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Conformationally-restricted Arginine Analogues as Alternative Substrates and Inhibitors of Nitric Oxide Synthases

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Abstract—Conformationally restricted arginine analogues (**1–5**) were synthesized and found to be alternative substrates or inhibitors of the three isozymes of nitric oxide synthase (NOS). A comparison of k_{cat}/K_m values shows that (*E*)-3,4-didehydro-D,L-arginine (**1**) is a much better substrate than the corresponding (*Z*)-isomer (**2**) and 3-guanidino-D,L-phenylglycine (**3**), although none is as good a substrate as is arginine; 5-keto-D,L-arginine (**4**) is not a substrate, but is an inhibitor of the three isozymes. Therefore, it appears that arginine binds to all of the NOS isozymes in an extended (*E*-like) conformation. None of the compounds exhibits time-dependent inhibition of NOS, but they are competitive reversible inhibitors. Based on the earlier report that N^{ω}-propyl-L-arginine is a highly selective nNOS inhibitor (Zhang, H. Q.; Fast, W.; Marletta, M.; Martasek, P.; Silverman, R. B. *J. Med. Chem.* **1997**, *40*, 3869), (*E*)-N^{ω}-propyl-3,4-didehydro-D,L-arginine (**5**) was synthesized, but it was shown to be weakly potent and only a mildly selective inhibitor of NOS. Imposing conformational rigidity on an arginine backbone does not appear to be a favorable approach for selective NOS inhibition.

Introduction

Nitric oxide synthase (NOS, EC 1.14.13.39) is a family of three heme-containing enzymes that catalyzes the conversion of L-arginine to L-citrulline and nitric oxide. The three isozymes are expressed differently and play different physiological roles; the endothelial enzyme (eNOS) is involved in the regulation of smooth muscle relaxation and blood pressure, in neuronal tissue (nNOS) NO serves as an important intracellular second messenger involved in neurotransmission, and in macrophage cells an inducible form of the enzyme (iNOS) is produced by inflammatory stimuli.[1] The uncontrolled production of NO has been implicated in numerous disease states, therefore, the design of isoform-specific NOS inhibitors to regulate NO synthesis in specific cells has potential therapeutic benefits.[1]

Many different inhibitors of NOS are known.[2] Substrate modification is a well-tested strategy for the discovery of inhibitors of an enzyme, and this approach has been successful in the discovery of early inhibitors of NOS. These inhibitors include a variety of L-arginine derivatives in which the terminal guanidine group of the arginine is modified to give methylarginine[3] and nitroarginine.[4] Non-amino acid NOS inhibitors also have been reported, and these include isothioureas,[5,6] amidines,[7,8] guanidines,[9] imidazole,[10] indazoles,[11] and 2-iminohomopiperidinium salts.[12] Although some selective inhibitors of the enzyme have been described, the selectivity is modest in most cases. However, a recent report by the GlaxoWellcome group showed that *N*-[3-(aminomethyl)benzyl]acetamidine is a potent inhibitor of NOS with a selectivity of 5000 for iNOS versus eNOS.[13] We have found that N^{ω}-propyl-L-arginine is about 3000-fold selective for nNOS over iNOS and about 150-fold selective for nNOS versus eNOS.[14] These findings constitute the largest selectivities reported for the inhibition of NOS and suggest that the design of selective inhibitors of the enzyme is achievable.

Until the three-dimensional structure of the oxygenase domain of iNOS was determined by X-ray crystallography,

Key words: Arginine analogues; nitric oxide synthase; conformationally-restricted; enzyme inhibition.

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† Designed and carried out all of the experiments in this study.

‡ Provided the recombinant *Escherichia coli* cells expressed with murine macrophage iNOS used in this study.

§ Provided the recombinant eNOS and the *Escherichia coli* cells expressed with bovine neuronal nNOS used in this study.

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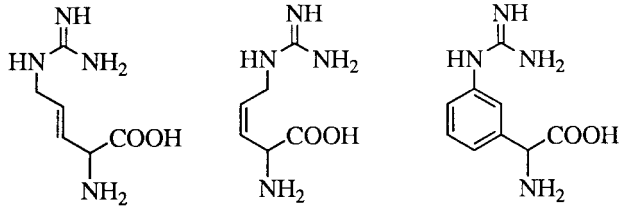
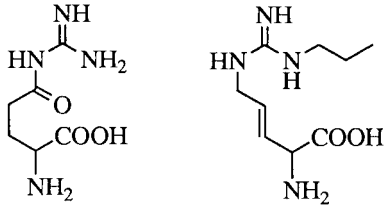
little information of the active site of any of the isozymes of the enzyme was available.[15] A novel approach to probe the active site topologies of the three NOS isozymes using various aryldiazenes suggested that the sizes of the active sites of these enzymes decrease in the order of nNOS > iNOS > eNOS, although they are very similar in their global topology.[16] The fact that most of the L-arginine-derived inhibitors have modest isoform selectivity supports this result.

We envisioned that some conformationally-restricted arginine analogues, however, may be alternative substrates and/or inhibitors of the isozymes of NOS, thereby allowing us to determine the preferred orientation of the natural substrate in each of the isozymes. If a difference in the preferred geometry exists, this would be a key component in the design of selective inhibitors of the NOS isozymes. On that basis we designed five conformationally-restricted arginine analogues, (*E*)-(**1**)- and (*Z*)-3,4-didehydro-D,L-arginine (**2**), *m*-guanidino-D,L-phenylglycine (**3**), and 5-keto-D,L-arginine (**4**), as potential alternative substrates or inhibitors. Compounds **1–3** were both substrates and inhibitors of all three isozymes of NOS, and **4** and **5** were only inhibitors; little selectivity ($\leqslant$25-fold) was displayed by any of these compounds. While this work was in progress, a different set of conformationally-restricted arginine analogues was reported that had no better than a 12-fold selectivity of eNOS and nNOS over iNOS.[17]



1 **2** **3**



4 **5**

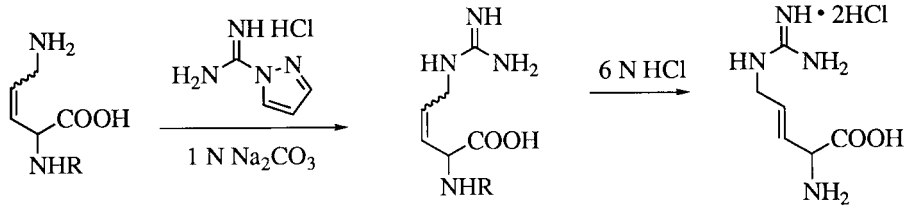
Results and Discussion

Syntheses of conformationally-restricted analogues

(*E*)-3,4-Didehydro-D,L-arginine (**1**) was synthesized by the treatment of (*E*)-2-acetamido-5-amino-3-pentenoic acid (**6a**)[18] with 1*H*-pyrazole-1-carboxamidine[19] in aqueous Na_2CO_3 followed by deprotection of the *N*-acetyl group in HCl (Scheme 1). (*Z*)-3,4-Didehydro-D,L-arginine (**2**) was prepared from (*Z*)-3,4-didehydroornithine (**6b**)[18] employing the same guanylation reaction as described for the synthesis of **1** (Scheme 1). *m*-Guanidino-D,L-phenylglycine (**3**) was prepared by the route shown in Scheme 2. *N*-Boc protection of 3-nitrophenylglycine (**8**)[20] followed by catalytic hydrogenation of the nitro group provided *N*-Boc protected 3-aminophenylglycine (**10**). Guanylation of **10** with 1*H*-pyrazole-1-carboxamidine in the presence of diisopropylethylamine at elevated temperature, and subsequent *N*-Boc deprotection, afforded **3**. 5-Keto-L-arginine (**4**) was prepared by the reaction of L-glutamic acid 5-methyl ester with guanidine. (*E*)-*N*-Propyl-3,4-didehydro-D,L-arginine (**5**) was prepared by guanylation of **6a** with 1*H*-pyrazole-*N*-propyl-1-carboxamidine in aqueous Na_2CO_3. 3-Ureido-D,L-phenylglycine (**13**), the potential oxidation product of **3**, was synthesized by conversion of *N*-Boc-(*m*-aminophenyl)glycine (**10**) to the corresponding cyanamide derivative (**12**), followed by simultaneous hydrolysis and deprotection (Scheme 3).

Inhibition of NOS isoforms by the conformationally-restricted analogues

Table 1 shows the substrate and inhibitory activities of compounds **1–5**. Compounds **1–3** are both substrates and inhibitors of all three NOS isozymes. Under initial velocity conditions with the (*E*)-didehydro arginine analogue **1**, the maximal rate of NO formation by nNOS and eNOS is observed at 5 and 10 μM, respectively, and the rate of NO formation is approximately 30% that of when 10 μM L-arginine was used. The K_m values with nNOS (1.5 μM) and eNOS (1 μM) are lower than the respective K_m values for L-arginine (2.7 and 1.1 μM, respectively) with those isozymes; the k_{cat}/K_m values for **1** are only slightly lower than those with L-arginine. In the case of iNOS, the maximal rate of NO formation, which was observed at a 300 μM concentration, corresponds to only 4% of that when L-arginine was used. The K_m value for **1** with iNOS was much higher (35 μM) than those for the other two isoforms and for L-arginine, and the k_{cat}/K_m value is an

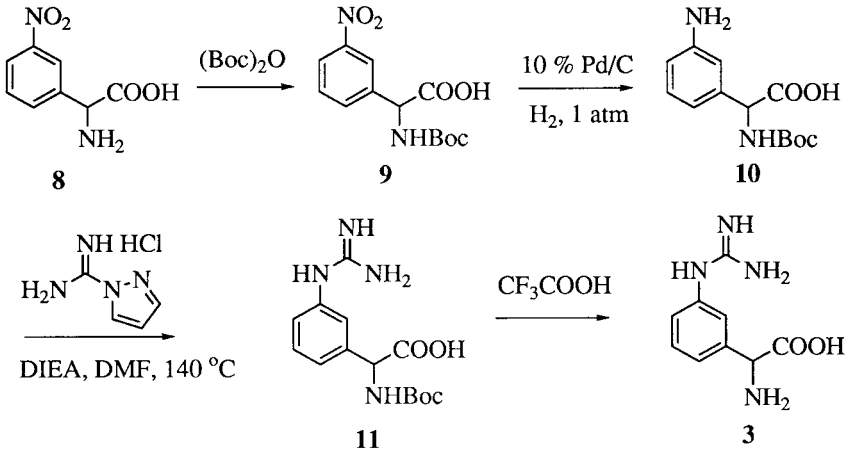


6a : R = acetyl, *trans*
6b : R = H, *cis*

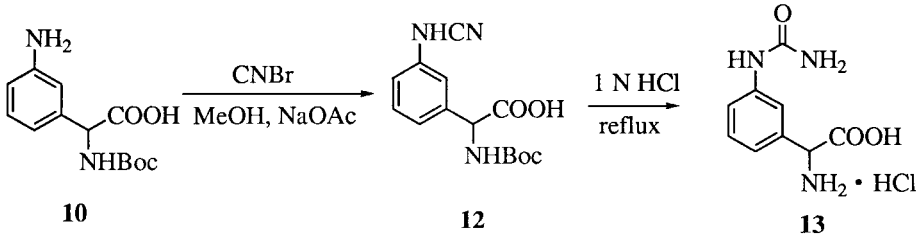
7a : R = acetyl, *trans*
2 : R = H, *cis*

1

Scheme 1.



Scheme 2.



Scheme 3.

Table 1. Kinetic constants for substrate and inhibitor activity of L-arginine and compounds **1–5**

Compound	K_m	K_i	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m	K_i	k_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m	K_i	k_{cat}/K_m ($s^{-1}mM^{-1}$)
L-Arginine	2.7 μM		180	9.5 μM		110	1.1 μM		73
1	1.5 μM	25 μM	110	35 μM	45 μM	13	1.0 μM	50 μM	26
2	5 mM	6.5 mM	1.8×10^{-3}	20 mM	13 mM	6.9×10^{-3}	15 mM	10 mM	8.0×10^{-4}
3	40 mM	2.0 μM	1.8×10^{-2}	20 mM	8 μM	4.4×10^{-3}	25 mM	50 μM	7.9×10^{-2}
4		770 μM			900 μM			3 mM	
5		640 μM			9 mM			2.5 mM	

order of magnitude lower than that for L-arginine. On the basis of K_m alone, the selectivities of **1** for nNOS and eNOS over iNOS are 23- and 35-fold, respectively (Table 1); however, on the basis of k_{cat}/K_m values the selectivities are a much more modest 8- and 4-fold, respectively. As a competitive inhibitor, **1** shows little or no selectivity.

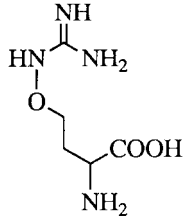
The *Z*-isomer (**2**) was found to be a much poorer substrate for all three isoforms of NOS than was **1**, having K_m values of 3–4 orders of magnitude greater than **1** and k_{cat}/K_m values 3–5 orders of magnitude lower than those for **1** (Table 1)! Although there are many conformers that **1** and **2** can assume, these results suggest that the binding orientation of the 2–5 carbon atoms of arginine in the active site is better mimicked by an extended geometry rather than a folded one. The maximal velocities with **2** were 12–17% that for L-arginine with the respective isoforms, and substrate selectivities for nNOS and eNOS over iNOS were 4- and 1.3-fold, respectively, if only K_m is considered, but the selectivities reverse in favor of iNOS (4- and 9-fold, respectively), when k_{cat}/K_m values are compared.

To further rigidify **1** in an attempt to increase the binding effectiveness, analogue **3** was synthesized, which can be drawn in a conformer with the same geometry as **1**, but has additional atoms from the aromatic ring. However, this compound is a much poorer substrate than **1**; its K_m and k_{cat}/K_m values are 3–4 orders of magnitude greater than those for **1** (Table 1), suggesting a possible steric hindrance to its binding at the arginine binding site by the aromatic ring or that **1** and **3** bind in different geometries. Interestingly, the K_i values for **3** with nNOS, iNOS, and eNOS are much lower (by 3–4 orders of magnitude) than the corresponding K_m values. This suggests that nonproductive binding is important and that this alternative substrate can bind to the active site, but poorly elicit oxidation.

While monitoring turnover with **3**, the NO formation was found to diminish with time. Compound **3** was

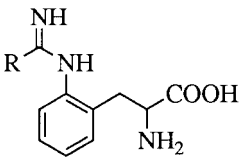
shown not to be a time-dependent inhibitor, so product inhibition was suspected. As monitored by HPLC, the major product (other than NO) formed was shown to be 3-ureido-D,L-phenylglycine (**13**), the expected oxidation product. However, **13** was found to be a poor inhibitor of nNOS; at a concentration of 5 mM (the highest concentration allowable because of its low solubility), less than 10% of enzyme activity was inhibited. Compound **13** also was not a time-dependent inhibitor of nNOS at 1 mM concentration for 1 h. Possibly, other products, formed in much smaller concentrations, are responsible for the inhibitory activity of **3** or the release of **13** from the enzyme is slow.

5-Ketoarginine (**4**) also was investigated as a potential conformationally-restricted analogue of arginine because of the potential intramolecular hydrogen bonding properties of the ketone carbonyl with the terminal NH_2 group. This compound was not a substrate at all for any of the isozymes of NOS, and showed only weak inhibitory properties (Table 1). In addition to its possible cyclic structure, as a result of hydrogen bonding, the carbonyl group will strongly decrease the pK_a of the guanidino group. In fact, L-canavadine (**14**), with an oxygen in place of the carbonyl, has a guanidino pK_a of **7**, and it is not a substrate for NOS either.[21]

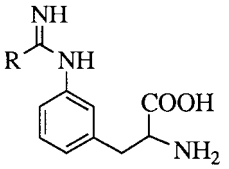


14

Two conformationally-restricted series of arginine analogues (**15** and **16**, R = NH_2, $NHCH_3$, and CH_3) were recently reported to be NOS inhibitors by the Glaxo-Wellcome group.[17]

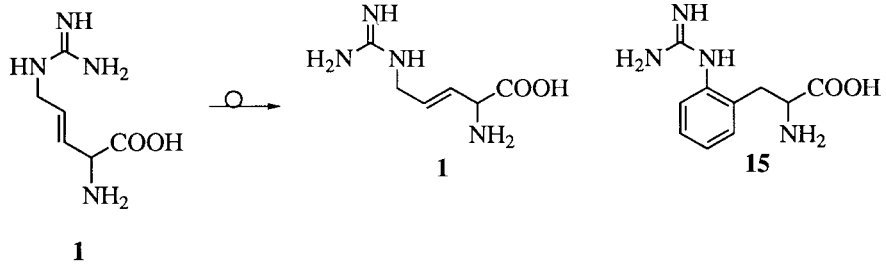


15



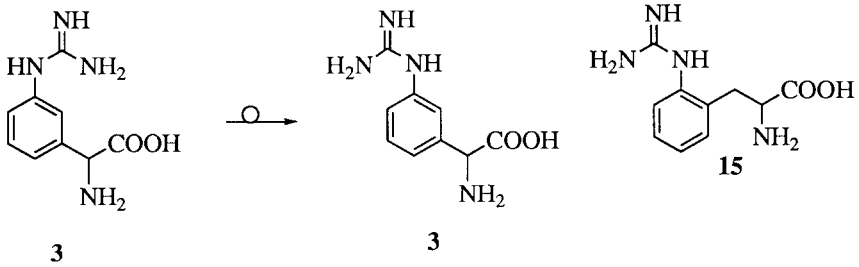
16

The arginine analogue **15** (R = NH_2), in which the amino acid group is *ortho* to that of the guanidine group, inhibits all three human NOS isoforms with K_i values for iNOS, eNOS, and nNOS of 2.60, 0.25, and 0.37 μM, respectively. Arginine analogue **16** (R = NH_2) also inhibits all isoforms of NOS, but the inhibitory potencies are lower (100, 46, and 44 μM, respectively) than those of **15**. On the basis of these results, it was proposed that arginine based inhibitors preferentially bind to the active sites of all of the NOS isoforms in an orientation where the arginine alkyl chain is in a 'folded conformation.' This hypothesis appears to be different from what we proposed above using **1–3**, in which the 'extended' *E*-arginine analogue is a better substrate than the 'folded' *Z*-isomer. However, this is really just semantics, because **15** can be drawn in a conformer that is identical to that of **1** (Fig. 1). On the other hand, **3** cannot be drawn in a conformer that is identical to that of **15**, but it can be drawn in a conformer that is similar to it (Fig. 2). This may account for our observation that **3** is a poor substrate but a comparable inhibitor to **1**; it may be bound appropriately for inhibition, but not for oxygenation. However, with regard to selectivities for the NOS isozymes (Table 2), **1** is more similar to **15** than **3** is to **15**. Structure **16**, however, is not really an analogue of arginine, but rather, is an analogue of homoarginine



1 **1** **15**

Figure 1. Comparison of specific conformations of **1** and **15**.



3 **3** **15**

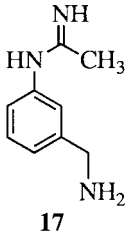
Figure 2. Comparison of specific conformations of **3** and **15**.

Table 2. Comparison of the selectivities of NOS isozyme inhibition by conformationally-rigid analogues

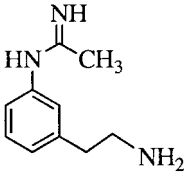
Compound	Selectivity nNOS/eNOS[a]	Selectivity nNOS/iNOS[a]
1	2	1.8
3	25	4
15[b]	0.68	7
16[b]	1	22
17[c]	155	50
18[c]	33	10

[a]Ratio of the inverse of the K_i values (i.e. a ratio > 1 means that nNOS is a more potent inhibitor).
[b]Data taken from ref 17.
[c]Data taken from ref 22.

(there is an extra carbon in **16** relative to arginine), whereas **1–3** are all analogues of arginine. Therefore, **1** and **3** are not similar in structure to **16**, although the low selectivities of **1** for the NOS isozymes are similar to those of **16**; **3** is much more selective than either **1** or **16**. Better structural comparisons are made between **3** and *N*-[3-(aminomethyl)benzyl]acetamidine[13] (**17**) and between **16** and *N*-[3-(aminoethyl)benzyl]acetamidine[22] (**18**). On the basis of selectivities (Table 2), **3** appears to be closer to **18** than to **17**. However, **3** is a guanidine and **17** and **18** are amidines, and that could be very important to the binding interactions. The fact that the selectivities of **16** are not similar to either **17** or **18** may indicate that they bind in different conformations than those depicted in the structures drawn. Relatively minor structural changes can have major consequences with regard to isozyme selectivities. For example, the deletion of one methylene group from *N*-[3-(aminomethyl) benzyl]acetamidine[13] transforms the compound from an iNOS-selective inhibitor into an nNOS-selective inhibitor.[22]



17



18

Since **1** is a very good substrate, we attempted to convert it into a potent and selective inactivator of the isoforms of NOS by N^{ω}-substitution. Earlier, we found that N^{ω}-propyl-L-arginine is a potent and selective inhibitor of nNOS.[14] However, (*E*)-*N*-propyl-3,4-didehydro-D,L-arginine (**5**) is a poor, low selective inhibitor of the nNOS isoform (Table 1). There, obviously, are subtle binding interactions that are disturbed when substitution of **1** by a propyl group is made.

Conclusion

On the basis of the results reported here and those by Shearer et al.[17] conformational restriction appears to prevent the molecules from assuming the appropriate discriminatory binding orientations needed for high selectivity of the isozymes of NOS.

Experimental

Materials

NADPH, HEPES, DTT, calmodulin, and human ferrous hemoglobin were purchased from Sigma Chemical Co. Tetrahydrobiopterin (H_4B) was obtained from B. Schircks Laboratories (Jona, Switzerland) or from Alexis Biochemicals (San Diego, CA). Acids, bases, and conventional organic solvents were purchased from Fisher. All chemicals were purchased from Aldrich unless otherwise stated. TLC plates (silica gel 60-F254, 250 μM), and silica gel 60 (230–400 mesh) were purchased from VWR Scientific.

Analytical methods

Optical spectra and enzyme assays were recorded on either a Perkin–Elmer Lambda 10 UV–vis spectrophotometer. NMR spectra were recorded on a Varian Gemini-300 300-MHz spectrometer (75 MHz for ^{13}C spectra). Chemical shifts are reported as δ values in parts per million downfield from Me_4Si as the internal standard in $CDCl_3$, unless stated otherwise. HPLC was performed on a Beckman System Gold instrument (Model 125P solvent module and Model 166 detector). Samples were analyzed by elution from an Alltech Econosil C_{18} 10 μ (4.6×250 mm) column. Mass spectra were recorded on a VG Instruments VG70-250SE high-resolution spectrometer. Combustion analyses were performed by the Department of Geological Sciences at Northwestern University. Melting points were obtained with a Fisher–Johns melting point apparatus and are uncorrected.

(*E*)-3,4-Didehydro-D,L-arginine (1). A mixture of (*E*)-2-acetoamido-5-amino-3-pentenoic acid[18] (344 mg, 2.5 mmol), 1*H*-pyrazole-1-carboxamidine hydrochloride[19] (366 mg, 2.5 mmol), and 1 M Na_2CO_3 (4 mL) was stirred for 2 days at room temperature. Concentrated NH_4Cl (1 mL) was added, and the mixture was concentrated. The residue was loaded onto Dowex 50W-X8 (1×2 cm), washed with H_2O, then eluted with a gradient of 0.5 to 1 N HCl. The combined fractions of HCl elution were concentrated, the residue was loaded onto Dowex 1X2-100, washed with H_2O, then eluted with 3% NH_4OH. After evaporation of the combined fractions containing product, the colorless foam (265 mg, 62%) of N^{α}-acetyl (*E*)-3,4-didehydro-D,L-arginine (**7a**) was used in the next reaction without further purification; ^1H NMR (D_2O) δ 1.88 (s, 3*H*), 3.68 (d, *J* = 4.61 Hz, 1*H*), 4.57 (m, 1*H*), 5.57 (dt, *J* = 16.00, 4.61 Hz, 1*H*), 5.65 (dd, *J* = 16.00, 5.62 Hz, 1*H*).

A solution of **7a** (420 mg, 2.0 mmol; two preps were combined) in 6 N HCl (7 mL) was refluxed for 2 h. After concentration of the reaction mixture, the residue was loaded onto Dowex 50W-X8 and eluted with a gradient of 1–2.5 N HCl. The fractions containing product were combined, concentrated, then lyophilized to a foamy solid (132 mg, 27%) of **1**; ^1H NMR (D_2O) δ 3.89 (d, *J* = 3.30 Hz, 2*H*), 4.64 (d, *J* = 8.01 Hz, 1*H*), 5.77 (app dd, *J* = 15.72, 8.01 Hz, 1*H*), 6.05 (dt, *J* = 15.72, 4.54 Hz, 1*H*); ^{13}C NMR (75 MHz, D_2O) 41.7, 54.1, 121.4, 134.2,

156.9, 170.4. Anal. calcd for $C_6H_{12}N_4O_2 \cdot 2HCl$: C, 29.40; H, 5.76; N, 22.86. Found: C, 29.22; H, 6.08; N, 22.69.

(*Z*)-3,4-Didehydro-D,L-arginine (2). A mixture of (*Z*)-3,4-didehydro-D,L-ornithine dihydrochloride[18] (120 mg, 0.59 mmol), 1*H*-pyrazole-1-carboxamidine hydrochloride[19] (87 mg, 0.59 mmol), and 0.9 mL of 1.0 M Na_2CO_3 was stirred for 3 days at room temperature. Concentrated NH_4Cl (1 mL) was added to the mixture, and the solution was concentrated to dryness. The residue was loaded onto a column of Dowex 50 (1×2 cm), and eluted with a gradient of 0.1–2 M HCl. The fractions containing product were combined and concentrated to give a sticky solid (66 mg, 46%), which was recrystallized from a mixture of H_2O:EtOH (5:95); mp 170–172 °C; ^{1}H NMR (D_2O) δ 4.04 (dt, J = 6.59, 2.05 Hz, 2*H*), 4.86 (dd, J = 9.84, 0.88 Hz, 1*H*), 5.66 (ddt, J = 10.86, 9.84, 1.78 Hz, 1*H*), 5.96 (dtd, J = 10.86, 6.59, 0.88 Hz, 1*H*); ^{13}C NMR (D_2O) 38.9, 50.5, 122.5, 133.6, 156.7, 170.0; Anal. calcd for $C_6H_{12}N_4O_2$(2HCl): C, 29.40; H, 5.76; N, 22.86. Found: C, 29.67; H, 5.98; N, 22.70.

N^{α}-Boc-*m*-nitro-D,L-phenylglycine (9). To a stirred solution of di-*tert*-butyl dicarbonate (32 g, 0.146 mol) and *m*-nitro-D,L-phenylglycine[20] (8.26 g, 0.133 mol) in dichloromethane (400 mL) was added triethylamine (37 mL, 0.266 mol). After being stirred overnight, the reaction mixture was concentrated under reduced pressure, applied to a silica gel column (6×25 cm), and eluted with hexane:ethyl acetate (3:1). Fractions containing the desired compound were combined and concentrated to give **9** as a pale yellow foamy solid (28.3 g, 72%); ^{1}H NMR ($CDCl_3$) δ 1.19 (s, 9*H*), 5.27 (d, J = 4.35 Hz, 1*H*), 7.60 (app t, J = 7.97 Hz, 1*H*), 7.80 (d, J = 7.41 Hz, 1*H*), 8.18 (d, J = 8.25 Hz, 1*H*), 8.32 (bs 2*H*), 12.23 (bs, 1*H*); ^{13}C NMR ($CDCl_3$) 28.0 (3C) 58.3, 82.8, 122.4, 123.2, 129.7, 133.2, 140.6, 148.3, 157.0, 172.3; HRMS (EI) calcd for $C_{13}H_{16}N_2O_6$ 296.1009; found 296.1018.

N^{α}-Boc-*m*-amino-D,L-phenylglycine (10). A mixture of **9** (9.24 g, 0.081 mol) and 10% Pd/C (1.5 g) in EtOH (400 mL) was flushed with hydrogen. After being stirred vigorously under a hydrogen atmosphere (1 atm) with frequent refill of hydrogen for 7 h, the reaction mixture was filtered through Celite and concentrated to a foamy solid which was recrystallized from chloroform to give **10** as a pale yellow solid (15 g, 70%); mp 148–149 °C; ^{1}H NMR ($CDCl_3$) δ 1.34 (s, 9*H*), 4.88 (d, J = 8.07 Hz, 1*H*), 6.53–6.59 (2*H*), 7.01 (app t, J = 7.61 Hz, 1*H*), 7.27 (d, J = 8.31 Hz, 1*H*); HRMS (EI) calcd for $C_{13}H_{18}N_2O_4$ 266.1267; found 266.1271.

***m*-Guanidino-D,L-phenylglycine (3).** A mixture of **10** (1.33 g, 5 mmol), 1*H*-pyrazole-1-carboxamidine hydrochloride[19] (733 mg, 5 mmol), and diisopropylethylamine (870 μL) in DMF (2 mL) was stirred overnight at 140 °C. DMF was removed under reduced pressure, and the crude mixture was stirred in trifluoroacetic acid:CH_2Cl_2 (1:2, 20 mL) for 2 h at room temperature. After evaporation of the volatile material, the mixture was treated with 2 N NH_4OH (3 mL), loaded on the Dowex 1X2-100, washed with water (100 mL), then eluted with 2 N HCl. The fractions stained by ninhydrin treatment were combined and concentrated. The resulting crude solid was loaded on Dowex 50W-X8 and eluted with a gradient of 1–2.5 N HCl. The fractions containing **3** were combined and concentrated to give *m*-guanidino-D,L-phenylglycine dihydrochloride (280 mg, 20%) as a colorless solid; ^{1}H NMR (D_2O) δ 5.18 (s, 1*H*), 7.35–7.43 (3*H*), 7.50–7.54 (m, 1*H*); ^{13}C NMR (D_2O) 56.1, 125.6, 127.3, 127.5, 131.4, 133.3, 135.4, 156.2, 170.4; HRMS (FAB, M + 1) calcd for $C_9H_{13}N_4O_2$ 209.1038; found 209.1072. Anal. calcd for $C_9H_{13}N_4O_2$ (0.2 H_2O): C, 37.96; H, 5.09; N, 19.67. Found: C, 37.65; H, 5.12; N, 19.71.

5-Keto arginine (4). To a solution of sodium ethoxide (20 mmol) in 40 mL of EtOH was added guanidine hydrochloride (1.91 g, 20 mmol) portionwise at room temperature. After the mixture had been stirred for 1 h, the solution was filtered, and the filtrate was transferred to the flask containing L-glutamic acid 5-methyl ester (1.61 g, 10 mmol). The mixture was stirred overnight at room temperature. The homogeneous solution was concentrated under reduced pressure, and the residue was loaded on Dowex 1X2-100 and eluted with water. The combined fractions containing the product was concentrated to give **4** as a white crystalline solid which was recrystallized from a mixture of H_2O:EtOH (322 mg, 17%); mp 88–89 °C; ^{1}H NMR (D_2O) δ 1.87 (m, 1*H*), 2.26 (dd, J = 8.78, 6.89 Hz, 2*H*), 2.35 (m, 1*H*), 4.03 (dd, J = 9.03, 5.76 Hz, 1*H*); ^{13}C NMR (D_2O) 25.3, 29.6, 58.2, 157.9, 180.2, 181.5; HRMS (M + 1) calcd for $C_6H_{13}N_4O_3$ 189.0988; found 189.1001. Anal. calcd for $C_6H_{13}N_4O_3$: C, 38.29; H, 6.42; N, 29.82. found: C, 38.02; H, 6.57; N, 29.82.

(*E*)-N^{ω}-Propyl-3,4-didehydro-D,L-arginine (5). A mixture of (*E*)-2-acetoamido-5-amino-3-pentenoic acid[18] (861 mg, 5 mmol), 1*H*-pyrazole-*N*-propyl-1-carboxamidine hydrochloride[23] (940 mg, 5 mmol) in 1 N Na_2CO_3 (10 mL) was stirred for 6 days at room temperature. Concentrated NH_4Cl (2 mL) was added, and the mixture was concentrated, then the residue was loaded on Dowex 50W-X8 (2.5×6 cm) and washed with water (200 mL). The amino acid was eluted with a HCl gradient (0.5–2.5 N), and the eluate was concentrated to give **5** as a colorless foam (210 mg, 18%); ^{1}H NMR (D_2O) δ 0.86 (t, J = 7.38 Hz, 3*H*), 1.53 (m, 2*H*), 3.12 (t, J = 7.00 Hz, 2*H*), 3.91 (d, J = 4.11 Hz, 2*H*), 4.6 (d, J = 7.95 Hz, 1*H*), 5.75 (ddt, J = 15.69, 8.10, 1.65 Hz, 1*H*), 6.05 (dt, J = 15.69, 4.41 Hz, 1*H*); ^{13}C NMR (D_2O) 10.5, 21.6, 41.7, 43.0, 54.0, 120.9, 134.6, 155.7, 170.2; HRMS (FAB, M + 1) calcd for $C_9H_{19}N_4O_2$ 215.1508; found 215.1512. Anal. calcd for $C_9H_{19}N_4O_2$ (0.3 H_2O): C, 37.64; H, 7.09; N, 19.14. found: C, 37.46; H, 7.17; N, 19.12.

N^{α}-Boc-*m*-cyanamido-D,L-phenylglycine (12). To a mixture of **10** (700 mg, 2.63 mmol) and sodium acetate (220 mg, 2.63 mmol) stirred in MeOH (40 mL) at 0 °C was added cyanogen bromide (276 mg, 2.63 mmol) portionwise. After being stirred for 3 h at room temperature the volatile materials were removed under reduced pressure, and the residue was dissolved in ethyl acetate (50 mL) and extracted with 0.5 N HCl (30 mL). The organic layer was concentrated, and the crude mixture

was purified by flash silica gel chromatography using ethyl acetate as eluant to give **12** as a white foam (420 mg, 55%); ^{1}H NMR (DMSO-d_6) δ 1.38 (s, 9*H*), 5.01 (d, J = 7.74 Hz, 1*H*), 6.85 (dd, J = 7.98, 1.41 Hz, 1*H*), 6.97 (s, 1*H*), 7.03 (d, J = 7.88 Hz, 1*H*), 7.29 (t, J = 7.83 Hz, 1*H*), 7.51 (d, J = 7.89 Hz, 1*H*); ^{13}C NMR (DMSO-d_6) 28.1 (3C), 57.4, 111.9, 114.1, 114.4, 122.0, 129.7, 138.6, 139.2, 155.1, 171.9; HRMS (FAB, M + 1) calcd for $C_{14}H_{18}N_3O_4$ 292.1297; found 292.1303.

***m*-Ureido-D,L-phenylglycine (13).** Compound **12** (260 mg, 0.89 mmol) in 1 N HCl (20 mL) was heated under reflux for 10 min. After evaporation of the solvent, the residue was loaded on Dowex 50W-X8, washed with water (40 mL), then eluted with 1 N NH_4OH (150 mL). The fractions stained by ninhydrin treatment were combined and concentrated to yield **13** as a colorless solid (133 mg, 82%), which was recrystallized from water; ESMS (M + 1) 210.0; ^{1}H NMR (D_2O) δ 4.71 (s, 1*H*), 7.13 (m, 1*H*), 7.27 7.40 (3*H*); ^{13}C NMR (D_2O) 58.3, 120.8, 122.4, 123.5, 130.2, 135.0, 138.7, 159.3, 173.1; HRMS (FAB, M + 1) calcd for $C_9H_{12}N_3O_3$ 210.0878; found 210.0819. Anal. calcd for $C_9H_{12}N_3O_3$: C, 51.67; H, 5.30; N, 20.09. found: C, 51.64; H, 5.57; N, 20.09.

Enzymes

All of the enzymes used were recombinant enzymes overexpressed in *Escherichia coli*. The murine macrophage iNOS was expressed[24] and isolated[25] as reported; the bovine endothelial eNOS was prepared as previously described,[26] and the rat neuronal nNOS was expressed and purified as described.[27]

Initial velocity measurements via the hemoglobin assay

The generation of nitric oxide by NOS was measured using the hemoglobin capture assay,[28] which utilizes the rapid oxidation of oxyhemoglobin by NO to produce methemoglobin, which is detected spectrophotometrically at 401 nm (ε = 19,700 M^{-1} cm^{-1}) on a Perkin–Elmer Lamda 10 UV–vis spectrophotometer. A typical assay mixture for nNOS and eNOS contained 10 μM L-arginine, 1.6 mM $CaCl_2$, 11.6 mg/mL calmodulin, 100 μM NADPH, 6.5 μM BH_4, and 3 mM oxyhemoglobin in 100 mM HEPES buffer (pH 7.5). The reaction mixture for iNOS contained 10 μM of L-arginine, 100 μM NADPH, 6.5 μM BH_4, and 3 mM oxyhemoglobin in 100 mM HEPES buffer (pH 7.5). All assays were in a final volume of 600 μL and were initiated with enzyme.

Substrate activity/reversible inhibition measurements

Substrate activity was measured as described above using varying concentrations of the alternative substrate (**1–3**) instead of L-arginine. K_i values were determined by the method of Dixon.[29]

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23. This compound was prepared as follows: To a stirred solution of *n*-propylamine (8.2 mL, 100 mmol) in diethyl ether (150 mL) at 0 °C was added cyanogen bromide (5.2 g, 50 mmol) in diethyl ether (30 mL) over a period of 10 min. The

mixture was stirred further for 30 min at room temperature, then the precipitated solid was filtered off, and the filtrate was concentrated under reduced pressure to afford *N*-propylcyanamide as an oil which was used directly for the next step. To this oil in dioxane (20 mL) were added pyrazole (2.7 g, 40 mmol) and 4 N HCl in dioxane (10 mL, 40 mmol), and the mixture was stirred overnight at room temperature. The precipitated white crystals were filtered, washed with cold dioxane, and dried to a constant weight (6.5 g, 69%); mp 154–156 °C; ^{1}H NMR (D_2O) δ 0.96 (t, J = 7.41 Hz, 3H), 1.72 (m, 2H), 3.43 (t, J = 6.90 Hz, 2H), 6.66 (m, 1H), 7.91 (s, 1H), 8. 26 (d, J = 2.94 Hz, 1H); ^{13}C NMR (D_2O) 10.6, 20.9, 44.5, 111.7, 130.5, 146.0, 150.7.

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