Recognition of α-Amino Acids Bearing Various C═NOH Functions by Nitric Oxide Synthase and Arginase Involves Very Different Structural Determinants†

Catherine Moali,‡ Maurice Brollo,‡ Julien Custot,‡ Marie-Agnès Sari,‡ Jean-Luc Boucher,‡ Dennis J. Stuehr,§ and Daniel Mansuy*,‡

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601 CNRS, Université Paris V, 45 Rue des Saints-Pères, 75270 Paris Cedex 06, France, and Department of Immunology, Lerner Research Institute, The Cleveland Clinic Fundation, 9500 Euclid Avenue, Cleveland, Ohio 44195

Received December 30, 1999; Revised Manuscript Received April 27, 2000

ABSTRACT: Several α-amino acids bearing a C═NOH function separated from the Cα carbon by two to five atoms have been synthesized and tested as substrates or inhibitors of recombinant nitric oxide synthases (NOS) I and II and as inhibitors of rat liver arginase (RLA). These include four *N*-hydroxyguanidines, N^{ω}-hydroxy-L-arginine (NOHA) and its analogues homo-NOHA, nor-NOHA, and dinor-NOHA, two amidoximes bearing the $-NH-C(CH_3)=NOH$ group, and two amidoximes bearing the $-CH_2-C(NH_2)=NOH$ group. Their behavior toward NOS and RLA was compared to that of the corresponding compounds bearing a C═NH function instead of the C═NOH function. The results obtained clearly show that efficient recognition of these α-amino acids by NOS and RLA involves very different structural determinants. NOS favors molecules bearing a $-NH-C(R)=NH$ motif separated from Cα by three or four CH_2 groups, such as arginine itself, with the necessary presence of δ-NH and ω-NH groups and a more variable R substituent. The corresponding molecules with a C═NOH function exhibit a much lower affinity for NOS. On the contrary, RLA best recognizes molecules bearing a C═NOH function separated from Cα by three or four atoms, the highest affinity being observed in the case of three atoms. The presence of two ω-nitrogen atoms is important for efficient recognition, as in the two best RLA inhibitors, N^{ω}-hydroxynorarginine and N^{ω}-hydroxynorindospicine, which exhibit IC_{50} values at the micromolar level. However, contrary to what was observed in the case of NOS, the presence of a δ-NH group is not important. These different structural requirements of NOS and RLA may be directly linked to the position of crucial residues that have been identified from crystallographic data in the active sites of both enzymes. Thus, binding of arginine analogues to NOS particularly relies on strong interactions of their δ-NH and ω-NH_2 groups with glutamate 371 (of NOS II), whereas binding of C═NOH molecules to RLA is mainly based on interactions of their terminal OH group with the binuclear Mn(II)···Mn(II) cluster of the enzyme and on possible additional bonds between their ω-NH_2 group with histidine 141, glutamate 277, and one Mn(II) ion. The different modes of interaction displayed by both enzymes depend on their different catalytic functions and give interesting opportunities to design useful molecules to selectively regulate NOS and arginase.

L-Arginine (Arg)[1] is metabolized in cells by two major pathways (*1*). The first one involves arginases that hydrolyze Arg into urea and L-ornithine. This reaction is catalyzed by the binuclear Mn(II) cluster of arginases (*2*). It serves two major purposes depending on the cell type: either it allows the disposal of the nitrogenous waste from protein catabolism through the urea cycle or it provides polyamine synthesis from the amino acid precursor ornithine (*3*). These two functions seem to correlate with two distinct arginase genes, which are termed AI and AII (*3*). The second pathway involves other metalloenzymes called nitric oxide synthases (NOS), which are responsible for the oxidation of Arg to L-citrulline (Cit) and nitric oxide (NO) with intermediate formation of N^{ω}-hydroxy-L-arginine (NOHA) (*4*−*7*). NO is a messenger molecule with extremely diverse functions in

† We are grateful to the Ministère de l'Enseignement Supérieur et de la Recherche and the Institut de Formation Supérieure Bio-Médicale (Villejuif, France) for providing grants to C.M. and to the National Institutes of Health for Grant CA 53914 to D.J.S.

* To whom correspondence should be addressed: fax +33 1 42 86 83 87; e-mail Daniel.Mansuy@biomedicale.univ-paris5.fr.

‡ Université Paris V.

§ The Cleveland Clinic Fundation.

[1] Abbreviations: AcOH, acetic acid; Arg, L-arginine; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; Boc, *tert*-butyloxycarbonyl; BTEAC, benzyltriethylammonium chloride; CaM, calmodulin; CI, chemical ionization; Cit, L-citrulline; DEAD, diethylazodicarboxylate; DMAC, *N*,*N*-dimethylacetamide; EI, electron impact; FAB, fast atom bombardment; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; homo-Arg, homo-L-arginine; homo-NOHA, N^{ω}-hydroxyhomo-L-arginine; HRMS, high-resolution mass spectrometry; LDA, lithium diisopropylamide; NIL, N^{ϵ}-(1-iminoethyl)-L-lysine; NIO, N^{δ}-(1-iminoethyl)-L-ornithine; NOHA, N^{ω}-hydroxy-L-arginine; nor-NOHA, N^{ω}-hydroxynor-L-arginine; NOHI, N^{ω}-hydroxy-D,L-indospicine; dinor-NOHA, N^{ω}-hydroxydinor-L-arginine; nor-NOHI, N^{ω}-hydroxynor-D,L-indospicine; NOS, nitric oxide synthase; OH-NIL, N^{ϵ}-(1-*N*-hydroxyiminoethyl)-L-lysine; OH-NIO, N^{δ}-(1-*N*-hydroxyiminoethyl)-L-ornithine; RLA, rat liver arginase; SOD, superoxide dismutase; ZCl, benzyloxycarbonyl chloride.

10.1021/bi992992v CCC: $19.00
Published on Web 06/16/2000

mammals, ranging from regulation of blood pressure to neurotransmission and host defense (*8*). Three main isoforms of NOS have been isolated and characterized. NOS I and NOS III are expressed constitutively and require Ca^{2+} and calmodulin (CaM) for activity whereas NOS II is induced by cytokines and bacterial lipopolysaccharides and is mostly Ca^{2+}- and CaM-independent (*9*, *10*). All forms of NOS require NADPH and dioxygen as cosubstrates and heme, tetrahydrobiopterin (BH_4), FAD, and FMN as cofactors (*5*−*7*).

Identification of potent and selective inhibitors of NOS I and II has recently been a subject of intense interest because of their therapeutic potential for the treatment of diseases caused by excessive production of NO (*11*). Of the hundreds of molecules that have been tested, Arg competitors seem to be promising targets and numerous studies have been performed to determine the influence of substrate modifications on the interaction with the NOS active site. Thus, it appears that the α-amino acid moiety of Arg can be removed without detrimental consequences, while the integrity of the guanidine function must be partially retained, as evidenced by the large majority of NOS inhibitors (guanidines, amidines, and isothioureas) that contain the amidine function −C(NHR)═NH (*12*).

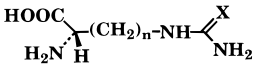
By comparison, far fewer inhibitors of arginases have been described. All those that exhibit K_i at the micromolar level involve an L-α-amino acid function and an OH group separated from the C_α atom of the amino acid by five or six atoms (*13*, *14*). In that regard, NOHA, a NOS product that can be released into the extracellular medium (*15*, *16*), and its shorter analogue, N^ω-hydroxynor-L-arginine (nor-NOHA), appear to be particularly interesting for comparing the active sites of NOSs and arginases. NOHA is well recognized by both enzymes as a good endogenous substrate of NOS ($K_m \sim 10\ \mu$M) (*4*, *17*) and a medium inhibitor of arginases ($K_i \sim 50\ \mu$M) (*18*−*21*), whereas nor-NOHA is a highly potent inhibitor of arginases ($K_i \sim 0.5\ \mu$M) (*13*) but a very bad substrate of NOS (*22*).

In an effort to exploit these preliminary results for a better understanding of the different structures recognized by NOS and arginase, we have synthesized several α-amino acids bearing a C═N−OH function separated from the C_α amino acid carbon by two to five atoms (Chart 1). These include four *N*-hydroxyguanidines, NOHA and its analogues homo-NOHA, nor-NOHA, and dinor-NOHA, two amidoximes of the −NH−C(CH_3)═NOH type, and two amidoximes of the −CH_2−C(NH_2)═NOH type. The activity of these compounds as substrates and/or inhibitors of NOS I and NOS II and of rat liver arginase (RLA) has been studied and compared to that of the corresponding molecules in which the C═NOH function has been replaced with a C═NH function (Chart 1). The very different behaviors of some of the tested compounds toward NOS and RLA allow us to identify the main structural factors that are important for differential recognition by the active sites of NOS and RLA.

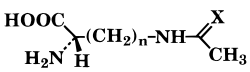
MATERIALS AND METHODS

Chemistry, General. N^ϵ-*Z*-L-Lysine, N^δ-*Z*-L-ornithine, L-glutamine, L-serine, and Boc-glycine were purchased from Sigma. Other chemicals and reagents of the highest grade commercially available were obtained from Aldrich, Fluka, or Janssen and used without further purification. Dioxane and diethyl ether were distilled over sodium benzophenone. Chemical reactions were monitored by TLC on Merck precoated silica gel $60F_{254}$ (0.25 mm thickness) plates. The products on the plates were visualized by 254-nm UV light or ninhydrin spray. Merck Kieselgel 60 (70−230 mesh ASTM) was utilized for flash chromatography. Solvent A was $CHCl_3$/MeOH/H_2O/acetic acid(AcOH) 7/3/0.6/0.3, solvent B was acetone/H_2O/*i*PrOH/AcOH 2/2/2/1, and solvent C was acetone/H_2O/*i*PrOH/AcOH 2/1/2/2. Analytical HPLC was carried out after treatment of the samples with naphthalene dicarboxaldehyde and sodium cyanide (*23*) on a Spectra Physics apparatus equipped with a Nova-Pak C_{18} column (150 mm × 3.9 mm, 4 μm particle size, Waters Associate Inc.). The HPLC retention times reported thereafter are those of naphthalene dicarboxyaldehyde derivatives. Proton (^{1}H) and carbon (^{13}C) NMR were recorded on a Bruker ARX 250 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane with peak multiplicities abbreviated as follows: singlet, s; broad singlet, br s; doublet, d; triplet, t; multiplet, m. Coupling constants (*J*) are reported in hertz. IR spectra were recorded on a Perkin-Elmer 783 spectrometer (wavenumbers in reciprocal centimeters). Mass spectra were recorded at Ecole Normale Superieure, Paris, on a RiberMag system with fast atom bombardment (FAB), electron impact (EI), and chemical ionization (CI) capabilities. Electrospray mass spectra were recorded at the University Paris VI mass spectra facility. High-resolution mass spectrometry is indicated as HRMS. Specific optical rotations were measured at the sodium D line at 20 °C on a Perkin-Elmer model 241C polarimeter (1-dm and 0.1-dm cells).

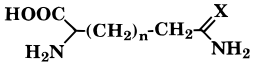
Chart 1: Formula of the Compounds Studied in This Article



X = NH	n = 4	homo-Arg	**1**
	n = 3	Arg	**2**
X=NOH	n = 4	homo-NOHA	**3**
	n = 3	NOHA	**4**
	n = 2	nor-NOHA	**5**
	n = 1	dinor-NOHA	**6**
X = O	n = 4	homo-Cit	**7**
	n = 3	Cit	**8**
	n = 2	nor-Cit	**9**
	n = 1	dinor-Cit	**10**



X = NH	n = 4	NIL	**11**
	n = 3	NIO	**12**
X=NOH	n = 4	OH-NIL	**13**
	n = 3	OH-NIO	**14**



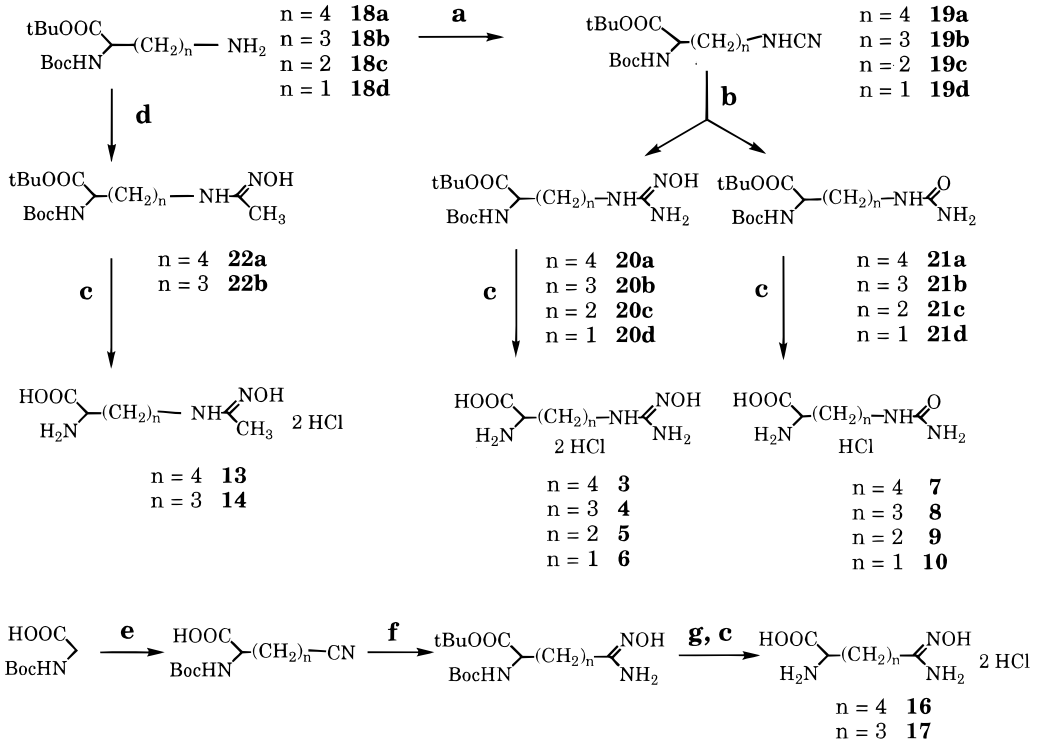
X =NH	n = 3	Indospicine	**15**
X = NOH	n = 3	NOHI	**16**
	n = 2	nor-NOHI	**17**

Syntheses of Compounds **3**−**10**, **13**, **14**, **16**, *and* **17**

These syntheses are described below by following the steps indicated in Schemes 1 and 2. Preparation of precursors **18a**−**d** (Scheme 2) is reported first, then reactions leading from **18a**−**d** to **3**−**10**, **13**, and **14**, and finally reactions leading to **16** and **17** (Scheme 1).

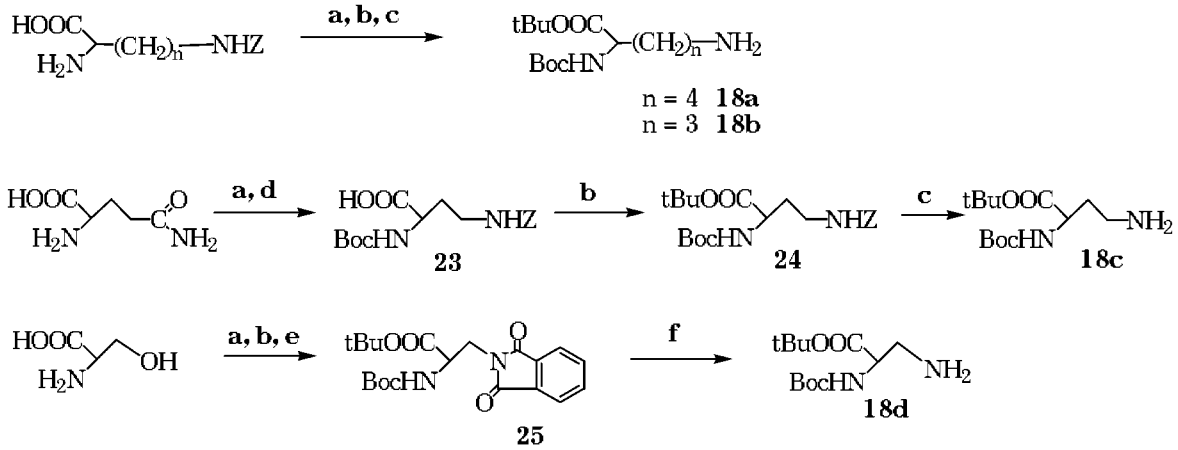
All the products described were found to be pure by TLC, HPLC, and ^{1}H and ^{13}C NMR (for the most important ones)

Scheme 1: Synthetic Pathways Used for the Preparation of Compounds **3**−**10**, **13**, **14**, **16**, and **17**[a]



[a] Molecules **18**−**22** are intermediates in the synthesis and are described under Materials and Methods. Steps a−g refer to the following conditions for each step of the synthesis. These conditions are described under Materials and Methods. (a) BrCN, AcONa, CH_3OH; (b) NH_2OH−HCl, Na_2CO_3, dioxane, 60 °C; (c) HCl gas, dioxane, 20 °C; (d) EtO−C(CH_3)=NOH, EtOH, reflux; (e) (i) LDA, THF, 0 °C, (ii) Br(CH_2)$_n$CN, −78 °C, (iii) H_3O^+; (f) tBuBr, BTEAC, K_2CO_3, DMAC, 55 °C; (g) NH_2OH−HCl, Na_2CO_3, EtOH, reflux.

Scheme 2: Synthetic Pathways Used for the Synthesis of Intermediates **18a**−**d**[a]



[a] The starting compounds N^{ϵ}-*Z*-L-lysine, N^{δ}-*Z*-L-ornithine, L-glutamine, and L-serine were obtained from commercial sources. Molecules **23**−**25** are intermediates that are described under Materials and Methods. Steps a−f refer to the following conditions used for each step: (a) $(Boc)_2O$, dioxane + H_2O, $NaHCO_3$, 20 °C; (b) tBuBr, BTEAC, K_2CO_3, DMAC, 55 °C; (c) H_2, Pd/C, CH_3OH, 20 °C; (d) Br_2, NaOH in H_2O, 0 °C for 30 min, then 80 °C for 30 min, then ZCl, toluene, 20 °C; (e) phthalimide, $P(C_6H_5)_3$, DEAD, dioxane, 0 °C; (f) NH_2NH_2, EtOH, 60 °C.

spectroscopy. Their purity was confirmed by quantitative analysis of the ^{1}H NMR spectrum of a mixture of each product with a known amount of a standard (purity > 95%). Their $[\alpha]_D$ values are indicated below; they should be optically pure as a similar synthesis procedure has been reported not to racemize the C_α of the starting, commercially available L-α amino acids (*24*).

*Synthesis of the Protected Amino Acid Precursors **18a**−**d***. Protection of the α-NH_2 function of N^{ϵ}-*Z*-L-lysine, N^{δ}-*Z*-L-ornithine, L-glutamine, and L-serine with a Boc group (steps a in Scheme 2) was performed by a classical procedure (*24*).

*Synthesis of N^{α}-Boc-N^{γ}-Z-nor-L-ornithine **23*** (*Step d of Scheme 2) (25).* A solution of N^{α}-Boc-L-glutamine (1 equiv, 21 mmol) and NaOH (2 equiv) in 30 mL of water was added dropwise to a solution of bromine (1.3 equiv) and NaOH (7 equiv) in 90 mL of water maintained at 0 °C. The green mixture was then rapidly heated to 80 °C and allowed to stir at this temperature until the green color disappeared (about 10 min). After cooling to room temperature, 20 mL of toluene and a 50% solution of ZCl in toluene (1.1 equiv) were added. The resulting mixture was allowed to stir at room temperature for 18 h before extraction of the aqueous

phase with EtOAc. The aqueous phase was then acidified to pH 7 with NaH_2PO_4, extracted with EtOAc, and acidified again to pH 5 and pH 3 successively. The title compound **23** was isolated as a pure product in the organic layers obtained at pH 7 and 5. These phases were combined, dried over $MgSO_4$, filtered, and stripped under reduced pressure. White foam (60%); TLC (EtOAc/solvent A 1/1) R_f 0.70; ^{1}H NMR ($CDCl_3$) δ 7.31 (m, 5H), 5.65 (br s, 1H), 5.42 (br s, 1H), 5.10 (s, 2H), 4.33 (m, 1H), 3.43 (br s, 1H), 3.11 (br s, 1H), 2.03 (m, 1H), 1.70 (m, 1H), 1.41 (s, 9H).

Protection of the COOH function of intermediates (steps b in Scheme 2) as *tert*-butyl esters was done with slight changes of a previously reported method (*26*). The Boc-protected amino acid (1 equiv) was dissolved in *N*,*N*-dimethylacetamide (DMAC; 8 mL/mmol of amino acid) and 1 equiv of benzyltriethylammonium chloride (BTEAC), 46 equiv of *tert*-butyl bromide, and 26 equiv of K_2CO_3 were added. The mixture was heated for 18 h at 55 °C and cooled to room temperature. The salts were filtered and washed with AcOEt, and the resulting mixture was evaporated under vacuum. Water was added to the residue and the mixture was extracted with EtOAc. The combined organic layers were dried ($MgSO_4$), filtered, and stripped of solvent under reduced pressure to provide the crude *tert*-butyl esters, which were further purified by flash chromatography (gradient of EtOAc/cyclohexane from 1/4 to 1/1).

N$^\alpha$-*Boc*-*N*$^\epsilon$-*Z*-L-*Lysine tert-Butyl Ester*. Colorless oil (99%); TLC (EtOAc/cyclohexane 3/7) R_f 0.50; ^{1}H NMR ($CDCl_3$) δ 7.32 (m, 5H), 5.06 (br s, 3H), 4.80 (br s, 1H), 4.08 (m, 1H), 3.15 (m, 2H), 1.50−1.70 (m, 6H), 1.43 (s, 9H), 1.41 (s, 9H); MS (CI + NH_3) 437 (MH^+).

N$^\alpha$-*Boc*-*N*$^\delta$-*Z*-L-*Ornithine tert-Butyl Ester*. Spectral characteristics were as previously described (*20*, *27*).

N$^\alpha$-*Boc*-*N*$^\gamma$-*Z*-*Nor*-L-*ornithine tert-Butyl Ester*. Colorless oil (99%); TLC (EtOAc/cyclohexane 1/4) R_f 0.30; ^{1}H NMR ($CDCl_3$) δ 7.31 (m, 5H), 5.45 (br s, 1H), 5.20 (br s, 1H), 5.10 (s, 2H), 4.20 (m, 1H), 3.45 (m, 1H), 3.00 (m, 1H), 2.10 (m, 1H), 1.60 (m, 1H), 1.43 (s, 9H), 1.41 (s, 9H).

N$^\alpha$-*Boc*-L-*Serine tert-Butyl Ester*. White solid (74%); mp 80 °C [lit. (*28*) 76−78 °C]; TLC (EtOAc/cyclohexane 3/7) R_f 0.25; ^{1}H NMR ($CDCl_3$) δ 5.37 (br s, 1H), 4.22 (br s, 1H), 3.87 (m, 2H), 2.30 (br s, 1H), 1.46 (s, 9H), 1.43 (s, 9H); MS (FAB) 262 (MH^+); $[\alpha]_D = -21°$ (EtOH) [lit. (*28*) $[\alpha]_D = -20°$].

Compounds **18a**−**c** were obtained from selective deprotection of the NH_2 function protected with a Z group (steps c of Scheme 2) by a classical procedure (*27*).

N$^\alpha$-*Boc*-L-*Lysine tert-Butyl Ester* **18a**. Colorless oil (99%); TLC (MeOH) R_f 0.05; ^{1}H NMR ($CDCl_3$) δ 5.00 (d, 1H, J = 7.6 Hz), 4.12 (br s, 1H), 2.68 (t, 2H, J = 6.4 Hz), 2.26 (m, 2H), 1.60−1.90 (m, 6H), 1.44 (s, 9H), 1.41 (s, 9H).

N$^\alpha$-*Boc*-L-*Ornithine tert-Butyl Ester* **18b**. Spectral characteristics were as previously described (*20*, *27*).

N$^\alpha$-*Boc*-*Nor*-L-*Ornithine tert-Butyl Ester* **18c**. Colorless oil (99%); TLC (EtOAc/solvent A 1/1) R_f 0.18; ^{1}H NMR ($CDCl_3$) δ 5.37 (d, 1H, J = 7.2 Hz), 4.20 (m, 1H), 2.80 (t, 2H, J = 6.8 Hz), 1.85 (m, 3H), 1.72 (m, 1H), 1.45 (s, 9H), 1.42 (s, 9H).

Synthesis of N$^\alpha$*-Boc-N*$^\beta$*-Phthalimidodinor*-L-*ornithine tert-Butyl Ester* **25** (*step e of Scheme 2*). *N*$^\alpha$-Boc-L-Serine *tert*-butyl ester (1 equiv, 7.3 mmol) dissolved in 50 mL of dioxane was stirred at 0 °C in the presence of triphenylphosphine (1.3 equiv) and phthalimide (1.3 equiv). Diethylazodicarboxylate (DEAD, 1.3 equiv) was added dropwise over 10 min and the solution was allowed to warm to room temperature. After 2 h, the mixture was evaporated, chromatographed on silica gel with EtOAc/cyclohexane 1/9, and recrystallized from the same solvent. White solid (50%); TLC (EtOAc/cyclohexane 3/7) R_f 0.38; ^{1}H NMR ($CDCl_3$) δ 7.83 (m, 2H), 7.68 (m, 2H), 5.28 (d, 1H, J = 6.8 Hz), 4.56 (m, 1H), 3.98 (m, 2H), 1.41 (s, 9H), 1.28 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 169.0, 168.0, 155.2, 132.0, 134.0, 123.3, 82.8, 52.9, 39.2, 28.0, 27.8; MS (CI + NH_3) 391 (MH^+).

Synthesis of N$^\alpha$*-Boc-Dinor*-L-*ornithine tert-Butyl Ester* **18d** (*Step f of Scheme 2*). *N*$^\alpha$-Boc-*N*$^\beta$-Phthalimidodinor-L-ornithine *tert*-butyl ester **25** (3.8 mmol) was dissolved in 5 mL of MeOH with 1.5 mL of hydrazine hydrate. The mixture was heated at 60 °C for 2 h, filtered, evaporated to dryness, and dissolved in EtOAc. Extraction with water and brine and evaporation under reduced pressure afforded the title compound **18d**, which was used for the next step without further purification.

Formation of Cyanamides **19a**−**d** *(Step a of Scheme 1 According to Reference 27)*

N$^\alpha$*-Boc-N*$^\epsilon$*-Cyano*-L-*lysine tert-Butyl Ester* **19a**. Yellow oil (72%); TLC (EtOAc/cyclohexane 1/9) R_f 0.55; ^{1}H NMR ($CDCl_3$) δ 5.11 (br s, 1H), 4.72 (br s, 1H), 4.13 (m, 1H), 2.97 (m, 2H), 1.60−1.80 (m, 6H), 1.38 (s, 9H), 1.35 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.6, 155.5, 116.6, 81.9, 79.7, 53.5, 45.5, 32.4, 28.9, 28.2, 27.8, 21.8; IR (neat) 2220 cm^{-1}; MS (CI + NH_3) 328 (MH^+).

N$^\alpha$*-Boc-N*$^\delta$*-Cyano*-L-*ornithine tert-Butyl Ester* **19b**. Spectral characteristics were as previously described (*20*, *27*).

N$^\alpha$*-Boc-N*$^\gamma$*-Cyanonor*-L-*ornithine tert-Butyl Ester* **19c**. Yellow oil (68%); TLC (EtOAc/cyclohexane 1/1) R_f 0.50; ^{1}H NMR ($CDCl_3$) δ 5.26 (d, 1H, J = 4.9 Hz), 5.06 (br s, 1H), 4.19 (br s, 1H), 3.17 (m, 1H), 3.09 (m, 1H), 2.16 (m, 1H), 1.53 (m, 1H), 1.45 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.1, 156.5, 116.3, 83.0, 80.7, 50.7, 42.7, 29.6, 28.2, 27.9; IR (neat) 2220 cm^{-1}; MS (CI + NH_3) 300 (MH^+).

N$^\alpha$*-Boc-N*$^\beta$*-Cyanodinor*-L-*ornithine tert-Butyl Ester* **19d**. Yellow oil (46%); TLC (EtOAc/cyclohexane 3/7) R_f 0.20; ^{1}H NMR ($CDCl_3$) δ 5.38 (d, 1H, J = 5.6 Hz), 4.98 (br s, 1H), 4.18 (br s, 1H), 3.37 (m, 2H), 1.48 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 168.7, 155.6, 116.2, 83.5, 80.5, 54.6, 48.2, 28.2, 27.8; IR (neat) 2230 cm^{-1}; MS (CI + NH_3) 286 (MH^+).

General Procedure for N-Hydroxyguanidine **20a**−**d** *and Urea* **21a**−**d** *Synthesis (Steps b of Scheme 1) (20, 27).* A mixture containing one of the above compounds **19a**, **19b**, **19c**, or **19d** (1 equiv), NH_2OH, HCl (5 equiv), Na_2CO_3 (2.5 equiv) and dioxane (9 mL/mmol of amino acid) was heated at 60 °C for approximately 1 h. After filtration, the crude product was chromatographed on silica gel with EtOAc/solvent A 1/1 or EtOAc/EtOH 7/3 + 1% AcOH.

N$^\alpha$*-Boc-N*$^\omega$*-Hydroxyhomo*-L-*arginine tert-Butyl Ester* **20a**. Yellow oil (68%); TLC (EtOAc/solvent A 1/3) R_f 0.36; ^{1}H NMR ($CDCl_3$) δ 8.20 (br s, 1H), 7.32 (br s, 1H), 7.06 (br s, 1H), 5.89 (br s, 1H), 5.30 (d, 1H, J = 7.2 Hz), 4.07 (m, 1H), 3.26 (m, 2H), 1.60−1.90 (m, 6H), 1.44 (s, 9H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.1, 158.3, 155.8, 81.9, 79.8,

54.0, 41.5, 32.1, 30.1, 28.3, 28.0, 22.5; HRMS (EI) 360.2398 (M; calcd 360.2373).

N^{α}-Boc-N^{ω}-Hydroxy-L-*arginine tert-Butyl Ester* **20b**. Spectral characteristics were as previously described (*20*, *27*).

N^{α}-Boc-N^{ω}-Hydroxy-nor-L-*arginine tert-Butyl Ester* **20c**. White foam (57%); TLC (EtOAc/solvent A 1/1) R_f 0.20; ^{1}H NMR ($CDCl_3$) δ 9.6 (br s, 1H), 7.40 (br s, 1H), 7.20 (br s, integration masked by solvent), 5.57 (br s, 1H), 4.08 (br s, 1H), 3.40 (m, 2H), 2.10 (m, 1H), 1.90 (m, 1H), 1.43 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.5, 158.4, 156.3, 82.8, 80.4, 52.1, 38.8, 28.3, 28.0, 26.9; HRMS (CI + NH_3) 333.2166 (MH^+; calcd 333.2138).

N^{α}-Boc-N^{ω}-Hydroxydinor-L-*arginine tert-Butyl Ester* **20d**. White foam (27%); TLC (EtOAc/solvent A 1/1) R_f 0.18; ^{1}H NMR ($CDCl_3$) δ 7.45 (br s, 2H), 5.60 (br s, 1H), 4.05 (m, 1H), 3.50 (m, 2H), 1.51 (s, 9H), 1.42 (s, 9H); MS (CI + NH_3) 319 (MH^+).

N^{α}-Boc-Homo-L-*citrulline tert-Butyl Ester* **21a**. Colorless oil (23%); TLC (EtOAc/solvent A 1/3) R_f 0.64; ^{1}H NMR ($CDCl_3$) δ 5.18 (m, 2H), 4.74 (s, 2H), 4.10 (m, 1H), 3.12 (m, 2H), 1.45−1.80 (m, 6H), 1.43 (s, 9H), 1.41 (s, 9H).

N^{α}-Boc-L-*Citrulline tert-Butyl Ester* **21b**. This product was not isolated since it is the precursor of L-citrulline.

N^{α}-Boc-Nor-L-*citrulline tert-Butyl Ester* **21c**. Colorless oil (30%); TLC (EtOAc/solvent A 1/1) R_f 0.55; ^{1}H NMR ($CDCl_3$) δ 5.54 (br s, 1H), 5.25 (d, 1H, J = 8.0 Hz), 4.38 (s, 2H), 4.20 (m, 1H), 3.60 (m, 1H), 2.90 (m, 1H), 2.00 (m, 1H), 1.60 (m, 1H), 1.45 (s, 9H), 1.43 (s, 9H); MS (CI + NH_3) 318 (MH^+).

N^{α}-Boc-Dinor-L-*citrulline tert-Butyl Ester **21d***. White foam (27%); TLC (EtOAc/solvent A 1/1) R_f 0.50; ^{1}H NMR ($CDCl_3$) δ 5.60 (br s, 1H), 5.30 (br s, 1H), 4.85 (br s, 2H), 4.14 (m, 1H), 3.50 (m, 2H), 1.45 (s, 9H), 1.41 (s, 9H); MS (CI + NH_3) 304 (MH^+).

General Procedure for Preparation of **22a** *and* **22b** *(Step d of Scheme 1).* One equivalent of amino acid **18a** or **18b** and 2 equiv of ethyl *N*-hydroxyacetimidate were refluxed in EtOH for 48 h. After evaporation, the mixture was poured onto a column of silica gel and eluted with EtOAc/EtOH 7/3 + 1% AcOH.

N^{α}-Boc-N^{ϵ}-[1-(N-Hydroxyimino)ethyl]-L-*lysine tert-Butyl Ester* **22a**. Yellow oil (53%); TLC (EtOAc/EtOH 7/3 + 1% AcOH) R_f 0.60; ^{1}H NMR ($CDCl_3$) δ 6.43 (s, 1H), 5.06 (d, 1H, J = 7.6 Hz), 4.14 (br s, 1H), 3.10 (br s, 2H), 1.84 (s, 3H), 1.50−1.80 (m, 6H), 1.44 (s, 9H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.9, 155.4, 152.8, 81.9, 79.7, 53.7, 42.2, 32.7, 30.6, 28.3, 28.0, 22.4, 14.7; HRMS (EI) 359.2426 (M; calcd 359.2420).

N^{α}-Boc-N^{δ}-[1-N-Hydroxyimino)ethyl]-L-*ornithine tert-Butyl Ester* **22b**. Yellow oil (27%); TLC (EtOAc/EtOH 7/3 + 1% AcOH) R_f 0.41; ^{1}H NMR ($CDCl_3$) δ 6.19 (br s, 1H), 5.18 (d, 1H, J = 7.3 Hz), 4.12 (br s, 1H), 3.21 (br s, 2H), 1.99 (s, 3H), 1.60−1.80 (m, 4H), 1.42 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$ + CD_3OD) δ 171.6, 155.6, 154.3, 82.1, 79.8, 53.3, 41.8, 29.5, 28.0, 27.7, 26.3, 13.8; HRMS (EI) 329.2315 (M − 16; calcd 329.2314).

Preparation of **3**−**10**, **13** *and* **14** *from Deprotection (20, 24, 27) of Compounds* **20a**−**d**, **21a**−**d**, *and* **22a,b** *(Steps c of Scheme 1)*

N^{ω}-Hydroxyhomo-L-*arginine Dihydrochloride* **3**. White solid (66%; 32% from *N^{ϵ}-Z*-L-lysine); TLC (solvent C) R_f 0.35; HPLC 12.7 min; ^{1}H NMR (D_2O) δ 4.10 (t, 1H, J = 6.2 Hz), 3.29 (t, 2H, J = 6.6 Hz), 2.00 (m, 2H), 1.70 (m, 2H), 1.54 (m, 2H); ^{13}C NMR (D_2O) δ 175.3, 161.6, 55.9, 43.5, 32.0, 30.3, 24.3; MS (electrospray) 205 (MH^+); $[\alpha]_D$ = +19° (MeOH, c = 0.6).

N^{ω}-Hydroxy-L-*arginine Dihydrochloride* **4**. Spectral characteristics were as previously described (*20*, *24*, *27*).

N^{ω}-Hydroxynor-L-*arginine Dihydrochloride* **5**. White solid (67%; 15% from L-glutamine); TLC (solvent B) R_f 0.40; HPLC 8.7 min; ^{1}H NMR (D_2O) δ 4.12 (t, 1H, J = 6.6 Hz), 3.52 (t, 2H, J = 7.4 Hz), 2.28 (m, 2H); ^{13}C NMR (D_2O) δ 174.2, 161.5, 53.2, 40.1, 31.8; MS (electrospray) 177 (MH^+); $[\alpha]_D$ = +16° (H_2O, c = 1.1).

N^{ω}-Hydroxydinor-L-*arginine Dihydrochloride* **6**. White solid (96%; 4% from L-serine); TLC (solvent B) R_f 0.35; HPLC 5.3 min; ^{1}H NMR (D_2O) δ 4.23 (t, 1H, J = 5.6 Hz), 3.87 (d, 2H, J = 5.6 Hz); ^{13}C NMR (D_2O) δ 172.7, 161.7, 55.2, 43.5; MS (electrospray) 163 (MH^+); $[\alpha]_D$ = +14° (H_2O, c = 0.2).

Homo-L-*citrulline* **7**. White powder (60% from **21a** and 10% from *N^{ϵ}-Z*-L-lysine). Spectral characteristics were as previously described (*22*).

Nor-L-*citrulline Hydrochloride* **9**. White solid (90%; 11% from L-glutamine); TLC (solvent B) R_f 0.55; HPLC 3.9 min; ^{1}H NMR (D_2O) δ 4.15 (t, 1H, J = 6.6 Hz), 3.36 (td, 2H, J = 6.6 and 1.2 Hz), 2.20 (m, 2H); ^{13}C NMR (D_2O) δ 174.8, 164.6, 53.2, 38.2, 33.8; MS (electrospray) 162 (MH^+); $[\alpha]_D$ = +19° (2 N HCl, c = 0.6).

Dinor-L-*citrulline Hydrochloride* **10**. White solid (100%; 4% from L-serine); TLC (*n*BuOH/AcOH/H_2O 4/1/1) R_f 0.31 [lit. (*29*) R_f 0.34]; HPLC 3.1 min; ^{1}H NMR (D_2O) δ 4.23 (dd, 1H, J = 5.6 and 4.2 Hz), 3.72 (2 dd, ABX, 2H, J = 15.3, 5.6, and 4.2 Hz); ^{13}C NMR (D_2O) δ 173.0, 164.4, 56.5, 42.4; MS (electrospray) 148 (MH^+); $[\alpha]_D$ = −17° (H_2O, c = 1).

N^{ϵ}-[1-(N-Hydroxyimino)ethyl]-L-*lysine Dihydrochloride* **13**. White solid (70%; 37% from *N^{ϵ}-Z*-L-lysine); TLC (solvent B) R_f 0.35; HPLC 11.3 min; ^{1}H NMR (D_2O) δ 4.12 (t, 1H, J = 6.1 Hz), 3.47 (t, 2H, J = 7.0 Hz), 2.24 (s, 3H), 2.02 (m, 2H), 1.74 (m, 2H), 1.56 (m, 2H); ^{13}C NMR (D_2O) δ 175.1, 165.4, 55.7, 45.2, 32.3, 31.1, 24.3, 15.5; MS (electrospray) 204 (MH^+); $[\alpha]_D$ = +19° (MeOH, c = 0.6).

N^{δ}-[1-(N-Hydroxyiminoethyl)-L-*ornithine Dihydrochloride* **14**. White solid (60%; 16% from *N^{δ}-Z*-L-ornithine); TLC (solvent B) R_f 0.38; HPLC 9.5 min; ^{1}H NMR (D_2O) δ 4.13 (t, 1H, J = 6.0 Hz), 3.50 (t, 2H, J = 6.9 Hz), 2.34 (s, 3H), 2.01 (m, 2H), 1.80 (m, 2H); ^{13}C NMR (D_2O) δ 174.8, 164.2, 55.5, 44.9, 29.7, 27.5, 15.5; MS (electrospray) 190 (MH^+); $[\alpha]_D$ = +21° (MeOH, c = 0.6).

Synthesis of compounds **16** *and* **17** *(Steps e−g of Scheme 1)*

N^{α}-Boc-6-Cyanonor-D,L-*leucine.* This compound was synthesized as previously described (*30*).

N^{α}-Boc-5-Cyanonor-D,L-*valine*. Boc-glycine (1 equiv, 11.4 mmol) dissolved in 30 mL of dry THF was added to a solution of lithium diisopropylamide (LDA, 2 M in THF; 3 equiv) in 90 mL of THF at 0 °C under Ar. After 90 min at 0 °C, the mixture was cooled to −78 °C and 4-bromobutyronitrile (1.5 equiv) was added dropwise. The solution was then allowed to warm to room temperature for 2 h and the

reaction was quenched with 10 mL of saturated NaH_2PO_4 and 50 mL of water. The aqueous phase was acidified with concentrated HCl and extracted with EtOAc, and the combined organic layers were extracted with water and brine, dried ($MgSO_4$), filtered, and evaporated. The crude product contained a mixture of Boc-glycine and of the title compound (40%), which was used without further purification. TLC (EtOAc/solvent A 1/1) R_f 0.58; ^{1}H NMR ($CDCl_3$) δ 4.30 (br s, 1H), 2.39 (t, 2H, $J = 6.4$ Hz), 2.10 (m, 2H), 1.60 (m, 2H), 1.43 (s, 9H).

*N^{α}-Boc-6-Cyanonor-*D,L*-leucine tert-Butyl Ester*. Spectral characteristics were as previously described (*30*).

*N^{α}-Boc-5-Cyanonor-*D,L*-valine tert-Butyl Ester*. Yellow oil (46%); TLC (EtOAc/cyclohexane 1/4) R_f 0.23; ^{1}H NMR ($CDCl_3$) δ 5.05 (br s, 1H), 4.17 (br s, 1H), 2.38 (t, 2H, $J =$ 6.0 Hz), 1.60−2.00 (m, 4H), 1.45 (s, 9H), 1.42 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.1, 155.3, 119.1, 82.1, 79.6, 53.0, 31.7, 28.1, 27.8, 21.4, 16.6; MS (CI + NH_3) 299 (MH^+).

*N^{α}-Boc-N^{ω}-Hydroxy-*D,L*-indospicine tert-Butyl Ester*. This compound was synthesized as previously described (*30*).

*N^{α}-Boc-N^{ω}-Hydroxynor-*D,L*-indospicine tert-Butyl Ester*. N^{α}-Boc-5-Cyanonor-D,L-valine *tert*-butyl ester (1 equiv; 0.3 mmol), NH_2OH−HCl (6.4 equiv), Na_2CO_3 (4.3 equiv), and 5 mL of absolute EtOH were stirred at 110 °C (external temperature) in a sealed glass tube for 1 h. The mixture was then filtered, washed with EtOH, and concentrated under vacuum to yield crude product, which was purified on silica gel (EtOAc/cyclohexane 1/4). White foam (64%); TLC (EtOAc/solvent A 1/1) R_f 0.40; ^{1}H NMR ($CDCl_3$) δ 5.93 (m, 2H), 5.40 (d, 1H, $J = 7.5$ Hz), 4.00 (br s, 1H), 2.18 (m, 2H), 1.50−1.70 (m, 4H), 1.34 (s, 9H), 1.31 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 171.7, 155.9, 155.0, 82.0, 80.8, 53.3, 31.9, 29.3, 28.2, 27.8, 22.5; MS (EI) 331 (M).

*N^{ω}-Hydroxy-*D,L*-indospicine Dihydrochloride* **16**. Spectral characteristics were as previously described (*30*).

*N^{ω}-Hydroxynor-*D,L*-indospicine Dihydrochloride* **17**. White solid (80%; 5% from **34**); TLC (solvent B) R_f 0.40; HPLC 6.4 min; ^{1}H NMR (D_2O) δ 4.05 (t, 1H, $J = 6.2$ Hz), 2.59 (t, 2H, $J = 7.4$ Hz), 2.10 (m, 2H), 1.70 (m, 2H); ^{13}C NMR (D_2O) δ 174.8, 166.5, 55.5, 31.7, 30.8, 24.6; MS (electrospray) 176 (MH^+).

Biochemistry, General. Arg, Cit, homo-Arg, bovine brain CaM, and bovine erythrocyte superoxide dismutase (SOD) were purchased from Sigma. NADPH was from Boehringer Mannheim Biochemicals. [*guanidino*-^{14}C]Arg (55 mCi/mmol) was from DuPont−NEN. BH_4, N^{δ}-(1-iminoethyl)-L-ornithine (NIO) and N^{ϵ}-(1-iminoethyl)-L-lysine (NIL) were purchased from Alexis. Recombinant rat brain NOS I was overexpressed in *Saccharomyces cerevisiae* and purified with a CaM−agarose (Sigma) column as previously described (*31*). NOS I was filtered over a Bio-Spin column (Bio-Rad) just prior to use. Recombinant mouse macrophage inducible NOS II with a His_6 tag attached to the C-terminus of the protein was overexpressed in *Escherichia coli* and purified with a Ni^{2+}−nitrilotriacetic acid−Sepharose CL 4B column as described elsewhere (*32*). RLA was purified from rat liver homogenates with an Amicon Green (Bio-Rad) column following a previously described procedure (*33*). Protein concentrations were determined by the Bradford assay with bovine serum albumin as a standard. Proteins were more than 80% pure as judged by SDS−PAGE.

Assays of NOS Activity. Nitrite detection was performed on incubated mixtures containing 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (HEPES) (pH 7.4), 500 μM substrate, 100 μM NADPH, 4 μM FAD, 4 μM FMN, 6 μM BH_4, 0.4−1 mM dithiothreitol (DTT), 50 units of SOD/mL, and 0.5−1 μg of NOS in a 100 μL final volume. For NOS I, 1 mM $CaCl_2$ and 10 μg/mL CaM were also added. After 10 min at 37 °C, reactions were stopped with 25 μL of EtOH. Griess reagent [250 μL; 125 μL of 1% sulfanilamide in 0.5 N HCl and 125 μL of 0.1% *N*-(1-naphthyl-)ethylenediamine] was added and absorbances were measured at 543 nm (*22*, *34*). Calibration curves were obtained from identical incubated mixtures (without NOS) containing increasing concentrations of $NaNO_2$.

NOS Inhibition Assay. Inhibition constants (IC_{50}) were determined with at least eight concentrations of inhibitor added to a mixture containing, in a 100 μL final volume: 50 mM HEPES (pH 7.4), 10 μM Arg, 0.2−1 mM NADPH, 4 μM FAD, 4 μM FMN, 6 μM BH_4, 0.4−1 mM DTT, 1 mM $CaCl_2$ (NOS I only), 10 μg/mL CaM (NOS I only), 0.1 μg of NOS, and 0.05 μCi of [*guanidino*-^{14}C]Arg (final concentration of Arg 11.5 μM). Following incubation at 37 °C for 5−15 min, the reactions were stopped by the addition of 500 μL of cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-Cit, 2 mM EDTA, and 0.2 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid. Samples (500 μL) were applied to columns containing 1 mL of Dowex AG-50W-X8 resin (Na^+ form, prepared from the H^+ form) preequilibrated with stop buffer, and a total of 1.5 mL of stop buffer was added to elute [*ureido*-^{14}C]Cit. Aliquots were then counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer after addition of Pico-Fluor 40 scintillation cocktail. Control samples without NOS, NADPH, or CaM were included for background determinations.

Assays of Rat Liver Arginase Activity. Assays quantitated the [^{14}C]urea produced from [*guanidino*-^{14}C]Arg following a previously described method (*35*). A typical assay was performed in 100 μL of 0.2 M Tris-HCl (pH 7.4) containing 20 mM L-Arg, 0.1 μCi of [*guanidino*-^{14}C]Arg, and variable concentrations of inhibitors. The reactions were initiated by the addition of RLA. Protein amounts were adjusted to yield less than 15% substrate conversion. After 10 min at 37 °C, 150 μL of cold stop buffer (0.25 M AcOH and 7 M urea) were added and [^{14}C]urea was separated from unreacted [*guanidino*-^{14}C]Arg by mixing with 250 μL of a 1:1 slurry of Dowex AG-50W-X8 (H^+ form) and centrifugation. Aliquots of the supernatant were then counted after addition of Pico-Fluor 40.

RESULTS

Synthesis of the Target Molecules. All the target *N*-hydroxyguanidines and amidoximes, except **16** and **17**, were synthesized from the amine precursor of the desired chain length protected on the α-COOH function by a *tert*-butyl ester and on the α-NH_2 function by a *tert*-butyloxycarbonyl (Boc) group, **18a**−**d** (Scheme 1). Synthesis of the *N*-hydroxyguanidines **3**−**6** followed the procedure described by Wallace and Fukuto (*27*) for the synthesis of NOHA **4**. The key step of this method was the nucleophilic addition of hydroxylamine on a cyanamide compound **19a**−**d** resulting from the reaction of the starting amine **18a**−**d** with

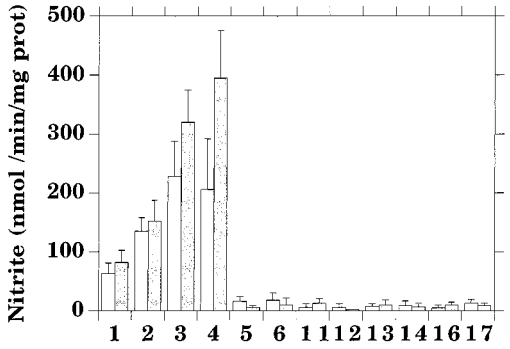


FIGURE 1: Nitrite production by NOS I (open bars) and NOS II (solid bars) in the presence of the guanidines, *N*-hydroxyguanidines, amidines, and amidoximes shown in Chart 1. Nitrite production was measured by the Griess assay, as described under Materials and Methods, from incubations containing 500 μM potential substrate and run at 37 °C for 10 min. Means ± SD ($n = 3$) are shown.

cyanogen bromide. This procedure yielded a mixture of the desired *N*-hydroxyguanidines **20a**−**d** and of the corresponding ureas **21a**−**d** (*36*, *37*). Careful control of the reaction time was needed since *N*-hydroxyguanidines were labile under the reaction conditions. Removal of the protecting groups on *N*-hydroxyguanidines and ureas was achieved in the presence of gaseous HCl dissolved in anhydrous dioxane, yielding white solids that were thoroughly dried. The resulting compounds were highly hygroscopic and needed to be handled in a dry atmosphere.

The reactions described in Scheme 1 were straighforward with good to moderate yields. More difficulties were encountered during the preparation of the starting amines **18a**−**d**. Actually, whereas compounds **18a** and **18b** were easily obtained from the commercially available N^{ϵ}-*Z*-L-lysine and N^{δ}-*Z*-L-ornithine by a well-described procedure (Scheme 2), this was not the case for the shorter derivatives of L-ornithine **18c** and **18d** (*26*, *27*, *30*). Thus, the latter compounds were synthesized from L-glutamine and L-serine by totally different procedures that are shown in Scheme 2 and completely described under Materials and Methods.

Finally, nor-NOHI **17** was prepared as a racemate from Boc-glycine and 4-bromobutyronitrile (Scheme 1), following the alkylation procedure already described for the synthesis of *N*-hydroxyindospicine, NOHI, **16** (*30*, *38*).

As far as the eight *N*-hydroxyguanidines and amidoximes shown in Chart 1 are concerned, only the syntheses of NOHA (*20*, *24*, *27*, *30*) and NOHI (*30*) have been completely described so far. Only preliminary indications about the synthesis method of homo-, nor-, and dinor-NOHA have been previously mentioned (*13*). The first complete description of the synthesis and characteristics of these compounds, as well as that of the new products OH-NIL **13**, OH-NIO **14**, and nor-NOHI **17**, is presented under Materials and Methods.

Study of the Various Compounds as NOS Substrates. Formation of nitrite was followed upon incubation of each compound with recombinant NOS I or NOS II in the presence of NADPH and under the usual conditions for NOS activity measurements (*22*). Figure 1 shows that only four compounds led to significant formation of nitrite under those conditions, namely, Arg **2** and NOHA **4**, the endogenous NOS substrates, and homo-Arg **1** and its N^{ω}-hydroxy derivative **3**. As reported recently (*22*), the shorter analogues of NOHA, nor-NOHA **5** and dinor-NOHA **6**, failed to produce significant amounts of nitrite and did not act as substrates of NOS I and II under the conditions used. Compounds **11**, **12**, **13**, and **14**, that simply derive from homo-Arg, Arg, homo-NOHA, and NOHA by replacement of their ω-NH_2 group with a CH_3 group, were also inactive. This was also the case for NOHI **16** and nor-NOHI **17**, which derive from NOHA and nor-NOHA by replacement of their δ-NH group with a CH_2 group (Chart 1).

Table 1: IC_{50} Values for Inhibition of NOS I, NOS II, and RLA by Various Analogues of Arg and NOHA

	IC_{50}[a] (μM)		
	NOS I	NOS II	RLA
homo-Arg **1**	23[b]	33[b]	60 000
Arg **2**	2.7[b]	13[b]	6000[c]
homo-NOHA **3**	68[b]	146[b]	200
NOHA **4**	13[b]	59[b]	20
nor-NOHA **5**	1150	>1500	1
dinor-NOHA **6**	360	465	40
NIL **11**	35	2.5	20 000
NIO **12**	2	2	>10 000
OH−NIL **13**	>1500	410	3000
OH−NIO **14**	170	290	1400
NOHI **16**	1200	>1500	5
nor-NOHI **17**	>1500	>1500	2

[a] IC_{50} values were determined by testing each compound at eight concentrations. NOS activities were measured in the presence of a final Arg concentration of 11.5 μM by monitoring the conversion of [*guanidino*-^{14}C]Arg to [*ureido*-^{14}C]Cit as described under Materials and Methods. Similarly, RLA activity was measured by monitoring the conversion of [*guanidino*-^{14}C]Arg to [^{14}C]urea in the presence of a final Arg concentration of 20 mM. The data are reported as means of at least three separate experiments (SD < 20%). [b] K_m values previously described for the NOS-dependent oxidation of these substrates (*22*). [c] K_m value for RLA-dependent hydrolysis of Arg (*55*).

These results once again illustrate the strict substrate specificity for NOS-dependent formation of NO. The only change in the structure of Arg or NOHA accepted by the enzyme for NO_2^- formation is an increase of their chain lengths by one CH_2, whereas any shortening of the chain or any replacement of an NH group of the guanidine (or *N*-hydroxyguanidine) function with a CH_2 group are not tolerated.

Properties of the Studied Compounds as NOS and Arginase Inhibitors. Table 1 summarizes the results obtained from inhibition studies with recombinant NOS I and II and purified RLA with Arg as the substrate.

The best inhibitor for both NOSs was NIO **12** with IC_{50} values around 2 μM. Its longer analogue NIL **11** also acted as a good inhibitor, although with a markedly larger IC_{50} value in the case of NOS I. However, all the other analogues that involve a *N*-hydroxyguanidine (**5**, **6**) or an amidoxime (**13**, **14**, **16**, **17**) function were very bad inhibitors of NOS I and II, with IC_{50} values higher than 170 μM. It is noteworthy that introduction of a N^{ω}-hydroxy substituent generally leads to a marked decrease of the affinity of the corresponding compound for NOSs. This has been previously reported for NOHA and homo-NOHA, whose K_m values are 3−9-fold higher than those of Arg and homo-Arg (*22*). Table 1 shows that this is also true for OH-NIL **13** and OH-NIO **14** when compared to NIL **11** and NIO **12**. In fact, the IC_{50} values found for these two amidoximes are 2 orders of magnitude higher than those of the corresponding amidines **11** and **12**.

Our results on NOS I and II also further illustrate that, although many compounds are recognized by NOS and act as strong inhibitors of this enzyme, only very few of them are oxidized by NOS with formation of NO (Figure 1). Clearly, additional structural factors are required for a molecule well recognized by NOS, such as those described above, to become a substrate producing NO. Further studies are necessary to clarify this point.

Very different results were obtained with purified RLA. Here, on the contrary, N^{ω}-hydroxylated compounds are very good inhibitors of RLA, with IC_{50} values between 1 and 40 μM for the N^{ω}-hydroxyguanidines nor-NOHA, NOHA, and dinor-NOHA. The two amidoximes, NOHI **16** and nor-NOHI **17**, are among the best RLA inhibitors known so far (IC_{50} of 5 and 2 μM). However, interestingly enough, the two amidoximes bearing a terminal CH_3 group, OH-NIL **13** and OH-NIO **14**, are bad inhibitors of RLA with IC_{50} values 3 orders of magnitude higher than amidoximes **16** and **17**. The difference in the behavior of the studied compounds toward NOS and RLA is particularly striking if one considers that the two best inhibitors of RLA, nor-NOHA **5** and nor-NOHI **17** (IC_{50} around 1 μM), are among the worst inhibitors of NOS I and II (IC_{50} > 1 mM), whereas the compounds that show the highest affinities for NOSs, Arg, NIL and NIO (IC_{50} or K_m at the micromolar level) exhibit a very low affinity for RLA (IC_{50} or K_m in the case of Arg > 1 mM) (Table 1).

Finally, the strongest RLA inhibitors in the studied α-amino acid series, nor-NOHA **5** and nor-NOHI **17**, are compounds with a chain of five atoms between the Cα amino acid atom and the terminal OH function.

DISCUSSION

The results of Table 1 clearly show that the main structural determinants for efficient recognition of α-amino acids by NOS and arginase are very different. In the case of NOS, the recognition is based on the presence of a −NH−C(R)═NH or −NH−C(NH_2)═NOH motif separated from the amino acid C_α atom by three or four CH_2 groups (Figure 2A), whereas in the case of RLA it involves the presence of a N−OH function separated from the amino acid C_α carbon by four or five atoms, the highest affinities being observed in the case of four atoms (Figure 2B).

The best structural motif recognized by NOS, depicted in Figure 2A, is found in Arg itself, which has a relatively high affinity for NOS I and II (K_m or K_s values at the micromolar level) (*5*−*7*), and in some of its close analogues such as NIO and NIL (R = CH_3) and NOHA and homo-NOHA (R = NH_2 and presence of a C═NOH function) (Table 1). This is in agreement with literature data previously reported for these compounds (*39*, *40*), as well as on other Arg analogues in which R (see Figure 2A) is a slightly longer alkyl chain (NIO analogues) (*41*), a NHR′ group (N^{ω}-substituted arginines) (*41*−*43*), or a SR″ group (α-amino acids bearing an isothiourea function) (*44*, *45*). These data are easily explained by considering the X-ray structure published recently for the Arg complex of the oxygenase domain of NOS II (*46*) and III (*47*, *48*), which shows the existence of key interactions of the protein with the Arg δ-NH and ω-NH_2 groups, in addition to specific bonds with the Arg α-NH_2 and α-COOH functions. Figure 3 illustrates these interactions and indicates

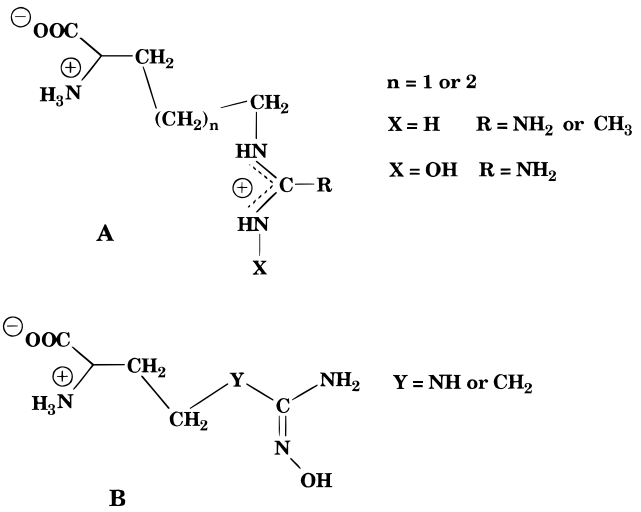


FIGURE 2: Structural determinants of the studied α-amino acid series best recognized by NOS (A) and RLA (B).

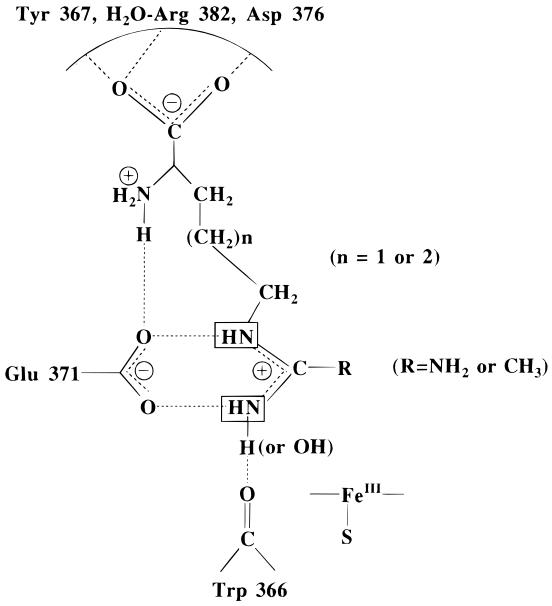


FIGURE 3: Modes of interaction between NOS and α-amino acids studied in this article. These are based on the X-ray structure published for NOS−Arg complexes (*46*−*48*). Labeling of NOS residues corresponds to that of NOS II.

how α-amino acids bearing the −NH−C(R)═NH or −NH−C(NH_2)═NOH motif could be bound in the NOS active site. The R substituent of the guanidine carbon is less restricted and may be an alkyl (*39*−*41*), NHR′ (*41*−*43*), or SR″ (*44*, *45*) group.

Our data show that N^{ω}-hydroxylation of the α-amino acid derivatives always leads to a marked decrease in the affinity for NOS I and II. This is true not only for NOHA compared to Arg but also for homo-NOHA, OH-NIL, and OH-NIO compared to homo-Arg, NIL, and NIO, respectively. However, it is noteworthy that the decrease in affinity upon N^{ω}-hydroxylation is clearly larger in the case of the amidines NIL and NIO (about 100-fold increase in IC_{50}) than in the case of the guanidines homo-Arg and Arg (3−4-fold increase in K_m). The much lower affinity of the amidoximes OH-NIL, OH-NIO, and NOHI when compared to the corresponding *N*-hydroxyguanidines (homo-NOHA and NOHA) could be due, at least in part, to the different pK_a values of

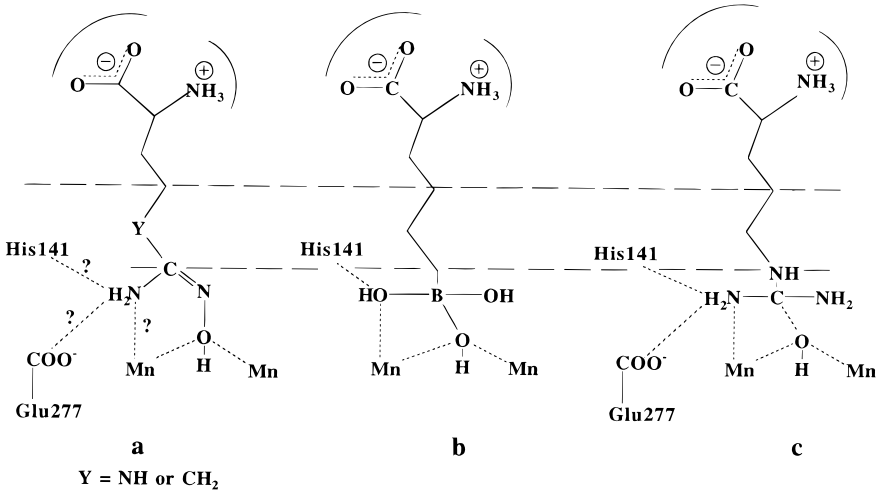


FIGURE 4: Comparison between the structure of the RLA−(2-amino-6-boronohexanoic acid) complex (*54*) (b) and those proposed for the transition state of RLA-catalyzed Arg hydrolysis (*54*) (c) and the RLA-nor-NOHA (or -NOHI) complex (this article) (a).

these two functions. Aliphatic *N*-hydroxyguanidines have pK_a values around 8 (*49*−*51*) and should mainly exist as their protonated form at physiological pH, as aliphatic guanidines such as Arg and amidines such as NIO exhibit pK_a values around 13 (*49*, *50*). On the contrary, aliphatic amidoximes such as OH-NIL, OH-NIO, NOHI, and nor-NOHI appear to have lower pK_a values between 4 and 6 (*49*, *50*) and should be not protonated at physiological pH. These data suggest that the presence of a positive charge coming from protonation of the guanidine, amidine, or *N*-hydroxyguanidine function plays an important role in recognition of such α-amino acids by NOS (Figure 3). An exception to this rule is thiocitrulline, a well-known strong inhibitor of NOS (*52*), whose thiourea function is not protonated. However, the high affinity of this compound for NOS could be due, at least in part, to an additional bond between its C═S function and the NOS iron (*46*, *52*), which does not exist in the α-amino acids studied here.

Interestingly, two isomers, OH-NIO and NOHI, that both involve an amidoxime function exhibit markedly different IC_{50} values (Table 1). The lower affinity of NOSs for NOHI than for OH-NIO further illustrates the importance of the bond between glutamate 371 and the δ-NH group of the inhibitor (or substrate), which is lacking in NOHI (Figure 3). A similar result showing that indospicine (**15**, Chart 1), which is derived from Arg by replacement of the δ-NH group with a δ-CH_2 group, is a much weaker inhibitor of NOS II than its isomer NIO has been reported previously (*53*).

Finally, the crucial importance of the chain length between the α-amino acid and −NH−C(R)═NOH function, is clearly shown by the IC_{50} or K_m values found for NOHA and its homo, nor, and dinor analogues, which are low for NOHA, slightly higher in the case of homo-NOHA, and greatly increased for nor-NOHA (Table 1).

Efficient recognition of the same α-amino acids by RLA is based on very different structural characteristics. Here the presence of a N−OH group separated from C_α by four or five atoms appears to be crucial. The lowest IC_{50} values (at the micromolar level) are observed for nor-NOHA and nor-NOHI that involve a four atom chain between C_α and N−OH, exactly as found in N^ω-hydroxylysine, a previously described good inhibitor of RLA (IC_{50} = 15 μM) (*20*). An increase or a decrease in the chain length of nor-NOHA by one CH_2 group leads to a 20-fold or 40-fold increase of the IC_{50} value, respectively. The same increase in the chain length of nor-NOHI by one carbon leads to a smaller (2.5-fold) increase of IC_{50} (Table 1). The higher affinity of RLA for NOHI than for NOHA could be due to the greater flexibility of the $-(CH_2)_4-$ chain of NOHI as compared to the $-(CH_2)_3-NH-$ chain of NOHA. The presence of a positive charge, due to protonation of the −NH−C(R)═NOH function, does not seem to be as important as in the case of NOS, since NOHI that bears an amidoxime function not protonated at physiological pH exhibits a higher affinity for RLA than the corresponding *N*-hydroxyguanidine NOHA. Finally, contrary to what was found in the case of NOS, the presence of a δ-NH group is not important for RLA recognition (see, for instance, the almost identical IC_{50} values of nor-NOHI and nor-NOHA), whereas the presence of two terminal N^ω nitrogen atoms is very important for RLA recognition, as shown by the much higher IC_{50} value found for OH-NIO than for its isomer NOHI (1.4 mM instead of 5 μM for NOHI).

In its three main characteristics for efficient recognition of α-amino acids, i.e. (i) the greater preference for a C═N^ω−OH group than for a C═NH group, (ii) the importance of the presence of two N^ω nitrogen atoms, and (iii) the lack of recognition of δ-NH groups, RLA exhibits a behavior opposite to that of NOS. This can be explained by considering the X-ray structure published for RLA (*2*). The much higher affinity of compounds bearing a terminal N−OH group than that of their N−H analogues could be due to a strong interaction between the OH group and the Mn(II)···Mn(II) cluster of the RLA active site.

In the structure postulated for the transition state of RLA-catalyzed hydrolysis of Arg (*2*, *54*) (Figure 4c), the μ-hydroxo ligand that bridges the two Mn(II) ions interacts with the electrophilic guanidino carbon, establishing a six-atom $O-C-N-(CH_2)_3-$ chain bound to the C_α atom. A very similar motif with a six-atom $O-B-C-(CH_2)_3-$ chain has been found very recently in the X-ray structure of the complex between RLA and the boronic isostere of Arg, 2(*S*)-amino-6-boronohexanoic acid, a strong RLA inhibitor of the transition-state analogue type (*54*). This compound binds as the tetrahedral boronate anion with one hydroxyl oxygen bridging the binuclear manganese cluster (Figure 4b). The

structure postulated for the RLA complex with nor-NOHA (*13*) (or nor-NOHI) is strikingly similar if one considers that the OH group of nor-NOHA replaces the μ-hydroxo bridging ligand of the Mn cluster (Figure 4a). This structure is based on an $-O-N-C-N-(CH_2)_2-$ motif between the amino acid C_α atom and the Mn cluster, involving an identical number of atoms and a length similar to those of the RLA−boronic acid complex and the transition state of the RLA-catalyzed reaction (Figure 4). This could explain why nor-NOHA is a much better RLA inhibitor than its longer (NOHA and homo-NOHA) or shorter (dinor-NOHA) analogues.

The importance of the presence of two N^ω nitrogen atoms (point (ii) mentioned above) for efficient recognition of α-amino acids by RLA is also easily explained when the X-ray structures of RLA (*2*) and of the RLA−boronic acid (*54*) complex are considered. Actually, one ω-OH group of the boronic acid that corresponds to the ω-NH_2 group of Arg is bound to RLA via a hydrogen bond with the CO function of His 141 and via its oxygen atom to Mn_A, whereas the second ω-OH group is only bound to a water molecule (*54*). Modeling experiments on the possible transition state of RLA-catalyzed hydrolysis of Arg (Figure 4c) suggest the existence of hydrogen bonds between an ω-NH_2 group of Arg and the CO function of His 141 and the COO^- function of Glu 277, whereas the second ω-NH_2 group of Arg would only interact with a water molecule (*2*). The much higher affinity of RLA for NOHI than for its isomer OH-NIO (Table 1) indicates that relatively strong bonds could also exist between the ω-NH_2 group of NOHI, nor-NOHI, or nor-NOHA and residues of RLA, possibly Mn_A, Glu 277, and His 141.

The proposed model for the transition state of the RLA-catalyzed hydrolysis of Arg does not show any bond with the δ-NH group of Arg. Our results showing that the IC_{50} values of nor-NOHI and nor-NOHA are almost identical strongly suggest that the lack of interaction with δ-NH groups postulated from model studies (*2*) is likely to be real.

Finally, comparison of Figures 3 and 4 also explains why optimal chain length (between C_α and terminal N^ω) involves five atoms in NOS but four atoms in RLA. The five-atom motif is found in Arg and NIO, which are the best ligands in the studied series with K_m or IC_{50} values at the micromolar level, because they fit best into the Arg recognition site of NOS. The four-atom motif corresponds to nor-NOHA and nor-NOHI (IC_{50} at the micromolar level), a situation of optimal interaction of RLA not with Arg itself, which has a high K_m value (at the millimolar level), but with the transition state of Arg hydrolysis, which involves a presumably strong additional interaction with the Mn cluster.

The different modes of interaction displayed by both enzymes depend on their different catalytic functions and give interesting opportunities to design useful molecules to selectively regulate NOS and arginase. However, it is noteworthy that the main modes of interactions described in this paper are based on a particular class of compounds that all contain an α-amino acid function. These compounds have a marked tendency to strongly interact with the protein residues that specifically recognize the NH_3^+ and COO^- α-amino acid functions. This appears to be crucial in the case of arginase since all its strong inhibitors known so far contain this function. This could be less important in the case of NOS, as many strong NOS inhibitors (K_i between 10 and 1000 nM) are not α-amino acids (*12*). With inhibitors such as *N*-alkylisothioureas, other sites of the protein than those discussed in this article could be involved in inhibitor recognition.

ACKNOWLEDGMENT

We thank Dr. S. Vadon-Le Goff for helpful discussions and Qian Wang, Christine Curran, and Abby Meade for excellent technical assistance.

REFERENCES

1. Wu, G., and Morris, S. M. (1998) *Biochem. J. 336*, 1−17.
2. Kanyo, Z. F., Scolnick, L. R., Ash, D. E., and Christianson, D. W. (1996) *Nature 383*, 554−557.
3. Jenkinson, C. P., Grody, W. W., and Cederbaum, S. D. (1997) *Comp. Biochem. Physiol. Biochem. Mol. Biol. 114B*, 107−132.
4. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991) *J. Biol. Chem. 266*, 6259−6263.
5. Kerwin, J. F., Lancaster, J. R., and Feldman, P. L. (1995) *J. Med. Chem. 38*, 4343−4362.
6. Masters, B. S. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P. (1996) *FASEB J. 10*, 552−558.
7. Pfeiffer, S., Mayer, B., and Hemmens, B. (1999) *Angew. Chem., Int. Ed. Engl. 38*, 1714−1731.
8. Moncada, S., and Higgs, E. A. (1995) *FASEB J. 9*, 13619−13630.
9. Knowles, R. G., and Moncada, S. (1994) *Biochem. J. 298*, 249−258.
10. Förstermann, U., Closs, E. I., Pollock, J. S., Nakane, M., Schwarz, P., Gath, I., and Kleinerth, H. (1994) *Hypertension 23*, 1121−1131.
11. Marletta, M. A. (1994) *J. Med. Chem. 37*, 23790−23795.
12. Babu, B. R., and Griffith, O. W. (1998) *Curr. Opin. Chem. Biol. 2*, 491−500.
13. Custot, J., Moali, C., Brollo, M., Boucher, J. L., Delaforge, M., Mansuy, D., Tenu, J. P., and Zimmermann, J. L. (1997) *J. Am. Chem. Soc. 119*, 4086−4087.
14. Baggio, R., Elbaum, D., Kanyo, Z. F., Carroll, P. J., Cavalli, R. C., Ash, D. E., and Christianson, D. W. (1997) *J. Am. Chem. Soc. 119*, 8107−8108.
15. Chenais, B., Yapo, A., Lepoivre, M., and Tenu, J. P. (1991) *J. Chromatogr. 539*, 433−441.
16. Hecker, M., Schott, C., Bucher, B., Busse, R., and Stoclet, J. C. (1995) *Eur. J. Pharmacol. 275*, R1−R3.
17. Klatt, P., Schmidt, K., Uray, G., and Mayer, B. (1993) *J. Biol. Chem. 268*, 14781−14787.
18. Daghigh, F., Fukuto, J. M., and Ash, D. E. (1994) *Biochem. Biophys. Res. Commun. 202*, 174−180.
19. Boucher, J. L., Custot, J., Vadon, S., Delaforge, M., Lepoivre, M., Tenu, J. P., Yapo, A., and Mansuy, D. (1994) *Biochem. Biophys. Res. Commun. 203*, 1614−1621.
20. Custot, J., Boucher, J. L., Vadon, S., Guedes, C., Dijols, S., Delaforge, M., and Mansuy, D. (1996) *J. Biol. Inorg. Chem. 1*, 73−82.
21. Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkinson, C. P., Cederbaum, S. D., and Ignarro, L. J. (1996) *Am. J. Physiol. 271*, H1988−H1998.
22. Moali, C., Boucher, J. L., Sari, M. A., Stuehr, D. J., and Mansuy, D. (1998) *Biochemistry 37*, 10453−10460.
23. Clague, M. J., Wishnok, J. S., and Marletta, M. A. (1997) *Biochemistry 36*, 14465−14473.
24. Feldman, P. L. (1991) *Tetrahedron Lett. 32*, 875−878.
25. Rudinger, J., Poduska, K., and Zaoral, M. (1960) *Czech. Chem. Commun. 25*, 2022−2028.
26. Chevallet, P., Garrouste, P., Malawska, B., and Martinez, J. (1993) *Tetrahedron Lett. 34*, 7409−7412.

27. Wallace, G. C., and Fukuto, J. M. (1991) *J. Med. Chem. 34*, 1746−1748.
28. Gibson, F. S., Bergmeier, S. C., and Rapoport, H. (1994) *J. Org. Chem. 59*, 3216−3218.
29. Inouye, S., Shomura, T., Tsuruoka, T., Ogawa, Y., Watanabe, H., Yoshida, J., and Niida, T. (1975) *Chem. Pharm. Bull. 23*, 2669−2677.
30. Vadon, S., Custot, J., Boucher, J. L., and Mansuy, D. (1996) *J. Chem. Soc., Perkin Trans. 1*, 645−648.
31. Renodon, A., Boucher, J. L., Sari, M. A., Delaforge, M., Ouazzani, J., and Mansuy, D. (1997) *Biochem. Pharmacol. 54*, 1109−1114.
32. Wu, C., Zhang, J., Abu-Soud, H., Ghosh, D. K., and Stuehr, D. J. (1996) *Biochem. Biophys. Res. Commun. 222*, 439−444.
33. Reczkowski, R. S., and Ash, D. E. (1992) *J. Am. Chem. Soc. 114*, 10992−10994.
34. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem. 126*, 131−138.
35. Cavalli, R. C., Burke, C. J., Kawamoto, S., Soprano, D. R., and Ash, D. E. (1994) *Biochemistry 33*, 10652−10657.
36. Bailey, D. M., DeGrazia, C. G., Lape, H. E., Frering, R., Fort, D., and Skulan, T. (1973) *J. Med. Chem. 16*, 1561−1569.
37. Belzecki, C., Hintze, B., and Kwiatowska, S. (1970) *J. Chem. Soc., Chem. Commun.*, 806−807.
38. DeNicola, A., Einhorn, J., and Luche, J. L. (1992) *Tetrahedron Lett. 33*, 6461−6464.
39. Grant, S. K., Green, B. G., Stiffey-Wilusz, J., Durette, P. L., Shah, S. K., and Kozarich, J. W. (1998) *Biochemistry 37*, 4174−4180.
40. Moore, W. M., Webber, R. K., Jerome, G. M., Tjoeng, F. S., Misko, T. P., and Currie, M. G. (1994) *J. Med. Chem. 37*, 3886−3888.
41. Babu, B. R., Frey, C., and Griffith, O. W. (1999) *J. Biol. Chem. 274*, 25218−25226.
42. Zhang, H. Q., Fast, W., Marletta, M. A., Martasek, P., and Silverman, R. B. (1997) *J. Med. Chem. 40*, 3869−3870.
43. Komori, Y., Wallace, G. C., and Fukuto, J. M. (1994) *Arch. Biochem. Biophys. 315*, 213-218.
44. Furfine, E. S., Harmon, M. F., Paith, J. E., Knowles, R. G., Salter, M., Kiff, R. J., Duffy, C., Hazelwood, R., Oplinger, J. A., and Garvey, E. P. (1994) *J. Biol. Chem. 269*, 26677−26683.
45. Narayanan, K., Spack, L., McMillan, K., Kilbourn, R. G., Hayward, M. A., Masters, B. S. S., and Griffith, O. W. (1995) *J. Biol. Chem. 270*, 11103−11110.
46. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) *Science 279*, 2121−2126.
47. Raman, C. S., Li, H. Y., Martasek, P., Kral, V., Masters, B. S. S., and Poulos, T. L. (1998) *Cell 95*, 939−950.
48. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999) *Nat. Struct. Biol. 6*, 233−242.
49. Häfelinger, G., and Kuske, F. K. H. (1991) in *The Chemistry of Amidines and Imidates* (Patai, S., and Rappoport, Z., Eds.) pp 75−79, John Wiley and Sons Ltd., New York.
50. Taylor, P. J., and Wait, A. R. (1986) *J. Chem. Soc., Perkin Trans. 2*, 1765−1770.
51. Everett, S. A., Dennis, M. F., Patel, K. B., Stratford, M. R. L., and Wardman, P. (1996) *Biochem. J. 317*, 17−21.
52. Frey, C., Narayanan, K., McMillan, K., Spack, L., Gross, S. S., Masters, B. S. S., and Griffith, O. W. (1994) *J. Biol. Chem. 269*, 26083−26091.
53. Feldman, P. L., Chi, S., Sennequier, N., and Stuehr, D. J. (1996) *Bioorg. Med. Chem. Lett. 6*, 111−114.
54. Cox, J. D., Kim, N. N., Traish, A. M., and Christianson, D. W. (1999) *Nat. Struct. Biol. 6*, 1043−1047.
55. Reczkowski, R. S., and Ash, D. E. (1994) *Arch. Biochem. Biophys. 312*, 31−37.

BI992992V