Nitroaromatic Amino Acids as Inhibitors of Neuronal Nitric Oxide Synthase

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Nitric oxide (NO·) is an important biomodulator of many physiological processes. The inhibition of inappropriate production of NO· by the isoforms of nitric oxide synthase (NOS) has been proposed as a therapeutic approach for the treatment of stroke, inflammation, and other processes. In this study, certain 2-nitroaryl-substituted amino acid analogues were discovered to inhibit NOS. Analogues bearing a 5-methyl substituent on the aromatic ring demonstrated maximal inhibitory potency. For two selected inhibitors, investigation of the kinetics of the enzyme showed the inhibition to be competitive with L-arginine. Additionally, functional NOS inhibition in tissue preparations was demonstrated.

Introduction

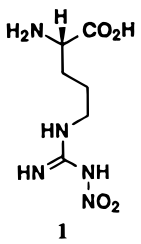
Nitric oxide (NO·) plays an important role in many physiological and pathological processes.[1,2a,b] NO· and its coproduct citrulline are produced by the enzymatic oxidation of L-arginine by nitric oxide synthase (NOS).

Three isoforms of NOS have been identified to date: iNOS, the inducible isoform found in macrophages and other tissues; eNOS, the constitutive isoform found in endothelial cells which is important in the regulation of blood pressure; and nNOS, the constitutive isoform found in neurons. When intracellular calcium levels are elevated in response to extracellular or other signals, constitutive NOS isoforms are activated to produce NO·. This NO· can act as a messenger molecule by binding to guanylate cyclase and thereby stimulating cGMP accumulation.

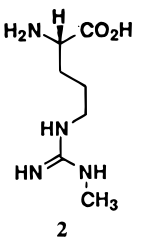
NO· plays an important role in the nervous system and appears to act as a neurotransmitter or neuromodulator. It has been implicated in the induction of long-term potentiation (LTP) in neurons[3] and in memory formation.[4] However, NO· may be a "two-edged sword",[5] in that excessive NO· production following conditions such as cerebral ischemia may contribute to cell death. Both nNOS and eNOS are found in the brain, and experiments to elucidate the physiological role of these isoforms have been reported recently. Transgenic mice lacking nNOS exhibited almost normal LTP induction, suggesting that eNOS is the physiological regulator of this process.[6] However, transgenic mice lacking nNOS exhibited greatly reduced infarct volume following middle cerebral artery occlusion, suggesting that NO produced by nNOS appears to exacerbate ischemic brain damage.[7] These findings support the potential clinical utility of selective inhibitors for nNOS versus eNOS and the utility of selective inhibitors as probes of the role of NO· in neuronal pathology.

The classical NOS inhibitors, most significantly N^G-nitroarginine (L-NNA, **1**) and N^G-methylarginine (L-NMA, **2**), are analogues of the endogenous NOS substrate L-arginine. These inhibitors, however, display low selectivity for inhibition of nNOS versus eNOS (Table 1) and thus may not be appropriate molecules to probe the relative roles of nNOS and eNOS in central nervous system (CNS) pathophysiology. Recently, several groups[8] have described arginine- and non-arginine-based analogues as NOS inhibitors with enhanced selectivity.

Table 1. Inhibition of NOS Isoforms by Nitroaromatic Amino Acids[a]



1



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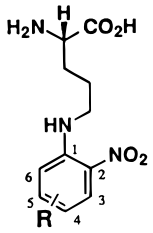

compd	substituent	IC$_{50}$ values (μM) nNOS	eNOS	iNOS
1	L-NNA	0.6	1.2	14
2	L-NMA	7.5	7	25
3	4-H	100	100	>100
4	4-F	>100	>100	>100
5	4-NO_2	100		
6	4-CH_3	>100		>100
7	4-CF_3	>100		>100
8	4-CO_2H	>100		>100
9	5-OH	>100		>100
10	5-CH_3	2.5	5	11
11	5-OCH_3	20		
12	6-NO_2	30	60	
13	6-Cl	100		

[a] NOS activity was assessed by measuring the inhibition of the conversion of radiolabeled arginine to citrulline.

The work which follows describes the synthesis and activity of a series of N^{ω}-2-nitroaryl amino acid analogues. One rationale for the preparation of N^{ω}-2-nitroaryl amino acid analogues such as **3** was to create a molecule combining features of the substrate amino acid arginine with an aromatic moiety having the possibility of an interaction (hydrophobic, charge transfer, etc.) with one of the known NOS enzyme cofactors such as flavin adenine dinucleotide (FAD), tetrahydrobiopterin, flavin adenine mononucleotide (FMN),

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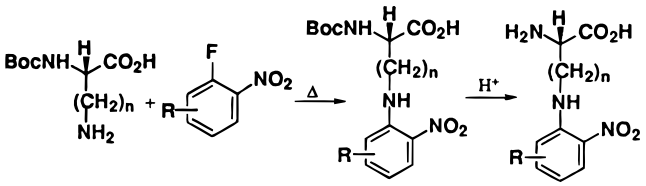
Scheme 1



NADPH, or heme. Nitroarylated amino acids are readily synthesized by N-alkylation of N^{α}-Boc-protected amino acids with 2-fluoro nitroaromatics, followed by acid-catalyzed removal of the protecting group. After the start of the work reported here, another 2-amino-substituted nitroaromatic compound, 7-nitroindazole, was reported to inhibit NOS.[9]

Chemistry

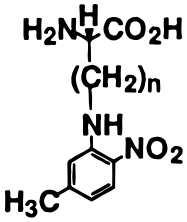
The synthesis of the 2-nitroaryl amino acids (compounds **3**−**19**) was effected as depicted in Scheme 1 by the nucleophilic aromatic substitution reaction of Boc-protected amino acid analogues (ornithine, α,*β*-diaminobutyric acid, or lysine) with 2-halonitrobenzene under basic conditions at elevated temperature, followed by the acid-catalyzed deprotection to give the target compounds.

The alternative synthetic methodology required for the synthesis of the amino acid analogues **20**−**23** is shown in Scheme 2. A compound of central importance was the alcohol **24**.[9] Compound **25** was prepared as an unstable oil from **24**[10] and immediately reacted with 2-nitro-5-methylphenol to give **20** or with 3-methylthiophenol to give **22**. The mesylate could also be converted to the mercaptan by reaction with thiolacetic acid and then converted to compound **21** by a nucleophilic aromatic substitution reaction with 2-fluoro-4-methylnitrobenzene. The alcohol **24** could be oxidized to the aldehyde **26** and reductively aminated with *m*-toluidine to give compound **23**.

Results and Discussion

A variety of N^{ω}-arylated derivatives of L-ornithine were prepared and found to inhibit NOS (Table 1). The first 2-nitrophenyl derivative of ornithine prepared (**3**) was found to be a weak inhibitor of nNOS, much less potent than L-NNA or L-NMA. When the 2-nitrophenyl ring was modified with a variety of substituents, it was found that substitution at the 4-position led to a loss of activity. However, substitution of the aromatic ring of **3** at the 5-position with a methyl group led to compound **10** (2.5 μM) with a 40-fold increase in potency at nNOS. The inhibitory potency of **10** was found to lie between that of L-NNA (**1**) and L-NMA (**2**). Substitution of a nitro (**12**) or chloro (**13**) at the 6-position provided a more modest or no improvement of nNOS inhibitory activity compared to **3**. Thus 5-methyl substitution of **3** to give **10** provided the greatest improvement in nNOS inhibitory potency.

Table 2. Effect of Chain Length on Potency of a Series of Nitroaryl Amino Acids



		IC_{50} values (μM)		
compd	n	nNOS	eNOS	iNOS
14	2	>100		>100
10	3	2.5	5	11
15	4	40		

The effect of varying the number of methylene units between the amino acid portion and the nitroaniline moiety in **10** was then examined (Table 2). It was determined that in the 5-methyl-2-nitroaryl series, the three-methylene bridge found in the ornithine derivative **10** was optimal, when compared to compounds containing a two-methylene (**14**) or four-methylene (**15**) bridge. This should perhaps not be surprising, since the natural substrate arginine also has a three-methylene spacer between the amino acid and the guanidine moieties.

The effect of modifying the amino acid moiety of **10** was examined (Table 3). Compound **16**, which is devoid of the amino acid moiety, showed greatly reduced activity, which in conjunction with findings from enzyme kinetics investigations (vide infra) supports the conten-

Scheme 2

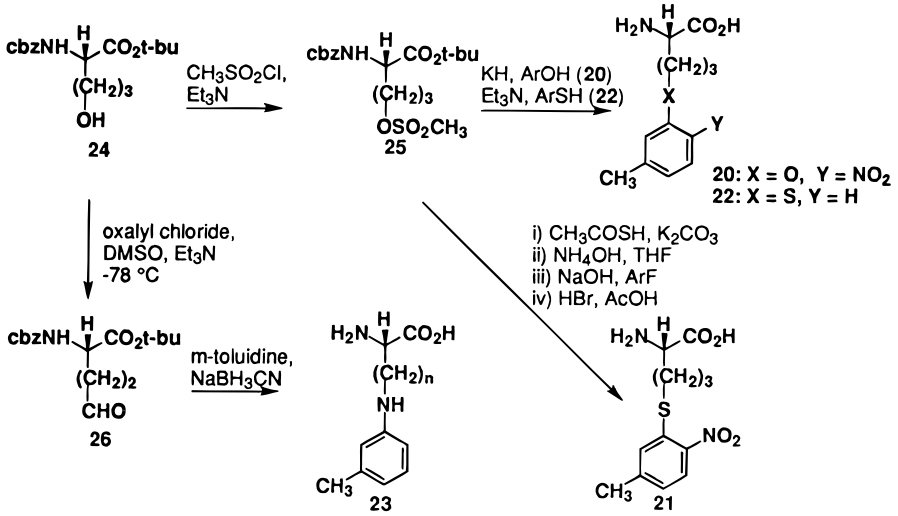
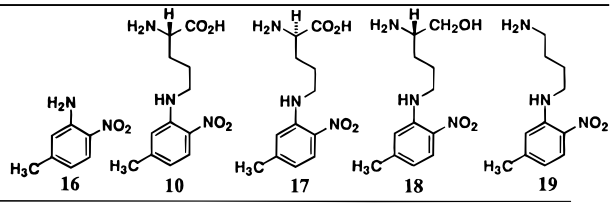
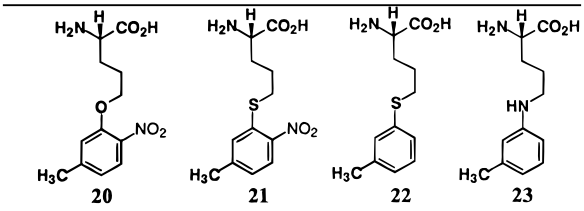


Table 3. Effect of Variation of the Amino Acid Portion of a Series of 5-Methyl-2-nitroanilines (IC_{50} values, μM)



	16	**10**	**17**	**18**	**19**
nNOS	70	2.5	70	3	2.5
eNOS		5		40	20
iNOS		11		5	9

Table 4. SAR of a Series of Substituted Aryl Amino Acids as NOS Inhibitors (IC_{50} values, μM)



	20	**21**	**22**	**23**
nNOS	4	5	>100	>100
eNOS	10	9		
iNOS	80	4	>100	>100

tion that the analogues bind at the same site in the enzyme as the substrate arginine.

It was seen that the L-amino acid enantiomer corresponding to that seen in the substrate arginine was by far the more potent inhibitor. The L-enantiomer of the most potent compound (**10**) was about 30-fold more potent at nNOS inhibition than the D-enantiomer **17**, suggestive of stereoselective inhibition.

The carboxylic acid moiety of the amino acid analogues was found to be unnecessary for activity, because inhibitory potency was found to be almost unchanged when **10** was compared with the alcohol **18** and with **19**, which lacks the carboxylic acid of **10**. Interestingly, compounds **18** and **19** show reduced inhibition of eNOS compared to **10** and are therefore more nNOS-selective (relative to eNOS) than L-NNA and L-NMA.

It was found that the aniline nitrogen in **10** could be replaced with oxygen (**20**) or sulfur (**21**) without a significant loss of potency at nNOS (Table 4), so the N^{ω} anilino nitrogen seems not to be required for activity. Interestingly, in compound **20** activity at iNOS was decreased, and this compound was the most nNOS-selective (versus iNOS) compound in the series.

In contrast, the nitro substituent at the 2-position of the aromatic ring appears to be required for enzyme inhibitory activity since the desnitro analogues (compounds **22** and **23**) were found to be devoid of inhibitory activity.

The kinetics of inhibition of rat brain cytosolic nNOS-catalyzed citrulline formation was determined for two of the most potent analogues, **10** and **21**, and compared to that of L-NMA. Double-reciprocal plots indicated competitive inhibition of nNOS with respect to L-arginine for both **10** and **21**, with apparent K_i values of 1.4 and 0.4 ($\pm$0.1) μM, respectively, suggesting that the compounds may displace the substrate L-arginine from

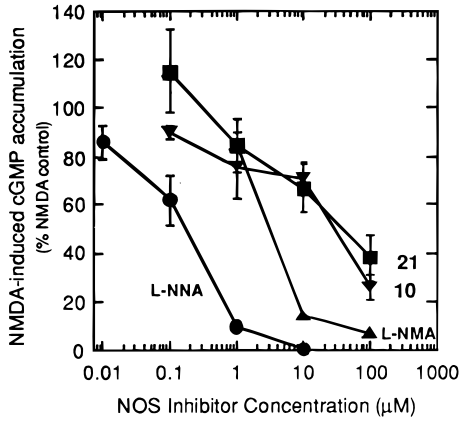


Figure 1. Two of the more potent analogues were investigated for functional inhibition of the constitutive NOS isoforms, nNOS. Compounds **10** ($IC_{50} = 33 \pm 10$ μM) and **21** ($IC_{50} = 37 \pm 29$ μM) inhibited NMDA-induced cGMP accumulation in rat cerebellar slices, as did the classical NOS inhibitors L-NNA ($IC_{50} = 0.2 \pm 0.1$ μM) and L-NMA ($IC_{50} = 3$ μM), suggesting functional inhibition of nNOS. nNOS-catalyzed NO· formation has been shown to mediate the elevation of cGMP levels by NMDA receptor activation in the cerebellum.

the enzyme. Coupled with the observation that the natural L-enantiomer is a more potent inhibitor than the D-enantiomer, this finding supports a hypothesis that the nitroaromatic amino acid analogues bind at the enzyme substrate binding site. For comparison, L-NMA also was found to inhibit nNOS competitively with respect to L-arginine with an apparent K_i value of 1.3 μM.

Compounds **10** and **21** inhibited a functional response mediated by nNOS in a tissue preparation (Figure 1). It has previously been shown that nNOS-catalyzed NO· formation mediates the elevation of cyclic GMP levels by *N*-methyl-D-aspartate (NMDA)-induced glutamate receptor activation in the cerebellum.[13,14] Compounds **10** ($IC_{50} = 33 \pm 10$ μM) and **21** ($IC_{50} = 37 \pm 29$ μM) inhibit NMDA-induced cyclic GMP accumulation in rat cerebellar slices, as did the known NOS inhibitors L-NNA ($IC_{50} = 0.2 \pm 0.1$ μM) and L-NMA ($IC_{50} = 3$ μM). These observations indicate that compounds **10** and **21** can penetrate the cell membrane to inhibit nNOS intracellularly.

Compounds **10** and **18** were also found to partially reverse the NO·-mediated endothelium-dependent relaxation of phenylephrine-preconstricted isolated rat aortic rings by acetylcholine (ACh), suggesting functional inhibition of eNOS (Figure 2). Endothelium-dependent relaxation by ACh is mediated by activation of eNOS to generate NO·.[14,15]

ACh-induced relaxation was reversed by L-NNA ($EC_{50} = 3 \pm 2$ μM) and L-NMA ($EC_{50} = 14 \pm 4$ μM), as well as by two of our compounds. Compound **10** showed an EC_{50} of about 100 μM in this assay (with 41 $\pm$ 8% relaxation at 100 μM). Compound **18** showed an EC_{50} of 17 $\pm$ 5 μM. The observation that the amino alcohol **18** showed greater activity in this assay than the amino acid **10** may be due to an enhanced ability of the less polar **18** to traverse membranes and inhibit NOS at intracellular sites. It is also interesting that **18** seemed to be more potent in the functional assay in aortic rings than in the inhibition assay with the eNOS in a solubilized particulate fraction. This might be due to

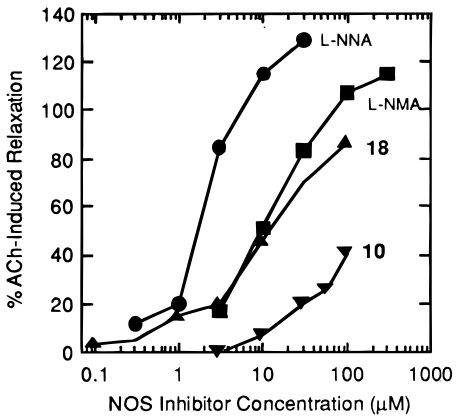


Figure 2. Compounds **10** and **18** partially reversed the NO·-mediated endothelium-dependent relaxation of phenylephrine-preconstricted isolated rat aortic rings by ACh, suggesting functional inhibition of eNOS. Endothelium-dependent relaxation by ACh is mediated by activation of eNOS to generate NO·.[12,13] ACh-induced relaxation was also reversed by L-NNA and L-NMA.

the different enzyme sources in the assays (eNOS inside cells versus eNOS in the CHAPS-solubilized particulate fraction of disrupted cells), differing concentrations of enzyme cofactors, or selective concentration of the compound into cells.

Conclusions

Certain nitroaromatic amino acid analogues have been found to be potent inhibitors of nNOS, with five analogues (**10** and **18**−**21**) possessing an IC_{50} of 5 μM or less. These lipophilic inhibitors are unusual in that they do not possess the polar guanidino moiety often seen in previously published NOS inhibitors, which are analogues of the substrate L-arginine. Additionally, two compounds (**18** and **19**) displayed significantly enhanced selectivity for nNOS over eNOS in an in vitro assay, as compared to L-NNA and L-NMA. The competitive inhibition of two of the more potent analogues (**10** and **21**) with respect to the substrate L-arginine, coupled with the findings that the natural L-enantiomer is preferred, supports a hypothesis that the nitroaromatic amino acid analogues displace arginine and bind at the enzyme substrate binding site. Three of the more potent analogues demonstrated functional inhibition of the constitutive nNOS and eNOS isoforms in tissue-based assays (Table 5).

Experimental Section

Chemistry. All solvents were of anhydrous reagent grade from Aldrich Chemical Co. The ^{1}H NMR spectra were obtained at 300 MHz on a Nicolet/GE QE300 spectrometer, and the same instrument was used to obtain the ^{13}C NMR spectra at 75 MHz. Chemical shifts are reported in parts per million (ppm, δ) relative to TMS or TSP as internal standard. Mass spectra were obtained on a Kratos MS-50 instrument. High-resolution mass spectra were recorded by Midwest Analytical Services. Elemental analyses were performed by Abbott Laboratories Pharmaceutical Products Division Structural Chemistry Department or by Robertson Microlit Laboratories, Inc., Madison, NJ. Flash chromatography was carried out using silica gel 60 (E. Merck, 230−400 mesh), and thin-layer chromatography was performed on 250-μm silica-coated glass plates from EM Science. Melting points were determined on a Buchi 510 melting point apparatus and are uncorrected. Unless otherwise noted, all chemical and reagents were obtained commercially and used without purification.

Preparation of Nitroaromatic Amino Acid Analogues 3−15, 17, and 19. 10: A solution of α-*N*-Boc-ornithine (2.37 g, 10 mmol), 2-nitro-5-methylfluorobenzene (1.65 g, 11 mmol), and 10 mL of 1 M aqueous NaOH in 10 mL of ethanol was heated at reflux for 12 h, at which time a further 1.65 g of 2-nitro-5-methylfluorobenzene and 10 mL of 1 M aqueous NaOH were added, and heating at reflux continued for another 12 h. The residue was diluted with 50 mL of water, acidified to pH 2 with 1 N HCl, and extracted with dichloromethane. The organic phase was dried (Na_2SO_4), and concentrated in vacuo to a solid, which was dissolved in 20 mL of 1:1:1 water/acetic acid/12 M HCl. After 1 h, the solvent was removed in vacuo and the residue recrystallized from ethanol/hexane to give 1.5 g (56%) of N^G-(2-nitro-5-methylphenyl)ornithine (**10**) as a yellow powder: mp 263−264 °C dec; ^{1}H NMR (1 M NaOD/D_2O) δ 7.58 (d, J = 8.1 Hz, 1 H), 6.43 (s, 1H), 6.22 (d, J = 8.1 Hz, 1 H), 3.30 (t, J = 4.5 Hz, 1 H), 3.12 (m, 2 H), 1.64 (m, 4 H); ^{13}C NMR (1 M NaOD/D_2O) δ 185.88, 152.28, 148.71, 128.71, 120.22, 116.11, 58.73, 45.24, 35.40, 27.73, 24.24; MS (DCI/NH_3) 268 $[M + H]^+$. Anal. ($C_{12}H_{17}N_3O_4$·0.4HCl·0.1H_2O) C, H, N.

3: yield 81%; orange crystals; mp 240−241 °C (ethanol/2-propanol); R_f 0.37 (silica gel, 6:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (CD_3OD) δ 8.09 (dd, J = 1.8, 8.4 Hz, 1 H), 7.49 (td, J = 1.8, 7.5 Hz, 1 H), 7.02 (dd, J = 0.6, 8.6 Hz, 1 H), 6.65 (td, J = 0.6, 7.5 Hz, 1 H), 4.05 (t, J = 5.7 Hz, 1 H), 3.43 (t, J = 6.0 Hz, 1 H), 1.8−2.1 (m, 4 H); ^{13}C NMR (CD_3OD) δ 171.65, 146.54, 137.52, 132.98, 127.50, 115.21, 53.64, 42.92, 28.97, 25.59; MS $(FAB)^+$ m/z calcd for $C_{11}H_{16}N_3O_4$ 254.1141, found 254.1142. Anal. ($C_{11}H_{15}N_3O_4$·1.5H_2O) C, H, N.

4: yield 94%; orange solid; mp 252−255 °C dec (water/methanol/ethanol); R_f 0.43 (95% ethanol/5% triethylamine); ^{1}H NMR (D_2O/NaOD) δ 7.62 (dd, J = 9.3, 2.7 Hz, 1 H), 7.29 (m, 1 H), 6.88 (dd, J = 9.3, 3.6 Hz, 1 H), 3.29 (m, 3 H), 1.69 (m, 4 H); ^{13}C NMR (D_2O/NaOD) δ 186.01, 154.89 (d, J = 236 Hz), 146.07, 131.56 (d, J = 9.8 Hz), 128.82 (d, J = 24 Hz), 118.89 (d, J = 7.5 Hz), 113.47 (d, J = 26 Hz), 58.63, 45.49, 35.09, 27.59; MS (FAB) m/z $[M + H]^+$ 272. Anal. ($C_{11}H_{14}N_3O_4F$) C, H, N.

5: yield 47%; amorphous yellow powder; mp 223−225 °C (ethanol/ethyl acetate); R_f 0.05 (10% triethylamine/ethanol); ^{1}H NMR (CD_3OD) δ 8.9 (d, J = 1 Hz, 1 H), 8.2 (dd, J = 3, 1 Hz, 1 H), 7.15 (d, J = 3 Hz, 1 H), 4.00 (t, J = 2 Hz, 1 H), 3.50 (t, J = 2 Hz, 2 H), 1.8−2.1 (m, 4 H); ^{13}C NMR (CD_3OD) δ 170.25, 148.19, 135.50, 130.16, 129.72, 123.35, 114.56, 52.10, 42.20, 27.36, 23.98; MS (FAB^+) m/z calcd for $C_{11}H_{15}N_4O_6$ 299.0991, found 299.1007. Anal. ($C_{11}H_{14}N_4O_6$·H_2O·HCl) C, H, N.

6: yield 46%; yellow powder; mp 260 °C dec; R_f 0.35 (6:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (D_2O/DCl) δ 7.81 (s, 1 H), 6.72 (d, J = 10.5 Hz, 1 H), 6.12 (d, J = 9 Hz, 1 H), 4.20 (t,

Table 5. Summary of Pharmacology of NOS Inhibitors in Tissue Preparations

	citrulline assay (IC_{50}, μM)			inhibn of NMDA-induced	reversal of ACh-induced relaxation	
compd	nNOS	eNOS	iNOS	cGMP (IC_{50}, μM) accumulation	EC_{50}[a] (μM)	E_{max}[a] (%)
L-NNA	0.6	1.2	14	0.2 ± 0.1	3 ± 2	138 ± 14
L-NMA	7.5	7	25	3	14 ± 4	119 ± 3
10	2.5	5	11	33 ± 10	41 ± 8% relaxation @ 100 μM	
18	3	40	5		17 ± 5	106 ± 11
21	5	9	4	37 ± 29		

[a] Mean ± SE (n = 3−5).

J = 6 Hz, 1 H), 3.13 (m, 2 H), 1.7−2.1 (m, 4 H); ^{13}C NMR (D_2O/DCl) δ 174.41, 166.91, 150.37, 132.18, 132.06, 128.00, 110.19, 55.44, 45.03, 30.15, 26.48; MS m/z [M + H]$^+$ 270. Anal. ($C_{12}H_{17}N_3O_4$·HCl) C, H, N.

7: yield 63%; yellow solid; mp 246−249 °C (water/ethanol); ^{1}H NMR (D_2O/NaOD) δ 8.02 (s, 1 H), 7.42 (dd, 5.7 H, J = 1.5 Hz, 1 H), 6.83 (d, J = 5.7 Hz, 1 H), 3.29 (m, 2 H), 1.7 (m, 4 H); ^{13}C NMR (D_2O/NaOD) δ 185.43, 149.43, 131.93, 126.69, 126.13 (q, J = 271 Hz), 119.23 (q, J = 34 Hz), 117.59, 58.72, 45.47, 35.49, 27.58; MS m/z calcd for $C_{12}H_{15}N_3O_4F_3$ 322.1015, found 322.1022. Anal. ($C_{12}H_{14}N_3O_4F_3$) C, H, N.

8: yield 50%; amorphous yellow powder; mp 253−255 °C dec (ethanol); R_f 0.25 (6:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (D_2O/NaOD) δ 8.52 (d, J = 0.6 Hz, 1 H), 7.88 (dd, J = 5.7, 0.6 Hz, 1 H), 6.89 (d, J = 5.7 Hz, 1 H), 3.30 (m, 3 H), 1.71 (m, 4 H); ^{13}C NMR (D_2O/NaOD) δ 186.13, 176.06, 149.80, 139.62, 132.47, 130.91, 125.68, 116.68, 58.68, 45.41, 35.16, 27.56; MS m/z [M + H]$^+$ 298. Anal. ($C_{12}H_{15}N_3O_6$·0.8 HCl) C, H, N.

9: yield 46%; mp 240 °C dec (H_2O); ^{1}H NMR (DCl/D_2O) δ 7.81 (bs, 1 H), 6.72 (d, J = 9 Hz, 1 H), 6.12 (d, J = 9 Hz, 1 H), 4.20 (t, J = 6 Hz, 1 H), 3.13 (m, 2 H), 1.7−2.1 (m, 4 H); ^{13}C NMR (HCl/D_2O) δ 174.41, 166.91, 150.37, 132.18, 132.06, 128.00, 110.19, 55.44, 45.03, 30.15, 26.48; MS [M + H]$^+$ 270.

11: yield 40%; yellow crystals; mp 245−246 °C (acetic acid/water); ^{1}H NMR (D_2O/NaOD) δ 7.33 (dd, J = 8.4, 5.7 Hz, 1 H), 5.69 (m, 1 H), 5.52 (m, 1 H), 3.52 (s, 3 H), 3.10 (m, 1 H), 2.81 (m, 2 H), 1.45 (m, 4 H); ^{13}C NMR (D_2O/NaOD) δ 185.47, 168.65, 150.74, 131.00, 127.20, 108.64, 96.78, 58.48, 45.27, 35.37, 27.37; MS (DCI/NH_3) m/z [M + H]$^+$ 284. Anal. ($C_{12}H_{17}N_3O_5$·0.2H_2O) C, H, N.

12: yield 38%; yellow amorphous powder; mp 215−220 °C dec (ethanol/ethyl acetate/hexane); R_f 0.53 (12:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (300 MHz, D_2O) δ 8.0 (d, J = 7.8 Hz, 2 H), 6.92 (t, J = 7.8 Hz, 1 H), 4.09 (t, J = 6.0 Hz, 1 H), 3.08 (m, 2 H), 1.6−2.1 (m, 4 H); MS (DCI/NH_3) m/z [M + H]$^+$ 299. Anal. ($C_{11}H_{14}N_4O_6$·0.9HCl) C, H, N.

13: yield 30%; red-orange powder; mp 205−207 °C dec (water); R_f 0.62 (6:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (300 MHz, D_2O/DCl) δ 8.02 (dd, J = 7.8, 1 Hz, 1 H), 7.76 (dd, J = 7.8, 1 Hz, 1 H), 7.11 (t, J = 7.8 Hz, 1 H), 3.42 (td, J = 6.6, 0.8 Hz, 2 H), 1.8−2.0 (m, 4 H); ^{13}C NMR (75.5 MHz, D_2O/DCl) δ 178.20, 142.08, 139.04, 138.88, 128.79, 127.57, 125.13, 54.46, 49.66, 28.95, 26.49; MS (FAB$^+$) m/z calcd for $C_{11}H_{15}N_3O_4Cl$ 288.0751, found 288.0751. Anal. ($C_{11}H_{14}N_3O_4Cl$·0.3HCl) C, H, N.

14: yield 22%; yellow needles; mp >260 °C dec (2-propanol/acetic acid); ^{1}H NMR (CD_3OD) δ 8.05 (dd, J = 5.1, 9.0 Hz, 1 H), 6.9 (br s, 1 H), 6.73 (d, J = 1.2 Hz, 1 H), 6.53 (dd, J = 1.2, 9.0 Hz, 1 H), 4.21 (t, 6.3 Hz, 1 H), 3.70 (t, J = 6.3 Hz, 2 H), 3.59 (m, 2 H); ^{1}H NMR (1 M NaOD/D_2O) δ 7.58 (d, J = 8.1 Hz, 1 H), 6.43 (s, 1 H), 6.22 (d, J = 8.1 Hz, 1 H), 3.30 (t, J = 4.5 Hz, 1 H), 3.12 (m, 2 H), 1.64 (m, 4 H); MS (DCI/NH_3) [M + H]$^+$ 254. Anal. ($C_{11}H_{15}N_3O_4$·1.0i-PrOH·0.25AcOH) C, H, N.

15: yield 42%; bright-orange prisms; mp 234−235 °C dec (water/methanol); R_f 0.24 (12:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (300 MHz, D_2O) δ 7.32 (d, J = 7.8 Hz, 1 H), 6.07 (s, 1 H), 5.88 (d, J = 7.8 Hz, 1 H), 2.99 (t, J = 6.0 Hz, 1 H), 2.78 (t, J = 6.6 Hz, 2 H), 1.1−1.45 (m, 6 H); ^{13}C NMR (75.5 MHz, D_2O) δ 185.73, 151.31, 148.37, 131.02, 119.60, 116.09, 58.83, 45.20, 37.87, 31.19, 25.86; MS (DCI/NH_3) m/z [M + H]$^+$ 282. Anal. ($C_{13}H_{19}N_3O_4$) C, H, N.

17: yield 95%; yellow needles; mp 240−243 °C dec (hydrochloride salt); ^{1}H NMR (300 MHz, D_2O/DCl) δ 8.11 (d, J = 9.3 Hz, 1 H), 7.12 (s, 1 H), 6.95 (d, J = 9.3 Hz, 1 H), 4.20 (t, J = 6.6 Hz, 1 H), 3.55 (t, J = 7.5 Hz, 2 H), 1.7−2.2 (m, 4 H); MS (DCI/NH_3) [M + H]$^+$ 268. Anal. ($C_{12}H_{17}N_3O_4$·0.5HCl) C, H, N.

18: A solution of 384 mg (1.05 mmol) of N-Boc-protected **10** in 4 mL of THF was cooled to 0 °C, and 1.3 mL of 1 M solution of borane in THF was added dropwise over 2 min. After 1 h, 1.3 mL more of borane−THF was added. After 1 h, 4 mL of methanol was added, and the mixture was poured into 125 mL of dichloromethane. The organic phase was washed with 125 mL of water, dried over $MgSO_4$, and concentrated in vacuo to a foam. The foam was dissolved in 20 mL of 1:1:1 acetic acid/water/12 M HCl and stirred at 25 °C for 12 h. Concentration in vacuo gave a yellow powder, which was recrystallized from ethyl acetate/ethanol to give 55 mg (15%) of a yellow powder: mp 202−204 °C dec; ^{1}H NMR (D_2O) δ 8.00 (d, J = 8.4 Hz, 1 H), 6.82 (s, 1 H), 6.55 (dd, J = 8.4, 0.6 Hz, 1 H), 3.35−3.45 (m, 3 H), 2.33 (s, 3 H), 1.80 (m, 4 H); HRMS (FAB$^+$) calcd for $C_{12}H_{20}N_3O_3$ 254.1505, found 254.1505. Anal. ($C_{12}H_{19}N_3O_3$·2.8HCl) C, H, N.

19: yield 90%; orange powder; mp 55−56 °C, purified by sublimation (50 °C, 0.07 mm); R_f 0.66 (6:1:1 acetonitrile/water/acetic acid); ^{1}H NMR (300 MHz, CD_3OD) δ 8.04 (d, J = 8.4 Hz, 1 H), 6.61 (s, 1 H), 6.43 (dd, J = 1, 8.4 Hz, 1 H), 3.31 (m, 2 H), 2.8 (t, J = 7.5 Hz, 2 H), 1.5−1.7 (m, 6 H); ^{13}C NMR (75.5 MHz, CD_3OD) δ 147.56, 145.55, 130.30, 126.78, 116.82, 113.28, 42.85, 41.75, 31.11, 26.44, 22.04. Anal. ($C_{11}H_{17}N_3O_2$) C, H, N.

20: To a suspension of 571 mg of potassium hydride (5.0 mmol of a 35% suspension in mineral oil) in 5 mL of THF at 0 °C was added dropwise a solution of 842 mg (5.5 mmol) of 2-nitro-5-methylphenol in 5 mL of THF. After 15 min at 0 °C, a solution of 1.644 g (4.1 mmol) of mesylate **25** in 5 mL of diethyl ether was added, and the reaction was heated at reflux for 14 h. The reaction was concentrated in vacuo and purified by flash chromatography, eluting with ethyl acetate/dichloromethane. The product was dissolved in 25 mL of a 30% (w/v) solution of HBr in acetic acid. After 5 h at room temperature, the solvent was removed in vacuo and purified by flash chromatography, eluting with a 3:1:1 mixture of acetonitrile/water/acetic acid. Product-containing fractions were combined and concentrated in vacuo to a white foam, 210 mg (18%): ^{1}H NMR (CD_3OD) δ 7.76 (d, J = 8.7 Hz, 1 H), 7.11 (d, J = 0.5 Hz, 1 H), 6.92 (dd, J = 8.7, 0.5 Hz, 1 H), 4.23 (t, J = 6.0 Hz, 2 H), 4.05 (t, J = 7.5 Hz, 1 H), 2.0−2.2 (m, 4 H); ^{13}C NMR (D_2O) δ 175.31, 155.23, 151.30, 138.95, 129.06, 124.61, 118.49, 112.10, 71.87, 29.96, 26.97, 24.01; MS (DCI/NH_3) [M + H]$^+$ 269. Anal. ($C_{12}H_{16}N_2O_5$·2.0HBr·0.2H_2O) C, H, N.

21: A mixture of 485 mg (1.21 mmol) of mesylate **25**, 206 μL of mercaptoacetic acid, and 400 mg of potassium carbonate in 3 mL of THF was stirred at 25 °C for 18 h. The mixture was then treated with 5 mL of THF and 3 mL of 12 M ammonium hydroxide solution. After 15 min at 25 °C, the mixture was poured into 80 mL of water and brought to pH 7 with 12 M HCl. The aqueous phase was extracted with dichloromethane. The organic phase was washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4, and concentrated in vacuo to give a white solid. The white solid mercaptan was combined with 107 mg (0.69 mmol) of 2-nitro-5-methylfluorobenzene and heated at reflux in a mixture of 0.69 mL of 1 N NaOH and 2 mL of ethanol for 15 min. The mixture was poured into 80 mL of dichloromethane, washed with 120 mL of water, dried over Na_2SO_4, and concentrated in vacuo to a yellow powder. The yellow powder was dissolved in 5 mL of 1.1 M HBr in acetic acid and stirred at 25 °C for 48 h. Removal of solvent and purification by flash chromatography, eluting with 10:5:1:1 ethanol/ether/acetic acid/water, gave a yellow solid that was recrystallized from water to give 118 mg of a yellow powder (34%): mp 212−214 °C dec; ^{1}H NMR (DCl/D_2O) δ 8.2 (d, J = 8.7 Hz, 1 H), 7.37 (s, 1 H), 7.19 (d, J = 8.7 Hz, 1 H), 4.05 (t, J = 6.0 Hz, 1 H), 3.30 (t, J = 7.5 Hz, 2 H), 2.42 (s, 3 H), 2.15 (m, 2 H), 1.7−1.9 (m, 2 H); MS (FAB$^+$) [M + H]$^+$ 285. Anal. ($C_{12}H_{16}N_2O_4S$·3.0HBr·2.0AcOH) C, H, N.

22: To a solution of 621 mg (1.55 mmol) of mesylate **25** in 5 mL of THF were added 432 μL of triethylamine and 369 μL of *m*-thiocresol. After 12 h, the reaction was concentrated in vacuo and purified by flash chromatography, eluting with 2:1 hexane/ethyl acetate to give 351 mg of a thick oil; 200 mg of the oil was treated with 1.6 M HBr in acetic acid for 30 min. The reaction was diluted with ether, and the precipitated solid was collected by filtration and triturated with hot ethanol to give 46 mg (41%) of the target compound. A portion was purified by ion exchange on Dowex 50W (H^+ form), eluting with 1 N ammonium hydroxide to give a solid: mp 263−264 °C dec; ^{1}H NMR (1 M NaOD/D_2O) δ 6.5−7 (m, 3 H) 6.74 (m, 1

H), 3.20 (m, 1 H), 2.80 (m, 2 H), 2.07 (s, 3 H), 1.76 (m, 1 H), 1.47−1.66 (m, 3 H); ^{13}C NMR (1 M NaOD/D_2O) δ 184.53, 140.50, 138.53, 131.22, 130.95, 128.59, 127.60, 58.17, 37.27, 35.12, 27.58, 23.07; HRMS (FAB$^+$) calcd for $C_{12}H_{18}NO_2S$ 240.1058, found 240.1057. Anal. ($C_{12}H_{17}NSO_2 \cdot 0.5NH_3 \cdot 0.5H_2O$) C, H, N.

23: To a stirred solution of 0.85 mL (1.7 mmol) of oxalyl chloride in 10 mL of dichloromethane at −78 °C was added dropwise 0.26 mL of DMSO. After 2 min, a solution of 500 mg (1.74 mmol) of **24** in 1 mL of dichloromethane was added dropwise. After 15 min, a solution of 1 mL of triethylamine in dichloromethane was added. After 5 more minutes at −78 °C, the reaction was allowed to warm to 0 °C, and the reaction was partitioned between dichloromethane and water. The organic phase was dried over Na_2SO_4 and concentrated in vacuo to give the aldehyde, which was combined with 0.28 mL of *m*-toluidine (2.6 mmol) and 99 mg (1.6 mmol) of sodium cyanoborohydride in 5 mL of ethanol. After stirring for 3 days at 25 °C, the reaction was concentrated in vacuo and purified by flash chromatography, eluting with 126 mg (23%) of a clear foam (HRMS calcd for [M + H]$^+$ $C_{24}H_{33}N_2O_4$ 412.2362, found 412.2379). A solution of 62 mg of the aforementioned foam in 2 mL of 30% (w/v) hydrogen bromide in acetic acid was stirred for 1 h at 25 °C to remove cBz and Boc groups. The reaction was added to 50 mL of ether and the supernatant decanted to provide a hygroscopic oil. The oil was purified by chromatography on Dowex 50W ion-exchange resin (H$^+$ form), eluting with 0.25 M ammonium hydroxide to give 19 mg (54%) of a white foam: ^{1}H NMR (D_2O/DCl) δ 7.4 (m, 4 H), 4.14 (t, J = 6 Hz, 1 H), 3.52 (t, J = 7 Hz, 2 H), 2.40 (s, 3 H), 1.9−2.1 (m, 4 H); HRMS calcd for $C_{12}H_{19}N_2O_2$ 223.1446, found 223.1442. Anal. ($C_{12}H_{18}N_2O_2 \cdot 0.9H_2O$) C, H, N.

25: To a solution of alcohol[10] **24** (323 mg, 1 mmol) in 2 mL of dichloromethane at 0 °C was added 202 mg (2 mmol) of triethylamine and 114 mg (1 mmol) of methanesulfonyl chloride. After 1 h, the reaction was diluted with 20 mL of dichloromethane, washed with 20 mL of water, 20 mL of 1 M HCl, and 20 mL of saturated $NaHCO_3$, dried ($MgSO_4$), and concentrated in vacuo to give **25** as a thick oil. Chromatography (1:1 hexane/ethyl acetate) of the residue provided pure product, which was used immediately in the next step.

Biological Data. NOS activity was assayed by measuring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline according to Bredt and Snyder,[12] with slight modifications. Standard conditions for measuring citrulline formation by nNOS and eNOS utilized samples of enzyme incubated for 20 min at ambient temperature in the presence of 10 μM L-[2,3-^{3}H]-arginine (55 C_i/mmol), 1 mM NADPH, 2 mM $CaCl_2$, and varying concentrations of inhibitors, in a final volume of 100 μL. Similar conditions were used in standard incubations for measuring citrulline formation by iNOS except that Ca^{2+} was omitted from the assays. The reaction was stopped by adding 0.5 mL of stop buffer (2 mM EGTA, 2 mM EDTA, 20 mM HEPES, pH 5.5). The total volume was then applied to a 1-mL Dowex AG 50WX-8 column (Na$^+$ form; Bio-Rad) that had been preequilibrated with the stop buffer. L-[2,3-^{3}H]Citrulline was eluted with two 0.5-mL aliquots of stop buffer, and radioactivity of the eluent was determined by liquid scintillation counting. The L-[2,3-^{3}H]arginine (DuPont NEN) was purified prior to use on Dowex AG-1-X8 ($CH_3CO_2^-$ form; Bio-Rad), eluted with distilled water, acidified, concentrated on a Speed-Vac (Savant), and stored at −20 °C.

A rat brain cytosolic preparation was used as the source of nNOS. The cytosolic preparations of iNOS were prepared from RAW 264.7 cells (a murine monocyte-macrophage cell line), induced for 16 h with 10 mg/mL medium of lipopolysaccharide. The 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS)-solubilized particulate fraction of cultured bovine aortic endothelial cells was used as a source of eNOS.[15]

The NMDA-induced cyclic GMP accumulation in neonatal rat cerebellar slices was measured as described by Bredt and Snyder[12] with some modifications. Cerebella from 8−10-day-old rats were cut at 0.3-mm intervals coronally and sagitally. Slices were preincubated for 105 min in Krebs buffer gassed with 95% O_2/5% CO_2 at 37 °C. Aliquots of gravity-packed slices were then transferred to prewarmed 24-well plates containing Krebs buffer equilibrated with 95% O_2/5% CO_2 at 37 °C. After 15 min of equilibration, slices were exposed to test compound, followed by 3 min of 500 μM NMDA. Slices were inactivated by addition of 50 mM sodium acetate, followed by rapid freezing with liquid nitrogen. After samples were thawed and sonicated, cyclic GMP was determined by standard radioimmunoassay.

The ACh-induced relaxation of rat thoracic aorta was measured in aortae isolated from male Sprague−Dawley rats (350−375 g), taking care to minimize damage to the endothelium and cut into transverse rings. Aortic rings were mounted under 1.25-g resting tension in 25-mL tissue baths containing Krebs buffer with added indomethacin (5 μM), maintained at 37 °C, and gassed continuously with 5% CO_2 in O_2. Krebs buffer consisted of 120 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM dextrose, 25 mM $NaHCO_3$, and 2.5 mM $CaCl_2$ (pH 7.4). After 1 h of equilibration, aortic rings were precontracted with 1 μM phenylephrine to a plateau contraction and then relaxed with 1 μM acetylcholine (ACh). When the ACh-induced relaxation had reached a plateau, the cumulative concentration−response curve for the reversal of the relaxation was determined by successive additions of increasing concentrations of the NOS inhibitor, allowing a plateau to be reached between additions.

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