Mechanism of Inactivation of Neuronal Nitric Oxide Synthase by N^{ω}-Allyl-L-Arginine

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Abstract: N^{ω}-Allyl-L-arginine is shown to be a competitive reversible inhibitor and time-dependent inactivator of bovine brain nitric oxide synthase (nNOS). The enzyme is protected against inactivation by the presence of the substrate, the absence of O_2, or if $NADP^+$ is substituted for NADPH. The NADPH absorption spectrum is converted to that of $NADP^+$ concomitant with inactivation. The latter two results indicate that redox chemistry is required for inactivation. N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine is synthesized and shown also to be a competitive inhibitor and time-dependent inactivator of nNOS, suggesting that it is a viable intermediate in the inactivation process. Inactivation of nNOS with N^{ω}-allyl-[^{14}C]-L-arginine or N^{ω}-[^{3}H]allyl-L-arginine followed by gel filtration or dialysis results in no radioactivity bound to the enzyme. It is shown spectrophotometrically as well as by HPLC that the heme is modified to four different species during inactivation. Inductively coupled plasma atomic emission spectrophotometry is used to show that 1 equiv of ferric ion is present in the modified hemes. When the heme is isolated after inactivation by the two radiolabeled inactivators, it is found that no ^{14}C is associated, but 0.9 equiv of ^{3}H is bound to the heme. This indicates that only the allyl part of the inactivator is bound to the heme. HPLC−electrospray mass spectrometry is used to show that the four modified hemes have the same mass which corresponds to heme plus an allyl group plus a hydrogen. The fact that the modified hemes no longer have an absorption at 400 nm but, instead, absorb at 280 nm suggests that four reduced and allylated hemes are produced (such as **34** or **35**). N^{ω}-Propyl-L-arginine also is shown to be a competitive inhibitor and time-dependent inactivator of nNOS; inactivation requires O_2 and NADPH, and the substrate protects the enzyme from inactivation. Twenty-six equivalents of citrulline and nitric oxide is produced during inactivation with N^{ω}-propyl-L-arginine. This suggests that the double bond of the allyl group is not important to the inactivation mechanism. Possible mechanisms that rationalize these results are suggested. No N^{ω}-allyl-L-citrulline (**39**) is detected from the inactivation of nNOS by N^{ω}-allyl-L-arginine, which indicates that initial hydroxylation of the guanidino imine nitrogen does not occur. The only radioactive metabolite generated from N^{ω}-allyl-[^{14}C]-L-arginine inactivation is citrulline (13 equiv). Approximately 13 equiv of nitric oxide also are generated. When N^{ω}-allyl-N^{ω}-hydroxy-L-arginine is the inactivator, about 20 equiv of citrulline and nitric oxide is produced. When N^{ω}-[^{3}H]allyl-L-arginine is the inactivator, approximately equal amounts of $^{3}H_2O$ and acrolein are produced (8−9 equiv); arginine also is a product. Acrolein does not inactivate nNOS. These last few findings support cleavage of the α-C−H bond of the allyl group as a turnover pathway which does not lead to inactivation.

Introduction

Nitric oxide, which is produced by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39), is now known to be an important cell signaling agent that regulates many different physiological functions, including blood pressure by regulation of smooth muscle relaxation;[1] platelet aggregation, thereby acting as an antithrombotic agent;[2] antitumor, antibacterial, and antiviral action of macrophages;[3] brain development, learning, and memory;[4] and it is the neuronal mediator of penile erection.[5] All of these functions, except for the macrophage activities, are the result of nitric oxide activation of soluble guanylate cyclase and the consequent increase in the concentration of cGMP.[6] However, since nitric oxide is a free radical, an excess of nitric oxide can cause deleterious effects, including post ischemic stroke damage;[7] septic shock;[8] seizures;[9] schizophrenia;[10] migraine headaches;[11] Alzheimer's disease;[12] tolerance to and

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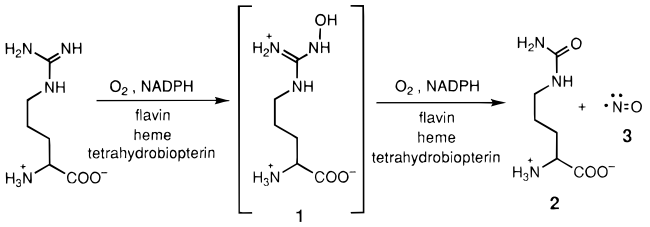
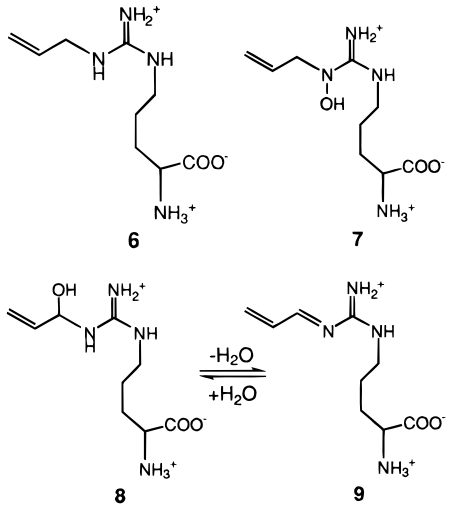
Scheme 1



dependence on morphine;[13] development of colitis;[14] tissue damage and inflammation;[15] overproduction of osteoclasts, leading to osteoporosis, Paget's disease, and rheumatoid arthritis;[16] destruction of photoreceptors in the retina;[17] long-term depression;[18] and priapism.[19] Because of the harmful effects of excess nitric oxide, inhibition of NO biosynthesis should be an important approach to the design of new drugs to treat these afflictions. However, its inhibition also could be detrimental to the other essential functions of nitric oxide. This dilemma has been somewhat abated because nitric oxide is not produced from a single source. There are (at least) three isoforms of nitric oxide synthase that appear to be present in three different types of cells. Neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are constitutive and Ca^{2+} ion- and calmodulin-dependent enzymes; macrophage nitric oxide synthase (iNOS) is inducible, requires transcription and translation, and copurifies with calmodulin bound.[20] Therefore, selective inhibition of one of the isoforms may lead to selective effects on nitric oxide action.

Nitric oxide synthases are unusual in the number of coenzymes that are associated with the apoenzyme: tetrahydrobiopterin, NADPH, FAD/FMN, and heme have all been identified as functional coenzymes.[21] The reaction catalyzed by NOS, the conversion of L-arginine to L-citrulline (**2**) and nitric oxide (**3**), is shown in Scheme 1. The oxygen atoms in nitric oxide and the urea carbonyl of L-citrulline are derived from molecular oxygen;[22] the nitrogen atom of nitric oxide is derived from the guanidine nitrogen of L-arginine.[23] The first step in the reaction appears to be *N*-hydroxylation of the guanidine group of L-arginine to give **1**.[24] The nitrogen atom of N^{ω}-hydroxy-L-arginine (**1**) ends up as the nitrogen in nitric oxide.[24,25] A variety of reasonable mechanisms for NOS have been suggested, but many are minor modifications of each other.[26] A mechanism that encompasses some aspects of each is shown in Scheme 2. The first step mimics standard heme-dependent hydroxylation reactions to give **1**, although it is not clear if heme is responsible for this oxygenation.[26e] The second step is similar to heme-dependent enzymes that catalyze reactions with carbonyl compounds.[27] Hydrogen atom abstraction followed by nucleophilic addition of the heme peroxide to the carbon−nitrogen double bond of **4** gives radical anion **5**, which can be written in two ways. Breakdown would give citrulline and nitric oxide.

Several years ago, Olken and Marletta[28] reported the inactivation of macrophage NOS by N^{ω}-allyl-L-arginine (**6**). It was suggested that either *N*-hydroxylation (**7**) or *C*-hydroxylation (**8**) may be important; dehydration of **8** would give the imine **9**. These hydroxylations could lead to typical pathways for



inactivation of an enzyme that catalyzes an oxidation reaction by an allyl-substituted compound. There appear to be four general inactivation mechanisms that allyl-substituted inactivators of redox enzymes follow. One mechanism (Scheme 3) is depicted by the inactivation of monoamine oxidase by allylamine.[29] This mechanism, also proposed for the inactivation of sarcosine oxidase by allylglycine,[30] involves an oxidation of the olefinic amine to an α,β-unsaturated imine, a Michael acceptor, which is a potent electrophile. A second inactivation mechanism involves radical abstraction followed by radical combination, as shown in Scheme 4 for dopamine β-monooxygenase inactivation by 3-arylpropenes.[31] This also is a possible pathway for the inactivation of monoamine oxidase by allylamine (Scheme 3). A third possibility, originally proposed for heme-dependent enzymes such as cytochrome P-450, is epoxidation of the alkene followed by nucleophilic attack of the heme

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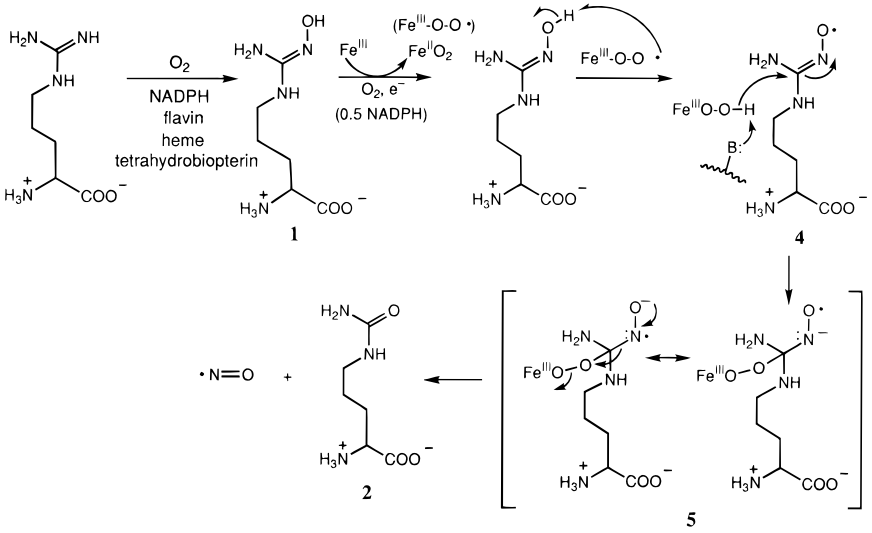
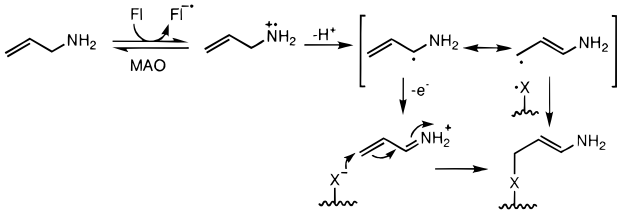
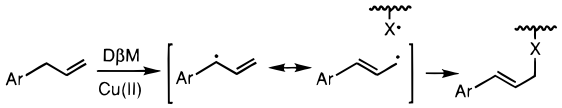
Scheme 2



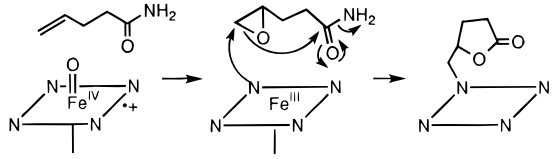
Scheme 3



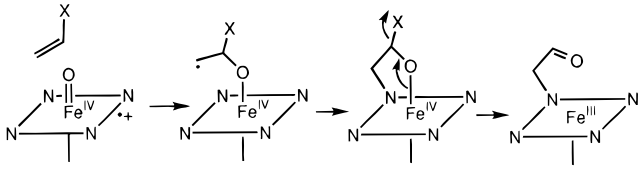
Scheme 4



Scheme 5



Scheme 6



at the epoxide (Scheme 5);[32a] however, epoxides have been shown not to react with cytochrome P-450.[32b] Instead, the more accepted mechanism for alkene inactivation of heme-dependent enzymes is oxygenation of the double bond followed by radical insertion into the heme, as shown for a series of vinyl halides in Scheme 6.[33]

On the basis of the mechanisms in Schemes 2−6 and the evidence that with N^{ω}-methyl-L-arginine[34,35] C−H bond cleavage is an important inactivation event, several potential mechanisms for the inactivation of NOS by N^{ω}-allyl-L-arginine can be considered (Schemes 7 and 8; the initial part of some of these routes were mentioned previously as possibilities[28,35]). Scheme 7 follows the proposed mechanism of substrate oxygenation, giving N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**7**), and then, after heme peroxo radical addition (pathway a), **10** breaks down to the allyl nitroso cation radical (**11**), similar to the methyl nitroso cation radical intermediate proposed as a possible cause for inactivation of NOS by N^{ω}-methyl-L-arginine.[35] Attack by an active-site nucleophile on the allyl double bond of **11** leads to displacement of nitric oxide and produces the allyl enzyme (**12**). Alternatively, from intermediate **7** (pathway b), the elements of water could be eliminated to give the Michael acceptor **13**, which could be attacked by an active-site nucleophile to give **14**. Hydrolysis of **14** gives **15** and arginine. Scheme 8 depicts a mechanism that is initiated by C−H bond cleavage (either by hydrogen atom abstraction or by single electron/proton transfer) and oxygen rebound to give **17**. Loss of water gives **13**, which can proceed as in pathway b of Scheme 7. Alternatively, the other resonance structure of **16** could undergo radical combination with an active site radical, leading to the same adduct as was obtained from nucleophilic addition to **13**. The results of the study described here indicate that none of these mechanisms is correct; an alternative pathway is suggested.

Results

Inhibition and Time-Dependent Inactivation of nNOS by N^{ω}-Allyl-L-arginine. N^{ω}-Allyl-L-arginine is a competitive inhibitor of nNOS with $K_i = 200$ nM against L-arginine (Figure 1). It also was shown to be a time-dependent inactivator of NOS (Figure 2). The kinetic constants are $K_I = 470$ nM and the $k_{inact} = 0.05$ min^{-1} at 0 °C. Because of the low stability of the enzyme in dilute solutions at 25 °C, all of the inactivation assays were carried out at 0 °C. No time-dependent inactivation occurred in the presence of 1.5 mM L-arginine and all cofactors (same as the control line). No inactivation occurred when $NADP^+$ was substituted for NADPH or in the absence of oxygen (both same as the control line). The addition of catalase or superoxide dismutase had no effect on the kinetics of inactivation.

Change in the NADPH Absorption Spectrum During Inactivation of nNOS by N^{ω}-Allyl-L-arginine. When excess

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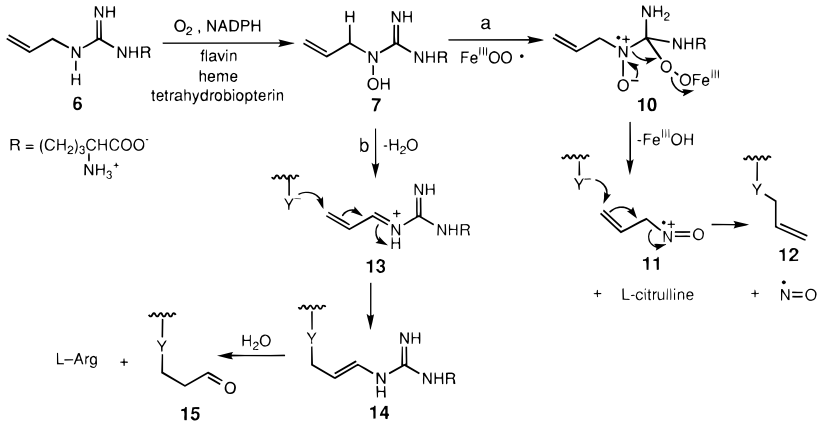
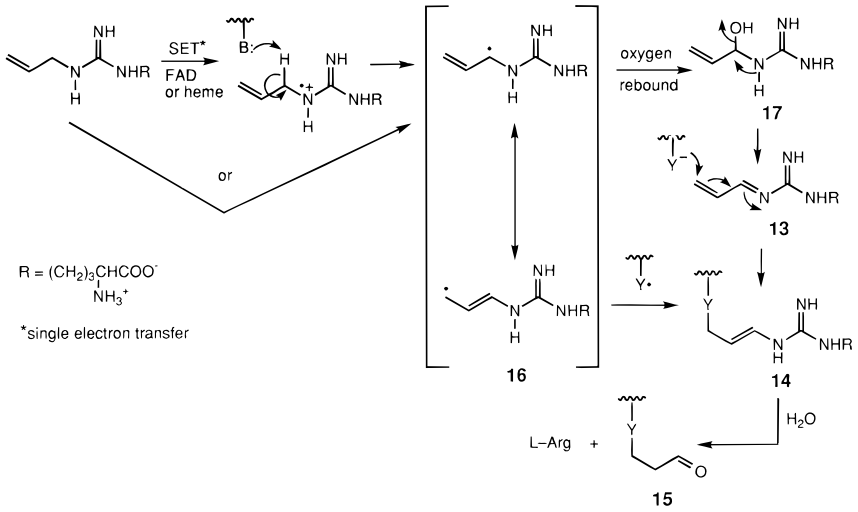
Scheme 7



Scheme 8



N^{ω}-allyl-L-arginine was used, the change in the absorption spectrum from that of NADPH to $NADP^+$ diminished as the enzyme became inactivated (Figure 3A); when enzyme activity was lost (Figure 3B), there was no further change in the NADPH absorption spectrum.

Inhibition and Time-Dependent Inactivation of nNOS by N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine. N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine is a competitive inhibitor of nNOS with a $K_i = 90$ nM (data not shown). It also was shown to be a time-dependent inactivator of NOS. The kinetic constants are $K_I = 29$ nM and $k_{inact} = 0.04\ min^{-1}$ at 0 °C (data not shown). No time-dependent inactivation occurred in the presence of 1.5 mM L-arginine and all cofactors, when $NADP^+$ was substituted for NADPH, or in the absence of oxygen. The addition of catalase or superoxide dismutase had no effect on the kinetics of inactivation.

Inactivation of nNOS with N^{ω}-Allyl-L-[1-^{14}C]arginine and N^{ω}-[1-^{3}H]Allyl-L-arginine. Inactivation of nNOS by N^{ω}-allyl-L-[1-^{14}C]arginine or N^{ω}-[1-^{3}H]allyl-L-arginine followed by rapid gel filtration[36] or dialysis to remove the excess inactivator led to the incorporation of no radioactivity bound to the enzyme.

Difference Absorption Spectrum of nNOS before and after Incubation with N^{ω}-Allyl-L-arginine. The type I difference binding spectrum of nNOS and nNOS in the presence of N^{ω}-allyl-L-arginine is shown in Figure 4, consistent with the interaction of N^{ω}-allyl-L-arginine at the substrate-binding site.

Change in the Heme Absorption Spectrum During Inactivation of nNOS by N^{ω}-Allyl-L-arginine. The heme absorption spectrum between 340 and 520 nm diminished in a time-dependent manner during inactivation of nNOS by N^{ω}-allyl-L-arginine (Figure 5).

Heme Destruction During Inactivation of nNOS by N^{ω}-Allyl-L-arginine. HPLC analysis (400 nm detection) of nNOS after inactivation by N^{ω}-allyl-L-arginine indicated that the heme was mostly destroyed (Figure 6).

Heme Modification after Inactivation of nNOS by N^{ω}-Allyl-L-arginine. HPLC with detection at 280 nm of nNOS after inactivation by N^{ω}-allyl-L-arginine showed what appear to be four peaks with t_R between 48 and 59 min (Figure 7), suggestive of four modified hemes. Figure 9A shows a clearer chromatogram with four distinct peaks.

Determination of the Iron Content of the Modified Hemes. To ascertain whether the compounds isolated in the 48−59 min fractions are really hemes, the iron content was determined by inductively coupled plasma atomic emission spectroscopy. On the basis of a calibration curve for iron concentrations, the iron content of the control (fractions 48−59 min when buffer was injected into the HPLC) was 0.02 ppm and the iron content of the modified hemes was 0.29 ppm. Therefore, the total iron content of fractions 48−59 min is 0.27 ppm. A 1:1 adduct with the heme should contain 0.25 ppm iron. Therefore, the total iron content of the four modified hemes is 1 equiv.

Radioactivity Bound to Modified Hemes after Inactivation of nNOS with N^{ω}-Allyl-L-[1-^{14}C]arginine and N^{ω}-[1-^{3}H]Allyl-L-arginine. The four modified hemes produced by inactivation of nNOS by N^{ω}-allyl-L-[1-^{14}C]arginine or N^{ω}-[1-^{3}H]allyl-L-arginine (fractions 48−59 min) were isolated by HPLC and counted for radioactivity. When N^{ω}-allyl-L-[1-^{14}C]arginine was

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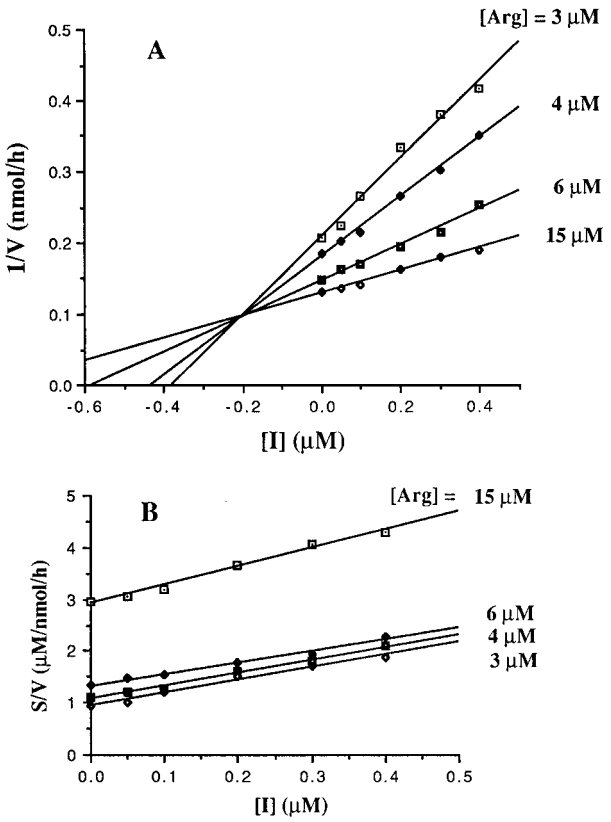


Figure 1. Dixon[52] (A) and Cornish-Bowden[58] (B) plots for the inhibition of nNOS by N^{ω}-allyl-L-arginine (**6**). See the Experimental Section for details.

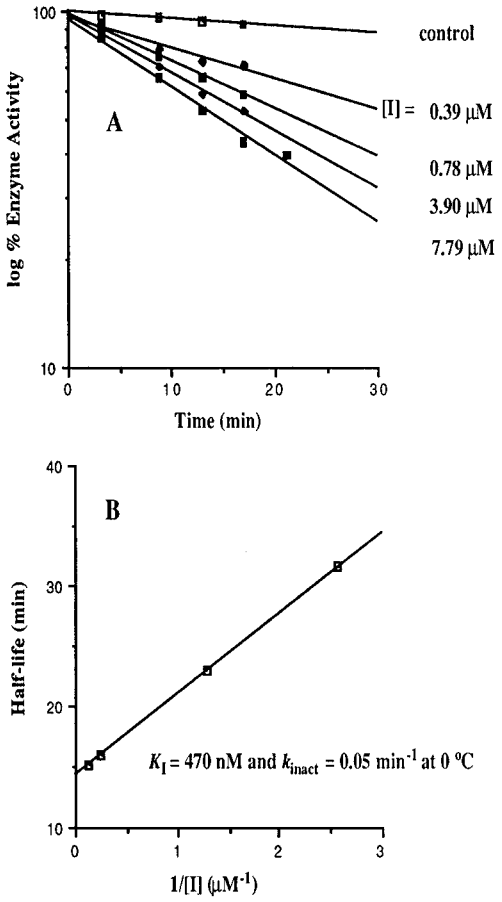


Figure 2. Time-dependent inactivation of nNOS by N^{ω}-allyl-L-arginine (A) and Kitz and Wilson[53] replot of the inactivation data. See the Experimental Section for details.

the inactivator, no radioactivity comigrated with the modified hemes, but when N^{ω}-[1-^{3}H]allyl-L-arginine was the inactivator, 0.9 equiv (425 dpm) of radioactivity comigrated.

HPLC−Electrospray Ionization Mass Spectra of Unmodified and Modified Hemes. nNOS was inactivated by N^{ω}-allyl-L-arginine and the modified hemes in the 48−59 min fractions of the HPLC were isolated and then subjected to HPLC−electrospray ionization mass spectrometry (under different elution conditions than in Figure 7). A control was run in which $NADP^+$ was substituted for NADPH, which does not lead to modified hemes. The HPLC trace (A) and the mass spectrum (B) of the control are shown in Figure 8; the $(m + 1)/z$ for the isolated heme is 616. The HPLC of the modified hemes (A) and the mass spectra of each of the four peaks (B) are presented in Figure 9; all four of the peaks corresponds to the same $(m + 1)/z$ of 658.

Turnover of N^{ω}-Allyl-L-arginine and N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine. The only metabolite detected after inactivation of nNOS by N^{ω}-allyl-L-arginine or N^{ω}-allyl-N^{ω}-hydroxy-L-arginine was L-citrulline. When N^{ω}-allyl-L-[1-^{14}C]arginine was used as the inactivator, the only two radioactive fractions detected by HPLC were citrulline (7000 dpm) and N^{ω}-allyl-L-arginine (**6**) (9500 dpm) (Figure 10). When NADPH was replaced by $NADP^+$, no citrulline was formed. The addition of catalase or superoxide dismutase had no effect on the formation of citrulline. With unlabeled N^{ω}-allyl-L-arginine, citrulline formation was observed by conversion first to the *o*-phthalaldehyde (OPA) derivative and detection by fluorescence monitoring. Under the same conditions, the amount of citrulline formed from inactivation of nNOS by N^{ω}-allyl-N^{ω}-hydroxy-L-arginine was 50% larger than that produced by N^{ω}-allyl-L-arginine (Table 1).

Production of Nitric Oxide During Inactivation of nNOS by N^{ω}-Allyl-L-arginine, N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine. Nitric oxide, measured as the sum of nitrite ion and nitrate ion, was generated during inactivation of nNOS by N^{ω}-allyl-L-arginine and N^{ω}-allyl-N^{ω}-hydroxy-L-arginine. A comparison of the amount of nitric oxide and citrulline generated during inactivation is shown in Table 1. For N^{ω}-allyl-L-arginine, the amount of citrulline was determined by scintillation counting because the inactivator was radiolabeled. For N^{ω}-allyl- N^{ω}-hydroxy-L-arginine, the amount of citrulline formed was determined from an HPLC analysis. On the basis of the amount of citrulline formed, the partition ratio (the amount of product generated per inactivation event) for N^{ω}-allyl-L-arginine is 13 and that for N^{ω}-allyl-N^{ω}-hydroxy-L-arginine is 20.

Inhibition and Time-Dependent Inactivation of nNOS by N^{ω}-Propyl-L-arginine. N^{ω}-Propyl-L-arginine was found to be a competitive inhibitor of nNOS with a $K_i = 57$ nM (data not shown). It also was a time-dependent inactivator of NOS (data not shown). The kinetic constants are $K_I = 19.8$ nM and the $k_{inact} = 0.0059$ min^{-1} at 0 °C. No time-dependent inactivation occurred in the presence of 1.5 mM L-arginine, when $NADP^+$ was substituted for NADPH, or in the absence of oxygen.

Production of Citrulline and Nitric Oxide During Inactivation of nNOS by N^{ω}-Propyl-L-arginine. Under the same conditions as with N^{ω}-allyl-L-arginine and N^{ω}-allyl-N^{ω}-hydroxy-L-arginine, 4000 pmol of citrulline and 3921 pmol of NO (measured as NO_2^- and NO_3^-) were generated during inactivation of NOS by N^{ω}-propyl-L-arginine; the partition ratio, therefore, is 26.

Production of [^{3}H]Water During Inactivation of nNOS by N^{ω}-[1-^{3}H]Allyl-L-arginine. Following inactivation of nNOS by N^{ω}-[1-^{3}H]allyl-L-arginine, Dowex separation of the nonamines, and bulb-to-bulb distillation of the tritiated eluent, 8

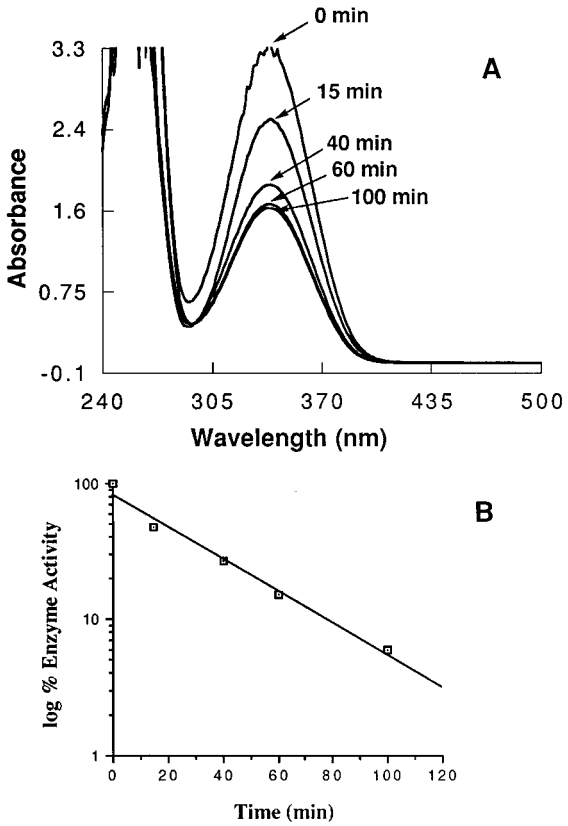


Figure 3. (A) Change in the NADPH absorption spectrum during incubation of nNOS with excess N^{ω}-allyl-L-arginine. (B) Concomitant loss of enzyme activity corresponding to A. See the Experimental Section for details.

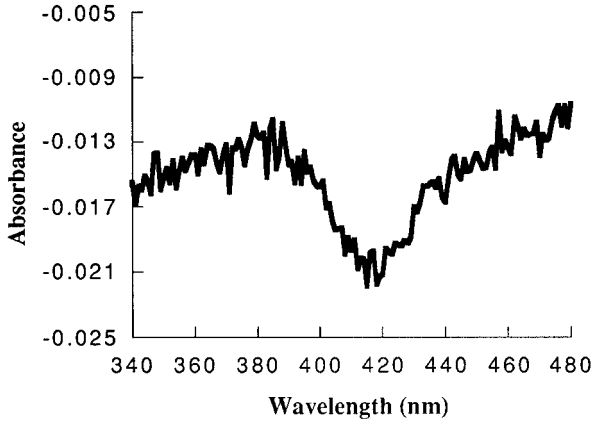
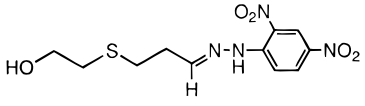


Figure 4. Type I difference spectrum for incubation of nNOS with **6**. See the Experimental Section for details.

equiv (1800 dpm) of tritiated water was recovered, as analyzed by HPLC and scintillation counting.

Production of Acrolein During Inactivation of nNOS by N^{ω}-[1-^{3}H]Allyl-L-arginine. Following inactivation of nNOS by N^{ω}-[1-^{3}H]allyl-L-arginine, 9 equiv (1800 dpm) of acrolein was isolated by HPLC (Figure 11) as the 2,4-dinitrophenylhydrazone of the β-mercaptoethanol Michael adduct (**18**).



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Production of Arginine During Inactivation of nNOS by N^{ω}-Allyl-L-[1-^{14}C]arginine. When the inactivation was carried out with N^{ω}-allyl-L-[1-^{14}C]arginine in the presence of L-arginine to dilute the L-[1-^{14}C]arginine that may be produced, HPLC analysis showed that L-[1-^{14}C]arginine (427 dpm) was generated (Figure 12).

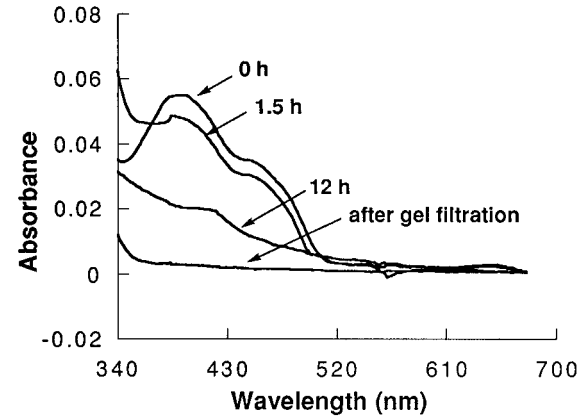


Figure 5. Change in the heme absorption spectrum during inactivation of nNOS by N^{ω}-allyl-L-arginine. See the Experimental Section for details.

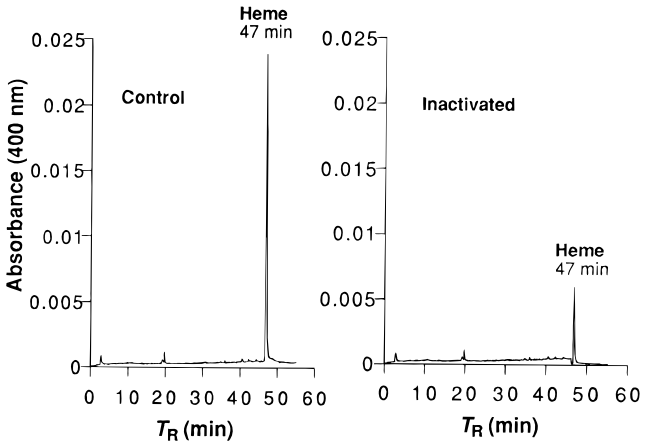


Figure 6. HPLC of heme after inactivation of nNOS by N^{ω}-allyl-L-arginine with all cofactors present (Inactivated) and when NADPH was replaced by NADP$^+$ (Control). See the Experimental Section for details.

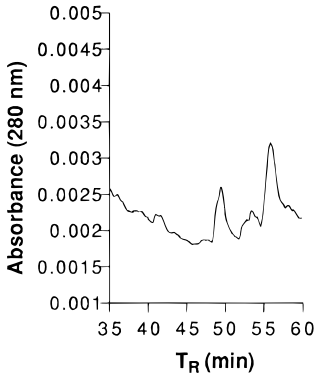


Figure 7. HPLC of modified hemes after inactivation of nNOS by N^{ω}-allyl-L-arginine. See the Experimental Section for details.

Effect of Incubation of nNOS with Acrolein. Incubation of nNOS with 850 μM acrolein (more than is generated by the N^{ω}-[1-^{3}H]allyl-L-arginine and more than double the excess over the DTT present) showed no inactivation over a period of an hour, time sufficient to produce complete inactivation of nNOS by incubation with N^{ω}-[1-^{3}H]allyl-L-arginine.

Discussion

N^{ω}-Allyl-L-arginine was reported to be a reversible inhibitor (K_i = 2.1 μM) and a time-dependent inactivator (K_I = 3.4 μM;

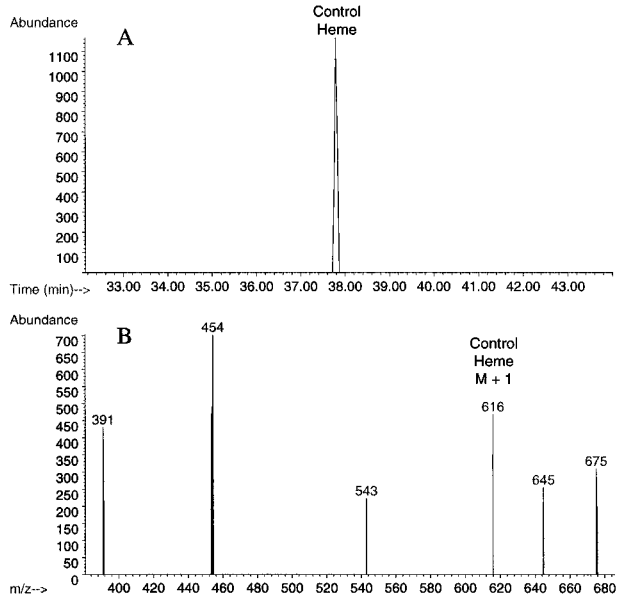


Figure 8. (A) HPLC and (B) electrospray mass spectrum of the control for inactivation of nNOS by **6**. See the Experimental Section for details.

$k_{inact} = 0.026$ min^{-1}) of macrophage NOS.[28] We have found that it also is a competitive reversible inhibitor (Figure 1; $K_i = 0.2$ μM) and time-dependent inactivator (Figure 2; $K_I = 0.47$ μM; $k_{inact} = 0.05$ min^{-1}) of neuronal NOS. This indicates that there is a modest selectivity (10.5) in favor of inhibition of the neuronal enzyme. Inactivation also was active-site directed, since it was prevented by the presence of L-arginine. The fact that no inactivation occurred in the absence of oxygen or when $NADP^+$ was substituted for NADPH suggests that inactivation requires an electron transfer and/or oxidation process. The oxidation of NADPH was followed spectrophotometrically in the presence of excess inactivator over enzyme (Figure 3A) to show that the spectral change correlates with the loss of enzyme activity. When the enzyme is completely inactivated (Figure 3B), the absorption change ceases.

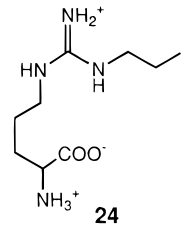
According to the reaction for the oxidation of L-arginine, the first intermediate is N^{ω}-hydroxy-L-arginine (**1**). To determine if a similar mechanism is involved in the inactivation of nNOS by N^{ω}-allyl-L-arginine, N^{ω}-allyl-N^{ω}-hydroxy-L-arginine (**7**) was synthesized by the route shown in Scheme 9. Hydroxylamine hydrochloride was diacetylated, and then the nitrogen was alkylated with allyl bromide to give *N*-allyl-*O*-acetylhydroxylamine (**19**). N^{α}-Boc-N^{δ}-Cbz-L-ornithine was converted to the *tert*-butyl ester, and then the Cbz protecting group was removed. Cyanogen bromide converted this diprotected ornithine to N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester (**20**). Reaction of **19** with **20** followed by deprotection gave **7**. Compound **7** also was a competitive reversible inhibitor of nNOS and a time-dependent inactivator. As in the case of **6**, inactivation by **7** was blocked by L-arginine and it required molecular oxygen; no inactivation occurred when $NADP^+$ was substituted for NADPH, indicating that all of the components needed for enzyme turnover are required for inactivation.

According to the mechanisms in Scheme 7, pathway a (giving **12**) and pathway b after hydrolysis (**15**) would result in only allyl group modification of the enzyme, but pathway b before hydrolysis (**14**) would result in attachment of the entire molecule to the enzyme. In Scheme 8, after hydrolysis (**15**), only the allyl group would be attached, but before hydrolysis (**14**), the entire molecule would be attached. Synthesis of radiolabeled analogues of **6**, then, would provide information regarding what part of the inactivator molecule remained bound to the enzyme. N^{ω}-Allyl-L-[1-^{14}C]arginine (**21**) was synthesized from allylamine as shown in Scheme 10, using [1-^{14}C]ornithine to incorporate the radioactivity. N^{ω}-[1-^{3}H]Allyl-L-arginine (**23**) was prepared by the reaction of N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester (**20**) with [1-^{3}H]allylamine[29] followed by deprotection (Scheme 11).

Inactivation of nNOS by N^{ω}-allyl-L-[1-^{14}C]arginine and N^{ω}-[1-^{3}H]allyl-L-arginine followed by either dialysis or gel filtration leads to incorporation of no radioactivity into the enzyme by either compound. In a control experiment using native nNOS, it was found that although rapid gel filtration did not remove the heme cofactor by comparative UV−vis spectroscopy (data not shown), overnight dialysis did result in removal of the heme. This indicates that heme binding is noncovalent and that a modified heme may bind very poorly to the apoenzyme. To determine if there is any interaction of the inactivator with the heme, the absorption spectrum was recorded in the absence and presence of N^{ω}-allyl-L-arginine. The type I difference spectrum shown in Figure 4 indicates that N^{ω}-allyl-L-arginine interacts with the active site. During inactivation the heme absorption spectrum changes with time (Figure 5); however, after gel filtration the heme is lost completely. It appears that, upon modification, the heme is released from the active site. When the heme content was monitored at 400 nm by HPLC ($t_R = 47$ min) before and after inactivation, a sharp decrease was observed (Figure 6). The residual heme may result from inactive enzyme at the beginning of the experiment. Monitoring at 280 nm by HPLC showed the formation of what could be construed as four new peaks between retention times of 48 and 59 min (Figure 7; in Figure 9A the four peaks are distinct). It is not unusual for heme-dependent enzymes to be alkylated at each of the four pyrrole rings of the heme, giving four different modified hemes.[37] To support the contention that these peaks in the HPLC are modified hemes, inductively coupled plasma atomic emission spectroscopy was carried out on the combined 48−59 min fractions. One equivalent of iron was measured in those fractions, suggesting that these are modified hemes with the iron still bound.

When N^{ω}-allyl-L-[1-^{14}C]arginine was used as the inactivator and the fractions with retention times between 48 and 59 min were collected and counted, no radioactivity was detected, but when N^{ω}-[1-^{3}H]allyl-L-arginine was the inactivator, 0.9 equiv of radioactivity per enzyme was detected. These results suggest that inactivation is caused by attachment of the allyl group of the inactivator to the heme, which causes the heme to be released from the enzyme. Additional structural information about the modified hemes is described below (*vide infra*).

All of these results appear to be consistent with the mechanisms in Scheme 7, leading to either **12** or **15** (where Y is the heme), both of which proceed by initial *N*-hydroxylation via **7**. In pathway a, citrulline and nitric oxide are generated directly; in pathway b, arginine is produced, which would be converted into citrulline and nitric oxide by the enzyme. Both of these mechanisms require the allyl group for inactivation. To test the importance of the double bond of the allyl group for inactivation, N^{ω}-propyl-L-arginine (**24**) was synthesized by hydrogenolysis of N^{ω}-allyl-L-arginine. However, this compound



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also is a competitive inhibitor ($K_i = 57$ nM) and a time-

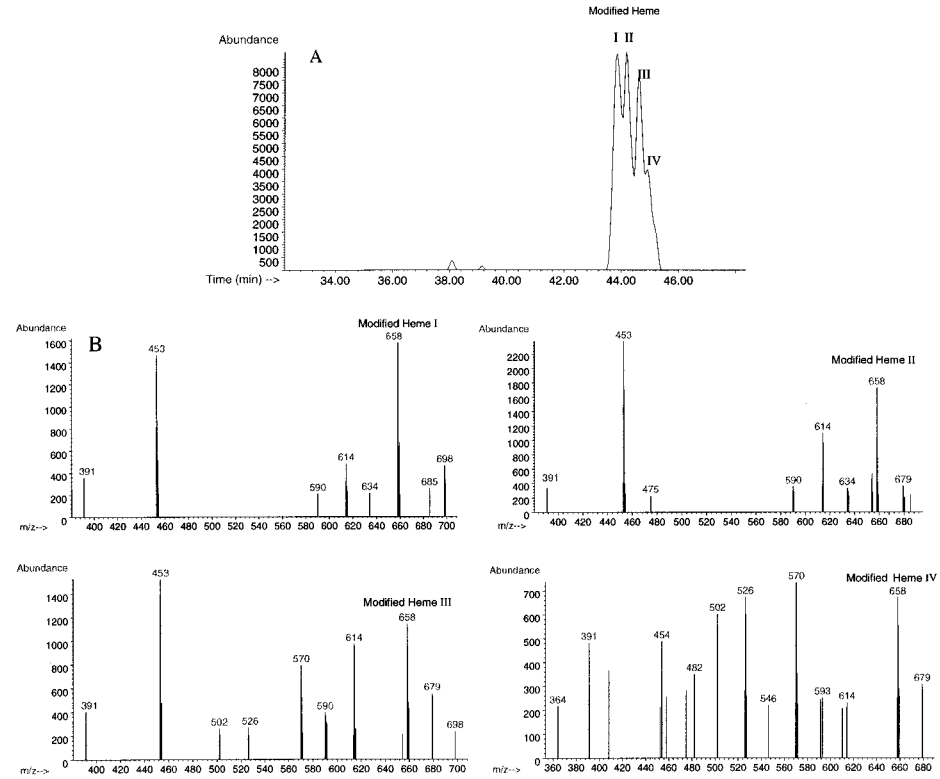


Figure 9. (A) HPLC and (B) electrospray mass spectrum of each of the four modified hemes produced from inactivation of nNOS by **6**. See the Experimental Section for details.

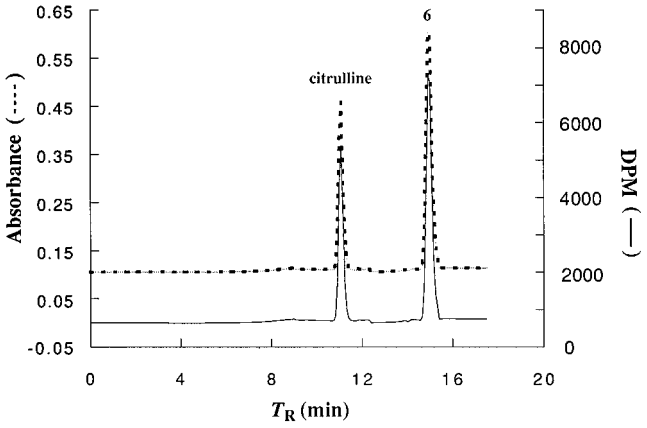


Figure 10. HPLC of metabolites generated by inactivation of nNOS by N^{ω}-allyl-L-[1-^{14}C]arginine. See the Experimental Section for details.

Table 1. Quantification of Citrulline and NO Produced from NOS-Catalyzed Oxidation of N^{ω}-Allyl-L-arginine and N^{ω}-Allyl-N^{ω}-hydroxy-L-arginine (See Experimental Section for details)

compound	citrulline (pmol)	nitric oxide (pmol)
N^{ω}-allyl-L-arginine	2016	1803
N^{ω}-allyl-N^{ω}-hydroxy-L-arginine	3007	3262

dependent inactivator ($K_I = 19.8\ \mu$M; $k_{inact} = 0.0059\ min^{-1}$) of nNOS. As for N^{ω}-allyl-L-arginine, L-arginine blocks inactivation, the inactivation requires molecular oxygen, and $NADP^+$ cannot substitute for NADPH. Furthermore, an equivalent amount of citrulline and nitric oxide are produced. These results indicate that N^{ω}-propyl-L-arginine acts similarly to the action of N^{ω}-allyl-L-arginine as an inactivator of nNOS. On the assumption that N^{ω}-allyl-L-arginine and N^{ω}-propyl-L-arginine act by related mechanisms (*vide infra*), the double bond of N^{ω}-allyl-L-arginine is unnecessary for inactivation and inactivation

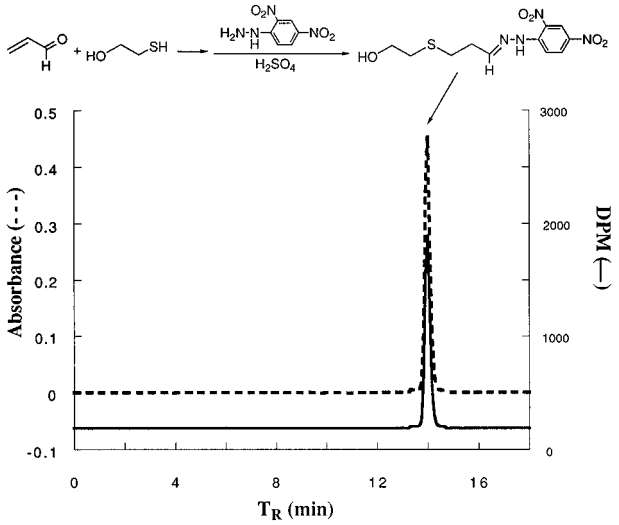


Figure 11. HPLC of **18** formed by quenching of the reaction of nNOS with N^{ω}-[1-^{3}H]allyl-L-arginine with β-mercaptoethanol and 2,4-dinitrophenylhydrazine solution. See the Experimental Section for details.

cannot be the result of the reaction depicted in Scheme 7, pathway a or b, because both **11** and **13** require the double bond for their respective reactions. Also, inactivation cannot be caused by the reactions in Scheme 8, because one route proceeds via **13** and the other depends upon resonance structures **16**, which would be irrelevant to the inactivation with N^{ω}-propyl-L-arginine. Although this dilemma cannot be avoided with **13** or **16**, it is possible to modify pathway a in Scheme 7 to account for the inactivation with N^{ω}-propyl-L-arginine. If the attack by heme (the data suggest that Y^- in Schemes 7 and 8 is the heme) on **11** occurs by an S_N2 reaction instead of by the S_N2' reaction shown in Scheme 7, then the double bond would not be required

(37) Ortiz de Montellano, P. R. *Biochimie* **1995**, *77*, 581−93. (b) Ortiz de Montellano, P. R.; Grab, L. A. *J. Am. Chem. Soc.* **1986**, *108*, 5584−9.

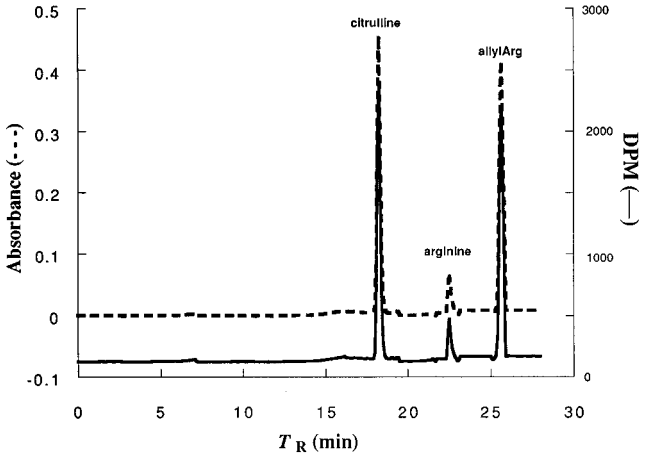
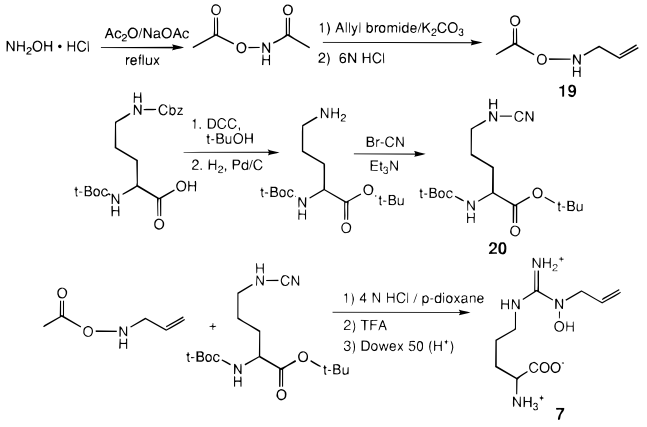
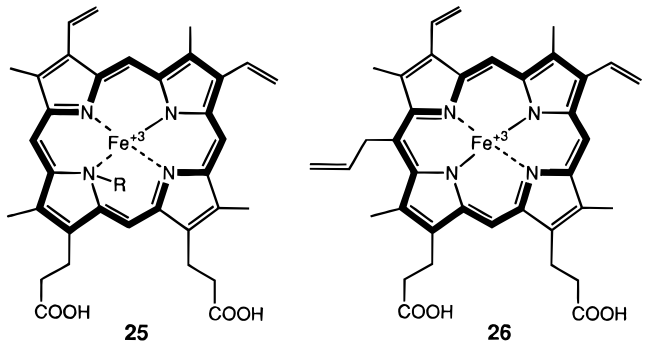


Figure 12. HPLC of the products of the reaction of nNOS with N^{ω}-allyl-L-[1-^{14}C]arginine in the presence of L-arginine. See the Experimental Section for details.

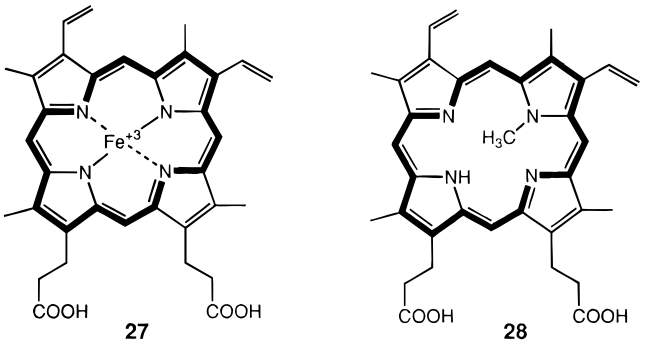
Scheme 9



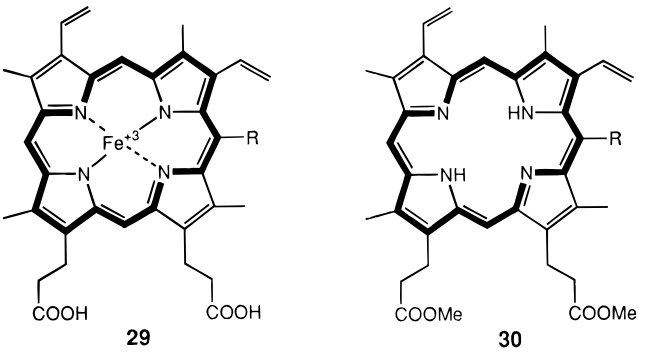
for inactivation (Scheme 12). By an S_N2 reaction, *N*-substituted heme (**25**, R = allyl) should result; however, if alkylation occurs at the porphyrin ring, which is equivalent to electrophilic aromatic substitution, then a *meso*-substituted heme (**26**) should result. However, we measured the absorption spectrum of heme



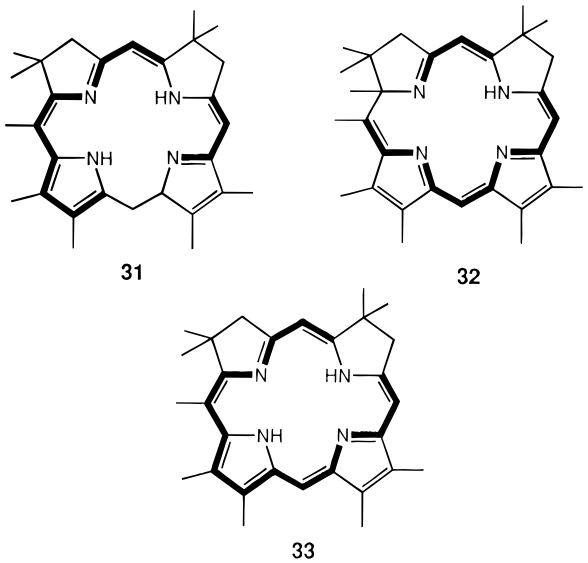
(**27**) (purchased from Aldrich) and *N*-methylprotoporphyrin IX (**28**) (purchased from Porphyrin Products, Inc., Logan, UT) and found that the λ_{max} values are 410 and 412 nm, respectively, even though the *N*-methylprotoporphyrin IX was not bound to Fe(III). Furthermore, the λ_{max} value for both *meso*-alkylprotoporphyrin IX bound to Fe(III)[38] (**29**) and the dimethyl ester



of *meso*-alkylprotoporphyrin IX not bound to Fe(III)[39] (**30**) is 404 nm. This indicates that as long as the aromatic porphyrin ring persists (shown as the bold lines in **27**−**30**), whether iron is bound or not, the λ_{max} should appear at about 400 nm.



However, as shown in Figures 6 and 7, the modified hemes produced by inactivation of nNOS by N^{ω}-allyl-L-arginine do not have an absorption at 400 nm; rather, they absorb at 280 nm. This suggests that the modified hemes are not aromatic. Reduced porphyrins are known to have lower λ_{max} values. For example, **31** and **32** have λ_{max} values of 284 nm and **33** has a λ_{max} value of 371 nm (note that the bold bonds are discontinuous, indicating interrupted aromaticity).[40] It appears that when



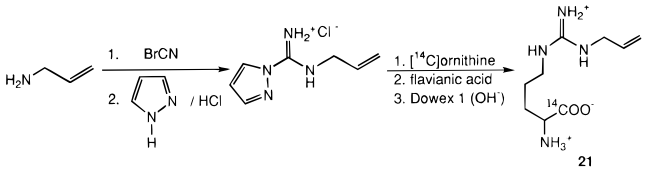
interruption of the aromaticity occurs at one atom, e.g., **33**, a higher wavelength is observed, but interruption at more than

(38) Ator, M.; David, S. K.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1987**, *262*, 14954−60.

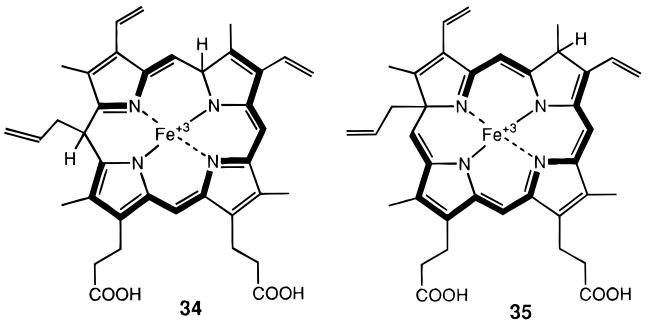
(39) Torpey, J. W.; Ortiz de Montellano, P. R. *J. Org. Chem.* **1995**, *60*, 2295−9.

(40) Hilpert, K.; Leumann, C.; Davis, A. P.; Eschenmoser, A. *J. Chem. Soc., Chem. Commun.* **1983**, 1401−4.

Scheme 10



one atom (**31** and **32**) produces a λ_{max} at about 280 nm. This suggests, then, that the structure of the modified hemes may be related to either **34** or **35**, in which alkylation occurs at four different sites. These structures correspond to heme plus an allyl group plus a hydrogen atom. To provide support for this



hypothesis LC−electrospray ionization mass spectrometry was carried out on the modified hemes isolated in the 48−59 min fractions. Figure 8 shows the control experiment in which inactivation was carried out in the presence of $NADP^+$ instead of NADPH, conditions that do not lead to heme modification. The mass (actually $(m + 1)/z$) of the heme is 616. Each of the proposed four modified hemes (Figure 9A) was shown to have the identical $(m + 1)/z$ peak at 658 (Figure 9B), which corresponds to heme plus an allyl group plus a hydrogen, consistent with **34** or **35** (*inter alia*). Interestingly, no peak was observed at $(m + 1)/z$ of 674, which corresponds to heme plus CH_2CH_2-CHO plus hydrogen. This product would be related to structure **15** (Schemes 7 and 8), where Y is reduced heme. The formation of a reduced heme by enzyme inactivation is rare, if not unique.

But how can structures **34** or **35** (or a related structure) be rationalized from a mechanistic point of view? It would appear that a radical mechanism needs to be invoked; a cation mechanism would lead to the equivalent of electrophilic aromatic substitution and produce an oxidized heme. As a working hypothesis, consider the mechanism shown in Scheme 13, which leads to **34** (although the same mechanism could produce **35** or other related structures as well). The mechanism begins the same as that in Scheme 12 up to intermediate **10**. At this point a double bond of the heme may react with the allyl group with concomitant loss of nitric oxide and citrulline to give allylated heme having a highly resonance-stabilized radical and a ferric oxo radical. Two single-electron transfers plus three protons would give **34** from **36** via **37**. Two possible mechanisms for these electron and proton transfers are shown in Scheme 13. In going from **36** to **37**, either one electron and one proton could come from the flavin semiquinone (and another proton from the solvent) or a hydrogen atom could be abstracted from an active-site amino acid (and another proton donated from the solvent). Likewise, in going from **37** to **34**, either one electron and one proton could come from the flavin semiquinone or a hydrogen atom could come from an active-site amino acid. Precedence for the addition of an alkyl radical to the heme is found in the inactivation of cytochrome P-450 by 4-alkyl-3,5-dicarbethoxy-2,6-dimethyl-1,4-dihydropyridine.[41] Furthermore, four isomers of *N*-alkyl protoporphyrin IX are formed during this inactivation process.[42] Since the conversion of arginine to nitric oxide requires 1.5 NADPH, but NADPH can only donate an even number of electrons per catalytic cycle, NOS must store the extra electron for use in the next catalytic cycle. Both FAD and FMN can accept and retain up to two electrons each from the NADPH; therefore, they probably serve as the electron storage resevoirs between cycles.[21] Precedence for the retention of an electron between catalytic cycles by flavins can be found in cytochrome P-450 reductase.[43] In fact, it has been found that NOS in its native state exists in the semiquinone form,[44] supporting the resevoir hypothesis for the flavin.

Another pathway that was considered is one that involves initial hydroxylation of N^ω-allyl-L-arginine at the imino group rather than at the secondary (allyl-substituted) amino group. Hydroxylation at the imino nitrogen (**38**, Scheme 14), via the same mechanism as is shown for substrate turnover, leads to N^ω-allyl-L-citrulline (**39**) and nitric oxide. When nNOS was inactivated with N^ω-allyl-L-[1-^{14}C]arginine and the reaction products derivatized with *o*-phthalaldehyde (OPA), no OPA-derivatized **39** could be detected by HPLC. This excludes imino nitrogen hydroxylation and suggests that initial hydrogen atom abstraction occurs, leading to the more stabilized secondary amino radical rather than the imino radical.

To complete the picture of reactions of N^ω-allyl-L-arginine with nNOS, the products that are generated were determined. HPLC separation of products generated from inactivation of nNOS by N^ω-allyl-L-[^{14}C]arginine showed the formation of only one amine-containing radioactive metabolite, namely, citrulline (Figure 10). Nitric oxide formation was measured by the Griess reaction[45] for nitrite and nitrate ions. A comparison of the amount of citrulline produced and the amount of nitric oxide formed showed about a 1:1 stoichiometry (Table 1). An approximate stoichiometric amount of citrulline and NO also were produced during inactivation of nNOS by N^ω-allyl-N^ω-hydroxy-L-arginine (Table 1). With N^ω-allyl-L-arginine, the partition ratio (number of product molecules per inactivation event) is 13, and with N^ω-allyl-N^ω-hydroxy-L-arginine, the partition ratio is 20. The larger partition ratio for the intermediate may explain why this intermediate is not detected; the rate-determining step appears to be conversion of N^ω-allyl-L-arginine into N^ω-allyl-N^ω-hydroxy-L-arginine.

When the inactivation was carried out with N^ω-[1-^{3}H]allyl-L-arginine followed by bulb-to-bulb distillation to trap the volatiles and then injection of the volatiles into the HPLC, collection of the radioactive fractions, and scintillation counting, 8 equiv of ^3H_2O (t_R = 2.5 min) was obtained; no [^{3}H]acrolein (t_R = 8.9 min) was detected, either because it was too volatile to be trapped by this method or it polymerized under these conditions. Another approach used to detect [^{3}H]acrolein was by quenching the enzyme reaction with β-mercaptoethanol to trap the acrolein by Michael addition and then conversion to the 2,4-dinitrophenylhydrazone (**18**). HPLC (Figure 11) showed that the 2,4-dinitrophenylhydrazone of the β-mercaptoethanol adduct of acrolein was produced; scintillation counting indicated the formation of 9 equiv. Given the complexity of these experiments, the amounts of ^3H_2O and acrolein produced are

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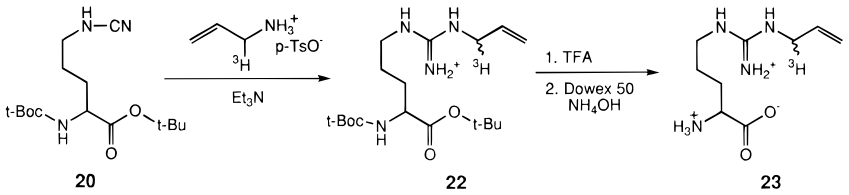
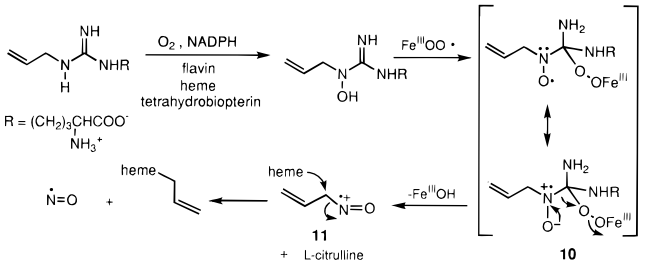
Scheme 11



Scheme 12



the same. These experiments indicate that the α-C−H bond of the allyl group is broken during turnover, which is reminiscent of the turnover of N^{ω}-methyl-L-arginine by iNOS, from which formaldehyde was isolated.[46] There are several mechanisms by which loss of tritium from N^{ω}-[1-^{3}H]allyl-L-arginine may occur involving either initial C−H bond cleavage or initial N−H bond cleavage (Scheme 15). These pathways also indicate that arginine should be produced, but HPLC of the products formed by inactivation of nNOS with N^{ω}-allyl-L-[^{14}C]arginine showed no arginine. However, because arginine is an excellent substrate, it may be rapidly converted into citrulline and nitric oxide. By carrying out the inactivation with N^{ω}-allyl-L-[1-^{14}C]arginine in the presence of unlabeled L-arginine, any [^{14}C]arginine produced would be diluted with the unlabeled arginine, and the degradation of [^{14}C]arginine would be slowed. When this was done and the reaction was stopped before complete inactivation, [^{14}C]arginine was detected as a product (Figure 12). Since L-arginine is the substrate for nNOS, quantitation of the [^{14}C]-arginine produced is meaningless.

Since acrolein is a reactive molecule, it was necessary to show that inactivation was not the secondary result of acrolein formation. Treatment of nNOS with acrolein produced no inactivation under conditions that caused rapid inactivation by N^{ω}-allyl-L-arginine. Therefore, the deallylation of N^{ω}-allyl-L-arginine is not on the pathway to inactivation.

Finally, the question must be asked whether the inactivation of NOS by N^{ω}-propyl-L-arginine follows the same mechanism as that by N^{ω}-allyl-L-arginine or whether they are unrelated. If they are different, then the fact that N^{ω}-propyl-L-arginine is an inactivator does not necessarily imply that the double bond is unnecessary for inactivation. Our preliminary evidence is that inactivation of NOS by N^{ω}-propyl-L-arginine results in loss of the 400 nm absorption of the heme, as for N^{ω}-allyl-L-arginine. The mechanism of N^{ω}-propyl-L-arginine is under investigation.

In summary, the evidence presented here supports an inactivation mechanism that involves reductive allylation of the heme to produce four isomeric modified hemes without attachment to the protein; possible structures of the modified hemes are given by **34** or **35**, and possible inactivation mechanisms are described in Scheme 13. The fact that N^{ω}-propyl-L-arginine also inactivates NOS suggests that the double bond is not important to inactivation, which is highly unusual, if not unique, for an allyl-substituted inactivator of a heme- or flavin-containing enzyme. Reduction of the heme by enzyme inactivation also is rare, if not unique.

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Experimental Section

Analytical Methods. Optical spectra and enzyme assays were recorded on either a Cary 1E, a Perkin-Elmer Lambda 1, or Perkin-Elmer Lambda 10 UV−vis spectrophotometer. NMR spectra were recorded on a Varian Gemini-300 300 MHz instrument. Chemicals shifts are reported as δ values in parts per million downfield from Me_4-Si as the internal standard in $CDCl_3$, unless stated otherwise, and coupling constants (J) are given in hertz. Mass spectra were recorded on a VG Instruments VG70-250SE high-resolution spectrometer. HPLC−electrospray ionization mass spectra were obtained with a Hewlett-Packard series 1050 HPLC and a Hewlett-Packard 5989B mass spectrometer. Inductively coupled plasma atomic emission spectra were obtained with an AtomScan 25 spectrophotometer from Thermo Jarrell Ash Corporation (Franklin, MA). Melting points were obtained on a Fisher-Johns melting point apparatus and are uncorrected. Flash chromatography was performed with Merck silica gel (230−400 mesh). All chemicals were purchased from Aldrich or Sigma Chemical Companies and used without further purification. THF and ether were freshly distilled from sodium metal. The radioactivity was counted with a Packard TRI-CARB 2100TR liquid scintillation analyzer. The amino acids were detected with a Spectra/glo filter fluorometer detector using an *o*-phthalaldehyde filter. 6(*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland).

***N*$^{\omega}$-Allyl-*N*$^{\omega}$-hydroxy-L-arginine (7).** To a mixture of *N*-allyl-*O*-acetylhydroxylamine[47] (26.0 mg, 0.23 mmol) and N^{α}-Boc-N^{δ}-cyano-L-ornithine *tert*-butyl ester[48] (62.6 mg, 0.20 mmol) in *p*-dioxane (0.1 mL) was added 4 N HCl (0.05 mL, 0.20 mmol). The mixture was stirred for 26 h at room temperature, ether (0.3 mL) was then added, and the mixture was shaken and cooled to −30 °C. The ether layer was removed, and the ether washing process was repeated twice. To the solid residue (63 mg) was added trifluoroacetic acid (3.5 mL), and the mixture was stirred for 1.5 h at room temperature. A crude oil was obtained after removal of the trifluoroacetic acid. The crude oil was loaded onto a cation exchange column (Dowex 50W-X8 resin, 200−400 mesh, H^+ form, Bio-Rad Laboratories), the column was washed first with water, and the product was then eluted off with NH_4-OH. The NH_4OH solution was collected, concentrated in vacuo, and then lyophilized to give a solid (56.0 mg). This solid was further purified by silica gel chromatography ($CHCl_3$:MeOH:NH_4OH = 3:3:1); after lyophilization, **7** (20.0 mg, 43%) was obtained as a white solid: ^{1}H NMR (D_2O) δ 5.77 (m, 1 H), 5.25 (d, 1 H, J = 10.0), 5.20 (d, 1 H), 4.11 (d, 2 H, J = 5.0), 3.66 (t, 1 H, J = 5.1), 3.22 (t, 2 H, J = 6.6), 1.55−1.85 (m, 4 H); ^{13}C NMR (D_2O) 175.5, 153.5, 130.6, 117.6, 54.4, 54.0, 40.9, 28.1, 24.7; HRMS (FAB) calcd for $C_9H_{19}N_4O_3$ (MH^+) 231.1457, found 231.1485.

6-Hydroxy-4-thiahexanal, 2,4-Dinitrophenylhydrazone (18). This was synthesized by the known procedure.[29]

***N*$^{\omega}$-Allyl-L-[1-^{14}C]arginine (21).** This compound was prepared on

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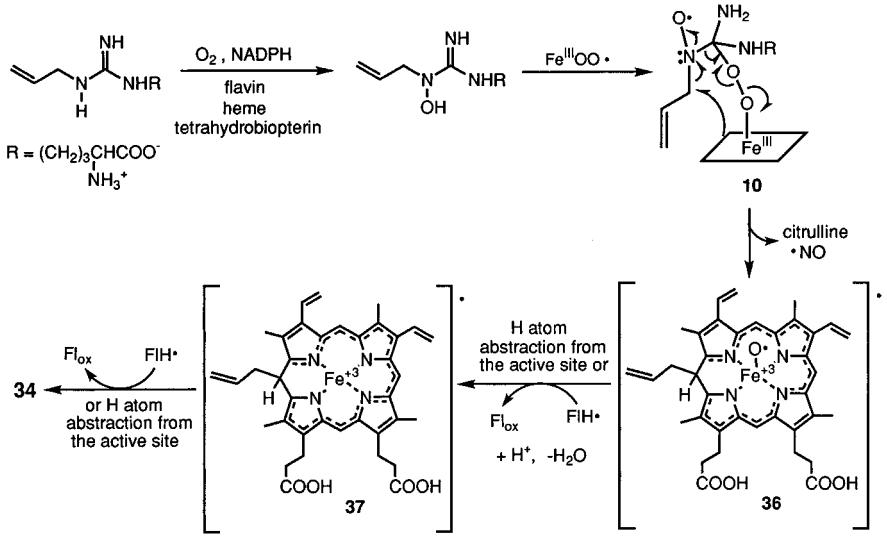
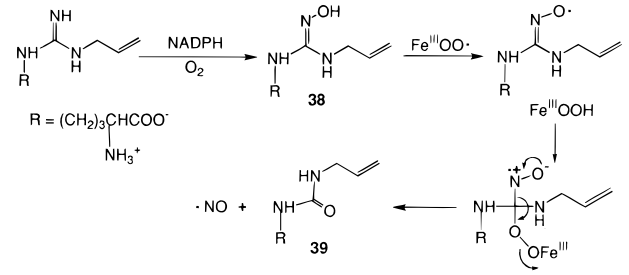
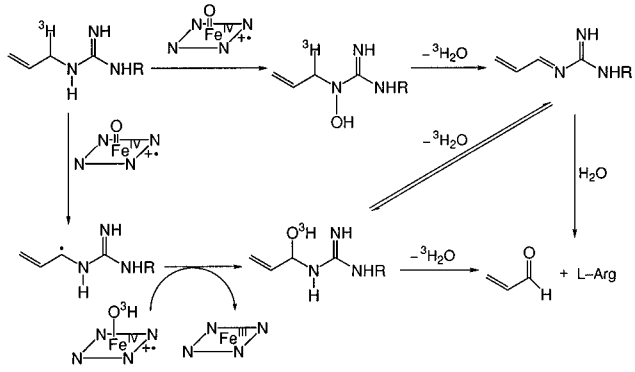
Scheme 13



Scheme 14



Scheme 15



a 0.10 mmol scale by the route used to make the unlabeled analogue,[49] except L-[1-^{14}C]ornithine (American Radiolabeled Chemicals; 56 mCi/mmol, 0.0067 mmol) was mixed with L-ornithine (15.6 mg, 0.093 mmol). The TLC and HPLC analyses demonstrated that the compound was 98% radiochemically pure. The specific radioactivity was 3.75 mCi/mmol.

***N*$^{\omega}$-[1-^{3}H]Allyl-L-arginine (23).** To a stirred solution of *N*$^{\alpha}$-Boc-*N*$^{\delta}$-cyano-L-ornithine *tert*-butyl ester[48] (**20**, 0.147 g, 0.47 mmol) and [1-^{3}H]allylamine hydrochloride[29] (0.040 g, 0.43 mmol) in ethanol (7 mL) was added triethylamine (96 μL, 0.69 mmol), and the reaction mixture was refluxed for 96 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 (60 g, 6:1 methylene chloride:methanol), yielding 96 mg (0.24 mmol, 55%) of *N*$^{\alpha}$-Boc-*N*$^{\omega}$-[1-^{3}H]allyl-L-arginine *tert*-butyl ester (**22**). To **22** (0.093 g, 0.23 mmol) was added trifluoroacetic acid (5.0 mL, 65 mmol), 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 Dowex 50W-X8 cation-exchange column (resin 200−400 mesh, H^+ form) by washing the column with water (50 mL) and then eluting the inactivator off the column with 0.25 M ammonium hydroxide. The water was removed by lyophilization, yielding 15 mg (0.07 mmol, 30%) of **23**. TLC and HPLC analyses demostrated that the compound was 98% radiochemically pure. The specific radioactivity was 0.75 mCi/mmol.

***N*$^{\omega}$-Propyl-L-arginine (24).** To *N*$^{\omega}$-allyl-L-arginine (**6**)[49] (10.0 mg) in MeOH (HPLC grade, 3 mL) was added palladium (10% on activated carbon, 10 mg). The mixture was stirred under hydrogen (1 atm) for 5 h at room temperature. The palladium and carbon were removed by filtration, and the filtrate was concentrated in vacuo to give a white solid. Water (HPLC grade, 3 mL) was added, and the aqueous layer was washed with ethyl acetate (2 mL) three times and then lyophilized to give **24** (9.0 mg, 89%) as a white solid: ^{1}H NMR (D_2O) δ 3.23 (m, 1 H), 3.15 (m, 2 H), 3.08 (t, 2 H, J = 7.2), 1.48−1.60 (m, 6 H), 0.85 (t, 2 H, J = 7.8); ^{13}C NMR (D_2O) 179.5, 155.7, 56.3, 55.0, 42.9, 40.7, 24.3, 21.5, 10.4; HRMS (FAB) calcd for $C_9H_{21}N_4O_2$ (MH^+) 217.1665, found 217.1658.

***N*$^{\omega}$-Allyl-L-citrulline (39).** To *N*$^{\alpha}$-Boc-L-ornithine *tert*-butyl ester[48] (90.0 mg, 0.31 mmol) in $CHCl_3$ (3 mL) was added allyl isocyanate (26 mg, 0.31 mmol) in $CHCl_3$ (0.05 mL) at 0 °C dropwise. The mixture was stirred for 20 min at 0 °C and then concentrated in vacuo to give an oil. To this oil was added trifluoroacetic acid (2 mL) at room temperature. The mixture was stirred for 1.5 h at room temperature. The crude oil that was obtained after removal of the trifluoroacetic acid was then loaded onto a cation exchange column (Dowex 50W-X8 resin, 200−400 mesh, H^+ form, Bio-Rad Laboratories). The column was washed with water, and the product was eluted off with NH_4OH. The NH_4OH solution was collected, concentrated in vacuo first, and then lyophilized to give the **39** (58.0 mg, 86%): ^{1}H NMR (D_2O) δ 5.70 (m, 1 H), 5.00 (d, 1 H, J = 16.0), 4.95 (d, 1 H, J = 9.0), 3.95 (t, 1 H, J = 6.0), 3.55 (m, 2 H), 3.02 (t, 2 H, J = 6.5), 1.80 (m, 2 H), 1.45 (m, 2 H); ^{13}C NMR (D_2O) 198.1, 171.5, 134.6, 114.6, 52.3, 41.9, 38.9, 26.8, 24.7; HRMS (FAB, M + 1) calcd for $C_9H_{17}N_3O_3$ 216.1348, found 216.1368.

Enzyme Purification. nNOS was obtained from bovine brain as described.[50] The specific activity was 250 nmol/min/mg, and it exhibited a single band on $NaDodSO_4$-PAGE.

Initial Velocity Measurements via the Hemoglobin Assay. The generation of nitric oxide by nNOS was measured by utilizing the rapid oxidation of oxyHb to metHb by nitric oxide.[51] The assay mixture contained nNOS (5.4−54 nM), L-arginine (12−50 μM), $CaCl_2$ (1.6 mM), calmodulin (0.68 mM), NADPH (104 μM), tetrahydrobiopterin

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(6.5 μM), DTT (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 nitric oxide synthesis was determined by monitoring the NO-mediated conversion of oxyhemoglobin (oxyHb) to methemoglobin (metHb) at 401 nm on a Perkin-Elmer Lambda 1 UV−vis spectrophotometer. All assays were performed at 30 °C.

Reversible Inhibition Kinetics (Figure 1 shows the results for 6). The type of reversible inhibition of nNOS by **6**, **7**, and **24** was studied under initial rate conditions with the hemoglobin assay[50] as described above. The concentrations were 3, 4, 6, and 15 μM for L-arginine; 0, 0.05, 0.10, 0.20, 0.30, and 0.40 μM for **6**; 0, 0.05, 0.10, 0.15, and 0.20 μM for **7** and **24**. Data were analyzed by the method of Dixon.[52]

Irreversible Inhibition Kinetics (Figure 2 shows inactivation by 6). nNOS (0.32−0.37 μM) was incubated at 0 °C in Hepes buffer (100 mM, pH 7.5) containing $CaCl_2$ (2.1 mM), calmodulin (1.8 mM), NADPH (0.6 mM), tetrahydrobiopterin (50 μM), DTT (700 μM), glycerol (20% v/v), and **6** or **7** or **24** (0.39−50 μM) in a total volume of 70−140 μL. The reactions were initiated by the addition of NADPH, and 10−25 μL aliquots were removed to assay for enzyme activity at various times. A control was performed by either replacing inactivators with L-arginine (1 mM)[34,35] or by replacing NADPH with $NADP^+$. The inactivation constants K_I and k_{inact} were determined by the method of Kitz and Wilson.[53] The same inactivation experiments were performed except with addition of catalase (1000 units/mL) and superoxide dismutase (100 units/mL).[35]

Substrate Protection of the Irreversible Inhibition of nNOS by 6 or 7. nNOS (0.32−0.37 μM) was incubated at 0 °C in Hepes buffer (100 mM, pH 7.5) containing $CaCl_2$ (2.1 mM), calmodulin (1.8 mM), NADPH (0.6 mM), tetrahydrobiopterin (50 μM), DTT (700 μM), glycerol (20% v/v), **6** or **7** (50 μM), and L-arginine (1.5 mM) in a total volume of 70−140 μL. The reactions were initiated by the addition of NADPH, and 10−25 μL aliquots were removed to assay for enzyme activity at various times. A control was performed by replacing inactivators with L-arginine (1 mM).[34,35]

Oxygen Dependence of the Irreversible Inhibition of nNOS by 6 or 7. Dependence of the inactivation by **6** or **7** on molecular oxygen was examined by carrying out the reaction under an argon atmosphere using protocatechuic acid (PCA)/protocatechuate 3,4-dioxygenase (PCD) as the oxygen-scavenging system.[54] A preincubation mixture containing cofactors as described above, nNOS (0.79 μM), **6** or **7** (100 μM), and PCA (250 μM) in a 0.5 mL Eppendorf tube sealed with a rubber septum was frozen in liquid nitrogen, and then the system was pumped under vacuum through a needle. After 0.5 min, the tube was thawed at 0 °C, and the system was filled with argon. The freeze−pump−thaw−argon process was repeated twice. PCD (0.5 units) was added, and the mixture was warmed to room temperature. Aliquots (25 μL) were removed for assay of enzyme activity at various times. Simultaneously, a control experiment was carried out by replacing the PCD with Hepes buffer (100 mM, pH 7.5) and exposing the incubation mixture with air instead of argon.

NADPH Dependence of the Irreversible Inhibition of nNOS by 6 or 7. nNOS (0.32−0.37 μM) was incubated at 0 °C in Hepes buffer (100 mM, pH 7.5) containing $CaCl_2$ (2.1 mM), calmodulin (1.8 mM), $NADP^+$ (0.6 mM), tetrahydrobiopterin (50 μM), DTT (700 μM), glycerol (20% v/v), and **6** or **7** (50 μM) in a total volume of 70−140 μL. A 10−25 μL aliquot was removed to assay for enzyme activity at various times. A control was performed by replacing inactivators with L-arginine (1 mM).

Changes in NADPH Absorption Spectra with Excess of 6 (Figure 3). An incubation mixture in a cuvette containing nNOS (0.23 μM), $CaCl_2$ (12 mM), calmodulin (48 μg/mL), BH_4 (36 μM), DTT (517 μM), NADPH (0.35 mM), and **6** (8 μM) in Hepes buffer (100 mM, pH 7.5) was scanned at room temperature over time (0, 15, 40, 60, and 100 min) (Figure 3A). Aliquots (25 μL) from the identical incubation mixture were removed to assay enzymatic activity at various times (0, 15, 40, 60, and 100 min) (Figure 3B).

Stoichiometry of Radiolabeling of nNOS by 21 and 23. A preincubation mixture containing nNOS (1.8 μM), $CaCl_2$ (6.25 mM), calmodulin (16 mM), NADPH (7.8 mM), tetrahydrobiopterin (18 μM), DTT (250 μM), and either **21** or **23** (75 μM) in Hepes buffer (100 mM, pH 7.5), in a total volume of 480 μL was incubated at room temperature for 23 h until inactivation was complete as determined via the hemoglobin assay. Aliquots (468 μL) were placed in a dialysis membrane (Spectra/Por, MWCO 12−14000) and dialyzed against 4 × 1 L of Hepes buffer (100 mM, pH 7.5) for a total of 40 h at 4 °C, until there was no radioactivity detectable in the buffer solution. Half of the total volume of the contents of the dialysis bag was analyzed for protein concentration and radioactivity. The other half of inactivated nNOS was denatured by adding urea to a final concentration of 8 M and incubating for 14 h at 56 °C. The incubation mixture was then dialyzed against 8 M urea in Hepes buffer (100 mM, pH 7.5) at room temperature until there was no radioactivity detectable in the buffer solution. The contents of the dialysis bag were analyzed for protein concentration and radioactivity. A control was performed by replacing NADPH with $NADP^+$. In another experiment an aliquot (100 μL) was gel filtered by the method of Penefsky.[36]

Heme Absorption Difference Spectrum for Incubations of nNOS with 6 (Figure 4). A preincubation mixture in a cuvette containing nNOS (5 μM), $CaCl_2$ (12 mM), calmodulin (2.9 mM), BH_4 (36 μM), and DTT (517 μM) in Hepes buffer (100 mM, pH 7.5) was scanned at room temperature on a Cary 1E UV−vis spectrophotometer. Another preincubation mixture in a cuvette containing nNOS (5 μM), the same cofactors as described above, and **6** (645 μM) was also scanned. Subtraction of the former spectrum from the latter one resulted in a type I binding spectrum similar to that for L-arginine,[55] N^{ω}-hydroxy-L-arginine,[56] and N^{ω}-methyl-L-arginine.[34]

Changes in the Heme Absorption Spectrum During the Inactivation of nNOS with 6 (Figure 5). An incubation mixture in a cuvette containing nNOS (1.3 μM), $CaCl_2$ (5.7 mM), calmodulin (1.5 mM), BH_4 (16 μM), DTT (2.3 mM), and **6** (100 μM) in Hepes buffer (100 mM, pH 7.5) was scanned at room temperature on a Perkin-Elmer Lambda 1 or 10 UV−vis spectrophotometer. The inactivation was initiated by the addition of NADPH to a final concentration of 7 mM. The heme absorption was monitored at 0, 1.5, and 12 h. A control without **6** lost only 10% of its activity over the 12 h period. An aliquot (265 μL) was applied to a Penefsky[36] column with Sephadex G-50 gel (Sigma), and the collected effluent was scanned.

Heme Analysis by Reversed-Phase HPLC (Figures 6 and 7). A preincubation mixture containing nNOS (0.56 μM), cofactors, and **6** (100 μM) in Hepes buffer (100 mM, pH 7.5) was incubated at room temperature for 26 h until inactivation was completed as determined by the hemoglobin assay. Samples (50 μL, containing 28 pmol of nNOS) were analyzed by reversed-phase HPLC with a Vydac C18 (46 × 250 mm) column, eluting at 1 mL/min with solvent A (0.1% trifluoroacetic acid (TFA) in H_2O) for 5 min, followed by a linear gradient from 0 to 75% solvent B (0.1% trifluoroacetic acid in CH_3CN) over 60 min (Figure 7). Absorbance was monitored at 400 (Figure 6) or 280 nm (Figure 7) with the use of a Beckman System Gold Model 125P solvent module and a Model 166 detector with a second-order filter. A control was performed by replacing NADPH with $NADP^+$.

An aliquot (300 μL) of the inactivated enzyme solution was placed in a dialysis membrane (Spectra/Por, MWCO 12−14000) and was dialyzed against 4 × 200 mL of Hepes buffer (100 mM, pH 7.5) for a total of 25 h at 4 °C; the contents of the dialysis bag were analyzed for protein concentration, and a sample (20 μL, containing 87 pmol of protein) was analyzed by reversed phase-HPLC as described above.

Heme Loss During the Dialysis of Native nNOS. A mixture of nNOS (233 μg), $CaCl_2$ (12 mM), calmodulin (48 μg/mL), BH_4 (36 μM), and DTT (5.3 mM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 0.3 mL was placed in a dialysis membrane (Spectra/Por, MWCO 12−14000) and was dialyzed against 5 × 200 mL of Hepes buffer (100 mM, pH 7.5) for a total of 15 h at 4 °C. An aliquot (100 μL) of the dialysis bag was analyzed by reversed-phase HPLC (400 nm detection) as described in the Heme Analysis by Reversed-Phase HPLC section.

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Stoichiometry of Radiolabeling of Heme by N^{ω}-Allyl-L-[1-^{14}C]-arginine and N^{ω}-[1-^{3}H]Allyl-L-arginine. A preincubation mixture containing nNOS (0.56 μM), $CaCl_2$ (8.3 mM), calmodulin (2.1 mM), NADPH (10 mM), BH_4 (23 μM), DTT (3.3 mM), and either N^{ω}-[1-^{3}H]allyl-L-arginine or N^{ω}-allyl-L-[1-^{14}C]arginine (100 μM) in Hepes buffer (100 mM, pH 7.5), in a total volume of 720 μL was incubated at room temperature for 36 h until inactivation was complete, as determined by the hemoglobin assay. The mixtures (720 μL) were analyzed by reversed-phase HPLC with a Vydac C18 (46 × 250 mm) column and eluted at 1 mL/min with solvent A (0.1% TFA in H_2O) for 5 min, followed by a linear gradient from 0 to 75% solvent B (0.1% TFA in CH_3CN) over 60 min (Figure 8). Absorbance was monitored at 280 nm with the use of a Beckman System Gold Model 125P solvent module and a Model 166 detector. Fractions of 2 min each were collected, and the radioactivity was monitored by liquid scintillation counting. The total radioactivity of fractions t_R = 48−59 min was added together (425 dpm) to determine the equivalence of inactivator binding to the modified heme. A control was performed by replacing NADPH with $NADP^+$.

Determination of the Iron Content in the Modified Hemes by Inductively Coupled Plasma Atomic Emission Spectrophotometry. The incubation mixture of nNOS with unlabeled N^{ω}-allyl-L-arginine (see the Stoichiometry of Radiolabeling of Heme by N^{ω}-Allyl-L-[1-^{14}C]arginine and N^{ω}-[1-^{3}H]Allyl-L-arginine section) was injected into the HPLC (monitoring at 280 nm), and the 48−59 min fractions were collected. The solvent was removed by evaporation, and the residue was dissolved in water (2.2 mL, HPLC grade) and then injected into the inductively coupled plasma atomic emission spectrophotometer. A control was performed with the same conditions as described above except buffer was injected into the HPLC instead of the incubation mixture, and the 48−59 min fractions were treated as in the case of the inactivated enzyme sample. Samples containing known concentrations of Fe(III) were used to obtain the calibration curve.

HPLC−Electrospray Ionization Mass Spectra of Unmodified and Modified Hemes (Figures 8 and 9). A preincubation mixture containing nNOS (216 μg), $CaCl_2$ (6.25 mM), calmodulin (260 μg/mL), NADPH (7.8 mM), BH_4 (18 μM), DTT (5.3 mM), and N^{ω}-allyl-L-arginine (75 μM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 1.2 mL was incubated at room temperature for 20 h. A 0.6 mL aliquot was then removed, and glacial acetic acid (0.2 mL) was added. The heme was extracted with diethyl ether (4 × 3 mL) as previously described.[38] The ethereal solution was washed with water and dried in vacuo. The residue was dissolved in 50 μL of dilute aqueous ammonia (0.1 *N*) and was injected into the HPLC−mass spectrometer (280 nm detection) (Figure 9). A control was carried out the same as above except with the replacement of NADPH by $NADP^+$ and 400 nm detection (Figure 8). The reaction samples were analyzed by reversed-phase HPLC with a Vydac C_{18} column (250 mm × 2.1 mm) and eluted at 0.25 mL/min. The mobile phase was a gradient from 90% buffer A (water, 0.1% formic acid) and 10% buffer B (acetonitrile, 0.1% formic acid) to 75% buffer B over 60 min.

Identification of Amino Acid Products from N^{ω}-Allyl-L-[1-^{14}C]-arginine-Induced Inactivation of nNOS (Figure 10). A preincubation mixture containing nNOS (1.6 μM), $CaCl_2$ (6.25 mM), calmodulin (15 mM), NADPH (7.8 mM), tetrahydrobiopterin (18 μM), DTT (250 μM), and N^{ω}-allyl-L-[1-^{14}C]arginine (**6**) (75 μM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 60 μL was incubated at room temperature for 20 h. Samples were analyzed for amino acid content by reversed-phase HPLC. Identification of amino acids was established by coinjection of samples with amino acid standards. Fractions of 0.5 min each were collected, and the radioactivity was monitored by liquid scintillation counting. When the same inactivation was performed with the addition of catalase (1000 units/mL) and superoxide dismutase (100 units/mL), there was no effect on the formation of citrulline. A control was carried out by the replacement of NADPH with $NADP^+$.

Quantitative Determination of Citrulline Formation During nNOS Inactivation by N^{ω}-Allyl-L-[1-^{14}C]arginine, 7, and 24 (Table 1). A preincubation mixture containing nNOS (0.8 μM, 153 pmol), the cofactors as described above, and N^{ω}-allyl-L-[1-^{14}C]arginine (75 μM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 120 μL was incubated at room temperature for 20 h. Samples (55 μL) were combined at room temperature with Fluoraldehyde (*o*-phthalaldehyde;[57] Pierce, 55 μL) for 1 min. Aliquots (100 μL) were analyzed by reversed-phase HPLC with an Econosil C_{18} column (Alltech, 10 μm, 250 mm × 4.6 mm), eluting at 1 mL/min. The mobile phase was a gradient from 80% buffer A (95% 0.1 M NaOAc, pH 7.2, 4.5% methanol, and 0.5% THF) and 20% buffer B (methanol) to 30% buffer A and 70% buffer B over 15 min and then to 0% buffer A and 100% buffer B over 5 min. When N^{ω}-allyl-L-[1-^{14}C]arginine was used, all fractions (0.5 min each) were collected, and the radioactivity was measured by liquid scintillation counting. With **7** and **24** the area of the citrulline peak was determined by cutting and weighing the chromatogram peak (Table 1).

Production of [^{3}H]Water During Inactivation of nNOS by N^{ω}-[1-^{3}H]Allyl-L-arginine. A preincubation mixture containing nNOS (30 μg), $CaCl_2$ (6.25 mM), calmodulin (260 μg/mL), NADPH (7.8 mM), BH_4 (18 μM), DTT (5.3 mM), and N^{ω}-[1-^{3}H]allyl-L-arginine (75 μM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 120 μL was incubated at room temperature for 20 h. The incubation mixture was bulb-to-bulb distilled under vacuum at −78 °C as described previously.[29] The distillate was analyzed by HPLC (214 nm detection) with an Econosil C_{18} column (Alltech, 10 μm, 250 mm × 4.6 mm) and eluted at 1 mL/min. The mobile phase was a gradient from 0% buffer A (water, 0.1% TFA) to 100% buffer B (acetonitrile, 0.1% TFA) over 45 min. Fractions (1 min each) were collected, and the radioactivity was measured by liquid scintillation counting. A control was carried out with the replacement of NADPH by $NADP^+$ or with the replacement of nNOS with Hepes buffer. $^{3}H_2O$ and acrolein were injected into the HPLC separately as stardards to determine their retention times.

Production of Acrolein During Inactivation of nNOS by N^{ω}-[1-^{3}H]Allyl-L-arginine (Figure 11). A preincubation mixture containing nNOS (120 μg), $CaCl_2$ (6.25 mM), calmodulin (260 μg/mL), NADPH (7.8 mM), BH_4 (18 μM), DTT (5.3 mM), β-mercaptoethanol (118 mM), and N^{ω}-[1-^{3}H]allyl-L-arginine (75 μM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 480 μL was incubated at room temperature for 2 days. The incubation mixture was then microdialyzed against 3 mL of Hepes buffer solution for 6 h.[29] To the dialysis solution was added 2,4-dinitrophenylhydrazine solution (20 μL, 94 mM), and the mixture was stirred for 2 days. The mixture was extracted with ethyl acetate (3 × 1 mL), the organic phase was washed with water (1 mL) and concentrated in vacuo. The residue was dissolved in acetonitrile (0.3 mL). Aliquots (50 μL) were analyzed by HPLC (360 nm detection) with an Econosil C_{18} column (Alltech, 10 μm, 250 mm × 4.6 mm) and eluted at 1 mL/min. The mobile phase was a gradient from 40% buffer A (water, 0.1% TFA) and 60% buffer B (40% water, 60% acetonitrile, 0.1% TFA) to 100% buffer B over 15 min and then at 100% buffer B over 10 min. Fractions of 0.5 min each were collected, and the radioactivity of products was monitored by liquid scintillation counting. Identification of products was established by coinjection of samples with **18**.[29] A control was carried out with the replacement of NADPH by $NADP^+$.

Production of Arginine During Inactivation of nNOS by N^{ω}-Allyl-L-[1-^{14}C]arginine (Figure 12). A preincubation mixture containing nNOS (30 μg), $CaCl_2$ (6.25 mM), calmodulin (260 μg/mL), NADPH (7.8 mM), BH_4 (18 μM), DTT (5.3 mM), N^{ω}-allyl-L-[1-^{14}C]arginine (75 μM), and L-arginine (62 μM) in Hepes buffer (100 mM, pH 7.5) in a total volume of 120 μL was incubated at room temperature for 20 h. Samples (25 μL) were combined at room temperature with Fluoraldehyde (*o*-phthalaldehyde;[57] Pierce, 25 μL) for 1 min. Aliquots (50 μL) were analyzed by HPLC (340 nm detection) with an Econosil C_{18} column (Alltech, 10 μm, 250 mm × 4.6 mm) and eluted at 1 mL/min. The mobile phase was a gradient from 100% buffer A (95% 0.1 M NaOAc, pH 7.2, 4.5% methanol, and 0.5% THF) to 50% buffer A and 50% buffer B (methanol) over 20 min and then to 0% buffer A and 100% buffer B over 25 min. Fractions of 0.5 min each were collected, and the radioactivities of citrulline and arginine were monitored by liquid scintillation counting. Identification of amino acids was established by coinjection of samples with amino acid standards. A control was carried out with the replacement of NADPH by $NADP^+$ or with the replacement of nNOS with Hepes buffer.

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NO_2^- and NO_3^- Determinations (Table 1). The sum of NO_2^- and NO_3^- (NO_x) was measured via the Griess reaction[45] as follows. An aliquot (120 μL) of sample in water (300 μL) was placed in a cuvette, and then nitrate reductase (15 μL, Cayman's nitrate/nitrile assay kit from Alexis Corp., San Diego, CA) was added. The cuvette was covered, and the mixture was incubated for 4.5 h at room temperature. Griess reagent (100 μL, 1% sulfanilamide, 0.1% (naphthyl)ethylenediamine dihydrochloride, and 5% H_3PO_4 from the Cayman's nitrate/nitrite assay kit from Alexis Corp.) was added. After 10 min at room temperature, absorbances were recorded at 540 nm. A standard curve was constructed with known concentrations of NO_2^- and NO_3^- with inactivation cofactors in buffer as described above.

Effect of Incubation of nNOS with Acrolein. nNOS (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), BH_4 (50 μM), DTT (385 μM), glycerol (20% v/v), and acrolein (850 μM) in a total volume of 130 μL. The reactions were initiated by the addition of NADPH, and 25 μL of aliquots were removed to assay enzymatic activity over a 1 h period. A control was performed by replacing acrolein with Hepes buffer (100 mM, pH 7.5).

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