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Deuterium Isotope Effects and Product Studies for the Oxidation of N^{ω}-Allyl-L-arginine and N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine by Neuronal Nitric Oxide Synthase

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Abstract—The nitric oxide synthases (NOS), which require heme, tetrahydrobiopterin, FMN, FAD, and NADPH, catalyze the O_2-dependent conversion of L-arginine to L-citrulline and nitric oxide. N^{ω}-Allyl-L-arginine, a mechanism-based inactivator of neuronal NOS, also is a substrate, producing L-arginine, acrolein, and H_2O (Zhang, H. Q.; Dixon, R. P.; Marletta, M. A.; Nikolic, D.; Van Breemen, R.; Silverman, R. B. *J. Am. Chem. Soc.* **1997**, *119*, 10888). Two possible mechanisms for this turnover are proposed, one initiated by allyl C–H bond cleavage and the other by guanidino N–H cleavage, and these mechanisms are investigated with the use of N^{ω}-allyl-L-arginine (**1**), N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (**7**), N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**) and N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (**8**) as substrates. Significant isotope effects on the two kinetic parameters, k_{cat} and k_{cat}/K_m, are observed in case of **1** and **7** during turnover, but not with **2** and **8**. No kinetic isotope effects are observed for either compound in their role as inactivators. These results support a mechanism involving initial C–H bond cleavage of N^{ω}-allyl-L-arginine followed by hydroxylation and breakdown to products. © 2000 Published by Elsevier Science Ltd.

Introduction

Nitric oxide synthase (NOS, EC 1.14.13.39) requires five different coenzymes, NADPH, FAD, FMN, heme, and tetrahydrobiopterin, in a mixed function oxidase reaction that uses molecular oxygen to convert L-arginine to L-citrulline and the second messenger, nitric oxide.[1] Nitric oxide is implicated in a variety of biological functions[2] that can be correlated with one of three isoforms of NOS: inducible NOS (iNOS, immune response), endothelial NOS[3] (eNOS, smooth muscle relaxation), and neuronal NOS[4] (nNOS, neuronal signaling). However, overproduction of nitric oxide has been a factor in numerous disease states,[5] including cerebral ischemia, migraines, septic shock, Parkinson's disease, and arthritis; therefore, the design of selective NOS inhibitors has been of widespread interest.

Arginine analogues comprise the first class of potent reversible and irreversible inhibitors of NOS and include a variety of N^{ω}-alkyl substituted arginines with varying potencies and isoform selectivities.[6] N^{ω}-Allyl-L-arginine (**1**) was reported to be an inhibitor and inactivator of iNOS by Olken and Marletta,[7] and it was shown to be not only a competitive inhibitor, but a mechanism-based irreversible inactivator of nNOS.[8] Inactivation is initiated by *N*-hydroxylation of N^{ω}-allyl-L-arginine (**1**) to give N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**), which causes reductive allylation of the heme coenzyme. In addition to this inactivation reaction, N^{ω}-allyl-L-arginine also acts as a substrate for nNOS. N^{ω}-[1-^{3}H] Allyl-L-arginine and N^{ω}-allyl-L-[1-^{14}C]arginine were used to show that acrolein, water, and L-arginine are generated, and the amount of acrolein produced corresponds to the amount of water generated by loss of the α-^{3}H.[8] Two pathways were proposed for the formation of these turnover products (Scheme 1). Pathway (a) involves hydrogen atom abstraction from the α-C-H bond to give radical **3** and oxygen rebound to carbinolamine **4** (these may not be discrete steps), a typical pathway for cytochrome P450-catalyzed amine oxidation;[9] **4** decomposes to acrolein (**6**) and L-arginine. Pathway (b) follows the inactivation route of initial *N*-hydroxylation

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†Carried out all of the chemical and biological work except for the overexpression of nNOS.

‡Developed the nNOS overexpression system in *Escherichia coli* and the purification of the enzyme.

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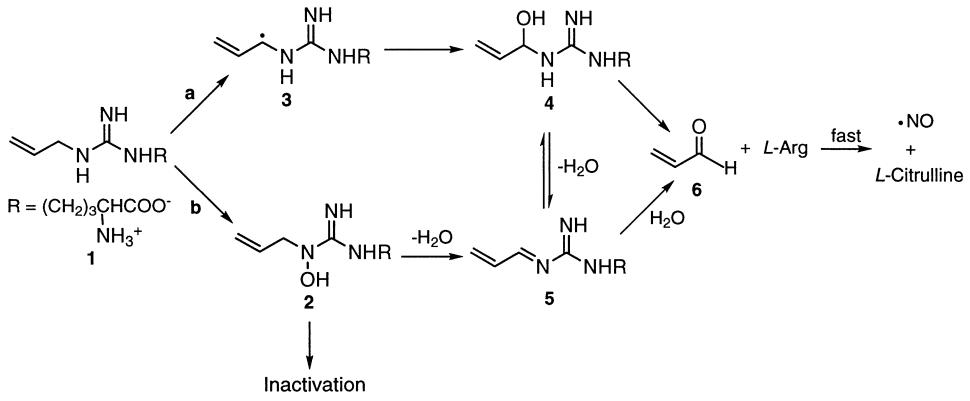
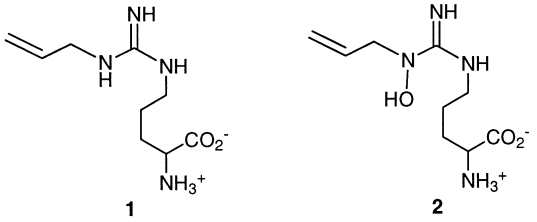


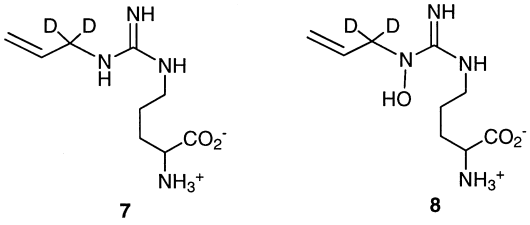
Scheme 1.

to **2**, which loses a molecule of water to give the conjugated guanidine **5**; hydrolysis of **5** produces acrolein and L-arginine.



Heme-dependent enzymes, such as cytochrome P450s, are known to catalyze both aliphatic and aromatic hydroxylations as well as *N*-hydroxylations, particularly of amines with low oxidation potentials.[9] Tetrahydrobiopterin-dependent enzymes are known to catalyze both aliphatic and aromatic hydroxylation.[10–12] Because most tetrahydrobiopterin enzymes also contain Fe^{2+}, it is thought that the actual hydroxylating species may be a hypervalent iron-oxo species, possibly generated by the tetrahydrobiopterin[13,14] (originally, it was thought that a tetrahydrobiopterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* did not require a metal ion for phenylalanine hydroxylation,[12] but that appears not to be the case[15]). To the best of our knowledge, the only tetrahydrobiopterin-dependent enzyme that is known to catalyze an *N*-hydroxylation reaction is NOS, but there has been some debate as to whether the hydroxylation is heme dependent[16] or tetrahydrobiopterin dependent.[17] Recently, Marletta and co-workers observed a tetrahydrobiopterin radical in the presence of L-arginine with the heme domain of inducible NOS.[18] The conclusion was that the tetrahydrobiopterin is an intermediary electron transfer cofactor between a flavin in the reductase domain and the heme in the oxygenase domain, but the tetrahydrobiopterin is not directly involved in substrate hydroxylation.

We report a NOS-catalyzed reaction of a substrate that undergoes both *C*- and *N*-oxidation. If the turnover of N^{ω}-allyl-L-arginine proceeds via pathway (a) in Scheme 1, the α-C–H bond cleavage step would be the rate-determining step in the entire process.[19] However, if pathway (b) is important, then *N*-hydroxylation or dehydration could be the rate-determining step. To differentiate whether pathway (a) or (b) is the principal pathway of NOS-catalyzed *N*-deallylation, N^{ω}-allyl-L-arginine (**1**), N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (**7**), N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**), and N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (**8**) were synthesized and the kinetics of nNOS-catalyzed oxidation of each were determined. The results support a mechanism via both pathways (a) and (b), but principally by pathway (a).



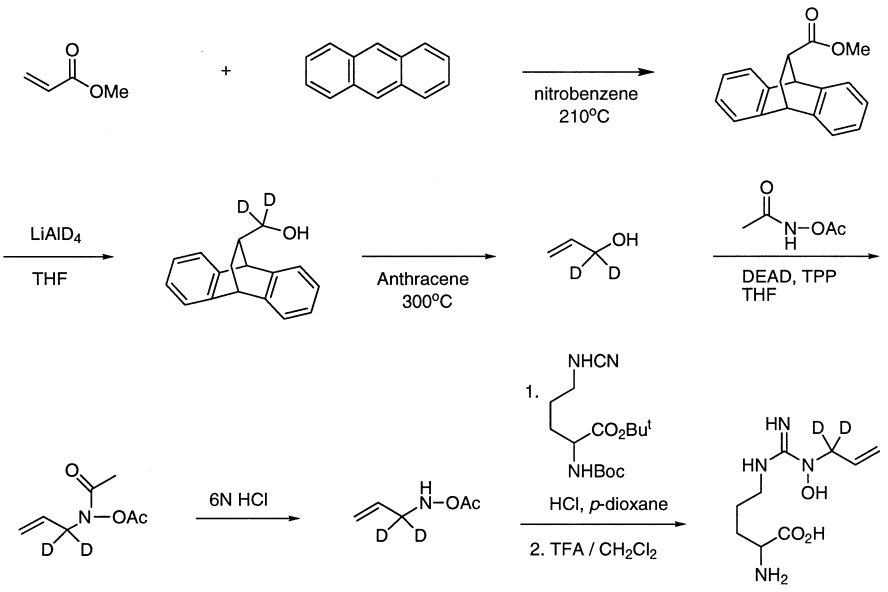
Results

Syntheses of N^{ω}-[1,1-$^2H_2$]Allyl-L-arginine (7) and N^{ω}-[1,1-$^2H_2$]Allyl-N^{ω}-hydroxy-L-arginine (8)

N^{ω}-[1,1-$^2H_2$]Allyl-L-arginine (**7**) was synthesized by the same route employed for N^{ω}-allyl-L-arginine (**1**),[8] except using [1,1-$^2H_2$]allyl amine.[20] N^{ω}-[1,1-$^2H_2$]Allyl-N^{ω}-hydroxy-L-arginine (**8**) was synthesized as previously reported for N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**),[8] using *N*-[1,1-$^2H_2$]allyl acetyl hydroxylamine, which was prepared by a Mitsunobu reaction (Scheme 2).

Kinetic constants for N^{ω}-allyl-L-arginine (1), N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (2), N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (7), and N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (8)

N^{ω}-Allyl-L-arginine (**1**) and N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**) are alternate substrates for nNOS. The apparent K_m and k_{cat} values for **1**, **2**, **7** and **8**, determined by the method of Hanes and Woolf[21] (Fig. 1), are given in Table 1. The isotope effect on k_{cat} for **7** is 3.4 (Table 2), but there is no isotope effect on the k_{cat} for **8**. The corresponding isotope effects on k_{cat}/K_m are 4.6 and 1.2, respectively. The products, NO_2^- and NO_3^-, were measured by the Griess method.[22] Nonenzymatically produced NO_2^-/NO_3^-, determined from identical assays without nNOS, was subtracted from each concentration. Inactivation kinetic constants also are given in Table 1.



Scheme 2.

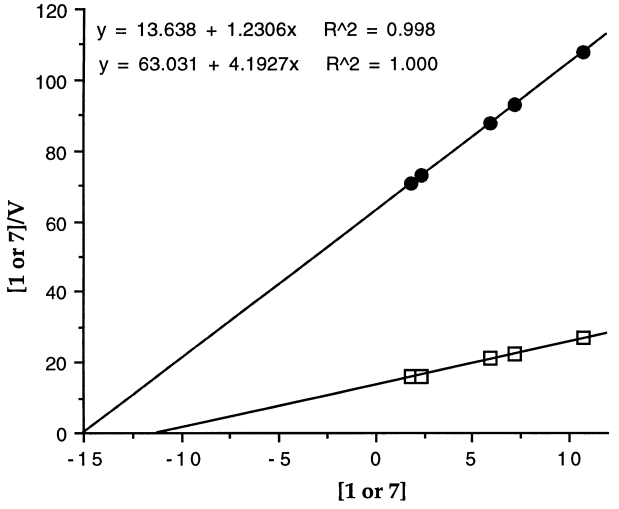


Figure 1. Hanes–Woolf plot for N^{ω}-allyl-L-arginine (**1**, □) and N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (**7**, ●).

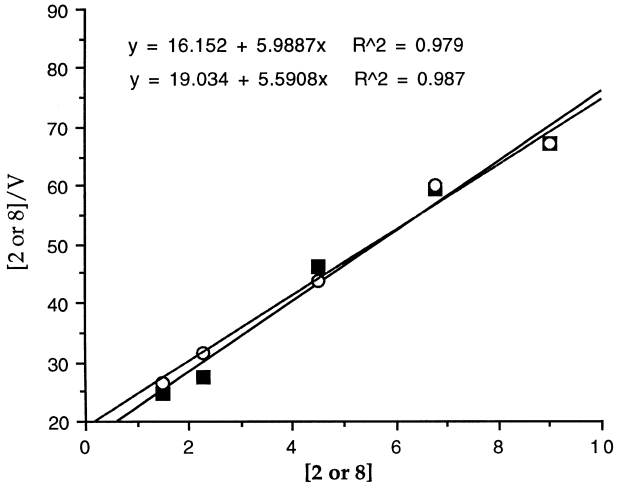


Figure 2. Hanes–Woolf plot for N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**, ■) and N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (**8**, ○).

Table 1. Kinetic constants for N^{ω}-allyl-L-arginine (**1**), N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (**7**), N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**), and N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (**8**)

Compound	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	K_I (μM)	k_{inact} (min^{-1})
1	11.1	3.48	0.31	19.0	0.095
7	15.0	1.03	0.07	20.0	0.096
2	2.70	0.73	0.27	3.49	0.091
8	3.40	0.77	0.23	3.47	0.090

Table 2. Kinetic deuterium isotope effects on kinetic parameters for the reaction of N^{ω}-allyl-L-arginine (NAA) and N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (NAHA) with neuronal nitric oxide synthase

Kinetic constants	Kinetic isotope effect	
	NAA	NAHA
$(K_m)_D/(K_m)_H$	1.351±0.003	1.271±0.148
$(k_{cat})_H/(k_{cat})_D$	3.379±0.042	1.055±0.028
$[k_{cat}/K_m]_H/[k_{cat}/K_m]_D$	4.604±0.082	1.207±0.173

Detection of acrolein as a coproduct of turnover

Following incubation of nNOS with **1** or **2**, detection of acrolein was accomplished by LC-ESMS as the 2,4-dinitrophenylhydrazone of the β-mercaptoethanol Michael adduct ($m+1/z$ of 315).

Discussion

Substrate turnover was measured by following the formation of nitric oxide (and its oxidation products)[22] from N^{ω}-allyl-L-arginine (**1**) and N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**). This assay is based on earlier work,[8] in

which it was observed that the oxidation of L-arginine, the initial product of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine oxidation, is faster than the oxidation of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine. Therefore, the rate-determining step is N^{ω}-allyl-N^{ω}-hydroxy-L-arginine oxidation, not L-arginine oxidation, and the rate of formation of nitric oxide is a measure of the rate of oxidation of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine. It also was found that oxidation of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine is faster than oxidation of N^{ω}-allyl-L-arginine, indicating that nitric oxide formation from N^{ω}-allyl-L-arginine also is a measure of the rate of allyl oxidation.[8] We were not able to monitor the rate of formation of acrolein, because it involves trapping the acrolein with β-mercaptoethanol followed by derivatization with 2,4-dinitrophenylhydrazine and HPLC detection, and accurate time-dependent formation of acrolein was not dependable.

N^{ω}-Allyl-L-arginine (**1**) and N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (**7**) are moderately good substrates for recombinant nNOS;[23] the ratio of k_{cat} values for formation of nitric oxide and L-citrulline by L-arginine relative to that by **1** is 8.2. There is a significant deuterium isotope effect on k_{cat} and k_{cat}/K_m (Tables 1 and 2). Isotope effects of 3.4 and 4.6 on the k_{cat} and k_{cat}/K_m values, respectively, suggest that cleavage of the α-C–H bond is at least partially rate-determining; isotope effects of about four are large enough to be consistent with complete rate control by C–H bond cleavage, for example, in the case of early or late transition states.

As a control for the isotope effect on turnover, and to corroborate the mechanism of inactivation of NOS by N^{ω}-allyl-L-arginine, which was shown to occur by initial N-H bond cleavage and formation of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**),[8] the isotope effect on inactivation of nNOS by **1** and **7** was investigated as well as inactivation by **2** and **8**. No isotope effect was observed on inactivation (either on K_I or k_{inact}) by either **7** or **8** (Table 1), consistent with the observed formation of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine as an intermediate to inactivation and the proposed inactivation mechanism, which does not involve α-C–H bond cleavage.[8]

Furthermore, no isotope effect was observed on the turnover of N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (**8**), indicating that the dehydration step in pathway (b) is not the rate-limiting step in the turnover of **2** and **8**. This is reasonable because *N*-hydroxylation to **2** should be an energetic step, but loss of water to the conjugated **5** should be facile. Compound **2** was stable in the incubation medium in the absence of NOS, implying that dehydration of **2** to **5** is an active site-directed enzymatic process. Therefore, the lack of an isotope effect observed on turnover of **2** suggests that pathway (a) in Scheme 1 is the major pathway for turnover of **1**. However, the fact that acrolein (as the 2,4-dinitrophenylhydrazone of the β-mercaptoethanol adduct) and nitric oxide are observed as products of nNOS-catalyzed oxidation of **2** indicates that pathway (b) is a minor pathway for oxidation of **1**.

Previously, it was found that N^{ω}-methyl-L-arginine also was a substrate and inactivator of NOS; in that case, the corresponding oxidation product, formaldehyde, was isolated,[24] indicating that this *N*-dealkylation reaction is general for N^{ω}-substituted-L-arginines. As in the case of N^{ω}-allyl-L-arginine, however, formation of formaldehyde from NOS-catalyzed oxidation of N^{ω}-methyl-L-arginine can occur by either of the mechanisms shown in Scheme 1.

Because there is an isotope effect on k_{cat} for **7** relative to **1**, but no isotope effect on k_{inact} for **7** relative to **1**, the partition ratio (the ratio of turnover to inactivation or k_{cat}/k_{inact}) decreases from 37 to 11 (i.e. more inactivation occurs), as expected for partitioning from a common ES complex. α-Deuteration shifts the flux from C–H cleavage toward an increase in *N*-oxidation (metabolic switching), leading to **2** and inactivation (a lower partition ratio), instead of C–H cleavage to **3** and turnover. Further support that pathway (b) is a minor pathway for turnover comes from the lack of an effect on the partition ratio by **8** versus **2**.

The ratio of the k_{cat} for **1**, which represents *C*-hydroxylation (actually $k_{cat}-k_{inact}$, because one equivalent of nitric oxide is produced per inactivation event) to the k_{cat} (again $k_{cat}-k_{inact}$) for **2**, which represents *N*-hydroxylation, is 5.3. This number represents the lower limit for the formation of nitric oxide via pathway (a) relative to pathway (b) in Scheme 1, because starting from **2** gives the saturated rate, but in starting from **1** there is some leakage via pathway (b).

Conclusion

The lack of a deuterium isotope effect on product formation by N^{ω}-[1,1-$^2H_2$]allyl-N^{ω}-hydroxy-L-arginine (**8**) versus N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**) suggests that the dehydration step in pathway (b) of Scheme 1 is not rate determining. Therefore, the kinetic isotope effect displayed by the oxidation of N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (**7**) relative to N^{ω}-allyl-L-arginine (**1**) supports a *C*-hydroxylation mechanism (pathway (a)) as the major pathway in the turnover. However, observation of the products nitric oxide and acrolein from NOS-catalyzed oxidation of **2** and **8** indicates that *N*-hydroxylation (pathway (b)) is a minor pathway.

Experimental

General methods

NMR spectra were recorded on either a Varian Inova 500 MHz or a Varian Gemini 300 MHz spectrometer. Chemical shifts are reported as δ values in parts per million downfield from Me_4Si as the internal standard in $CDCl_3$. Thin-layer chromatography was performed on EM/UV silica gel plates with a UV indicator. Mass spectra were obtained on a VG Instruments VG70-250E high-resolution spectrometer. Electrospray mass spectra were measured with a Micromass Quattro II mass spectrometer. UV spectra were recorded on a Perkin–Elmer Lambda 10 UV–vis spectrometer. Column chromatography was

performed with Merck silica gel (230–400 mesh). Chemicals were purchased from Aldrich Chemical Co. Biochemicals and coenzymes were purchased from Sigma Chemical Co.

N^{ω}-[1,1-$^2H_2$]Allyl-L-arginine (7). To a stirred solution of N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester[8] (480 mg, 1.53 mmol) and [1,1-$^2H_2$] allyl amine toluic acid salt (598 mg, 3.06 mmol)[20] in ethanol (20 mL) was added triethylamine hydrochloride (421.2 mg, 3.06 mmol), and the reaction mixture was heated to reflux for 48 h. After the reaction was completed, the volatile materials were removed by rotary evaporation, resulting in a yellowish oil, which was chromatographed on silica gel (180 g, 6:1 methylene chloride:methanol), yielding 403 mg (1.08 mmol, 35%) of N^{α}-Boc-N^{ω}[1,1-$^2H_2$]-L-arginine *tert*-butyl ester. To 390 mg (1.05 mmol) of this ester was added trifluoroacetic acid (5.5 mL) and methylene chloride (5.5 mL), and the reaction mixture was allowed to stir for 2 h. After the reaction was completed, the volatile materials were removed by rotary evaporation, resulting in a yellowish oil, which was chromatographed on a Dowex 50W-X8 cation exchange column (resin 200–400 mesh, H+ form), washing the column with water (50 mL) and then eluting the product from the column with 0.25 M ammonium hydroxide. The water was removed by lyophilization, yielding 150 mg (0.69 mmol, 65%) of **7**. TLC, ^{1}H NMR, and HPLC analyses demonstrated that the compound was 98% chemically and isotopically pure; ^{1}H NMR (D_2O) δ 5.761 (dd, 1H, $J=17$, 10.25 Hz), 5.148 (m, 2H), 3.436 (t, 1H, $J=10.0$ Hz), δ 3.137 (t, 2H, $J=7.0$ Hz) 1.486–1.680 (m, 4H). HRMS (APCI) calcd for $C_9H_{16}N_4O_2D_2$ (MH^+) 217.1628, found 217.1603.

N^{ω}-[1,1-$^2H_2$]Allyl-N^{ω}-hydroxy-L-arginine (8). Compound **8** was synthesized and purified following the synthesis of N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**2**),[8] except using *N*-[1,1-$^2H^2$]allyl acetyl hydroxylamine instead of *N*-allyl acetyl hydroxylamine. *N*-[1,1-$^2H_2$]allyl acetyl hydroxylamine was prepared from *N*-[1,1-$^2H_2$]allyl *N*,*O*-diacetyl hydroxylamine (see below).[25] TLC, ^{1}H NMR, and HPLC analyses demonstrated that the the compound was 98% chemically and isotopically pure; ^{1}H NMR (D_2O) δ 5.781 (m, 1H), 5.234 (m, 2H), 3.887 (t, 1H, $J=6.0$ Hz), 3.246 (t, 2H, $J=6.5$ Hz) 1.822–1.905 (m, 2H), 1.592–1.712 (m, 2H). HRMS (APCI) calcd for $C_9H_{16}N_4O_3D_2$ (MH^+) 233.1577, found 233.1584.

***N*-[1,1-$^2H_2$]Allyl-*N*,*O*-diacetyl hydroxylamine.** To a magnetically-stirred solution of [1,1-$^2H_2$]allyl alcohol[26] (1 g, 16.7 mmol), triphenylphosphine (4.37 g, 16.7 mmol), and diethylazodicarboxylate (3.49 g, 16.7 mmol) in dry tetrahydrofuran, was added a solution of *N*,*O*-diacetylhydroxyl amine (1.95 g, 16.7 mmol)[27] over a period of 15 min and stirred for about 24 h. When the solvent was removed from the reaction mixture on a rotary evaporator under reduced pressure, a thick, oily, yellow liquid was obtained which was chromatographed on silica gel (900 g, EtOAc:*n*-hexane 1:3), yielding 1.425 g (8.96 mmol, 54%) of *N*-[1,1-$^2H_2$]allyl *N*,*O*-diacetyl hydroxylamine. ^{1}H NMR ($CDCl_3$) δ 5.78 (m, 1H), 5.20 (m, 2H), 2.20 (s, 3H), 2.05 (s, 3H).

Expression and purification of recombinant nNOS. A recombinant rat neuronal NOS overexpression system in *E. coli* was used,[23] and the enzyme was purified as described.[28]

Assay for enzymatic activity by analysis of nitrite and nitrate. A pre-incubation mixture containing nNOS (1.2 nmol), $CaCl_2$ (10 mM), calmodulin (70 μg/mL), NADPH (0.93 mM), tetrahydrobiopterin (0.07 μM), DTT (1 mM), Hepes buffer (50 mM, pH 7.5), and N^{ω}-allyl-L-arginine or N^{ω}-[1,1-$^2H_2$]allyl-L-arginine (1.78, 2.37, 5.94, 7.13, 10.7 μM) in a total volume of 700 μL was incubated at room temperature. After the homogenization was completed (around 1 min of stirring), 100 μL of the reaction mixture was removed every minute (seven times) and was quenched by heating in hot water (90 °C). The production of nitric oxide under each condition was measured by analyzing the concentration of the sum of the nitrite and nitrate in these seven reaction aliquots. Nitric oxide was analyzed using the LDH method of the nitrate/nitrite assay kit from Cayman Chemicals, Ann Arbor, MI. An aliquot (90 μL from 100 μL) of the sample was diluted in water (HPLC grade, 300 μL) and was transferred to a cuvette, and then nitrate reductase (15 μL, nitrate/nitrite assay kit, LDH Method from Cayman Chemicals, Ann Arbor, MI) and NADPH (1 mM, 15 μL) were added. The cuvette was covered, and the mixture was incubated for 4.5 h at room temperature. Lactate dehydrogenase (from the same kit, 15 μL) and its cofactors (from the same kit, 15 μL) were added to destroy the excess amount of NADPH, which could interfere with the chemistry of the Griess reagents, then it was incubated for another 30 min. The Griess reagents (150 μL, 1% sulfanilamide, 0.1% (naphthyl)ethylene diamine dihydrochloride, and 5% H_3PO_4 from the same kit) were added to the cuvette, the color was allowed to develop for 10 min at room temperature, and absorbances were recorded at 540 nm. A standard curve was constructed with known concentrations of nitrate and nitrite to convert the change of absorbances to nitrite concentrations.

Acknowledgements

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References and Notes

1. (a) Nathan, C. *FASEB J.* **1992**, *6*, 3051. (b) Marletta, M. A. *J. Biol. Chem.* **1993**, *268*, 12231.
2. Kerwin, J. F.; Lancaster, J. R.; Feldman, P. L. *J. Med. Chem.* **1995**, *38*, 4343.
3. Stuehr, D. S.; Fashehun, O. A.; Kwon, N. S.; Gross, S. S.; Gonzalez, J. A.; Levi, R.; Nathan, C. F. *FASEB J.* **1991**, *5*, 98.
4. Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. *Biochemistry* **1993**, *32*, 8512.
5. Kerwin, J. F.; Heller, M. *Med. Res. Rev.* **1994**, *14*, 23.
6. (a) Zhang, H. Q.; Fast, W.; Marletta, M. A.; Martasek,P.; Silverman. R. B. *J. Med. Chem.* **1997**, *40*, 3869. (b) Stuehr, D. J.; Griffith, O. W. *Adv. Enzymol.* **1992**, *65*, 287.

7. Olken, N. M.; Marletta, M. A. *J. Med. Chem.* **1992**, *35*, 1137.
8. Zhang, H. Q.; Dixon, R. P.; Marletta, M. A.; Nikolic, D.; Van Breeman, R.; Silverman, R. B. *J. Am. Chem. Soc.* **1997**, *119*, 10888.
9. Silverman, R. B. *The Organic Chemistry of Drug Design and Drug Action*. Academic: San Diego, 1992, p 289.
10. Guroff, G.; Daly, J.; Jerina, D. M.; Renson, J.; Witkop, B.; Udenfriend, S. *Science* **1967**, *157*, 1524.
11. Siegmund, H.; Kaufman, S. *J. Biol. Chem.* **1991**, *266*, 2903.
12. Carr, R. T.; Balasubramanian, S.; Hawkins, P. C. D.; Benkovic, S. J. *Biochemistry* **1995**, *34*, 7525.
13. Dix, T. A.; Benkovic, S. J. *Acc. Chem. Res.* **1988**, *21*, 101.
14. Hillas, P. J.; Fitzpatrick, P. F. *Biochemistry* **1996**, *35*, 6969.
15. Chen, D.; Frey, P. A. *J. Biol. Chem.* **1998**, *273*, 25594.
16. (a) Presta, A.; Weber-Main, A. M.; Stankovich, M. T.; Stuehr, D. J. *J. Am. Chem. Soc.* **1998**, *120*, 9460. (b) Clague, M. J.; Wishnok, J. S.; Marletta, M. A. *Biochemistry* **1997**, *36*, 14465.
17. Perry, J. M.; Marletta, M. A. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11101.
18. Hurshman, A. R.; Krebs, C.; Edmondson, D. E.; Huynh, B. H.; Marletta, M. A. *Biochemistry* **1999**, *38*, 15689.
19. (a) Miwa, G. T.; Walsh, J. S.; Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1983**, *258*, 14445. (b) Guengerich, F. P.; Yun, C.-H; Macdonald, T. L. *J. Biol. Chem.* **1996**, *271*, 27321. (c) Okazaki, O.; Guengerich, F. P. *J. Biol. Chem.* **1993**, *268*, 1546. (d) Manchester, J. I.; Dinnocenzo, J. P.; Higgins, L.; Jones, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 5069. (e) Karki, S. B.; Dinnocenzo, J. P.; Jones, J. P.; Korzekwa, K. R. *J. Am. Chem. Soc.* **1995**, *117*, 3657.
20. Coustumer, F. L.; Lautie, M. F.; Dizabo, P. *J. Labelled Comp. Radiopharm* **1973**, *4*, 355.
21. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*. Portland: London, 1995.
22. Green, L. C.; Wagner, D. A.; Glogowaski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **1982**, *126*, 131.
23. Roman, L. J.; Sheta, E. A.; Martasek, P.; Gross, S. S.; Liu, Q.; Masters, B. S. S. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8428.
24. Olken, N. M.; Marletta, M. A. *Biochemistry* **1993**, *32*, 9677.
25. Synthesis of the non-deuterated analogue: Cheng, Y. S.; Lupo, A. T., Jr.; Fowler, F. W. *J. Am. Chem. Soc.* **1983**, *105*, 7696.
26. Salomon, R. G.; Reuter, J. M. *J. Am. Chem. Soc.* **1977**, *99*, 4372.
27. Exner, O.; Horak, M. *Collect. Czech. Chem. Commun.* **1959**, *24*, 2992.
28. Gerber, N. C.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1995**, *270*, 17791.