Synthesis of $^{15}N^{\omega}$-Hydroxy-L-arginine and ESR and ^{15}N-NMR Studies for the Elucidation of the Molecular Mechanism of Enzymic Nitric Oxide Formation from L-Arginine[+)++)]

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N^{ω}-Hydroxy-L-arginine (**2**) was prepared by a multi-stage synthesis; the key step was the addition of hydroxylamine to the protected cyanamide **8**. The presence of *N*-hydroxyguanidines was confirmed, above all, by ^{15}N-NMR investigations. $^{15}N^{\omega}$-Hydroxy-L-arginine (**2**) was converted quantitatively to ^{15}NO by NO synthases from macrophages. ^{15}NO was identified by ESR-spectroscopy. These experiments confirm that $^{15}N^{\omega}$-hydroxy-L-arginine (**2**) is an intermediate in the biosynthesis of NO from arginine (**1**) and that the *N*-hydroxylated N-atom is present in the NO formed.

Synthese, ESR- und ^{15}N-NMR-Untersuchungen von $^{15}N^{\omega}$-Hydroxy-L-arginin zur Aufklärung der enzymatischen Stickstoffmonoxidbildung aus L-Arginin

N^{ω}-Hydroxy-L-arginin (**2**) wurde in einer mehrstufigen Synthese gewonnen, wobei der entscheidende Schritt die Addition von Hydroxylamin an das geschützte Cyanamid **8** darstellt. Das Vorliegen von *N*-Hydroxyguanidinen wurde besonders durch ^{15}N-NMR-Studien bestätigt. $^{15}N^{\omega}$-Hydroxy-L-arginin (**2**) wurde von NO-Synthase aus Makrophagen quantitativ zu ^{15}NO umgesetzt. ^{15}NO wurde ESR-spektroskopisch nachgewiesen. Die Studien belegen, daß N^{ω}-Hydroxy-L-arginin (**2**) eine Zwischenstufe bei der Biosynthese von NO aus Arginin (**1**) darstellt und ausschließlich der N-hydroxylierte Stickstoff im gebildeten NO erscheint.

One of the most exciting biological discoveries of the last few years is the identification of nitric oxide (NO) as a practically universal cellular signalling molecule (for reviews, see refs.[1)2)]). This radical, previously localized only in non-living nature and a few bacteria, does indeed mediate numerous, widely differing biological activities. The NO formed in the endothelial cells of blood vessel walls plays a prominent role in the cardiovascular system by lowering the vascular tone and inhibiting the aggregation of thrombocytes. Some years before the discovery of the endogenous formation of NO[3)], the release of a non-prostanoid relaxing factor from endothelial cells (endothelium-derived relaxing factor, EDRF) was reported[4)]. New results consider a labile, NO-containing compound[5)], for example, *S*-nitrosothiols or nitrosyliron-complexes. The action of numerous drugs such as the so-called organic nitrates is also attributed to their content or metabolic formation of NO or R-NO[1)]. The NO formed in certain neurons appears to function as a retrograde neurotransmitter in the central nervous system and has recently been implicated in the explanation of phenomena associated with learning processes, such as potentiation and depression of long-term memory[6)]. Finally, NO has been used as a cytotoxic effector molecule for activated macrophages, granulocytes, and other cells of the immune system to inhibit the growth of bacteria, fungi, parasites, as well as tumors and exogenous cells, or to kill them and thus retain the immunological identity of the organism[7)8)].

Labeling experiments with $^{15}N^{\omega}_{2}$-L-arginine[**)] have shown that NO or R-NO in endothelial cells[9)10)] and macrophages[11)12)] is formed from one or both terminal N-atoms of the guanidine group of L-arginine (**1**). Citrulline (**3**) is formed as a further product[9-12)]. The urea oxygen atom of this citrulline (**3**) originates from molecular oxygen[13)]. The participating enzymes were initially designated as "NO synthases" [E.C. 1.14.13.39] and two major classes, a constitutive enzyme and an inducible one, were recognized (for a review, see ref.[2)]). The exact mechanism of the transformation of L-arginine (**1**) to NO and citrulline (**3**) by NO synthases is still unknown. However, all of the more favored proposals start from a common first intermediate, N^{ω}-hydroxy-L-arginine (**2**)[12)14)], (Scheme 1) and this possibility has received further support from investigations on N^{ω}-hydroxy-L-arginine[15)16)].

Even so, none of the currently proposed mechanisms for the enzymic formation of NO from L-arginine can account for the incorporation of molecular oxygen into the urea group of L-citrulline[12)14)15)16)]. Future investigations will require a complete characterization of N^{ω}-hydroxy-L-arginine (**2**). We have previously prepared *N*-monosubstituted *N''*-hydroxyguanidines[***)] and characterized this class of compounds by ^{15}N-NMR spectroscopy[17)]. We have also investigated the enzymic *N*-hydroxylation of some - albeit non-physiological - guanidines[18)]. On the basis of this experience, we have synthesized N^{ω}-hydroxy-L-arginine (**2**) and its $^{15}N^{\omega}$-labeled analogue. ^{15}N-NMR spectra were recorded for further structural characterization. ^{15}N-NMR spectroscopy is usually employed to clarify the questions of the

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[**)] In arginine-derivatives N^{ω} refers to the two terminal nitrogen atoms.

[***)] In guanidines *N* and *N'* refer to the single-bonded and *N''* to the double-bonded N-atom.

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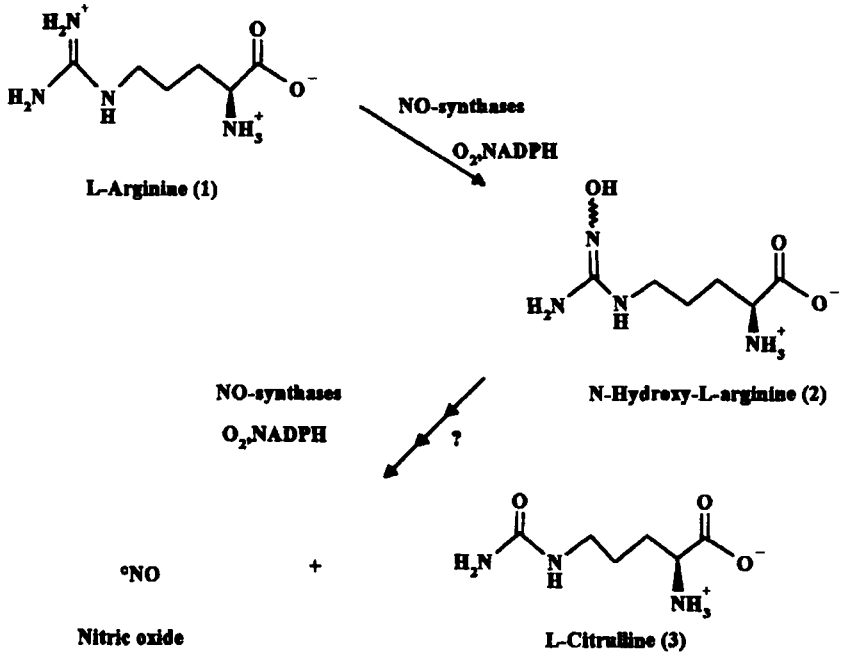


Scheme 1

constitutions, configurations, positions of tautomeric equilibria and the site of protonation in nitrogen containing functional groups[17]. These structural features have not been addressed before by other groups and can only be derived from ^{15}N-NMR spectra which provide additional information not available from ^{1}H- or ^{13}C-NMR data.

The biochemical activity of the reaction product was investigated with the help of partially purified NO synthase from macrophages.

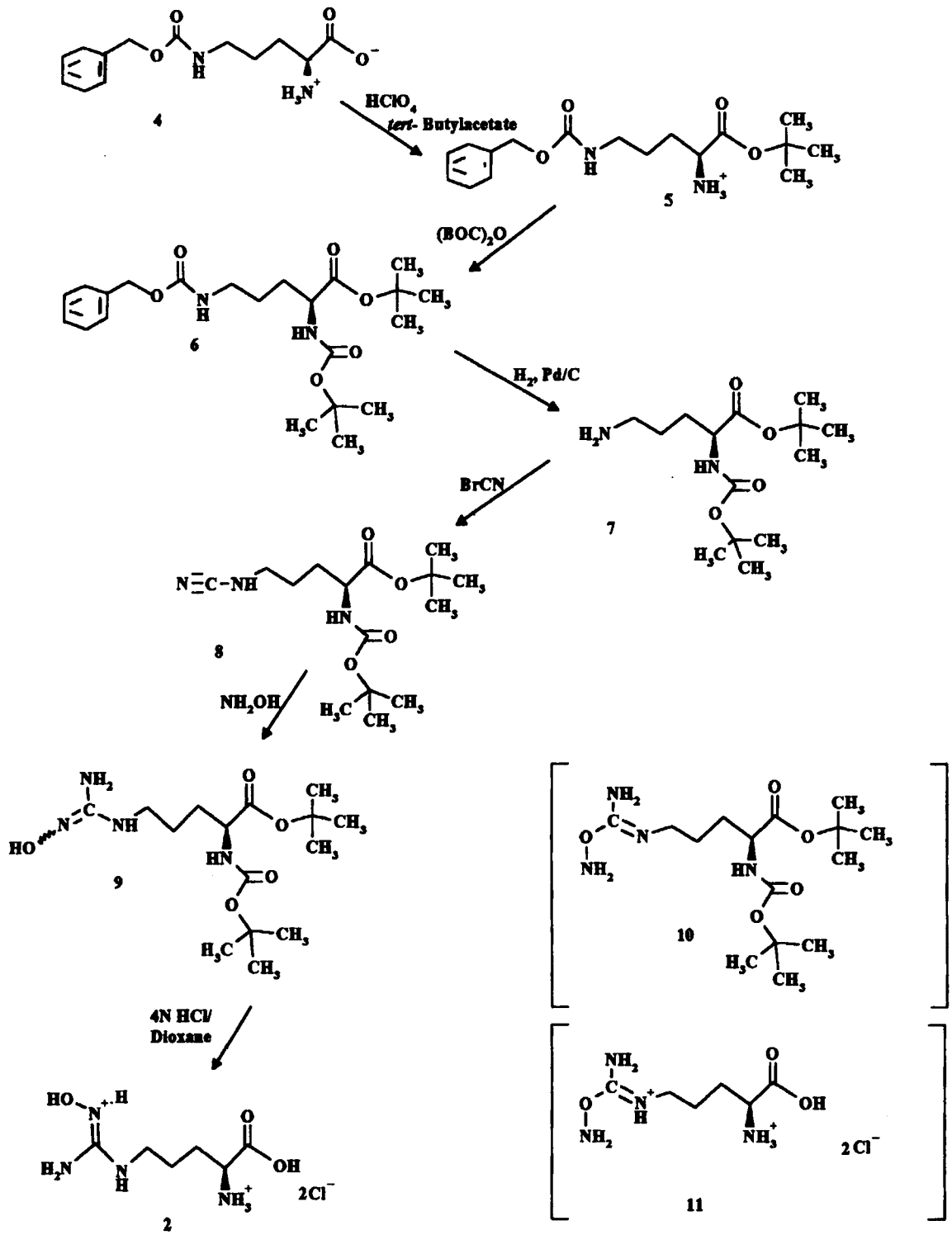
Chemistry

The most promising synthetic concept seemed to be that which had already been successfully employed for the preparation of other *N*-monosubstituted *N''*-hydroxyguanidines[17]. The final step in this approach is the reaction of a cyanamide with hydroxylamine[17]. Indeed, this route proved to be the most successful of all other feasible and tested alternative routes (Scheme 2). L-ornithine was chosen as the starting material. Prior to introduction of a cyanamide function, protection of its α-amino and carboxy groups was essential; this was realized by use of the commercially available *N*-δ-benzyloxycarbonyl-L-ornithine (**4**). This compound was converted to the known derivative **6**[19] by a somewhat modified procedure. The carboxy function of **4** was protected by transesterification with *tert*-butyl acetate to furnish the *tert*-butyl ester **5**[19], *tert*-butylcarbonyl azide[19] and, after its publication[20], *tert*-butyl pyrocarbonate [$(BOC)_2O$] were employed to introduce the α-amino protecting group. Compound **7** obtained after catalytic hydrogenation[19] was treated with BrCN to furnish the cyanamide **8**. This purified product was reacted with hydroxylamine base (obtained according to ref.[21]) in anhydrous dioxan to yield the desired N^{ω}-hydroxyguanidine derivative **9**. The analogous preparation of the protected $^{15}N^{\omega}$-hydroxy-L-arginine involved the direct reaction of ^{15}N-hydroxylammonium chloride and an equimolar amount of triethylamine with **8**. Cleavage of the protecting groups was achieved in anhydrous medium by treatment with HCl in dioxan. The product $^{15}N^{\omega}$-hydroxy-L-arginine dihydrochloride (**2**) is very hygroscopic and unstable; it was thus liberated from the protected precursor as required. The obtained ^{15}N- (see below) and ^{13}C-NMR spectral data are in accord with the structure but a satisfactory elemental analysis could not be obtained. However, the precursors **7** and **9** (characterized as their oxalates) did give satisfactory elemental analyses.

Although reports on biological investigations of **2** were available[9] when we started our work, no synthetic procedures for **2** had been given. In the course[20][22] and after the conclusion[23] of our investigations, synthetic procedures for N^{ω}-hydroxy-L-arginine (**2**) were published. Although *Feldman*[20] started from the same protected orthinine derivative **6**, he then required considerably more steps for the preparation of **2** and chose a route involving a protected thiocitrulline derivative. His data for **2** (mp., ^{13}C-NMR) were in agreement with our results. *Wallace* and *Fukuto*[22] as well as *Pufahl et al.*[23] followed the same synthetic concept as used in our work but gave different procedures for some of the steps. Also, the intermediates were not confirmed by elemental analysis and ^{15}N-NMR spectral data were not reported although we consider such analyses to be indispensable for the unambiguous structural characterization of **9** and **2**.

^{15}N-NMR Spectroscopic Investigations

The problem of the isomeric (aminooxy)formamidines **10** and **11** (Scheme 2) was also not mentioned in refs.[22][23]. It is known that, together with the hydroxyguanidines, (aminooxy)formamidines can also be formed by addition of hydroxylamines to cyanamides by attack of the oxygen atom of the ambident hydroxylamine[24]. In some cases the



Scheme 2

(aminooxy)formamidines were isolated[24], while in other additions of hydroxylamines to monosubstituted cyanamides[25] they have been postulated as unstable intermediates that decompose to give urea derivatives[25]. The *N''*-hydroxyguanidine **9** and the (aminooxy)formamidine **10** (only one of the possile tautomeric forms of the free base is shown in Scheme 2) as well as the unprotected compounds **2** and **11**, respectively, are extremely difficult to distinguish by ^{1}H- and ^{13}C-NMR spectroscopy. In such cases, ^{15}N-NMR spectroscopy seems again to be more useful[17]. The spectral data of the addition product from hydroxylamine (Experimental Section, unlabeled product) are in accord with the *N''*-hydroxyguanidine **9** and are also in good agreement with the chemical shifts reported for other

N''-hydroxyguanidines[17]. After cleavage of the protecting groups, compound **2** also exhibits the same chemical shift for the *N*''-hydroxyguanidine nitrogen atom (for data, see Experimental Section, labeled product).

From the data obtained we were able to conclude that (i) **2** and **9** are *N*''-hydroxyguanidines and not (aminooxy)formamidines, (ii) **2** and **9** exist in the oxime-type structure and not in the hydroxylamine-type tautomer and (iii) the possible *E*, *Z*-isomers rapidy interconvert.

Even when **9** is formed by addition of hydroxylamine to **8** in dioxan and **2** is subsequently liberated, this does not necessarily mean that the enzymic reaction of **2** in aqueous medium (Scheme 2) cannot proceed through a rearrangement to the (aminooxy)formamidine **11** and further transformation to citrulline (**3**) and NO. The fact that (aminooxy)formamidines are preferentially formed in protic solvents does, in fact, favor such an isomerization[24]. This isomerization can take place either through decomposition to hydroxylamine and cyanamide followed by a renewed attack of the hydroxylamine or through an intramolecular rearrangement. An intermediate **11** in the formation of NO and citrulline (**3**) (Scheme 1) could also explain why the urea oxygen atom of **3** originates from molecular oxygen and not from H_2O[13], by assuming that the (aminooxy)formamidine **11** is again hydroxylated at the O-NH_2 group with subsequent decomposition to citrulline and NO° or NO^-. However, it must be realized that the individual redox forms of NO (NO°, NO^+, and NO^-) undergo mutual interconversions very readily[5b].

Biological Results

Electron spin resonance (ESR) techniques for the measurement of ^{15}NO formed from one of the two guanidine N-atoms of $^{15}N_2^{\omega}$-L-arginine have already been reported[26][27]. The synthetically prepared $^{15}N^{\omega}$-L-arginine (**2**) was investigated in the same manner. For this purpose, the arginine derivative was incubated with partially purified NO synthase from activated murine macrophages[26] in the presence of a finely dispersed (diethyldithiocarbamato) iron(II) complex[28]. This iron complex binds NO with high affinity. The resultant diethyldithiocarbamatonitrosyliron(II) complex [$Fe^{II}(DETC)_2(^{15}NO)$] is paramagnetic and generates a typical, anisotropic ESR spectrum characterized by the $g\perp = 2.035$ and $g\parallel = 2.02$.

The ^{14}NO complex, on one hand, exhibits a triplet hyperfine structure at $g\perp = 2.035$ (Fig. 1b) whereas the signal for the ^{15}NO complex shows a doublet structure[28] (Fig. 1d). The incubate with ^{15}N-hydroxy-L-arginine exhibits an overlapping of the ESR signals of the two N-isotopes (Fig. 1c). The proportion of ^{14}NO, however, is not attributable to the activity of the macrophage NO synthase but to a genuine NO contaminaion of baker's yeast[26] used for the dispersion of the complex (Fig. 1a). On substraction of the background signal (Fig. 1a) from the ESR signal of the incubate with $^{15}N^{\omega}$-hydroxy-L-arginine (Fig. 1c), the pure ESR signal of the ^{15}NO complex is indeed obtained (Fig. 1d)[28]. This

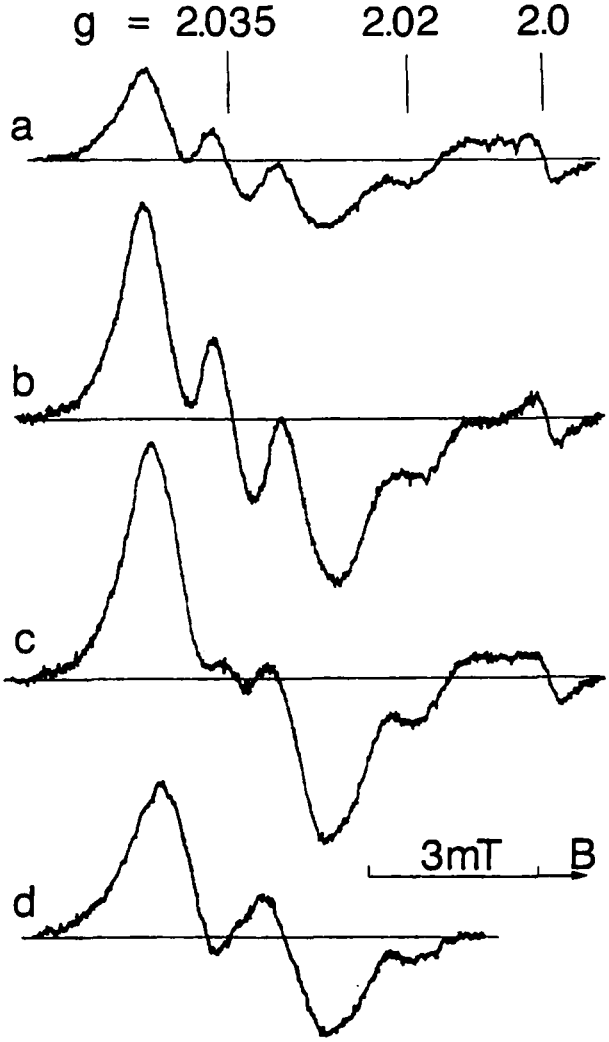


Figure 1: ESR Spectroscopic Detection of NO after Enzymic Reaction of N^{ω}-Hydroxy-L-arginine.

A yeast suspension loaded with [Fe^{II}(DETC)] was incubated a) without further additives, b) with NO synthase from macrophages and L-arginine, and c) with NO synthase and $^{15}N^{\omega}$-hydroxy-L-arginine. The incubates were frozen after 1 h and the ESR spectra recorded. The ESR signals are characteristic for the [$Fe^{II}(DETC)_2NO$] complex. Spectrum d) was obtained by subtraction of spectrum c) from spectrum a). The arrow under "B" indicates the direction and calibration (in milliTesla, mT) of the external magnetic field. The g factors are given above. $g\perp = 2.0$ represents the position of the ESR resonance of the free electron.

result demonstrates that exclusively the hydroxylated N^{ω}-guanidine N-atom of L-arginine is transformed to NO by NO synthase. In the case of equal reactivities of the guanidine nitrogen atoms ^{14}NO and ^{15}NO would have been formed in the same amounts. Thus, we may exclude the possibility that the N^{ω}-hydroxy-L-arginine is merely an alternative (artificial) substrate of NO synthase which is transformed to NO and citrulline (**3**) after reduction to L-arginine. This conclusion is in agreement with mass spectrometric investigations[23]. In this study[23], ^{15}NO was not identified directly, but by further transformation into nitrobenzene.

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Experimental Part

Mps: Büchi 510 apparatus, uncorrected.- Elemental analyses: Mikroanalytisches Labor I. Beetz, Kronach, and Institut für anorganische Chemie der Christian-Albrechts-Universität Kiel.- IR-spectra: Perkin-Elmer FT-IR 1600 PC.- NMR: ^{1}H- and ^{13}C-NMR spectra: Bruker AM 100 and Bruker AM 400 instruments [TMS Int. Stand., δ (ppm) scale]; ^{15}N-NMR spectra: Jeol JNM GX-400 spectrometer (NH_3 as external standard), INEPT-technique for NH coupled spectra; for details of the measurement conditions, see ref.[17].- ESR spectra: Bruker e.p.r. 420 instrument, see refs.[27][28].- Hydrogenation apparatus: Gehard Y 10.- Column chromatography: silica gel Merck G 60 (particle size: 0.063-0.2 mm).- TLC on silica gel microplates (Polygram SIL G/UV_{254}, Macherey and Nagel) 0.25 mm.- TLC eluents (when not otherwise stated): CH_2Cl_2/MeOH/glacial acetic acid, 8.5:1.5:0.5 (v/v/v).- Detection: ninhydrin spray reagent, 2% $FeCl_3$-solution.- Rf-values: **5** = 0.45, **6** = 0.95, **7** = 0.5, **8** = 0.9, **9** = 0.5, **10** = 0.1.

N^{α}-(tert-Butyloxycarbonyl)-Nδ-(benzyloxycarbonyl)-L-ornithine tert-Butyl Ester (**6**)

(adapted from refs.[19][20]). Compound **5** (11.4 g; 35.4 mmol; obtained according to ref.[19]) was taken up in 80 mL of dioxan; after addition of 80 mL of saturated aqueous $NaHCO_3$ solution, $(BOC)_2O$ (11.6 mL; 53.1 mmol) was added dropwise to the suspension within 1 h. After being stirred at room temp. for 12 h, dioxan was removed and the aqueous phase extracted several times with diethyl ether. The combined ether phase was dried (Na_2SO_4) and evaporated to afford 14.2 g (95%) of the protected ornithine derivative as an oil. The spectroscopic data agree with those reported[19], satisfactory elemental analyses were obtained.

N^{α}-tert-Butyloxycarbonyl-L-ornithine tert-Butyl Ester (**7**)

Compound **6** (2.4 g; 5.69 mmol) was hydrogenated in 24 mL of anhydrous methanol containing 600 mg Pd/C until H_2 uptake ceased. After filtration and concentration, the residue was suspended in a small volume of water and the pH adjusted to 3 with glacial acetic acid. A by-product was separated by several extractions with n-hexane/diethyl ether, 9:1 (v/v). The remaining solution was basified with conc. ammonia solution, rapidly extracted with diethyl ether under ice cooling, the extract dried (Na_2SO_4), and the solvent removed. The isolated amine (1.39 g, 85%) solidified on cooling and could be used directly for further reactions. The oxalate of **7** was precipitated from the ether solution by addition of a saturated solution of oxalic acid in diethyl ether: mp. (oxalate) 117°C.- ^{1}H-NMR ([D_6]DMSO, oxalate): δ = 1.39 (s, 9H), 1.4 (s, 9H), 1.58-1.69 (m, 4H), 2.76 (m, 2H), 3.76 (t, J = 5 Hz, 1H), 6.00-7.00 (br. m, 4H), 7.15 (d, J = 8 Hz, 1H).- ^{13}C-NMR ([D_6]DMSO, oxalate): δ = 23.9 (CH_2-$\underline{C}H_2$-CH_2), 27.6 [$C(\underline{C}H_3)_3$], 27.9 (CH-$\underline{C}H_3$)$_3$], 27.9 (CH-$\underline{C}H_2$), 28.2 [$C(\underline{C}H_3)_3$], 40.2 (CH_2-NH_2), 54.0 ($\underline{C}H$-CH_2), 78.1 [$\underline{C}(CH_3)_3$], 80.4 [$\underline{C}(CH_3)_3$], 155.0 (NH-CO), 164.6 (COOH, oxalate), 171.5 (CO).- IR (KBr, cm^{-1}) 1710; 1730.- $C_{16}H_{30}N_2O_8$, oxalate (378.4) Calcd. C 50.8 H 7.99 N 7.4 Found C 50.6 H 8.10 N 7.2.

N^{α}-tert-Butyloxycarbonyl-Nδ-cyano-L-ornithine tert-Butyl Ester (**8**)

Compound **7** (2.00 g; 6.94 mmol) was dissolved in 15 mL of anhydrous diethyl ether; 1.5 g of anhydrous Na_2CO_3 were added to a solution of BrCN (750 mg; 7.1 mmol) in 3 mL of anhydrous diethyl ether. Under ice cooling, the amine component was added dropwise, the mixture was stirred for 16 h, filtered, the ether removed, and the crude product subjected to chromatography on a silica gel G 60 column with n-hexane/ethyl acetate, 1:4 (v/v). Fractions were analyzed by TLC, those containing **8** (R_f 0.59) were combined, dried (Na_2SO_4), and evaporated to afford 1.6 g C 74%) of an oil: ^{1}H-NMR ([D_6]DMSO): δ = 1.39 (s, 9H), 1.40 (s, 9H), 1.46-1.70 (m, 4H), 3.33 (t, J = 6 Hz, 2H), 3.77 (m, 1H), 6.71 (br, 1H), 7.16 (br. d, 1H).- ^{13}C-NMR ([D_6]DMSO): δ = 26.1 (CH_2-$\underline{C}H_2$-CH_2), 27.6 [$C(\underline{C}H_3)_3$], 27.9 (CH-$\underline{C}H_2$), 28.2 [$C(\underline{C}H_3)_3$], 44.4 (CH_2-NH), 54.0 ($\underline{C}H$-CH_2), 78.1 [$\underline{C}(CH_3)_3$], 80.3 [$\underline{C}(CH_3)_3$], 117.3 (C≡N), 155.5 (NH-CO), 171.6 (CO).- IR (KBr, cm^{-1}): 1690; 1730; 2230.- $C_{15}H_{27}N_3O_4$ (313.4) Calcd. C 57.5 H 8.68 N 13.4 Found C 57.0 H 8.48 N 13.3.

N^{α}-tert-Butyloxycarbonyl-N^{ω}-hydroxy-L-ornithine tert-Butyl Ester (**9**)

To a solution of 150 mg (4.5 mmol) of hydroxylamine base[21] in anhydrous dioxan was added dropwise the cyanamide **8** (0.97 g, 3.1 mmol) in 15 mL of anhydrous dioxan. The mixture was stirred for 20 h, the solvent was carefully removed, and the residue subjected to column chromatography (CC) on silica gel G 60 (CH_2Cl_2/MeOH/HOAc, 8.5:1.5:0.5, v/v/v). After TLC monitoring, the appropriate fractions were combined and concentrated. The resultant oil was characterized as its oxalate which was recrystallized from anhydrous diethyl ether to furnish 0.87 g (64%) of a white powder.

For preparation of the ^{15}N-labeled product, the cyanamide (0.6 g, 1.9 mmol) was added dropwise to a solution (dioxan) of 150 mg (2.12 mmol) of ^{15}*N*-hydroxylammonium chloride ($^{15}NH_2OH \cdot HCl$, 99% ^{15}N, Cambridge Isotope Laboratories, Massachusetts, USA) and of an equimolar amount of triethylamine. CC as described above furnished the product which was characterized as the oxalate: yield: 0.48 g (58%), mp. (of oxalate) 103-104°C; ^{1}H-NMR ([D_6]DMSO, oxalate): δ = 1.36-1.41 (s, 18H), 1.52 (m, 2H), 1.56 (m, 2H), 3.17 (t, J = 6 Hz, 2H), 3.76 (t, J = 5 Hz, 1H), 4.60-5.00 (br, 2H), 7.13 (d, J = 8 Hz, 1H), 7.78 (s, 2H), 8.19 (s, 1H), 10.80 (br, 1H).- ^{13}C-NMR ([D_6]DMSO, oxalate): δ = 25.1 (CH_2-$\underline{C}H_2$-CH_2), 27.6 [$C(\underline{C}H_3)_3$], 27.8 (CH-$\underline{C}H_2$), 28.2 [$C(\underline{C}H_3)_3$], 40.4 (CH_2-NH), 54.1 ($\underline{C}H$-CH_2), 78.1 [$\underline{C}(CH_3)_3$], 80.3 [$\underline{C}(CH_3)_3$], 155.5 (NH-CO), 158.4 (C=N), 164.1 (COOH, oxalate), 171.6 (CO).- ^{15}N-NMR ([D_6]DMSO, oxalate of unlabeled compound): δ = 72.2 [t, ^{1}J (^{15}N, ^{1}H = 95.8 Hz, NH_2], 80.7 [d, ^{1}J (^{15}N, ^{1}H) = 89.4 Hz, NH], 89.8 [d, ^{1}J (^{15}N, ^{1}H) = 94.8 Hz, NH], 136.2 (NH-OH, only a very broad signal in the INEPT spectrum).- IR (KBr, cm^{-1}): 1660; 1690; 1730; 3200.- $C_{17}H_{32}N_4O_9$ oxalate (463.4) Calcd. C 46.8 H 7.39 N 12.8 Found C 46.5 H 7.57 N 12.1.

N^{ω}-Hydroxy-L-arginine Dihydrochloride (**2**)

Compound **9** (0.54 g, 1.34 mmol) in anhydrous dioxan was stirred with 8 mL of 4 N HCl in anhydrous dioxan for 20 h. The mixture was diluted with about 10 mL of anhydrous diethyl ether. The product thus precipitated was collected under N_2, washed several times with anhydrous ethyl acetate, and dried. The product **2** · HCl is very hygroscopic and must be stored under N_2 in a dessicator; for the biochemical experiments, it was liberated from the protected derivative immediately prior to use. Yield: 0.25 g (71%).- mp. 180°C.- ^{1}H-NMR (MeOD): δ = 1.60-1.90 (m, 2H), 1.90-2.10 (m, 2H), 3.20 (t, J = 6 Hz, 2H), 4.05 (t, J = 6 Hz, 1H).- ^{13}C-NMR (MeOD): δ = 25.8 (CH_2-$\underline{C}H_2$-CH_2), 28.6 (CH-$\underline{C}H_2$), 41.6 (CH_2-NH), 53.5 ($\underline{C}H$-CH_2), 160.3 (C=N), 171.5 (CO).- ^{15}N-NMR ([D_6]DMSO, ^{15}N-labeled compound): δ = 136.4 [d, ^{1}J (^{15}N, ^{1}H) = 98.1 Hz, NH-OH].- ^{15}N-NMR (D_2O/H_2O, ^{15}N-labeled compound): δ = 133.9 (s, NH-OH).

Biological Tests

These investigations were carried out as described in detail in ref.[26][27] and are only summarized briefly here. A yeast suspension (commercial

baker's yeast, 200 mg/mL in 15 mM HEPES buffer, pH 7.5) was boiled for 30 min and then loaded with DETC (2.5 mg/mL) at 37°C during 30 min. This suspension (final concentration 40 mg/mL) was incubated with the NO synthase preparation from macrophages (100 µg of protein)[26] at 37°C for 1 h. The incubation mixture contained additionally 0.1 mM NADPH, 0.1 µM 6*R*-tetrahydrobiopterine, 2 mM GSH, 15 mM HEPES buffer, pH 7.5, 4 mM $MgCl_2$, 1 µM superoxide dismutase, 0.1 mg/mL bovine γ-globulin as well as 0.3 mM of L-arginine or $^{15}N^{\omega}$-hydroxy-L-arginine as the respective substrate. After addition of sodium dithionite (1 mg/mL) to reduce the oxidized DETC complex,[27] the samples were immediately frozen in cylindrical teflon tubes in liquid N_2. These solid samples fitted exactly in the ESR cuvette, a quartz Dewar vessel filled with liquid N_2. The ESR spectra were recorded at -196°C with a microwave frequency of 9.330 GHz and a microwave power of 5 mW.

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