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N^{ω}-Propargyl-L-arginine and N^{ω}-Hydroxy-N^{ω}-propargyl-L-arginine are Inhibitors, but not Inactivators, of Neuronal and Macrophage Nitric Oxide Synthases

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Abstract—N^{ω}-Propargyl-L-arginine (**7**) was synthesized as a potential mechanism-based inactivator of neuronal nitric oxide synthase (nNOS) and macrophage nitric oxide synthase (iNOS). Compound **7** is a potent reversible competitive inhibitor for both isoforms, having K_i values of 430 ± 50 nM and 620 ± 30 nM for nNOS and iNOS, respectively. These values are 12 and 32 times lower than the K_m for L-arginine with nNOS and iNOS, respectively; however, **7** does not exhibit time-dependent inhibition with either. It also only undergoes oxidation very slowly. N^{ω}-Hydroxy-N^{ω}-propargyl-L-arginine also was synthesized to determine if the initial proposed enzyme-catalyzed hydroxylation of N^{ω}-propargyl-L-arginine was problematic. This compound also is a potent reversible inhibitor of both nNOS and iNOS, but is not a time-dependent inactivator and is oxidized only very slowly. These results are in sharp contrast with the corresponding olefins, N^{ω}-allyl-L-arginine and N^{ω}-allyl-N^{ω}-hydroxy-L-arginine recently reported to be potent time-dependent, irreversible inhibitors of nNOS (Zhang, H. Q.; Dixon, R. P.; Marletta, M. A.; Silverman, R. B., *J. Am. Chem. Soc.* **1997**, *119*, in press); N^{ω}-allyl-L-arginine also was reported to be an inactivator of iNOS (Olken, N. M.; Marletta, M. A. *J. Med. Chem.* **1992**, *35*, 1137). This suggests that the active site of both isoforms of NOS can accommodate a variety of structures, but binding must have the appropriate juxtaposition for hydroxylation; otherwise, no oxidation occurs. © 1997 Elsevier Science Ltd.

Introduction

The relatively recent implication of nitric oxide (NO) in numerous biological functions has fueled an increasing interest in this unlikely biological messenger and its biosynthesis by nitric oxide synthase (NOS, EC 1.14.13.39). NOS has been isolated as three distinct isoforms, each associated with a particular physiological process: endothelial (eNOS), which regulates smooth muscle relaxation and blood pressure, neuronal (nNOS), which is involved in long-term potentiation, and inducible (iNOS), which is produced by activated macrophage cells in the immune response.[1] Overproduction of NO has been implicated in many disease states including strokes;[2] septic shock;[3] seizures;[4] schizophrenia;[5] migraine headaches;[6] Alzheimer's disease;[7] tolerance to and dependence on morphine;[8] development of colitis;[9] tissue damage and inflammation;[10] overproduction of osteoclasts, leading to osteoporosis, Paget's disease, and rheumatoid arthritis;[11] destruction of photoreceptors in the retina;[12] long-term depression;[13] and priapism.[14] Considering the diverse roles of NO in healthy physiology, design of isoform-specific NOS inhibitors to treat the pathological production of NO is of obvious interest.

NOS converts L-arginine (**1**) and O_2 to L-citrulline (**3**) and NO with the concomitant oxidation of NADPH (Scheme 1). N^{ω}-Hydroxy-L-arginine (**2**) has been shown to be a catalytically competent intermediate.[15] The reaction also shows a dependence on tetrahydrobiopterin and thiols to reach maximum activity.[16] The labeling pattern in Scheme 1 indicates the origin of the atoms in the intermediate and products.

White and Marletta have characterized NOS as a cytochrome P450 type hemoprotein containing a *C*-terminal domain which has an amino acid sequence highly similar to that of cytochrome P-450 reductase, and an *N*-terminal domain which contains protoporphyrin IX and a binding site for tetrahydrobiopterin.[17] Like cytochrome P-450, each step of the hydroxylation can be blocked by carbon monoxide, implicating a role for the heme in each oxidation. Marletta[18] suggests that the mechanism may be more like that proposed for aromatase[19] in which the first hydroxylation proceeds by an electrophilic high-energy iron-oxo species, but the second step involves a nucleophilic attack on N^{ω}-hydroxy-L-arginine radical (**4**) by a ferric peroxide (Fe(III)OOH) (Scheme 2).

A few compounds, including N^{ω}-methyl-L-arginine and its metabolite N^{ω}-hydroxy-N^{ω}-methyl-L-arginine,[20] N^{ω}-

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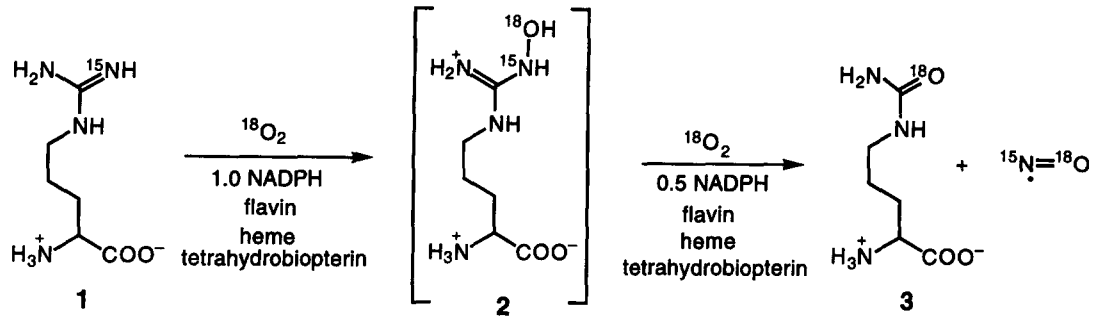


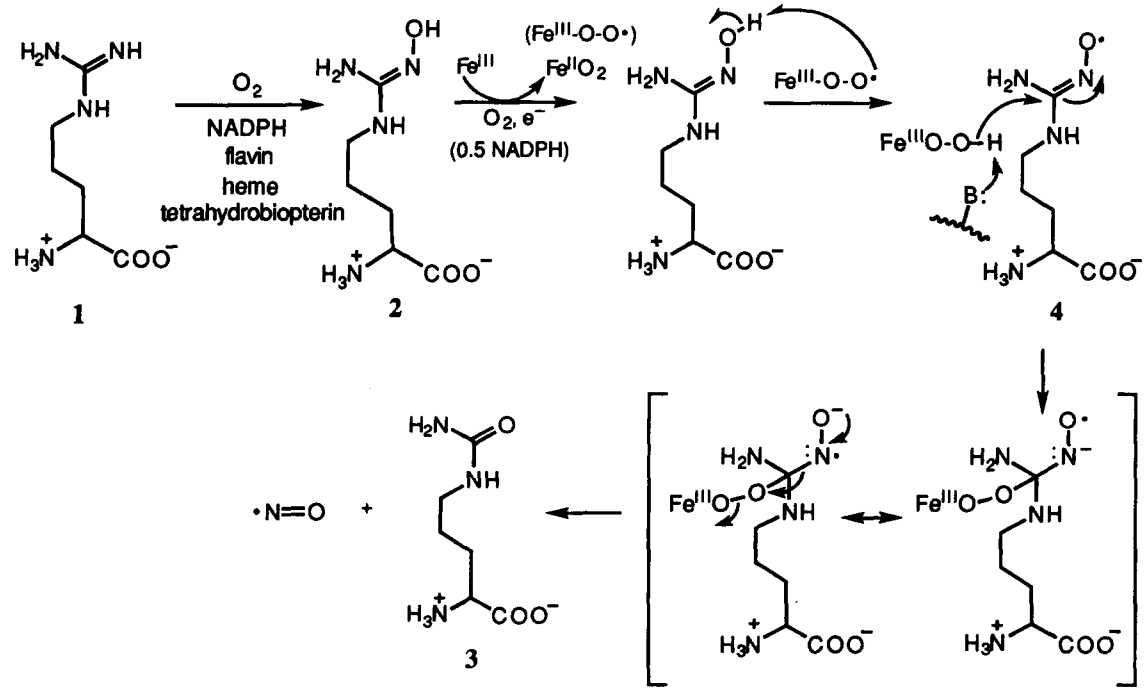
Scheme 1.

allyl-L-arginine,[21] aminoguanidines,[22] N^{ω}-nitro-L-arginine,[23] phencyclidine,[24] and N^{5}-(1-iminoethyl)-L-ornithine[25] and the corresponding lysine analogue,[26] have demonstrated time-dependent inhibition of NOS activity. Because of the success of N^{ω}-allyl-L-arginine as a time-dependent inactivator of macrophage[21] and brain[27] NOS, it was reasoned that the corresponding acetylenic analogue, N^{ω}-propargyl-L-arginine (**7**, Scheme 3), also should be an inactivator. Substituted acetylenes, in general, are known to inactivate other heme-dependent enzymes, such as cytochrome P-450,[28] by the mechanism shown in Scheme 4. If inactivation of NOS by N^{ω}-propargyl-L-arginine does not proceed by the mechanism in Scheme 4, it may operate by a mechanism related to that proposed for N^{ω}-allyl-L-arginine.[27] In this mechanism it does not matter what the N^{ω}-arginine substituent is, so a propargyl group should be just as effective as is the allyl substituent. Furthermore, the corresponding hydroxylated analogue, N^{ω}-hydroxy-N^{ω}-propargyl-L-arginine (**11**, Scheme 5) also should inactivate the enzyme, if the mechanism is like that shown for the substrate in Scheme 2 or like that proposed for N^{ω}-allyl-L-arginine.[27] Here we show that N^{ω}-propargyl-L-arginine (**7**) and N^{ω}-hydroxy-N^{ω}-propargyl-L-arginine (**11**) are potent inhibitors of both the neuronal and inducible NOS, but, surprisingly, neither exhibits time-dependent inactivation.

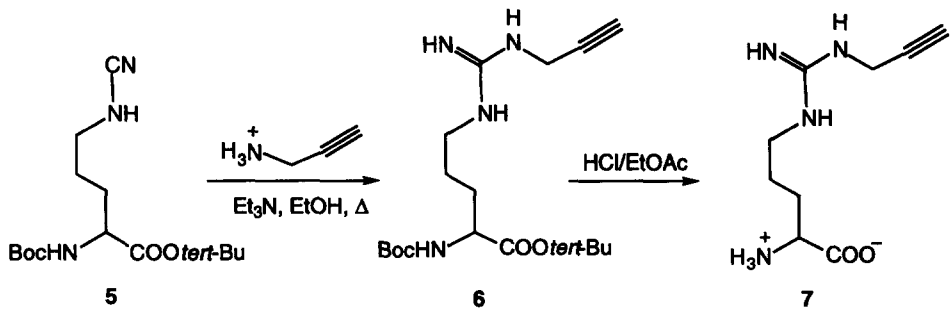
Results

Reversible inhibition of nNOS and iNOS by N^{ω}-propargyl-L-arginine (7) and N^{ω}-hydroxy-N^{ω}-propargyl-L-arginine (11)

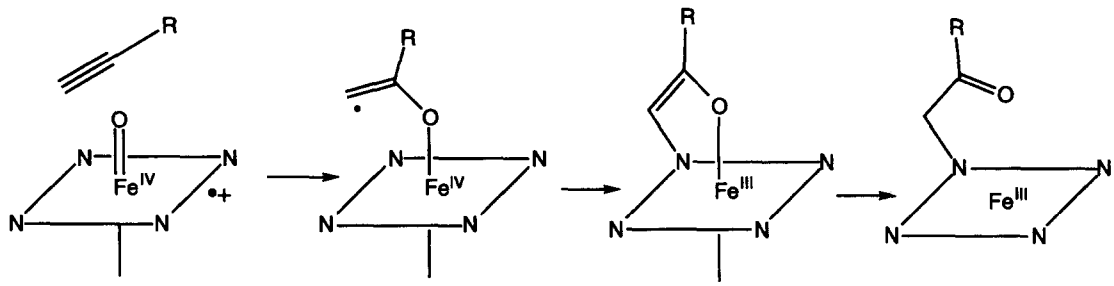
N^{ω}-Propargyl-L-arginine (**7**) and N^{ω}-hydroxy-N^{ω}-propargyl-L-arginine (**11**) were found to be potent, competitive inhibitors of both neuronal and macrophage NOS. Dixon analyses ($1/V$ vs [I] at varying substrate concentrations)[29] indicated that **7** and **11** were either competitive or mixed (Figs 1A and 2A are for inhibition of nNOS and iNOS, respectively, with **7**); Cornish-Bowden replots ([S]/V vs [I])[30] demonstrated that inhibition by



Scheme 2.



Scheme 3.



Scheme 4.

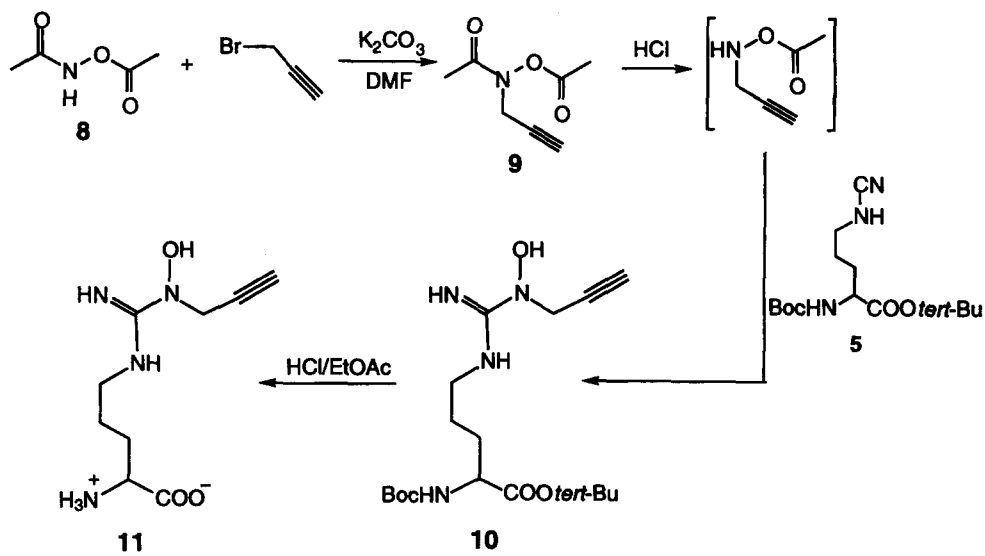
both analogues was competitive for both nNOS and iNOS (Figs 1B and 2B, respectively for **7**). As derived from the Dixon plots, the K_i values for **7** were 430 ± 50 nM and 620 ± 30 nM with nNOS and iNOS, respectively, and for **11**, 1.33 ± 0.04 μM and 1.10 ± 0.20 μM with nNOS and iNOS, respectively.

Time-dependent inhibition of nNOS and iNOS by N^{ω}-propargyl-L-arginine (7) and N^{ω}-hydroxy-N^{ω}-propargyl-L-arginine (11)

No time-dependent inhibition was observed for either **7** or **11** (data not shown) with either nNOS (Fig. 3A) or iNOS (Fig. 3B) for up to an hour.

Metabolite formation during incubation of nNOS with 1, 7, or 11

Using HPLC assays, standards of **1**, **3**, **7**, **11**, and NADPH were separated with retention times of 14.8, 6.6, 16.2, 11.6, and 2.2 min, respectively (Fig. 4); $NADP^+$ shows no fluorescence under these conditions. Control injections of nNOS with either NADPH or $NADP^+$ show an additional unknown peak at 2.8 min, which can probably be attributed to other cofactors present in the enzyme preparation (Fig. 4). After 1 h of incubation with **1**, HPLC assays show turnover dependent loss (65%) of substrate and production of a metabolite with the same retention time as **3** (Fig. 5). Fluorescence of arginine standards is linear over the



Scheme 5.

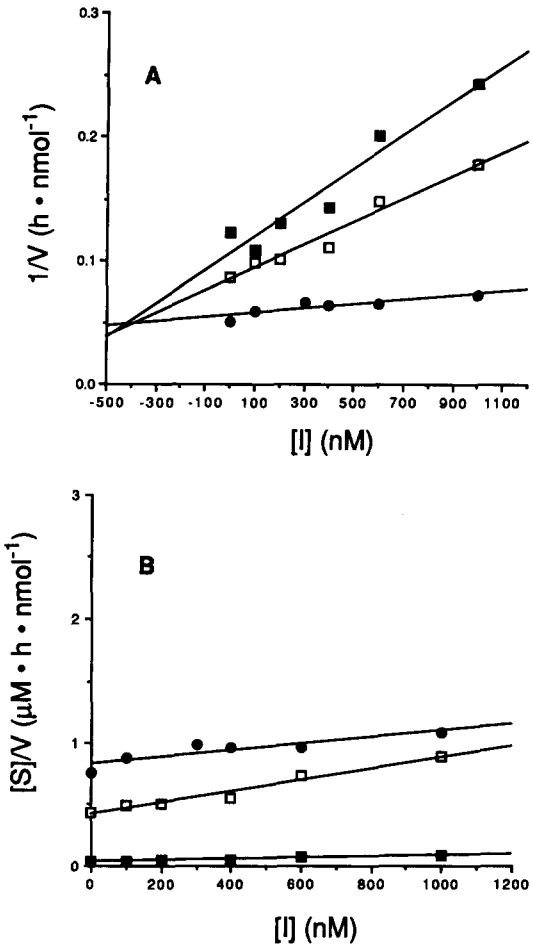


Figure 1. (A) Dixon[29] plot of nNOS inhibition by **7**. Closed circles, open squares and closed squares represent assay points at 15, 5, and 3 μM arginine, respectively. (B) Cornish-Bowden[30] replot of the data in (A). Closed circles, open squares and closed squares represent assay points at 15, 5, and 3 μM arginine, respectively. See the Experimental section for details.

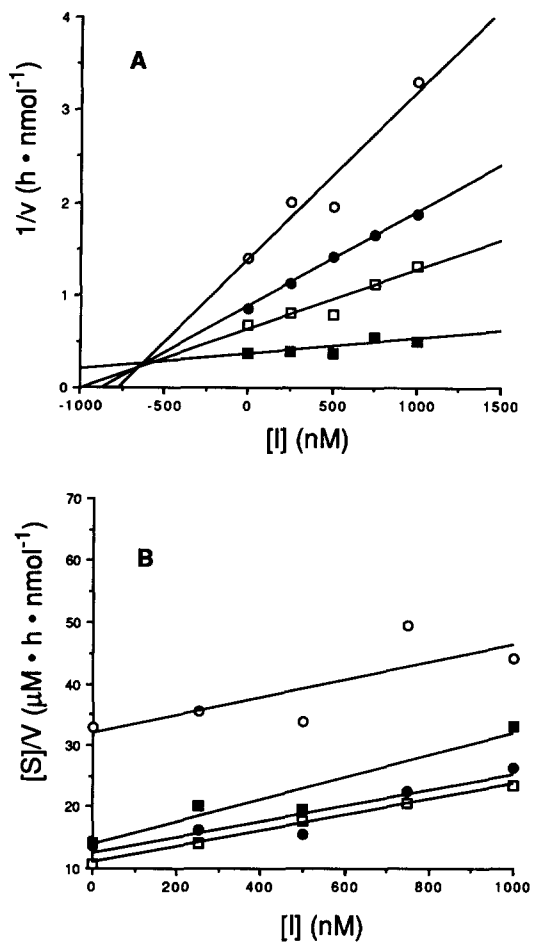


Figure 2. (A) Dixon plot of iNOS inhibition by 7. Open circles, closed circles, open squares and closed squares represent assay points at 90, 20, 12.5, and 10 μM arginine, respectively. (B) A Cornish-Bowden[30] replot of the data in (A). Open circles, closed circles, open squares and closed squares represent assay points at 90, 20, 12.5, and 10 μM arginine, respectively. See the Experimental section for details.

range of concentrations studied (data not shown). After 1 h of incubation with **7** (Fig. 6A) or **11** (Fig. 7), little or no production of metabolites is apparent. A 16 h incubation with **7** produces a small, turnover-dependent loss of inhibitor (about 12%) and production of a small amount of metabolite with the same retention time as citrulline (**3**) (Fig. 6B, standards not shown).

Discussion

N^{ω}-Propargyl-L-arginine (**7**) has been found to be a potent competitive reversible inhibitor of both nNOS (K_i = 430 nM) and iNOS (K_i = 620 nM) (Figs 1 and 2), but it shows no time-dependent inhibition over a period of an hour for either isoform (Fig. 3). Given the proclivity of acetylene analogues to inactivate heme-dependent enzymes,[31] and the potency of inactivation of nNOS[27] and iNOS[21] by N^{ω}-allyl-L-arginine and by N^{ω}-propyl-L-arginine,[27] it is quite surprising that no time-dependent inactivation of either isoform occurs with N^{ω}-propargyl-L-arginine. The only apparent differences between N^{ω}-propargyl-L-arginine, N^{ω}-allyl-L-arginine, and N^{ω}-propyl-L-arginine are the redox potentials and the geometry of the substituent; the alkyl substituent is tetrahedral, the alkenyl part of allyl is trigonal, and the ethynyl moiety of propargyl is linear. One possibility is that the difference in geometry of the substituents affects the ability of the enzyme to catalyze the proposed initial N^{ω}-hydroxylation, but it may not be important once N^{ω}-hydroxylation has occurred. To address that possibility, N^{ω}-hydroxy-N^{ω}-propargyl-L-arginine (**11**), the product of N^{ω}-hydroxylation, was synthesized, but it also was shown to be a potent reversible competitive inhibitor that exhibits no time-dependent inactivation (data not shown). Apparently, the geometric constraint is important throughout the reaction pathway. Although the K_i values for **7** and **11** are lower than the K_m for L-arginine with nNOS (5 μM) and iNOS (2.3 μM), these compounds appear to be involved in a nonproductive (or low-productive) binding, because little or no citrulline could be detected

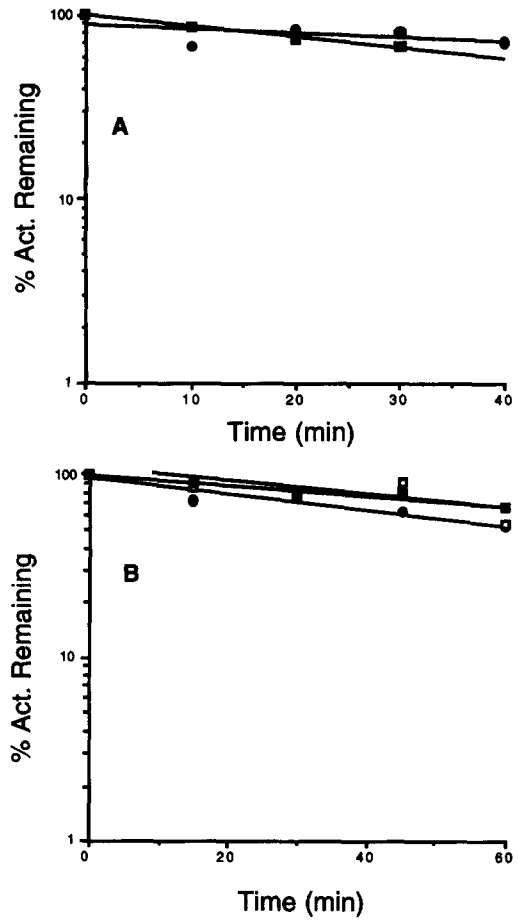


Figure 3. (A) Time-dependent inhibition of nNOS by **7**. Closed circles and closed squares represent assay points at 0 and 200 μM (**7**), respectively. (B) Time-dependent inhibition of iNOS by **7**. Closed circles, open squares and closed squares represent assay points at 0 μM, 15 μM, and 5 mM (**7**), respectively. See the Experimental section for details.

(Figs 6A and 7, respectively) until after long reaction times (Fig. 6B). This suggests that the active site of both isoforms of NOS can accommodate a variety of structures, but binding must have the appropriate juxtaposition for hydroxylation; otherwise, no oxidation occurs. The geometry of the substituent also affects the relative binding efficiency for the isoforms. Whereas N^{ω}-propargyl-L-arginine exhibits essentially no isoform selectivity (620/430 nM = 1.4), N^{ω}-allyl-L-arginine shows a selectivity of 10.5 (2100/200 nM) in favor of the neuronal isoform.[21,27]

Conclusion

These results suggest that the propargyl group in bound N^{ω}-propargyl-L-arginine is not appropriately positioned for oxygenation, possibly because of a geometric constraint in the active site. This constraint may be alleviated by deleting part of the binding interactions, as would be the case with propargylguanidine or propargylornithine. Both of these approaches are being pursued.

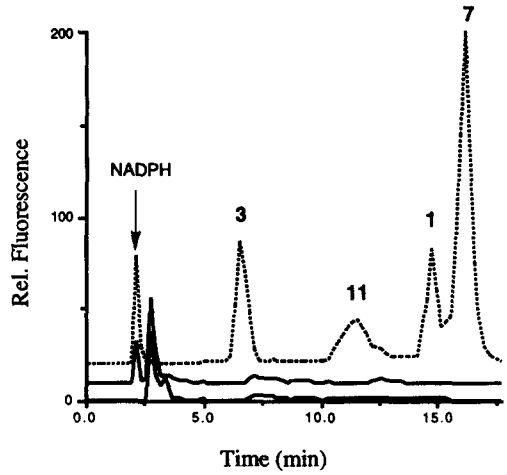


Figure 4. HPLC of control experiments and standards. Incubation with $NADP^+$ and nNOS is represented by the thin solid line; incubation with NADPH and nNOS is represented with a thick solid line; and standards of NADPH, **1**, **3**, **7**, and **11**, are given by a dotted line. See the Experimental section for details.

Experimental

Materials

N-Boc-*N*-Cbz-L-ornithine, 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). *tert*-Butanol was purchased from Malinckrodt. D_2O was purchased from Cambridge Isotope Laboratories, and $CDCl_3$ was purchased from Aldrich Chemical Co. Acids, bases, and conventional organic solvents were purchased from Fischer. DCC, DMAP, 10% Pd–C, cyanogen bromide, and propargylamine hydrochloride were purchased from Aldrich. 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 1 or Perkin–Elmer Lambda 10 UV–vis spectrophotometer. NMR spectra were recorded on a Varian Gemini-300 300-MHz. Chemicals shifts are reported as δ values in parts per million downfield from Me_4Si as the internal standard in $CDCl_3$, unless stated otherwise. Mass spectra were recorded on a VG Instruments VG70-250SE high-resolution spectrometer. Melting points were obtained with a Fisher–Johns melting point apparatus and are uncorrected.

N^{α}-Boc-N^{ω}-propargyl-L-arginine *tert*-butyl ester (6). N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester[32] (**5**, 1.9 g, 6 mmol) was dissolved in 25 mL of absolute EtOH, and propargylamine hydrochloride (0.77 g, 8.4 mmol) was added with triethylamine (0.98 g, 1.35 mL, 9.7 mmol). The reaction mixture was refluxed at 85 °C

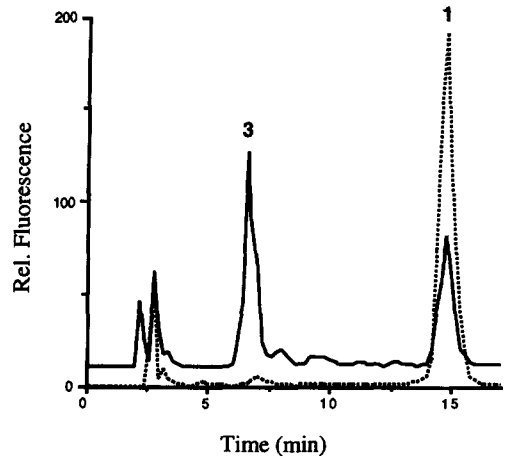


Figure 5. HPLC of **1** incubated with nNOS for 1 h. Incubation with $NADP^+$, **1**, and nNOS is represented by a dotted line, and incubation with NADPH, **1**, and nNOS by a solid line. See the Experimental section for details.

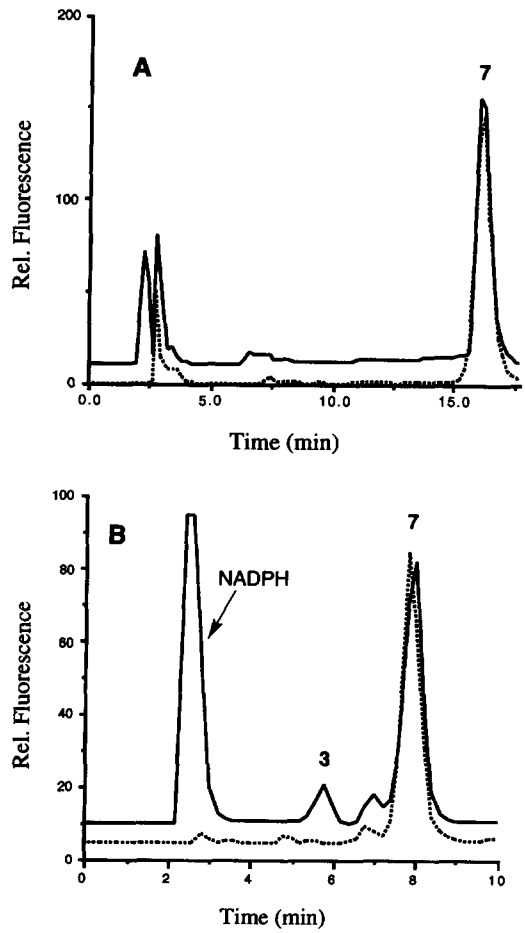


Figure 6. (A) HPLC of **7** incubated with nNOS for 1 h. Incubation with $NADP^+$, **7**, and nNOS is represented by a dotted line, and incubation with NADPH, **7**, and nNOS by a solid line. (B) HPLC of nNOS incubated with **7** for 16 h. Incubation with $NADP^+$, nNOS and **7** is represented by a dotted line, and incubation with NADPH, nNOS and **7** by a solid line. Note that compounds in B are eluted with a different gradient and solvent system than in A. See the Experimental section for details.

under a N_2 blanket for 140 h, at which time the relative sizes of reactant and product spots by TLC had stopped changing. The resulting dark-brown solution was dried by rotary evaporation, and was chromatographed twice on silica-gel (3 cm × 45 cm) to yield N^{α}-Boc-N^{ω}-propargyl-L-arginine *tert*-butyl ester (**6**, 707 mg, 32%); R_f = 0.36 in 4:1 $CHCl_3$:MeOH; R_f = 0.84 in 2:2:1 $CHCl_3$:MeOH:NH_4OH; ^1H NMR ($CDCl_3$) δ 1.40 (s, 9H), 1.43 (s, 9H), 1.60–1.90 (m, 4H), 2.43 (m, 1H), 3.20–3.50 (m, 2H), 4.09 (m, 1H), 4.14 (m, 2H), 5.51 (m, 1H), 7.2 (br s, 1H), 8.03 (br s, 2H).

N^{ω}-Propargyl-L-arginine dihydrochloride (7). N^{α}-Boc-N^{ω}-propargyl-L-arginine *tert*-butyl ester (**6**, 95.3 mg, 0.259 mmol) was dissolved in 5 mL of ethyl acetate and chilled on ice. A 4 N solution of HCl in ethyl acetate (5 mL) was prepared from aqueous 12 N HCl and ethyl acetate. The two solutions were then combined and stirred on ice for 135 min. This yielded a mixture of mono- and di- deprotected product (R_f = 0.66 for monodeprotected, R_f = 0.29 for dideprotected product, R_f = 0.74 for the starting material in 2:2:1 $CHCl_3$:MeOH:NH_4OH). The mixture was then subjected to rotary evaporation under low, then high vacuum. It was found that during the rotary evaporation process the deprotection was completed. The light-yellow solid was then redissolved in H_2O (1 mL) and was loaded onto a prewashed Dowex 50W-X8 plug (2 mL). The sample did not elute with H_2O (15 mL) or with 1 N HCl (10 mL), but did elute with 4 N HCl (8 mL). The product-containing fractions were combined, subjected to rotary evaporation under high vacuum, and lyophilized overnight to yield 6.26 mg (0.022 mmol, 9%) of the desired compound; R_f = 0.30 in 2:2:1 $CHCl_3$:MeOH:NH_4OH; ^1H NMR (D_2O) δ 1.40–1.90 (m, 4H), 2.52 (t, 1H), 3.08 (t, 2H), 3.81 (d, 2H), 3.86 (t, 1H). The terminal propargyl proton (2.52 ppm) was found to be exchangeable in D_2O, on a time scale of 24 h at neutral-acidic pH, and on a time scale

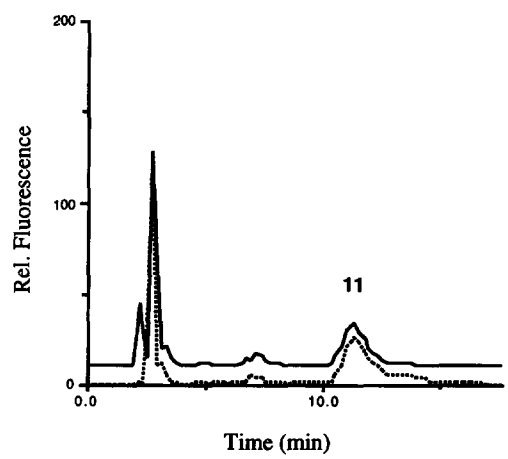


Figure 7. HPLC of **11** incubated with nNOS for 1 h. Incubation with $NADP^+$, **11**, and nNOS is represented by a dotted line, and incubation with NADPH, **11**, and nNOS by a solid line. See the Experimental section for details.

of 1 h at basic pH (~12); HRMS (FAB^+): calcd for $C_9H_{16}N_4O_2$ (M+1) *m/z* 213.1352, found *m/z* 213.1384.

***N*-Propargyl-*N,O*-diacetylhydroxylamine (9).** The synthetic procedure of Cheng et al.[33] for *N*-allyl-*N,O*-diacetylhydroxylamine was followed directly, starting with N,O-diacetylhydroxylamine (14.99 g, 128.1 mmol) in DMF (30 mL). The product was purified by silica-gel column chromatography (1:1; hexane:ether) to yield **9** (10.63 g, 68.8 mmol, 54% yield) as an off-white solid; ^1H NMR ($CDCl_3$) δ 1.92 (s, 3H), 2.12 (s, 3H), 2.20 (t, 1H), 4.32 (d, 2H); ^{13}C NMR ($CDCl_3$) 18.07, 20.04, 37.27, 72.66, 76.26, 77.00 (t, $CDCl_3$), 167.96. EI-HRMS calcd for $C_9H_9NO_3$ *m/z* 155.0582, found *m/z* 155.0599.

***N*$^{\alpha}$-Boc-*N*$^{\omega}$-Propargyl-*N*$^{\omega}$-hydroxy-L-arginine *tert*-butyl ester (10).** *N*-Propargyl-*N,O*-diacetylhydroxylamine (**9**, 4g, 25.8 mmol) was dissolved in 10 mL of 6 N HCl (aq) and was allowed to stir at room temperature for 3.5 h after which time the solution was neutralized by the addition of solid Na_2CO_3 and extracted into ether. The ether extract was acidified by addition of HCl(g) and washed several times with water. The organic layer was concentrated by rotary evaporation to approximately 4 mL and was added to **5** (1.02 g, 3.25 mmol). The resulting solution was allowed to stir at room temperature for 48 h, then was chromatographed on a silica-gel column (4:1 $CHCl_3$: MeOH), yielding **10** (70 mg, 0.18 mmol, 6% yield) as a pale-yellow oil; ^1H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.49 (s, 9H), 1.55–1.90 (m, 4H), 2.38 (s, 1H) 3.38–3.65 (m, 2H), 4.1 (m, 1H), 4.68 (m, 2H), 5.48 (m, 1H) 7.60–8.16 (m, 3H); ^{13}C NMR ($CDCl_3$) 24.82, 28.12, 28.45, 30.25, 42.16, 44.94, 53.33, 74.12, 76.10, 77.15 (t, $CDCl_3$), 80.28, 82.48, 156.19, 159.40, 171.82. HRMS (FAB^+): calcd for $C_{18}H_{32}N_4O_5$ (M+1) *m/z* 385.2451, found *m/z* 385.2493.

***N*$^{\omega}$-Propargyl-*N*$^{\omega}$-hydroxy-L-arginine (11).** *N*$^{\alpha}$-Boc-*N*$^{\omega}$-propargyl-*N*$^{\omega}$-hydroxy-L-arginine *tert*-butyl ester (**10**, 20 mg, 0.05 mmol) was treated with 3 N HCl in 5 mL of ethyl acetate at room temperature for 3.5 h. Volatile solvents were removed by rotary evaporation to yield **11** (12.5 mg, 0.04 mmol, 80 % yield); ^1H NMR (D_2O) δ 1.60–2.02 (m, 4H), 2.75 (m, 1H), 3.33 (t, 2H), 3.94 (t, 1H) 4.35 (d, 2H); ^{13}C NMR (D_2O) 23.61, 27.20, 41.11, 43.69, 53.28, 74.80, 75.75, 158.96, 173.17. HRMS (FAB^+): calcd for $C_9H_{16}N_4O_3$ (M+1) *m/z* 229.1301, found *m/z* 229.1355.

Enzyme purification

nNOS was obtained from bovine brain as described.[34] iNOS was purified and assayed according to the procedures of Hevel et al.[35]

Initial velocity measurements via the hemoglobin assay

The generation of NO by nNOS was measured by the rapid oxidation of oxyHb to metHb by nitric oxide.[36] The assay mixture contained nNOS (0.5–5 μg), L-arginine (12–50 μM), $CaCl_2$ (1.6 mM), calmodulin (11.6 μg/mL), NADPH (104 μM), tetrahydrobiopterin (6.5 μM), dithiothreitol (100 μM), and oxyhemoglobin (3 mM), diluted to a total volume of 600 μL with Hepes buffer (100 mM, pH 7.5). The relative rate of NO synthesis was determined by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm on a Perkin–Elmer Lambda 1 UV–vis spectrophotometer. All assays were performed at 30 °C.

Reversible inhibition kinetics

The type of reversible inhibition of nNOS by **7** and **11** was studied under initial rate conditions with the hemoglobin assay as described above. Data were analyzed by the methods of Dixon[29] and Cornish-Bowden.[30]

Irreversible inhibition kinetics

The nNOS (3.5–8.0 μg) was incubated at 0 °C in Hepes buffer (100 mM, pH 7.5), containing $CaCl_2$ (2.1 mM), calmodulin (30 μg/mL), NADPH (0.6 mM), tetrahydrobiopterin (50 μM), dithiothreitol (700 μM), glycerol (20% v/v), **7** or **11** in a total volume of 70–140 μL. The reactions were initiated by the addition of enzyme, and 10 μL aliquots were removed to assay for enzyme activity at various times. A control was performed by omitting the inhibitors. Preincubations for time-dependent assays of purified iNOS were conducted under the same conditions as nNOS except that $CaCl_2$ and calmodulin were omitted.

HPLC assay for metabolites

For all experiments, except that depicted in Figure 6B, nNOS (12 μg) was incubated for 1 h at 0 °C in Hepes buffer (100 mM) supplemented with $CaCl_2$ (4.6 mM), calmodulin (1.9 μM), DTT (12.3 mM), glycerol (16%), and tetrahydrobiopterin (1 mM). NADPH (0.49 mM), $NADP^+$ (0.49 mM), **1** (300 μM), **7** (300 μM), or **11** (300 μM) also were included depending on the experiment. After incubation, each sample was filtered through a microcentrifuge filter (Sigma, 10,000 NMWL), derivatized with OPA reagent (Pierce), and injected onto an Alltech Econosil C_{18} HPLC column (10 μm × 250 mm × 4.6 mm). Using a Beckman System Gold 125P solvent module, the sample was eluted with a gradient of 35% solvent B: 65% solvent A for 6 min followed by 35–45% solvent B over 1 min. The gradient was held at 45% solvent B: 55% solvent A for 11 min and then reset with a 45-35% solvent B gradient over 2 min. Solvent A is 95% 0.1 M sodium acetate pH 7.2, 4.5% methanol, and 0.5% tetrahydrofuran. Solvent B is 95% methanol and 5% 0.1 M sodium acetate pH 7.2. All solvents were HPLC grade and were filtered and degassed before use. Sample elution was detected by a Spectra/glo filter fluorimeter fitted with *o*-phthalaldehyde fluorescence

filters. For the experiment depicted in Figure 6B, nNOS (3.5 μg) was incubated overnight at room temperature in Hepes buffer (100 mM) supplemented with $CaCl_2$ (6.25 mM), calmodulin (1.5 μM), DTT (250 μM), glycerol (7.5%), 7 (100 μM) and tetrahydrobiopterin (25 μM). NADPH (1.9 mM) or $NADP^+$ (1.9 mM) also was included depending on the experiment. After incubation, each sample was filtered through a microcentrifuge filter (Sigma, 10,000 NMWL) and then through a 0.2 μm filter (Gelman PVDF acrodisk), derivatized with OPA reagent (Pierce), and injected onto the same column as described above. The sample was eluted with a gradient of 45% solvent B: 55% solvent A for 5 min followed by 45–80% solvent B over 5 min. The gradient was held at 80% solvent B: 20% solvent A for 8 min and then reset with a 80–45% solvent B gradient over 2 min. Solvent A is 95% 0.1 M sodium acetate pH 7.2 and 5% methanol. Solvent B is 95% methanol and 5% 0.1 M sodium acetate pH 7.2. All solvents were HPLC grade and were filtered and degassed before use. Sample elution was detected as described above.

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Note added in proof

Propargylguanidine was synthesized and was shown to be a time-dependent inactivator of neuronal NOS.[37]

References

1. Kerwin, J. F.; Heller, M. *Med. Res. Rev.* **1994**, *14*, 23.

2. (a) Choi, D. W. *J. Neurosci.* **1990**, *10*, 2493. (b) Choi, D. W.; Rothman, S. M. *Annu. Rev. Neurosci.* **1990**, *13*, 171.

3. Crossin, K. L. *Trends Biochem. Sci.* **1991**, *16*, 81.

4. Ferrendelli, J. A.; Blank, A. C.; Gross, R. A. *Brain Res.* **1980**, *200*, 93.

5. Das, I.; Khan, N. S.; Puri, B. K.; Sooranna, S. R.; de Belleroche, J.; Hirsch, S. R. *Biochem. Biophys. Res. Commun.* **1995**, *212*, 375.

6. Thomsen, L. L.; Iversen, H. K.; Lassen, L. H.; Olesen, J. *CNS Drugs* **1994**, *2*, 417.

7. Dorheim, M.-A.; Tracey, W. R.; Pollock, J. S.; Grammas, P. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 659.

8. Bhargava, H. N. *Gen. Pharmacol.* **1995**, *26*, 1049.

9. Seo, H. G.; Takata, I.; Nakamura, M.; Tatsumi, H.; Suzuki, K.; Fujii, J.; Taniguchi, N. *Arch. Biochem. Biophys.* **1995**, *324*, 41.

10. Kubes, P.; Suzuki, M.; Granger, D. N. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4651.

11. MacIntyre, I.; Zaidi, M.; Towhidul Alam, A. S. M.; Datta, H. K.; Moonga, B. S.; Lidbury, P. S.; Hecker, M.; Vane, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 2936.

12. Ross, C. A.; Bredt, A.; Snyder, S. H. *Trends Neurosci.* **1990**, *13*, 216.

13. Shibuki, K.; Okada, D. *Nature (London)* **1991**, *349*, 326.

14. Burnett, A. L.; Lowenstein, C. J.; Bredt, D. S.; Chang, T. S. K.; Snyder, S. H. *Science* **1992**, *257*, 401.

15. Feldman, P. L.; Griffith, O. W.; Stuehr, D. J. *Chem. Eng. News* **1993**, *71* , 26.

16. Hofmann, H.; Schmidt, H. H. H. W. *Biochemistry* **1995**, *34*, 13443.

17. White, K.; Marletta, M. A. *Biochemistry* **1992**, *31*, 6627.

18. Marletta, M. A. *J. Biol. Chem.* **1993**, *268*, 12231.

19. Akhtar, M.; Corina, D.; Miller, S.; Shyadehi, A. Z.; Wright, J. N. *Biochemistry* **1994**, *33*, 4410.

20. (a) Olken, N. M.; Marletta, M. A. *Biochemistry* **1993**, *32*, 9677. (b) Olken, N. M.; Osawa, Y.; Marletta, M. A. *Biochemistry* **1994**, *33*, 14784.

21. Olken, N. M.; Marletta, M. A. *J. Med. Chem.* **1992**, *35*, 1137.

22. (a) Wolff, D. J.; Lubeskie, A. *Arch. Biochem. Biophys.* **1995**, *316*, 290. (b) Wolff, D. J.; Lubeskie, A. *Arch. Biochem. Biophys.* **1996**, *325*, 227.

23. Dwyer, M. A.; Bredt, D. S.; Snyder, S. H. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 1136.

24. Osawa, Y.; Davila, J. C. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 1435.

25. McCall, T. B.; Feelisch, M.; Palmer, R. M. J.; Moncada, S. *Br. J. Pharmacol.* **1991**, *102*, 234.

26. Moore, W. M.; Webber, R. K.; Jerome, G. M.; Tjoeng, F. S.; Misko, T. P.; Currie, M. G. *J. Med. Chem.* **1994**, *37*, 3886.

27. Zhang, H. Q.; Dixon, R. P.; Marletta, M. A.; Silverman, R. B., *J. Am. Chem. Soc.* **1997**, *119*, in press..

28. (a) Ortiz de Montellano, P. R.; Kunze, K. L. *J. Biol. Chem.* **1980**, *255*, 5578. (b) Ortiz de Montellano, P. R.; Komives, E. A. *J. Biol. Chem.* **1985**, *260*, 3330.

29. Dixon, M. *Biochem. J.* **1953**, *55*, 170.

30. Cornish-Bowden, A. *Biochem. J.* **1974**, *137*, 143.

31. Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC: Boca Raton, FL, 1988.

32. Pufahl, R. A.; Nanjappan, P. G.; Woodard R.; Marletta, M. A. *Biochemistry* **1992**, *31*, 6822.

33. Cheng, Y.; Lupo, A. T.; Fowler, F. W. *J. Am. Chem. Soc.* **1983**, *105*, 7696.

34. Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. *Biochemistry* **1993**, *32*, 8512.

35. Hevel, J. M., White, K. A., Marletta, M. A. *J. Biol. Chem.* **1991**, *266*, 22789.

36. Hevel, J. M.; Marletta, M. A. *Methods Enzymol.*, **1994**, *233*, 250.

37. Fast, W.; Huff, M. E., Silverman, R. B. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1449.

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