Aromatic Reduced Amide Bond Peptidomimetics as Selective Inhibitors of Neuronal Nitric Oxide Synthase

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Nitric oxide synthase inhibitors could act as important therapies for disorders arising from overstimulation or overexpression of individual nitric oxide synthase (NOS) isoforms. But preservation of physiologically important nitric oxide functions require the use of isoform-selective inhibitors. Recently we reported reduced amide bond pseudodipeptide analogues as potent and selective neuronal nitric oxide synthase (nNOS) inhibitors (Hah, J.-M.; Roman, L. J.; Martasek, P.; Silverman, R. B. *J. Med. Chem.* **2001**, *44*, 2667−2670). To increase the lipophilicity a series of aromatic, reduced amide bond analogues (**6**−**25**) were designed and synthesized as potential selective nNOS inhibitors. The hypothesized large increase in isoform selectivity of nNOS over inducible NOS was not obtained in this series. However, the high potency with nNOS as well as high selectivity of nNOS over endothelial NOS was retained in some of these compounds (**15**, **17**, **21**), as well as good selectivity over inducible NOS. The most potent nNOS inhibitor among these compounds is *N*-(4*S*)-{4-amino-5-[2-(2-aminoethyl)-phenylamino]-pentyl}-*N*′-nitroguanidine (**17**) (K_i = 50 nM), which also shows the highest selectivity over eNOS (greater than 2100-fold) and 70-fold selectivity over iNOS. Further modification of compound **17** should lead to even more potent and selective nNOS inhibitors.

Introduction

Nitric oxide (NO)[1] is involved in a number of physiological functions, acting as an inter- and intracellular signaling agent. A family of enzymes, the nitric oxide synthases (NOS, EC 1.14.13.39), catalyzes the stepwise oxidation of L-arginine to L-citrulline and nitric oxide. Three distinct NOS isoforms have been isolated and characterized, each associated with different physiological functions: blood-vessel dilation (endothelial NOS or eNOS),[2] neuronal signal transmission (neuronal NOS or nNOS),[3] immune response such as cytotoxicity against pathogens and tumors (inducible NOS or iNOS).[4]

Overstimulation or overexpression of individual nitric oxide synthase isoforms plays a role in a wide range of disorders including septic shock, arthritis, diabetes, ischemia-reperfusion injury, pain, and various neurodegenerative diseases.[5,6] High levels of sustained nitric oxide generated by nNOS activation as a result of disrupted calcium homeostasis are associated with numerous neurodegenerative disorders such as ischemic brain, Parkinson's disease, and Huntington's disease.[7] Studies with nNOS knockout mice have indicated that nitric oxide produced by nNOS during neuronal injury is associated with glutamate toxicity in the brain.[8] Additionally, the nNOS-derived nitric oxide or peroxynitrite (a reaction of nitric oxide with superoxide anion)[9] exacerbates acute ischemic injury. Animal studies and early clinical trials suggest that nitric oxide synthase inhibitors could be therapeutic in many of these disorders,[10] but preservation of physiologically important nitric oxide functions would require the use of isoform-selective inhibitors. Because eNOS plays a key role in maintaining normal blood pressure, most investigators believe therapeutically useful NOS inhibitors must not inhibit eNOS.[11]

Our aim is to develop nNOS-selective inhibitors. In our labs libraries of nitroarginine-containing dipeptides,[12] dipeptide esters,[12] and dipeptide amides[13] were synthesized as possible candidates for nNOS-selective inhibition based on two observations. First, L-nitroarginine itself is a potent nNOS inhibitor (K_i = 15 nM),[14] and it has about a 250-fold selectivity over iNOS, but only a 2.5-fold selectivity over eNOS. Second, L-arginine-containing dipeptides are good substrates for the various isoforms of NOS,[15,16] suggesting that the active site of NOS is flexible enough to accommodate larger molecules than arginine. Indeed, crystal structures of the oxygenase domains of eNOS[17] and iNOS[18] showed that there is a large opening, which allows the diffusion of both the substrate and the product (L-arginine and L-citrulline). Furthermore, there are significant structural differences between isoforms just outside of the substrate-binding pocket. From these two aspects, it was hypothesized that dipeptides containing nitroarginine could fit into the active site of NOS and at the same time utilize the structural differences to achieve isoform selectivity. Twelve nitroarginine (L or D)-containing dipeptides and dipeptide esters were synthesized in the standard way,[12]

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§ Carried out all of the experiments in this study.

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

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

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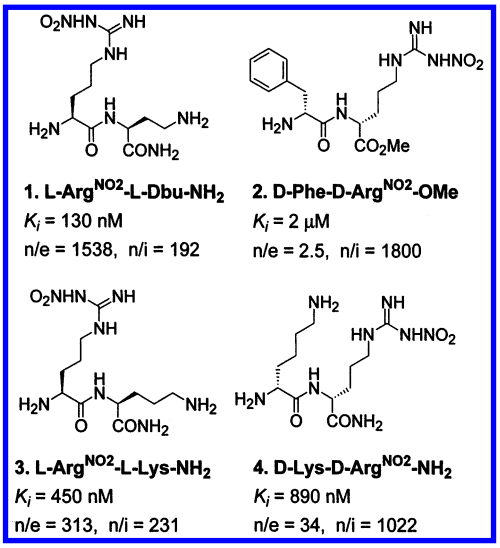
Chart 1. Dipeptides Containing Nitroarginine as Selective Inhibitors of nNOS



and 152 dipeptide amides were prepared using a solid-phase synthesis method.[13] They were tested against each isoform of NOS. Among these libraries of compounds, several dipeptides were found to be potent and nNOS-selective inhibitors (Chart 1). The dipeptide amides containing amino acids with a nitrogen-containing side chain, such as Lys, Orn, and Dbu, were relatively potent inhibitors of nNOS. They also have great selectivity over eNOS (**1**, **3** in Chart 1), implying that the terminal amine group is significant for selectivity over eNOS.13 Among the dipeptide esters, D-Phe-D-Arg^{NO2}-OMe (2) showed excellent selectivity for nNOS over iNOS (1800-fold), although the potency is weak, and the selectivity for nNOS over eNOS is minimal.

Even though there are several exceptions, when L-Arg^{NO2} is at the N-terminus of the dipeptide inhibitors, an L-amino acid is also favored at the C-terminus. But when Arg^{NO2} is at the C-terminus, the amino acid was more selective as the D-isomer. From these observations, a *retro-inverso* dipeptide model was proposed.[13] In this case, the nitroarginine residue binds to the same binding site, regardless of its position in the dipeptide (Figure 1). For example, D-Arg^{NO2} of **4** may flip over 180° to assume an L-Arg^{NO2} configuration (like **3**) at the N-terminus for binding (Figure 1). Molecular modeling and energy minimization of the *retro-inverso* dipeptide amides **3** and **4** demonstrate perfect overlap; furthermore, recent ENDOR spectroscopic results[19] confirmed that the dipeptides bind to holo-nNOS similarly from the point of view of the nitroguanidino functionality.

If the *retro-inverso* binding model holds for D-Phe-D-Arg^{NO2}-OMe (**2**) at the active site of nNOS, then it can be expected to bind shown in Figure 2. The important terminal nitrogen interaction of **1** (or **3** or **4**) in the active site is lost; instead, the phenyl ring moiety replaces the C-terminal residue active site. This can explain the weaker potency and minimal selectivity for nNOS over eNOS of **2**. However, the replacement of the phenyl ring could also attribute to the high selectivity of nNOS over iNOS (1800-fold); it is not as effective as the amine group moiety at the nNOS active site, but much more unfavorable in the active site of iNOS.

From this hypothesis (selectivity for nNOS over eNOS is from the terminal nitrogen, and selectivity over iNOS

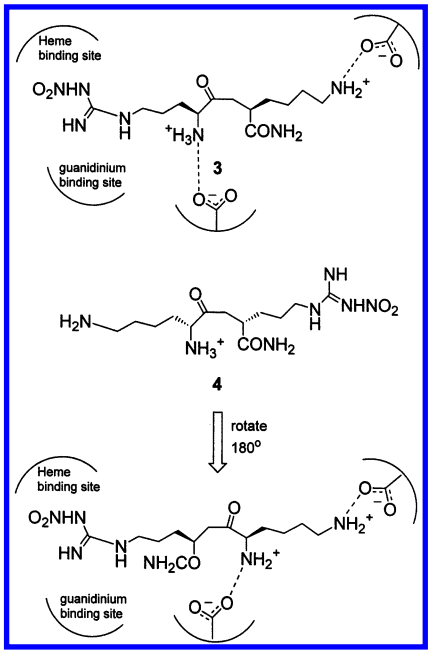


Figure 1. A proposed model for binding of the *retro-inverso* dipeptide amide **3** and **4** at the active site of nNOS, taken from ref 10.

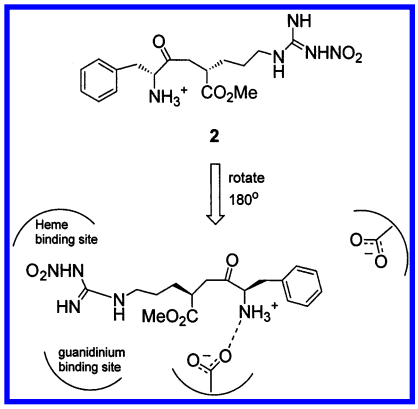


Figure 2. A proposed model for binding of the *retro-inverso* dipeptide amide **2** at the active site of nNOS.

is from the aromatic ring moiety), it was thought that great nNOS-selective inhibitors could be achieved by combining the aromatic ring and the terminal nitrogen moieties into a dipeptide inhibitor. This also would increase the lipophilicity of the molecule for enhanced absorption properties. Finally, the successful surrogate of the amide bond, namely, the reduced-amide bond,[20] could be incorporated. Based on all of these observations, two series of aromatic, reduced amide bond peptidomimetic compounds as possible selective inhibitors of nNOS were designed (Charts 2 and 3).

Chemistry

All of the desired compounds (**5**−**25**, Charts 2 and 3) were prepared by reductive amination, using *N*-Boc-L-nitroarginal (**26**)[21] as the key intermediate, which was reductively coupled to various aromatic diamines. Compounds **8**−**13** were synthesized according to Scheme 1. N-Boc-L-nitroarginal (**26**) was coupled with (2-, 3-, or 4-aminomethyl)anilines (**27**−**29**), and [2-,3-, or 4-(2-aminoethyl)]anilines (**30**−**32**), respectively. The reductive amination was carried out in good yields without protection of the aniline group because alkylamines are

Chart 2. Phenyl Reduced Amide Bond Peptidomimetics

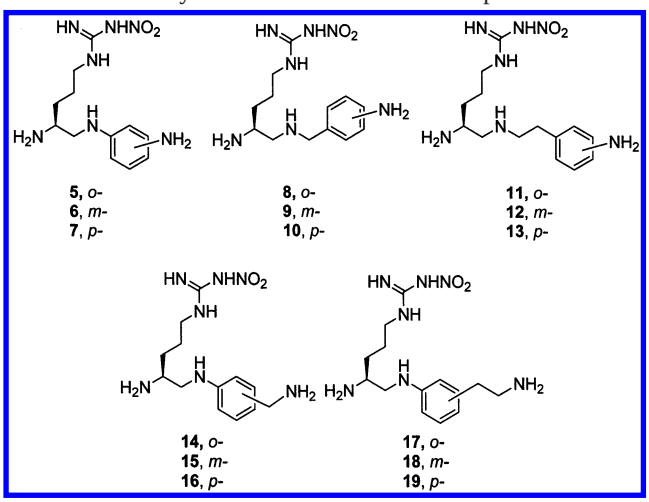
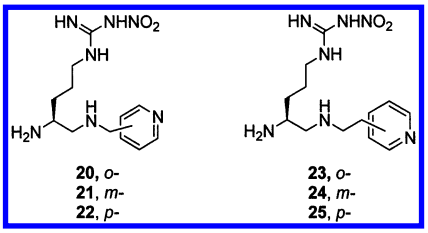
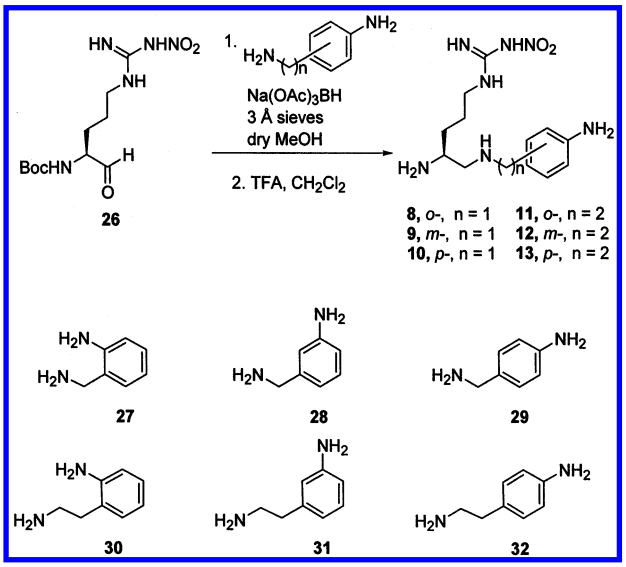


Chart 3. Pyridinyl Reduced Amide Bond Peptidomimetics

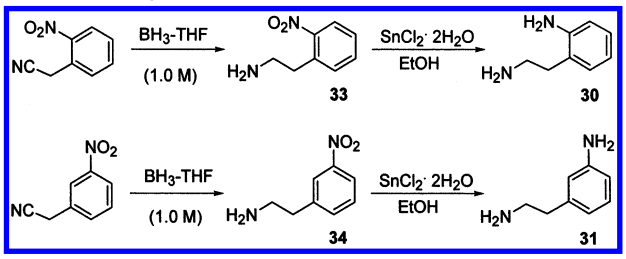


Scheme 1. Synthesis of the Substituted Phenyl, Reduced Amide Bond Analogues (**8**−**13**)

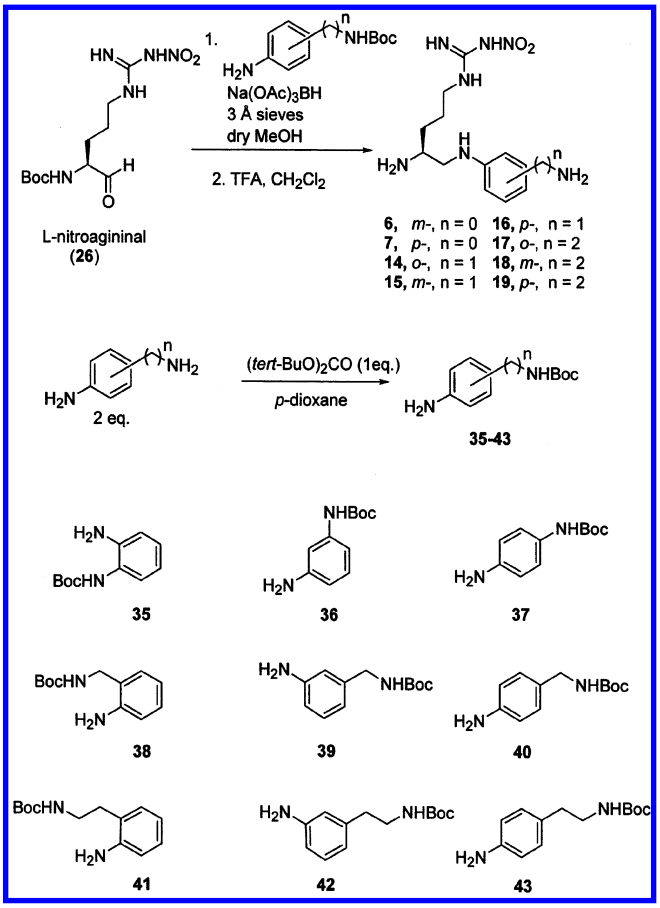


much more reactive than aniline. Because of the relative acidity of the α-proton of *N*-Boc-L-nitroargininal, it is reasonable that reductive amination could lead to racemization. Chiral chromatography was carried out to determine if loss of chirality occurred. Chiral normal-phase HPLC of the free base (Daicel ChiralPak AD, ChiralPakAS, ChiralPak OD; solvents system: hexanes/isopropyl alcohol (30−80%) with 0.5% diethylamine, flow rate = 1 mL/min), reverse-phase HPLC with *o*-phthaldialdehyde, *N*-acetyl-L-cysteine derivatization (Alltech, Econosil C18; solvent system: 80% methanol + 20% 50 mM sodium acetate pH 9, flow rate = 1 mL/min), and reverse-phase chiral HPLC of the salts (Regis ChirosilSCA; solvent system: water/methanol (40−70%)

Scheme 2. Syntheses of **30** and **31**



Scheme 3. Synthesis of the Substituted Phenyl, Reduced Amide Bond Analogues (**6**−**7**, and **14**−**19**)

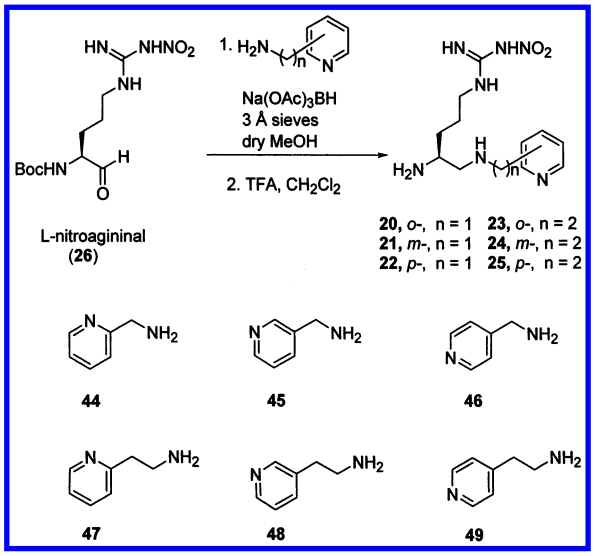


with 0.05% acetic acid, flow rate = 1 mL/min) gave one peak for **13**, **17**, and **25**. It is not clear if the enantiomers were not separated by these procedures or if there is only one isomer formed, but the racemization of nitroarginal is known to be limited (only 9% at room temperature after 22 h.[22] The specific rotations for all three of these compounds were greater than zero, so, if the stereochemistry of the compounds was compromised, it was not to the degree of racemization.

Most of the corresponding aromatic diamines were commercially available, but 2- and 3-(2-aminoethyl)-aniline (**30**, **31**) were synthesized (Scheme 2). The starting materials were the corresponding (nitrophenyl)-acetonitriles, which were sequentially reduced by BH_3−THF[23] complex and tin chloride hydrate[24] to give **30** and **31**.

The aniline amino group of the (aminoalkyl)anilines (**27**−**32**) were coupled with *N*-Boc-L-nitroargininal (Scheme 3). In this case, protection of the alkylamine moieties preceded the reductive amination (except **36** and **39** were commercially available).[25] Using 2 equiv

Scheme 4. Synthesis ofthe Pyridyl, Reduced-amide Bond Analogues (**20**−**25**)



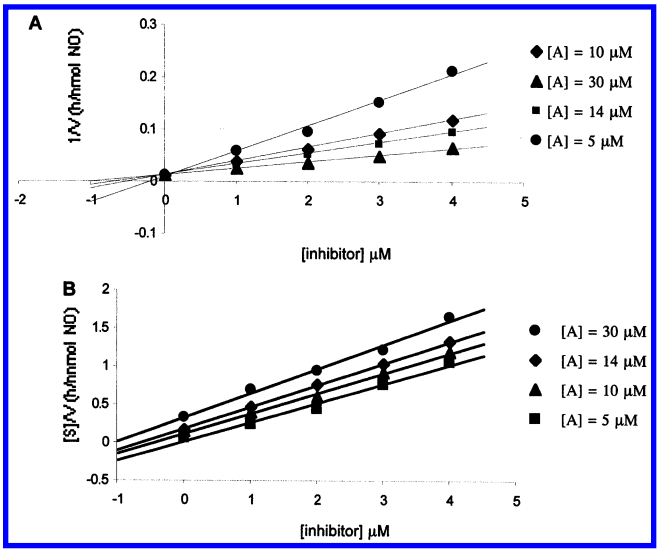


Figure 3. Inhibition of nNOS by **17**. A: Dixon plot for the inhibition of nNOS by **17**. B: Cornish−Bowden plot of competitive inhibition.

of (aminoalkyl)aniline was sufficient to attain monoprotection, while 8 equiv of the (aminoalkyl)aniline was necessary for the monoprotection of α,ω-dialkylamines.

Among these target molecules, **5** (Chart 2) could not be made using this same synthetic method. The reductive coupling of mono-Boc-protected benzene-1,2-diamine (**34**) with *N*-Boc-L-nitroargininal was not successful; the bulky Boc-group was thought to be the reason. However, it turned out that the low reactivity of benzene-1,2-diamine, not the steric factor, was the cause, because reductive aminations with either free benzene-1,2-diamine or monomethylcarbamoyl-protected benzene-1,2-diamine proved to be fruitless. Furthermore, *N*-Boc-L-nitroargininal is also somewhat unreactive, because it exists in two forms in solution: cyclized hemiaminal and aldehyde.[12] To accomplish this reaction, different reaction conditions, other than reductive amination, are needed. However, further investigation of this reaction was postponed until enzyme tests could show whether this family of compounds was potent enough to warrant synthesis of this analogue.

Scheme 4 shows the synthetic route for the pyridyl, reduced amide bond analogues. All of the aminoalkyl pyridines (**44**−**49**) were commercially available, and in each case the reductive amination with *N*-Boc-L-nitroargininal worked well.

All products from the coupling reactions were purified by column chromatography using various solvent systems, and the Boc group was deprotected in 30% TFA/methylene chloride.

Enzymology. All of the aromatic, reduced amide analogues are competitive inhibitors of the three isoforms of NOS. Representative plots for the competitive inhibition of nNOS by **17** are shown in Figure 3. Dixon analysis (Figure 3A)[26] and the method of Cornish and Bowden (Figure 3B)[27] were used to determine the type of inhibition.

The K_i data for the aromatic, reduced amide bond analogues (**6**−**25**) are given in Table 1 along with the data for the D-Phe-D-Arg^{NO2}-OMe (**2**) and the reduced amide ethyl analogue (**50**).[20]

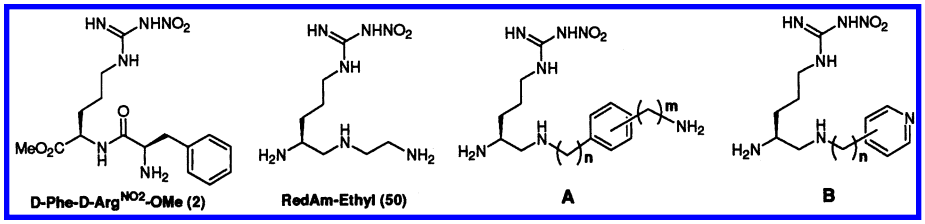
Results and Discussion

As shown in the Table 1, all of the aromatic, reduced amide bond analogues were found to be nNOS-selective inhibitors. When compared to the lead compound, RedAm-ethyl (**50**), the potencies on nNOS are decreased (except **17**), but most of the compounds showed still higher potency than D-Phe-D-Arg^{NO2}-OMe (**2**). However, the potencies with iNOS also were decreased from those of **50**, except for **14** and **17**, but not as low as **2**, giving good nNOS/iNOS selectivities. Disappointingly, the K_i values with iNOS are closer to that of **50** or L-Arg^{NO2}-L-Dbu-NH$_2$ (**1**) than to that of D-Phe-D-Arg^{NO2}-OMe (**2**), indicating that the desired unfavorable interaction of the aromatic moiety in the iNOS active site did not occur in this series, and the nNOS/iNOS selectivities were not as high as expected.

The first two benzenediamine compounds (**6**, **7**) were found to be poorer inhibitors of nNOS and iNOS than RedAm-ethyl (**50**), while having little difference toward eNOS inhibition. This results in the dramatic decrease of isoform selectivity of nNOS over eNOS (from 2577-fold to 36- and 51-fold). This implies the amino residue of the C-terminal phenyl ring is not long enough for an effective interaction with the enzyme.

The members of the (aminomethyl)aniline series (**8**−**10**) were also poor inhibitors of nNOS and iNOS. The rigid C-terminal aniline structure does not seem to be favored for interaction with the enzyme in the nNOS active site. This is clear in the case of the ortho-isomers (**8** and **14**), which showed almost the same potency with all of the isozymes, thereby resulting in the loss of isoform selectivity. However, when the (aminomethyl)aniline moiety was attached at the anilineamino group (but with the same length, **14**−**16**), a favorable result was obtained for the meta-isomer (**15**), indicating that a flexible amino residue is better for selective interaction with nNOS and that spatial geometry seems to be very important. Compound **15** showed almost the same potency and selectivity as RedAm-ethyl (**50**) implying that the spatial geometry in the enzyme active site is similar.

Table 1. NOS Inhibition by the Aromatic, Reduced Amide Analogues[a]



compound	K_i (μM)[b]			selectivity[c]	
	nNOS	iNOS	eNOS	iNOS/nNOS	eNOS/nNOS
D-Phe-D-Arg^{NO2}-OMe (**2**)[d]	2	3600	5	1800	2.5
RedAm-Ethyl (50)	0.12	39	314	320	2577
6 (**A**, $n = 0$, $m = 0$, meta)	2.20	279	78.6	127	36
7 (**A**, $n = 0$, $m = 0$, para)	2.28	339	116	149	51
8 (**A**, $n = 1$, $m = 0$, ortho)	20.3	86.3	86.1	4.25	4.24
9 (**A**, $n = 1$, $m = 0$, meta)	2.06	572	185	278	90
10 (**A**, $n = 1$, $m = 0$, para)	1.60	326	303	204	189
11 (**A**, $n = 2$, $m = 0$, ortho)	1.36	184	30.1	135	22.1
12 (**A**, $n = 2$, $m = 0$, meta)	1.36	166	47.6	122	35
13 (**A**, $n = 2$, $m = 0$, para)	0.74	339	170	458	230
14 (**A**, $n = 0$, $m = 1$, ortho)	5.55	29.3	39.8	5.28	7.17
15 (**A**, $n = 0$, $m = 1$, meta)	0.21	80	194	381	924
16 (**A**, $n = 0$, $m = 1$, para)	1.37	335	409	245	299
17 (**A**, $n = 0$, $m = 2$, ortho)	0.05	3.51	105	70.2	2121
18 (**A**, $n = 0$, $m = 2$, meta)	2.21	260	100	118	45.2
19 (**A**, $n = 0$, $m = 2$, para)	1.66	360	414	217	249
20 (**B**, $n = 1$, ortho)	2.90	154	443	53	153
21 (**B**, $n = 1$, meta)	0.55	141	523	256	951
22 (**B**, $n = 1$, para)	1.59	392	589	247	370
23 (**B**, $n = 2$, ortho)	2.21	72.6	123	33	254
24 (**B**, $n = 2$, meta)	2.06	178	275	86	133
25 (**B**, $n = 2$, para)	0.76	395	193	520	56

[a] The K_i values are calculated from the measured IC_{50} values. The enzymes used are recombinant rat nNOS, recombinant murine iNOS, and recombinant bovine eNOS. [b] The K_i values represent at least duplicate measurements; standard deviations of ±8−12% were observed. [c] The ratio of K_i (eNOS or iNOS) to K_i (nNOS); all are nNOS selective. [d] Data taken from ref 10.

The inhibitory potency of the (aminoethyl)aniline series **11**−**13** and **17**−**19** also varied depending on the location of the rigid phenyl ring. It appears that the para-isomer is preferred when the phenyl ring is at the C-terminus (**13**), but the ortho-isomer is much more preferred when the flexible alkylamine residue is at the C-terminus (**17**) in the same length of compounds. Compound **17** was the best nNOS inhibitor ($K_i = 50$ nM) of all of the compounds this series, and the isoform selectivity over eNOS was also excellent (>2000-fold); however, the selectivity over iNOS is only 70-fold.

The variation of the inhibitory potencies with nNOS as a result of a small structural change was also found in the pyridinyl series (**20**−**25**). They are generally weak inhibitors of nNOS; **21** was the best of these compounds, with good isoform selectivity over iNOS and eNOS.

In conclusion, the hypothesized great increase in isoform selectivity of nNOS over iNOS was not obtained in the aromatic, reduced amide bond peptidomimetic series. However, the high potency with nNOS as well as high selectivity of nNOS over eNOS was retained in some of these compounds (**15**, **17**, **21**), as well as good selectivity over iNOS. Further modification of compound **17**, which was the best nNOS-inhibitor in these series of compounds, and the most potent nNOS inhibitor of all of the dipeptide or dipeptidomimetic compounds so far reported, should be an effective way to develop more potent and selective nNOS-inhibitors.

Experimental Section

General Methods. NOS assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Inova 500-MHz or Varian Mercury 400-MHz NMR spectrometer. Chemical shifts are reported as δ values in parts per million downfield from TMS (δ 0.0) as the internal standard in $CDCl_3$. For samples run in D_2O, the HOD resonance was arbitrarily set at 4.80 ppm, and the CD_3-OH resonance was set at 4.87 ppm in CD_3OD. An Orion Research model 701 pH meter with a general combination electrode was used for pH measurements. Electrospray mass spectra were obtained on a Micromass Quattro II spectrometer. Elemental analyses were obtained from Oneida Research Services, Inc., Whiteboro, NY. Thin-layer chromatography was carried out on E. Merck precoated silica gel 60 F_{254} plates. Amino acids were visualized with a ninhydrin spray reagent or a UV/vis lamp. E. Merck silica gel 60 (230−400 mesh) was used for flash column chromatography.

High-performance liquid chromatography was performed on a Beckman System Gold (Model 125P solvent module and Model 166 detector). In case of analytical HPLC, samples were analyzed by elution from a Hypersil ODS C_{18} column (Agilent, 5 μm, 4.0 × 250 mm), with a flow rate of 1 mL/min. The mobile phase was a gradient from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% of TFA in CH_3CN) to 60% solvent A and 40% solvent B over 5 min, then to 30% solvent A and 70% solvent B over 20 min and to 100% solvent A over 10 min. Sample elution was detected by absorbance at 254 nm. For the purification of final products, a Whatman Partsil C_{18} semi-prep HPLC column (9.4 × 125 mm) was used. Samples were eluted using a gradient of 100% solvent A (0.1% TFA in water) to 70% of solvent B (0.1% of TFA in CH_3CN) over 30 min at a flow rate of 4 mL/min.

Reagents and Materials. Amino acids were purchased from Advanced ChemTech, Inc. 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. 2-, 3-, and 4-(amino-

methyl)aniline were purchased from TCI, America. All other chemicals were purchased from Aldrich, unless otherwise stated.

Chemical Synthesis. General Procedure for the Reductive Amination of 6−25. To a solution of N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal[21] (1 equiv) in dry methanol were added aromatic diamine (1.5 equiv) and 3 Å molecular sieves, and the mixture was stirred at room temperature. After being stirred for 1 h, the reaction mixture was treated with sodium triacetoxyborohydride (2 equiv) and was stirred overnight. The reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel to afford the *N*-Boc product as a solid, which was treated with 30% TFA in CH_2Cl_2 for 2 h. Excess trifluoroacetic acid and solvent were removed by evaporation. The residue was dissolved in a small amount of water, which was washed with ether and lyophilized.

***N*-(4*S*)-{[4-Amino-5-(3-amino)phenylamino]pentyl}-*N*′-nitroguanidine (6).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (500 mg, 1.66 mmol) and (3-aminophenyl)carbamic acid *tert*-butyl ester (514.9 mg, 2.49 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 3:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-{[4-amino-5-(3-amino)-phenylamino]pentyl}-*N*′-nitroguanidine (300 mg, 37%). Removal of the Boc-group yielded 170 mg of **6** (95%, purple solid): ^{1}H NMR (500 MHz, D_2O) δ 7.11 (t, J = 8 Hz, 1H), 6.60 (d, J = 8 Hz, 1H), 6.52 (d, J = 8 Hz, 1H), 6.47 (s, 1H), 3.31 (m, 1H), 3.22 (m, 2H), 3.10 (brs, 2H), 1.41−1.73 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{12}H_{22}N_7O_2$ 296.1835, found 296.1846.

***N*-(4*S*)-{[4-Amino-5-(4-amino)phenylamino]-pentyl}-*N*′-nitroguanidine (7).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (500 mg, 1.66 mmol) and (4-aminophenyl)carbamic acid *tert*-butyl ester (515 mg, 2.49 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 2:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-{[4-amino-5-(4-amino)-phenylamino]pentyl}-*N*′-nitroguanidine (400 mg, 49%). Removal of Boc-group from 360 mg yielded 197 mg of **7** (92%, orange solid): ^{1}H NMR (500 MHz, D_2O) δ 7.04 (d, J = 8 Hz, 2H), 6.65 (d, J = 8 Hz, 2H), 3.34 (m, 1H), 3.30 (m, 2H), 3.14 (brs, 2H), 1.40−1.81 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{12}H_{22}N_7O_2$ 296.1835, found 296.1848.

***N*-(4*S*)-[4-Amino-5-(2-aminobenzylamino)pentyl]-*N*′-nitroguanidine (8).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (359 mg, 1.19 mmol) and (2-aminomethyl)aniline (204 μL, 2.49 mmol). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 3:2) to afford N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(2-amino)benzylamino]pentyl}-*N*′-nitroguanidine (270 mg, 56%) as a white solid. Removal of the Boc-group (30% TFA) gave a yellow foam (204 mg, 95% yield): ^{1}H NMR (400 MHz, D_2O) δ 7.57 (m, 2H), 7.46 (d, J = 3.6 Hz, 1H), 7.29 (m, 1H), 3.44 (m, 1H), 3.31 (m, 2H), 3.17 (m, 2H), 1.46−1.90 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{23}N_7O_2$ 310.1991, found 310.1920.

***N*-(4*S*)-[4-Amino-5-(3-aminobenzylamino)pentyl]-*N*′-nitroguanidine (9).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (500 mg, 1.66 mmol) and (3-aminomethyl)aniline (284 μL, 2.49 mmol). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 3:2) to afford N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-[4-amino-5-(2-aminobenzylamino)pentyl]-*N*′-nitroguanidine (400 mg, 58.9%) as a white solid. Removal of Boc-group gave a light brown foam of **9** (278 mg, 92% yield): ^{1}H NMR (500 MHz, D_2O) δ 7.23 (m, 2H), 7.18 (brs, 1H), 7.13 (d, J = 3 Hz, 1H), 4.01 (s, 2H), 3.39 (q, J = 6 Hz, 1H), 3.12 (d, J = 5.5 Hz, 2H), 2.93 (brs, 2H), 1.25−1.65 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{23}N_7O_2$ 310.1991, found 310.2008.

***N*-(4*S*)-[4-Amino-5-(4-aminobenzylamino)pentyl]-*N*′-nitroguanidine (10).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (500 mg, 1.66 mmol) and (4-aminomethyl)aniline (284 μL, 2.49 mmol). The residue was purified by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 3:2) to afford N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-[4-amino-5-(2-aminobenzylamino)pentyl]-*N*′-nitroguanidine (410 mg, 62%) as a white solid. An aliquot of 304 mg was treated with 30% TFA in CH_2Cl_2 to give **10** (204 mg, 89%, brown solid): ^{1}H NMR (500 MHz, D_2O) δ 7.43 (d, J = 8.5 Hz, 2H), 7.28 (d, J = 8.5 Hz, 2H), 4.16 (s, 2H), 3.52 (q, J = 5.5 Hz, 1H), 3.26 (d, J = 5.5 Hz, 2H), 3.10 (brs, 2H), 1.45−1.73 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{23}N_7O_2$ 310.1991, found 310.1992.

***N*-(4*S*)-{[4-Amino-5-(2-aminophenyl)ethylamino]-pentyl}-*N*′-nitroguanidine (11).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (400 mg, 1.32 mmol) and 2-(2-aminoethyl)-aniline (361 mg, 2.65 mmol). The purification by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 1:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-{[4-amino-5-(2-aminophenyl)ethylamino]pentyl}-*N*′-nitroguanidine (415 mg, 74%). Removal of the Boc-group yielded 282 mg of **11** (89%, brown solid): ^{1}H NMR (500 MHz, D_2O) δ 7.26 (m,4H), 3.53 (m, 1H), 3.26 (m, 4H), 3.12 (brs, 2H), 2.95 (t, J = 6.8 MHz, 2H), 1.36−1.80 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{14}H_{26}N_7O_2$ 324.2148, found 324.2151

***N*-(4*S*)-{[4-Amino-5-(3-aminophenyl)ethylamino]-pentyl}-*N*′-nitroguanidine (12).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (400 mg, 1.32 mmol) and 3-(2-aminoethyl)-aniline (361 mg, 2.65 mmol). The purification by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 1:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-[4-amino-5-(3-amino-phenyl)-ethylamino)pentyl]-*N*′-nitroguanidine (250 mg, 45%). Removal of the Boc-group yielded 189 mg of **12** (99%, brown solid): ^{1}H NMR (400 MHz, D_2O) δ 7.30 (d, J = 7.2 Hz, 1H), 7.21 (d, J = 7.2 Hz, 1H), 7.11 (m, 2H), 3.49 (m, 1H), 3.20 (m, 4H), 3.11 (brs, 2H), 2.90 (t, J = 7.2 MHz, 2H), 1.33−1.77 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{14}H_{26}N_7O_2$ 324.2148, found 324.2147.

***N*-(4*S*)-{[4-Amino-5-(4-aminophenyl)ethylamino]-pentyl}-*N*′-nitroguanidine (13).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (500 mg, 1.66 mmol) and 4-(2-aminoethyl)-aniline (338 μL, 2.49 mmol). The purification by flash column chromatography on silica gel (CH_2Cl_2:MeOH = 2:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-[4-amino-5-(4-aminophenyl)ethylamino)pentyl]-*N*′-nitroguanidine (380 mg, 54%). Removal of the Boc-group yielded 218 mg of **13** (95%, yellow solid): ^{1}H NMR (500 MHz, D_2O) δ 7.28 (d, J = 8 Hz, 2H), 7.21 (d, J = 8 Hz, 2H), 3.53 (m, 1H), 3.24 (m, 4H), 3.15 (brs, 2H), 2.94 (t, J = 7.5 MHz, 2H), 1.43−1.77 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{14}H_{26}N_7O_2$ 324.2148, found 324.2150. Anal.Calcd for $C_{14}H_{25}N_7O_2 \cdot 3TFA \cdot H_2O$: C, 35.15,. H, 4.42,. N,14.35; Found: C, 35.65, H, 4.25, N, 14.30. $[\alpha]^{24}_D$ +2.96 (c = 1.15, MeOH).

***N*-(4*S*)-{4-Amino-5-[(2-aminomethyl)phenylamino]-pentyl}-*N*′-nitroguanidine (14).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (200 mg, 0.73 mmol) and (2-aminobenzyl)-carbamic acid *tert*-butyl ester (324 mg, 1.46 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 2:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(3-aminomethyl)phenylamino]pentyl}-*N*′-nitroguanidine (160 mg, 43%). Removal of the Boc-group yielded 92.3 mg of **14** (95%, orange solid): ^{1}H NMR (400 MHz, D_2O) δ 7.55 (m, 2H), 7.46 (m, 1H), 7.27 (m, 1H), 3.63 (m, 1H), 3.31 (m, 2H), 3.23 (m, 4H), 1.40−1.88 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{23}N_7O_2$ 310.1991, found 310.1931.

***N*-(4*S*)-{4-Amino-5-[(3-aminomethyl)phenylamino]-pentyl}-*N*′-nitroguanidine (15).** This compound was prepared as described above using N^{α}-(*tert*-butoxycarbonyl)-L-nitroargininal (458.5 mg, 1.52 mmol) and (3-aminobenzyl)-carbamic acid *tert*-butyl ester (675 mg, 3.04 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 1:1) gave N^{α}-(*tert*-butoxycarbonyl)-(4*S*)-{4-

amino-5-[(3-aminomethyl)phenylamino]pentyl}-*N*′-nitroguanidine (320 mg, 41%). Removal of the Boc-group yielded 120 mg of **15** (62%, brown solid): ^{1}H NMR (400 MHz, D_2O) δ 7.18 (t, J = 7.5 Hz, 1H), 6.79 (m, 3H), 3.91 (s, 2H), 3.55 (m, 1H), 3.37 (m, 2H), 3.09 (m, 2H), 1.36−1.82 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{23}N_7O_2$ 310.1991, found 310.1992. Anal.Calcd for $C_{13}H_{23}N_7O_2$·3TFA·2H_2O: C, 33.19,· H, 4.40,· N,-14.26; Found: C, 33.62, H, 4.38, N, 14.19.

***N*-(4*S*)-{4-Amino-5-[(4-aminomethyl)phenylamino]-pentyl}-*N*′-nitroguanidine (16).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (500 mg, 1.66 mmol) and (4-aminobenzyl)-carbamic acid *tert*-butyl ester (569 mg, 2.49 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 2:1) gave N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(4-aminomethyl)phenylamino]pentyl}-*N*′-nitroguanidine (390 mg, 46%). Removal of the Boc-group yielded 234 mg of **16** (99%, pale yellow solid): ^{1}H NMR (400 MHz, D_2O) δ 7.12 (d, J = 8 Hz, 2H), 6.65 (d, J = 8 Hz, 2H), 3.89 (s, 2H), 3.36 (m, 1H), 3.29 (m, 2H), 3.14 (brs, 2H), 1.36−1.62 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{23}N_7O_2$ 310.1991, found 310.1992.

***N*-(4*S*)-{4-Amino-5-[2-(2-aminoethyl)phenylamino]-pentyl}-*N*′-nitroguanidine (17).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (463.5 mg, 1.53 mmol) and [2-(2-aminophenyl)-ethyl]carbamic acid *tert*-butyl ester (544 mg, 2.30 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 2:1) gave N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[2-(2-aminoethyl)phenylamino]pentyl}-*N*′-nitroguanidine (300 mg, 38%). Removal of the Boc-group yielded 176 mg of **17** (95%, brown solid): ^{1}H NMR (400 MHz, D_2O) δ 7.06 (t, J = 6.8 Hz, 1H), 6.94 (d, J = 6.8 Hz, 1H), 6.60 (m, 2H), 3.37 (m, 1H), 3.26 (m, 2H), 3.10 (brs, 2H), 3.04 (t, J = 7.2 Hz, 2H), 2.72 (t, J = 7.2 Hz, 2H), 1.38−1.78 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{14}H_{26}N_7O_2$ 324.2148, found 324.2137. Anal.Calcd for $C_{14}H_{25}N_7O_2$·3TFA·H_2O: C, 35.15, H, 4.42, N,-14.35; Found: C, 35.59,· H, 4.32, ·N, 14.41. $[\alpha]^{24}_D$ +2.70 (c = 0.89, MeOH).

***N*-(4*S*)-{4-Amino-5-[3-(2-aminoethyl)phenylamino]-pentyl}-*N*′-nitroguanidine (18).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (433 mg, 1.43 mmol) and [3-(2-aminophenyl)-ethyl]carbamic acid *tert*-butyl ester (508 mg, 2.15 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 2:1) gave N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[3-(2-aminoethyl)phenylamino]pentyl}-*N*′-nitroguanidine (314 mg, 42%). Removal of the Boc-group yielded 174 mg of **18** (90%, brown solid): ^{1}H NMR (400 MHz, D_2O) δ 7.07 (t, J = 7.6 Hz, 1H), 6.59 (m, 3H), 3.55 (m, 1H), 3.45 (m, 2H), 3.31 (m, 2H), 3.09 (m, 4H), 1.34−1.74 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{14}H_{26}N_7O_2$ 324.2148, found 324.2137.

***N*-(4*S*)-{4-Amino-5-[4-(2-aminoethyl)phenylamino]-pentyl}-*N*′-nitroguanidine (19).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (477 mg, 1.58 mmol) and [4-(2-amino-phenyl)-ethyl]carbamic acid *tert*-butyl ester (560 mg, 2.37 mmol). The purification by flash column chromatography on silica gel (EtOAc:hexane = 2:1) gave N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[4-(2-aminoethyl)phenylamino]pentyl}-*N*′-nitroguanidine (300 mg, 36%). Removal of the Boc-group yielded 176 mg of **19** (95%, light brown solid): ^{1}H NMR (500 MHz, D_2O) δ 7.01 (d, J = 8 Hz, 2H), 6.64 (d, J = 8 Hz, 2H), 3.60 (m, 1H), 3.49 (m, 1H), 3.29(m, 3H), 3.15 (m, 2H), 2.72 (t, J = 7.5 Hz, 2H), 1.42−1.81 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{14}H_{26}N_7O_2$ 324.2148, found 324.2154.

***N*-(4*S*)-{4-Amino-5-[(pyridin-2-yl)methyl]aminopentyl}-*N*′-nitroguanidine (20).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (400 mg, 1.32 mmol) and 1-(pyridin-2-yl)methylamine (204.8 μL, 1.98 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:MeOH = 1:1) to afford N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(pyridin-2-yl-methyl)amino]pentyl}-*N*′-nitroguanidine (340 mg, 65%) as a yellow solid. Removal of the Boc-group gave **20** as a pale yellow foam (251 mg, 99%, brown solid): ^{1}H NMR (500 MHz, D_2O) δ 8.64 (d, J = 5.5 Hz, 1H), 8.37 (t, J = 8 Hz, 1H), 7.91 (d, J = 8 Hz, 1H), 7.83 (t, J = 6.5 Hz, 1H), 4.51 (s, 2H), 3.57 (m, 1H), 3.37 (m, 2H), 3.12 (brs, 2H), 1.42−1.80 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{12}H_{22}N_7O_2$ 296.1385, found 296.1837.

***N*-(4*S*)-{4-Amino-5-[(pyridin-3-yl)methyl]amino]pentyl}-*N*′-nitroguanidine (21).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (400 mg, 1.32 mmol) and 1-(pyridin-3-yl)methylamine (202 μL, 1.98 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:MeOH = 1:1) to afford N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(pyridin-3-ylmethyl)amino]pentyl}-*N*′-nitroguanidine (300 mg, 58%) as a yellow solid. Removal of the Boc-group gave **21** as a greenish yellow foam (217 mg, 97%): ^{1}H NMR (500 MHz, D_2O) δ 8.78 (brs, 1H), 8.68 (d, J = 5.5 Hz, 1H), 8.56 (d, J = 7.5 Hz, 1H), 7.96 (t, J = 7.5 Hz, 1H), 4.39 (s, 2H), 3.55 (m, 1H), 3.36 (m, 2H), 3.08 (brs, 2H), 1.39−1.82 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{12}H_{22}N_7O_2$ 296.1835, found 296.1839. Anal.Calcd for $C_{12}H_{21}N_7O_2$·3TFA·2H_2O: C, 32.10, H, 4.19, N,14.56; Found: C, 31.62, H, 4.16, N, 14.47.

***N*-(4*S*)-{4-Amino-5-[(pyridin-4-yl)methyl]amino]pentyl}-*N*′-nitroguanidine (22).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (420 mg, 1.39 mmol) and 1-(pyridin-4-yl)methylamine (211 μL, 2.09 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:MeOH = 5:2) to afford N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(pyridin-4-ylmethyl)aminopentyl}-*N*′-nitroguanidine (419 mg, 76%) as a yellow solid. Removal of the Boc-group gave **22** as a orange yellow foam (310 mg, 99%): ^{1}H NMR (500 MHz, D_2O) δ 8.72 (d, J = 6 Hz, 2H), 8.01 (d, J = 6 Hz, 2H), 4.50 (s, 2H), 3.62 (m, 1H), 3.43 (m, 2H), 3.15 (brs, 2H), 1.46−1.83 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{12}H_{22}N_7O_2$ 296.1835, found 296.1848.

***N*-(4*S*)-{4-Amino-5-[(2-pyridin-2-yl)ethyl]aminopentyl}-*N*′-nitroguanidine (23).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (415 mg, 1.37 mmol), 2-(pyridin-2-yl)ethylamine (190 μL, 1.51 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:MeOH = 1:1) to afford N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(2-pyridin-2-yl)ethyl]-aminopentyl}-*N*′-nitroguanidine (400 mg, 71%) as a yellow solid. Removal of the Boc-group gave **23** as a light brown foam (296 mg, 98%): ^{1}H NMR (500 MHz, D_2O) δ 8.38 (d, J = 5.5 Hz, 1H), 8.21 (t, J = 8 Hz, 1H), 7.66 (d, J = 8 Hz, 1H), 7.63 (m, 1H), 3.41 (m, 1H), 3.30 (m, 2H), 3.24 (m, 2H), 3.16 (m, 2H), 2.95 (brs, 2H), 1.26−1.62 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{24}N_7O_2$ 310.1991, found 310.1995.

***N*-(4*S*)-{4-Amino-5-[(2-pyridin-3-yl)ethyl]aminopentyl}-*N*′-nitroguanidine (24).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (460 mg, 1.52 mmol), 2-(pyridin-3-yl)ethylamine dihydrobromide salt (509 mg, 1.82 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:MeOH = 1:1) to afford N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(2-pyridin-3-yl)ethyl]aminopentyl}-*N*′-nitroguanidine (404 mg, 65%) as a yellow solid. Removal of the Boc-group gave **24** as a pale yellow foam (290 mg, 95%): ^{1}H NMR (500 MHz, D_2O) δ 8.49 (brs, 1H), 8.45 (d, J = 4.5 Hz, 1H), 8.30 (d, J = 6.5 Hz, 1H), 7.77 (ddd, J = 4.5, 6 Hz, 1H), 3.46 (m, 1H), 3.25 (m, 2H), 3.20 (m, 2H), 3.07 (m, 2H), 3.02 (brs, 2H), 1.35−1.71 (m, 4H). HRMS (ES) (*m*/*z*): M + H$^+$ calcd for $C_{13}H_{24}N_7O_2$ 310.1991, found 310.1995.

***N*-(4*S*)-{4-Amino-5-[(2-pyridin-4-yl)ethylamino]pentyl}-*N*′-nitroguanidine (25).** This compound was prepared as described above using N^α-(*tert*-butoxycarbonyl)-L-nitroargininal (400 mg, 1.32 mmol), 2-(pyridin-4-yl)ethylamine (249 μL, 1.98 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:MeOH = 1:1) to afford N^α-(*tert*-butoxycarbonyl)-(4*S*)-{4-amino-5-[(2-pyridin-4-yl)ethylamino]pentyl}-*N*′-nitroguanidine (400 mg, 74%) as a yellow solid. Removal of the Boc-group gave **25** as a white foam (287 mg, 95%): ^{1}H NMR (500 MHz, D_2O) δ 8.45 (d, J = 6 Hz, 1H),

7.73 (d, J = 6 Hz, 2H), 3.49 (q, J = 6 Hz, 1H), 3.30 (m, 2H), 3.21 (m, 1H), 3.15 (t, J = 7.5 Hz, 2H), 3.03 (brs, 2H), 1.33−1.76 (m, 4H). HRMS (ES) (m/z): M + H^+ calcd for $C_{13}H_{24}N_7O_2$ 310.1991, found 310.1994. $[\alpha]^{24}_D$ +5.30 (c = 0.88, MeOH).

2-(2-Aminoethyl)aniline (30). To a solution of (2-nitrophenyl)acetonitrile (3 g, 18.5 mmol) was added dropwise a 1 M BH_3−THF solution (125 mL) at 0 °C. After 4 h of stirring at 25 °C, 6 N HCl solution (125 mL) was added to the reaction mixture at 0 °C. After evaporation of the organic solvent in vacuo, the aqueous phase was basified with a 4 N NaOH solution to pH 10. The product was extracted with EtOAc, and the organic phase was dried over $MgSO_4$ and concentrated in vacuo. A brown liquid (**32**, 2 g, 10.8 mmol) was isolated. Without further purification, it was mixed with $SnCl_2 \cdot 2H_2O$ (13.57 g, 60.2 mmol) and absolute ethanol (20 mL). The suspension was heated at 70 °C under nitrogen. After being stirred for 30 min, the starting material disappeared, the solution was allowed to cool, then poured into ice (100 g). The pH was made slightly basic (pH 8) by the addition of a 5% aqueous $NaHCO_3$ solution, and the resulting basic mixture was stirred for 1 h. The precipitate was extracted with ethyl acetate (100 mL × 3), then the extract was washed with water (50 mL × 3) and dried over $MgSO_4$. The product was obtained in a yield of 52% (1.3 g, brown liquid, over two steps) after evaporation of the solvent: ^{1}H NMR (400 MHz, CD_3OD) δ 6.97 (t, J = 7.2 Hz, 2H), 6.72 (d, J = 7.2 Hz, 2H), 6.64 (t, J = 7.2 Hz, 1H), 2.82 (t, J = 7.2 Hz, 2H), 2.66 (t, J = 7.2 Hz, 2H). HRMS (EI) m/z (M^+) calcd for $C_8H_{12}N_2$ 136.1000, found 136.1002.

3-(2-Aminoethyl)aniline (31). This compound was prepared as described above using (3-nitrophenyl)acetonitrile (2.2 g, 13.6 mmol). Evaporation of the solvent gave the product in a yield of 81% (1.5 g, brown liquid, over two steps): ^{1}H NMR (400 MHz, CD_3OD) δ 7.02 (t, J = 7.2 Hz, 1H), 6.59 (m, 2H), 6.56 (m, 1H), 2.84 (t, J = 7.2 Hz, 2H), 2.64 (t, J = 7.2 Hz, 2H). HRMS (EI) m/z (M^+) calcd for $C_8H_{12}N_2$ 136.1000, found 136.0980.

(3-Aminophenyl)carbamic Acid *tert*-Butyl Ester (36). A solution of di-*tert*-butyl dicarbonate (2 g, 9.16 mmol, 1 equiv) in dioxane (25 mL) was added over a period of 30 min to a solution of 1,3-phenylenediamine (2 g, 18.5 mmol, 2 equiv) in dioxane (25 mL). The mixture was allowed to stir for 22 h, and the solvent was removed using a rotary evaporator. The residue was purified by flash column chromatography on silica gel (EtOAc:hexane = 2:1) to afford 1.7 g of product (89% based on dicarbonate) as a peach color solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 (t, J = 8 Hz, 1H), 6.60 (d, J = 8 Hz, 1H), 6.52 (t, J = 8 Hz, 1H), 6.47 (s, 1H), 4.79 (s, 1H), 1.44 (s, 9H). HRMS (EI) m/z (M^+) calcd for $C_{11}H_{16}N_2O_2$ 208.1211, found 208.1201.

(2-Aminobenzyl)carbamic Acid *tert*-Butyl Ester (38). This compound was prepared as described above using di-*tert*-butyl dicarbonate (2 g, 9.16 mmol) and 2-aminomethylaniline (2 g, 18.5 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:hexane = 2:1) to afford 1.5 g of product (79% based on dicarbonate) as a light yellow solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (t, J = 7.6 Hz, 1H), 7.02 (d, J = 6.8 Hz, 1H), 6.67 (m, 2H), 4.80 (s, 1H), 1.44 (s, 9H). HRMS (EI) m/z (M^+) calcd for $C_{12}H_{18}N_2O_2$ 222.1369, found 222.1357.

(3-Aminobenzyl)carbamic Acid *tert*-Butyl Ester (39). This compound was prepared as described above using di-*tert*-butyl dicarbonate (0.89 g, 4.07 mmol) and 3-aminomethylaniline (1 g, 8.18 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:hexane = 2:1) to afford 0.95 g of brown liquid (quantitative yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (t, J = 7.6 Hz, 1H), 6.64 (d, J = 7.6 Hz, 1H), 6.58 (m, 2H), 4.91 (s, 2H), 1.45 (s, 9H). HRMS (EI) m/z (M^+) calcd for $C_{12}H_{18}N_2O_2$ 222.1369, found 222.1357.

[(2-Aminophenyl)ethyl]carbamic Acid *tert*-Butyl Ester (41). This compound was prepared as described above using di-*tert*-butyl dicarbonate (0.3 g, 2.2 mmol) and 2-(2-aminoethyl)aniline (481 mg, 2.2 mmol). The product was obtained as a pale yellow solid in a quantitative yield (544 mg): ^{1}H NMR (400 MHz, CD_3OD) δ 6.97 (t, J = 7.2 Hz, 2H), 6.71 (d, J = 8 Hz, 1H), 6.63 (t, J = 7.2 Hz, 1H), 3.18 (t, J = 8 Hz, 2H), 2.67 (t, J = 8 Hz, 2H), 1.43 (s, 9H). HRMS (EI) m/z (M^+) calcd for $C_{13}H_{20}N_2O_2$ 236.1524, found 236.1537.

[(3-Aminophenyl)ethyl]carbamic Acid *tert*-Butyl Ester (42). This compound was prepared as described above using di-*tert*-butyl dicarbonate (401 mg, 1.84 mmol) and 3-(2-aminoethyl)aniline (401 mg, 1.84 mmol). The residue was purified by flash column chromatography on silica gel (EtOAc:hexane = 2:1) to afford 508 mg of product (quantitative yield, yellow solid): ^{1}H NMR (400 MHz, CD_3OD) δ 7.00 (t, J = 8 Hz, 1H), 6.58 (m, 3H), 3.21 (t, J = 7.6 Hz, 2H), 3.63 (t, J = 7.6 Hz, 2H), 1.42 (s, 9H). HRMS (EI) m/z (M^+) calcd for $C_{13}H_{20}N_2O_2$ 236.1524, found 236.1508.

[(4-Aminophenyl)ethyl]carbamic Acid *tert*-Butyl Ester (43). This compound was prepared as described above using di-*tert*-butyl dicarbonate (1 g, 7.34 mmol) and 4-(2-aminoethyl)-aniline (0.8 g, 3.67 mmol). The product was obtained in a yield of 98% (850 mg, pale yellow solid): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.96 (d, J = 8.4 Hz, 2H), 6.62 (d, J = 8.4 Hz, 2H), 3.29 (t, J = 6.4 Hz, 2H), 2.66 (t, J = 6.4 Hz, 2H), 1.43 (s, 9H). HRMS (EI) m/z (M^+) calcd for $C_{13}H_{20}N_2O_2$ 236.1524, found 236.1555.

Enzyme and Assay. All of the NOS isoforms used are 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.[28] The rat nNOS was expressed[29] and purified as described.[30] The bovine eNOS was isolated as reported.[31] Nitric oxide formation from NOS was monitored by the hemoglobin capture assay as described at 30 °C.[32] A typical assay mixture for nNOS contained 3−15 μM l-arginine, 1.6 mM CaCl2, 11.6 μg/mL calmodulin, 100 μM DTT, 100 μM NADPH, 6.5 μM BH4, and 3 mM oxyhemoglobin in 100 mM Hepes (pH 7.5). The reaction mixture for the iNOS assay included 10 μM L-arginine, 100 μM DTT, 100 μM NADPH, 6.5 μM BH4, and 3 mM oxyhemoglobin in 100 mM Hepes (pH 7.5). The eNOS assay mixture contained 3−25 μM l-arginine, 10 μM CaCl2, 1 μg/mL calmodulin, 100 μM DTT, 100 μM NADPH, 5 μM BH_4, and 80 μM oxyhemoglobin in 50 mM Hepes (pH 7.5). All assays were in a final volume of 600 μL and were initiated by enzyme. Nitric oxide reacts with oxyHb to yield methemoglobin which was detected at 401 nm (ϵ = 19700 M^{-1} cm^{-1}) on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer.

Determination of K_i Values. The reversible inhibition of NOS by reduced-amide bond analogues was studied under initial rate conditions with the hemoglobin assay as described above. The type of competitive inhibition of nNOS was determined from Dixon plots[33] with various L-arginine and inhibitor concentrations. 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[33] 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), 8.3 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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References

(1) Kerwin, J. F., Jr.; Lancaster, J. R. Jr. Nitric Oxide; A New Paradigm for Second Messengers. *Med. Res. Rev.* **1994**, *14*, 23−74.

(2) Forstermann, U.; Pollock, J. S.; Schmidt, H. H. H. W.; Heller, M.; Murad, F. Calmodulin Dependent Endothelium-Derived Relaxing Factor/Nitric Oxide Synthase Activity is Present in the Particulate and Cytosolic Fractions of Bovine Aortic Endothelial Cells. Prot. *Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1788−1792.
(3) Schmidt, H. H. H. W.; Walter, U. NO at Work. *Cell* **1994**, *78*, 919−925.
(4) MacMicking, J.; Xie, Q. W.; Nathan, C. Nitric Oxide and Macrophage Function. *Annu. Rev. Immunol.* **1997**, *15*, 323−350.
(5) Marletta, M. A. Approaches Toward Selective Inhibition of Nitric Oxide Synthase. *J. Med. Chem.* **1994**, *37*, 1899−1907.
(6) Moncada, S.; Higgs, E. A. Molecular Mechanism and Therapeutic Strategies related to Nitric Oxide. *FASEB J.* **1995**, *9*, 1319−1330.
(7) Dawson, V. L.; Dawson, T. M.; London, E. D.; Bredt, D. S.; Snyder, S. H. Nitric Oxide Mediates Glutamate Neurotoxicity in Primary Cortical Cultures. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 6368−6371. (b) Mayer, B.; Hemmens, B. Biosynthesis and Action of Nitric Oxide in Mammalian Cells. *Trends Biochem. Sci.* **1997**, *22*, 477−481.
(8) Nelson, R. J.; Demas, G. E.; Huang, P. L.; Fishman, M. C.; Dawson, V. L.; Dawson, T. M.; Snyder, S. H. Behavioral Abnormalities in Male Mice Lacking Neuronal Nitric Oxide Synthase. *Nature* **1995**, *378*, 383−386.
(9) Crow, J. P.; Beckman, J. S. The Role of Peroxynitrite in Nitric Oxide-Mediated Toxicity. *Curr. Top. Microbiol. Immunol.* **1995**, *196*, 57−73.
(10) Kilbourn, R. G.; Jubran, A.; Gross, S. S.; Griffith, O. W.; Levi, R.; Adams, J.; Lodato, R. F. Reversal of Endotoxin-Mediated Shock by *N*G-Methyl-L-Arginine, an Inhibitor of Nitric Oxide Synthesis. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 1132−1138.
(11) Babu, B. R.; Griffith, O. W. Design of Isoform-Selective Inhibitors of nitric oxide synthase. *Curr. Opin. Chem. Biol.* **1998,** *2*, 491−500.
(12) Silverman, R. B.; Huang, H.; Marletta, M. A.; Martesek, P. Selective Inhibition of Neuronal Nitric Oxide Synthase by *N*ω-Nitroarginine- and Phenylalanine-Containing Dipeptides and Dipeptide Esters. *J. Med. Chem.* **1997**, *40*, 2813−2817.
(13) Huang, H; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *N*ω- Nitroarginine-Containing Dipeptide Amides. Potent and Highly Selective Inhibitors of Neuronal Nitric Oxide Synthase. *J. Med. Chem.* **1999**, *42*, 3147−3153.
(14) Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. Selective Inhibitor of Constitutive Nitric Oxide Synthase by *NG*-Nitroarginine. *Biochemistry* **1993**, *32*, 8512−8517.
(15) Thiemermann, C.; Mustafa, M.; Mester, P. A.; Mitchell, J. A.; Hecker, M.; Vane, J. R. Inhibition of the release of endothelium-derived relaxing factor in vitro and in vivo by dipeptides containing NG-nitro-L-arginine. *Br. J. Pharmacol.* **1991**, *104*, 31−38.
(16) Hecker, M.; Walsh, D. T.; Vane, J. R. On the substrate specificity of nitric oxide synthase. *FEBS Lett.* **1991**, *294*, 221−224.
(17) Fischmann, T. O.; Hruza, A.; Niu, X. D.; Fossetta, J. D.; Lunn, C. A.; Dolphin, E.; Prongay, A. J.; Reichert, P.; Lundell, D. J.; Narula, S. K.; Weber, P. C. Structural Characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nature Struct. Biol.* **1999**, *6*, 233−242.
(18) Crane, B. R.; Arval, A. S.; Gachuhui, R.; Wu, C.; Ghosh, D. K.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **1997**, *278*, 425−431.
(19) Tierney, D. L.; Huang, H.; Martasek, P.; Roman, L. J.; Silverman, R. B.; Hoffman, B. M. ENDOR Spectroscopic Evidence for the Geometry of Binding of retro-inverso-Nω- Nitroarginine-Containing Dipeptide Amides to Neuronal Nitric Oxide Synthase. *J. Am. Chem. Soc.* **2000**, *122*, 7869−7875.
(20) Hah, J.-M.; Roman, L. J.; Martasek, P.; Silverman, R. B. Reduced Amide Bond Peptidomimetics. *N*- (4S)-[4-Amino-5-(2-aminoalkylamino)-pentyl]-*N*′-nitroguanidines, Potent and Highly Selective Inhibitors of Neuronal Nitric Oxide Synthase. *J. Med. Chem.* **2001**, *44*, 2667−2670.
(21) Tamura, S. Y.; Semple, J. E.; Ardecky, R. J. Novel and General Method for the Preparation of Peptidyl Argininals. *Tetrahedron Lett.* **1996**, *37*, 4109−4112.
(22) Ito, A.; Takahashi, R.; Baba, Y. A New Method to Synthesize α-Aminoaldehydes. *Chem. Pharm. Bull.* **1975**, *23*, 3081−3087.
(23) Peng, L.; Goeldner, M. Synthesis and Characterization of Photolabile Chlorine Precursors as Reversible Inhibitors of Cholinesterases: Release of Choline in the Microsecond Time Range. *J. Org. Chem.***1996**, *61*, 185−191.
(24) Bellamy, F. D.; Ou, K. Selective Reduction of Aromatic Nitro Compounds with Stannous Chloride in Non Acidic and Non Aqueous Medium. *Tetrahedron Lett.* **1984**, *25*, 839−842.
(25) Krapcho, A. P.; Kuell, C. S. *N-tert*-Butoxycarbonyl-α,ω-Alkanediamines from α,ω-Alkanediamines. *Synth. Commun.* **1990**, *20*, 2559−2564.
(26) Dixon, M. Determination of enzyme−inhibitor constants. *Biochem. J.* **1953**, *55*, 170−171.
(27) Cornish-Bowden, A. Simple graphical method for determining the inhibition constants of mixed, uncompetitive, and noncompetitive inhibitors. *Biochem. J.* **1974**, *137*, 143−144.
(28) Hevel, J. M.; White, K. A.; Marletta, M. Purification of the Inducible Murine Macrophage Nitric Oxide Synthase. *J. Biol. Chem.* **1991**, *266*, 22789−22791.
(29) Roman, L. J.; Sheta, E. A; Martasek, P.; Gross, S. S.; Liu, Q.; Masters, B. S. S. High-Level Expression of Functional Rat Neuronal Nitric Oxide Synthase in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8428−8432.
(30) Gerber, N. C.; Montelloano, P. R. Neuronal nitric oxide synthase. Expression in *Escherichia coli*, irreversible inhibition by phenyldiazene, and active site topology. *J. Biol. Chem.* **1995**, *270*, 17991−17996.
(31) Martasek, P.; Liu, Q.; Roman, L. J; Gross, S. S.; Sessa, W. C.;Masters, B. S. S. Characterization of Bovine Endothelial Nitric Oxide Synthase Expressed in *Escherichia coli. Biochem. Biophys. Res. Commun.* **1996**, *219*, 359−365.
(32) Hevel, J. M.; Marletta, M. Nitric Oxide Synthase Assays. *Methods Enzymol.* **1994**, *133*, 250−258.
(33) Segel, I. H. *Enzyme Kinetics*; John Wiley and Sons: New York, 1975; p 105.

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