N^{ω}-Nitroarginine-Containing Dipeptide Amides. Potent and Highly Selective Inhibitors of Neuronal Nitric Oxide Synthase

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Selective inhibition of the isoforms of nitric oxide synthase (NOS) could be therapeutically useful in the treatment of certain disease states arising from the overproduction of nitric oxide (NO). Recently, we reported the dipeptide methyl ester, D-Phe-D-Arg^{NO_2}-OMe (**19**), as a modest inhibitor of nNOS ($K_i = 2\ \mu$M), but with selectivity over iNOS as high as 1800-fold (Silverman, R. B.; Huang, H.; Marletta, M. A.; Martasek, P. *J. Med. Chem.* **1997**, *40*, 2813−2817). Here a library of 152 dipeptide amides containing nitroarginine and amino acids other than Phe are synthesized and screened for activity. Excellent inhibitory potency and selectivity for nNOS over eNOS and iNOS is achieved with the dipeptide amides containing a basic amine side chain (**20**−**24**), which indicates a possible electrostatic (or hydrogen bonding) interaction at the enzyme active site. The most potent nNOS inhibitor among these compounds is L-Arg^{NO_2}-L-Dbu-NH_2 (**23**) ($K_i = 130$ nM), which also exhibits the highest selectivity over eNOS (>1500-fold) with a 192-fold selectivity over iNOS. These compounds do not exhibit time-dependent inhibition. The order and the chirality of the amino acids in the dipeptide amides have profound influences on the inhibitory potency as well as on the isoform selectivity. These dipeptide amide inhibitors open the door to the design of potent and highly selective inhibitors of nNOS.

Introduction

Nitric oxide (NO) is an important biological messenger involved in a variety of physiological processes.[1] A family of enzymes, the nitric oxide synthases (NOS, EC 1.14.13.39), catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide in a NADPH- and O_2-dependent process.[2] There are three paradigms of NO biological functions which correlate with three distinct isoforms of NO synthase.[3] The constitutive endothelial isoform (eNOS) is involved in the regulation of smooth muscle relaxation and blood pressure and in the inhibition of platelet aggregation. Another constitutive isoform is neuronal NOS (nNOS), which is important for neurotransmission and long-term potentiation. NO produced by the inducible isoform (iNOS) in activated macrophage cells acts as a cytotoxic agent in normal immune responses. All of the isoforms utilize NADPH, FAD, FMN, tetrahydrobiopterin, and heme as cofactors. The constitutive isoforms also require added Ca^{2+} and calmodulin for activity, while the inducible isoform has tightly bound Ca^{2+} and calmodulin. Cloning of the isoforms of NOS has revealed that they share only approximately 50% of primary sequence homology, suggesting that they may differ from each other in regulatory aspects.[4] However, interspecies similarity for each isoform is quite high (81−93% identical);[4] therefore, the source of the NOS used in various studies may not be important.

Overproduction of NO has been implicated in a wide variety of diseases. NO overproduction by nNOS has been associated with strokes,[5] migraine headaches,[6] and Alzheimer's disease.[7] Enhanced formation of NO following the induction of iNOS appears to be important in the tolerance to and dependence on morphine,[8] development of colitis,[9] and tissue damage and inflammation.[10] Because the three isoforms of NOS have unique roles in separate tissues, selective inhibition of one isoform over the others is essential.[11] Numerous pharmaceutical companies have initiated programs to identify potent and selective inhibitors of the isoforms of NOS as potential treatments for some of these disease states. In particular, nNOS inhibition has been targeted for the treatment of strokes,[12] and iNOS inhibition for the treatment of septic shock[13] and arthritis.[14] It is very important not to inhibit eNOS because of its important role in maintaining blood flow.

Many of the NOS inhibitors are analogues of the substrate L-arginine (Table 1) and include N^{ω}-methyl-L-arginine (**1**, L-NMA),[15] N^{ω}-nitro-L-arginine (**2**, L-NA),[16] N^{δ}-(iminoethyl)-L-ornithine (**3**, L-NIO),[17] N^5-(imino-3-butenyl)-L-ornithine (**4**, L-VNIO),[18] N^{ω}-alkyl-L-arginines (**5**, **6**),[19] and *S*-alkyl-L-thiocitrullines (**7**, **8**).[20] Most of these L-arginine analogues are inactivators of NOS, but they have minimal selectivity among the isozymes (Table 1), except for N^{ω}-propyl-L-arginine (**5**)[19,21] and N^5-(imino-3-butenyl)-L-ornithine (**4**),[18] which are highly selective inhibitors for nNOS over iNOS and eNOS. There also are numerous non amino acid based NOS inhibitors, for example (Table 2), aminoguanidine (**9**),[22] *S*-ethylisothiourea (**10**),[23] substituted *N*-phenylisothio-

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[§] Carried out all of the chemical and biological work except for the overexpression of nNOS and eNOS into *E. coli* and the purification of eNOS used in these studies.
[||] Provided the purified recombinant bovine eNOS used in these studies.
[⊥] Provided the overexpressed nNOS used in these studies.

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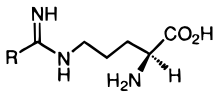
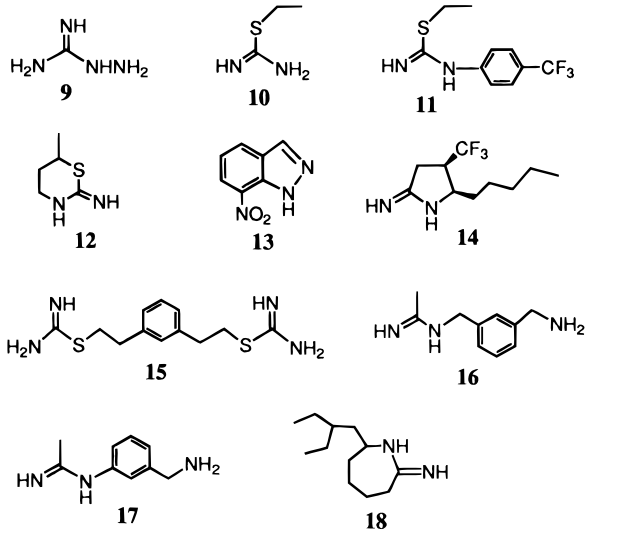
Table 1. Isoform Selectivity of Some Amino Acid Based NOS Inhibitors (L-Arginine Analogues)



compd[ref]	R	selectivity[a] eNOS/nNOS	eNOS/iNOS
1 L-NMA[15]	$NHCH_3$	0.6	0.5
2 L-NA[16]	$NHNO_2$	2.5	0.01
3 L-NIO[17]	CH_3	0.3	0.1
4 L-VNIO[18]	$CH_2CH{=}CH_2$	120	0.2
5 L-NPA[19,21]	$NHCH_2CH_2CH_3$	149	0.05
6 L-AA[19]	$NHCH_2CH{=}CH_2$	15	1.5
7 L-SMTC[20]	SCH_3	10	0.4
8 L-SETC[20]	SCH_2CH_3	50	1.5

[a] Defined as the ratio of the K_i or IC_{50} values.

Table 2. Isoform Selectivity of Some Non Amino Acid Based NOS Inhibitors



compd[ref]	selectivity[a] eNOS/nNOS	eNOS/iNOS
9[22]	10	500
10[23]	1.2	2
11[24]	29	0.3
12[25]	13	40
13[26]	5−10	5−10
14[27]	90	900
15[23]	38	180
16[14b]	25	5000
17[13a]	155	3
18[14a]	130	314

[a] The ratio of the IC_{50} or K_i values.

urea (**11**),[24] 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (**12**),[25] 7-nitroindazole (**13**),[26] (+)-*cis*-5-pentyl-4-(trifluoromethyl)pyrrolidin-2-imine (**14**),[27] bis(isothioureas) (**15**),[23] *N*-(3-(aminomethyl)benzyl)acetamidine (**16**),[14b] *N*-(3-(aminomethyl)phenyl)acetamidine (**17**),[13a] and 7-(2-ethylbutyl)hexahydro-1*H*-azepin-2-imine (**18**).[14a] As shown in Table 2, the selectivity has been significantly improved by these non amino acid based inhibitors, especially iNOS selective ones. In some cases, the selectivity is as high as 5000- (**16**), 900- (**14**), and 500-fold (**9**) in favor of iNOS over eNOS. However, such high selectivity has not been observed for the inhibition of nNOS over eNOS.

Our interest has been in the selective inhibition of nNOS. Since L-nitroarginine (**2**) is reported to have about a 250-fold selectivity in favor of nNOS over iNOS, and L-nitroarginine-containing dipeptides have been reported to be inhibitors of the release of endothelium-derived relaxing factor (now known to be nitric oxide),[28] we synthesized and evaluated a library of nitroarginine-containing dipeptides to see if the incorporation of nitroarginine into a dipeptide could increase the inhibitory potency and selectivity of L-nitroarginine. We recently reported that D-Phe-D-Arg^{NO_2} methyl ester (**19**) was an 1800-fold more selective inhibitor of nNOS than of iNOS; unfortunately, its selectivity for nNOS over eNOS was minimal,[29] as is the case with nitroarginine itself. We now report the first family of molecules that exhibits high selectivity for inhibition of nNOS over both iNOS and eNOS.

Chemistry

All of the dipeptide amides were synthesized by automated solid-phase peptide synthesis. Dipeptide amides were prepared as a result of the use of Rink resin as the solid support. All four isomers of each dipeptide combination (L−L; L−D; D−L; D−D) were synthesized with N^{ω}-nitroarginine at either the N-terminus or the C-terminus except for isoleucine and cysteine, which were used only as the L-isomers. In addition to the standard 19 natural amino acids (excluding Arg, which was replaced by Arg^{NO_2}), D- and L-ornithine (Orn), L-2,4-diaminobutyric acid (Dbu), and L-2,3-diaminopropionic acid (Dpr) also were used in the construction of the dipeptide amides with N^{ω}-nitroarginine. Each of the dipeptide amides was >80% pure after cleavage from the resin, as determined by NMR analysis, and was used directly for IC_{50} activity screening. Ten compounds (**20**−**29**), which showed either excellent potency, high selectivity, or both during the screening process, were selected for further purification and characterization. K_i values of these compounds were determined after they were purified by HPLC. The analytical data for these compounds are shown in Table 3.

Because of the limited synthetic scale by automated solid-phase peptide synthesis and the relatively long purification time by HPLC, the most promising dipeptide amide, L-Arg^{NO_2}-L-Dbu-NH_2 (**23**), was synthesized on a larger scale by a solution-phase synthesis. In the initial attempts to make this dipeptide amide from the corresponding dipeptide methyl ester, the nitro group on the Arg^{NO_2} was removed during the aminolysis reaction in a pressure bottle. This problem was overcome by first synthesizing L-Dbu amide for use in the peptide coupling step (Scheme 1). The best route to the amide was via treatment of the corresponding *p*-nitrophenyl ester of Dbu with dry ammonia. The inhibition constants for all three isozymes measured with the compound obtained by solution-phase synthesis were the same as those obtained with the compound from the solid-phase synthesis.

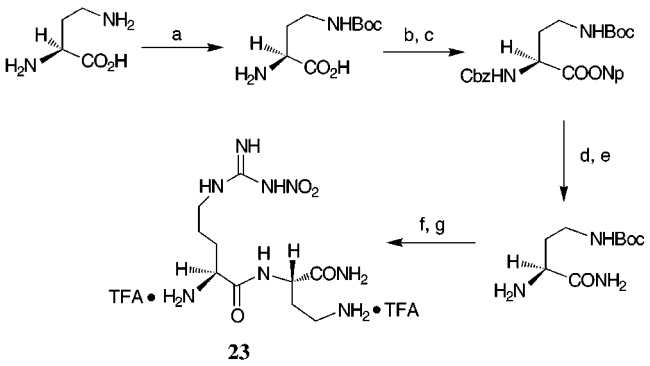
Results and Discussion

Initially, the 152 dipeptide amides prepared were screened by measuring the percentage of remaining enzyme activity in the presence of 100 μM inhibitors when compared to an untreated enzyme control. A rapid onset of inhibition was observed, and the dipeptide

Table 3. Analytical Data for the Selected N^{ω}-Nitroarginine-Containing Dipeptide Amides

compd	^{1}H NMR chemical shift (δ, D_2O)	HRMS (calcd)	anal. (C, H, N)
20 L-Arg^{NO_2}-L-Arg^{NO_2}-NH_2	4.32 (t, 1H), 4.05 (t, 1H), 3.28 (t, 4H), 1.56−1.98 (m, 8H)	420.2033 (420.2062)	$C_{12}H_{25}N_{11}O_6$·1.2TFA·1.5H_2O
21 L-Arg^{NO_2}-L-Lys-NH_2	4.30 (t, 1H), 4.05 (t, 1H), 3.29 (t, 2H), 2.98 (t, 2H), 1.87−2.01 (m, 2H), 1.58−1.86 (m, 6H), 1.35−1.50 (m, 2H)	347.2185 (347.2150)	$C_{12}H_{26}N_8O_4$·2TFA·0.8H_2O
22 L-Arg^{NO_2}-L-Orn-NH_2	4.34 (t, 1H), 4.08 (t, 1H), 3.29 (t, 2H), 3.00 (t, 2H), 1.89−1.98 (m, 2H), 1.62−1.89 (m, 6H)	333.1979 (333.1993)	$C_{11}H_{24}N_8O_4$·2TFA·H_2O
23 L-Arg^{NO_2}-L-Dbu-NH_2	4.47 (dd, 1H), 4.09 (t, 1H), 3.30 (t, 2H), 3.08 (m, 2H), 2.01−2.25 (m, 2H), 1.89−2.01 (m, 2H), 1.60−1.75 (m, 2H)	319.1819 (319.1837)	$C_{10}H_{22}N_8O_4$·2TFA·1.2H_2O
24 L-Arg^{NO_2}-L-Dpr-NH_2	4.12 (t, 1H), 3.49 (dd, 1H), 3.20−3.35 (m, 4H), 1.91−2.03 (m, 2H), 1.62−1.75 (m, 2H)	305.1692 (305.1680)	$C_9H_{20}N_8O_4$·2TFA·H_2O
25 L-Arg^{NO_2}-D-Asn-NH_2	4.77 (t, 1H), 4.06 (t, 1H), 3.29 (t, 2H), 2.87 (dd, 1H), 2.69 (dd, 1H), 1.85−1.96 (m, 2H), 1.56−1.72 (m, 2H)	333.1607 (333.1629)	$C_{10}H_{20}N_8O_5$·TFA·H_2O
26 D-Arg^{NO_2}-L-Ser-NH_2	4.45 (t, 1H), 4.10 (t, 1H), 3.85 (dd, 2H), 3.30 (t, 2H), 1.89−2.00 (m, 2H), 1.63−1.76 (m, 2H)	306.1551 (306.1520)	$C_9H_{19}N_7O_5$·TFA·0.6H_2O
27 L-Arg^{NO_2}-D-Orn-NH_2	4.34 (t, 1H), 4.08 (t, 1H), 3.29 (t, 2H), 3.00 (t, 2H), 1.89−1.98 (m, 2H), 1.62−1.89 (m, 6H)	333.1985 (333.1993)	$C_{11}H_{24}N_8O_4$·2TFA·H_2O
28 L-Lys-D-Arg^{NO_2}-NH_2	4.31 (t, 1H), 4.07 (t, 1H), 3.30 (t, 2H), 2.96 (t, 2H), 1.87−2.01 (m, 2H), 1.58−1.86 (m, 6H), 1.35−1.50 (m, 2H)	347.2186 (347.2150)	$C_{12}H_{26}N_8O_4$·2TFA·H_2O
29 D-Lys-D-Arg^{NO_2}-NH_2	4.31 (t, 1H), 4.07 (t, 1H), 3.30 (t, 2H), 2.96 (t, 2H), 1.87−2.01 (m, 2H), 1.58−1.86 (m, 6H), 1.35−1.50 (m, 2H)	347.2146 (347.2150)	$C_{12}H_{26}N_8O_4$·2TFA·H_2O

Scheme 1[a]



[a] Reagents: (a) $CuCO_3$, $Cu(OH)_2$, Boc_2O, 1 N $NaHCO_3$, dioxane; (b) benzyl chloroformate, 1 N $NaHCO_3$, H_2O; (c) *p*-nitrophenol, DCC, EtOAc; (d) dry NH_3, THF; (e) H_2, 10% Pd/C, EtOH; (f) Boc-L-Arg^{NO_2}, EDC, HOBt, DIEA, DMF; (g) 50% TFA in CH_2Cl_2.

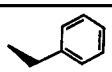
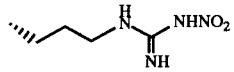
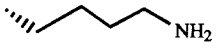
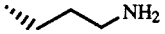
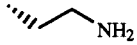
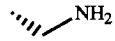
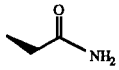
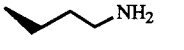
amides did not exhibit time-dependent inhibition, even though nitroarginine is a time-dependent inactivator.[16,29] The standard assay conditions for all three isoforms were used at a fixed L-arginine concentration (10 μM) as described previously.[29] Although the nNOS and eNOS used were derived from bovine tissue and the iNOS from murine tissue, the interspecies similarity for each isoform is quite high (81−93% identical), so the source of the enzyme should not be much of a factor in the evaluation of the inhibitors. The results of the screen for all 152 dipeptides are shown in the Supporting Information. By visual comparison of these results, the 10 most nNOS-selective inhibitors (**20**−**29**) were chosen for purification and more accurate K_i data measurements, which are listed in Table 4.

The initial screen with L-Arg^{NO_2} at the N-terminus revealed that the dipeptide amides containing amino acids with a nitrogen-containing side chain, such as Lys, Arg^{NO_2}, and His, were relatively potent and selective inhibitors of nNOS. In particular, L-Arg^{NO_2}-L-Lys-NH_2 (**21**) showed excellent inhibitory potency of nNOS over eNOS, which prompted us to investigate the optimal spatial preference of the free amino group with lysine analogues. Deletion of one methylene unit from the amino side chain of lysine, giving L-Arg^{NO_2}-L-Orn-NH_2 (**22**), increased the inhibitory potency of **21**, as well as the selectivity. Removal of one more methylene unit to give L-Arg^{NO_2}-L-Dbu-NH_2 (**23**) resulted in approximately 3-fold increases in the potency of both nNOS and iNOS inhibition. As the number of methylenes in the side chain decreased from four (**21**) to two (**23**), the inhibition of nNOS and iNOS increased, but that to eNOS decreased. This shows up in the dramatic increases in the selectivity of nNOS over eNOS (>1500-fold in the case of **23**), but there was little change in the relative selectivity of nNOS over iNOS, which was >190-fold in the case of **23**. However, by shortening the side chain by one more methylene to Dpr (**24**), there was a sharp reduction in inhibition of all isoforms (greater than a factor of 8 for nNOS). L-Arg^{NO_2} has a longer imine side chain than Lys, and L-Arg^{NO_2}-L-Arg^{NO_2} (**20**) produces a decreased nNOS inhibition and increased iNOS and eNOS inhibition, which results in significant reduction in selectivity of nNOS over iNOS and eNOS. The observed correlation between the length of the amine-containing side chain and the inhibitory potency and selectivity of nNOS suggests that both steric and electronic effects play critical roles in the binding of these dipeptide inhibitors to the enzyme. The basic nitrogen of these amine or imine side chains could act as ionic or hydrogen bond donors for potential interaction with the active site of nNOS. There may be an electrostatic interaction between the protonated basic nitrogen and nNOS, but not with eNOS. If the group is too close to (**20**, **21**, **22**) or too far away from (**24**) the hydrogen bond or ionic donor of the enzyme, it could result in a weaker interaction and, consequently, poorer binding.

All of the potent and selective dipeptide inhibitors mentioned above have L-Arg^{NO_2} at the N-terminus, except **26** (vide infra). When L-Arg^{NO_2} is at the N-terminus, the favored stereochemistry of the C-terminal amino acid also is L, except for L-Arg^{NO_2}-D-Asn-NH_2 (**25**; vide infra) and L-Arg^{NO_2}-D-Orn-NH_2 (**27**), a diastereomer of **22**, which shows a 6-fold reduction in both nNOS and eNOS inhibition while having minimal influence on iNOS inhibition.

In contrast to the above results, when Arg^{NO_2} is at the C-terminus, it is more selective as the D-isomer (L-Lys-D-Arg^{NO_2}-NH_2 (**28**) and D-Lys-D-Arg^{NO_2}-NH_2 (**29**)), except for L-Arg^{NO_2}-L-Arg^{NO_2}-NH_2 (**20**), which is mildly

Table 4. Nitric Oxide Synthase Inhibition by the Selected N^{ω}-Nitroarginine-Containing Dipeptide Amides[a]

Compound	R[d]	K_i (μM)[b] nNOS	iNOS	eNOS	Selectivity[c] eNOS/nNOS	iNOS/nNOS
19 D-Phe-D-L-Arg^{NO_2}-OMe[e]		2	3600	5	2.5	1800
20 L-Arg^{NO_2}-L-Arg^{NO_2}-NH_2		0.77	62	33	43	80
21 L-Arg^{NO_2}-L-Lys-NH_2		0.45	104	141	313	231
22 L-Arg^{NO_2}-L-Orn-NH_2		0.33	97	245	742	294
23 L-Arg^{NO_2}-L-Dbu-NH_2		0.13	25	200	1538	192
24 L-Arg^{NO_2}-L-Dpr-NH_2		1.1	61	261	237	55
25 L-Arg^{NO_2}-D-Asn-NH_2		0.32	8.9	412	1287	28
26 D-Arg^{NO_2}-L-Ser-NH_2		1.25	1180	218	174	944
27 L-Arg^{NO_2}-D-Orn-NH_2		2.0	103	1290	638	51
28 L-Lys-D-Arg^{NO_2}-NH_2		1.7	4700	229	135	2765
29 D-Lys-D-Arg^{NO_2}-NH_2		0.89	910	30	34	1022

[a] The enzymes used for the K_i determinations are bovine brain nNOS, recombinant murine iNOS, and recombinant bovine eNOS. See Experimental Section. [b] In most cases, the K_i values represent single measurements, but with 6 data points and correlation coefficients 0.980−0.996. When duplicate runs were made, 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] Side chains of the amino acids other than Arg^{NO_2}. [e] Data taken from ref 29.

selective. This may be the result of the binding of the nitroarginine residue to the same binding site, regardless of its position in the dipeptide. For example, when D-nitroarginine is at the C-terminus, it may flip over 180° to assume an L-nitroarginine-like configuration at the N-terminus for binding (Figure 1).[30] If that is the case, then D-Arg^{NO_2} should be favored at the C-terminus. Both L-Lys-D-Arg^{NO_2}-NH_2 (**28**) and D-Lys-D-Arg^{NO_2}-NH_2 (**29**) have excellent nNOS/iNOS selectivity because of their dramatic decreases in iNOS inhibition. Note that D-Phe-D-Arg^{NO_2}-OMe (**19**), which is an extremely poor inhibitor of iNOS but a much better inhibitor of nNOS, also has D-Arg^{NO_2} at the C-terminus. Since both L-Lys-D-Arg^{NO_2}-NH_2 (**28**) and D-Lys-D-Arg^{NO_2}-NH_2 (**29**) have similar selectivities, the stereochemistry of the amine-containing residue at the N-terminus does not appear to be highly critical. These observations indicate that the geometry of these dipeptide inhibitors at the active site have profound effects on their binding to the different isoforms of NOS; in particular, the position of Arg^{NO_2} in the sequence has a larger effect on the binding to iNOS than to nNOS or eNOS.

Only two nonamine side chain containing dipeptide amides were found to be highly selective, namely, L-Arg^{NO_2}-D-Asn-NH_2 (**25**) and D-Arg^{NO_2}-L-Ser-NH_2 (**26**). Asparagine is similar in structure to 2,4-diaminobutyric acid (Dbu), except for a carbonyl instead of a methylene adjacent to the terminal amino group. If the similarity between asparagine and Dbu is relevant, then the L−L isomer should be the most selective, since L-Arg^{NO_2}-L-Dbu-NH_2 (**23**) is the most selective of that series of compounds. However, this is not the case; L-Arg^{NO_2}-D-Asn-NH_2 (**25**) is the diastereomer that is the most potent inhibitor of nNOS with high selectivity over eNOS (>1200-fold). Since the amide amino group of the asparagine side chain is not basic and, therefore, not protonated, the prevalent interaction of the side chain with the enzyme is a hydrogen bond interaction, not an electrostatic one. However, it is unlikely that this isomer binds at the same site as **23** since the stereochemistry of the C-terminal amino acid is epimeric with **23**.

D-Arg^{NO_2}-L-Ser-NH_2 (**26**) also is a selective nNOS inhibitor, but, as in the case of **25**, the stereochemistry is epimeric compared with that for **23**. The side chain of serine is the same as that of Dpr, except for a hydroxyl group instead of the amino group of Dpr. The hydroxyl group of serine is a good hydrogen bond donor, but is not charged like the amino group of Dpr would be. Nonetheless, **24** and **26** have similar K_i values for both nNOS and eNOS and, therefore, similar nNOS/eNOS selectivities. However, binding of **26** to iNOS decreases significantly compared with **24**, which results in the sharp increase in selectivity of nNOS over iNOS for **26** compared with **24**. Possibly, the different stereochemistry at the N-terminus prevents the appropriate interaction with iNOS.

Some of the dipeptide amides with an alkyl side chain show eNOS inhibition preference. In these cases, D−D isomers with D-Arg^{NO_2} at the C-terminus, such as Gly-D-Arg^{NO_2}-NH_2, D-Val-D-Arg^{NO_2}-NH_2, and D-Leu-D-Arg^{NO_2}-NH_2, are favored. The acidic amino acid (Asp or Glu) containing dipeptide amides exhibited little or no inhi-

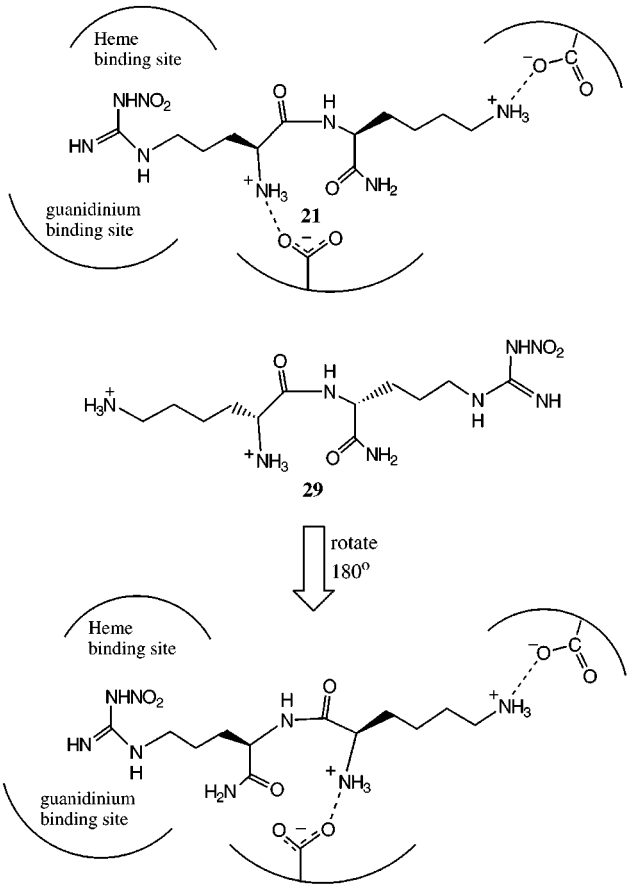


Figure 1. Proposed model to rationalize the binding of epimers to the active site of nNOS.

bition of any of the NOS isoforms, which supports the involvement of an anionic group at the active site with which **20**−**24** can interact. The histidine-containing dipeptide amides are not as potent as the other amine-containing dipeptide amides, but a trend similar to that observed with the amine-containing dipeptide amides is seen, namely, that L-Arg^{NO_2}-L-His-NH_2 and L-His-D-Arg^{NO_2}-NH_2 are the most potent and selective of the eight isomers. Similar to D-Phe-D-Arg^{NO_2} methyl ester (**19**), D-Tyr-D-Arg^{NO_2}-NH_2 and D-Trp-D-Arg^{NO_2}-NH_2 showed modest inhibitory potency toward both nNOS and eNOS.

Conclusion

A series of dipeptide amides containing Arg^{NO_2} were synthesized and screened for biological activity in vitro against the three isoforms of NOS. Several conclusions can be drawn from these studies: (1) the active sites of NOS are large enough to accommodate inhibitors as large as dipeptides; (2) there are very subtle differences in the active sites of the three isoforms of NOS, as evidenced by the observation that relatively minor structural modifications can produce highly divergent inhibition results; (3) the dipeptides containing an amino group containing side chain show high inhibitory potency with nNOS and excellent selectivity over both eNOS and iNOS, which supports a possible electrostatic interaction or hydrogen bonding interaction between the dipeptide side chain and nNOS, but not eNOS and iNOS; (4) there may be multiple hydrogen bond donors at the active site of nNOS; and (5) both steric and electronic factors greatly influence inhibitory potency and isoform selectivity of these dipeptide inhibitors. Among the compounds tested, maximal inhibition potency (130 nM) and selectivity (1538-fold) for nNOS over eNOS, with high selectivity (192-fold) for nNOS over iNOS, was achieved with L-Arg^{NO_2}-L-Dbu-NH_2 (**23**); maximal selectivity (2765-fold) for nNOS over iNOS with high selectivity (135-fold) for nNOS over eNOS was realized with L-Lys-D-Arg^{NO_2}-NH_2. This investigation opens the door to the design of potent and highly selective inhibitors of nNOS. Further peptidomimetic modifications are currently under investigation for potential therapeutically useful compounds to treat neuronal disorders caused by excess NO production.

Experimental Section

Chemistry. All Fmoc protected amino acids and coupling reagents were purchased from Advanced ChemTech, Inc. The dipeptide amides were synthesized on an automated peptide synthesizer (ACT model 357, Advanced ChemTech, Inc.) using established protocols[31] and purified on a Whatman Partisil C18 semi-prep HPLC column (9.4 × 125 mm). The dipeptides were eluted from the column using a gradient of 100% solvent A (0.1% TFA in H_2O) to 100% of solvent B (0.08% TFA in 60% CH_3CN/H_2O) over 30 min at a flow rate of 2 mL/min. ^{1}H NMR spectra were recorded on a Varian Gemini-300 spectrometer in the solvent indicated. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane (TMS, in $CDCl_3$) or sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS, in D_2O). Electrospray mass spectra were performed 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} (0.25 mm thickness) plates. Amino acids were visualized with a ninhydrin spray reagent or a UV/vis lamp. ICN silica gel 60 (230−400 mesh) was used for flash chromatography.

4-[*N*-(*tert*-Butoxycarbonyl)amino]-L-2,4-diaminobutyric Acid (L-Dbu(Boc)-OH). This compound was prepared in a 40% yield as a white solid from L-2,4-diaminobutyric acid (2.5 g, 21.2 mmol) according to the procedure of Scott et al.:[32] ^{1}H NMR (D_2O) δ 3.69 (dd, 1H), 3.20 (m, 2H), 2.01 (m, 2H), 1.43 (s, 9H).

2-[*N*-(Benzoxycarbonyl)amino]-4-[*N*-(*tert*-butoxycarbonyl)amino]-L-2,4-diaminobutyric Acid (Cbz-L-Dbu(Boc)-OH). L-Dbu(BOC)-OH (0.5 g, 2.3 mmol) was dissolved in 10 mL of 1 M Na_2CO_3 at 0 °C. To the stirred solution was added dropwise 0.55 mL of benzyl chloroformate (4.6 mmol). The solution was stirred at 0 °C for 30 min and at room temperature for 1 h. TLC (*n*-butanol:acetic acid:water, 5:1:1) showed that the reaction was complete. Citric acid (20%) was used to acidify the mixture, and then the white precipitate was extracted twice with EtOAc. The organic layer was washed with 10% Na_2CO_3 solution, which was acidified with 20% citric acid again, and extracted with EtOAc twice. The EtOAc layer was washed with water and saturated NaCl and dried over $MgSO_4$. After evaporation of the solvent, the product was obtained in a 90% yield as a white solid: ^{1}H NMR ($CDCl_3$) δ 7.35 (m, 5H), 5.58 (d, 1H), 5.12 (s, 2H), 4.43 (m, 1H), 3.20 (m, 2H), 2.05 (m, 1H), 1.83 (m, 1H), 1.45 (s, 9H).

2-[*N*-(Benzoxycarbonyl)amino]-4-[*N*-(*tert*-butoxycarbonyl)amino]-L-2,4-diaminobutyric *p*-Nitrophenyl Ester (Cbz-L-Dbu(Boc)-ONp). This compound was prepared from Cbz-L-Dbu (Boc)-OH (1.6 g, 4.5 mmol) according to the procedure of Tesser et al.[33] and was used in the next step without purification.

2-[*N*-(Benzoxycarbonyl)amino]-4-[*N*-(*tert*-butoxycarbonyl)amino]-L-2,4-diaminobutyric Amide (Cbz-L-Dbu(Boc)-NH_2). Cbz-L-Dbu (Boc)-ONp was dissolved in THF (20 mL, freshly distilled), and dry NH_3 was passed near the surface of the stirred solution in an ice bath. The precipitate was collected after 1 h and was recrystallized from ethanol−

ether, giving the product as a white solid in a 40% yield:[34] $[\alpha]_D = -13.8$ ($c = 1.45$, methanol, 24 °C); ^{1}H NMR (DMSO-d_6) δ 7.35 (m, 7H), 7.02 (s, 1H), 6.77 (brs, 1H), 5.02 (s, 2H), 3.92 (m, 1H), 2.95 (m, 2H), 1.52−1.83 (m, 2H), 1.36 (s, 9H).

L-N^{ω}-Nitroarginine-2,4-L-diaminobutyric Amide (L-Arg^{NO_2}-L-Dbu-NH$_2$·2TFA, 23). The above amide (400 mg) was dissolved in methanol and hydrogenated in the usual manner. The catalyst was filtered through a Celite pellet, and the methanol was evaporated in vacuo. The resulting oil, L-Dbu (Boc)-NH_2 (244 mg, 1.12 mmol) was treated with EDC (236 mg, 1.23 mmol), HOBt (166 mg, 1.23 mmol), Boc-L-Arg^{NO_2}-OH (394 mg, 1.23 mmol), and DIEA (215 μL, 1.23 mmol) in 20 mL of cold DMF. After 1 h of stirring in an ice bath, the reaction mixture was warmed to room temperature. DMF was evaporated under vacuum for another hour, and the residue was treated with 20 mL of EtOAc. The precipitate was dissolved in water, and the aqueous solution was extracted three times with EtOAc. The combined extracts were washed with 5% $NaHCO_3$, H_2O, and brine and dried over $MgSO_4$. The product was purified by silica gel chromatography (12:1 $CHCl_3$:CH_3-OH, R_f 0.25). The dipeptide was treated with 5 mL of TFA/CH_2Cl_2 (1:1 v/v) to remove the Boc group. About 100 mg of pure dipeptide TFA salt was obtained: ^{1}H NMR (D_2O) δ 4.47 (dd, 1H), 4.09 (t, 1H), 3.30 (t, 2H), 3.08 (m, 2H), 2.01−2.25 (m, 2H), 1.89−2.01 (m, 2H), 1.60−1.75 (m, 2H). HRMS (ES) Calcd: 319.1837 (MH^+). Found: 319.1819. Anal. ($C_{10}H_{22}N_8O_4$·2TFA·1.2H_2O) Calcd: C, 29.58; H, 4.64; N, 19.71. Found: C, 29.58; H, 4.39; N, 19.61.

Biology. All of the NOS isoforms used in the initial screens are recombinant enzymes overexpressed in *Escherichia coli* from different sources. The murine macrophage iNOS was expressed and isolated according to the procedures of Hevel et al.[35] The rat neuronal nNOS was expressed[36] and purified[37] as described. The bovine endothelial eNOS was isolated and purified as reported.[38] However, the K_i values of the most selective inhibitors were determined with bovine brain nNOS[39] instead of the recombinant nNOS. Nitric oxide formation was monitored by the hemoglobin capture assay as described previously.[40] The K_i values were measured from Dixon plots[41] with various L-arginine and inhibitor concentrations.

Supporting Information Available: Bar graphs of the effect of each of the 152 dipeptide amides on nNOS, eNOS, and iNOS activity, presented as the percent inhibition compared to control (0% inhibition). This information is available free of charge via the Internet at http://pubs.acs.org.

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