanto Med.J. 47 (3) : 133~139, 1997)

INTRODUCTION

In 1985, Stuehr and Marletta[1], who studied the biosynthesis of nitrite/nitrate induced by lipopolysaccharide in the mouse macrophages, suggested that synthesis of nitrite/nitrate was essential for macrophage function. It was soon reported that L-arginine was also required for the synthesis of nitric oxide (NO)[2~5]. The biochemical pathway for generation of NO and L-citrulline from L-arginine was thas established.

Acetylcholine (ACh) produces vascular relaxation that is dependent on the endothelium[6]. Palmer et al[7]. found that vascular endothelial cells generate NO and L-citrulline from L-arginine, and established NO as the endothelium-derived relaxing factor. It appears that L-arginine and the related amino acids are involved in the pathophysiology of cardiovascular disease. The infusion of L-arginine analogues that inhibit NO synthase increases the blood pressure of animals[8,9]. The infusion of L-arginine reduces blood pressure of hypertensive patients[10]. L-Arginine supplements reportedly prevent the development of atherosclerosis[11].

N^{ω}-Hydroxy-L-arginine (OHArg) has been proposed as an intermediate in the pathway from L-arginine (Arg) to L-citrulline[4,5]. OHArg is reportedly a substrate for NO synthase[12,13]. The initial step in NO synthesis is the hydroxylation of the guanidinium nitrogen of Arg yielding OHArg. The second step is

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the oxidation of OHArg to L-citrulline and NO. This pathway has been confirmed indirectly by the kinetics of substrate-enzyme reactions[12,13] and by radioactive tracers in cell culture[14,15]. Differences in substrate-enzyme kinetics suggest they may possess differing potencies for inducing vascular relaxation. OHArg has been reported to dilatate the arteries[16~18]. However, it is unclear whether such vascular relaxation is dependent on endothelium and NO synthase. In the present study, high-purity OHArg was synthesized, and the effects of Arg and OHArg on vascular relaxation were determined.

MATERIALS AND METHODS

Synthesis of OHArg

OHArg was synthesized from N^{ω}-carbobenzyloxy (Cbz)-L-ornitine (Sigma Chemical Co., St.Louis, USA) through five intermediates ; (i) t-butyl N^{δ}-carbobenzoxy (Cbz)-L-ornithinate, (ii) t-butyl N^{α}-Boc-N^{δ}-Cbz-L-ornithinate, (iii) t-butyl N^{α}-Boc-L-ornith-inate, (iv) t-butyl N^{α}-Boc-N^{δ}-cyano-L-ornithinate, and (v) t-butyl N^{α}-Boc-N^{δ}-hydroxy-L-arginine. The synthetic procedures described by Wallace and Fukuto[19] and Wagenaar and Kerwin Jr[20]. were modified in this study (see appendix). The synthesized OHArg was stored in brown light-protected ampules with argon gas.

Tissue Preparation and Tension Measurement

Male Wistar rats (250–300g) were individually caged and fed rat chow (Oriental Yeast Co.Ltd, Tokyo, Japan) and water *ad libitum*. They were exanguinated while anesthetized by the intraperitoneal injection of pentobarbital (50mg/kg). The thoracic aorta was immediately excised, loose connective tissue was removed without damaging the endothelium. The aorta was cut into two or three cylindrical segments 3mm long. These aortic rings were suspended in individual 10ml baths of Krebs' bicarbonate solution (120mM NaCl, 5.2mM KCl, 2.4mM $CaCl_2$, 1.2mM $MgSO_4$, 25mM $NaHCO_3$, 0.03mM Na_2EDTA, and 11mM dextrose ; pH 7.4). The solution was kept at 37°C and continuously aerated with 95% O_2/ 5 % CO_2. After the rings had equilibrated for 20min under 2 g tension, the changes in isometric tension were recorded through force-displacement transducers (UR-50GR Minebea, Japan) after the addition of drugs to the bath. Endothelium was removed gently with a soft plastic stick to prepare endothelium-denuded rings. Some aortic rings with intact endothelium were treated for 20min before tension measurement with 1mM N^{ω}-nitro-L-arginine methyl ester (LNAME ; Sigma Chemical Co.) to inhibit NO synthase. The functional integrity of the rings with intact endothelium, denuded endothelium, and rings exposed to LNAME to inhibit NO synthase was confirmed by contraction with 10^{-7}M norepinephrine (NE ; Sankyo Co., Tokyo, Japan) followed by relaxation by treatment with 10^{-7}M ACh (Sigma Chemical Co.). The aortic rings were again contracted with 10^{-7}M NE and the relaxation response resulting from 5-min exposures to increasing concentrations of Arg or OHArg was measured. Final drug concentrations (10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}M) were achieved by addition of concentrated stock solutions (100μl) to the bath. Stock solutions were prepared in water and stocked in a light-protected freezer box at -20°C for no more than one week prior to use.

Statistics

Data are expressed as the mean±SEM of n measurements. For comparison of the effects of OHArg or Arg on vascular relaxation, the tension developed by 10^{-7}M NE was defined as 100%. To compare the concentration/response curves, ED_{80} values (the molar concentration of drug resulting in relaxation to 80% of the initial value) were calculated. Statistical comparisons were made using ANOVA for repeated measures. Student's t-test was used to compare two values. A level of $P<0.05$ was considered statistically significant.

RESULTS

The purity of OHArg was confirmed by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (HRFABMS) (Fig. 1 and 2, respectively). Details of synthesis are described in the appendix.

Values in Table 1 confirmed the functional integrity of aortic rings selected for use in the study. The contraction induced by NE in endothelium-intact rings was abolished by subsequent treatment with ACh. Denudation of endothelium or inhibition of NO synthase were confirmed by the absence of ACh-induced vascular relaxation. Rather, NE-induced contractions were slightly increased by ACh in treated tissures.

Arg appeared to reduce the tension in the rings with intact endothelium in a concentration-dependent

Table 1. Confirmation of endothelium denudation or NO synthase inhibition by ACh-induced vascular relaxation of aortic rings contracted with NE

	Isometric Tension	
	NE(10^{-7}M)	ACh(10^{-7}M)
Endothelium-intact (n=10)	2.45±0.30g	0.02±0.05g
Endothelium-denuded (n=10)	3.25±0.26g	3.71±0.31g
NO synthase-inhibited[a] (n=10)	3.64±0.16g	4.28±0.18g

Values are mean±SEM of n determinations. a : Nitric oxide synthase was inhibited by 1mM N^{ω}-nitro-L-arginine methyl ester. NE=norepinephrine. ACh=acetylcholine.

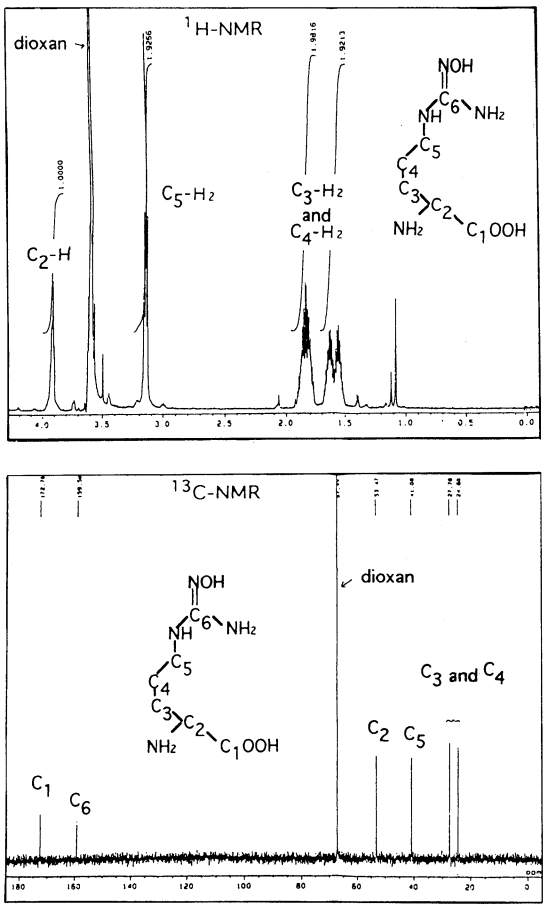

Fig. 1. ^{1}H-NMR and ^{13}C-NMR spectra of synthesized N$^\omega$-hydroxy-L-arginine. There are no unexpected spectra in the charts.

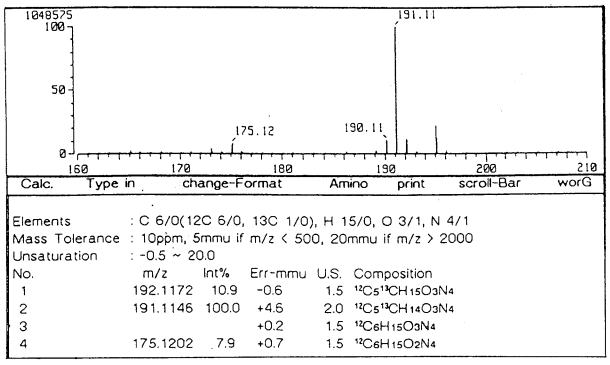

Fig. 2. FABMS spectra of synthesized N$^\omega$-hydroxy-L-arginine. The major spectrum is $^{12}C_5{}^{13}CH_{14}O_3N_4$ which is correspond to N$^\omega$-hydroxy-L-arginine. The spectra indicating other compositions are negligible.

manner between 10^{-8}M and 10^{-5}M (Fig. 3). However, only at 10^{-5}M Arg was the difference statistically significant. OHArg was more potent than Arg at decreasing the NE-induced tension. The difference from 100% contraction was significant at concentrations of OHArg greater than 10^{-8}M. The relaxation induced by OHArg was significantly greater than that induced by Arg 10^{-6}M or 10^{-5}M. Also, the ED_{80} for OHArg ($10^{-7.4\pm0.4}$M) was significantly less than the ED_{80} for Arg ($10^{-5.5\pm0.5}$M).

Neither Arg nor OHArg produced vascular relaxation in any preparation of endothelium-denuded or NO synthase-inhibited rings (Fig. 4). A trend toward values greater than 100% (increased tension) was observed as the dose of Arg or OHArg was increased. However, the difference did not attain statistical significance.

DISCUSSION

It must be emphasized that the purity of the OHArg used to induce vascular relaxation is important. Analyses by NMR and FABNMS confirmed the purity of OHArg used in this study. OHArg was stable in water for at least a week when stored at 4°C.

OHArg and Arg each produced a significant relaxation of rat aorta that had been contracted with NE. At concentration greater than 0.1μM, OHArg relaxed aortic rings to a greater extent than Arg. Denudation of the endothelium or inhibition of NO synthase by LNAME abolished the effect of OHArg, indicating that such relaxation was both endothelium- and NO synthase-dependent.

Arginine is a substrate of at least four enzymes identified in mammals (arginase, arginine decarboxylase, arginine-glycine transaminase and kyotorphine synthase) that can deplete Arg without generating NO. In contrast, OHArg is metabolized by NO synthase and

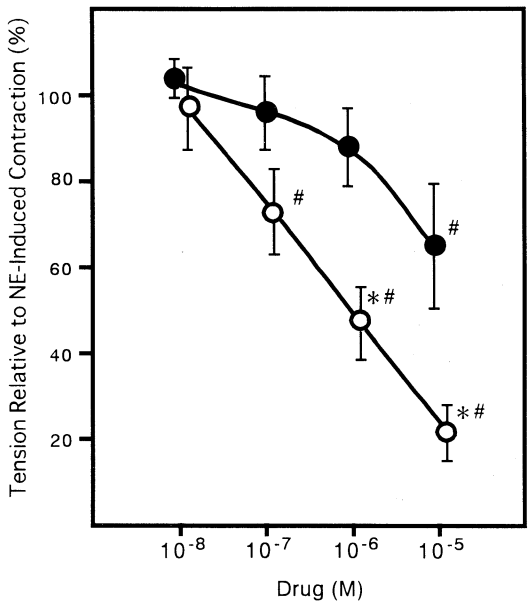

Fig. 3. Drug-induced relaxation of aortic rings contracted by norepinephrin. N$^\omega$-hydroxy-L-arginine (○); L-arginine (●). Values are the mean±SEM from 5 experiments using aortic rings with intact endothelium from 5 rats. and the endothelium is intact. *: a value of $p<0.05$ vs L-arginine at the indicated concentration. #: a value of $p<0.05$ from 100% for each drug.

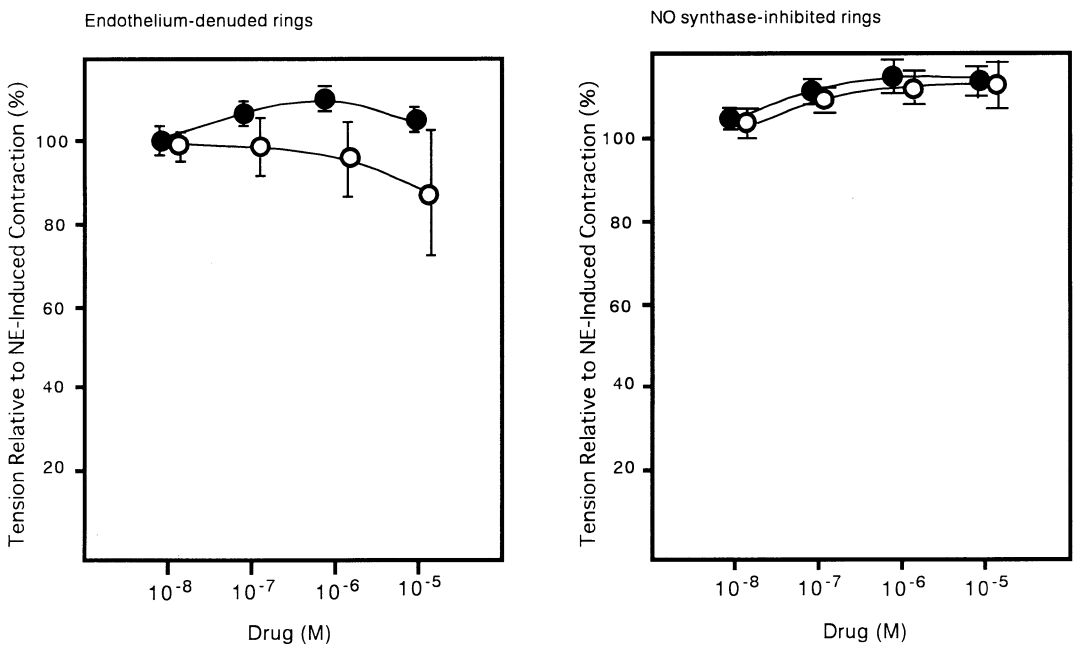

Fig. 4. Drug-induced relaxation of pretreated aortic rings contracted by norepinephrine. N^{ω}-Hydroxy-L-arginine (○); L-arginine (●). Values are the mean±SEM of five experiments with each type of pretreated ring.

cytochrome P450s, both of which catalyze production of NO[21,22]. OHArg is also oxidised to citrulline and NO by non-organic agents[23]. Recently, Daghigh et al[24]. and Boucher et al[25]. reported that OHArg is a potent inhibitor of arginase, one of the major enzymes in the urea cycle that deplete Arg. Thus, several mechanisms exist to explain the enhanced potency of OHArg over Arg with respect to vascular relaxation.

Discrepancies appear to exist between the present study and previous reports. In a semi-quantitative study, Wallace et al[16]. also reported that OHArg was a more potent vascular relaxant than Arg; such relaxation could be inhibited by NO synthase inhibitors. However, the relaxation appeared to be endothelium-independent. Zembowicz et al[17,18]. demonstrated that OHArg reacted with NO to generate potent, long-lasting vasorelaxing agents in a cascade assay system. OHArg itself did not produce vascular relaxation at concentrations less than 10μM in endothelium-intact aortic strips. High concentrations of OHArg produced an endothelium-independent vascular relaxation. The apparent differences in potency are probably due to the enhanced purity of OHArg prepared for the present study. The observation in previous reports that relaxation appeared to be endothelium-independent, but dependent on NO synthase, suggests that some endothelium remained, or that the NO synthase in smooth muscle had been induced. Additional study is necessary to resolve these issues.

Interest in Arg is increasing because it is both antihypertensive[10] and antiatherogenic[11]. Arg improves endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolemic humans[26], decreses the ACh-induced vasocontraction of human atheromatous coronary arteries[27], and restores the blunted, endothelium-dependent vascular relaxation in coronary arterioles downstream from atherosclerotic lesions[28]. Although the clinical effects of OHArg have not been investigated, results of this study suggest that this intermediate in the NO-generating pathway may be useful in treating hypertension and in preventing atherosclerosis.

APPENDIX

OHArg was synthesized from N^{ω}-carbobenzoxy (Cbz)-L-ornithine (Sigma Chemical Co., St. Louis, USA) through five intermediates, (i) to (v) below. Other reagents were purchased from Nacalai Co. Ltd., (Kyoto, Japan) or Aldrich Chemical Company Inc. (Milwaukee, USA). Reagents were used without further purification except: tetrahydrofuran (THF) was purified by refluxing with sodium and benzophenone, and dioxane was distilled in the presence of sodium. Two grades of silica gel, No 7734 (Merck, Darmstadt, Germany) and No 60 (Nacalai), were used for column chromatography and flash chromatography, respectively.

(i). A suspension of N^{ω}-Cbz-L-ornithine (9.78g, 36.7mM) in t-butyl acetate (520ml, 3.86M) containing perchloric acid (2.6ml, 40.1mM) was stirred at 70°C for

15 days. After addition of a large amount of water, the reaction mixture was made alkaline (pH 8-9) with 10% NaOH and extracted with ethyl acetate. The ethyl acetate solution was dried over K_2CO_3 and evaporated to dryness under reduced pressure to give t-butyl N^{δ}-Cbz-L-ornithinate (i) as a brownish yellow oil (6.37g, 53.8%). IR (JASCO, IR-700) ν^{max} ($CHCl_3$) cm^{-1} : 3454 (NH), 1719 (CO) ; ^1H NMR [JOEL, JNM GXS-500α (500MHz, in $CDCl_3$)] δ1.45 (9H, s, t-Bu), 1.50-1.65 (3H, m, 3-H, 4-H_2), 1.70-1.80 (1H, m, 3-H), 2.10 (2H, s, NH_2), 3.22 (2H, m, 5-H_2), 3.34 (1H, t like, 2-H), 5.09 (2H, s, OCH_2Ph), 5.16 (1H, s, NH), 7.29-7.34 (1H, m, OCH_2Ph), 7.34 (2H, s, OCH_2Ph), 7.36 (2H, s, OCH_2Ph) ; $[\alpha]^{D26}$(JASCO DIP-30)+3.44 (c = 0.387, AcOEt). The starting material, N^{ω}-Cbz-L-ornithine, was recovered (3.62g, 37.0% yield) from the aqueous layer.

(ii). A solution of $(Boc)_2O$ (0.817g, 3.74mM) in methylene chloride (1.4ml) was added dropwise to a stirred solution of t-butyl N^{δ}-Cbz-L-ornithinate (i) (1.036g, 3.21mM) in CH_2Cl_2 (3.9ml) at 0°C. The reaction mixture was stirred at 0°C for 1 h and then stirring continued at room temperature for 17h. Evaporation of the solvent under reduced pressure gave a brownish yellow oil. This was purified by column chromatography using 4 : 1 hexane : ethyl acetate to give t-butyl N^{α}-Boc-N^{δ}-Cbz-L-ornithinate (ii) as a pale yellow oil (1.293g, 95.1% yield). IR ν^{max} ($CHCl_3$) cm^{-1} : 3450 (NH), 1712 (CO) ; ^1H NMR δ1.44 (9H, s, t-Bu), 1.46 (9H, s, t-Bu), 1.50-1.65 (3H, m, 3-H, 4-H_2), 1.75-1.85 (1H, br s, 3-H), 3.22 (2H, q like, J = 5.0Hz, 5-H_2), 4.17 (1H, br, 2-H), 4.88 (1H, s, NH), 5.09 (3H, s, NH, OCH_2Ph), 7.31 (1H, m, OCH_2Ph), 7.35 (2H, s, OCH_2Ph), 7.36 (2H, s, OCH_2Ph) ; $[\alpha]^{D24}$-0.25 (c = 0.794, AcOEt) ; ORD (JASCO J-20) $[\alpha]^{589}$-1.68 (c = 0.954, AcOEt).

(iii). A suspension of the fully protected ornithinate (ii) (2.007g, 4.76mM) and 10% palladium on carbon (0.401g) in ethanol (123ml) was hydrogenated at room temperature for 4 h. The catalyst was removed by filtration and the filtrate was evaporated under reduced pressure to give t-butyl N^{α}-Boc-L-ornithinate (iii) (1.397g, quantitative yield) as a yellow oil. IR ν^{max} ($CHCl_3$) cm^{-1} : 3440 (NH), 1706 (CO) ; ^1H NMR δ1.40 (9H, s, t-Bu), 1.42 (9H, s, t-Bu), 1.40-1.65 (3H, m, 3-H, 4-H_2), 1.70-1.82 (1H, br, 3-H), 1.90-2.00 (2H, br, NH_2), 2.66 (2H, t like, 5-H_2), 4.12 (1H, br, 2-H), 5.17 (1H, br, NH).

(iv). A solution of cyanogen bromide (95%, 0.612g, 5.78mM) in dry methanol (1.96ml) was added to an ice-cooled solution of t-butyl N^{α}-Boc-L-ornithinate (iii) (0.697g, 2.42mM) in dry methanol (4.8ml) containing anhydrous sodium acetate (0.443g, 5.40mM). The reaction mixture was stirred at 5°C for 7 h. After evaporation of methanol, the residue was suspended in ether and filtered. The filtrate was evaporated to dryness under reduced pressure. Flash chromatography of the residue using 2 : 1 hexane : ethyl acetate gave t-butyl N^{α}-Boc-N^{δ}-cyano-L-ornithinate (iv) as a yellow oil (0.311g, 41.0% yield). IR ν^{max} ($CHCl_3$) cm^{-1} : 3432 (NH), 2224 (CN), 1706 (CO) ; ^1H NMR δ1.44 (9H, s, t-Bu), 1.48 (9H, s, t-Bu), 1.65-1.75 (4H, m, 3-, 4-H_2), 3.10-3.17 (2H, br t like, 5-H_2), 4.15 (1H, dif. d, J=5.8 Hz, 2-H), 4.53 (1H, br, NH), 5.20 (1H, d like, J=7.0 Hz, NH) ; ^{13}C NMR (125MHz) δ25.20 (4-CH_2), 27.93 ($C(CH_3)_3$), 28.25 ($C(CH_3)_3$), 29.98 (3-CH_2), 45.55 (5-CH_2), 53.08 (2-CH), 80.03 ($C(CH_3)_3$), 82.41 ($C(CH_3)_3$), 116.32 (C=N), 155.54 (NHCO), 171.33 (COO) ; HRFABMS m/z : 314.2076 (calculated for $C_{15}H_{28}N_3O_4$: 314.2080) ; $[\alpha]^{D16}$-12.3 (c=0.902, EtOH) {$[\alpha]^{D23}$-19.6 (c=0.85, EtOH) in Ref. 19}.

(v). A mixture of the cyanamide (iv) (0.279g, 0.89mM), hydroxylamine hydrochloride (0.134g, 1.92mM), and triethylamine (0.13ml, 0.90mM) in EtOH (2.5ml) was stirred at room temperature for 3 h. After evaporation of solvent, the residue was purified by flash chromatogarphy using 6 : 1 CH_2Cl_2 : EtOH to give t-butyl N^{α}-Boc-N^{δ}-hydroxy-L-arginine (v) as a colorless oil (0.189g, 61.1% yield). IR ν^{max} ($CHCl_3$) cm^{-1} : 3158 (NH), 1690 (CO), 1660 (C=N) ; ^1H NMR (CD_3OD) δ1.44 (9H, s, t-Bu), 1.46 (9H, s, t-Bu), 1.55-1.67 (3H, m, 3-H, 4-H_2), 1.77-1.89 (1H, m, 3-H), 3.01-3.04 (2H, m, 5-H_2), 3.93-3.95 (1H, m, 2-H) ; ^{13}C NMR (CD_3OD) δ27.16 (4-CH_2), 28.25 ($C(CH_3)_3$), 28.73 ($C(CH_3)_3$), 30.10 (3-CH_2), 41.74 (5-CH_2), 55.68 (2-CH), 80.45 ($C(CH_3)_3$), 82.58 ($C(CH_3)_3$), 158.12 (NHCO), 159.08 (7-CH), 173.66 (CO) ; HRFABMS m/z : 347.2289 (calculated for $C_{15}H_{31}N_4O_5$: 347.2294).

(vi). t-Butyl N^{α}-Boc-N^{δ}-hydroxy-L-arginine (v) (0.172g, 0.49mM), was dissolved in dry dioxane (2.4ml) saturated with dry hydrogen chloride gas and kept at room temperature for 3 h. A pale yellow solid was separated and collected by decantation. Washing with dry dioxane gave OHArg (vi) as a colorless powder (0.100g, 77.2% yield), mp 172-174°C (mp 178-180°C in Ref. 19). ^1H NMR (D_2O) δ1.64-1.81 (2H, m, 3- or 4-H), 1.89-2.01 (2H, m, 4- or 3-H), 3.29 (2H, t, J=6.8 Hz, 5-H_2), 3.93 (1H, diffused t, J=6.8Hz, 2-H) ; ^{13}C NMR (D_2O) δ24.60 (4-CH_2), 27.78 (3-CH_2), 41.08 (5-CH_2), 53.47 (2-CH), 159.58 (C=N), 172.76 (CO) ; HRFABMS m/z : 191.1146 (calculated for $C_6H_{15}N_4O_3$: 191.1144) ; $[\alpha]^{D21}$+13.5 (c=1.01, MeOH) {$[\alpha]^{D23}$+21.1 (c=1.07, MeOH) in Ref. 20}.

ACKNOWLEDGEMENT

The authors appreciate the excellent technical assis-

tance of Ms. Shizuko Saiki and Ms. Yoshie Numata.

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