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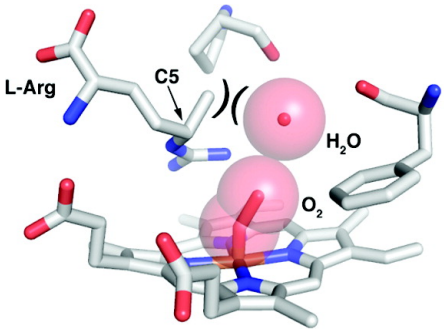
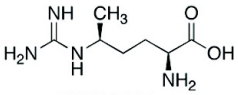
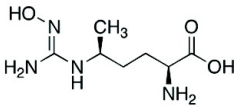




iNOS inhibitor



iNOS substrate

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Design and Synthesis of C5 Methylated L-Arginine Analogues as Active Site Probes for Nitric Oxide Synthase

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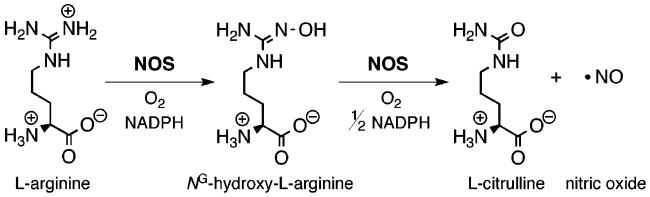
Abstract: The role of nitric oxide (NO) as a biological signaling molecule is well established. NO is produced by the nitric oxide synthases (NOSs, EC 1.14.13.39), a class of heme proteins capable of converting L-arginine to NO and L-citrulline. Despite the large body of knowledge associated with the NOSs, mechanistic details relating to the unique oxidative chemistry performed by these enzymes remain to be fully elucidated. Furthermore, a number of disease states are associated with either the over- or underproduction of NO, making the NOS pathway an attractive target for the development of therapeutics. For these reasons, molecular tools capable of providing mechanistic insights into the production of NO and/or the inhibition of the NOSs remain of interest. We report here the stereospecific synthesis and testing of a number of new L-arginine analogues bearing a minimal substitution, methylation at position 5 of the amino acid side chain (such analogues have not been previously reported). The synthetic approach employed a modified photolysis procedure whereby irradiation of the appropriate diacylperoxide precursors at 254 nm gave access to the required unnatural amino acids in good yields. A heme domain construct of the inducible NOS isoform ($iNOS_{heme}$) was used to assess the binding of each compound to the enzyme active site. The compounds were also investigated as either inhibitors of, or alternate substrates for, the inducible NOS isoform. The results obtained provide new insight into the steric and stereochemical tolerance of the enzyme active site. These findings also further support the role of a conserved active site water molecule previously proposed to be necessary for NOS catalysis.

The nitric oxide synthases (NOSs) are a family of enzymes that catalyze the conversion of L-arginine to L-citrulline and nitric oxide (NO) via the intermediate N^G-hydroxy-L-arginine (Scheme 1).[1,2] Three distinct isoforms of NOS have been identified, products of different genes, with different subcellular localization, regulation, catalytic properties, and inhibitor sensitivity. The human NOSs show 51−57% homology[3] and include two constitutive isoforms, neuronal nitric oxide synthase (nNOS), which generates NO in the CNS[4] and is involved in neurotransmission and long-term potentiation,[5] and endothelial nitric oxide synthase (eNOS), where the product NO is involved in the regulation of smooth muscle relaxation and vascular tone.[6,7] A third, inducible isoform in macrophages (iNOS) is important in immune system defense against pathogens and tumor cells.[8]

Scheme 1. Reaction Catalyzed by NOS



NOS is active as a homodimer with each subunit containing a C-terminal reductase domain (with binding sites for NADPH, FAD, and FMN) and a N-terminal oxygenase domain containing the heme prosthetic group. The substrate, L-arginine, and a redox cofactor, (6*R*)-5,6,7,8 tetrahydro-L-biopterin (H_4B), both bind near the heme center in the oxygenase domain.[9] The overall mechanism of the NOS reaction occurs via the two discreet reactions shown in Scheme 1. In the first, L-arginine undergoes two-electron oxidation (mediated by the redox active H_4B cofactor) with consumption of molecular oxygen to yield N^G-

(1) Griffith, O. W.; Stuehr, D. J. *Annu. Rev. Physiol.* **1995**, *57*, 707−736.
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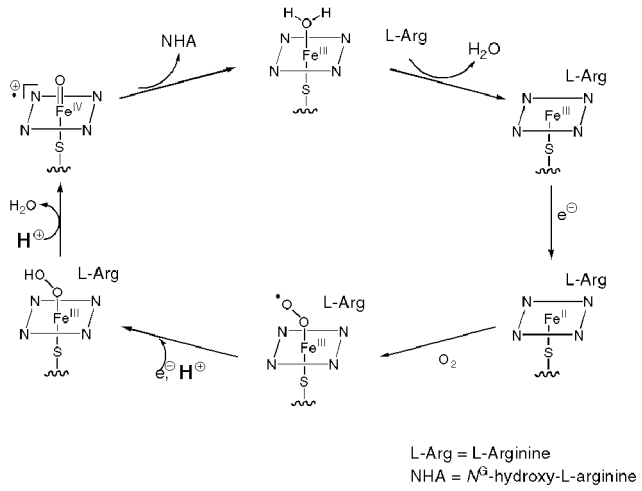
Scheme 2. Oxygen Rebound Mechanism for the Hydroxylation of L-Arginine[a]



[a] Indicated in bold are two mechanistically relevant protons which may be provided via an active site water molecule.

hydroxy-L-arginine. This is proposed to occur via an oxygen-rebound mechanism analogous to that of the cytochrome P450-type enzymes (Scheme 2).[10−12] The second NOS reaction, however, is unique in both its redox chemistry and substrate specificity — there are no other enzymes known to perform one-electron oxidations of N^G-hydroxyguanidines to yield a urea and NO as products. While the mechanistic details of the second half-reaction have been the subject of a number of investigations,[13−19] the precise mechanism of this process remains unclear. One proposal for the second reaction involves a transient tetrahedral intermediate formed by nucleophilic addition of the Fe^{III}-peroxo species (generated as in the first reaction) to N^G-hydroxy-L-arginine.[20] The collapse of this addition complex is most often proposed to proceed along one of two possible pathways yielding L-citrulline and NO, with the concomitant one-electron reduction of H_4B, to return NOS to its resting state (Scheme 3).

While the two half-reactions by which NOS operates are clearly distinct, there are common features. As seen in Schemes 2 and 3, both reactions involve the binding and reduction of oxygen. While a proton source in the first NOS reaction step is indicated, the need for externally supplied protons in the second NOS reaction step is less clear. It has been recently speculated that a specifically oriented water molecule in the NOS active site may serve as such a proton source or act as a "shuttle" by which protons from bulk solvent might be supplied in the first NOS reaction.[21,22] While a structure of the full-length protein is not yet available, extensive crystallographic studies of the

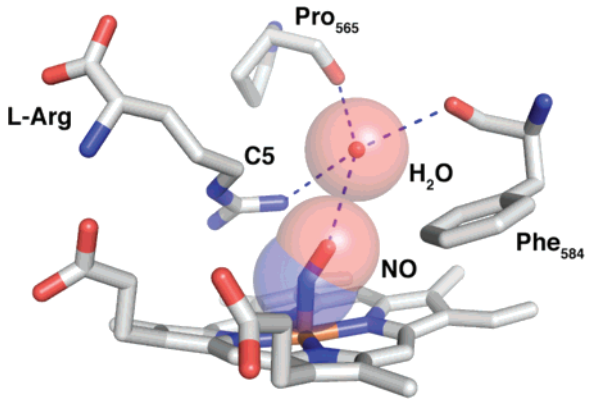
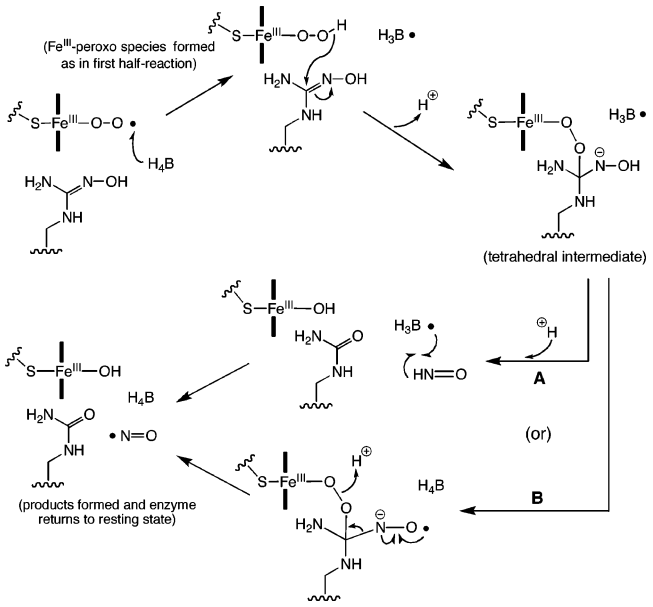


Figure 1. $nNOS_{heme}$ active site crystal structure (PDB ID 2GSK). A water molecule is specifically oriented and within hydrogen-bonding distance of the substrate, L-arginine, three active site residues (Val_{567} not shown for clarity), and NO as the heme ligand (the three active site residues that contribute to the orientation of the active site water molecule are completely conserved for all three NOS isoforms).

Scheme 3. Proposed Mechanisms for the Oxidation of N^G-Hydroxy-L-arginine to L-Citrulline and NO (an Exogenous Proton Source Is Not Required)



NOS oxygenase domain of each isoform have been reported.[21,23−28] These structures consistently show a water molecule in a defined location, held in place by a hydrogen-bonding network with the substrate, active site residues, and the diatomic molecule used as the heme ligand (Figure 1).

These considerations led us to speculate that modifications to the substrate L-arginine at the δ-position (C5, adjacent to the guanidine moiety) could provide useful molecular tools by which to probe the NOS active site and catalytic mechanism. A minimal modification was envisioned whereby either, or both, of the enantiotopic protons at C5 are replaced with a methyl

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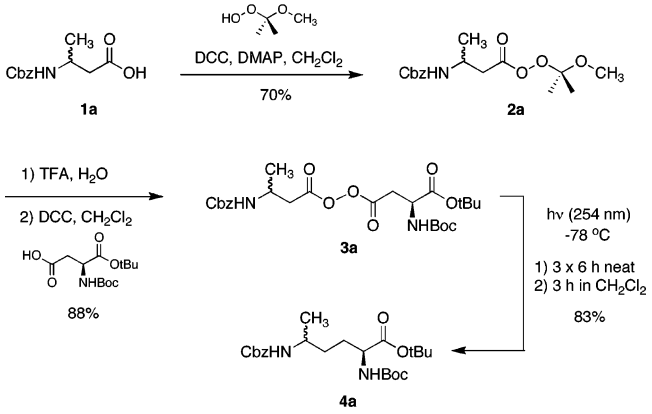
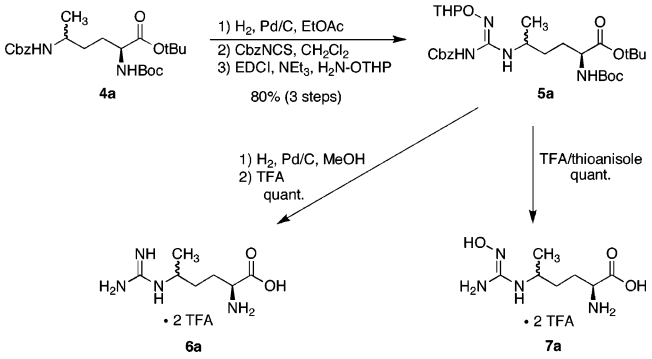
Scheme 4. Synthesis of Orthogonally Protected C5-Methylated L-Ornithine



Scheme 5. Conversion of Orthogonally Protected C5-Methylated L-Ornithine to (5*R/S*)-Methyl-L-arginine and N^G-Hydroxy-(5*R/S*)-methyl-L-arginine



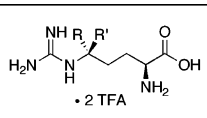
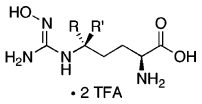
group. Analogues wherein the *pro-S* hydrogen is replaced with a methyl group may experience steric crowding with the active site water molecule. Alternatively, compounds with substitution of the *pro-R* hydrogen may interfere with or prevent binding of heme ligands.

While no syntheses of C5 substituted L-arginine analogues have been described, the preparation of analogues with methyl substitution at positions C2(α), C3(β), and C4(γ) have been reported, albeit as diastereomeric mixtures.[29] The effects of these compounds on purified NOS were not thoroughly investigated, and the synthetic approach utilized is not applicable to the preparation of C5 substituted L-arginine analogues. We here report the stereospecific synthesis of all possible C5 methylated L-arginine analogues and the effects observed when testing these compounds with iNOS.

Results

The synthesis of C5 substituted L-arginines requires access to the appropriately substituted and orthogonally protected L-ornithine precursors. From here, existing methods can be used to install either the guanidine or N^G-hydroxy guanidine moieties.[30] The required orthogonally protected L-ornithines were envisioned to be accessible via the diacylperoxide photolysis method recently described by Vederas and co-workers.[31] Initial investigation of this synthetic route made use of racemic 3-aminobutyric acid as starting material (Scheme 4). Carbamate protection of the amine gave compound **1a** which was coupled with 2-methoxyprop-2-yl hydroperoxide[32] to provide the perester **2a**. Upon treatment with TFA the perester was converted to

Table 1. 5-Substituted L-Arginines and N^G-Hydroxy-L-arginines Prepared

 6a-d	 7a-d
6a. R = CH_3/H, R' = CH_3/H (5*R/S*)	7a. R = CH_3/H, R' = CH_3/H (5*R/S*)
6b. R = CH_3, R' = H (5*R*)	7b. R = CH_3, R' = H (5*R*)
6c. R = H, R' = CH_3 (5*S*)	7c. R = H, R' = CH_3 (5*S*)
6d. R = CH_3, R' = CH_3 -	7d. R = CH_3, R' = CH_3 -

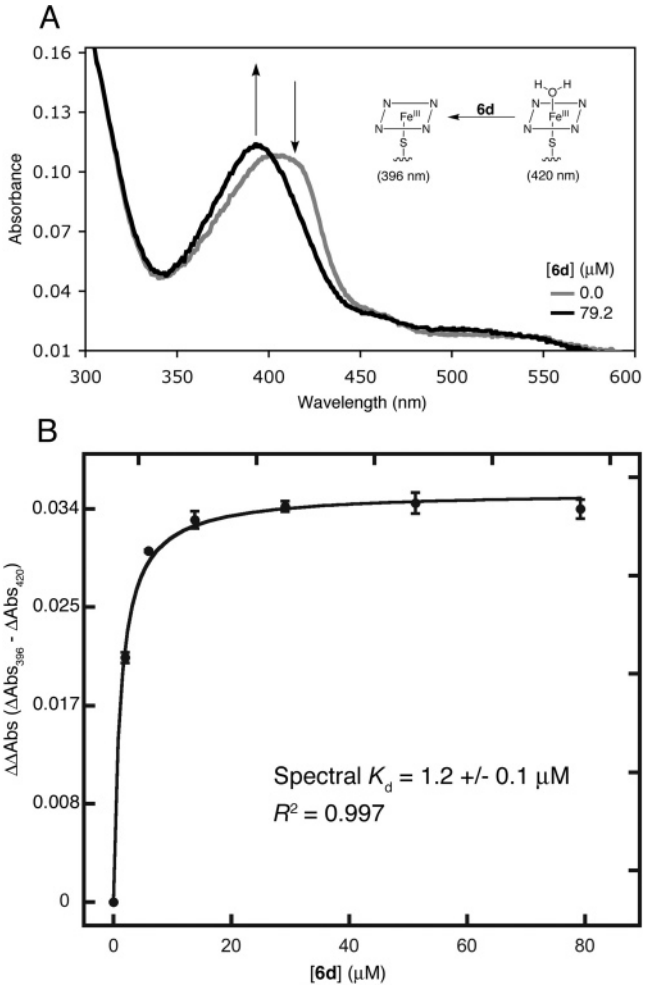


Figure 2. Spectral K_d determination for compound **6d**. (A) Low- to high-spin state transition effected by addition of **6d** to $iNOS_{heme}$. (B) Titration data and fit used in determination of the spectral K_d for **6d**.

(29) *The Biology of Nitric Oxide (4) Enzymology, Biochemistry and Immunology*; Narayanan, K., Frey, C., Griffith, O. W., Eds.; Portland Press: London, 1993.
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Table 2. Spectral K_d Values for **6b**−**d** and **7b**−**d**

compound	spectral K_d[a] (μM)
L-arginine[b]	7.0 ± 0.7 (5.8 ± 1.6)
N^G-hydroxy-L-arginine[b]	5.1 ± 0.4 (1.5 ± 0.3)
(5*R*)-methyl-L-arginine (**6b**)	1.6 ± 0.3
(5*S*)-methyl-L-arginine (**6c**)	165 ± 35
5,5-dimethyl-L-arginine (**6d**)	1.2 ± 0.1
N^G-hydroxy-(5*R*)-methyl-L-arginine (**7b**)	1.8 ± 0.5
N^G-hydroxy-(5*S*)-methyl-L-arginine (**7c**)	270 ± 57
N^G-hydroxy-5,5-dimethyl-L-arginine (**7d**)	2.1 ± 0.5

[a] Spectral K_d values reported for **6b**−**d** and **7b**−**d** are averages obtained from triplicate analysis. [b]Previously reported values given in parentheses (ref 20).

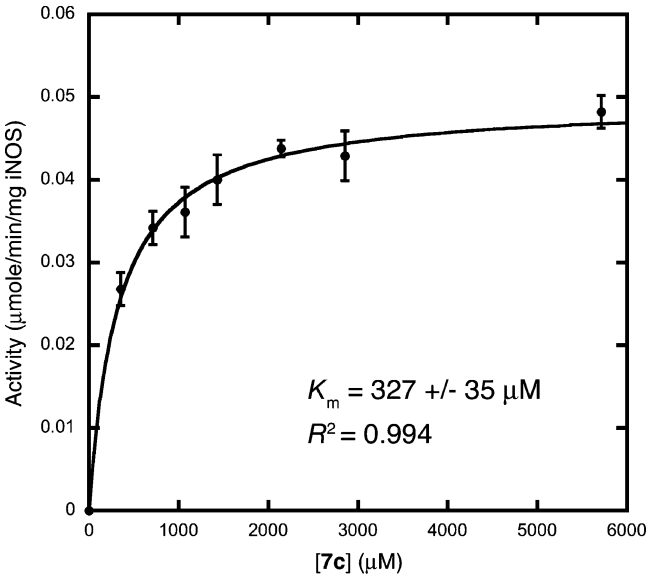

Figure 3. K_m determination for compound **7c**.

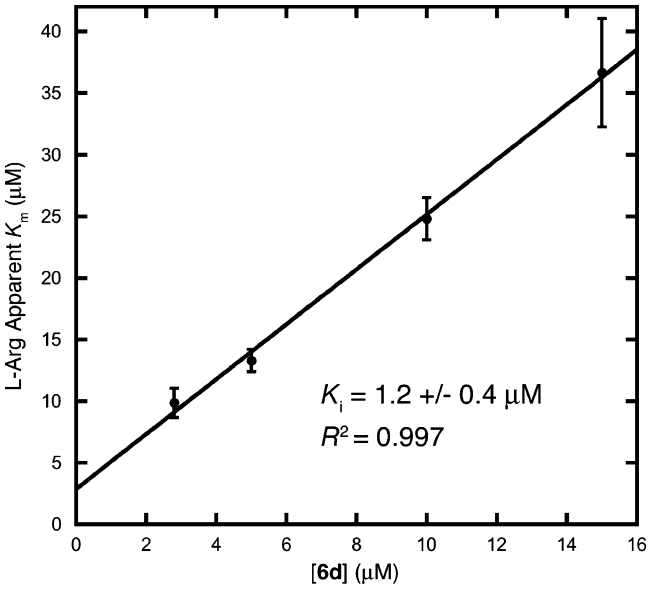

Figure 4. K_i determination for compound **6d**. Apparent K_m for L-arginine plotted as a function of inhibitor concentration.

the peracid followed by coupling with the appropriately protected L-aspartic acid to give diacylperoxide **3a** in good yield. Photolysis of **3a** provided access to the required L-ornithine analogue **4a** with methyl substitution at position C5.

As shown in Scheme 4, the photolytic conversion of diacylperoxide **3a** to the orthogonally protected (5*R*/*S*)-methyl-L-ornithine **4a** was critical to the construction of the amino acid backbone. The reaction conditions described in the literature[31] for this type of conversion, however, provided mixed results (low yields of **4a** requiring extended reaction times of up to 5 days). Optimization of the process led us to a modified protocol whereby the desired photolysis products were reliably obtained in yields of 73−83% and in a much shorter period of time. In the modified protocol, dissolution/evaporation cycles are performed every 6 h allowing for unreacted diacylperoxide to be evenly redistributed. Also of note is the inclusion of a short, solution-phase photolysis step to end the reaction. We found that this did not complicate formation of the desired products (via the presumed radical cage-recombination process) as very little of the undesired homocoupling products were detected. Furthermore, as described in detail in the Experimental Section, the equipment and setup used in this modified photolysis procedure requires no custom glassware or instrumentation; all necessary components are inexpensive or common in the laboratory.

With access to orthogonally protected L-ornithine compounds like **4a**, their conversion to the desired guanidine or N^G-hydroxyguanidines was then performed following literature methods (Scheme 5).[30]

The OTHP protected N^G-hydroxyguanidine **5a** was prepared in a one-pot process by converting the free amine (obtained after hydrogenation of **4a**) to the intermediate thiourea by treatment with CbzNCS. The thiourea was then directly converted to **5a** by treatment with EDCI and H_2N−OTHP. Owing to the reductive instability of OTHP protected N^G-

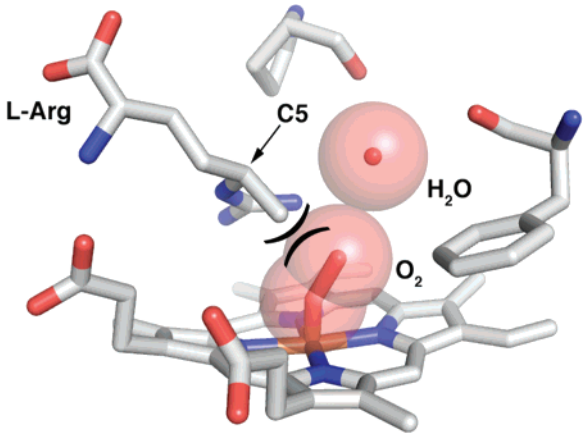

Figure 5. NOS_{heme} active site crystal structure with 5*R*-methyl-L-arginine modeled in place of L-arginine and oxygen in place of NO. The distance between the carbon atom of the methyl substituent in the substrate analogue and the oxygen molecule modeled at the heme center is calculated to be 2.23 Å (within van der Waals contact distance).

Table 3. K_i Values Determined for **6b**−**d**, **7b**, **7d** and K_m Value Determined for **7c**

compound	K_i[a] (μM)
N^G-amino-L-arginine[b]	2.2 ± 0.5 (1.7 ± 0.6)
(5*R*)-methyl-L-arginine (**6b**)	6.3 ± 0.1
(5*S*)-methyl-L-arginine (**6c**)	570 ± 130
5,5-dimethyl-L-arginine (**6d**)	1.2 ± 0.4
N^G-hydroxy-(5*R*)-methyl-L-arginine (**7b**)	3.4 ± 0.6
N^G-hydroxy-(5*S*)-methyl-L-arginine (**7c**)	327 ± 35[c]
N^G-hydroxy-5,5-dimethyl-L-arginine (**7d**)	12.0 ± 1.5

[a] Values reported are averages obtained from triplicate analysis. [b] K_i value determined for N^G-amino-L-arginine as a control; previously reported value given in parentheses.[33] [c] K_m reported.

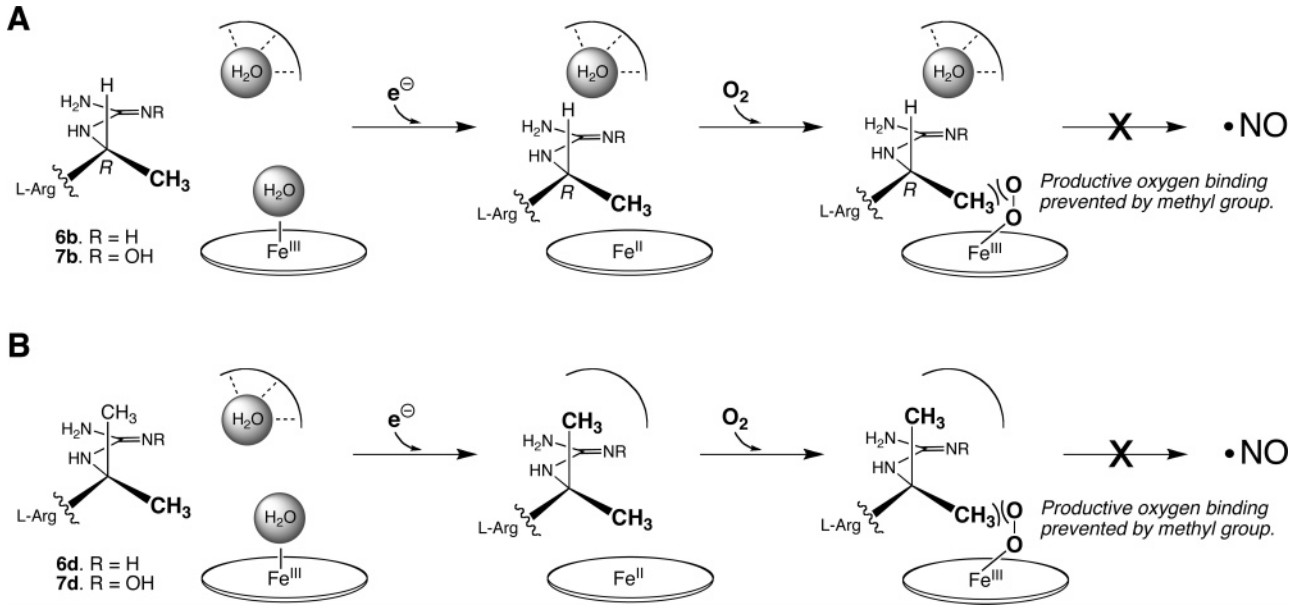

Figure 6. Proposed interactions of (A) **6b**, **7b** and (B) **6d**, **7d** with the iNOS active site. The presence of a methyl substituent in place of the *pro-R* hydrogen may interfere with oxygen binding and prevent catalysis.

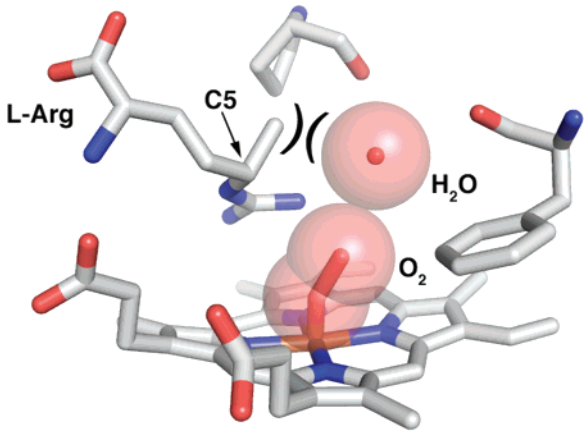

Figure 7. NOS_{heme} active site crystal structure with 5*S*-methyl-L-arginine modeled in place of L-arginine and oxygen in place of NO. The distance between the carbon atom of the methyl substituent in the substrate analogue and the active site water molecule is calculated to be 2.88 Å (within van der Waals contact distance) and may lead to its extrusion.

hydroxyguanidines,[30] compound **5a** could be used as a precursor to both the guanidine **6a** and N^G-hydroxyguanidine **7a** final products.

After establishing the validity of the approach, each diastereomer of 5-methyl-L-arginine and N^G-hydroxy-5-methyl-L-arginine was prepared in pure form from the appropriate enantiomer of 3-aminobutryric acid. The 5,5-dimethyl substituted compounds were prepared using 3-amino-3-methylbutyric acid as starting material (Table 1).

Binding affinities for the active site of iNOS were determined using a previously described spectral binding assay.[20] For this analysis, a truncated construct of iNOS ($iNOS_{heme}$, comprised of amino acids 1−490) was used. This construct has been previously shown to bind heme, H_4B, and substrate(s) in identical fashion to that for the full-length enzyme and is catalytically competent when provided with an exogenous source of electrons such as sodium dithionite.[20] Addition of **6b**−**d** and **7b**−**d** to H_4B-bound $iNOS_{heme}$ induces a low- to high-spin state conversion at the heme center resulting from loss of water (Fe^{III}-aqua → Fe^{III}-unligated) upon binding. This transition involves a concomitant decrease in absorbance at ∼420 nm and an increase at ∼396 nm in the UV−visible spectrum and is depicted for compound **6d** in Figure 2A. The magnitude of the spectral change, ΔΔAbs (where $\Delta\Delta Abs = \Delta Abs_{396} - \Delta Abs_{420}$), is dependent on the concentration of the added analogue. Spectral K_d values for each compound were then determined by fitting the titration data to the saturation binding equation: $\Delta\Delta Abs = (\Delta\Delta Abs_{max} \times [compound])/(K_d + [compound])$ (Figure 2B depicts this analysis for compound **6d**). Table 2 summarizes the results of the spectral binding assay for each of the 5-methylated and 5,5-dimethylated L-arginine and N^G-hydroxy-L-arginine analogues.

Working from the spectral K_d values determined for **6b**−**d** and **7b**−**d**, the ability of each compound to behave as either an inhibitor or substrate for full-length iNOS was next investigated. NOS activity was measured using a spectral assay making use of the NO-induced oxidation of oxymyoglobin to metmyoglobin observable at 405 nm. Initial investigation revealed that one of the six compounds tested, **7c**, led to detectable NO production when added at saturating concentrations (>10× the spectral K_d). The kinetics of NO production with **7c** was further investigated and showed the compound to be a substrate for iNOS. The K_m value for **7c** was determined to be 327 ± 35 μM with a k_{cat} value approximately 35% of that observed for L-arginine (Figure 3).

The five compounds that did not lead to NO production were investigated as competitive inhibitors of iNOS. Figure 4 illustrates the K_i determination as performed for compound **6d**, and Table 3 summarizes the NOS inhibition/NO production assay results for all of compounds **6b**−**d** and **7b**−**d**.

The five compounds displaying NOS inhibition (**6b**−**c**, **7b**, **7d**) were also investigated as time-dependent inactivators of the enzyme. In all cases NOS activity was fully restored upon addition of L-arginine to mixtures of each compound preincubated with iNOS under turnover conditions, indicating no time dependent or irreversible inactivation (data not shown).

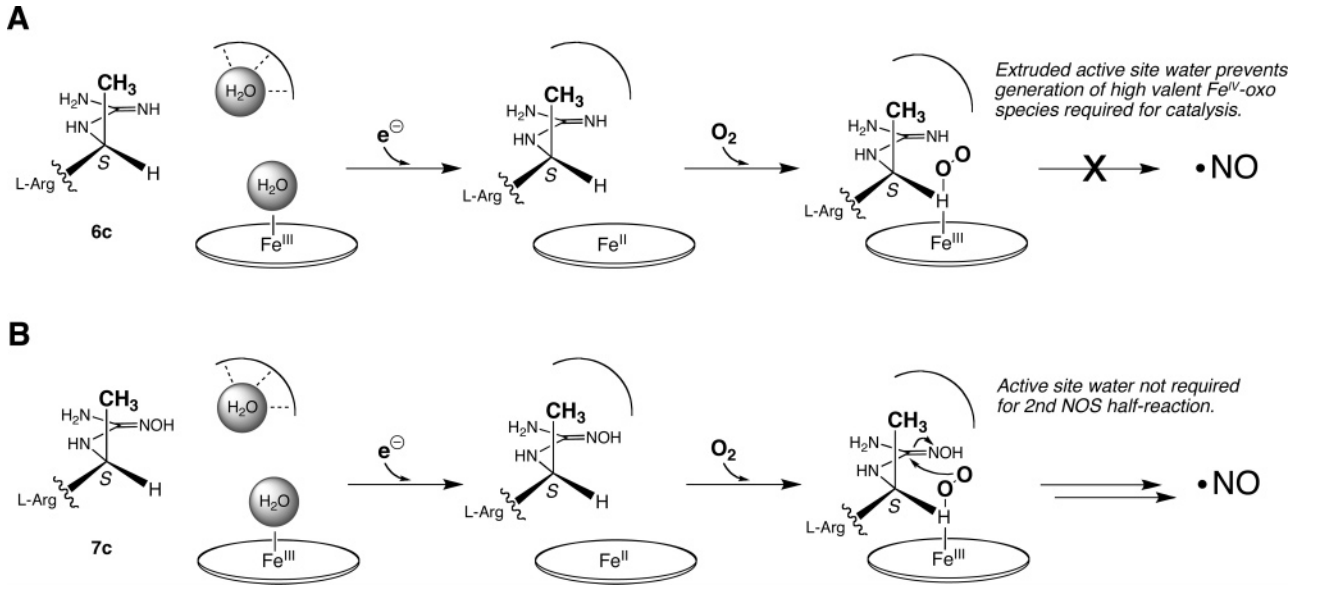

Figure 8. Proposed interactions of compounds **6c** and **7c**. (A) The methyl group in place of the *pro-S* hydrogen leads to extrusion of an active site water molecule required for the first step of the NOS reaction. (B) While bearing a methyl group in place of the *pro-S* hydrogen, the N^G-hydroxylated compound **7c** is converted to NO, suggesting that an active site water molecule is not required for the second step of the NOS reaction.

Discussion

The results obtained with the various C5-methylated analogues of L-arginine and N^G-hydroxy-L-arginine point to a stereochemical preference in the ability of these compounds to bind to the NOS active site with high affinity. In addition, as discussed below, these results also provide further support for the recently proposed role of an active site water molecule in NOS catalysis.[21,22]

The K_i and K_m values obtained for the six analogues tested coincide very well with the spectral K_d values obtained. Compounds **6b**, **6d**, **7b**, and **7d** all bear methyl substitution in the *pro-R* position of C5 in the side chain of L-arginine and N^G-hydroxy-L-arginine and bind with high affinity. Despite the tight binding of **6b**, **6d**, **7b**, and **7d** for iNOS, enzymatic formation of NO was not detected. Substitution of the *pro-R* hydrogen (as in **6b**, **6d**, **7b**, and **7d**) introduces a methyl group that may be positioned above the heme upon binding of the compound to NOS (Figure 5). In order for normal catalysis to proceed in both steps of the NOS reaction (Schemes 2 and 3), oxygen must coordinate to Fe in the heme. The presence of a methyl substituent in this space may block or prevent oxygen from binding in its required orientation and inhibit catalysis (Figure 6).

Methyl substitution in the *pro-R* position (as for **6b**, **6d**, **7b**, and **7d**) appears to have a stabilizing effect on the enzyme−substrate analogue complex as reflected in the low K_d and K_i values. The nature of this stabilization is not clear at this time. We speculate that methyl substitution of the *pro-R* proton facilitates the displacement of the heme iron coordinated water molecule present in the resting state of the enzyme. The overall effect of this on the detailed interactions at the active site awaits structural studies that are underway. However, further support for the stabilizing role of a methyl substituent at the *pro-R* position is suggested by the observation that **6c** and **7c**, each bearing methyl substitution solely in the *pro-S* position of C5, bind much less tightly than the other four analogues tested. Of special interest, however, is the observation that while **6c** is a weak inhibitor, **7c** is a substrate for iNOS. As compounds **6c** and **7c** do not possess a methyl substituent in the *pro-R* position, oxygen binding might not be affected in the manner proposed for the other four analogues (Figure 6). While the affinity of **6c** and **7c** for $iNOS_{heme}$ is relatively weak, we speculate that, upon binding, both compounds lead to extrusion of the hydrogen-bonded water molecule from the active site (Figure 7).[21,23,28]

Furthermore, the observation that the N^G-hydroxylated compound **7c** leads to NO formation while the non-hydroxylated compound **6c** does not suggests that the proposed hydrogen-bonded active site water molecule is required only for the first step of the NOS reaction (conversion of L-arginine to N^G-hydroxy-L-arginine) (Figure 8). As described earlier, an active site water molecule is proposed to be involved in the generation of the high valent Fe^{IV}-oxo species required for the hydroxylation of L-arginine to N^G-hydroxy-L-arginine (Scheme 2). The same process need not be invoked in a mechanistic explanation for the conversion of N^G-hydroxy-L-arginine to NO and L-citrulline (Scheme 3).

These results further support the idea that an active site water molecule is necessary for only the first reaction in the NOS catalytic cycle. This interpretation is in agreement with previously suggested mechanisms involving the role of water in NOS catalysis.[21,23,28]

Conclusion

In summary, analogues of L-arginine and N^G-hydroxy-L-arginine, bearing methyl substitution at the C5 position, have shed new light on the steric and stereochemical tolerances of the NOS active site. The construction of these analogues required the use of an optimized diacylperoxide photolysis procedure that should be of general synthetic use and applicability. Methyl substitutions adjacent to the guanidinium group in both L-arginine and N^G-hydroxy-L-arginine have here been shown to elicit varying effects on the enzyme. Of note is the implication that disruption of an organized water molecule in the NOS active site interferes with only the first reaction in the

two-step NOS catalytic cycle. These findings may also serve as a blueprint toward the design of new compounds capable of acting as inhibitors or alternate substrates for NOS. Continuing work is aimed at further characterizing the oxygen binding capabilities of the enzyme when bound to the C5 methylated analogues. Also, crystallographic studies with these methylated analogues bound to the NOS active site are under way to help establish the proposed modes of action for these compounds.

Experimental Section

All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. 2-Methoxyprop-2-yl hydroperoxide was prepared as described in the literature.[32] ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker AVQ-400 spectrometer. High-resolution FAB and ESI mass spectra were obtained using VG ZAB2-EQ and Q-Tof Premier (Waters) instruments, respectively. Detailed here is the representative synthesis of compounds **1a**−**7a**. Full experimental details and data for all other compounds are given in the Supporting Information.

(3*R*/*S*)-Benzyloxycarbonylamino-butyric Acid (1a). (3*R*/*S*)-Aminobutyric acid (2.0 g, 19.4 mmol) was dissolved in 20 mL of 2 M NaOH and cooled on ice. Benzyl chloroformate (1.2 equiv, 23.3 mmol, 3.4 mL) was slowly added, and 30 min after complete addition the mixture was warmed to room temperature and stirred for an additional 2 h. The pH of the mixture was adjusted to 2 with concentrated HCl and extracted with ethyl acetate (3 × 40 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum to a volume of 30 mL. Hexanes where then added and the product precipitated. Compound **1a** was isolated as a white solid with spectral data identical to those reported previously for the same compound.[34]

3(*R*/*S*)-Benzyloxycarbonylamino-butaneperoxoic Acid 1-Methoxy-1-methyl-ethyl Ester (2a). Compound **2a** was prepared by coupling compound **1a** to 2-methoxyprop-2-yl hydroperoxide. Compound **1a** (5.0 mmol, 1.2 g), DCC (6.0 mmol, 1.3 g), and DMAP (0.1 eq, 0.50 mmol, 61 mg) were added sequentially to a solution of 2-methoxyprop-2-yl hydroperoxide (1.5 equiv, 7.5 mmol, 0.80 g) in CH_2Cl_2 (30 mL) at −20 °C. The reaction was slowly warmed to room temperature and stirred for an additional 2 h. The precipitated urea was removed by filtering through Celite and washed with several volumes of CH_2Cl_2, and the filtrate was concentrated under vacuum. The desired product (R_f = 0.25) was purified as a colorless oil (1.14 g, 70%) by flash chromatography on silica gel (80:20 hexane/ethyl acetate). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.54−7.30 (m, 5H), 5.20 (br m, 1H), 5.10 (s, 2H), 4.15 (m, 1H), 3.33 (s, 3H), 2.58 (m, 2H), 1.46 (s, 6H), 1.28 (d, J = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.4, 155.4, 136.4, 128.5, 128.1, 128.0, 107.1, 66.7, 49.9, 44.0, 37.3 22.6, 22.4, 20.1; HRMS (ESI, M + Na) Calcd for $C_{16}H_{23}NO_6Na$ 348.1423, found 348.1430.

3-((3*R*/*S*)-Benzyloxycarbonylamino-butyrylperoxycarbonyl)-(2*S*)-*tert*-butoxycarbonylamino-propionic Acid *tert*-Butyl Ester (3a). Compound **2a** (3.0 mmol, 0.98 g) was dissolved in $CHCl_3$ (40 mL) and treated with 50% aqueous TFA (14 mL) at 0 °C. The reaction was slowly warmed to room temperature and stirred for 30 min. The reaction was quenched through the addition of saturated aqueous $NaHCO_3$ (important to verify complete neutralization by checking pH). The peracid was then extracted into ether (3 × 40 mL), dried with Na_2SO_4, concentrated under reduced pressure, and used without further purification. The crude peracid was dissolved in CH_2Cl_2 (50 mL) and cooled on ice. Boc-Asp-O^{t}Bu (1 equiv, 3.0 mmol, 0.87 g) was added as a solution in CH_2Cl_2 (10 mL) followed by DCC (1.1 equiv, 3.3 mmol, 0.68 g) as a solution in CH_2Cl_2 (10 mL). The reaction was warmed to room temperature and stirred for 3 h at which time TLC indicated complete consumption of the peracid (R_f = 0.13, 7:3 hexane/ethyl acetate). The precipitated urea was removed by filtration through Celite and washed with CH_2Cl_2, and the combined filtrates were evaporated under vacuum. The product (R_f = 0.50) was purified as a clear oil (1.39 g, 88% over two steps) by flash chromatography (7:3 hexane/ethyl acetate). Diacylperoxide **3a** was found to be stable when stored over a desiccant at −80 °C in small (0.5 mmol) aliquots. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.38−7.29 (m, 5H), 5.47 (d, J = 7.6 Hz, 1H), 5.19 (m, 1H), 5.09 (m, 2H), 4.52 (m, 1H), 4.19 (m, 1H), 3.02 (m, 2H), 2.68 (d, J = 4.9 Hz, 2H), 1.45 (m, 18H), 1.28 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.8, 166.9, 166.5, 155.4, 155.2, 136.3, 128.5, 128.1, 128.0, 83.1, 80.2, 66.7, 50.3, 43.9, 36.4, 33.0, 28.2, 27.7, 19.7. HRMS (ESI, M + Na) Calcd for $C_{25}H_{36}N_2O_{10}Na$547.2268, found 547.2266.

5(*R*/*S*)-Benzyloxycarbonylamino-(2*S*)-*tert*-butoxycarbonylamino-hexanoic Acid *tert*-Butyl Ester (4a). Following a modified literature protocol,[31] diacylperoxide **3a** (225 mg, 0.43 mmol) was dissolved in CH_2Cl_2 (2 mL) and transferred to the photolysis vessel (see Supporting Information for detailed description of photolysis vessel and procedure). Solvent was removed by flowing dry N_2 through the vessel until a thin film of neat starting material remained. The vessel was placed in an acetone/dry ice bath and continuously purged with dry N_2 gas throughout the course of the reaction. The neat diacylperoxide was irradiated at 254 nm for 6 h after which the residue was redissolved, re-evaporated, and reirradiated for an additional 6 h. This process was repeated once more after which the mixture was dissolved in a minimum volume of CH_2Cl_2 (1 mL) and the solution irradiated for 3 h in order to ensure full consumption of starting material. Compound **4a** (R_f = 0.55) was purified as a colorless oil (162 mg, 83%) by flash chromatography on silica gel (3:1 hexane/ethyl acetate). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35−7.28 (m, 5H), 5.15−5.04 (m, 3H), 4.74−4.63 (m, 1H), 4.17 (m, 1H), 3.71 (m, 1H), 1.88−1.73 (m, 1H), 1.68−1.55 (m, 1H), 1.55−1.34 (m, 20H), 1.14 (m, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.7, 155.7, 155.4, 136.6, 128.5, 128.1, 81.9, 79.7, 66.5, 53.8, 47.1, 46.9, 32.7, 32.5, 29.7, 29.5, 28.3, 28.0, 21.2; HRMS (FAB, M + H) Calcd for $C_{23}H_{37}N_2O_6$ 437.2652, found 437.2657.

***N*^{G}-OTHP-*N*^{G}-benzyloxycarbonyl-(5*R*/*S*)-methyl-*N*-*tert*-butoxycarbonyl-L-arginine-*tert*-butyl Ester (5a).** Compound **5a** was produced using a one-pot procedure previously described in the literature.[30] Compound **4a** (150 mg, 0.34 mmol) was dissolved in ethyl acetate (6 mL) and treated with 10% Pd/C (100 mg). Hydrogen gas was administered throughout the reaction using a balloon until all the starting material had reacted (9 h). The deprotected amine was visualized with TLC (3:1 hexane/ethyl acetate), appearing as a red spot along the baseline using ninhydrin stain. Following concentration under vacuum, the crude amine was dissolved in 6 mL of CH_2Cl_2. CbzNCS[4] was added dropwise (as a 0.5 M solution in CH_2Cl_2) until TLC indicated consumption of the amine with concomitant production of the thiourea (rapid conversion). The mixture was next directly treated with triethylamine (1.1 equiv, 0.37 mmol, 53 μL), EDCI (1.1 equiv, 0.37 mmol, 71 mg), and OTHP-protected hydroxylamine (1.2 equiv, 0.41 mmol, 48 mg). The reaction mixture was stirred at room temperature, and additional half equivalents of the reagents were added sequentially every 30−45 min until complete consumption of the starting material had occurred, as indicated by TLC. The product, a colorless oil, was isolated (156 mg, 80%) as the expected mixture of diastereomers (R_f = 0.44) by flash chromatography on silica gel (3:1 hexane/ethyl acetate). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.89 (d, J = 4.0 Hz, 1H), 7.42−7.35 (m, 5H), 6.19 (br q, J = 6.0 Hz, 1H), 5.40−5.04 (m, 3H), 4.94 (m, 1H), 4.13 (m, 1H), 3.90 (m, 1H), 3.63 (m, 2H), 1.91−1.72 (m, 4H), 1.65−1.50 (m, 6H), 1.49−1.41 (m, 18H), 1.15 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.84, 171.76, 155.5, 155.4, 152.9, 148.4, 148.3, 148.2, 135.1, 128.7, 128.4, 128.1, 101.3, 101.2, 81.7, 81.6, 81.5, 79.5, 79.4, 67.6, 64.0, 63.9, 54.2, 54.1, 53.8, 46.0, 36.6, 32.3, 32.3, 32.0, 31.9,

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29.7, 29.4, 29.14, 29.07, 28.3, 28.0, 25.1, 20.8, 20.6, 20.5; HRMS (FAB, M + H) Calcd for $C_{29}H_{47}N_4O_8$ 579.3394, found 579.3404.

(5*R*/*S*)-Methyl-L-arginine Di-trifluoroacetate (6a). Compound **5a** (140 mg, 0.24 mmol) was dissolved in 5 mL of MeOH/H_2O (1% acetic acid), and the system was purged with N_2(g) prior to addition of 10% Pd/C (100 mg). Hydrogen gas was delivered with a balloon to the stirring reaction mixture throughout the course of the reaction (3 h, room temperature). Following reaction completion, the catalyst was removed by filtering through Celite and washed with several volumes of MeOH/H_2O (1% acetic acid). The filtrate was concentrated under vacuum followed by treatment with 10 mL of 25% thioanisole in TFA for 5 h at room temperature. The mixture was then concentrated under vacuum, and the residue was partitioned between ether (15 mL) and water (15 mL) to remove nonpolar impurities. The ether layer was further extracted with 5 mL of H_2O, and the aqueous layers were combined and concentrated under vacuum (final volume 5.0 mL). The amino acid product was purified using a Varian C18 megabond elution column (60 mL volume containing 10 g C18 silica). Prior to applying the crude product, the column was pre-equilibrated with methanol (50 mL) followed by 0.1% TFA in H_2O (50 mL). After loading the column, the product was eluted using 0.1% TFA in H_2O and collected in 10 mL fractions. Fractions 2−5 (positive ninhydrin stain) were pooled, concentrated, and lyophilized to give the product as a clear, glassy foam in quantitative yield. ^{1}H NMR (D_2O, 400 MHz) δ 4.00 (app dt, J_1 = 1.6 Hz, J_2 = 6.0 Hz, 1H), 3.51 (app sext, J = 6.4 Hz, 1H), 2.03−1.81 (m, 2H), 1.73−1.46 (m, 2H) 1.13 (app dd, J_1 = 0.8 Hz, J_2 = 6.4 Hz, 3H); ^{13}C NMR (D_2O, 100 MHz) δ 171.7, 156.1, 52.50, 53.46, 47.4, 30.9, 30.8, 26.1, 26.0, 19.4, 19.3; HRMS (FAB, M + H) Calcd for $C_7H_{17}N_4O_2$ 189.1352, found 189.1357.

***N*^{G}-Hydroxy-(5*R*/*S*)-methyl-L-arginine Di-trifluoroacetate (7a).** Compound **5a** (150 mg, 0.25 mmol) was dissolved in a 10 mL solution of TFA/25% thioanisole, and the mixture was stirred at room temperature under N_2 for 6 h during which time the solution's color progressed from bright green to dark red. The product was isolated as described above for compound **6a** and obtained in quantitative yield. ^{1}H NMR (D_2O, 400 MHz) δ 4.00 (app dt, J_1 = 1.6 Hz, J_2 = 6.2 Hz, 1H), 3.51 (app sext, J = 6.4 Hz, 1H), 2.03−1.81 (m, 2H), 1.76−1.49 (m, 2H), 1.13 (app d, J = 6.0 Hz, 3H); ^{13}C NMR (D_2O, 100 MHz) δ 171.7, 158.1, 52.51, 52.48, 47.6, 30.8, 30.7, 26.2, 26.1, 19.4, 19.3; HRMS (FAB, M + H) Calcd for $C_7H_{17}N_4O_3$ 205.1301, found 205.1298.

Spectral K_d Titrations. $iNOS_{heme}$ was expressed and purified as previously described.[20] In a typical assay H_4B-bound iNOSheme (500 μL of 0.5−1 μM) was titrated with the compound of choice working at concentrations appropriate to detect the spectral transition from 420 nm (unbound Fe^{III}-aqua) to 396 nm (saturated). Absorbance spectra were corrected for the dilution due to the volume of titrant added. As described in the text, absorbance changes ($\Delta\Delta Abs = \Delta Abs_{396} - \Delta Abs_{420}$) were plotted as a function of compound concentration, and the K_d was determined by fitting the data by a nonlinear least-squares fit to the saturation binding equation: $\Delta\Delta Abs = (\Delta\Delta Abs_{max} \times [compound])/(K_d + [compound])$ (KaleidaGraph, Abelbeck Software). All analyses were performed in triplicate, and the average value was reported.

NOS Inhibition/Activity Assays. A modified, high-throughput oxymyoglobin assay[35] using a 96-well plate reader was utilized to assess the NO production or inhibition exhibited by compounds **6b−d** and **7b−7d** with full-length iNOS. Described here is the K_i determination for **6d** and the K_m determination for **7c**. ***K*$_i$ Determination for 6d:** Assay reactions were prepared containing L-arginine (0, 10, 20, 50, 100, 200, 400, and 1000 μM), **6d** (2.8, 5.0, 10, and 15 μM), 12 μM H_4B, 5.0 μM DTT, 8.5 μM oxymyoglobin, and 15 nM iNOS in 100 mM Hepes (pH 7.4). The NOS reaction was initiated by addition of NADPH (final concentration 100 μM) to each well using a multichannel pipet. NO production was monitored by measuring the increase in absorbance at 405 nm corresponding to the NO-mediated conversion of oxymyoglobin to metmyoglobin. The data derived from the Michaelis−Menten plots of iNOS inhibition by **6d** were used to generate an apparent K_m versus[6d] replot from which the K_i for **6d** was determined to be 1.3 ± 0.4 μM. Each analysis was performed in triplicate, and the average was reported. ***K*$_m$ Determination for 7c:** Assay reactions were prepared containing **7c** (350, 700, 1050, 1400, 2100, 2800, and 5600 μM), 12 μM H_4B, 5 μM DTT, 8.5 μM oxymyoglobin, and 15 nM iNOS in 100 mM Hepes (pH 7.4). The NOS reaction was initiated by addition of NADPH (final concentration 100 μM) to each well using a multichannel pipet and NO production monitored as described above. The data were fit to a standard Michaelis−Menten plot (KaleidaGraph, Abelbeck Software) from which the K_m for **7c** was determined to be 327 ± 35 μM. Each analysis was performed in triplicate, and the average was reported.

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Supporting Information Available: Experimental procedures and spectroscopic data for compounds **6b−d**, and **7c−7d** as well as ^{1}H and ^{13}C NMR spectra for all compounds. Complete results of the spectral binding and inhibition assays for compounds **6b−d** and **7c−7d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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