Potent and Selective Conformationally Restricted Neuronal Nitric Oxide Synthase Inhibitors

José A. Gómez-Vidal,[§,⊥] Pavel Martásek,[‡,†] Linda J. Roman,[‡,∥] and Richard B. Silverman*[,§]

Department of Chemistry, Department of Biochemistry, Molecular Biology, and Cell Biology, and the Drug Discovery Program, Northwestern University, Evanston, Illinois 60208-3113, and Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284-7760

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Selective inhibition of the isoforms of nitric oxide synthase (NOS) in pathologically elevated synthesis of nitric oxide has great therapeutic potential. We previously reported nitroarginine-containing dipeptide amides (Huang, H.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *J. Med. Chem.* **1999**, *42*, 3147) and some peptidomimetic analogues (Huang, H.; Martasek, P.; Roman, L. J.; Silverman, R. B. *J. Med. Chem.* **2000**, *43*, 2938) as potent and selective inhibitors of neuronal NOS (nNOS). Here we report conformationally restricted dipeptides derived from the dipeptide L-Arg^{NO_2}-L-Dbu-NH_2 (**8**). The selectivities for nNOS over endothelial NOS and inducible NOS of the most potent nNOS inhibitor (**10a**) among these compounds are comparable to that of the parent compound. An unsubstituted amide bond is necessary for potency against nNOS. The stereochemistry of compound **10a** was optimum for potency and selectivity and thus provides the binding conformation of the parent compound with nNOS.

Introduction

Nitric oxide (NO), a ubiquitous biological messenger involved in a variety of physiological processes, 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 have also been described.[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 an NADPH- and O_2-dependent process (reaction I).[3]

$$\text{L-Arg} + 2O_2 + 1.5\text{NADPH} + 2H^+ \rightarrow \text{L-citrulline} + \bullet\text{NO} + 2H_2O + 1.5\text{NADP}^+ \quad \text{(I)}$$

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, inducible NOS (iNOS), 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 forms do not bind Ca^{2+}/calmodulin at basal levels of Ca^{2+}, while the inducible form does. All three NOS isoforms are functional only as tight homodimers through interactions between the heme domains. NOS monomers consist of a reductase domain that contains binding sites for NADPH, FAD, and FMN and an oxygenase domain that has binding sites for L-Arg, the heme prosthetic group, and tetrahydrobiopterin (BH_4). A zinc tetrathiolate center at the dimer interface aids in dimer stabilization and formation of the pterin binding site.[4−6] Dimerization of NOS is required for fully coupled enzyme activity because the flow of electrons during catalysis occurs from the reductase domain of one monomer subunit to the oxygenase domain of the other monomer.[7]

The crystal structures of the oxygenase domains of the murine iNOS monomer,[8] murine and human iNOS dimers,[9−12] and human and bovine endothelial NOS dimers[13] indicate a high degree of structural similarity within the catalytic center and dimer interface regions between the NOS isoforms. They share only approximately 50% of the primary sequence homology, suggesting that they may differ from each other in regulatory aspects. The N-terminal segment exhibits the largest variation in sequence. While the N-terminal segments are not essential for catalytic activity, they are involved in cellular targeting and protein−protein interactions. The ceiling heights directly above the iron atom of the heme sites differ, suggesting the size of the active sites decrease in the order nNOS > iNOS > eNOS.[14] The subtle differences among the substrate sites of the NOS isozymes can be used to design a second generation of selective NOS inhibitors with therapeutic potential.

The use of NOS inhibitors against pathologically elevated synthesis of NO has great therapeutic poten-

* To whom correspondence should be addressed at the Department of Chemistry. Phone: (847) 491-5653. Fax: (847) 491-7713. E-mail: Agman@chem.northwestern.edu.

§ Northwestern University.

⊥ Carried out all experiments in this study.

‡ The University of Texas Health Science Center.

† Developed the eNOS overexpression system in *E. coli* and isolated and purified the eNOS.

∥ Developed the nNOS overexpression system in *E. coli* and the purification of the enzyme.

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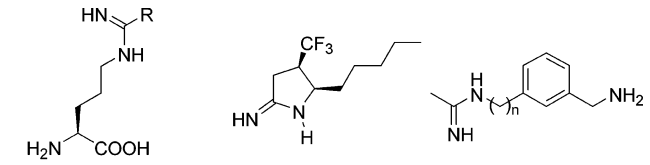


Figure 1. NOS inhibitors.

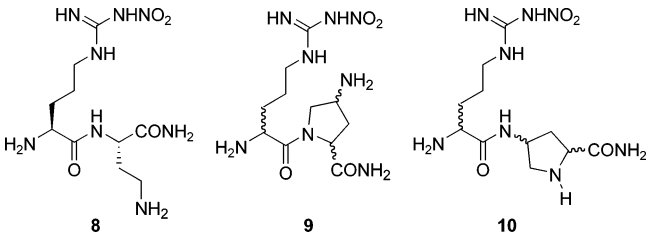
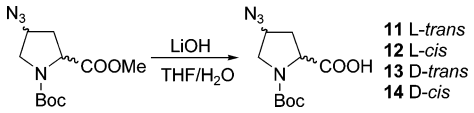


Figure 2. Compounds **9** and **10** are conformationally restricted analogues of **8**.

Scheme 1



tial.[15] NO overproduction by nNOS has been associated with neurodegeneration[16] during stroke,[17] chronic headaches,[18] and Alzheimer's disease.[19] Thus, nNOS represents a therapeutic target for inhibitors.[20] Enhanced formation of NO following the induction of iNOS appears to be important in arthritis,[21] development of colitis,[22] and tissue damage and inflammation.[23] 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 also be useful tools for investigating other biological functions of NO.[24]

Two broad categories of NOS inhibitors have been described: (a) substrate analogues and heme ligands and (b) pterin antagonists. Analogues of the substrate L-arginine include *N*-alkyl-L-arginines (**1**), N^{ω}-nitro-L-arginine (**2**), and N^5-(imino-3-butenyl)-L-ornithine (**3**) (Figure 1). Most of these have minimal selectivity among the isozymes, except for *N*-propyl-L-arginine[25] and **3**,[26] which are highly selective inhibitors for nNOS over iNOS and eNOS. There also are numerous non amino acid based NOS inhibitors, for example, (+)-*cis*-5-pentyl-4-(trifluoromethyl)pyrrolidin-2-imine (**5**), *N*-(3-(aminomethyl)phenyl)acetamidine (**6**), and *N*-(3-(aminomethyl)benzyl)acetamidine (**7**) (Figure 1). The iminopyrrolidine **5** belongs to a large group of cyclic amidine type inhibitors of NOS.[27] These non amino acid inhibitors have significantly improved selectivity. Arylamidine **6** is a highly potent and selective nNOS inhibitor (nNOS, IC_{50} = 11 nM; eNOS, IC_{50} = 1100 nM; iNOS, IC_{50} = 480 nM),[28] and arylamidine **7** has been characterized as a selective iNOS inhibitor.[29] In some cases, the selectivity is as high as 5000-fold (**5**) and 900-fold (**7**), in favor of iNOS over eNOS. However, such high selectivity has not normally been observed for the inhibition of nNOS over eNOS (the above few examples are exceptions). Recently, allosteric inhibitors of iNOS dimerization have been described.[30]

(*S*)-Alkyl-L-isothiocitrulline-containing dipeptides have been reported as selective iNOS competitive inhibitors.[31,32] (*S*)-Methyl-L-isothiocitrulline (**4**) was identified as a potent nNOS inhibitor (IC_{50} = 60 nM).[33] Thioimidates generally confer more NOS inhibitory potency than their corresponding amidines. The combination of a hydrophobic L-amino acid with the isothiourea derivative altered the inhibition pattern to give selective iNOS inhibitors.

Our interest has been in the selective inhibition of nNOS. Because **2** was reported to have more than 250-fold selectivity in favor of nNOS over iNOS,[34] we synthesized and evaluated nitroarginine-containing dipeptides to see if the incorporation of nitroarginine into a dipeptide could increase the inhibitory potency and selectivity of L-nitroarginine.[35,36] Hence, a library of 152 dipeptide amides containing nitroarginine was screened for activity. Excellent inhibitory potency and selectivity for nNOS over eNOS and iNOS was achieved with dipeptide amides containing a basic amine side chain. The most potent nNOS inhibitor among these initial compounds was L-Arg^{NO_2}-L-Dbu-NH_2 (**8**; K_i = 130 nM, Figure 2), which also displayed selectivity over eNOS (>1500-fold) and iNOS (192-fold).

The side chain amino group of **8** binds to the enzyme via a hydrogen bond to a water molecule, which interacts with the active-site tetrahydrobiopterin.[37] Because of free rotation in the aminoethyl side chain of L-Dbu, there are many conformations that are not ideally suited for this interaction. If the ideal conformation were incorporated, greater potency and selectivity would be attained.

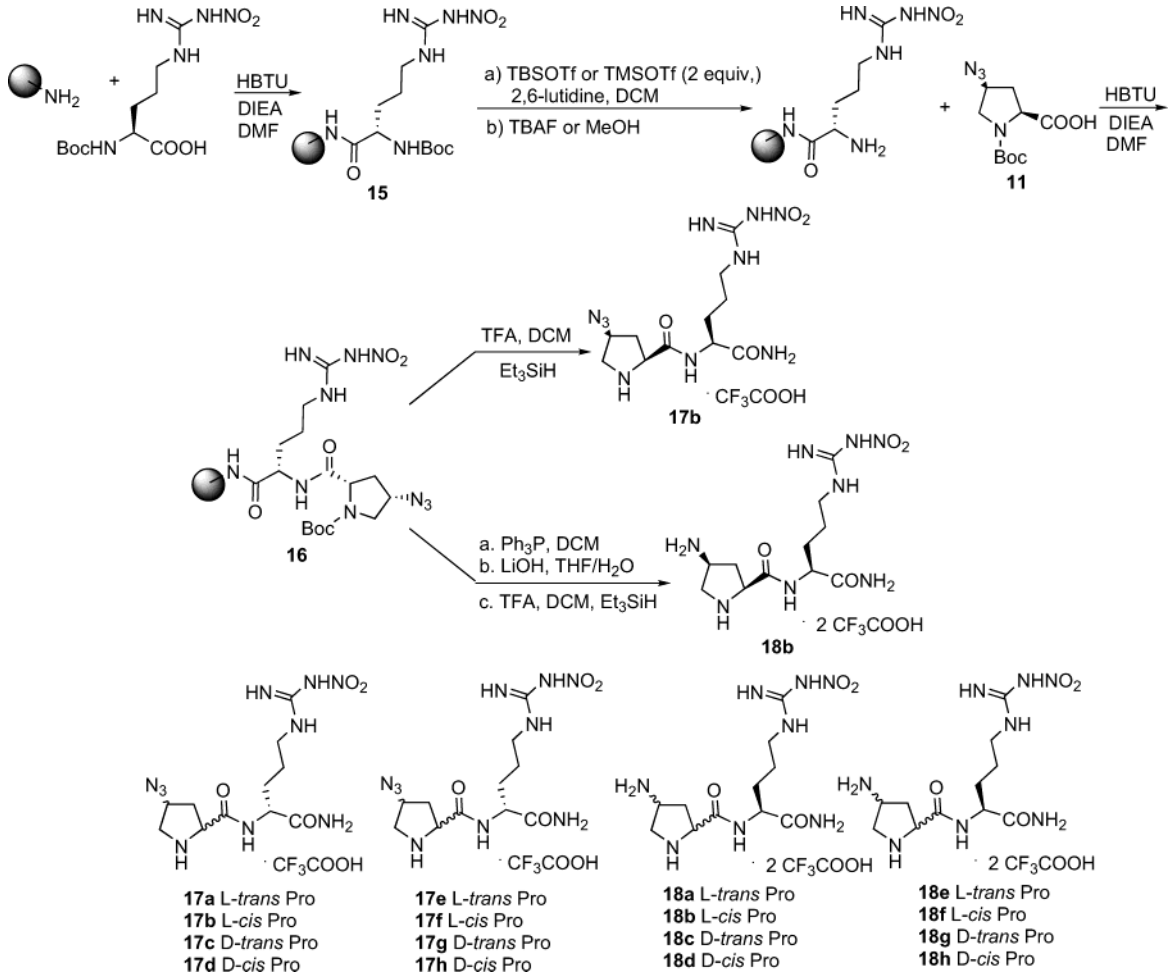
For these reasons, **8** has been used as a lead compound for the development of more potent and selective conformationally restricted dipeptide analogues. By this approach, a more optimal orientation of the amino group may be determined for better activity and selectivity. To that end, we have synthesized 4-aminoproline analogues **9** and **10**. The synthesis of these compounds was implemented on a solid phase, and the compounds were screened for activity against nNOS and iNOS. The most potent compounds were purified by HPLC, and the K_i values were determined against all three isozymes.

Chemistry

cis- and *trans*-4-Amino-L- and -D-proline derivatives were designed as constrained mimics to obtain conformationally restricted dipeptide amides derived from the dipeptide amide **8**. The 4-substituted proline precursors used in solid-phase synthesis were synthesized in 80−95% yield from the corresponding esters using LiOH in THF/H_2O (Scheme 1). The synthesis of the methyl esters has already been reported.[38]

The general solid-phase synthesis of L-Arg^{NO_2}-L-Dbu-NH_2 derivatives with *cis*- and *trans*-4-amino-L- and -D-proline at the N-terminus is depicted in Scheme 2. The Burgess methodology[39] to deprotect Boc-amines on a Rink resin was found not to be selective for *N*-Boc-L- and -D-Arg^{NO_2} (**15**). This synthetic procedure, which uses an excess of (TMS)OTf and 2,6-lutidine, afforded a pure dipeptide derivative but with different ^1H NMR and ESMS data; the nitroarginine moiety was affected under these conditions. It has been reported that nitroalkanes are silylated by an excess of (TMS)OTf and lead to a

Scheme 2



migration of the trialkylsilyloxy group to give oximes.[40] These compounds were not inhibitors of nNOS, and their complete structure determination was not pursued. The addition of only 2 equiv of (TMS)OTf or TBSOTf[41] (Scheme 2) instead of an excess of (TMS)OTf is critical to avoid a rearrangement of the *N*-nitroguanidino fragment during the deprotection reaction. TBAF is needed to cleave the intermediate silyl carbamate and to obtain the free amine if TBSOTf is used.

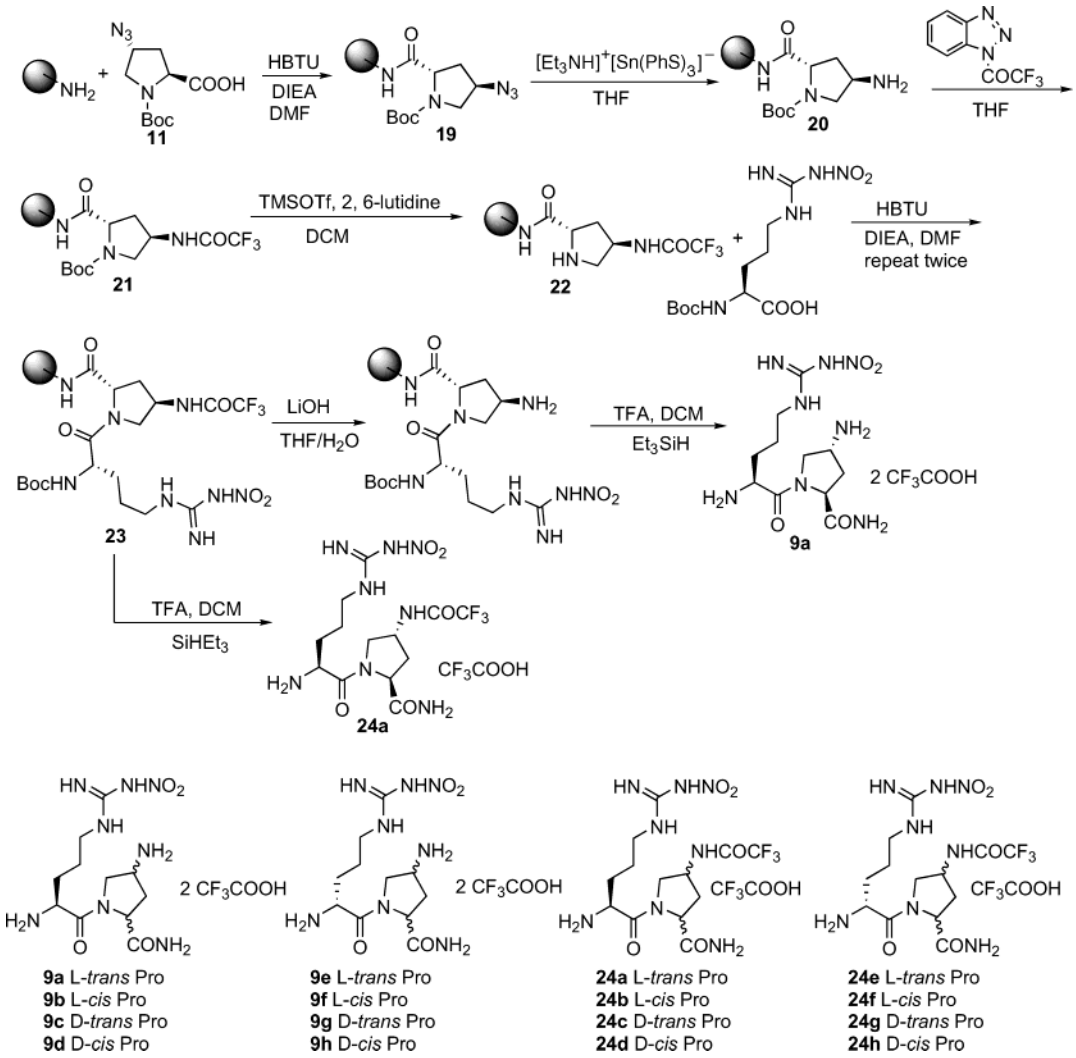
Analysis of the conditions of the reaction also showed that a nucleophilic hydroxide is needed to cleave the adduct intermediate during the Staudinger azide reduction on a solid phase (**16**, Scheme 2).[42] The use of lithium hydroxide in THF afforded the final products (**18a**−**h**) in high purity after resin cleavage. Azido compounds of type **17** also were synthesized for SAR studies.

The synthesis of dipeptide amides containing C-terminal 4-aminoprolinamide isomers with the proline nitrogen as part of the peptide bond also was carried out (**9** and **24**, Scheme 3). After azide reduction the resultant amine was protected as the trifluoroacetamide **21**. This additional step was found to be necessary because of the instability of azide **19** during *N*-Boc deprotection using (TMS)OTf. A variety of conditions with (TMS)OTf and other reagents were tried, but the purity of the products obtained was always lower than expected.

(TMS)I has been used for *N*-Boc deprotection,[43] but it had not been applied to a solid-phase synthesis. When applied to the *N*-Boc-L-Pro-loaded Rink resin, it allowed for the synthesis of L-Pro-containing dipeptides in high purity. This represents a new use of (TMS)I for the removal of *N*-Boc on the TFA-sensitive Rink resin. However, when (TMS)I was used to deprotect azide **19**, no product was obtained, showing the lack of selectivity of this reagent. These results demonstrate that the azide substituent in **19** is unstable during the deprotection step. This reaction with the azide is not general because it has been described previously with *N*-Boc deprotection in the presence of a secondary azide.[44] A possible explanation for this unusual reactivity has been offered recently.[45]

The reduction of azide **19** was initially accomplished under Staudinger conditions using triphenylphosphine. The dipeptide was obtained in 80% purity as shown in its ^{1}H NMR spectrum. Analysis of the ^{1}H NMR spectrum of the final compound showed that the main impurity was a derivative of the adduct intermediate during the Staudinger reaction. Consequently, trimethylphosphine was used in place of triphenylphosphine, but the purity did not improve. Triethylammonium tris(phenylthio)stannate has been described as a very efficient reducing agent with a high chemoselectivity toward azides.[46] This reagent has been applied successfully on a solid phase using Rink resin[47] and other resins,[48] and it afforded amine **20** in high purity (Scheme 3). Therefore, the azide group was reduced, and the resulting amine **20** was protected as its trifluoro-

Scheme 3



acetamide[49] using (trifluoroacetyl)benzotriazole[50] prior to *N*-Boc deprotection (Scheme 3).

N-Boc deprotection of intermediate **21** was studied to find the most mild conditions using (TMS)OTf. The results showed that an excess of 2,6-lutidine is necessary. The reaction did not work, and/or the resin was affected using a 1:1 ratio of (TMS)OTf and 2,6-lutidine. The best conditions were 4 equiv of (TMS)OTf and a ratio of 1:1.5 of the reagents.

Following the coupling reaction to give **23**, deprotection of **23** using lithium hydroxide in a THF/H_2O mixture and resin cleavage afforded **9a** (and its isomers **9b**−**h**) in high purity (Scheme 3). The corresponding trifluoroacetamido-protected analogues **24a**−**h** were also obtained for SAR studies.

The synthesis of nonnatural dipeptide amides **10a**−**h** containing L- or D-Arg^{NO_2} and isomers of 4-aminoprolinamide with the 4-amino group in the peptide bond was conducted as shown in Scheme 4. This third family of dipeptide amides was synthesized using the 4-amino group of resin-bound Boc-4-aminoproline (**20**) in the coupling step. These structures are rigid analogues of the lead compound but without a substituted peptide bond nitrogen. Thirty-two isomers were synthesized from Schemes 2 and 3, and eight isomers were synthesized from Scheme 4.

Biological Results

None of the 32 analogues containing the 4-aminoproline isomers at either the N- or C-terminus (**17**, **18**, **9**, and **24**) exhibited significant activity at 100 μM against iNOS (IC_{50} > 100 μM). Some compounds have an IC_{50} value of 100 μM or less against nNOS. Only three dipeptide amide analogues that contain a C-terminal 4-aminoprolinamide (**9a**, **9h**, **24a**) exhibited some nNOS inhibition at 100 μM ($IC_{50} \approx 100$ μM). This supports the importance of a nonsubstituted peptide bond for the interaction with the enzyme.[51]

The potency and selectivity of nitroarginine-containing dipeptides diminish dramatically when the peptide bond hydrogen is substituted by a methyl group.[51] This suggests a possible hydrogen bond at that position, which would be lost in compounds such as **9** and **24**. It is now known that, in fact, there is an important hydrogen bond between the peptide NH and a water molecule at the active site.[37] The fact that dipeptide analogue **24a**, having a protected 4-amino substituent, has an activity similar to that of the nonprotected **9a** suggests that this amino group is not interacting with the enzyme in this series of compounds.

Eleven dipeptide analogues (**17a**,**c**,**e**, **18a**−**h**) that contain 4-amino- or 4-azidoproline at the N-terminus showed some nNOS inhibition at 100 μM (IC_{50} = 50−

Scheme 4

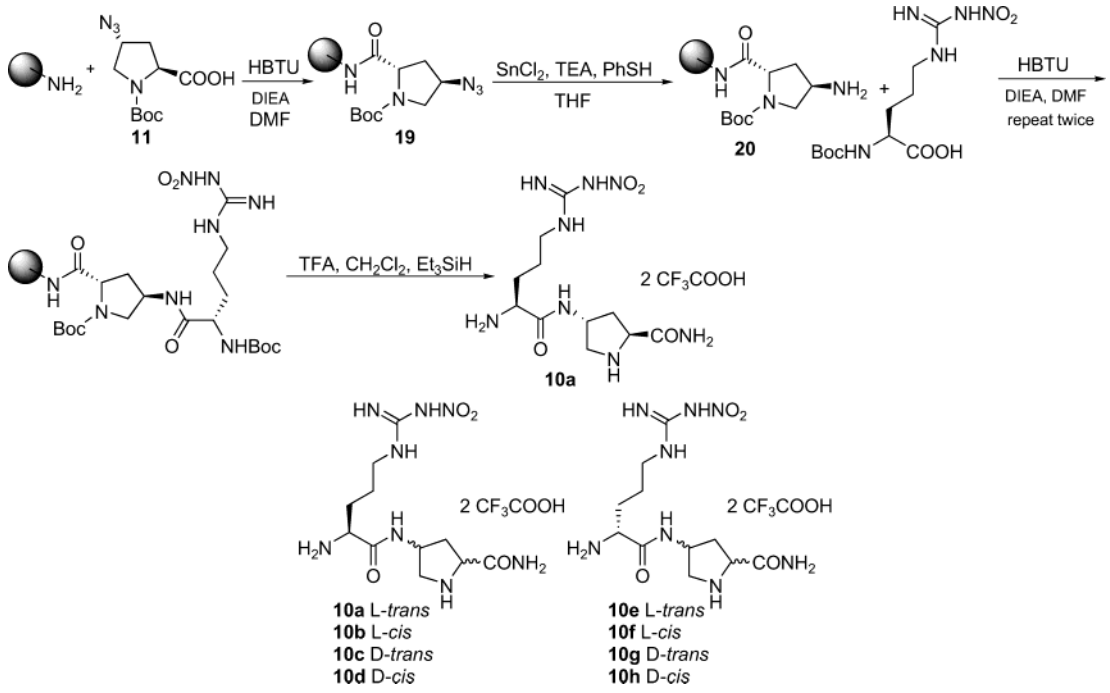
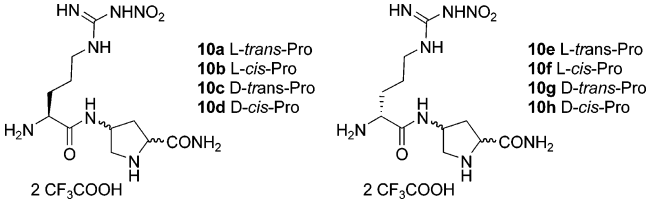


Table 1. nNOS and iNOS Inhibition by the C-Terminal 4-Aminoprolinamide-Containing Dipeptides in Which the 4-Amino Nitrogen Is in the Peptide Bond



	activity at 100 μM[a] (% enzyme activity)			activity at 100 μM[a] (% enzyme activity)	
compd	nNOS	iNOS	compd	nNOS	iNOS
10a	54[b]	10	**10e**	80	97
10b	50	95	**10f**	78	96
10c	49[c]	78	**10g**	52	96
10d	65	94	**10h**	83	97

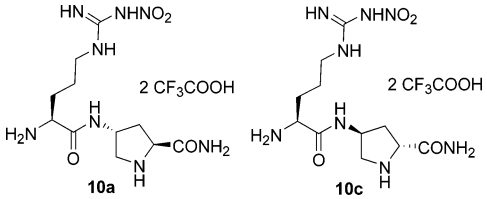
[a] Enzyme residual activity at 100 μM inhibitor. [b] Enzyme residual activity at 0.5 μM inhibitor. [c] Enzyme residual activity at 1 μM inhibitor.

100 μM). L-*trans*-Pro$^{4\text{-}NH_2}$-L-Arg^{NO_2}-NH_2 (**18a**) is the most potent compound of this second series with an IC_{50} value slightly lower than 50 μM. The azido analogues showed a decrease in inhibition, indicating either a steric problem with the azido group or a loss in hydrogen bonding of the amino group. The stereochemistry of **18a** is important for its activity. The introduction of D-Arg^{NO_2} instead of L-Arg^{NO_2} and the inversion of configuration at the 4-aminoproline Cα position in **18a** caused a decrease in potency.

A third family of dipeptides has been synthesized (Scheme 4) using the 4-amino group of the 4-aminoproline to form the dipeptide bond. These structures are rigid analogues of the lead compound and without a substituted peptide bond. Some of these compounds were the most potent compounds of the three series (Table 1).

Trans derivatives are more potent than *cis* derivatives in this series. L-Arg^{NO_2}-4-*N*-(L-*trans*-Pro$^{4\text{-}NH_2}$-NH_2 (or nitro-L-argininyl-4-*N*-(4(*R*)-amino-L-prolinamide, **10a**) is the most potent compound, having an IC_{50} value of 0.64 μM (Table 2). This compound represents the first conformationally restricted analogue of **8** with a similar K_i and selectivity over iNOS and eNOS. L-Arg^{NO_2}-containing dipeptide amides were more potent than the D-Arg^{NO_2} analogues. The diastereomer L-Arg^{NO_2}-4-*N*-(D-*trans*-Pro$^{4\text{-}NH_2}$-NH_2 (also, nitro-L-argininyl-4-*N*-(4(*S*)-amino-D-prolinamide, **10c**) showed a greater selectivity over iNOS although it was 15-fold less potent against nNOS than **10a**. No time dependence of inhibition was observed, suggesting that these compounds do not act as slow-binding inhibitors or irreversible inhibitors. In general, L-*trans*-4-aminoproline-containing dipeptide amides are the most potent compounds in each of the three series.

Table 2. IC_{50} and K_i Values for **10a** and **10c**



10a **10c**

	IC_{50} (μM)			K_i (μM)		
compd	nNOS	iNOS	eNOS	nNOS	iNOS	eNOS
10a	0.64	65	880	0.10[a]	29	128
10c	9.6	441	760	1.10	199	110
8				0.13[b]	25[b]	200[b]

[a] Experimentally determined. [b] From ref 27.

Conclusions

It is apparent from the results described here that the stereochemistry at each of the three stereocenters in **10a** strongly affects the potency and selectivity displayed with nNOS over iNOS (290-fold) and eNOS (1280-fold). The stereochemistry of **10a** suggests a

conformation of **8** that binds effectively with nNOS. From the crystal structures of **8** and **10a** bound to both nNOS and eNOS, it is apparent that these two compounds bind essentially identically in each of the isozymes; however, L-nitroarginine binds differently to these isozymes than does either **8** or **10a**.[37] This could account for the increased selectivity of the dipeptide amide analogues relative to L-nitroarginine.

Experimental Section

General Methods, Reagents, and Materials. All reagents were purchased from Aldrich or Advanced ChemTech and were used without further purification unless stated otherwise. 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 was distilled under nitrogen from sodium/benzophenone. Sodium sulfate was used to dry organic layers during workup. Thin-layer chromatography was carried out on E. Merck precoated silica gel 60 F_{254} plates. E. Merck silica gel 60 (230−400 mesh) was used for flash chromatography. An Orion Research model 701 pH meter with a general combination electrode was used for pH measurements. Combustion analyses were performed by Oneida Research Laboratories, New York. ^{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 values in parts per million relative to the peak for TMS in $CDCl_3$. For samples run in D_2O, the HOD resonance was arbitrarily set at 4.80 ppm. Mass spectra were obtained on a Micromass Quattro II spectrometer (ESI) or on a VG Instrument (VG70-250SE) high-resolution mass spectrometer. NOS assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer.

Triethylammonium Tris(phenylthio)stannate. *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 solution containing particulate matter was drawn into a dry syringe using a 0.45 μM filter on line. This solution was used immediately.

General Method for the Synthesis of *cis*- and *trans*-4-Amino-D- and -L-prolinyl-D- and -L-nitroargininamides (Scheme 2). Rink amide resin (0.1 mmol) was treated twice with 20% piperidine in DMF (5 mL) at room temperature for 5 and 20 min, respectively. The resin was washed (5 mL each time) with DMF (3×), DCM (2×), and MeOH (3×). The deprotected Rink amide resin (0.1 mmol) was suspended in DMF (1 mL). DIEA (0.3 mmol) and a solution of Boc-D- or L-ArgNO_2 (0.3 mmol) and HBTU (0.3 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 (5 mL each time) with DMF (3×), DCM (2×), and MeOH (3×). The resulting resin-bound amino acid was dried under vacuum for 2 h. The Boc-L-ArgNO_2-loaded resin was dried overnight under nitrogen and then was 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 (0.4 mmol) and (TMS)OTf (0.2 mmol) were sequentially dropwise injected under argon. The suspension was warmed to room temperature after 10 min and was allowed to stir for 2 h under argon. The reaction was quenched using a saturated solution of ammonium chloride in water. The resin was washed (5 mL each time) with DCM (3×), MeOH (3×), water (3×), MeOH (3×), and DCM (3×). Rink amide resin loaded with the deprotected L-ArgNO_2 isomer (0.1 mmol) was suspended in DMF (1 mL). A solution of the *N*-Boc-4-azidoproline isomer (0.2 mmol), HBTU (0.2 mmol), and DIEA (0.3 mmol) in 4 mL of DMF was added. The resulting mixture was shaken for 6 h at room temperature and washed (5 mL each time) with DMF (3×), DCM (2×), and MeOH (3×). The resulting resin-bound dipeptide was dried under vacuum for 2 h. A portion of the azidodipeptide-loaded resin (0.05 mmol) was cleaved to obtain the corresponding azidodipeptides. The rest of the resin (0.05 mmol) was suspended in DCM (3 mL), and triphenylphosphine (0.15 mmol) was added. The suspension was shaken at room temperature overnight. The resin was washed (5 mL each time) with MeOH (3×) and DCM (3×), and a solution of LiOH (0.5 mmol) in THF/H_2O (5:1) was added. The resin was shaken for 4 h at room temperature. The resin was washed (5 mL each time) with THF (3×), H_2O (3×), and MeOH (3×) and dried under vacuum for 2 h. The resulting Rink amide resin loaded with the dipeptide (0.05 mmol) was swollen in DCM and filtered. A solution of TFA and DCM (50:50, 3 mL) containing 1% Et_3SiH was added, and the resulting mixture was shaken for 30 min at room temperature. The suspension was filtered, and the resin was washed (5 mL each time) 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 dipeptide was prepurified by solid-phase extraction using a C-18 preconditioned column in water. The dipeptide amide (100 μL injections of a solution of 5 mg of dipeptide) was purified further by HPLC using a semipreparative column [Phenomenex, Luna, 250 × 10 mm, C18(2)] with a precolumn [Phenomenex, Luna 50 × 10 mm, C18(2)] at a flow rate of 4 mL/min at 214 nm. The elution program was as follows: (A, water + 0.1% TFA; B, MeCN + 0.1% TFA) 5 min using 1% B, then a gradient to 10% B over 15 min followed by 5 min with 10% B, and then a gradient to 1% B over 5 min. The total run time was 30 min in a Beckman HPLC instrument (System Gold 125, UV detector 166). Fractions containing the pure dipeptide were concentrated to dryness, and the residue was lyophilized.

General Method for the Synthesis of D- and L-Nitroargininyl-2-*N*-(*cis*- and *trans*-4-amino-D- and -L-prolinamides) (Scheme 3). Deprotected Rink amide resin (prepared as above, 0.1 mmol) was suspended in DMF (1 mL). DIEA (0.2 mmol) and a solution of the *N*-Boc-4-azidoproline isomer (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 (5 mL each time) with DMF (3×), DCM (2×), and MeOH (3×). The resulting resin-bound amino acid was dried under vacuum for 2 h. Rink amide resin loaded with the azidoproline isomer (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 (5 mL each time) with water (3×) and THF (3×). Then the resin was treated twice with a mixture of THF/saturated aqueous K_2CO_3 (2:1) for 15 min. The resin was filtered and washed (5 mL each time) with water (3×), MeOH (3×), DCM (3×), and methanol (3×). Rink amide resin loaded with the 4-aminoproline isomer **20** (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, and then the resin was washed (5 mL each time) with THF (3×), MeOH (3×), DCM (3×), and MeOH (3×). The resulting resin-bound bisprotected amino acid was dried under vacuum for 2 h. The bisprotected-4-aminoproline-loaded resin 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 (TMS)OTf (0.4 mmol) were sequentially injected dropwise under argon. The suspension was warmed to room temperature after 10 min and stirred for 2 h under argon. The reaction was quenched using a saturated solution of ammonium chloride in water. The resin was washed (5 mL each time) with DCM (3×), MeOH (3×), water (3×), MeOH (3×), and DCM (3×). Rink amide resin loaded with the Boc-deprotected aminoproline derivative (0.1 mmol) was suspended in DMF (1 mL). DIEA (0.4 mmol) and a solution of Boc-D- or -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 (5 mL each time) with DMF (3×), DCM (2×), and MeOH (3×). This coupling reaction was repeated for a second time. The resulting resin-bound protected dipeptide was dried under vacuum for 2 h. A portion of the protected-dipeptide-loaded Rink resin (0.05 mmol) was cleaved (see below) to obtain the corresponding trifluoroacetamide derivative. The rest of the protected-dipeptide-loaded resin (0.05 mmol) was preswollen with THF, and a solution of LiOH (0.5 mmol) in THF/H_2O (5:1) was added. The resin was shaken at room temperature for 3 h and washed (5 mL each time) with THF (3×), MeOH (3×), H_2O (3×), and MeOH (3×). The resulting 4-amino-deprotected-dipeptide-loaded resin was dried under vacuum for 2 h. The resulting Rink amide resin loaded with the dipeptide (0.05 mmol) was cleaved, and the dipeptides were purified as described for Scheme 2.

General Method for the Synthesis of D- and L-Nitroargininyl-4-*N*-(4-aminoprolinamide) Dipeptides (Scheme 4). The resin loaded with the aminoproline isomer **20** (Scheme 3, 0.1 mmol) was suspended in DMF (1 mL). DIEA (0.4 mmol) and a solution of Boc-D- or -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 then filtered. The resin was washed (5 mL each time) with DMF (3×), DCM (2×), and MeOH (3×). This coupling reaction was repeated a second time, and then the resulting resin-bound dipeptide was dried under vacuum for 2 h. The resulting resin loaded with the protected dipeptide (0.1 mmol) was cleaved, and the dipeptides were purified as described for Scheme 2.

4-*N*-(Nitro-L-argininyl)-4*R*-amino-L-prolinamide (10a). ^{1}H NMR (D_2O): δ 4.66 (t, 1H), 4.58 (q, 1H), 4.05 (t, 1H), 3.83 (dd, 1H), 3.41 (dd, 1H), 3.35 (br s, 2H), 2.56−2.47 (m, 2H), 1.98 (br s, 2H), 1.72 (br s, 2H). ^{13}C NMR (D_2O): δ 170.8, 169.8, 162.8 (c, TFA), 159.2, 116.3 (c, TFA), 58.9, 53.0, 50.0, 49.1, 40.3, 34.6, 28.0. MS (ES): [$C_{11}H_{22}N_8O_4$] *m*/*z* 331.1 (MH^+). Anal. Calcd: C, 31.23; H, 4.01; N, 18.21. Found: C, 31.17; H, 3.95; N, 17.98 (+2.5TFA).

4-*N*-(Nitro-L-argininyl)-4*S*-amino-D-prolinamide (10c). ^{1}H NMR (D_2O): δ 4.58 (t, 1H), 4.52 (q, 1H), 3.98 (t, 1H), 3.77 (dd, 1H), 3.32 (dd, 1H), 3.27 (br s, 2H), 2.46 (dd, 2H), 1.90 (br s, 2H), 1.65 (br s, 2H). ^{13}C NMR (D_2O): δ 170.8, 169.8, 162.8 (c, TFA), 159.0, 116.3 (c, TFA), 59.0, 52.9, 49.9, 49.1, 40.4, 34.7, 28.0. MS (ES): [$C_{11}H_{22}N_8O_4$] *m*/*z* 331.1 (MH^+). Anal. Calcd: C, 31.72; H, 4.16; N, 19.09. Found: C, 32.03; H, 4.12; N, 18.86 (+2.25TFA).

Enzyme and Assay. All of the NOS isoforms used were recombinant enzymes overexpressed in *E. coli* from different sources; there is very high sequence identity for the isoforms from different sources. The murine macrophage iNOS was expressed and isolated according to the procedure of Hevel et al.[52] The rat nNOS was expressed[53] and purified as described. The bovine eNOS was isolated as reported.[54] Nitric oxide formation from NOS was monitored by the hemoglobin capture assay as described.[55]

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 deviation from the mean of the three measurements was ±5%. The parameters of the following inhibition equation[56] 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.

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