Conformationally Restricted Dipeptide Amides as Potent and Selective Neuronal Nitric Oxide Synthase Inhibitors

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Four new conformationally restricted analogues of a potent and selective neuronal nitric oxide synthase inhibitor, L-nitroargininyl-L-2,4-diaminobutyramide (**1**), have been synthesized. N^{α}-Methyl and N^{α}-benzyl derivatives (**3** and **4**, respectively) of 4-*N*-(L-Arg^{NO_2})-*trans*-4-amino-L-prolineamide (**2**) are also selective inhibitors, but the potency and selectivity of **3** are weak. Analogue **4** has only one-third the potency and one-half to one-third the selectivity of **2** against iNOS (inducible nitric oxide synthase) and eNOS (endothelial nitric oxide synthase), respectively. 3-*N*-(L-Arg^{NO_2})-*trans*-3-amino-L-prolineamide (**6**) is as potent an inhibitor of nNOS (neuronal nitric oxide synthase) as **2**; selectivity for nNOS over iNOS is half of that for **2**, but the selectivity for nNOS over eNOS is almost double that for **2**. The corresponding cis-isomer (**5**) is a weak inhibitor of nNOS. These results are supported by computer modeling.

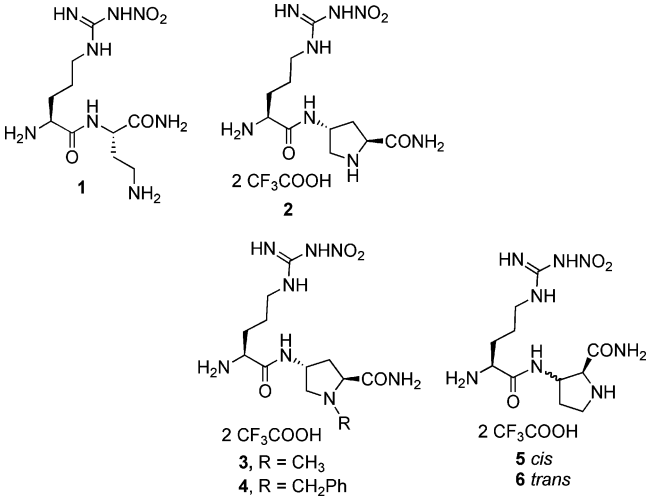
Introduction

Nitric oxide (NO) is a ubiquitous biological messenger involved in a variety of physiological processes that acts as a signal transducer but also exerts a variety of regulatory and cytostatic functions. In most instances NO mediates its biological effects by activating guanylate cyclase and increasing cyclic GMP synthesis. However, effects of nitric oxide that are independent of cyclic GMP are also known.[1]

Nitric oxide synthase (NOS, EC 1.14.13.39) isoforms[2] are homodimers that catalyze the oxidation of L-arginine to L-citrulline and nitric oxide in a NADPH- and O_2-dependent process.[3] There are at least three distinct isoforms of NOS. The constitutive endothelial isoform (eNOS) is involved in the regulation of smooth muscle relaxation and blood pressure and in the inhibition of platelet aggregation. A second constitutive isoform is the neuronal NOS (nNOS), which is important for neurotransmission. A third isozyme is the inducible NOS (iNOS), which is located in activated macrophage cells and acts as a cytotoxic agent in normal immune responses. All of the isoforms utilize NADPH, FAD, FMN, tetrahydrobiopterin, and heme as cofactors. The constitutive isoforms also require Ca^{2+} and calmodulin for activity, while the inducible isoform has tightly bound Ca^{2+} and calmodulin. The crystal structures of the oxygenase domains of murine iNOS monomer,[4] murine and human iNOS dimer,[5−8] human and bovine endothelial NOS dimers,[9] and rat neuronal NOS dimer[10] indicate a high degree of structural similarity within the catalytic center and the dimer interface regions between NOS isoforms. They share only approximately 50% of primary sequence identity, suggesting that they may differ from each other in regulatory aspects.

The use of NOS inhibitors in pathologically elevated synthesis of NO has great therapeutic potential.[11] NO overproduction by nNOS has been associated with neurodegeneration during stroke, spinal transmission of pain, migraine headaches, Parkinson's disease, and Alzheimer's disease. Thus nNOS represents an important therapeutic target for inhibitors.[12] Selective inhibition of one isoform over the others is essential, because the three isoforms of NOS have unique roles in separate tissues. Selective inhibitors may represent useful tools for investigating other biological functions of NO.[13]

Previously, we synthesized and evaluated nitroarginine-containing dipeptide esters and dipeptide amides and identified a family of compounds that had high potency and selectivity for inhibition of nNOS over both iNOS and eNOS.[14,15] The most potent nNOS inhibitor among these compounds was L-Arg^{NO_2}-L-Dbu-NH_2 (**1**, K_i = 130 nM); this compound exhibited high selectivity over eNOS (>1500-fold) and iNOS (192-fold). More recently we reported **2** (nNOS K_i = 103 nM) as a conformationally restricted analogue of L-Arg^{NO_2}-L-Dbu-NH_2 (**1**).[16] This compound was a little more potent than **1** with comparable selectivity for nNOS over eNOS and iNOS. Here we report the synthesis and in vitro evaluation of four new analogues of **2**, two that are N^{α}-substituted analogues (**3** and **4**) to increase lipophilicity and to determine if additional binding could enhance potency and selectivity and two that are positional



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|| Developed the eNOS overexpression system in *E. coli* and the purification of eNOS.

⊥ Developed the overexpression system for nNOS in *E. coli* and the purification of the nNOS.

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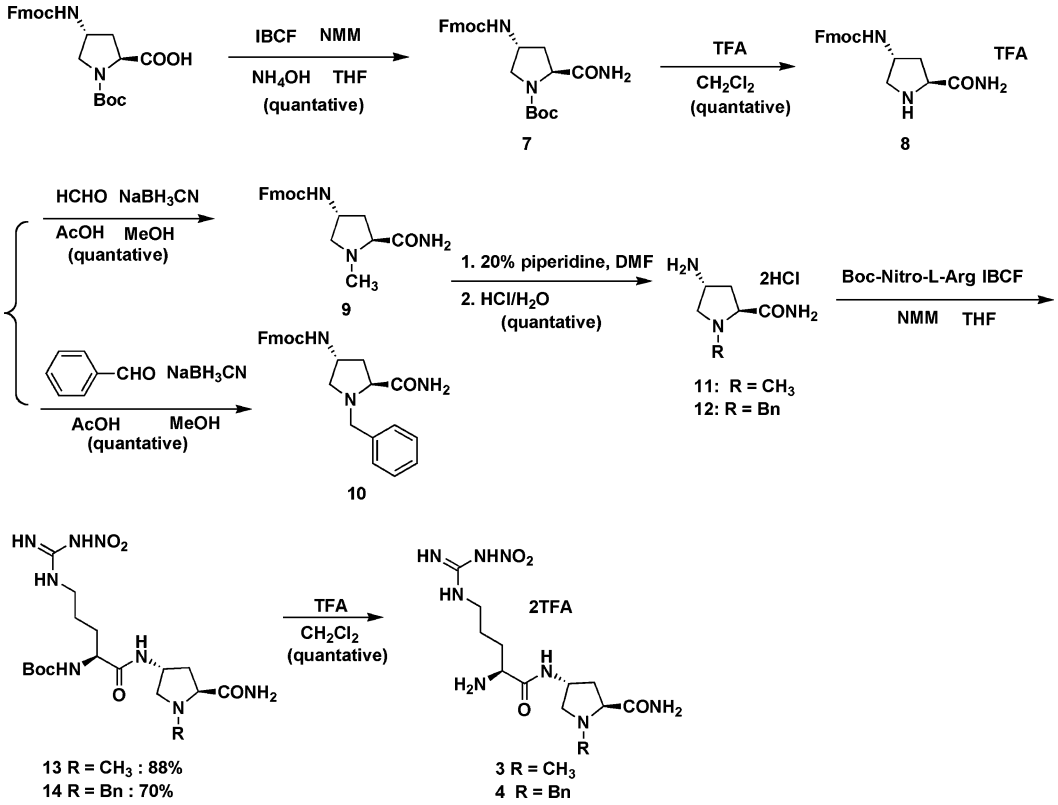
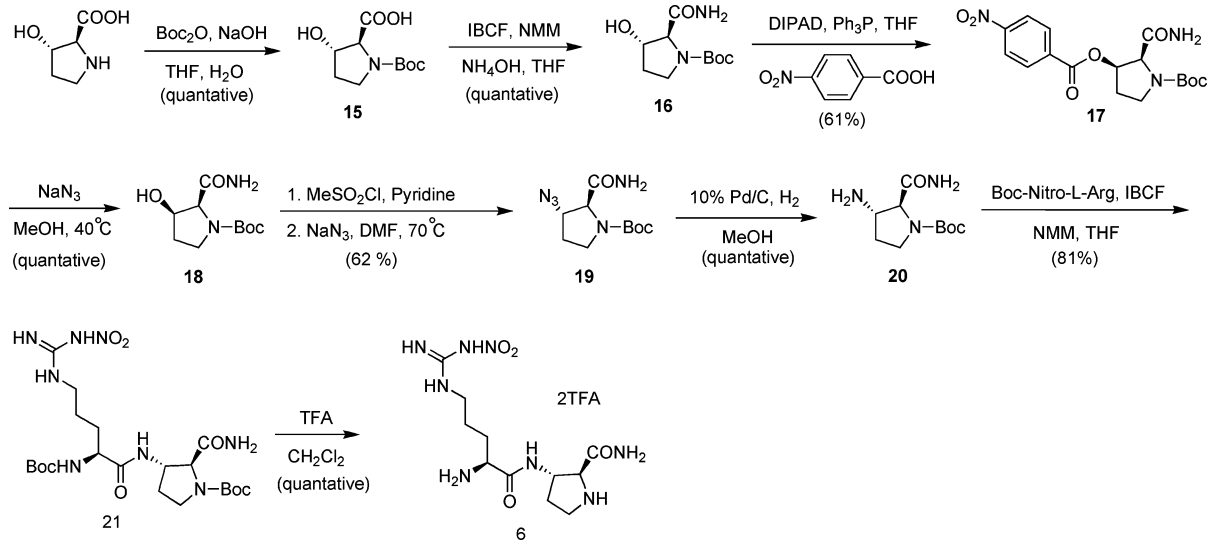
Scheme 1. Solution-Phase Synthesis of **3** and **4**



Scheme 2. Solution-Phase Synthesis of **6**



isomers of the pyrrolidine ring of **2** (**5** and **6**) to determine the optimal binding orientation.

Chemistry

The solution-phase syntheses of **3** and **4** are shown in Scheme 1. Starting from commercially available *N*-Boc-*trans*-4-*N*-Fmoc-amino-L-proline, only six steps were required to give the final product. All of the synthetic steps, except for the dipeptide coupling reaction, afforded quantitative yields of products. In the reductive amination reaction the starting material, *trans*-4-*N*-Fmoc-amino-L-proline-NH_2, has good solubility in MeOH, but the products, *N*-substituted-*trans*-4-*N*-Fmoc-amino-L-proline-NH_2, have poor solubility in MeOH, so they precipitated from the organic phase during the reaction. In the dipeptide coupling reaction the mixed anhydride method [isobutyl chloroformate (IBCF)/*N*-methylmorpholine (NMM)] provided a much higher yield (88%) than the active ester method (HBTU/HOBt/DIEA; 40%), especially when the starting material contained an amide group.

A solution-phase synthesis of **6**, shown in Scheme 2, started from commercially available *trans*-3-hydroxy-L-proline. In the Mitsunobu reaction for the conversion of **16** to **17** the use of *p*-nitrobenzoic acid as the nucleophile afforded pure product in a 61% yield, whereas with acetic acid as the nucleophile none of the product was isolated. The hydrolysis of **17** using sodium azide as the catalyst provided a quantitative yield of pure **18**.[17] A Mitsunobu reaction was also attempted to convert alcohol

Scheme 3. Solid-Phase Synthesis of **5** and **6**

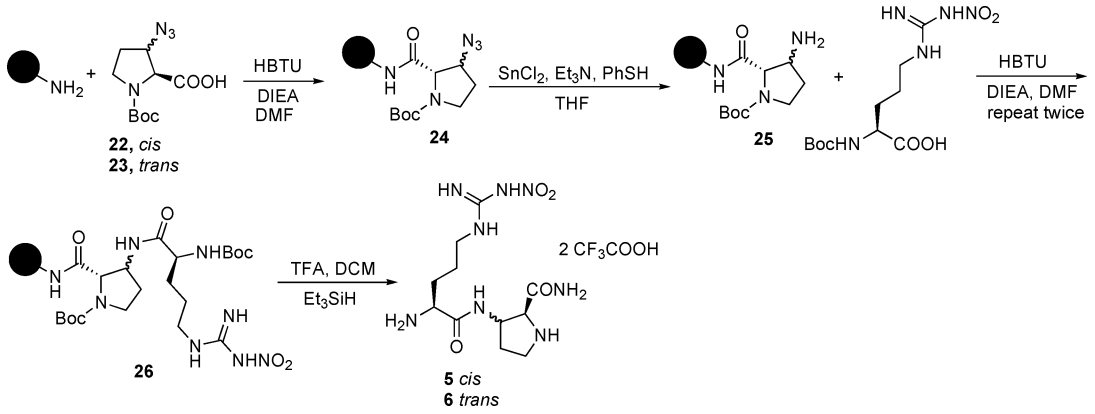
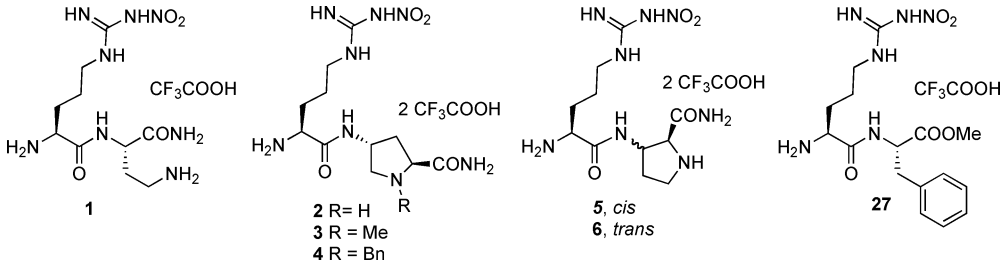


Table 1. In Vitro Assay Data for Dipeptide Inhibitors



	nNOS		iNOS		eNOS			
compd	IC_{50}[a] (μM)	K_i[a] (μM)	IC_{50}[a] (μM)	K_i[a] (μM)	IC_{50}[a] (μM)	K_i[a] (μM)	iNOS/ nNOS[g]	eNOS/ nNOS[g]
1[b]	1.1	0.13[c]	55.5	25[c]	1375	200	190	1540
2[d]	0.64	0.10[e]	65	29	880	128	290	1280
3[h]	73.5 ± 3.5	8.46 ± 0.40	83.5 ± 3.5	37.9 ± 1.6	1690 ± 100	246 ± 14	4.5	29
4[h]	2.85 ± 0.21	0.328 ± 0.024	87.5 ± 2.1	39.7 ± 1.0	1100 ± 90	159 ± 13	121	485
5	100	–	–	–	–	–	–	–
6[h]	0.75 ± 0.03	0.087 ± 0.004	12.7 ± 1.1	5.76 ± 0.61	1380 ± 80	200 ± 10	66	2299
27[f]	17.4	2[c]	8[a]	3600[c]	–	–	1800	–

[a] mM. [b] From ref 15. [c] Experimental K_i. [d] Drom ref 16. [e] Experimental K_i = 103 nM. [f] From ref 14. [g] Selectivity of inhibition in favor of nNOS inhibition obtained from the ratio of the K_i for iNOS or eNOS over that for nNOS. [h] The K_i values for **3**−**6** represent two separate measurements with five or six data points each and correlation coefficients of 0.947−0.992.

18 to amine **19**. Two different nucleophiles, diphenyl phosphoryl azide (DPPA) and phthalimide, were tried, but no product was produced. That might be because the hydroxyl group and the amide group in *cis*-**18** formed a strong intramolecular hydrogen bond, which prevented the triphenyl phosphine from reaching the alcohol. When the amide was changed to a methyl ester, the Mitsunobu reaction proceeded smoothly, as was demonstrated previously.[18] Alcohol **18** was activated with methanesulfonyl chloride to form the mesylate, which was converted to the azide with sodium azide in a 62% yield.

Compounds **3**−**6** were initially synthesized using a solid-phase synthetic method (Scheme 3 and Supporting Information Scheme 1), but **3**, **4**, and **6** were optimized by the solution-phase methods shown in Schemes 1 and 2. The solid-phase synthesis of 3-aminoproline-containing dipeptides **5** and **6** started from *N*-Boc protected *cis*- and *trans*-3-azido-L-proline (**22** and **23**), which were previously reported.[18] Triethylammonium tris(phenylthio)stannate has been described as a very efficient reducing agent with high chemoselectivity toward azides.[19] This reagent has been applied successfully on solid phase using Rink resin[20] or other resins[21] and afforded amine **25** in high purity.

Results and Discussion

Compound **2** was previously found to be the most potent conformationally restricted analogue of L-Arg^{NO_2}-L-Dbu-NH_2 (**1**), which is a highly selective inhibitor of nNOS over eNOS.[15] D-Arg^{NO_2}-D-Phe-OMe is a highly selective inhibitor of nNOS over iNOS.[14] Clearly, these two compounds interact differently with the NOS isozymes, as previously described.[10] *N*-Substituted analogues of **2** (**3** and **4**) were synthesized to determine if having both an amino group and a hydrophobic group would enhance binding for nNOS over the other two isoforms even more selectively than one or the other. Compounds **5** and **6** (the *cis*- and *trans*-3-amino-L-prolinamide derivatives) also were synthesized to determine if it was better to attach the L-Arg^{NO_2} at the 3-position or 4-position (**2**) of the proline ring to maximize potency and selectivity for nNOS.

These compounds were assayed against all three isozymes of NOS for potency and selectivity (Table 1). When compared to nonsubstituted analogue **2**, the *N*-substituted compounds (**3** and **4**) showed reduced nNOS potency (82- and 3-fold, respectively) and selectivity (73- and 44-fold for **3** against iNOS and eNOS, respectively; 2- and 3-fold for **4** against iNOS and eNOS, respectively). These results support the importance of a nonsubstituted pyrrolidine amino group for interaction with nNOS. Compound **4** showed a 2-fold decrease in potency against nNOS when compared to **1**. On the other hand, **4** was 6-fold more potent than the previously reported phenylalanine-containing dipeptide (**27**) against nNOS.[14] The sequence alignment and a schematic drawing of the active site of NOS were presented

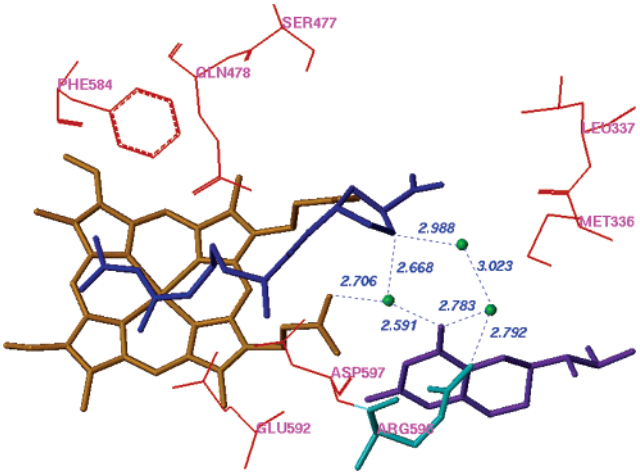


Figure 1. The H-bond network of the pyrrolidine nitrogen atom of **2** bound to rat nNOS (PDB ID 1p6j). The distances of the hydrogen bonds are shown in angstroms (Å).

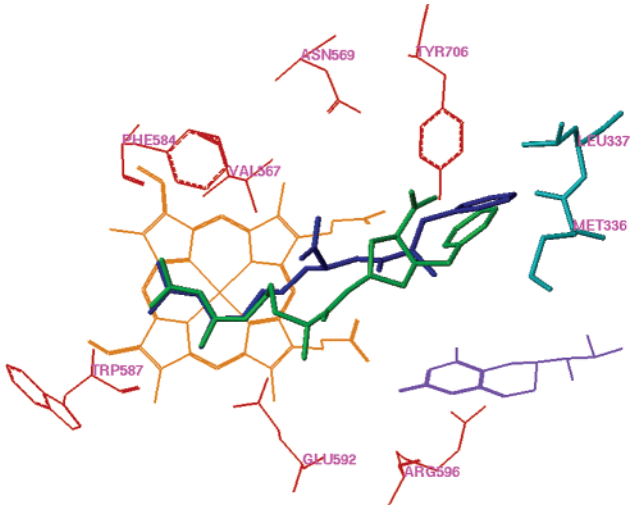


Figure 2. Superimposition of docked conformation of **4** and the crystallographic conformation of **27** in rat nNOS (PDB ID 1rs7). The blue conformation is the crystallographic binding conformation of **27**; the green structure is the docking conformation of **4**.

in an earlier paper.[22] The crystal structure of **2** bound in the active site of nNOS and eNOS[10a] shows that the pyrrolidine nitrogen is located between the heme propionate group and the carbonyl group of the tetrahydrobiopterin cofactor. Compound **27** does not have a nitrogen at that position. The loss of this charge−charge interaction between the positively charged nitrogen atom and the negatively charged heme propionate appears to be the main reason for the low nNOS potency of **27** compared to that of **2** or **6**.

Further analysis of the crystal structure of **2** bound to rat nNOS shows that the pyrrolidine nitrogen of **2**, as a secondary amine, forms a H-bond network interaction with R596, the heme propionate, and the carbonyl group of the tetrahydrobiopterin cofactor via three structural water molecules (Figure 1). Alkylation of this nitrogen atom would exclude these structural water molecules from the active site. An AutoDock calculation of **4** showed that its phenyl group has the same spatial orientation in the C1 pocket[22] of nNOS as that of **27**. The superimposition of the docked conformation of **4** and the crystallographic binding conformation of **27** with nNOS (PDB ID 1rs7) show, however, that the phenyl group of **27** binds more tightly to hydrophobic residues L337 and M336 of rat nNOS than does the phenyl group of **4** (Figure 2). The sum of the loss of the H-bond network interaction mentioned above and the partial compensation from the interaction of the benzyl group of **4** with the hydrophobic residues in the C1 pocket account for the low potency of **4** compared to **2**. Compound **3** is particularly poor because the methyl group interferes with the above H-bond network interactions and is not long enough to extend into the C1 pocket for a hydrophobic interaction (AutoDock data not shown). As described before,[10a,22] the charge−charge interaction between the α-amino group in our dipeptide nNOS inhibitors and D597 of rat nNOS accounts for the selectivity of these inhibitors for nNOS over eNOS. The stronger the interaction that the side chain of the depeptide inhibitors has with the active site, the more prone is the α-amino group to fix at the selective region defined by nNOS D597/eNOS N368.[22] That is most likely the reason **2** is more selective than **4** for binding to nNOS over eNOS.

The *cis*-3-amino-L-proline derivative (**5**) displayed very low potency against nNOS ($IC_{50} \approx 100$ μM). However, the *trans*-3-aminoprolinamide isomer (**6**) showed nearly the same potency against nNOS as the previously described *trans*-4-aminoprolinamide isomer (**2**) but with almost a doubling in selectivity for eNOS (Table 1). Therefore, the *trans*-pyrrolidine ring is the favored configuration for the interaction of these conformationally restricted dipeptides with nNOS, regardless of whether the L-Arg^{NO_2} is attached at the 3- or 4-position. Molecular modeling of **5** and **6** shows that the spatial orientation of the pyrrolidine ring of **6** is almost identical to that of **2**, except that the terminal amide groups protrude in different directions (Figure 3A). Since the terminal amide group of **2** only weakly interacts directly with the residues or cofactors in the active site, the nNOS potencies of these dipeptide inhibitors are primarily determined by the L-Arg^{NO_2} moiety and the pyrrolidine ring part of the proline. The superimposition of the docking conformation of **5** with the crystallographic binding conformation of **2** shows that the pyrrolidine ring of the cis compound (**5**) is out of the binding area of the heme propionate and the tetrahydrobiopterin (Figure 3B), which explains its low potency. The corresponding calculated binding free energies (Table 2) support the results of the above docking observations. All of the scoring functions arrived at the same conclusion, except for D_Score, that the binding of **5** should be less favorable than that for **6**. Others,[23] however, have found that D_Score gives the lowest correlation between calculated and experimental binding affinities.

In summary, a nonsubstituted proline nitrogen is necessary for high potency against nNOS. The L-Arg^{NO_2} moiety must be positioned as a *trans*-3- or *trans*-4-substituent on the proline ring for enhanced potency and selectivity against nNOS over eNOS and iNOS. Attachment at the 3-position favors nNOS over eNOS selectivity greater than at the 4-position but lower nNOS over iNOS selectivity than at the 4-position.

Experimental Section

General Methods, Reagents, and Materials. All reagents were purchased from Aldrich, Novabiochem, or Advanced ChemTech and were used without further purification unless stated otherwise. *trans*-3-Hydroxy-L-proline was purchased from Acros Organics. NADPH, calmodulin, and human ferrous hemoglobin were obtained from Sigma Chemical Co. Tetrahydrobiopterin (H_4B) was purchased from Alexis Biochemicals. HEPES, DTT, and conventional organic solvents were purchased from Fisher Scientific. Tetrahydrofuran (THF) was distilled under nitrogen from sodium/benzophenone. Sodium sulfate was used to dry organic layers during work up. ^{1}H NMR spectra were recorded on a Gemini 2000, Mercury 400, or Inova 500 spectrometer (75.4, 100.6, or 125.7 MHz, for ^{13}C NMR spectra) in $CDCl_3$. Chemical shifts are reported as δ values in parts per million with the $CDCl_3$ (TMS), $(CD_2H)_2SO$, CD_2HOD, and

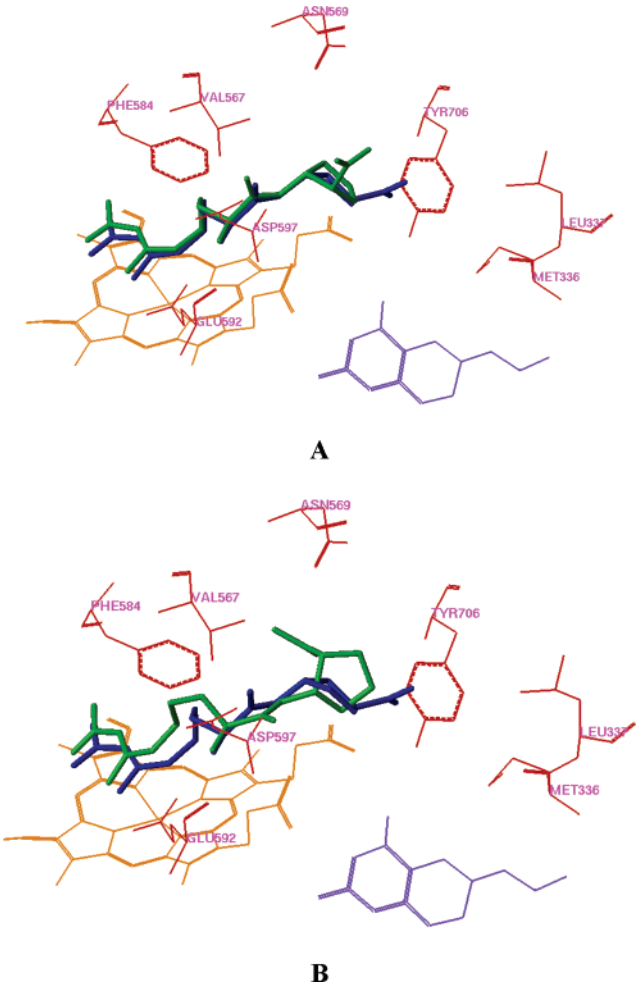


Figure 3. Superimposition of the crystallographic conformation of **2** (PDB ID 1p6j) and the docked conformations of **5** and **6** in rat nNOS. (A) The blue conformation is the crystallographic binding conformation of **2**; the green structure is the docking conformation of **6**. (B) The blue conformation is the crystallographic binding conformation of **2**; the green structure is the docking conformation of **5**.

Table 2. Predicted Binding Free Energies (kcal/mol) of **2**, **5**, and **6** with nNOS Using AutoDock, Chemscore, F_Score, PMF_Score, E_docked, G_Score, and D_Score Scoring Functions

	Chemscore	F_Score	PMF_Score	E_docked	G_Score	D_Score
2	−17.86	−18.38	−143.53	−11.94	−283.45	−23.95
5	−12.51	−7.30	−121.31	−9.26	−226.56	−25.21
6	−19.06	−19.18	−134.04	−10.14	−239.99	−21.25

HOD resonance peaks set at 0, 2.50, 3.31, and 4.80 ppm, respectively. E. Merck precoated silica gel 60 F254 plates were used for thin-layer chromatography (TLC), with visualization accomplished with the phosphomolybdic acid or ninhydrin spray reagent or with a UV−vis lamp. Flash chromatography was performed with E. Merck silica gel 60 (230−400 mesh) or Sorbent Technologies 250 mesh silica gel. Fractions containing pure compound were concentrated in vacuo, and the residue was lyophilized. pH measurements were conducted with an Orion Research model 701 pH meter with a general combination electrode. Elemental analyses were performed by Atlantic Microlab Inc. Mass spectra were performed on a Micromass Quattro II triple quadrupole mass spectrometer (ESI). High-resolution mass spectra were carried out at the University of Illinois at Urbana−Champaign using a Micromass Quatto tandem quadrupole/hexapole/quadrupole mass spectrometer. NOS assays were recorded on a Perkin-Elmer Lambda 10 UV−vis spectrophotometer.

***N*$^{\alpha}$-Boc-*trans*-4-*N*-Fmoc-amino-L-proline-NH_2 (7).** To a cooled solution (−15 °C) of *N*-Boc-*trans*-4-*N*-Fmoc-amino-L-proline (0.451 g, 0.001 mol) in anhydrous THF (10 mL) were added *N*-methylmorpholine (0.111 g, 0.12 mL, 0.0011 mol) and isobutyl chloroformate (0.150 g, 0.144 mL, 0.0011 mol), and stirring was continued for 30 min. NH_4OH (28% aqueous solution, 0.188 g, 0.21 mL, 0.0015 mol) was added, and the reaction mixture was stirred at −15 °C for 30 min and then warmed to room temperature over 1 h. The solvent was evaporated under reduced pressure. The residue was suspended in EtOAc (40 mL) and washed successively with 1 M $KHSO_4$ (2 × 20 mL), saturated NaCl (20 mL), 5% $NaHCO_3$ (2 × 20 mL), and saturated NaCl (20 mL). The solution was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, giving a residue, which was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 9:1) to afford a white solid (0.45 g, quantitative yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ (7.758 + 7.748) (d, 2H, J = 7.5 Hz), (7.569 +7.554) (d, 2H, J = 7.5 Hz), 7.389 (t, 2H, J = 7.5 Hz), 7.301 (t, 2H, J = 7.5 Hz), 4.421 (s, 2H), 4.383−4.095 (m, 3H), 3.856−3.653 (m, 1H), 3.408−3.008 (m, 1H), 2.523−2.427 (m, 1H), 2.122−1.728 (m, 1H), 1.448 (s, 9H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ (175.0 + 173.9) (1C), 155.9 (1C), (155.6 + 154.5) (1C), 143.9 (2C), 141.5 (2C), 127.9 (2C), 127.2 (2C), 125.1 (2C), 120.2 (2C), 81.2 (1C), 66.7 (1C), 58.3 (1C), 52.3 (1C), 50.1 (1C), 47.3 (1C), (37.1 + 34.0) (1C), 28.5 (3C). APCI (CH_2Cl_2) m/z = 452.1 ([M + H]$^+$).

***trans*-4-*N*-Fmoc-amino-L-proline-NH_2 (8).** **7** (0.451 g, 0.001 mol) was dissolved in anhydrous CH_2Cl_2 (1 mL), and trifluoroacetic acid (TFA, 1 mL) was added at 0 °C. The reaction mixture was stirred 1 h at 0 °C and 1 h at room temperature. After the reaction was completed, the solvent was evaporated under reduced pressure to afford a colorless oil (0.465 g, quantatitive yield): ^{1}H NMR (500 MHz, CD_3OD) δ (7.657 + 7.642) (d, 2H, J = 7.5 Hz), (7.500 + 7.485) (d, 2H, J = 7.5 Hz), 7.249 (t, 2H, J = 7.5 Hz), 7.170 (t, 2H, J = 7.5 Hz), 4.300 (m, 3H), 4.139−4.095 (m, 2H), 3.469 (m, 1H), 3.179 (m, 1H), 2.317−2.306 (m, 1H), 2.166 (m, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ (161.1 + 160.8) (1C), 158.3 (1C), 145.3 (2C), 142.8 (2C), 129.0 (2C), 128.3 (2C), 126.1 (2C), 121.1 (2C), 67.9 (1C), 60.3 (1C), 51.9 (1C), 51.5 (1C), 48.5 (1C), 36.7 (1C); ESI(CH_3OH) m/z = 352.3([M + H]$^+$).

***N*$^{\alpha}$-Methyl-*trans*-4-*N*-Fmoc-amino-L-proline-NH_2 (9).** To a solution of **8** (0.465 g, 0.001 mol) dissolved in MeOH (20 mL) at 0 °C were added a 37% formaldehyde solution (0.812 g, 0.745 mL, 0.01 mol), $NaBH_3CN$ (0.314 g, 0.005 mol), and acetic acid (0.120 g, 0.115 mL, 0.002 mol). The reaction mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure. The residue was dissolved in 5% $NaHCO_3$ (10 mL) and extracted with EtOAc (10 mL × 3). The combined organic layers were washed with brine and evaporated in vacuo. The residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 9.5:0.5) to afford a white solid (0.365 g, quantitative yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ (7.897 + 7.881) (d, 2H, J = 8 Hz), (7.694 + 7.680) (d, 2H, J = 7 Hz), 7.412 (t, 2H, J = 8 Hz), 7.331 (t, 2H, J = 7.5 Hz), (4.316 + 4.302) (d, 2H, J = 7 Hz), 4.204 (t, 1H, J = 7 Hz), 4.108−3.960 (m, 1H), 3.191 (t, 1H, J = 7 Hz), 2.781 (t, 1H, J = 8 Hz), 2.236 (s, 3H), 2.140 (t, 1H, J = 8.5 Hz), 1.986−1.896 (m, 2H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 174.9 (1C), 155.7 (1C), 143.9 (2C), 140.8 (2C), 127.6 (2C), 127.1 (2C), 125.2 (2C), 120.2 (2C), 67.6 (1C), 65.2 (1C), 61.1 (1C), 48.9 (1C), 46.7 (1C), 40.9 (1C), 36.7 (1C); ESI (CH_3CN) m/z = 366.6 ([M + H]$^+$).

***N*$^{\alpha}$-Benzyl-*trans*-4-*N*-Fmoc-amino-L-proline-NH_2 (10).** To a solution of **8** (0.465 g, 0.001 mol) dissolved in MeOH (20 mL) at 0 °C were added benzaldehyde (0.531 g, 0.505 mL, 0.005 mol), $NaBH_3CN$ (0.314 g, 0.005 mol), and acetic acid (0.120 g, 0.115 mL, 0.002 mol). The reaction mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure. The residue was dissolved in 5% $NaHCO_3$ (10 mL) and extracted with EtOAc (10 mL × 3). The combined organic layers were washed with brine and evaporated in vacuo. The residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 9.5:0.5) to afford a white solid (0.441 g, quantitative yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ (7.887 + 7.872) (d, 2H, J = 7.5 Hz), (7.672 +7.657) (d, 2H, J = 7.5 Hz), 7.462−7.241 (m, 9H),

(4.288 + 4.275) (d, 2H, J = 6.5 Hz), 4.182 (t, 1H, J = 6.5 Hz), 3.984−3.940 (m, 1H), 3.398−3.358 (m, 2H), 3.103 (t, 1H, J = 7.5 Hz), 3.014 (t, 1H, J = 8 Hz), 2.092 (t, 1H, J = 8.5 Hz), 1.986−1.951 (m, 2H); ^{13}C NMR (125.7 MHz, DMSO-d_6) δ 175.2 (1C), 155.7 (1C), 143.9 (2C), 140.8 (2C), 138.3 (1C), 128.8 (2C), 128.2 (2C), 127.6 (2C), 127.1 (3C), 125.1 (2C), 120.1 (2C), 67.7 (1C), 65.2 (1C), 58.2 (1C), 57.8 (1C), 48.9 (1C), 46.7 (1C), 36.7 (1C). ESI(CH_3OH) m/z = 442.6 ([M + H]$^+$).

N^{α}-Methyl-*trans*-4-*N*-amino-L-proline-NH_2 Dihydrochloride Salt (11). 9 (0.365 g, 0.001 mol) was treated with 20% piperidine (10 mL) in anhydrous DMF and stirred under a nitrogen atmosphere for 2 h. TLC monitored the complete conversion of the starting material to product. The reaction mixture was then concentrated under reduced pressure. The resulting residue was dissolved in a dilute HCl aqueous solution (four drops of concentrated HCl in 20 mL of H_2O) and washed with hexane to remove the piperidine−dibenzofulvene adduct and the free dibenzofulvene. The aqueous layer was concentrated in vacuo to yield the desired white solid (0.216 g, quantitative yield): ^{1}H NMR (500 MHz, D_2O) δ 3.921−3.861 (m, 1H), (3.557 + 3.542, 3.536 + 3.522) (dd, 1H, J = 7.5 Hz, J = 7 Hz), 3.340−3.306 (m, 1H), 2.566−2.529 (m, 1H), 2.405 (s, 3H), 2.368−2.236 (m, 2H); ^{13}C NMR (125.7 MHz, D_2O) δ 176.4 (1C), 66.6 (1C), 58.7 (1C), 48.0 (1C), 39.7 (1C), 34.3 (1C); ESI (CH_3OH) m/z = 144.2([M + H]$^+$).

N^{α}-Benzyl-*trans*-4-*N*-amino-L-proline-NH_2 Dihydrochloride (12). 10 (0.441 g, 0.001 mol) was treated with 20% piperidine (10 mL) in anhydrous DMF and stirred under a nitrogen atmosphere for 2 h. After complete conversion of the starting material to product (by TLC) the reaction mixture was concentrated under reduced pressure. The resulting residue was dissolved in the dilute aqueous HCl solution (four drops of concentrated HCl in 20 mL of H_2O) and washed with CH_2Cl_2 to remove the piperidine−dibenzofulvene adduct and the free dibenzofulvene. The aqueous layer was concentrated in vacuo to yield the desired white solid (0.292 g, quantitative yield): ^{1}H NMR (500 MHz, D_2O) δ 7.471−7.414 (m, 5H), 4.650−4.614 (m, 1H), (4.504 + 4.479) (d, 1H, J = 7.5 Hz), (4.459 + 4.434) (d, H, J = 7.5 Hz), 4.121−4.092 (m, 2H), 3.592−3.540 (m, 1H), 2.757−2.708 (m, 1H), 2.595−2.533 (m, 1H); ^{13}C NMR (125.7 MHz, D_2O) δ 168.7 (1C), 131.1 (2C), 130.8 (1C), 129.5 (2C), 128.6 (1C), 64.6 (1C), 58.7 (1C), 55.6 (1C), 46.9 (1C), 33.1 (1C); ESI (CH_3OH) m/z = 220.2 ([M + H]$^+$).

4-*N*-(N^{α}-Boc-L-Arg^{NO2})-N^{α}-methyl-*trans*-4-amino-L-proline-NH_2 (13). 11 (0.324 g, 0.0015 mol) was dissolved in 0.5 M NaOH aqueous solution (10 mL) and then was extracted with CH_2Cl_2 (10 mL × 2). The combined organic layers were washed with water (10 mL) to generate the free base form of **11**. A solution of N^{α}-Boc-L-Arg^{NO_2}-OH (0.319 g, 0.001 mol) in dry THF (20 mL) was cooled to −15 °C, and 4-methylmorpholine (0.111 g, 0.121 mL, 0.0011 mol) and isobutyl chloroformate (0.150 g, 0.144 mL, 0.0011 mol) were added. The reaction mixture was stirred for 30 min, and the free base form of **11** (0.157 mL, 0.0011 mol) in anhydrous THF (5 mL) was added via a cannula. The solution was stirred for 30 min at −15 °C and was then allowed to warm to room temperature over 30 min. The reaction mixture was stirred at room temperature for 1 h, and the solvent was evaporated in vacuo. The residue was purified directly by silica gel column chromatography (CH_2Cl_2:MeOH = 8.5:1.5) to afford 0.39 g of a colorless oil (88%): ^{1}H NMR (500 MHz, CD_3OD) δ 4.364−4.336 (m, 1H), 4.080−4.015 (m, 1H), 3.407−3.376 (m, 1H), 3.311−3.271 (m, 2H), 3.036−3.003 (m, 1H), 2.380 (s, 3H), 2.311 (t, 1H, J = 8.5 Hz), 2.209−2.152 (m, 1H), 2.094−2.055 (m, 1H), 1.800−1.636 (m, 4H), 1.437 (s, 9H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 179.1 (1C), 174.8 (1C), 161.0 (1C), 158.0 (1C), 80.8 (1C), 68.9 (1C), 61.9 (1C), 55.6 (1C), 41.8 (1C), 41.5 (2C), 38.3 (1C), 30.8 (1C), 28.8 (3C), 26.2 (1C); ESI (CH_3CN) m/z = 445.4 ([M + H]$^+$), m/z = 467.3 ([M + Na]$^+$).

4-*N*-(N^{α}-Boc-L-Arg^{NO2})-N^{α}-benzyl-*trans*-4-amino-L-proline-NH_2 (14). 12 (0.438 g, 0.0015 mol) was dissolved in 0.5 M NaOH aqueous solution (10 mL) and then extracted with CH_2Cl_2 (10 mL × 2). The combined organic layers were washed with water (10 mL) to generate the free base form of **12**. A solution of N^{α}-Boc-L-Arg^{NO_2}-OH (0.319 g, 0.001 mol) in dry THF (20 mL) was cooled to −15 °C, and 4-methylmorpholine (0.111 g, 0.121 mL, 0.0011 mol) and isobutyl chloroformate (0.150 g, 0.144 mL, 0.0011 mol) were added. The reaction mixture was stirred for 30 min, and the free base form of **12** (0.241 g, 0.0011 mol) in anhydrous THF (5 mL) was added via a cannula. The solution was stirred for 30 min at −15 °C and then allowed to warm to room temperature over 30 min. The reaction mixture was stirred at room temperature for 1 h, and the solvent was evaporated in vacuo. The residue was suspended in EtOAc (20 mL) and washed successively with 5% $NaHCO_3$ (2 × 20 mL) and saturated NaCl (20 mL). The solution was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 9:1) to afford 0.36 g of a colorless oil (70%): ^{1}H NMR (500 MHz, CD_3OD) δ 7.344−7.214 (m, 5H), 4.295−4.266 (m, 1H), 4.000−3.800 (m, 1H), (3.878 + 3.852) (d, 1H, J = 13 Hz), (3.521 + 3.495) (d, 1H, J = 13 Hz), 3.288−3.206 (m, 4H), 2.280 (t, 1H, J = 8.5 Hz), 2.177−2.123 (m, 1H), 2.087−2.026 (m, 1H), 1.696−1.525 (m, 4H), 1.400 (s, 9H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 179.8 (1C), 174.8 (1C), 161.1 (1C), 157.9 (1C), 139.2 (1C), 130.3 (2C), 129.6 (2C), 128.6 (1C), 80.8 (1C), 67.0 (1C), 60.2(1C), 58.9 (1C), 55.6 (1C), 41.8 (2C), 37.8 (1C), 30.8 (1C), 28.8 (3C), 26.1 (1C); ESI (CH_3CN) m/z = 521.1 ([M + H]$^+$), m/z = 543.1 ([M + Na]$^+$).

4-*N*-(L-Arg^{NO2})-N^{α}-methyl-*trans*-4-amino-L-proline-NH_2 Ditrifluoroacetate (3). 13 (0.222 g, 0.001 mol) was dissolved in anhydrous CH_2Cl_2 (1 mL), and TFA (1 mL) was added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 1 h at room temperature. After the reaction was done, the solvent was evaporated in vacuo. The residue was partitioned between water (10 mL) and ethyl acetate (10 mL). The aqueous layer was then washed with ethyl acetate (2 × 10 mL). The combined aqueous layers were evaporated by high-vacuum rotary evaporation to afford a white foamy solid (0.286 g, quantitative yield): ^{1}H NMR (500 MHz, D_2O) δ 4.459−4.446 (m, 1H), 4.382−4.354 (m, 1H), 4.040−4.018 (m, 1H), 3.933−3.922 (m, 1H), 3.220−3.151 (m, 3H), 2.914 (s, 3H), 2.460 (m, 2H), 1.855 (m, 2H), 1.597 (m, 2H); ^{13}C NMR (125.7 MHz, D_2O) δ 169.6 (1C), 169.4 (1C), (162.9 + 162.6) (2C, TFA-COOH), 158.9 (1C), (119.6 + 117.5 + 115.1 + 112.8) (2C, TFA-CF_3), 67.3 (1C), 59.0 (1C), 52.8 (1C), 47.4 (1C), 40.9 (1C), 40.3 (1C), 34.6 (1C), 27.9 (1C), 23.0 (1C). Comb. Anal. ($C_{12}H_{24}N_8O_4$·3TFA) C, H, N.

HPLC conditions for the purification of 4-*N*-(L-Arg^{NO2})-N^{α}-methyl-*trans*-4-amino-L-proline-NH_2 ditrifluoroacetate (3): column, Phenomenex Gemini 5 μm C18 110A preparative column; column size, 250 × 21.2 mm 5 μm; injection concentration, 20 mg/mL; flow rate, 12 mL/min; detection, 254 nm; mobile phase, H_2O (0.1% TFA):60% CH_3CN−40% H_2O (0.08% TFA) = 97:3; isocratic method. The retention time was 5 min.

4-*N*-(L-Arg^{NO2})-N^{α}-benzyl-*trans*-4-amino-L-proline-NH_2 ditrifluoroacetate (4). 14 (0.260 g, 0.5 mmol) was dissolved in anhydrous CH_2Cl_2 (1 mL), and TFA(1 mL) was added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and for 1 h at room temperature. After the reaction was completed, the solvent was evaporated in vacuo, and the residue was dissolved in water (10 mL). The color impurity was extracted with ethyl acetate (10 mL). The aqueous layer was then washed with ethyl acetate (2 × 10 mL), and the combined aqueous layers were evaporated by high-vacuum rotary evaporation to afford a white foamy solid (0.324 g, quantitative yield): ^{1}H NMR (500 MHz, D_2O) δ 7.279−7.242 (m, 5H), 4.329−4.183 (m, 4H), 3.807−3.772 (m, 2H), 3.151−3.074 (m, 3H), 2.338−2.215 (m, 2H), 1.725 (m, 2H), 1.466 (m, 2H); ^{13}C NMR (125.7 MHz, D_2O) δ 169.5 (1C), 169.4 (1C), (162.8 + 162.5 + 162.3 + 162.0) (2C, TFA-COOH), 158.6 (1C), 130.8 (2C), 130.4 (1C), 129.3 (2C), 128.7 (1C), (119.5 + 117.2 + 114.9 + 112.6) (2C, TFA-CF_3), (64.8 + 64.7) (1C), 58.9 (1C), 57.1 (1C), (52.8 + 52.7) (1C), (47.14 + 47.09) (1C), 40.2 (1C), 34.3 (1C), 27.9 (1C), 22.9 (1C); ESI (CH_3OH) m/z = 421.3 ([M + H]$^+$), m/z = 443.3 ([M + Na]$^+$); HRMS (ES+, CH_3OH) calcd for 421.2312, found 421.2319. Comb. Anal. ($C_{18}H_{28}N_8O_4$·3TFA) C, H, N.

HPLC conditions for the purification of 4-*N*-(L-Arg^{NO2})-*N*$^{\alpha}$-benzyl-*trans*-4-amino-L-proline-NH$_2$ ditrifluoroacetate (4): column, Phenomenex Gemini 5 μm C18 110A preparative column; column size, 250 × 21.2 mm 5 μm; injection concentration, 20 mg/mL; flow rate, 12 mL/min; detection, 254 nm; the mobile phase, H_2O (0.1% TFA):60% CH_3CN−40% H_2O (0.08% TFA) = 70:30; isocratic method. The retention time was 10 min.

***N*$^{\alpha}$-Boc-*trans*-3-hydroxy-L-proline (15).** A solution of *trans*-3-hydroxy-L-proline (1.311 g, 0.01 mol) in a 2:1 mixture of THF/H_2O (20 mL) was treated first with 10% aqueous NaOH (4.5 mL) and then with di-*tert*-butyl dicarbonate (3.274 g, 0.015 mol). The reaction mixture was stirred at room temperature overnight, and THF was evaporated in vacuo. The residue was adjusted to pH 2 by the addition of 10% aqueous $KHSO_4$. The acidic solution was extracted several times with ethyl acetate. The combined organic extracts were washed with H_2O and brine and then dried over anhydrous Na_2SO_4. Removal of the desiccant and evaporation of the solvent in vacuo gave the product as a solid, which was purified by silica gel column chromatography (EtOAc:MeOH:AcOH = 200:0.5:0.5) to afford a white solid (2.31 g, quantitative yield): ^{1}H NMR (500 MHz, CD_3OD) δ 4.371−4.330 (m, 1H), 4.181−4.029 (m, 1H), 3.542−3.439 (m, 2H), 2.002−1.968 (m, 1H), 1.862 (m, 1H), 1.458−1.333 (m, 9H); ^{13}C NMR (125.7 MHz, CD_3OD) δ (174.2 + 173.9) (1C), (156.3 + 155.9) (1C), (81.5 + 81.3) (1C), (75.7 + 74.9) (1C), (69.3 + 68.9) (1C), (45.8 + 45.3) (1C), (33.4 + 32.8) (1C), (28.9 + 28.7) (1C); ESI (CH_3OH) m/z = 232.1 ([M + H]$^+$), m/z = 463.1 ([2M + H]$^+$).

***N*$^{\alpha}$-Boc-*trans*-3-hydroxy-L-proline-NH$_2$ (16).** To a cooled solution (−15 °C) of **15** (0.231 g, 0.001 mol) in anhydrous THF (10 mL) was added 4-methylmorpholine (0.111 g, 0.121 mL, 0.0011 mol) and isobutyl chloroformate (0.150 g, 0.144 mL, 0.0011 mol); stirring continued for 30 min. NH_4OH (28% aqueous solution, 0.188 g, 0.206 mL, 0.0015 mol) was added, and the mixture was stirred at −15 °C for 30 min and then warmed to room temperature during 1 h. The solvent was removed under reduced pressure, and the residue was purified directly by silica gel column chromatography (CH_2Cl_2:MeOH = 9:1) to afford a white foamy solid (0.230 g, quantitative yield): ^{1}H NMR (500 MHz, CD_3OD) δ 4.315−4.285 (m, 1H), 4.103−4.000 (m, 1H), 3.569−3.517 (m, 2H), 2.062−1.996 (m, 1H), 1.851−1.842 (m, 1H), 1.474−1.401 (m, 9H); ^{13}C NMR (125.7 MHz, CD_3OD) δ (176.2 + 175.8) (1C), (156.7 + 156.2) (1C), (81.7 + 81.5) (1C), (76.5 + 75.5) (1C), (70.6 + 70.3) (1C), (46.2 + 45.7) (1C), (33.7 + 33.1) (1C), (28.82 + 28.76) (1C); ESI (CH_3OH) m/z = 231.2 ([M + H]$^+$), m/z = 253.2 ([M + Na]$^+$), m/z = 483.3 ([2M + Na]$^+$).

***N*$^{\alpha}$-Boc-*cis*-4-(4-nitrobenzoyloxy)-L-proline-NH$_2$ (17).** Diisopropyl azodicarboxylate (0.230 g, 0.227 mL, 0.0015 mol) was added slowly via syringe to a stirred ice-cooled solution of **16** (0.230 g, 0.001 mol), *p*-nitrobenzoic acid (0.250 g, 0.0015 mol), and triphenylphosphine (0.393 g, 0.0015 mol) in dry THF (10 mL) under nitrogen. The reaction temperature was allowed to rise to room temperature. After 36 h the solvent was evaporated under reduced pressure, and the residue was purified directly by silica gel column chromatography (hexanes:EtOAc = 3:7) to afford a pale yellowish oil (0.23 g, 61%): ^{1}H NMR (500 MHz, $CDCl_3$) δ (8.252 + 8.235) (d, 2H, J = 8.5 Hz), (8.166 + 8.149) (d, 2H, J = 8.5 Hz), 6.422 (s, 1H), 6.322 (s, 1H), 5.718 (m, 1H), 4.566 (m, 1H), 3.691 (m, 2H), 2.303 (m, 2H), 1.441 (s, 9H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 171.2 (1C), 164.0 (1C), 154.5 (1C), 150.8 (1C), 135.0 (1C), 131.0 (2C), 123.7 (2C), 81.3 (1C), (75.3 + 74.2) (1C), (63.5 + 62.2) (1C), 44.5 (1C), 30.2 (1C), 28.3 (1C); ESI (CH_3OH) m/z = 380.1 ([M + H]$^+$), m/z = 402.3 ([M + Na]$^+$), m/z = 780.9 ([2M + Na]$^+$).

***N*$^{\alpha}$-Boc-*cis*-3-hydroxy-L-proline-NH$_2$ (18).** A solution of **17** (0.379 g, 0.001 mol) and NaN_3 (0.195 g, 0.003 mol) in dry MeOH (10 mL) was warmed to 40 °C for 14 h under nitrogen. The solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 9:1) to afford a white solid (0.23 g, quantitative yield): ^{1}H NMR (500 MHz, CD_3OD) δ 4.497−4.485 (m, 1H), 4.172−4.159 (m, 1H), 3.586−3.538 (m, 1H), 3.415−3.351 (m, 1H), 2.016−1.897 (m, 2H), 1.416−1.393 (m, 9H); ^{13}C NMR (125.7 MHz, CD_3OD) δ (175.3 + 175.0) (1C), (156.4 + 156.2) (1C), (81.5 + 81.3) (1C), (72.9 + 72.2) (1C), (66.0 + 65.4) (1C), (45.8 + 45.3) (1C), (33.6 + 33.1) (1C), (28.8 + 28.7) (1C); ESI (CH_3OH) m/z = 231.2 ([M + H]$^+$), m/z = 253.3 ([M + Na]$^+$), m/z = 483.1 ([2M + Na]$^+$).

***N*$^{\alpha}$-Boc-*trans*-3-azido-L-proline-NH$_2$ (19).** To an ice-cooled solution of **18** (0.230 g, 0.001 mol) in dry pyridine (5 mL) was added dropwise methanesulfonyl chloride (0.172 g, 0.117 mL, 0.0015 mol). The reaction was stirred at 0 °C for 2 h and then kept in a refrigerator overnight. Pyridine was evaporated under reduced pressure, and the residue was partitioned between water (10 mL) and EtOAc (10 mL). The aqueous layer was extracted with EtOAc (10 mL × 3). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and evaporated to give the mesylate intermediate, which was used without further purification.

The dry mesylate intermediate was dissolved in 3 mL of dry DMF in the presence of NaN_3 (0.098 g, 0.0015 mol). The resulting mixture was gradually heated to 70 °C over 2 h and then stirred at 70 °C for 21 h. The solvent was removed under reduced pressure. The residue was partitioned between water (10 mL) and EtOAc (10 mL), and the aqueous layer was extracted with EtOAc (10 mL × 2). The combined organic layers were then washed with brine, dried over $MgSO_4$, filtered, and evaporated. The resulting crude oil was then purified by silica gel column chromatography (hexanes:EtOAc = 3:7) to provide a thick colorless oil (0.255 g, 62%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.479 (m, 1H), 4.310 (m, 1H), 3.556−3.500 (m, 2H), 2.223−2.007 (m, 2H), 1.478 (m, 9H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ (172.9 + 172.6) (1C), (155.7 + 154.4) (1C), (81.5 + 81.3) (1C), (66.5 + 65.1) (1C), (64.6 + 62.6) (1C), (45.2 + 44.8) (1C), (30.3 + 29.4) (1C), 28.4 (1C); ESI (CH_3OH) m/z = 256.2 ([M + H]$^+$), m/z = 278.5 ([M + Na]$^+$), m/z = 533.1 ([2M + Na]$^+$).

***N*$^{\alpha}$-Boc-*trans*-3-amino-L-proline-NH$_2$ (20). 19** (0.225 g, 0.001 mol) was dissolved in MeOH (10 mL) containing a catalytic amount of 10% Pd/C. The solution was stirred under a hydrogen atmosphere at room temperature for 21 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated in vacuo to provide a thick colorless oil (0.230 g, quantitative yield): ^{1}H NMR (500 MHz, CD_3OD) δ 4.745 (brs, 4H), 3.928−3.853 (m, 1H), 3.607−3.546 (m, 1H), 3.531−3.489 (m, 2H), 2.134−2.081 (m, 1H), 1.751−1.707 (m, 1H), 1.471−1.435 (m, 9H); ^{13}C NMR (125.7 MHz, CD_3OD) δ (177.0 + 176.6) (1C), (156.5 + 156.1) (1C), (81.6 + 81.3) (1C), (70.3 + 70.0) (1C), (58.4 + 57.4) (1C), (46.4 + 46.0) (1C), (33.5 + 33.0) (1C), (28.9+28.8) (1C); ESI (CH_3OH) m/z = 230.2 ([M + H]$^+$), m/z = 252.4 ([M + Na]$^+$), m/z = 459.3 ([2M + H]$^+$), m/z = 481.2 ([2M + Na]$^+$)

3-*N*-(*N*$^{\alpha}$-Boc-L-Arg^{NO_2})-*N*$^{\alpha}$-Boc-*trans*-3-amino-L-proline-NH$_2$ (21). A solution of *N*$^{\alpha}$-Boc-L-Arg^{NO_2}-OH (0.319 g, 0.001 mol) in dry THF (20 mL) was cooled to −15 °C, and *N*-methylmorpholine (0.111 g, 0.121 mL, 0.0011 mol) and isobutyl chloroformate (0.150 g, 0.144 mL, 0.0011 mol) were added. The reaction mixture was continued to stir for 30 min at −15 °C. **20** (0.252 g, 0.0011 mol) in anhydrous THF (5 mL) was added to the reaction mixture through a cannula. The solution was stirred for 30 min at −15 °C and then allowed to warm to room temperature over 30 min. The reaction mixture was stirred at room temperature for 1 h, and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 8.5:1.5) to afford a colorless oil (0.43 g, 81%): ^{1}H NMR (500 MHz, CD_3OD) δ 4.364 (m, 1H), 4.083−3.992 (m, 2H), 3.530−3.517 (m, 2H), 3.270−3.234 (m, 2H), 2.154−2.129 (m, 1H), 1.903−1.880 (m, 1H), 1.735−1.518 (m, 4H), 1.518−1.341 (m, 18H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 176.1 (1C), (175.6 + 174.8) (1C), 161.0 (1C), 157.9 (1C), (156.3 + 155.9) (1C), (81.9 + 81.6) (1C), 80.8 (1C), (67.1 + 66.9) (1C), (56.1 + 55.8) (1C), (55.5 + 55.4) (1C), (46.4 + 46.0) (1C), 41.9 (1C), (31.2 + 30.8) (1C), (30.6 + 30.5) (1C), (28.9 + 28.7) (6C), 26.0 (1C); ESI (CH_3OH) m/z = 531.5 ([M + H]$^+$), m/z = 553.5 ([M + Na]$^+$), m/z = 1061.4 ([2M + H]$^+$), m/z = 1083.2 ([2M + Na]$^+$).

3-*N*-(L-Arg^{NO_2})-*trans*-3-amino-L-proline-NH$_2$ (6). 21 (0.265 g, 0.001 mol) was dissolved in anhydrous CH_2Cl_2 (1 mL), and TFA (1 mL) was added at 0 °C. The reaction mixture was stirred for 1

h at 0 °C and 1 h more at room temperature. After the reaction was completed, the solvent was evaporated in vacuo, and the residue was dissolved in water (10 mL). The color impurity was extracted with ethyl acetate (10 mL × 2). The combined aqueous layers were evaporated under high-vacuum rotary evaporator to provide a white foamy solid (0.28 g, quantitative yield): ^{1}H NMR (500 MHz, D_2O) δ 4.465−4.429 (m, 1H), (4.210 + 4.200) (d, 1H, J = 5 Hz), 3.931 (t, 1H, J = 6.5 Hz), 3.451 (t, 2H, J = 7 Hz), 3.170 (m, 2H), 2.301−2.259 (m, 1H), 2.071−2.031 (m, 2H), 1.864−1.808 (m, 2H), 1.589 (m, 2H); ^{13}C NMR (125.7 MHz, D_2O) δ 170.3 (1C), (169.3 + 169.3) (1C), (163.0 + 162.7 + 162.4 + 162.1) (2C, TFA-COOH), 158.8 (1C), (119.7 + 117.4 + 115.1 + 112.8) (2C, TFA-CF_3), 64.2 (1C), (54.4 + 54.3) (1C), (52.9 + 52.8) (1C), 45.3 (1C), 40.8 (1C), 29.3 (1C), 28.0 (1C), 23.2 (1C); ESI (CH_3OH) m/z = 331.4 ([M + H]$^+$), m/z = 661.2 ([2M + H]$^+$); HRMS (ES+, CH_3OH) calcd 331.1829, found 331.1841. Comb. Anal. ($C_{11}H_{22}N_8O_4$·3TFA) C, H, N.

HPLC conditions for the purification of 3-*N*-(L-Arg^{NO_2})-*trans*-3-amino-L-proline-NH$_2$ (6): column, a Phenomenex Gemini 5 μm C18 110A preparative column; column size, 250 × 21.2 mm 5 μm; injection concentration, 20 mg/mL; flow rate, 12 mL/min; detection, 254 nm; mobile phase, H_2O (0.1% TFA):60% CH_3CN−40% H_2O (0.08% TFA) = 97:3; isocratic method. The retention time was 5 min.

Solid-Phase Synthesis of Dipeptide Analogue 5. Rink amide resin (0.1 mmol) was treated twice with 20% piperidine in DMF (5 mL) at room temperature for 5 and 20 min. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). Deprotected Rink amide resin (0.1 mmol) was suspended in DMF (1 mL). DIEA (0.2 mmol) and a solution of *N*-Boc-*cis*-3-azido-L-proline[19] (**22**, 0.2 mmol) and HBTU (0.2 mmol) in DMF (4 mL) were added sequentially. The resulting mixture was shaken at room temperature for 6 h and filtered. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). The resulting resin-bound azido proline *cis*-**24** was dried under vacuum for 2 h. Rink amide resin loaded with the azido proline derivative (*cis*-**24**, 0.1 mmol) was suspended in dry THF under nitrogen. A yellow solution of preformed $[Et_3NH]^+[Sn(PhS)_3]^-$ (0.2 mmol) was injected, and the mixture was stirred for 1 h at room temperature. The reaction was quenched with a saturated solution of K_2CO_3 in water. The resin was filtered and washed with water (3×) and THF (3×). Then the resin was treated twice with a mixture of THF/sat. solution of K_2CO_3 (2:1) for 15 min. The resin was filtered and washed with water (3×), methanol (3×), DCM (3×), and methanol (3×). Rink amide resin loaded with the amino proline derivative (*cis*-**25**, 0.1 mmol) was suspended in DMF (1 mL). DIEA (0.4 mmol) and a solution of Boc-L-Arg^{NO_2} (0.4 mmol) and HBTU (0.4 mmol) in DMF (4 mL) were added sequentially. The resulting mixture was shaken at room temperature for 3 h and filtered. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). This coupling reaction was repeated a second time. The resulting resin-bound dipeptide *cis*-**26** was dried under vacuum for 2 h. The resulting Rink amide resin loaded with the dipeptide (*cis*-**26**, 0.1 mmol) was swollen in DCM and filtered. A solution of TFA and DCM (50:50, 6 mL) containing 1% $SiHEt_3$ (TES) was added, and the resulting mixture was shaken for 30 min at room temperature. The suspension was filtered, and the resin was washed with DCM/TFA (8:2, 3×). The solvent was removed on the rotary evaporator, and the residue was treated with DCM and concentrated to dryness. This last step was repeated twice. The obtained dipeptide **5** was prepurified by solid-phase extraction (SPE) using a C-18 preconditioned column in water. The dipeptide was purified by HPLC using a semiprep column [Phenomenex, Luna, 250 × 10 mm, C18(2)] with a precolumn [Phenomenex, Luna 50 × 10 mm, C18(2)]. A 100 μL solution of 5 mg/100−200 μL was injected into a Beckman HPLC instrument (System Gold 125, UV detector 166) and chromatographed at a flow rate of 4 mL/min, with detection at 214 nm [program: (A) water + 0.1% TFA; (B) ACN + 0.1% TFA for 5 min using 1% of B, then 10% of B over 15 min., then 5 min using 10% of B, then 1% of B over 5 min; the total run time was 30 min]. Fractions containing the pure dipeptide were concentrated to dryness, and the residue was lyophilized: ^{1}H NMR (500 MHz, D_2O) δ 4.576−4.561 (d, 1H, J = 7.5 Hz), 4.024−4.000 (t, 1H, J = 6 Hz), 3.705−3.653 (m, 1H), 3.457−3.360 (m, 2H), 3.289 (m, 2H), 2.538−2.467 (m, 1H), 2.173−2.064 (m, 2H), 1.967−1.807 (m, 2H), 1.659 (m, 2H); ^{13}C NMR (100.7 MHz, D_2O) δ 169.9 (1C), 167.8 (1C), 159.1 (1C), 62.5 (1C), 52.8 (1C), 51.0 (1C), 44.4 (1C), 40.3 (1C), 29.7 (1C), 28.0 (1C), 23.9 (1C); ESI (CH_3OH) m/z = 331.5([M + H]$^+$), m/z = 661.3 ([2M + H]$^+$). Compound **6** also was made by this same solid-phase method except starting with **23**. The compound obtained was identical in all respects to that synthesized by the solution-phase method described above.

Method for the Preparation of Triethylammonium Tris(phenylthio)stannate. Preparation of $[Et_3NH]^+[Sn(PhS)_3]^-$ (*Caution*: Stench). $SnCl_2$ (0.2 mmol, 2 equiv) was solubilized in dry THF by sonicating the suspension under nitrogen. Thiophenol (0.8 mmol, 8 equiv) and TEA (1 mmol, 10 equiv) were sequentially injected under nitrogen. The obtained yellow suspension was filtered into a dry syringe using a 0.45 μM filter on line. This solution was used immediately.

General Method for the Solid-Phase Synthesis of *N*-Substituted Derivatives of 4-*N*-(L-Arg^{NO_2})-*trans*-4-amino-L-proline-NH$_2$ (3, 4) (see Supporting Information Scheme 1). Rink amide resin (0.1 mmol) was treated twice with 20% piperidine in DMF (5 mL) at room temperature for 5 and 20 min. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). The deprotected Rink amide resin (0.1 mmol) was suspended in DMF (1 mL). DIEA (0.2 mmol) and a solution of *N*-Boc-*trans*-4-azido-L-proline (0.2 mmol) and HBTU (0.2 mmol) in DMF (4 mL) were added sequentially. The resulting mixture was shaken at room temperature for 6 h and filtered. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). The resulting resin-bound amino acid **28** was dried under vacuum for 2 h. Rink amide resin loaded with the azido proline derivative **28** (0.1 mmol) was suspended in dry THF under nitrogen. A yellow solution of preformed $[Et_3NH]^+[Sn(PhS)_3]^-$ (0.2 mmol) was injected, and the mixture was stirred for 1 h at room temperature. The reaction was quenched with a saturated solution of K_2CO_3 in water. The resin was filtered and washed with water (3×) and THF (3×). Then the resin was treated twice with a mixture of THF/sat. solution of K_2CO_3 (2:1) for 15 min. The resin was filtered and washed with water (3×), methanol (3×), DCM (3×), and methanol (3×). Rink amide resin loaded with the amino proline derivative **29** (0.1 mmol) was suspended in NMP (1 mL). DIEA (0.4 mmol) and a solution of Fmoc-L-Arg^{NO_2} (0.4 mmol) and HBTU (0.4 mmol) in NMP (4 mL) were added sequentially. The resulting mixture was shaken at room temperature for 3 h and filtered. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). This coupling reaction was repeated for a second time. The resulting resin-bound dipeptide **30** was dried under vacuum for 2 h. The resulting Rink amide resin loaded with the dipeptide (**30**, 0.1 mmol) was treated twice with 20% piperidine in DMF (5 mL) at room temperature for 5 and 20 min. The resin was washed with DMF (3×), DCM (2×), and MeOH (3×). Rink amide resin **31** (0.1 mmol) was preswollen with dry THF and a solution of (trifluoroacetyl)-benzotriazole (1 mmol) in dry THF was added. The suspension was shaken at room temperature for 2 h. The resin was washed with THF (3×), MeOH (3×), DCM (3×), and MeOH (3×). The resulting resin-bound protected dipeptide **32** was dried under vacuum for 2 h. The Boc-protected proline-containing dipeptide loaded resin **32** was dried overnight under nitrogen and then suspended in dry DCM (0.1 mmol in 3 mL of dry DCM). The suspension was cooled in an ice bath, and 2,6-lutidine (0.6 mmol) and TMSOTf (0.4 mmol) were sequentially injected dropwise under argon. The suspension was warmed to room temperature after 10 min and left stirring for 2 h under argon. The reaction was quenched using a saturated solution of ammonium chloride in water. The resin **33** was washed with DCM (3×), MeOH (3×), water (3×), MeOH (3×), and DCM (3×). Deprotected intermediate **33** (0.1 mmol) was dried under vacuum, and a freshly prepared solution of formaldehyde (for **3**) or benzaldehyde (for **4**) (1 mmol) in trimethylorthoformate (4 mL) was added under nitrogen. The same solution (2 mL) was added again after 1 h at room temperature. The suspension

was stirred for 1 h at room temperature, and $NaBH_3CN$ (1 mmol) was added in 1 mL of TMOF. The suspension was washed after 15 h with MeOH (3×), DCM (3×), MeOH (3×), H_2O (2×), and MeOH (2×). Resin **34** was dried under vacuum for 2 h. The N^{α}-substituted loaded resin (**34**, 0.1 mmol) was preswollen with THF, and a solution of LiOH (1 mmol) in THF/H_2O (5:1) was added. The resin was shaken at room temperature for 3 h and washed with THF (3×), MeOH (3×), H_2O (3×), and MeOH (3×). The deprotected dipeptide loaded resin **35** was dried under vacuum for 2 h. The resulting Rink amide resin loaded with the dipeptide (**35**, 0.1 mmol) was swollen in DCM and filtered. A solution of TFA and DCM (50:50, 6 mL) containing 1% $SiHEt_3$ (TES) was added, and the resulting mixture was shaken for 30 min at room temperature. The suspension was filtered, and the resin was washed with DCM/TFA (8:2, 3×). The solvent was removed on a rotary evaporator, and the residue was treated with DCM and concentrated to dryness. This last step was repeated twice. The obtained dipeptide **3** or **4** was further purified by solid-phase extraction (SPE) using a C-18 preconditioned column in water.

Enzyme and Assay. All of the NOS isoforms used were recombinant enzymes overexpressed in *E. coli*. The murine macrophage iNOS was expressed and isolated according to the procedure of Hevel et al.[24] The rat nNOS was expressed[25] and purified as described. The bovine eNOS was isolated as reported.[26] Nitric oxide formation from NOS was monitored by the hemoglobin capture assay as described.[27]

Determination of K_i Values. The apparent K_i values were obtained by measuring percent inhibition in the presence of 10 μM L-arginine with at least three concentrations of inhibitor. The parameters of the following inhibition equation[28] were fitted to the initial velocity data: % inhibition $= 100[I]/\{[I] + K_i(1 + [S]/K_m)\}$. K_m values for L-arginine were 1.3 μM (nNOS), 8.2 μM (iNOS), and 1.7 μM (eNOS). The selectivity of an inhibitor was defined as the ratio of the respective K_i values.

Molecular Modeling. Docking simulations were carried out with AutoDock 3.0.5 as described by Ji et al.[22] The protein crystal structure used in the docking studies is that of (4*S*)-*N*-(4-amino-5-[aminoethyl] aminopentyl)-*N*′-nitroguanidine bound to nNOS (PDB ID: 1p6i). The ligands were built in SYBYL version 6.8[29] by modifying the molecular structure of **2**, which was extracted from the crystal structure of 1p6j (PDB ID). Energy minimizations were performed following the addition of hydrogen atoms and partial atom charge calculations by the Gasteiger−Marsili method.[30] Appropriate atom types were specified and labeled for physiological conditions, and ligand chirality was inspected. The grid maps were calculated using AutoGrid. The dimensions of the grid box was 31 × 28 × 31 Å. Default parameters as described in detail previously were used, and 100 docked conformations were yielded. A visual comparison of the superimposition of the guanidino moiety of the calculated conformations and that of **2** served as a preliminary identification of appropriate structures. The binding affinity of the inhibitors to the protein were then evaluated by the total AutoDock docking energies and further rescored using the CScore module of Sybyl 6.8 comprising the following scoring functions: F_Score, D_Score, PMF_Score, G_Score, and ChemScore. For rescoring docked poses, atomic coordinates of the target protein were unchanged, and relaxation of the bound ligand was performed.

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Supporting Information Available: Synthesis details for **3** and **4**; NMR, mass spectral, and elemental analysis data for **3**−**6**; and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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