Brief Articles

Synthesis and Evaluation of New Sulfur-Containing L-Arginine-Derived Inhibitors of Nitric Oxide Synthase

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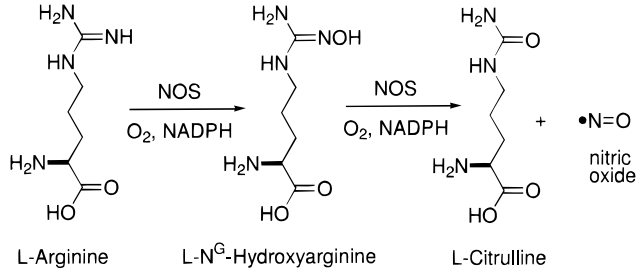
A series of compounds (**7**, **8**, **10**−**17**, **23**) containing new functional groups derived by the combination of the substrate, intermediate, product, and known inhibitors of nitric oxide synthase (NOS) were prepared and evaluated against NOS. While none of the compounds assayed acted as a nitric oxide-producing substrate, the sulfur-containing arginine derivatives **10**−**12** were competitive inhibitors of iNOS with K_i's of 202, 7, and 58 μM, respectively. Compound **11** demonstrated the greatest potency against NOS-mediated citrulline formation for each of the three isoforms with IC_{50}'s of 6.7, 19.7, and 13 μM for nNOS, eNOS, and iNOS, respectively. Compounds **10**−**12** each demonstrated a slight selectivity for inhibition of the neuronal isoform compared to the endothelial and inducible isoforms. These compounds also influenced the NADPH oxidase activity and heme iron spin state in a manner similar to structurally related compounds. Compound **10**, a thiocarbonyl-containing compound, decreased the NADPH oxidase activity of the enzyme (EC_{50} = 190 μM) and shifted the heme iron spin state toward a low-spin configuration, similar to that of L-thiocitrulline. Compounds **11** and **12**, *S*-alkylthiocitrulline derivatives, decreased the NADPH oxidase activity of the enzyme (EC_{50} = 6.6 and 180 μM, respectively) and shifted the heme iron spin state toward a high-spin configuration, similar to that of L-*S*-methylisothiocitrulline. Carbonyl-containing amino acid (**7**, **8**, **23**) and non-amino acid (**13**−**17**) analogues did not interact well with the enzyme.

Introduction

Nitric oxide (NO) plays various roles in a number of physiological processes.[1] The biochemical production of nitric oxide results from the stepwise oxidation of the terminal guanidino group of L-arginine to initially produce L-N^G-hydroxyarginine followed by a second oxidation to form nitric oxide and L-citrulline (Scheme 1).[2] The nitric oxide synthases (NOS) catalyze this conversion that requires 2 equiv of molecular oxygen and 1.5 equiv of reduced nicotine adenine dinucleotide phosphate (NADPH) as cosubstrates (Scheme 1).[3] Three distinct mammalian NOS isoforms exist: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed, and inducible NOS (iNOS).[3] All three isoforms require bound calmodulin for activity and (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4B), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and iron protoporphorin IX (heme) as cofactors.[3] Each isoform contains an N-terminal oxygenase domain with binding sites for L-arginine, H_4B, and the heme cofactor and a linked C-terminal reductase domain containing NADPH, FAD, FMN, and calmodulin binding sites.[3] Recent X-ray crystallographic structures of the iNOS oxygenase domain provide detailed active site structural information.[4,5]

Scheme 1



Much of the enzymology of NOS and the pharmacological roles of NO have been determined by experiments using various L-arginine-based NOS active site inhibitors including L-N^G-methylarginine (**1**),[6] L-N^G-nitroarginine (**2**) and its methyl ester[7] L-N^G-aminoarginine (**3**),[8] and L-(iminoethyl)lysine (**4**).[9] Sulfur-contain-

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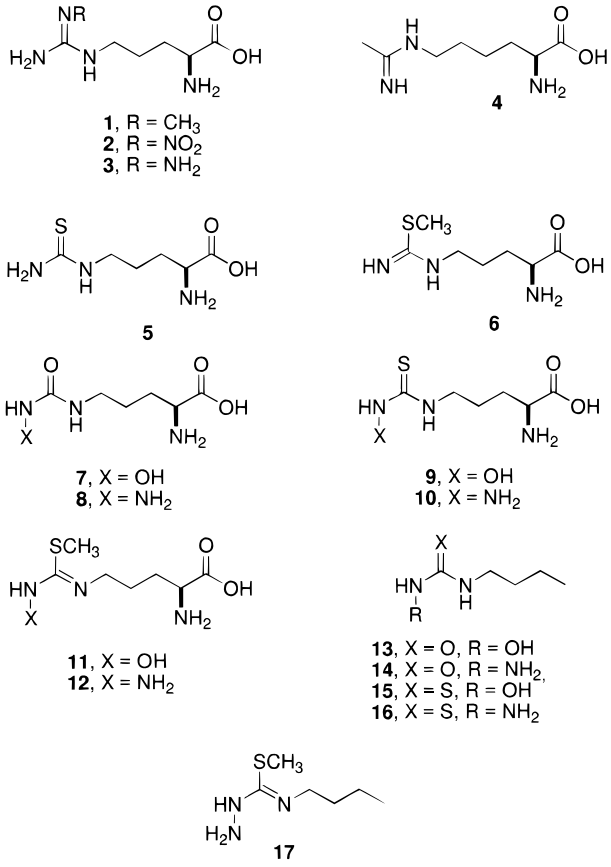
Chart 1



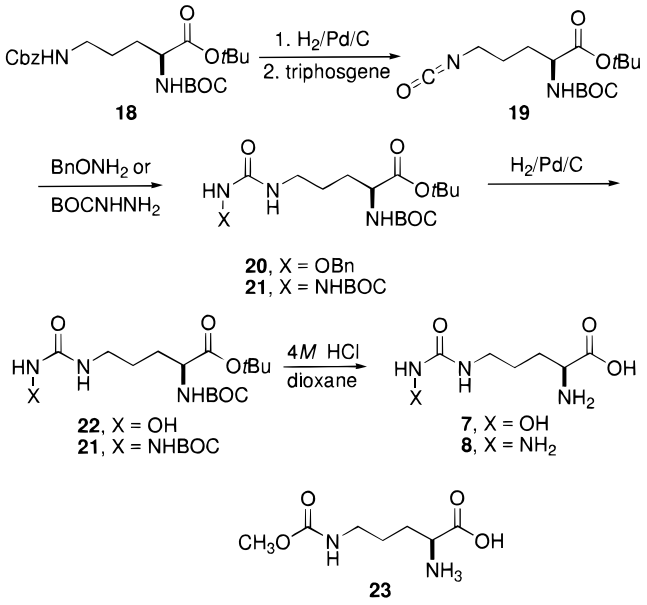
ing L-arginine derivatives, L-thiocitrulline (**5**) and L-*S*-alkylisothiocitrullines, such as **6**, also exhibit potent reversible inhibition of NOS.[10] The examination of new L-arginine derivatives remains a useful approach toward the identification of new functional groups capable of interaction with nitric oxide synthase. We describe the syntheses and biochemical evaluation with NOS of L-arginine derivatives **7**−**12**, which represent straightforward structural combinations of L-arginine, L-N^G-hydroxyarginine, L-citrulline, and known NOS inhibitors (**3**, **5**, **6**). In addition, compounds **13**−**17**, which contain the nonfunctionalized *n*-butyl group, were prepared and evaluated. (See Chart 1 for structures of compounds **1**−**17**.)

Results and Discussion

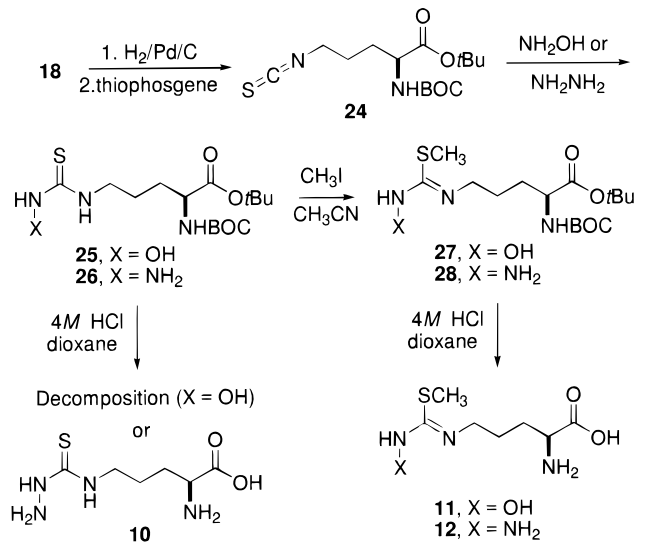
L-Arginine derivatives **7** and **8** were prepared from compound **18**,[11] synthesized in two steps from commercially available N^{δ}-Cbz-L-ornithine (Cbz, benzyloxycarbonyl). Hydrogenolysis of **18** followed by treatment of the resulting amine with triphosgene produced isocyanate **19** (Scheme 2), which was condensed without further purification with *O*-benzylhydroxylamine or *tert*-butyl carbazate to give **20** and **21** in 48% and 87% yields, respectively. Hydrogenolysis of **20** formed **22** in 94% yield, and acidic deprotection of **22** and **21** produced the L-arginine analogues **7** and **8** (67% and 78% yields, respectively).[12] The methyl carbamate **23**, isolated after acid deprotection, formed as a side product during the treatment of isocyanate **19** with a methanolic hydrazine solution.

The sulfur-containing L-arginine analogues **10**−**12** were also prepared from **18** (Scheme 3). Hydrogenolysis

Scheme 2



Scheme 3



of **18** followed by treatment of the resulting amine with thiophosgene afforded isothiocyanate **24** in 90% yield, which could be easily purified and characterized (Scheme 3). Condensation of **24** with hydroxylamine or hydrazine produced **25** and **26**, protected versions of the sulfur-containing L-arginine derivatives **9** and **10** in 66% and 63% yields, respectively. While the acid deprotection of **26** cleanly formed the desired compound **10**, the acidic or thermal deprotection of **25** resulted in the decomposition of this compound into a number of products. Control experiments demonstrated that this functional group is unstable to the conditions required for protecting group removal. Exposure of *N*-hydroxy-*N*′-*n*-butylthiohydroxyurea (**15**), a stable non-amino acid thiohydroxyurea, to 4 M HCl in dioxane resulted in the formation of a large number of products including *n*-butylthiourea, *n*-butylurea, and *n*-butyl isothiocyanate as determined by thin-layer chromatography and gas chromatography/mass spectrometry. Treatment of the thiocarbonyl-containing compounds **25** and **26** with methyl iodide produced the *S*-methylisothioureas **27** and **28**, and deprotection of

Table 1. Interaction of Sulfur-Containing L-Arginine Derivatives **10**−**12** with NOS

	IC_{50} (μM)			K_i, iNOS	NADPH oxidase	iron spin
compd	nNOS	eNOS	iNOS	(μM)	EC_{50} (μM)	state shift[a]
10	1621	3065	3713	202	190	LS
11	6.7	19.7	13	7	6.6	HS
12	327	752	320	58	180	HS

[a] LS, low-spin shift; HS, high-spin shift.

these compounds gave the desired L-arginine derivatives **11** and **12** (Scheme 3). The ability of compounds **26**−**28** to undergo acidic deprotection without decomposition further highlights the instability of the *N*-hydroxythiourea group to these reaction conditions.

Compounds **13**−**17** were prepared by the addition of hydroxylamine or hydrazine to commercially available *n*-butyl isocyanate or *n*-butyl isothiocyanate in good yields. Alkylation of **16** with methyl iodide formed the *S*-methylisothiourea **17**.

Compounds containing an NH-OH group (**7**, **11**, **13**, **15**) were expected to act as potential nitric oxide-producing substrates. The oxyhemoglobin (Fe^{2+}-O_2) oxidation of *N*-hydroxyureas to nitroxyl radicals, which decompose with nitric oxide formation, supports the possibility of nitric oxide synthase-catalyzed nitric oxide formation from *N*-hydroxyureas such as **7** and **13**.[13] This oxidation corresponds to the proposed initial one-electron oxidation of *N*-hydroxyarginine by NOS (Fe^{2+}-O_2) in the conversion of *N*-hydroxyarginine to nitric oxide and L-citrulline.[1] Despite the presence of the NH-OH group in a number of these compounds, none of the synthetic L-arginine derivatives (**7**, **8**, **10**−**12**, **23**) or *n*-butyl compounds (**13**−**17**) supported nitrite synthesis in the presence of iNOS. Nitrite identification provides evidence for nitric oxide formation, and these results demonstrate the inability of these compounds to act as nitric oxide-producing substrates of iNOS.

The sulfur-containing L-arginine analogues **10**−**12** displayed the greatest inhibition of iNOS-mediated nitrite formation at two concentrations (1 and 10 mM) in the presence of L-arginine (0.1 mM). The other amino acids (**7**, **8**, **23**) partially inhibited nitrite formation only at the higher concentration (10 mM), while the *n*-butyl derivatives either did not inhibit nitrite production at both inhibitor concentrations (**13**, **14**, **16**) or only inhibited it at 10 mM (**15**, **17**). While many potent non-amino acid inhibitors of NOS exist, these results indicate that the structural substitution of the amino acid group with the hydrophilic *n*-butyl group in these compounds could not be tolerated.

On the basis of these preliminary results, further kinetic analysis demonstrated that **10**−**12** inhibited iNOS in a competitive manner with K_i values of 202, 7, and 58 μM, respectively (Table 1). In comparison, the K_i values for iNOS inhibition of the structurally similar thiocitrulline (**5**) and *S*-methylisothiocitrulline (**6**) have been determined as 9 and 2.2 μM, respectively.[10,14] While 1-amino-*S*-methylisothiourea has recently been reported to irreversibly and selectively inhibit neuronal NOS, the structurally similar **12** did not exhibit either time- or concentration-dependent inactivation of iNOS.[15]

Table 1 also summarizes the ability of the sulfur-containing L-arginine derivatives **10**−**12** to inhibit citrulline production in the presence of the three isoforms of NOS and L-arginine. Compound **11** demonstrated the greatest potency against NOS-mediated citrulline formation for each of the three isoforms with IC_{50}'s of 6.7, 19.7, and 13 μM for nNOS, eNOS, and iNOS, respectively (Table 1). The IC_{50} values of **10**−**12** with the inducible isoform correspond in order of potency to the measured K_i values of these compounds with iNOS. In general, compounds **10**−**12** demonstrated a slight selectivity (1.9−2.9-fold, determined as a simple ratio of the IC_{50} values) toward the neuronal isoform over both the endothelial and inducible isoforms. Compound **12** inhibited nNOS and iNOS to a similar extent (Table 1).

Binding of substrates or inhibitors to the iNOS active site generally alters the NADPH oxidase activity of the enzyme.[16] The change in NADPH oxidation rates reflects the strength of the interaction between the compound and enzyme and also provides a method of estimating binding constants for analogues that occupy the substrate binding site but do not serve as substrates.[16] All of the synthetic analogues decreased the NADPH oxidase activity of iNOS with the exception of **7**, **13**, **14**, and **23**, which showed no effect on this activity. The sulfur-containing amino acids **10**−**12** again displayed the greatest interaction with the enzyme by decreasing the NAPDH oxidase activity at the lowest concentrations (EC_{50} values 190, 6.6, and 180 μM, respectively, Table 1). The EC_{50} values corresponded well to the K_i values determined for the inhibitors **10**−**11** but not for **12**. In comparison, the structurally similar inhibitors **5** and **6** also decrease the NADPH oxidase activity of iNOS.[16]

Characteristic changes in the visible spectrum of iNOS, indicative of a shift in the spin state equilibrium of the enzyme's heme iron, typically occur upon binding of either substrates or inhibitors.[17] Low-spin shifts of the iron spin state indicate the formation of a hexacoordinate heme iron possibly through inhibitor complexation and displacement of bound substrate from the active site.[17] Visible spectrophotometric experiments indicated that the thiocarbonyl-containing compound **10** shifted the enzyme's heme iron spin state toward a low-spin configuration (Table 1). These results are consistent with previous results for other thiocarbonyl-containing compounds, thiocitrulline (**5**), and thiourea.[14,16] The *S*-methylisothiourea derivatives **11** and **12** produced a high spin shift of the enzyme's heme iron similar to the known inhibitors *S*-methylthiocitrulline (**6**) and *S*-ethylisothiourea (Table 1).[16,18]

The recent NOS oxygenase−inhibitor X-ray structures provide some explanations regarding the interactions of these and other compounds with the enzyme.[4,5] From these studies, the mechanism-based inactivator *N*-aminoguanidine directly interacts with the carboxylate oxygen of Glu371 through hydrogen bonds to the *N*-amino group and one guanidino nitrogen, which place the other guanidino nitrogen near the iron heme site.[4] These results identify Glu371 as a critical binding point for both substrates and inhibitors of the enzyme and suggest the interaction of L-arginine with this group through hydrogen bonds from the bridging δ-nitrogen and one of the terminal guanidino nitrogens.[4] Similar results with L-arginine and thiocitrulline also indicate the importance of Glu371 as a binding point.[5]

By placing the L-amino acid derivatives from this

study in an arrangement capable of forming two hydrogen bonds to Glu371, some explanations regarding their activity can be made. Compound **11**, which was suggested as a possible substrate, can form two hydrogen bonds to Glu371 through the bridging nitrogen and the hydroxylamine group. This alignment places the *S*-methyl group and not the hydroxylamine group required for NO synthesis toward the catalytically active heme group, explaining the inability of this compound to support NO synthesis. Formation of two hydrogen bonds to Glu371 from the bridging nitrogen and the *N*-amino group of **12** also places the *S*-methyl group toward the heme site. Compound **10** and thiocitrulline (**5**) may represent molecules capable of simultaneous interaction with the heme iron and Glu371 of NOS. Formation of two hydrogen bonds to Glu371 with **10** must occur through the bridging nitrogen and the *N*-amino group directing the thiocarbonyl group toward the heme site. The low-spin shift observed upon binding of **10** and **5** to iNOS may indicate the direct interaction of the thiocarbonyl group with the heme iron. These results in combination with the X-ray crystallographic studies with aminoguanidine indicate that while the nonreactive Glu371 guanidinium binding site possesses some steric tolerance, the addition of atomic groups at this position of potential substrates or inhibitors generally decreases the interaction of these compounds with the enzyme.

Conclusion

A series of L-arginine derivatives containing new functional groups derived by the combination of the substrate, intermediate, product, and known inhibitors of NOS were prepared and evaluated against NOS. While none of the compounds assayed acted as NO-producing substrates, the sulfur-containing arginine derivatives **10**−**12** were competitive inhibitors of iNOS. The *S*-methyl-*N*-hydroxyisothiourea (**11**) demonstrated the greatest potency against NOS-mediated citrulline formation for each of the three NOS isoforms. Compounds **10**−**12** each displayed a slight selectivity for the neuronal isoform compared to the endothelial and inducible isoforms. These compounds also influenced the NADPH oxidase activity and heme iron spin state in a manner similar to structurally related compounds. Carbonyl-containing amino acid (**7**, **8**, **23**) and non-amino acid (**13**−**17**) analogues did not interact well with the enzyme.

Experimental Section

Chemistry−**General.** Melting points were determined on a Mel-Temp apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F-254 precoated plates obtained from E. Merck. Flash chromatography was performed on Mallinckrodt silica gel 60 (230−400 mesh). Optical rotations were taken on a Rudolph Autopol IV automatic polarimeter. Proton NMR spectra were taken in commercial deuterated solvents on a Varian VXR 200 multinuclear spectrometer with all chemical shifts being reported in δ scale in parts per million from Me_4Si. Carbon-13 NMR spectra were taken on a Varian VXR 200 multinuclear spectrometer (50 MHz). Infrared spectra were obtained on a Perkin-Elmer 1600 Series FTIR spectrometer. Analytical and preparative high-pressure liquid chromatographies were performed on a Shimadzu LC-10AT liquid chromatography system with a SPD-10AV UV/vis detector. Organic solvents were distilled from a drying agent prior to use. Commercially available reagents were used without further purification.

α-*N*-*tert*-Butoxycarbonyl-ω-*N*-hydroxy(*O*-benzyl)-L-citrulline *tert*-Butyl Ester (20). A mixture of **18**[18] (0.5875 g, 1.44 mmol) and 10% Pd−C (60 mg) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 12 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated under vacuum to give an oil. This oil was dissolved in CH_2Cl_2 (20 mL) and added to a mixture of Na_2CO_3 (0.3053 g, 2.88 mmol), H_2O (20 mL), CH_2Cl_2 (10 mL), and triphosgene (0.1566 g, 0.528 mmol). This mixture was vigorously stirred at 23 °C and after 1 h was added to a solution of *O*-benzylhydroxylamine (0.5818 g, 2.88 mmol) in MeOH (10 mL). The organic phase was separated, and the aqueous phase was extracted with 3 × 10 mL of CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The crude product was purified by flash chromatography (1:1 EtOAc:pentane) to afford **20** (0.2849 g, 48%) as a clear, colorless syrup: R_f 0.37 (1:1 EtOAc:pentane); $[\alpha]^{20}_D$ −12.59 (c = 1.85, EtOH); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.35 (s, 5H), 7.13 (br s, 1H), 5.63 (t, 1H, J = 4 Hz), 5.06 (d, 1H, J = 8 Hz), 4.75 (s, 2H), 4.13 (br m, 1H), 3.16 (br q, 2H, J = 6 Hz), 1.54−1.85 (br m, 4H), 1.43 (s, 9H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.5, 159.9, 155.1, 135.3, 128.9, 128.4, 128.3, 81.5, 79.2, 78.2, 53.4, 38.7, 29.6, 28.0, 27.7, 25.5; IR ($CDCl_3$) 3439, 3347, 2980, 1738−1674, 1538−1482 cm^{-1}; LRMS (FAB) m/z 438 $(M + H)^+$.

α-*N*-*tert*-Butoxycarbonyl-ω-*N*-hydroxy-L-citrulline *tert*-Butyl Ester (22). A mixture of **20** (0.2849 g, 0.70 mmol) and 10% Pd−C (30 mg) in MeOH (20 mL) was stirred vigorously under 1 atm of H_2 for 3 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated in vacuo. The crude product was purified by flash chromatography (3:1 EtOAc:pentane) to give **22** (0.2094 g, 94%) as a clear, colorless foam: R_f 0.22 (3:1 EtOAc:pentane): $[\alpha]^{20}_D$ −14.13 (c = 0.98, EtOH); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.41−8.44 (br, 1H), 7.29 (br s, 1H), 6.16 (br, 1H), 5.29 (d, 1H, J = 8 Hz), 4.08 (br m, 1H), 3.23 (br m, 2H), 1.56−1.74 (br m, 4H), 1.42 (s, 9H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.9, 162.3, 155.6, 82.0, 79.7, 53.8, 39.0, 29.9, 28.2, 27.9, 25.8; IR ($CDCl_3$) 3435, 3350 (br), 2982, 1722−1674, 1538, 1505 cm^{-1}; LRMS (FAB) m/z 348 $(M + H)^+$.

ω-*N*-Hydroxy-L-citrulline Trifluoroacetate (7). A solution of **22** (0.2094 g, 0.66 mmol) in HCl in dioxane (4 M, 10 mL; Aldrich) was stirred at 23 °C for 20 h under an argon atmosphere. The mixture was concentrated in vacuo, dissolved in 0.1% TFA (10 mL) solution, passed through a 6-mL Supelco LC-18 solid-phase extractor, and lyophilized to yield **6** (0.1341 g, 67%) as a white solid: $[\alpha]^{20}_D$ +9.21 (c = 0.63, H_2O); ^{1}H NMR (D_2O, 200 MHz) δ 3.92 (t, 1H, J = 5 Hz), 3.05 (t, 2H, J = 6 Hz), 1.77 (m, 2H), 1.47 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) δ 172.6, 163.6, 53.2, 38.5, 27.3, 25.0; IR (film, 3M Type 61 Disposable IR-card) 3370−2849, 1571, 1201, 1139 cm^{-1}; LRMS (FAB) m/z 192 (M^+); HRMS calcd for $C_6H_{14}N_3O_4$ 192.0984, found 192.0984.

δ-Carbomethoxy-L-ornithine (23). For **23**: ^{1}H NMR (D_2O, 200 MHz) δ 3.95 (t, 1H, J = 6 Hz), 3.54 (s, 3H), 3.07 (t, 2H, J = 7 Hz), 1.85 (m, 2H), 1.52 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) δ 172.3, 159.3, 52.9, 52.5, 39.6, 27.1, 24.6; LRMS (FAB) m/z 191 $(M + H)^+$; HRMS calcd for $C_7H_{15}N_2O_4$ 191.1031, found 191.1026.

α-*N*-*tert*-Butoxycarbonyl-ω-*N*-amino(*tert*-butoxycarbonyl)-L-citrulline *tert*-Butyl Ester (21). A solution of *tert*-butyl carbazate (0.6793 g, 5.14 mmol) in CH_2Cl_2 (10 mL) was added to the organic layers resulting from the hydrogenolysis and triphosgene condensation of **18** (1.090 g, 2.57 mmol). After 5 h the mixture was concentrated in vacuo and the crude product was purified by flash chromatography (2:1 EtOAc:pentane) to afford **21** (1.003 g, 87%) as a white solid: mp 60−65 °C; R_f 0.31 (2:1 EtOAc:pentane); $[\alpha]^{20}_D$ + 3.43 (c = 1.08, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.79 (m, 2H), 5.69 (t, 1H, J = 4 Hz), 5.19 (d, 1H, J = 8 Hz), 4.08 (br m, 1H), 3.19 (q, 2H, J = 6 Hz), 1.17−1.72 (m, 31H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.7, 159.0, 156.3, 155.4, 81.5, 81.2, 79.3, 53.7, 39.3, 29.6,

28.1, 27.9, 27.7, 25.9; IR ($CDCl_3$) 3690, 3605, 3439, 2983, 1703−1694 cm^{-1}; LRMS (FAB) *m/z* 447 $(M + H)^+$.

***ω*-*N*-Amino-L-citrulline Trifluoroacetate (8).** Deprotection of **21** (0.2500 g, 0.560 mmol), identical to deprotection of **22**, yielded **8** (0.1832 g, 78%) as a white solid: $[\alpha]^{20}_D$ +10.72 (*c* = 1.79, H_2O); ^1H NMR (D_2O, 200 MHz) *δ* 3.88 (t, 1H, *J* = 5 Hz), 3.07 (t, 2H, *J* = 6 Hz), 1.79 (m, 2H), 1.49 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) *δ* 172.0, 158.2, 52.7, 39.3, 27.2, 24.8; IR (film, 3M Type 61 Disposable IR-card) 3500−2500, 1748, 1658, 1558, 1428 cm^{-1}; LRMS (FAB) *m/z* 191 $(M + H)^+$; HRMS calcd for $C_6H_{15}N_4O_3$ 191.1144, found 191.1146.

α-*N*-*tert*-Butoxycarbonyl-*δ*-*N*-isothiocyanato-L-ornithine *tert*-Butyl Ester (24). A mixture of **18** (0.5018 g, 1.23 mmol) and 10% Pd−C (60 mg) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 12 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated under vacuum to give an oil. This oil was dissolved in CH_2Cl_2 (20 mL) and added to a mixture of $CaCO_3$ (0.3443 g, 3.44 mmol), H_2O (20 mL), CH_2Cl_2 (20 mL), and thiophosgene (0.1977 g, 1.72 mmol). This mixture was vigorously stirred at 23 °C, and after 1 h the organic phase was separated and the aqueous phase extracted with 3 × 10 mL of CH_2Cl_2. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The crude product was purified by flash chromatography (1:7 EtOAc:pentane) to afford **24** (0.3658 g, 90%) as a yellow oil: R_f 0.40 (1:7 EtOAc:pentane); $[\alpha]^{20}_D$ + 12.44 (*c* = 1.35, CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz) *δ* 5.09 (d, 1H, *J* = 7 Hz), 4.15 (br m, 1H), 3.51 (t, 2H, *J* = 6 Hz), 1.65−1.72 (br m, 4H), 1.42 (s, 9H), 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) *δ* 170.9, 155.0, 130.1, 81.8, 79.3, 52.7, 44.3, 29.6, 27.9, 27.6, 25.6; IR ($CDCl_3$) 3434, 2982, 2249, 1737−1694, 1504, 1495 cm^{-1}; LRMS (EI) *m/z* 331 $(M + H)^+$.

α-*N*-*tert*-Butoxycarbonyl-*ω*-*N*-hydroxy-L-thiocitrulline *tert*-Butyl Ester (25). A solution of hydroxylamine (0.1217 g, 3.69 mmol) in MeOH (20 mL) was added to **24** (0.3658 g, 1.10 mmol). The mixture was stirred for 30 min and concentrated in vacuo. The crude product was purified by flash chromatography (1:1 EtOAc:hexanes) to afford **25** (0.3658 g, 66%) as a clear, colorless foam: R_f 0.21 (1:1 EtOAc:hexanes); $[\alpha]^{20}_D$ −10.0 (*c* = 1.20, EtOH); ^1H NMR ($CDCl_3$, 200 MHz) *δ* 8.53 (br s, 1H), 8.20 (br s, 1H), 7.28 (br s, 1H), 5.28 (d, 1H, *J* = 7 Hz), 4.10 (br m, 1H), 3.60 (m, 2H), 1.65−1.70 (br m, 4H), 1.42 (s, 9H), 1.39 (s, 9H); ^{13}C NMR (CD_3OD, 50 MHz) *δ* 182.4, 173.5, 158.0, 82.6, 80.4, 55.6, 44.0, 29.9, 28.7, 28.2, 26.9; IR ($CDCl_3$) 3429−3141, 2980, 2935, 1737−1682, 1504, 1468 cm^{-1}; LRMS (FAB) *m/z* 364 $(M + H)^+$, 386 $(M + Na)^+$.

α-*N*-*tert*-Butoxycarbonyl-*ω*-*N*-amino-L-thiocitrulline *tert*-Butyl Ester (26). A solution of hydrazine (0.1058 g, 3.30 mmol) in MeOH (20 mL) was added to **24** (0.7286 g, 2.20 mmol). The mixture was stirred for 30 min and concentrated in vacuo. The crude product was purified by flash chromatography (1:1 EtOAc:hexanes) to afford **26** (0.5033 g, 63%) as a clear, yellow foam: R_f 0.35 (3:1 EtOAc:hexanes); $[\alpha]^{20}_D$ −10.36 (*c* = 3.00, EtOH); ^1H NMR ($CDCl_3$, 200 MHz) *δ* 7.52 (br s, 1H), 7.45 (br m, 1H), 5.09 (d, 1H, *J* = 8 Hz), 4.17 (br m, 1H), 3.62 (m, 2H), 1.61−1.73 (br m, 4H), 1.43 (s, 9H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) *δ* 181.5, 171.5, 155.2, 81.7, 79.4, 53.4, 43.1, 29.7, 28.0, 27.7, 25.0; IR ($CDCl_3$) 3435, 3400, 3350, 3206−3148, 2979, 2934, 1738−1682, 1538, 1504 cm^{-1}; LRMS (FAB) *m/z* 385 $(M + Na)^+$.

***ω*-*N*-Amino-L-thiocitrulline Trifluoroacetate (10).** Deprotection of **26** (0.5605 g, 1.55 mmol), identical to deprotection of **22**, yielded an oil that was dissolved in methanol and concentrated to give **10** (0.3230 g, 48%) as a yellow solid: $[\alpha]^{20}_D$ +11.85 (*c* = 2.60, H_2O); ^1H NMR (D_2O, 200 MHz) *δ* 4.13 (t, 1H, *J* = 5 Hz), 3.58 (t, 2H, *J* = 6 Hz), 2.01 (m, 2H), 1.79 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) *δ* 182.6, 171.7, 52.5, 43.8, 27.0, 23.6; IR (film, 3M Type 61 Disposable IR-card) 3600−2300, 1734, 1559, 1507 cm^{-1}; LRMS (FAB) *m/z* 207 (M^+); HRMS calcd for $C_6H_{15}N_4O_2S$ 207.0915, found 207.0915. Anal. ($C_6H_{14}N_4SO_2$·1.5HCl·1.7CH_3OH) C, H, N, S.

α-*N*-*tert*-Butoxycarbonyl-*ω*-*N*-hydroxy-*S*-methyl-L-isothiocitrulline *tert*-Butyl Ester (27). Iodomethane (0.0781 g, 0.550 mmol) was added to a solution of **25** (0.100 g, 0.275 mmol) in acetonitrile (15 mL). The mixture was stirred for 2 h and then concentrated in vacuo. The crude product was purified by flash chromatography (1:1 EtOAc:hexanes) to afford **27** (0.0742 g, 71%) as a yellow foam: R_f 0.32 (1:1 EtOAc:hexanes); $[\alpha]^{20}_D$ +11.0 (*c* = 0.300, CH_2Cl_2); ^1H NMR ($CDCl_3$, 200 MHz) *δ* 5.32 (t, 1H, *J* = 5 Hz), 5.09 (d, 1H, *J* = 8 Hz), 4.14 (br m, 1H), 3.21 (m, 2H), 2.28 (s, 3H), 1.46−1.81 (br m, 4H), 1.44 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) *δ* 171.6, 155.2, 154.3, 81.8, 79.4, 53.3, 42.3, 29.7, 28.1, 27.7, 26.4, 12.8; IR ($CDCl_3$) 3598, 3427, 3252−3007, 2980, 2934, 2872, 1738−1683, 1602, 1504 cm^{-1}; LRMS (FAB) *m/z* 378 $(M + H)^+$.

***ω*-*N*-Hydroxy-*S*-methyl-L-isothiocitrulline Trifluoroacetate (11).** Deprotection of **27** (0.2602 g, 0.69 mmol), identical to the deprotection of **22**, yielded **11** (0.2160 g, 90%) as a yellow solid: $[\alpha]^{20}_D$ +14.51 (*c* = 1.14, H_2O); ^1H NMR (D_2O, 200 MHz) *δ* 3.90 (t, 1H, *J* = 5 Hz), 3.28 (t, 2H, *J* = 6 Hz), 2.36 (s, 3H), 1.74 (m, 2H), 1.63 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) *δ* 172.2, 167.0, 53.0, 43.7, 27.5, 24.9, 13.5; IR (film, 3M Type 61 Disposable IR-card) 3629−2339, 1734, 1616 cm^{-1}; LRMS (FAB) *m/z* 222 (M^+); HRMS calcd for $C_7H_{16}N_3O_3S$ 222.0912, found 222.0913. Anal. ($C_7H_{15}N_3SO_3$·2HCl·0.25H_2O) C, H, N.

***ω*-*N*-Amino-*S*-methyl-L-isothiocitrulline Trifluoroacetate (12).** Iodomethane (0.2810 g, 1.98 mmol) was added to a solution of **26** (0.3585 g, 0.99 mmol) in acetonitrile (20 mL). The mixture was stirred for 2 h and then concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc:1% TEA) to afford **28** as a yellow foam: R_f 0.17 (EtOAc:1% TEA). The compound was dissolved in HCl in dioxane (4 M, 10 mL; Aldrich) and stirred at 23 °C for 1 h under an argon atmosphere. The mixture was concentrated in vacuo, dissolved in 0.1% TFA (10 mL) solution, passed through a 6-mL Supelco LC-18 solid-phase extractor, and lyophilized to yield **12** (0.2787 g, 63%) as a yellow solid: $[\alpha]^{20}_D$ +12.60 (*c* = 0.70, H_2O); ^1H NMR (D_2O, 200 MHz) *δ* 3.92 (t, 1H, *J* = 6 Hz), 3.28 (t, 2H, *J* = 6 Hz), 2.36 (s, 3H), 1.80 (m, 2H), 1.62 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) *δ* 171.4, 169.5, 52.3, 42.8, 26.7, 24.0, 12.6; IR (film, 3M Type 61 Disposable IR-card) 3600−2300, 1734, 1559 cm^{-1}; LRMS (FAB) *m/z* 221 (M^+); HRMS calcd for $C_7H_{17}N_4O_2S$ 221.1070, found 221.1072. Anal. ($C_7H_{16}N_4SO_2$·2HCl·1H_2O) C, H, N, S.

3-Butyl-1-hydroxyurea (13). To a solution of hydroxylamine (0.9979 g, 30.2 mmol) in MeOH (60 mL) was added butyl isocyanate (1.00 g, 10.1 mmol) at 0 °C. The mixture was stirred for 1 h and then concentrated in vacuo. The crude white solid was purified by flash chromatography (EtOAc) to afford **13** (1.12 g, 84%) as a white solid: mp 127−128 °C; R_f 0.40 (EtOAc); ^1H NMR (CD_3OD, 200 MHz) *δ* 3.07 (t, 2H, *J* = 7 Hz), 1.43−1.19 (m, 4H), 0.83 (t, 3H, *J* = 7 Hz); ^{13}C NMR (CD_3OD, 50 MHz) *δ* 164.4, 40.3, 33.2, 20.9, 14.1; IR (NaCl) 3896−2957, 2927, 2863, 1615, 1563 cm^{-1}; LRMS (FAB) *m/z* 133 $(M + H)^+$. Anal. ($C_5H_{12}N_2O_2$) C, H, N.

4-Butyl-3-semicarbazide (14). Condensation of hydrazine (0.9679 g, 30.2 mmol) with butyl isocyanate (1.00 g, 10.1 mmol) as described for **13** yielded **14** (1.12 g, 84%) as a white solid: mp 50−52 °C; R_f 0.08 (EtOAc); ^1H NMR (CD_3OD, 200 MHz) *δ* 3.05 (t, 2H, *J* = 7 Hz), 1.41−1.19 (m, 4H), 0.83 (t, 3H, *J* = 7 Hz); ^{13}C NMR (CD_3OD, 50 MHz) *δ* 163.1, 40.2, 33.5, 20.9, 14.1; IR ($CDCl_3$) 3407, 2962, 2933, 2875, 1687−1651, 1548−1532 cm^{-1}; LRMS (FAB) *m/z* 132 $(M + H)^+$. Anal. ($C_5H_{13}N_3O$) C, H, N.

3-Butyl-1-hydroxythiourea (15). Condensation of hydroxylamine (0.8580 g, 26.0 mmol) with butyl isothiocyanate (1.00 g, 8.70 mmol) as described for **13** yielded **15** (1.22 g, 95%) as a white solid: mp 99−101 °C dec; R_f 0.30 (1:1 EtOAc:hexanes); ^1H NMR (CD_3OD, 200 MHz) *δ* 3.47 (t, 2H, *J* = 7 Hz), 1.49 (m, 2H), 1.29 (m, 2H), 0.85 (t, 3H, *J* = 7 Hz); ^{13}C NMR (CD_3OD, 50 MHz) *δ* 182.4, 44.4, 32.5, 20.9, 14.2; IR ($CDCl_3$) 3522, 3449, 3420, 2962, 2935, 2875, 1682, 1594, 1532 cm^{-1}; LRMS (FAB) *m/z* 149 $(M + H)^+$. Anal. ($C_5H_{12}N_2OS$) C, H, N, S.

4-Butyl-3-thiosemicarbazide (16). Condensation of hydrazine (0.2785 g, 8.70 mmol) with butyl isothiocyanate (1.00 g, 8.70 mmol) yielded **16** (1.20 g, 94%) as a white solid: mp 70−73 °C; R_f 0.35 (2:1 EtOAc:hexanes); ^1H NMR (CD_3OD, 200

MHz) δ 3.50 (t, 2H, $J = 7$ Hz), 1.52 (m, 2H), 1.33 (m, 2H), 0.89 (t, 3H, $J = 7$ Hz); ^{13}C NMR (CD_3OD, 50 MHz) δ 182.3, 44.4, 32.5, 20.9, 14.2; IR ($CDCl_3$) 3400, 3352, 3201−3153, 2962, 2935, 2875, 1543, 1476, 1468 cm^{-1}; LRMS (FAB) *m*/*z* 148 $(M + H)^+$. Anal. ($C_5H_{13}N_3S$) C, H, N, S.

4-Butyl-*S*-methyl-3-isothiosemicarbazide Hydroiodide (17). Iodomethane (0.4826 g, 3.40 mmol) was added to a solution of **16** (0.5000 g, 3.40 mmol) in acetonitrile (15 mL). The mixture was stirred for 1 h and then concentrated in vacuo. To this oil was added EtOAc (25 mL), and the precipitate was collected by vacuum filtration yielding **17** (0.7828 g, 80%) as a white solid: mp 92−93 °C; ^{1}H NMR (CD_3OD, 200 MHz) δ 3.33 (t, 2H, $J = 7$ Hz), 2.55 (s, 3H), 1.54 (m, 2H), 1.32 (m, 2H), 0.87 (t, 3H, $J = 7$ Hz); ^{13}C NMR (CD_3OD, 50 MHz) δ 169.9, 44.7, 32.2, 20.6, 14.0, 13.9; IR ($CDCl_3$) 3316−2877, 1648, 1651, 1581 cm^{-1}; LRMS (FAB) *m*/*z* 162 $(M + H)^+$. Anal. ($C_6H_{15}N_3S$) C, H, N, S.

Biochemistry—General. Hemoglobin, myoglobin, dithiothreitol, NADPH, FAD, FMN, L-arginine, manganese superoxide dismutase (SOD), bovine serum albumin (BSA), and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma. Catalase (beef liver) was purchased from Boehringer Mannheim, Germany. (6*R*)-5,6,7,8-Tetrahydrobiopterin was obtained from Schircks, Switzerland. Mouse iNOS with a His_6 tag attached to the C-terminus of the protein was overexpressed in *Escherichia coli* (strain BL21-(DE3)) and purified with a Ni^{2+}-nitrotriacetic acid-Sepharose CL4B column as previously described.[19]

Measurement of Nitrite Production by iNOS. Inhibition of nitric oxide synthesis by the synthetic compounds was assayed by monitoring nitrite production using the Griess assay. iNOS (25 nM) was incubated at 37 °C for 30 min with 1 mM dithiothreitol, 4 μM each of FAD, FMN, and H_4B, 0.1 mM each of NADPH and L-arginine and either or 10 mM of test compound in a 96-well plate with a final volume of 100 μL. Reactions were initiated with the addition of NADPH/L-arginine mixture and were performed in triplicate. After any remaining NADPH was depleted by incubation with lactate dehydrogenase (770 unit/mL) and sodium pyruvate (50 mM) for 15 min at 37 °C, Griess reagent was added, and the absorbance difference between 550 and 650 nm was measured with a microplate reader (THERMOmax, Molecular Devices). Experiments to determine whether any of the synthetic analogues could be utilized by iNOS as substrates were performed using iNOS (150 nM) and substrate analogue (1 mM) in the absence of L-arginine. The other reaction conditions were the same as above, and nitrite production was analyzed by the Griess assay.

IC_{50} Determination of Compounds 10−12. A solution of NADPH (5 μL, 4 mM) was added to a 95-μL reaction mixture containing HEPES buffer (pH 7.4, 20 mM), eNOS (25 nM), nNOS (12.5 nM), or iNOS (2.5 nM), L-arginine containing 0.05 μCi of [^{14}C]-L-arginine (50 μM), FAD (4 μM), FMN (4 μM), DTT (1 mM), tetrahydrobiopterin (4 μM), bovine serum albumin (1 mg/mL), $CaCl_2$ (1 mM), and calmodulin (15 μg/mL), for eNOS and nNOS experiments, and variable inhibitor concentrations to initiate the reaction. After 10 min at room temperature, the reaction was terminated by the addition of HEPES buffer (pH 5.5, 200 μL, 100 mM) containing EDTA (5 mM). The [^{14}C]-L-citrulline was separated by ion-exchange chromatography with the addition of 250-μL aliquots of the reaction mixture on Dowex 50W X-8 (Na^+ form, 1.0-mL bed volume) cation-exchange resin and rinsed with 0.75-, 1.0-, and 1.0-mL aliquots of distilled water. Radioactivity was quantified with a liquid scintillation counter. Assays were performed in duplicate, average standard deviations were 5% or less, and the production of [^{14}C]-L-citrulline was linear over the reaction time for each isoform.

NADPH Oxidase Activity. The rate of iNOS NADPH oxidation was determined by monitoring the decrease in absorbance at 340 nm over time using a microplate reader. Assays were performed at 37 °C in 96-well plates containing 150 μL of 40 mM HEPES buffer, pH 7.4, supplemented with 0.5 μM iNOS, 0.3 mM dithiothreitol, 4 μM each of FAD, FMN, and H_4B, 10 units/mL each of catalase and SOD, various concentrations of synthetic substrate analogues, and 0.1 mM NADPH. Reactions were initiated by the addition of NADPH and performed in triplicate.

Spectral Shift of iNOS by Substrate Analogues. Optical spectra were recorded at room temperature on a Hitachi U-2000 spectrophotometer. After the baseline spectra were recorded, a 300-μL sample containing 1 μM iNOS was transferred to a cuvette and a first spectrum recorded; 5 μL of each substrate analogue (final concentration 1 mM) was added and the second spectrum recorded. A high-spin shift was denoted by a gain in absorbance at 385 nm and a corresponding loss at 420 nm, while a low-spin shift was denoted by the opposite results.

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Supporting Information Available: Details regarding the inhibition of nitrite formation, the determination of K_i values of **10**−**12** (including Lineweaver−Burk double-reciprocal plots), and the NADPH oxidase EC_{50} values and heme iron spin shifts of all synthetic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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