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Inhibitors of Human Nitric Oxide Synthase Isoforms with the Carbamidine Moiety as a Common Structural Element

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Abstract—Identification of potent and selective inhibitors of inducible nitric oxide synthase (NOS) is of great interest because of their therapeutic potential for treatment of diseases mediated by excess production of nitric oxide. We present here a comparison of potency and selectivity for amino acid and nonamino acid based compounds as inhibitors of human inducible, human endothelial constitutive and human neuronal constitutive NOS isoforms. In addition, a novel series of substituted amidines has been identified as NOS inhibitors. 2-Methylthioacetamidine and 2-thienylcarbamidine were the most potent of the series examined with IC_{50} values of 3.9 and 2.9 μM for human neuronal constitutive NOS. Cyclopropylcarbamidine and 2-thienylcarbamidine were the most potent inhibitors for human inducible NOS with IC_{50} values of 5.2 and 6.5 μM, respectively. These substituted amidines represent a new class of NOS inhibitors and provide a foundation for potential therapeutic agents. Copyright © 1996 Elsevier Science Ltd

Introduction

Nitric oxide synthase (NOS; EC 1.14.13.39) catalyzes the five-electron oxidation of L-arginine to produce L-citrulline and the biologically active free radical, nitric oxide. In addition to the co-substrates molecular oxygen and NADPH, NOS requires bound FAD, FMN, heme, calmodulin, and (6*R*)-tetrahydro-L-biopterin. A number of reviews have described in detail the rapid progress that has been made in the study of NOS and the biological activity of nitric oxide.[1–6] There are three distinct isoforms of NOS: endothelial NOS, neuronal NOS, and inducible NOS. The endothelial and neuronal isoforms are expressed constitutively, require Ca^{2+}/calmodulin for activity, and are found primarily in the vascular endothelium and brain, respectively. The endothelial enzyme generates nitric oxide which acts primarily to lower blood pressure and inhibit platelet aggregation, while the nitric oxide produced by the neuronal enzyme appears to function as a neurotransmitter. The inducible isoform is present in activated macrophages as well as numerous other cell types following induction with cytokines/endotoxin. The major function of inducible NOS is in host defense. It is this form that is implicated in the excessive production of nitric oxide that destroys functional tissue during acute and chronic inflammation. Due to the importance of the constitutive forms of NOS in regulating normal physiological processes, the identification of potent and selective inhibitors of inducible NOS should provide significant therapeutic value for the prevention and treatment of diseases mediated by the excessive production of nitric oxide.[7,8] Selective inhibition of neuronal NOS may be useful in reducing the neurotoxic actions of nitric oxide associated with cerebral ischemia.

Some of the most thoroughly studied inhibitors of NOS are structural analogues of L-arginine (Fig. 1). The most common examples of these are N^G-monomethyl-L-arginine (L-NMA, **5**)[9,10] and N^G-nitro-L-arginine (L-NNA, **8**),[11] which each inhibit the endothelial, neuronal, and inducible isoforms of NOS. L-NMA has similar IC_{50} values for the inducible and constitutive NOS isoforms (Table 1), which poses problems for its use as a therapeutic agent. Administration of L-NMA causes a marked and sustained increase in blood pressure, indicating the importance of nitric oxide synthesis by the vascular endothelium.[12] L-NNA has an IC_{50} for inducible NOS similar to that of L-NMA, but is a much more potent inhibitor for each of the constitutive NOS isoforms (Table 1). This observation indicates differences in the arginine binding site between the inducible and constitutive enzymes which can potentially be exploited for the development of selective inhibitors.

We have recently shown that L-N^6-(1-iminoethyl)lysine (L-NIL, **2**, Fig. 1) is a potent and selective inhibitor of mouse inducible NOS (IC_{50}=3.3 μM), with 28-fold selectivity for mouse inducible NOS compared with rat neuronal constitutive NOS.[13] L-N^6-(1-Iminoethyl) ornithine (L-NIO, **1**), which has one less methylene group than L-NIL, has similar potency for both mouse inducible NOS and rat neuronal constitutive NOS, thus lacking selectivity.[13] Such a difference in selectivity between two simple homologues is both interesting and exciting in its potential for the development of selective inhibitors. L-NIL suppressed the increase in plasma

Key words: nitric oxide synthase inhibitors, nitric oxide synthase, amidine, carbamidine.

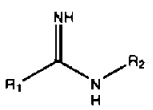
Table 1. Comparison of IC_{50} values for inhibition of human NOS isoforms by amino acid-based inhibitors

No.[a]		IC_{50} (μM)[b]			Selectivity[c]	
		hiNOS	hecNOS	hncNOS	hec/hi	hnc/hi
1	L-NIO	7.7	5.8	10	0.8	1.3
2	L-NIL	4.6	138	61	30	13
3	DL-homoNIL	48	2360	461	49	9.6
4	D-NIL	670	1540	3270	2.3	4.9
5	L-NMA	14	5.9	10	0.4	0.7
6	L-NMHA	375	63	51	0.17	0.14
7	L-NEA	6.1	9.5	16	1.6	2.6
8	L-NNA	7.6	0.5	0.52	0.07	0.07
9	L-NAA	3.8	3.7	4.8	1.0	1.3
10	S-CH_3-L-thiocitrulline	0.91	1.6	0.27	1.8	0.3
11	L-Thiocitrulline	3.6	8.4	7.4	2.3	2

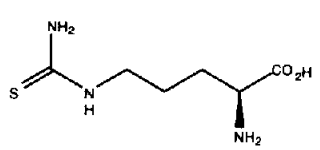
[a]Compound number corresponds to structure shown in Figure 1.
[b]Selectivity for hiNOS is defined as the ratio of the IC_{50} hecNOS or IC_{50} hncNOS to IC_{50}hiNOS.
[c]IC_{50} values were determined with hiNOS, hecNOS, and hncNOS by testing each compound at eight concentrations. NOS activity was measured in the presence of a final L-arginine concentration of 30 μM by monitoring the conversion of L-2,3-^{3}H-arginine to L-2,3-^{3}H-citrulline as described in the Experimental.

nitrite levels and joint inflammation associated with adjuvant-induced arthritis.[14] Importantly, blood pressure was not affected by the highest dose of L-NIL administered, indicating lack of inhibition of the endothelial constitutive NOS at doses which exhibited anti-inflammatory activity.[14]

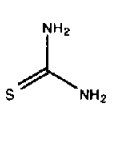
Nonarginine or nonamino acid-based inhibitors of NOS include aminoguanidine,[15] isothioureas,[16,17] bisisothioureas,[16] and 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine.[18] Aminoguanidine is a selective inhibitor of mouse inducible NOS in enzyme-, cell-, and organ-based assays.[15] The in vivo administration of aminoguanidine decreased the activity of inducible NOS in the post ischemic brain and reduced the extent of brain damage associated with focal ischemia in the rat.[19] Aminoguanidine also suppressed the increase in plasma nitrite levels and joint inflammation associated with adjuvant-induced arthritis.[14]



Name	#	R_1	R_2
L-NIO	1	CH_3	$CH_2(CH_2)_2CH(NH_2)CO_2H$
L-NIL	2	CH_3	$CH_2(CH_2)_3CH(NH_2)CO_2H$
DL-homoNIL	3	CH_3	$CH_2(CH_2)_4CH(NH_2)CO_2H$
D-NIL	4	CH_3	$CH_2(CH_2)_3CH(NH_2)CO_2H$
L-NMA	5	CH_3NH	$CH_2(CH_2)_2CH(NH_2)CO_2H$
L-NMHA	6	CH_3NH	$CH_2(CH_2)_3CH(NH_2)CO_2H$
L-NEA	7	CH_3CH_2NH	$CH_2(CH_2)_2CH(NH_2)CO_2H$
L-NNA	8	NO_2NH	$CH_2(CH_2)_2CH(NH_2)CO_2H$
L-NAA	9	NH_2NH	$CH_2(CH_2)_2CH(NH_2)CO_2H$
S-CH_3-L-thiocitrulline	10	CH_3S	$CH_2(CH_2)_2CH(NH_2)CO_2H$
	12	CH_3S	H
	13	CH_3CH_2S	H
	14	$CH_3(CH_2)_2S$	H
	15	CH_3S	CH_3
	16	CH_3CH_2S	Ph
	18	CH_3O	H
methylguanidine	19	CH_3NH	H
aminoguanidine	20	NH_2NH	H
agmatine	21	NH_2	$CH_2(CH_2)_3NH_2$



L-thiocitrulline **11**



thiourea **12**

Figure 1. Structures of compounds 1–21.

We present here a comparison of the potency and selectivity of L-NIL, L-NIL analogues, and amino acid based inhibitors (Fig. 1, **1**–**11**) for each of the major isoforms of human NOS: human inducible NOS (hiNOS), human endothelial constitutive NOS (hecNOS), and human neuronal constitutive NOS (hncNOS). For comparison, IC_{50} values with human NOS isoforms are shown for several nonamino acid inhibitors (Fig. 1, **12**–**21**), including alkylisothioureas, guanidines, and *O*-methylisourea. In addition, we present IC_{50} values with each of the human NOS isoforms for a new class of nonamino acid inhibitors, substituted amidines (Table 3). All of these compounds share the carbamidine moiety, which appears to be a common pharmacophore of NOS inhibitors.

Results and Discussion

L-NIO (**1**), L-NIL (**2**), DL-homoNIL (**3**), and D-NIL (**4**) were evaluated as inhibitors of each of the three major human NOS isoforms (Fig. 1). As shown in Table 1, L-NIL has an IC_{50} for hiNOS of 4.6 μM compared with 138 μM for hecNOS and 61 μM for hncNOS, indicating that L-NIL is 30-fold and 13-fold more selective for hiNOS than hecNOS and hncNOS, respectively. Selectivity of hiNOS compared with hecNOS is defined as the ratio of the IC_{50} for hecNOS to the IC_{50} for hiNOS. Similarly, selectivity for hiNOS compared with hncNOS is expressed as the ratio of the IC_{50} for hncNOS to the IC_{50} for hiNOS. L-NIO, which

differs from L-NIL by having one less methylene group, was slightly less potent than L-NIL in inhibition of hiNOS with essentially no selectivity for hiNOS compared with either hecNOS or hncNOS (Table 1). Incorporation of an additional methylene group to give DL-homoNIL (**3**) resulted in a substantial decrease in inhibitory potency for each of the human NOS isoforms as compared with L-NIL; however, the selectivity profile for DL-homoNIL was very similar to that obtained for L-NIL. D-NIL (**4**) was a very poor inhibitor of each of the three human NOS isoforms, indicating the importance of maintaining stereochemistry at the α-carbon. These results are consistent with data previously reported for these compounds using mouse inducible NOS and rat neuronal NOS and indicate that changes in the length of the amino acid side chain significantly affect potency and selectivity.[13]

Also shown in Table 1 is a comparison of several arginine analogs. N^G-monoethyl-L-arginine (L-NEA, **7**) was slightly more potent that L-NMA (**5**) for hiNOS. Both L-NMA (**5**) and L-NEA (**7**) had similar IC_{50} values for hecNOS and hncNOS. N^G-monomethyl-L-homoarginine (L-NMHA, **6**) was substantially less potent for hiNOS than L-NMA, with an IC_{50} for hiNOS of 375 μM, compared with an IC_{50} of 14 μM for L-NMA. The potency of L-NMHA (**6**) for hecNOS and hncNOS was also reduced. Increasing the length of L-NMA by one carbon to give L-NMHA results in a reduction of potency for each of the NOS isoforms and an increase in selectivity for the constitutive isoforms. This is in striking contrast to the increase in potency and selectivity for hiNOS that occurs following the identical increase in chain length in going from L-NIO to L-NIL. N^G-nitro-L-arginine (L-NNA, **8**) is slightly less potent for hiNOS and much more potent for hecNOS and hncNOS than L-NIL, making it substantially more selective for both constitutive isoforms. N^G-amino-L-arginine (L-NAA, **9**) was the most potent inhibitor for hiNOS of the arginine analogues tested, with an IC_{50} of 3.8 μM, similar to that for L-NIL. L-NAA gave similar IC_{50} values for each of the three human NOS isoforms, showing essentially no selectivity. IC_{50} values reported here for L-NMA, L-NNA, and L-NIO are in good agreement with those reported previously for human NOS isoforms.[20]

Also shown in Table 1 are the IC_{50} values obtained with *S*-methyl-L-thiocitrulline[21] (**10**) and L-thiocitrulline[21] (**11**), which are 0.91 and 3.6 μM, respectively, for hiNOS. The nonamino acid analogues of these inhibitors are compared in Table 2. *S*-methylisothiourea (**12**), which differs from S-methyl-L-thiocitrulline (**10**) by the absence of the amino acid component at the R_2 position (Fig. 1), has very similar potency for hiNOS and hecNOS, but is less potent for hncNOS (Table 2). Compound **15**, with a methyl substituent at the R_2 position, gave a dramatic reduction in potency for hiNOS and a substantial but smaller reduction for hecNOS and hncNOS in comparison to *S*-methylisothiourea (**12**), where R_2 is H. Similarly, compound **16**, with a phenyl group at the R_2 position was less potent than *S*-ethylisothiourea (**13**), where R_2 is H. These substituents at the R_2 position have a dramatic effect on decreasing the potency of the *S*-methyl and *S*-ethyl isothioureas, particularly for hiNOS, and tend to increase the selectivity for hncNOS. Thiourea (**17**), the nonamino acid analogue of L-thiocitrulline, is inactive as an inhibitor of the human NOS isoforms, indicating the importance of the amino acid side chain for inhibition by L-thiocitrulline.

Also evaluated in this series were nonamino acid guanidine and urea analogues (Table 2). Methylguanidine (**19**), the nonamino acid analogue of L-NMA, is a very weak inhibitor of the human NOS isoforms, indicating the importance of the amino acid side chain. Aminoguanidine (**20**) can be viewed as the nonamino acid analogue of L-NAA (**9**) and is considerably less potent than L-NAA for each human NOS isoform. While L-NAA shows essentially no selectivity for the human NOS isoforms, aminoguanidine has about

Table 2. Comparison of IC_{50} values for inhibition of human NOS isoforms by nonamino acid isothioureas, isourea and guanidines

No.[a]	IC_{50} (μM)[b]			Selectivity[c]	
	hiNOS	hecNOS	hncNOS	hec/hi	hnc/hi
12[16]	0.72	2.4	1.4	3.3	1.9
13[16]	0.17	1.1	0.55	6.5	3.2
14[16]	2.4	17	5	7	2.1
15[17]	1234	490	169	0.4	0.14
16	14	14	1.7	1	0.12
17	> 10 mM	> 10 mM	> 10 mM		
18	39	139	46	3.6	1.2
19	1940	2660	553	1.4	0.2
20	168	1680	358	10	2
21	1210	4888	278	4.0	0.2

[a]Compound number corresponds to structure shown in Figure 1.
[b]IC_{50} values were determined with hiNOS, hecNOS, and hncNOS by testing each compound at eight concentrations. NOS activity was measured in the presence of a final L-arginine concentration of 30 μM by monitoring the conversion of L-2,3-^{3}H-arginine to L-2,3-^{3}H-citrulline as described in the Experimental.
[c]Selectivity for hiNOS is defined as the ratio of the IC_{50} hecNOS or IC_{50} hncNOS to IC_{50}hiNOS.

10-fold selectivity for hiNOS compared with hecNOS. Agmatine (**21**), the des carboxyl arginine, is a poor inhibitor of the human NOS isoforms, indicating the importance of the carboxyl group. Both methylguanidine (**19**) and agmatine (**21**) are more potent for hncNOS than for the other isoforms.

O-Methylisourea (**18**) was tested to evaluate the effect of replacing sulfur with oxygen. While substantially less potent than *S*-methylisothiourea (**12**), *O*-methylisourea was a modest inhibitor of the human NOS isoforms, with a similar selectivity profile. Examination of the isosteric series consisting of methylguanidine (**19**), *O*-methylisourea (**18**), and *S*-methylisothiourea (**12**) demonstrates increasing potency for human NOS isoforms progressing from N to O to S (Table 2).

With the exception of the *N*-iminoethyl derivatives of ornithine, lysine, and homolysine (**1**–**4**), the compounds presented in Tables 1 and 2 can be viewed as having either N, O, or S bound to the carbamidine carbon and therefore are examples of ureas or guanidines. The selectivity and potency observed with the iminoethyl derivatives and the potency of the nonamino acid inhibitors described above suggested that the amidine group alone might inhibit human NOS isoforms. A series of substituted amidines were purchased or prepared and evaluated as NOS inhibitors. As shown in Table 3, acetamidine (**22**), the simplest member of this series, was a very weak inhibitor of the NOS isoforms, similar in potency and selectivity to methylguanidine (**19**, Table 2). Chloroacetamidine (**23**) was substantially more potent than acetamidine (**22**). The most potent inhibitors of hiNOS were cyclopropylcarbamidine (**26**) and 2-thienylcarbamidine (**33**), with IC_{50} values of 5.2 and 6.5 μM, respectively. 2-Methylthioacetamidine (**29**) and 2-thienylcarbamidine (**33**) were the most potent NOS inhibitors of the substituted amidine series, with IC_{50} values for hncNOS of 3.9 and 2.9 μM, respectively. The hecNOS/hiNOS selectivity ratios ranged from 0.6 to 5.1 for the substituted amidines. The hncNOS/hiNOS ratios ranged from 0.07 to 1 and indicate a trend in these examples toward greater selectivity for hncNOS compared with hiNOS and hecNOS.

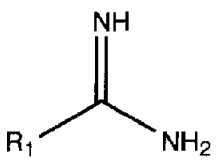
The loss of potency observed when comparing 2-methylthioacetamidine (**29**, Table 3) to its structural isomer, *S*-ethylisothiourea (**13**, Table 2) is most pronounced for hiNOS and hecNOS, but less so for hncNOS. Interestingly, oxidation of 2-methylthioacetamidine (**29**) to the corresponding sulfone (**30**) results in further loss in potency for hiNOS and hecNOS, with only a slight decrease in potency for hncNOS (Table 3).

The amidines shown in Table 3 share the carbamidine moiety as a common structural feature found in all the inhibitors described in this paper. These substituted amidines represent a new class of NOS inhibitors and provide a potential foundation for developing potent and selective NOS inhibitors.

Experimental

L-2,3-^{3}H-arginine was purchased from Dupont NEN (Boston, Massachussetts); (6*R*)-tetrahydro-L-biopterin

Table 3. Comparison of IC_{50} values for inhibition of human NOS isoforms by substituted amidines



No.	R_1	IC_{50} (μM)[a]			Selectivity[b]	
		hiNOS	hecNOS	hncNOS	hec/hi	hnc/hi
22	CH_3	1760	3030	598	1.7	0.34
23	$ClCH_2$	62	103	14	1.7	0.22
24	Isopropyl	148	203	29	1.4	0.2
25	Isobutyl	17	76	14	4.5	0.8
26	Cyclopropyl	5.2	16	5.0	3.1	1
27	2-Furanyl	17	10	4.8	0.6	0.28
28	3-Pyridyl	66	113	16	1.7	0.24
29	CH_3SCH_2	10	51	3.9	5.1	0.39
30	$CH_3SO_2CH_2$	91	218	6.6	2.4	0.07
31	$PhCH_2SCH_2$	91	179	8.0	2.0	0.09
32	CH_3OCH_2	54	200	34	3.7	0.63
33	2-Thienyl	6.5	4.2	2.9	0.6	0.44

[a] IC_{50} values were determined with hiNOS, hecNOS, and hncNOS by testing each compound at eight concentrations. NOS activity was measured in the presence of a final L-arginine concentration of 30 μM by monitoring the conversion of L-2,3-^{3}H-arginine to L-2,3-^{3}H-citrulline as described in the Experimental.

[b] Selectivity for hiNOS is defined as the ratio of the IC_{50} hecNOS or IC_{50} hncNOS to IC_{50} hiNOS.

was from Research Biochemicals, Inc. (Natick, Massachussetts); aminoguanidine hemisulfate, methylguanidine hydrochloride, *S*-methylisothiourea hemisulfate, L-NMA and L-NNA were from Sigma (St Louis, Missouri); *S*-methyl-L-thiocitrulline dihydrochloride, L-thiocitrulline, N^G-monoethyl-L-arginine monoacetate, N^G-monomethyl-L-homoarginine monoacetate, and N^G-amino-L-arginine hydrochloride were from Alexis Biochemicals (San Diego, California); *S*-ethylisothiourea hydrobromide, acetamidine hydrochloride, *O*-methylisourea hemisulfate, and *S*-methyl,*N*-phenylisothiopseudourea were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin); 2-chloroacetamidine hydrochloride and cyclopropylcarbamidine hydrochloride were from Lancaster (Windham, New Hampshire); 2-amidinothiophene hydrochloride and 3-amidinopyridine hydrochloride were from Ryan Scientific (Columbia, South Carolina). L-NIL, DL-homoNIL, L-NIO, and D-NIL were prepared and homogeneity assessed as previously described.[13] Purification of synthesized compounds was performed by crystallization or by preparative HPLC chromatography on a Waters Prep LC2000 using a Delta Pak C-18 column and a linear gradient over 30 min from 0–40% acetonitrile in 0.1% trifluoroacetic acid in water. ^{1}H NMR spectra were obtained at 300 MHz on a Varian VXR300 spectrometer in D_2O. MS were obtained either on a VG Model 250 or a Finnigan MAT 90 spectrometer. Elemental analyses were performed by Atlantic Microlabs, Inc. (Norcross, Georgia).

2-(1-Propyl)-2-thiopseudourea hydroiodide[16] (14). Thiourea (0.76 g, 0.01 mol) was dissolved in 30 mL of acetone and 1-iodopropane (5.1 g, 0.03 mol) was added. The reaction was run at room temperature for 12 h. After rotary evaporation, the residue was triturated with ethyl ether. The solids were crystallized from ethyl alcohol and ethyl ether to afford 1.4 g (57%) of a pale-yellow solid. ^{1}H NMR (D_2O): δ 0.88 (t, 3H), 1.6 (m, 2H), 2.95 (t, 2H). MS: 119 (MH^+). Anal. calcd for $C_4H_{11}N_2S_1I_1$: C, 19.52; H, 4.51; N, 11.38. Found: C, 19.41; H, 4.48; N, 11.29%.

***N,S*-Dimethylisothiourea[17] (15).** *N*-Methylthiourea (0.9 g, 0.01 mol) was dissolved in 20 mL of acetone. Iodomethane (4.3 g, 0.03 mol) was added dropwise. The reaction was run at room temperature for 18 h. After rotary evaporation, the residue was triturated with ethyl ether to afford 1 g (43%) of a pale-yellow solid. ^{1}H NMR (D_2O): δ 2.95 (S, 3H), 3.1 (S, 3H). MS: 105 (MH^+). Anal. calcd for $C_3H_9N_2S_1I_1$: C, 15.52; H, 3.91; N, 12.07. Found: C, 15.60; H, 3.95; N, 12.06%.

General procedure[22] for amidine formation

2-Methylthioacetamidine hydrochloride[23] (29). Methylthioacetonitrile (10 g, 0.11 mol) was dissolved in 30 mL of ethanol and cooled to 0 °C under nitrogen atmosphere. Anhydrous HCl gas was bubbled into the solution until saturated while maintaining the temperature at less than 5 °C. The solution was allowed to warm to 25 °C and after stirring at this temperature for 18 h, concentrated in vacuo to afford the *O*-ethyliminoether hydrochloride as a white solid, mp 88–90 °C. This solid was suspended in 20 mL of ethanol, cooled to 0 °C under nitrogen atmosphere and then added to a solution of 40 mL of ethanol saturated with anhydrous ammonia. This was stoppered and stirred at 25 °C for 3 days. This solution was filtered and concentrated to afford 14.5 g (94%) of a white solid, mp 104–107 °C. ^{1}H NMR (D_2O): δ 2.08 (s, 3H), 3.42 (s, 2H). MS: 105 (MH^+). Anal. calcd for $C_3H_9N_2Cl_1S_1$: C, 25.62; H, 6.45; N, 19.92. Found: C, 25.42; H, 6.53; N, 20.07%.

2-Furanylcarbamidine hydrochloride[24] (27). Reaction of 2-furanylcarbonitrile afforded 1.5 g (51%) of a brown solid, mp 83–90 °C. ^{1}H NMR (D_2O): δ 6.52 (m, 1H), 7.27 (d, 1H), 7.63 (d, 1H). MS: 111 (MH^+). Anal. calcd for $C_5H_7N_2O_1Cl_1$: C, 40.97; H, 4.81; N, 19.11. Found: C, 40.96; H, 4.85; N, 19.02%.

2-Methoxyacetamidine hydrochloride[25] (32). Reaction of 2-methoxyacetonitrile afforded 4 g (46%) of a white solid, mp 54–58 °C. ^{1}H NMR (D_2O): δ 3.35 (s, 3H), 4.22 (s, 2H). MS: 89 (MH^+). Anal. calcd for $C_3H_9N_2O_1Cl_1 \cdot 0.25\ H_2O$: C, 27.92; H, 7.42; N, 21.70. Found: C, 28.16; H, 7.23; N, 21.93%.

2-Methylsulfonylacetamidine hydrochloride (30). Reaction of 2-methanesulfonylacetonitrile afforded 4.7 g (65%) of a brown solid, mp 83–90 °C. ^{1}H NMR (D_2O): δ 3.13 (s, 5H). MS: 166 (MH^+). Anal. calcd for $C_3H_9N_2O_2S_1Cl_1 \cdot 0.1\ NH_4Cl$: C, 20.25; H, 5.32; N, 16.53. Found: C, 20.47; H, 5.36; N, 16.67%.

2-Propylcarbamidine hydrochloride[26] (24). Reaction of isobutyronitrile afforded 6.3 g (35%) of a white solid, mp 154–156 °C. ^{1}H NMR (D_2O): δ 1.15 (d, 6H), 2.66 (hep, 1H). MS: 87 (MH^+). Anal. calcd for $C_4H_{11}N_2Cl_1 \cdot 0.65\ NH_4Cl$: C, 30.53; H, 8.71; N, 23.59. Found: C, 30.69; H, 8.73; N, 23.31%.

1-(2-Methylpropyl)carbamidine hydrochloride[27] (25). Reaction of isovaleronitrile afforded 0.35 g (52%) of a white solid. ^{1}H NMR(DMSO-d_6): δ 0.9 (d, 6H), 2.05 (m, 1H), 2.25 (d, 2H). MS: 101 (MH^+). Anal. calcd for $C_5H_{13}N_2Cl_1 \cdot 0.1\ NH_4Cl$: C, 42.30; H, 9.51; N, 20.72. Found: C, 42.45; H, 9.47; N, 20.69%.

2-(Benzylthio)acetamidine trifluoroacetic acid[28] (31). Reaction of 2-benzylthioacetonitrile afforded 2.3 g (58%) of the hydrochloride salt as an off-white solid. ^{1}H NMR (D_2O): δ 3.47 (s, 2H), 3.72 (s, 2H), 7.22 (m, 5H). MS: 181 (MH^+). Purification by C-18 HPLC afforded the trifluoroacetic acid salt as a white solid. Anal. calcd for $C_{11}H_{13}N_2O_2S_1F_3$: C, 44.89; H, 4.45; N, 9.52. Found: C, 44.69; H, 4.37; N, 9.50%.

Assay of NOS activity. NOS activity was measured by monitoring the conversion of L-2,3-^{3}H-arginine to L-2,3-^{3}H-citrulline.[13] Human inducible NOS (hiNOS), human endothelial constitutive NOS (hecNOS), and human neuronal constitutive NOS (hncNOS) were each cloned from RNA extracted from human tissue.

The cDNA for human inducible NOS (hiNOS) was isolated from a λcDNA library made from RNA extracted from a colon sample from a patient with ulcerative colitis.[29] The cDNA for human endothelial constitutive NOS (hecNOS) was isolated from a λcDNA library made from RNA extracted from human umbilical vein endothelial cells (HUVEC) and the cDNA for human neuronal constitutive NOS (hncNOS) was isolated from a λcDNA library made from RNA extracted from human cerebellum obtained from a cadaver. The recombinant enzymes were expressed in Sf9 insect cells using a baculovirus vector.[29] Enzyme activity was isolated from soluble cell extracts and partially purified by DEAE-Sepharose chromatography.[15,29] Average specific activity values for hiNOS, hecNOS, and hncNOS DEAE preparations were 4600, 3600, and 2200 pmoles citrulline/minute/mg protein, respectively. K_m values for L-arginine were 7, 4, and 6 μM for hiNOS, hecNOS, and hncNOS, respectively. To measure NOS activity, 10 μL of enzyme was added to 40 μL of 50 mM Tris (pH 7.6) and the reaction initiated by the addition of 50 μL of a reaction mixture containing 50 mM Tris (pH 7.6), 2.0 mg/mL bovine serum albumin, 2.0 mM DTT, 4.0 mM $CaCl_2$, 20 μM FAD, 100 μM tetrahydrobiopterin, 0.4 mM NADPH and 60 μM L-arginine containing 0.9 μCi of L-2,3-^{3}H-arginine. The final concentration of L-arginine in the assay was 30 μM. For constitutive NOS, calmodulin was included at a final concentration of 40 nM. Following incubation at 37 °C for 15 min, the reaction was terminated by addition of 300 μL of cold buffer containing 10 mM EGTA, 100 mM HEPES (pH 5.5) and 1.0 mM L-citrulline. The ^{3}H-citrulline was separated by chromatography on Dowex 50W X-8 cation exchange resin and radioactivity quantified with a liquid scintillation counter. All assays were performed at least in duplicate; standard deviations were 10% or less. Production of ^{3}H-citrulline was linear with time for hiNOS, hecNOS, and hncNOS over the time period utilized for the assay.

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