Articles

Selective Inhibition of Neuronal Nitric Oxide Synthase by N^{ω}-Nitroarginine- and Phenylalanine-Containing Dipeptides and Dipeptide Esters

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A series of N^{ω}-nitroarginine (Arg^{NO_2})- and phenylalanine-containing dipeptides and dipeptide esters were synthesized as potential selective inhibitors of neuronal nitric oxide synthase (nNOS). All of the dipeptides and dipeptide esters are competitive inhibitors of nNOS, macrophage nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS), except for the ones that contain D-Arg^{NO_2} (**8**−**10**, **12**, **13**), which are uncompetitive inhibitors of iNOS but competitive inhibitors of nNOS and eNOS. None of the dipeptides or dipeptide esters tested (**1**, **2**, **12**, **13**) exhibited time-dependent inhibition of any of the NOS isoforms, unlike N^{ω}-nitro-L-arginine itself, which does, although it is reversible. The order of the amino acids in the dipeptide or dipeptide ester is important to selectivity, and the selectivity depends on the chirality of the amino acids. In the case of the corresponding benzyl esters (**5** vs **6**), both dipeptides favor iNOS over nNOS and eNOS inhibition. All of the dipeptide methyl esters containing a D-amino acid, however, exhibit an inhibitory preference for nNOS over iNOS and eNOS. The most impressive selectivities observed are 1800- and 800-fold for **12** and **13**, respectively, in favor of nNOS over iNOS; unfortunately, the selectivities of these compounds for nNOS over eNOS are only 2.5 and 5.3, respectively.

Introduction

Nitric oxide (NO) has been shown to be an important biological second messenger which is biosynthesized by a family of enzymes called nitric oxide synthases (EC 1.14.13.39). Three principal isoforms of this enzyme have been isolated and characterized, each associated with different physiological functions.[1] The isoform from endothelial cells (eNOS) is involved in the regulation of smooth muscle relaxation, blood pressure lowering, and inhibition of platelet aggregation. Neuronal nitric oxide synthase (nNOS) is important for long-term potentiation, and an inducible form (iNOS), which acts in host defense, is generated by activated macrophage cells during an immune response. The endothelial and neuronal isoforms are expressed constitutively and require Ca^{2+} and calmodulin for activity; the macrophage form is induced by cytokines or bacterial lipopolysaccharides and is Ca^{2+} and calmodulin independent. All forms of the enzyme require NADPH, tetrahydrobiopterin, heme, and FAD and FMN. Despite the extraordinary importance of NO to the apparent health of organisms, it also has been associated with a large number of harmful effects as a result of its reactive free radical properties; in fact, NO has a lifetime of only a few seconds at physiological pH and temperature.[2] Overproduction of NO, consequently, has been implicated in a wide variety of diseases.[3] NO overproduction by nNOS has been implicated in strokes,[4] septic shock,[5] seizures,[6] schizophrenia,[7] migraine headaches,[8] and Alzheimer's disease.[9] iNOS overproduction of NO has been associated with tolerance to and dependence on morphine,[10] development of colitis,[11] tissue damage and inflammation,[12] overproduction of osteoclasts, leading to osteoporosis, Paget's disease, and rheumatoid arthritis,[13] and destruction of photoreceptors in the retina.[14] This suggests that inhibition of NOS would have a significant beneficial effect on disease states arising from the overproduction of NO. A wide variety of compounds have been shown to inhibit NOS.[15,18] However, because of the general importance of NO to human health, *selective* inhibition of the isoforms of NOS is essential. Selectivity for the three isoforms already has been reported to some degree. The early inhibitors were analogues of L-arginine. N^{ω}-Methyl-L-arginine and N^{ω}-ethyl-L-arginine, however, show only about a factor of 2 selectivity; N^{ω}-methyl-L-arginine is selective for eNOS and nNOS over iNOS and N^{ω}-ethyl-L-arginine is selective for iNOS over nNOS and eNOS.[16] N^{ω}-Nitro-L-arginine is about 300-fold selective for nNOS over iNOS.[17] 2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine and *S*-ethylisothiourea had 10−40-fold selectivity in favor of iNOS,[18] and 2-iminoazaheterocycles showed selectivities of 1.1−9 in favor of iNOS over eNOS and nNOS.[19] Various indazole analogues had selectivities of 5−10 for either nNOS or iNOS.[20] Imidazole analogues exhibited selectivities in the range of 3−6-fold in favor of iNOS.[21] Aminoguanidine shows a 50-fold selectivity for iNOS over nNOS and 500-fold over

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|| Carried out all of the chemical and enzymological studies described here.
⊥ Supplied the recombinant *E. coli* cells expressed with murine macrophages iNOS.
▽ Supplied the recombinant eNOS used in these studies.
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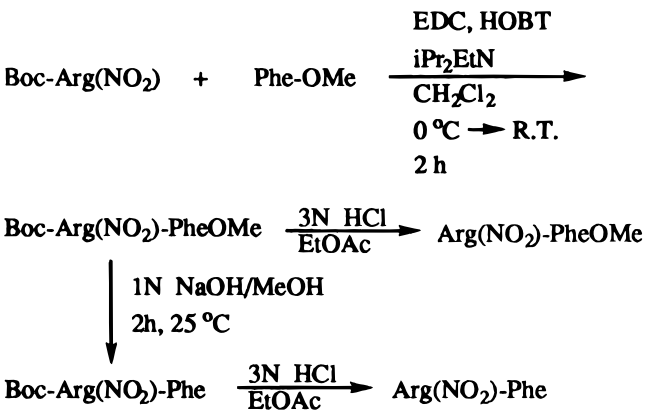
Scheme 1



eNOS.[22] Several series of isothiourea analogues favored inhibition of iNOS over eNOS by factors of between 2- and 6-fold with one analogue being 19-fold selective; bisisothioureas were more selective with one analogue showing a selectivity of 190-fold in preference for iNOS.[23] *S*-Methyl- and *S*-ethyl-L-thiocitrulline were 10- and 50-fold, respectively, more selective for nNOS than eNOS.[24] L-N^6-(1-Iminoethyl)lysine, another inhibitor of NOS, favors the inhibition of iNOS by a factor of 30 over eNOS and by 13 over nNOS.[25]

Because of the relatively high selectivity of N^ω-nitro-L-arginine for nNOS over iNOS,[19] and the observation that it is a time-dependent inhibitor of nNOS but a reversible inhibitor of iNOS,[19,26] we decided to determine if N^ω-nitroarginine-containing dipeptides and dipeptide esters would have increased selectivity for nNOS. Since L-arginine methyl ester, L-argininamide, and L-arginine-containing dipeptides are substrates for NOS,[27,28] it was thought that the dipeptide esters may be functional as well. Furthermore, the fact that N^ω-nitro-L-arginine inhibits nNOS in vivo following intraperitoneal injection[30] suggests that it crosses the blood−brain barrier. Here we describe results of our studies of selective inhibition of all three isoforms by N^ω-nitroarginine- and phenylalanine-containing dipeptides and dipeptide esters.

Results and Discussion

Chemistry. All of the dipeptides were synthesized manually by the route shown in Scheme 1. Chiral HPLC, which can separate all four epimers of each dipeptide, indicated the presence of only one epimer for each compound, and all dipeptides were pure by NMR and elemental analysis and were optically active (Table 1). A variety of other conditions led to epimerization in the product, which resulted in two product peaks (diastereomers) in the chiral HPLC system used.

Enzymology. All of the dipeptides and dipeptide esters are competitive inhibitors of the three isoforms of NOS, except for the ones that contain D-Arg^{NO_2} (**8**−**10**, **12**, **13**), which are uncompetitive inhibitors of iNOS but competitive inhibitors of nNOS and eNOS (Table 2). The kinetic data were obtained by Dixon analysis,[29] and a replot of these data was obtained by the method of Cornish and Bowden[30] to determine the type of inhibition. Plots for the competitive inhibition of nNOS by D-phenylalanyl-D-nitroarginine methyl ester (**12**) and for the uncompetitive inhibition of iNOS by D-nitroargininyl-D-phenylalanine methyl ester (**9**) can be found in the Supporting Information. None of the dipeptides or dipeptide esters tested (**1**, **2**, **12**, **13**) exhibited time-dependent inhibition of any of the isoforms, unlike N^ω-nitro-L-arginine itself, which we found had K_i and k_{inact} values of 9.3 μM and 0.132 min^{-1}, respectively, with nNOS. None of the kinetic plots showed curvature, suggesting that there was no time dependence to the inhibition.

The order of the amino acids in the dipeptide is important to selectivity, but it depends on the chirality of the amino acids. Thus, for the dipeptide and dipeptide methyl ester pairs **1** vs **2** and **3** vs **4**, having L-Arg^{NO_2} at the N-terminus favors nNOS over iNOS and eNOS inhibition, but when the L-Phe is at the *N*-terminus, it favors iNOS over nNOS and eNOS inhibition. In the case of the corresponding benzyl ester (**5** vs **6**), both dipeptides favor iNOS over nNOS and eNOS inhibition. All of the dipeptide methyl esters containing a D-amino acid, however, exhibit an inhibitory preference for nNOS over iNOS and eNOS. The most impressive selectivities observed are 1800- and 800-fold for **12** and **13**, respectively, in favor of nNOS over iNOS. These results indicate that there is a difference in the binding sites of nNOS vs iNOS that can be taken advantage of by appropriate design of D-amino acid-containing peptide esters. Unfortunately, the selectivity of these analogues for nNOS over eNOS is only 2.5 and 5.3, respectively. The selectivity of eNOS over iNOS for **12** and **13**, however, is 714- and 152-fold, respectively. Because of the unnatural chirality of the amino acids and the incorporation of an ester functionality, the most nNOS- vs iNOS-selective compounds are peptidomimetic analogues, which may make them orally bioavailable. The enormous selectivity of **12** and **13** suggests that other dipeptides and dipeptide esters of unnatural amino acids should be tested.

Experimental Section

General Chemical Methods. Reagents. N^α-Boc-ω-nitro-D-arginine and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NOVA Biochemicals (San Diego, CA). L-Arg^{NO_2}-L-Phe-OMe (**3**) was purchased from BACHEM Feinchemikalien AG (Bubendorf, Switzerland) and was used without further purification. All other *N*-Boc-L- or D-amino acids and L- or D-amino acid esters were purchased from Sigma (St. Louis, MO). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole hydrate (HOBT), *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acids, bases, and conventional organic solvents were purchased from Fischer Scientific Co.

Analytical Chemical Methods. Optical rotations were determined with an AA-100 polarimeter (Optical Activity Ltd., England). ^{1}H-NMR spectra were recorded on a Varian Gemini-300 spectrometer in the solvent indicated. ^{1}H-NMR chemical shifts in D_2O are reported relative to internal sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS). Fast atom bombardment (FAB) mass spectra were performed on a VG-Instruments 70-250-SE high-resolution mass spectrometer. Elemental analyses were obtained from Oneida Research Services, Inc., Whitesboro, NY. Thin-layer chromatography was carried out on Merck silica gel 60-F254, 0.25 mm thickness. Amino acids were visualized with a ninhydrin/pyridine spray reagent or a UV/vis lamp. High-performance liquid chromatography was performed on a Beckman System Gold instrument (Model 125P solvent module and Model 166 detector). Samples were analyzed by elution from a Vydac RP C_{18} 90A (4 × 250 mm) pharmaceutical column with a flow rate of 0.75 mL/min. Samples (10 μL) were injected and eluted using the following

Table 1. Analytical Data for the Nitroarginine-Containing Dipeptides and Dipeptide Esters

inhibitor	^{1}H-NMR chemical shift (δ, D_2O)	FAB HRMS (M + 1)	anal. (C, H, N)	$[\alpha]^{23.5}_D$
1, L-Arg^{NO_2}-L-Phe	7.25 (m, 5H), 4.62 (t, 1H), 3.94 (t, 1H), 3.18 (m, 2H), 3.05 (m, 2H), 1.79 (m, 2H), 1.53 (m, 2H)	367	$C_{15}H_{22}N_6O_5 \cdot 1.5CF_3COOH \cdot 0.5H_2O$	+17.9 (*c* 1.70, water)
2, L-Phe-L-Arg^{NO_2}	7.30 (m, 5H), 4.31 (t, 1H), 4.25 (t, 1H), 3.21 (m, 4H), 1.85 (m, 1H), 1.74 (m, 1H), 1.56 (m, 2H)	367	$C_{15}H_{22}N_6O_5 \cdot 2HCl$	+15.7 (*c* 1.5, water)
4, L-Phe-L-Arg^{NO_2}-OMe	7.30 (m, 5H), 4.36 (m, 1H), 4.26 (t, 1H), 3.70 (s, 3H), 3.19 (m, 4H), 1.82 (m, 1H), 1.71 (m, 1H), 1.54 (m, 2H)	381	$C_{16}H_{24}N_6O_5 \cdot 2\ CF_3COOH$	+6.4 (*c* 1, water)
5, L-Arg^{NO_2}-L-Phe-OBn	7.21 (m, 5H), 7.10 (m, 5H), 4.96 (d, 2H), 4.68 (t, 1H), 3.95 (t, 1H), 3.04 (m, 4H), 1.78 (m, 2H), 1.51 (m, 2H)	457	$C_{22}H_{28}N_6O_5 \cdot 2\ CF_3COOH$	+12.5 (*c* 1.2, MeOH)
6, L-Phe-L-Arg^{NO_2}-OBn	7.28 (m, 10H), 5.15 (d, 2H), 4.40 (t, 1H), 4.19 (t, 1H), 3.10 (m, 4H), 1.78 (m, 2H), 1.42 (m, 2H)	457	$C_{22}H_{28}N_6O_5 \cdot 2\ CF_3COOH$	−5.5 (*c* 1.6, MeOH)
7, L-Arg^{NO_2}-D-Phe-OMe	7.25 (m, 5H), 4.88 (dd, 1H), 3.87 (t, 1H), 3.75 (s, 3H), 3.38 (dd, 1H), 2.96 (t, 2H), 2.85 (dd, 1H), 1.52 (m, 2H), 0.99 (m, 2H)	381	$C_{16}H_{24}N_6O_5 \cdot 2\ CF_3COOH$	+27.0 (*c* 1, water)
8, D-Arg^{NO_2}-L-Phe-OMe	7.25 (m, 5H), 4.88 (dd, 1H), 3.87 (t, 1H), 3.75 (s, 3H), 3.36 (dd, 1H), 2.97 (t, 2H), 2.85 (dd, 1H), 1.52 (m, 2H), 0.99 (m, 2H)	381	$C_{16}H_{24}N_6O_5 \cdot 2\ CF_3COOH$	−27.2 (*c* 1, water)
9, D-Arg^{NO_2}-D-Phe-OMe	7.25 (m, 5H), 4.68 (m, 1H), 3.93 (t, 1H), 3.28 (s, 3H), 3.19 (m, 2H), 3.03 (m, 2H), 1.80 (m, 2H), 1.54 (m, 2H)	381	$C_{16}H_{24}N_6O_5 \cdot 2HCl\ H_2O$	−12.9 (*c* 1.40, MeOH)
10, L-Phe-D-Arg^{NO_2}-OMe	7.25 (m, 5H), 4.21 (m, 2H), 3.69 (s, 3H), 3.20 (dd, 1H), 3.01 (m, 3H), 1.63 (m, 1H), 1.42 (m, 1H), 1.08 (m, 1H), 0.94 (m, 1H)	381	$C_{16}H_{24}N_6O_5 \cdot 2\ CF_3COOH$	+56.8 (*c* 1, water)
11, D-Phe-D-Arg^{NO_2}-OMe	7.25 (m, 5H), 4.20 (m, 2H), 3.68 (s, 3H), 3.20 (dd, 1H), 3.01 (m, 3H), 1.64 (m, 1H), 1.43 (m, 1H), 1.09 (m, 1H), 0.95 (m, 1H)	381	$C_{16}H_{24}N_6O_5 \cdot 1.5\ CF_3COOH$	−56.6 (*c* 1, water)
12, D-Phe-D-Arg^{NO_2}-OMe	7.30 (m, 5H), 4.36 (m, 1H), 4.26 (t, 1H), 3.70 (s, 3H), 3.19 (m, 4H), 1.82 (m, 1H), 1.71 (m, 1H), 1.54 (m, 2H)	381	$C_{16}H_{24}N_6O_5 \cdot 2CF_3COOH$	−6.5 (*c* 1, water)
13, D-Phe-D-Arg^{NO_2}	7.30 (m, 5H), 4.31 (t, 1H), 4.25 (t, 1H), 3.21 (m, 4H), 1.85 (m, 1H), 1.74 (m, 1H), 1.56 (m, 2H)	367	$C_{15}H_{22}N_6O_5 \cdot 2HCl$	−15.6 (*c* 1.5, water)

Table 2. K_i Values for Inhibition of NOS Isoforms by Nitroarginine-Containing Dipeptides and Dipeptide Esters

				selectivity		
inhibitor	nNOS (μM)	iNOS (μM)	eNOS (μM)	nNOS/iNOS	nNOS/eNOS	iNOS/eNOS
1, L-Arg^{NO_2}-L-Phe	18	160	395	8.9	21.9	2.5
2, L-Phe-L-Arg^{NO_2}	140	93	490	0.66	3.5	5.3
3, L-Arg^{NO_2}-L-Phe-OMe	14	45	400	3.2	28.6	8.9
4, L-Phe-L-Arg^{NO_2}-OMe	370	204	1350	0.55	3.6	6.6
5, l-Arg^{NO_2}-L-Phe-OBn	55	18	125	0.33	2.3	6.9
6, L-Phe-L-Arg^{NO_2}-OBn	110	45	250	0.41	2.3	5.6
7, L-Arg^{NO_2}-D-Phe-OMe	92	100	525	1.1	5.7	5.3
8, D-Arg^{NO_2}-L-Phe-OMe	290	6400[a]	1400	22.1	4.8	0.22
9, D-Arg^{NO_2}-D-Phe-OMe	150	6500[a]	375	43.3	2.5	0.058
10, L-Phe-D-Arg^{NO_2}-OMe	90	7500[a]	150	83.3	1.7	0.02
11, D-Phe-L-Arg^{NO_2}-OMe	400	1200	8500	3	21.3	7.1
12, D-Phe-D-Arg^{NO_2}-OMe	2	3600[a]	5	1800	2.5	0.0014
13, D-Phe-D-Arg^{NO_2}	17	13600[a]	90	800	5.3	0.0066

[a] Uncompetitive inhibition.

gradient protocol: 0−5 min, solvent A (0.06% TFA in water); 5−60 min, linear gradient from solvent A to solvent B (0.05% TFA in acetonitrile).

Chiral HPLC was carried out on a Beckman System Gold HPLC with a 150 × 4.0 mm Crownpak CR (+) column (Chiral Technologies, Inc., Exton, PA) using a gradient of 100% solvent A to 90% solvent A/10% solvent B over 30 min at a flow rate of 0.5 mL/min. Solvent A is water taken to pH 2.0 with perchloric acid, and solvent B is methanol.

Chemical Synthesis. *N*-Boc-dipeptide Methyl Ester. *N*-Boc-protected amino acid (1.5 mmol), amino acid methyl ester (1.5 mmol), HOBT (1.5 mmol), and EDC (1.65 mmol) were mixed at 0 °C (ice−water bath) in freshly distilled methylene chloride (4 mL). The suspension was stirred for 10 min to which was added dropwise DIEA (3.0 mmol) under N_2. The mixture was stirred for 2 h from 0 °C to room temperature. Shorter reaction time and concentrated reaction solution helped to minimize the chance of racemization. A large amount of water (30 mL) was added to quench the reaction; then the crude product was extracted with ethyl acetate (30 mL). After being washed sequentially with water (30 mL), 5% (v/v) HCl solution (2 × 20 mL), water (20 mL), 5% (w/v) $NaHCO_3$ solution (2 × 20 mL), water (20 mL), and saturated NaCl solution (20 mL), the organic layer was dried over $MgSO_4$. Crude dipeptide was obtained as a white powder by rotary evaporation of the ethyl acetate solution. The powder was dissolved in $CHCl_3$ and loaded onto a silica gel column (3 × 50 cm). Pure *N*-Boc-dipeptide methyl ester was eluted with 20:1 $CHCl_3/CH_3OH$, monitoring by TLC. The yields were about 50%.

***N*-Boc-dipeptide Benzyl Ester.** To a stirred suspension of amino acid benzyl ester (1.5 mmol) and *N*-Boc-amino acid (1.3 equiv) in 2 mL of CH_2Cl_2 at 0 °C was added a methylene chloride solution (2 mL) of HBTU/HOBT (1.3 equiv each). After the mixture stirred for a few more minutes, 6 equiv of DIEA was added dropwise under N_2. The reaction mixture became homogenous after 2 h. Ethyl acetate was added to dilute the reaction solution, and the same workup procedure was performed as described above.

Dipeptide Esters 4−12. The Boc groups of the methyl ester- and benzyl ester-protected dipeptides were removed by TFA or HCl (3 N in ethyl acetate). The Boc-protected amino acid ester was treated with acid (3 mL). The solution was stirred for 1 h (shorter time for HCl deprotection) under N_2. The acid was evaporated at room temperature, and the residue was dissolved in water (10 mL). The aqueous solution was washed with ethyl ether (3 × 10 mL) and vacuum evaporated. When it was difficult to remove the water, the oily residue was dissolved in methanol and evaporated. The yield from the deprotection step was about 80%. The TFA or HCl salts of the dipeptide esters were used for all of the elemental analyses. The analytical data for the compounds are listed in Table 1.

Dipeptides 1, 2, and 13. To a solution of *N*-Boc-dipeptide methyl ester (1 mmol) in methanol (5 mL) was added 1 N NaOH solution (10 mL). The mixture was stirred for 2 h at room temperature, and then the methanol was evaporated. The aqueous solution was washed with ethyl acetate (2 × 10 mL) and acidified with 1 N citric acid. The precipitate that formed was extracted with ethyl acetate (2 × 20 mL). The extract was washed with water (2 × 20 mL) and dried over $MgSO_4$. The *N*-Boc-dipeptide acid was obtained in a yield of 70% after evaporation of the solvent. A further deprotection of the Boc group by TFA or HCl was carried out as described above. Only D-Phe-D-Arg^{NO_2} (**13**) was chromatographed on Dowex 50 X8 (H^+ form) 200−400 mesh. The zwitterion was eluted with 0.25 N NH_4OH, lyophilized, and used for elemental analysis. The analytical data for these compounds are listed in Table 1.

General Biochemical Methods. Reagents. L-Arginine, human ferrous hemoglobin A_0, NADPH, $CaCl_2$, and calmodulin were purchased from Sigma Chemical Co. (St. Louis, MO). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was obtained from B. Schircks Laboratories (Jona, Switzerland) or Alexis Biochemicals (San Diego, CA). Dithiothreitol (DTT) and Hepes were purchased from Fischer.

Enzyme Preparation. nNOS was obtained from bovine brain as described.[19] A typical preparation had a specific activity of 250 nmol of nitric oxide (mg of protein)$^{-1}$ min^{-1} at 30 °C. iNOS was purified according to the procedures of Hevel et al.[31] with a specific activity of 500 nmol of nitric oxide (mg of protein)$^{-1}$ min^{-1} at 30 °C. eNOS, expressed in *Escherichia coli*, was purified as described and supplemented with L-arginine and BH_4;[32] the specific activity was 235 nmol of NO mg^{-1} min^{-1} at 25 °C.

Analytical Biochemical Methods. Nitric oxide formation was measured using the hemoglobin capture assay.[33] A typical assay mixture for nNOS contained 3−15 μM L-arginine, 1.6 mM $CaCl_2$, 11.6 μg/mL calmodulin, 100 μM DTT, 100 μM NADPH, 6.5 μM BH_4, and 3 mM oxyhemoglobin in 100 mM Hepes (pH 7.5). The reaction mixture for iNOS assay included 10−60 μM L-arginine, 100 μM DTT, 100 μM NADPH, 6.5 μM BH_4, and 3 μM oxyhemoglobin in 100 mM Hepes (pH 7.5). The production of nitric oxide by eNOS was measured as described.[34] Briefly, the assay mixture contained 80 μM oxyhemoglobin, 3−25 μM L-arginine, 100 μM DTT, 10 μM $CaCl_2$, 1 μg/mL calmodulin, 5 μM BH_4, 100 μM NADPH, and 50 mM Hepes (pH 7.5). All assays were in a final volume of 600 μL and were initiated with enzyme. Nitric oxide reacts with oxyHb to yield methemoglobin which is detected at 401 nm (ϵ = 19 700 M^{-1} cm^{-1})[35] on a Perkin-Elmer Lambda 1 UV/vis spectrophotometer. The concentration of arginine is higher for iNOS than for nNOS or eNOS because there is substrate inhibition with nNOS and eNOS but not iNOS. Protein concentration of enzyme was determined with the Bradford Assay (Bio-Rad) using bovine serum albumin as the standard.

Inhibition Methods. The reversible inhibition of NOS by the dipeptide analogues was studied under initial rate conditions with the hemoglobin assay as described above. The K_i values were determined from Dixon plots[29] with various L-arginine and inhibitor concentrations. The type of reversible inhibition was determined from Cornish−Bowden replots[30] of the data in the Dixon plots.

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Supporting Information Available: Dixon and Cornish−Bowden plots for the competitive inhibition of nNOS by D-phenylalanyl-D-nitroarginine methyl ester (**12**) and for the uncompetitive inhibition of iNOS by D-nitroargininyl-D-phenylalanine methyl ester (**9**) (4 pages). Ordering information is given on any current masthead page.

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