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SYNTHESIS OF THE PUTATIVE L-ARGININE METABOLITE L-N^G-HYDROXYARGININE

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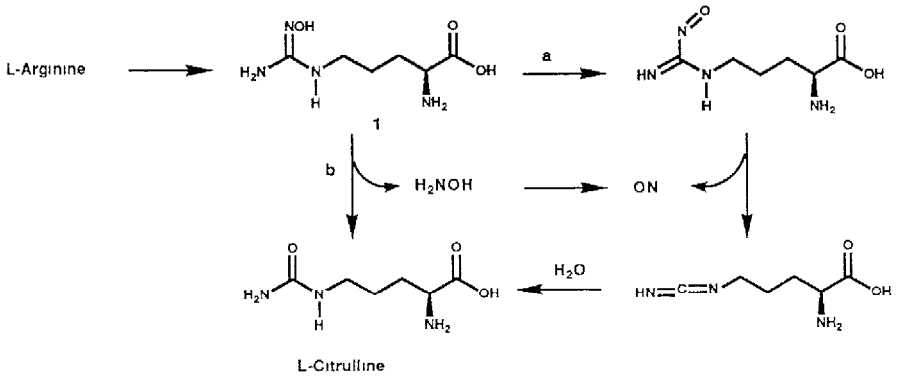
Summary: Syntheses of L-N^G-hydroxyarginine (**1**), the putative biosynthetic precursor of nitric oxide, and the ^{15}N labelled analogs **9** and **10** using L-ornithine as the enantiopure starting material is reported

The continued progress in defining the physiological role of nitric oxide (NO) as it relates to the regulation of cell function and communication has attracted considerable interest [1] NO was first demonstrated to be a second messenger in endothelium dependent smooth muscle relaxation and activated macrophage cytotoxicity Recent reports implicating its role as a second messenger in several other mammalian cell lines attest to its increasing importance in human biology.[1a]

It has been demonstrated that NO is synthesized from L-arginine (L-arg) in endothelial cells and activated macrophages via oxidation of one of the two terminal guanidino nitrogens [2] Three biosynthetic pathways have been proposed for the conversion of L-arg to NO and L-citrulline (L-cit) by the enzyme(s) NO synthase.[2b,3] One involves an enzyme catalyzed hydrolytic deimination of L-arg to generate L-cit and ammonia, with subsequent oxidation of the ammonia to give NO.[3a] In the two other postulated mechanisms, depicted in Scheme 1, L-N^G-hydroxyarginine (**1**) is implicated as the first oxidation product of L-arg. As shown in path a, further oxidation of **1** followed by a homolytic bond cleavage yields NO and the N^E-carbodiimide of ornithine, which upon hydration yields L-cit [2b] An alternative pathway to NO from **1**, path b, is via the hydrolysis of **1** to give hydroxylamine and L-cit followed by oxidation of hydroxylamine to NO [3b] All three of the proposed mechanisms require that the oxygen of the urea function of L-cit be derived from water Since it has recently been demonstrated using $^{18}O_2$ labelling experiments that the oxygen of the urea group comes from dioxygen rather than water, an alternative mechanism for this novel oxidation pathway must be operating.[4]

Scheme 1

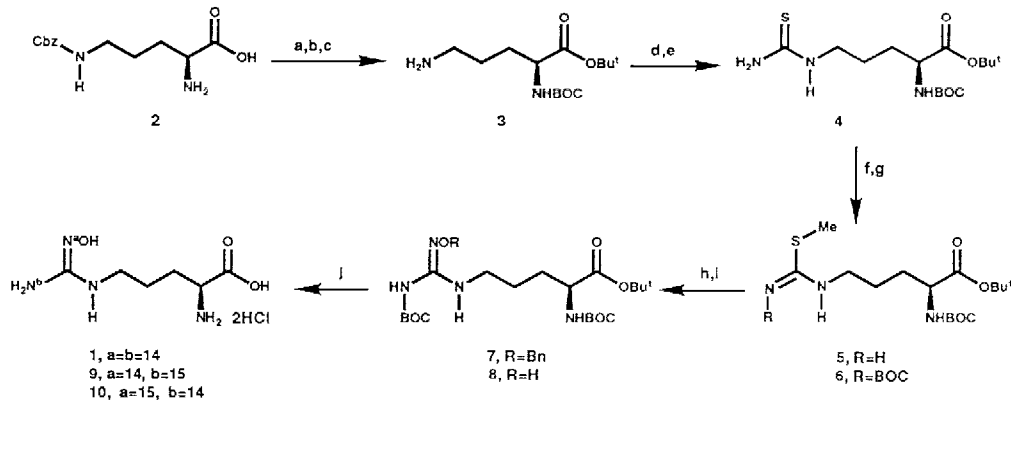


In order to facilitate studies to elucidate the mechanism by which L-arg is converted to NO and L-cit the initial putative oxidation product of L-arg, L-N^G-hydroxyarginine, and its ^{15}N labelled analogs, **9** and **10**, have been synthesized in enantiomerically pure form using L-ornithine as the enantiopure educt (See Scheme 2) Although direct oxidation of a protected L-arg derivative may

be the most expedient pathway to **1**, the route to **1** was chosen such that there would be no ambiguity as to which nitrogen of the guanidine was oxidized, N^G versus N^E, and ^{15}N labelling would be easily accommodated Furthermore, the methodology used to make **1** should be readily adaptable for synthesizing analogs of **1** that might serve as alternative substrates or inhibitors of NO synthase

The synthesis of **1** commenced with the conversion of **2** to the known protected L-ornithine derivative **3** in three steps using a modification of the literature protocol [5] Protection of the α-amino group of **2** yielded its t-butoxycarbonyl (BOC) derivative which was esterified using O-t-butylisopropylisourea[6] and subsequently hydrogenated to give **3** in 44% overall yield [6b] Addition of a solution of **3** in $CHCl_3$ to a suspension of thiophosgene in aqueous $CaCO_3$ rapidly afforded the isothiocyanate of **3** [7] This material could be purified by silica gel chromatography (sgc), but it was more efficient to treat the crude material with an excess of NH_3 in MeOH at 0°C for 3 h to give **4** in 89% yield from **3** following sgc Formation of isothiourea **5** was readily accomplished by alkylating the sulfur of thiourea **4** with methyl iodide in CH_3CN at 23°C Attempted displacement of the thiomethyl moiety of **5** by heating with O-benzylhydroxylamine in various solvents was unrewarding There is precedent that N-tosyl isothioureas are readily converted to tosyl protected guanidines at room temperature by reacting them with amines in the presence of $AgNO_3$ [8] These mild conditions were ideally suited for a sulphonyl protected derivative of **5**. In order to avoid the harsh conditions needed to remove most N-sulphonyl protecting groups, the BOC moiety was employed since it could be removed under mild conditions along with the other protecting groups at the end of the synthesis Protection of crude **5** with $(BOC)_2O$ in dioxane/sat $NaHCO_3$ yielded **6** in 69% from **4** after sgc. Replacement of the thiomethyl group of **6** with O-benzylhydroxylamine was achieved in 90% yield by adding a CH_3CN solution of $AgNO_3$ to a solution of **6**, triethylamine, and O-benzylhydroxylamine in CH_3CN at 0°C and stirring for 2 h. Hydrogenolysis of the benzyl group of the resulting arginine derivative, **7**, using 10% Pd-C and H_2 (1 atm) in MeOH for 30 min afforded **8** in 89% yield following sgc Removal of the BOC groups and hydrolysis of the t-butyl ester was achieved in one step by reacting **8** with 4 N HCl/dioxane at 23°C for 20 h The L-N^G-hydroxyarginine dihydrochloride salt precipitated from the reaction medium to give analytically pure material in 83% yield

Scheme 2



Scheme 2. a. $(BOC)_2$ O (150 mol %), dioxane, sat. $NaHCO_3$, 23°C, 24 h, 100 %; b. ref. 6b, 48 %; c. ref. 6b, 92%; d. thiophosgene (140 mol %), $CaCO_3$ (280 mol %), H_2O, $CHCl_3$, 23°C, 2 h; e. NH_3 (excess), MeOH, 0°C, 3 h, 89% from 3 f. MeI (200 mol %), CH_3CN, 23°C, 16 h, g. $(BOC)_2O$ (150 mol %), dioxane, sat. $NaHCO_3$, 23°C, 12 h, 69% from 4; h. O-benzylhydroxylamine (200 mol %), TEA (120 mol %), $AgNO_3$ (110 mol %), CH_3CN, 0°C, 2 h, 90%; i. 10% Pd-C (20 wt %), MeOH, H_2 (1 atm), 30 min, 89% j 4N HCl/dioxane, 23°C, 20h, 83%.

This sequence of reactions was demonstrated to yield optically pure **1** via the following analysis. Hydrogenolysis of **7** with 10% Pd-C under 50 psi H_2 followed by deprotection with 4 N HCl/dioxane gave L-arg dihydrochloride in 13% yield from **7**. This material was derivatized with Marfey's reagent[9], and subsequent HPLC analysis, using derivatized D, L-arg as a standard, demonstrated that the synthetic material was greater than 99.6% optically pure.

The two ^{15}N labelled samples, **9** and **10**, were readily prepared using the same sequence of reactions except $^{15}NH_3$ was substituted for NH_3 in the **3** to **4** conversion and $H_2{}^{15}NOBn$[10] for H_2NOBn in the **6** to **7** transformation.[11] These compounds, along with **1**, should serve as useful tools for determining whether **1** is an intermediate in the biosynthesis of NO [12,13] The results of the biochemical investigations using **1**, **9**, and **10** will be reported elsewhere [14]

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11 All new compounds gave satisfactory C, H, and N analyses

12 **α-N-t-Butoxycarbonyl-δ-(thioureido)-L-norvaline-t-butyl ester (4).** To a mixture of $CaCO_3$ (6 5 g, 65 2 mmol), H_2O (110

mL), and thiophosgene (2 5 mL, 32 6 mmol) was added a solution of **3** (6 72 g, 23 3 mmol) in $CHCl_3$ (110 mL) The reaction was vigorously stirred at 23°C for 2 h, then filtered and the organic phase separated. The aqueous phase was extracted with $CHCl_3$ and the combined organics washed with brine, dried ($MgSO_4$), and concentrated The oil was dissolved in dry MeOH (200mL), cooled to 0°C, and a stream of NH_3 passed through the solution for 10 min. The reaction was stirred at 0°C for 3 h, then concentrated and the residue chromatographed on silica gel (4/1 EtOAc/hexanes) to give **4** as a solid· 7.22 g, 89%, mp 122-124°C, $[\alpha]^{23}_D$ 4.1 (c 1 19, $CHCl_3$), ^{13}CNMR ($CDCl_3$) 183 4-180 4 (one carbon), 171.6, 155 9, 82 2, 80.0, 53 6-52 5 (one carbon), 44 6-43 1 (one carbon), 30 4, 28 1, 27 8, 24.6

α-N-t-Butoxycarbonyl-δ-[(N-t-butoxycarbonyl-S-methyl) isothioureido]-L-norvaline-t-butyl ester (6). A solution of **4** (2 92 g, 8 4 mmol) and iodomethane (1 0 mL, 16 8 mmol) in CH_3CN (16 mL) was stirred at 23°C for 16 h The solution was concentrated and the residue dissolved in dioxane (20 mL) To a mixture of sat. $NaHCO_3$ (20 mL) and the dioxane solution of **5** was added di-t-butylcarbonate (2 75 g, 12 6 mmol). The reaction was vigorously stirred at 23°C for 12 h. The dioxane was removed via evaporation and the aqueous solution extracted with EtOAc The organics were dried ($MgSO_4$), concentrated, and the residue chromatographed on silica gel (3/1 hexanes/EtOAc) to give **6** as an oil 2 68g, 69%, $[\alpha]^{23}_D$ 15 7° (c 1 26, $CHCl_3$), ^{13}CNMR ($CDCl_3$) 173 0, 171 1, 161 8, 155 0, 81 7, 79 3, 78 7, 53 0, 42 9, 29 7, 28 0, 27 9, 24 8, 13.2

α-N-t-Butoxycarbonyl-N^G-benzyloxy-N$^{G'}$-t-butoxycarbonyl-L-arginine-t-butyl ester (7). To a solution of **6** (1 1 g, 2.38 mmol), triethylamine (0 4 mL, 2 86 mmol), and O-benzylhydroxylamine (587 mg, 4.76 mmol) in CH_3CN (20 mL) at 0°C was added a solution of $AgNO_3$ (450 mg, 2 6 mmol) in CH_3CN (2 mL) dropwise The reaction was stirred at 0°C for 2 h and then filtered through celite to remove the yellow silver salts. The filtrate was concentrated and the residue dissolved in EtOAc. The organics were washed with 1 M H_3PO_4 and brine, then dried ($MgSO_4$) and concentrated The residue was chromatographed on silica gel (4/1 hexanes/EtOAc) to give the product as an oil· 1 05 g; 90%; $[\alpha]^{23}_D$ 8 4° (c 1 16, $CHCl_3$), ^{13}CNMR ($CDCl_3$) δ 171.7, 155 3, 152 1, 148 8, 137 6, 128 6, 127 8, 82 0, 81 7, 79 4, 75 7, 53 7, 40 3, 30 1, 28 2, 28 0, 27 9, 24 8.

α-N-t-Butoxycarbonyl-N^G-hydroxy-N$^{G'}$-t-butoxycarbonyl-L-arginine-t-butyl ester (8). A mixture **7** (2 29 g, 4 27 mmol) and 10% Pd C (200 mg) in MeOH (30 mL) was stirred under 1 atm of H_2 for 30 min. The mixture was filtered through celite to remove the catalyst and the filtrate concentrated The residue was chromatographed on silica gel (3/1 EtOAc/hexanes) to give the product as a low melting solid: 1 70 g, 89%, mp 50-54°C, $[\alpha]^{23}_D$ 8.0° (c 1 14, $CHCl_3$), ^{13}CNMR ($CDCl_3$) δ 171 7, 155 3, 152 3, 148 9, 82 0, 81 8, 79 5, 53 6, 40 4, 30 2, 28 2, 28 0, 27 8, 24 7

L-N^G-Hydroxyarginine dihydrochloride (1). A solution of **8** (7 51 g, 3 38 mmol) in HCl in dioxane (4 N, 13 mL) was stirred at 23°C for 20 h. The mixture was diluted with dry ether (approx 20 mL) and the solid collected by vacuum filtration The solid was washed with EtOAc, then dried at 60°C in a vacuum oven to give **1** as a white solid yield 737 mg, 83%, mp 183-184°C; $[\alpha]^{23}_D$ 19.5° (c 0 61, CH_3OH); ^{13}CNMR (CD_3OD) δ 171 3, 160 3, 53 5, 41 6, 28 5, 25 8.

13 A synthesis of L-N^G-hydroxyarginine has been claimed, and it was reported to be a substrate for NO synthase However, no details of the synthesis or evidence for its intermediacy in the L-Arg to NO pathway was advanced Tayeh, M A , Marletta, M A *J Biol Chem* **1989**, 33, 19654.

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