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# TIME-DEPENDENT INHIBITION OF NEURONAL NITRIC OXIDE SYNTHASE BY *N*-PROPARGYLGUANIDINE

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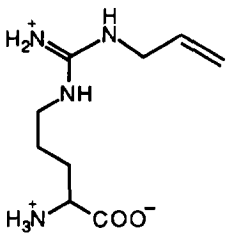
**Abstract:** *N*-Propargylguanidine was synthesized and found to be a time-dependent inhibitor of neuronal nitric oxide synthase from bovine brain. No inactivation occurs when $NADP^+$ is substituted for NADPH, inhibition is blocked by *L*-arginine, but not *D*-arginine, and no enzyme reactivation occurs by dilution of inactivated enzyme with excess *L*-arginine. © 1997 Elsevier Science Ltd.

Nitric oxide synthases (NOS, EC 1.14.13.39) comprise a family of enzyme isoforms that catalyze the conversion of L-arginine to L-citrulline and nitric oxide (NO) in various cells. Nitric oxide is now known to be an important second messenger molecule for the regulation of a variety of physiological functions; the endothelial cell enzyme (eNOS) is involved in the regulation of smooth muscle relaxation and blood pressure, neuronal nitric oxide synthase (nNOS) is important to long-term potentiation, and an inducible form (iNOS) is produced by activated macrophage cells during an immune response.[1] Despite the importance of NO, its overproduction can be hazardous to tissues because of its reactivity and free radical structure.[2] When this occurs, inhibitors of NOS would be an important approach to decrease the concentration of NO in the cell.

Many different inhibitors of NOS are known; some of the earliest inhibitors included the $N^{\omega}$-substituted arginine analogues.[3] $N^{\omega}$-Allyl-L-arginine (1) was designed as a mechanism-based inactivator[4] of NOS, and it was found to inactivate murine macrophage NOS.[5] Recently, we carried out a detailed study on the mechanism of inactivation of nNOS by $N^{\omega}$-allyl-L-arginine and concluded that it undergoes initial *N*-hydroxylation followed by further oxidation, leading to an allylated heme.[6] As an extension of this inactivation approach, the corresponding acetylenic analogue, $N^{\omega}$-propargyl-L-arginine (2), was synthesized, but, much to our surprise, it was found to be only a competitive reversible inhibitor of both nNOS and iNOS, not a time-dependent inactivator.[7] Furthermore, only very slow oxidation of this compound occurs upon incubation with nNOS. There are many potential inactivation mechanisms for an acetylenic inactivator of a heme- or flavin- containing enzyme. NOS, which contains both heme and flavin coenzymes, could be inactivated by the mechanisms for



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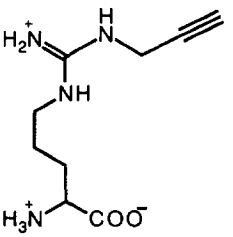

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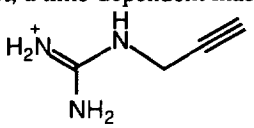
inactivation of heme-dependent cytochrome P-450 by acetylenes[8] or of flavin-dependent monoamine oxidase by acetylenes.[9] If not these mechanisms, then a mechanism analogous to inactivation of NOS by $N^{\omega}$-allyl-L-arginine[6] would be reasonable. However, all of these inactivation mechanisms require the inactivator to become oxidized by the enzyme to convert them into activated species. The fact that $N^{\omega}$-allyl-L-arginine does inactivate NOS, but $N^{\omega}$-propargyl-L-arginine does not, suggests that the difference is either the result of the geometric or redox potential difference of the $sp^2$-hybridized allyl group and the sp-hybridized propargyl group. Although both compounds bind to the enzyme, the propargyl analogue may be misoriented in the active site so that oxidation is prohibited. We sought to test this hypothesis by synthesizing a propargyl-containing compound that had less restrictive binding within the active site, namely, *N*-propargylguanidine (**3**), to see if inactivation could be induced. Here we show that **3** is, in fact, a time-dependent inactivator of nNOS.



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*N*-Propargylguanidine (**3**)[10] was synthesized by the general route to *N*-substituted guanidines,[11] using propargyl amine as the amine nucleophile. Incubation of purified bovine brain nNOS[12] with **3** resulted in time-dependent inactivation (Figure 1);[13] the $K_I$ and $k_{inact}$ values, determined by the method of Kitz and Wilson[14] (Figure 2), are 12 mM and 0.39 $min^{-1}$, respectively. No inactivation occurs when $NADP^+$ is substituted for NADPH (Figure 3), indicating that electron transfer is important to the inactivation mechanism. The presence of 1.2 mM *L*-arginine protects the enzyme from inactivation by 3 mM *N*-propargylguanidine; the same concentration of *D*-arginine has no effect on the inactivation rate (Figure 4), suggesting that inactivation is active-site directed. Activity does not return upon addition of 1.2 mM *L*-arginine to the inactivated enzyme (Figure 5), supporting the irreversible nature of the reaction. To demonstrate that possible trace amounts of residual 1-*H*-pyrazole-1-carboxamidine hydrochloride, the reagent used in the synthesis of **3**, was not responsible for the inactivation, NOS was incubated with a 100 μM concentration of the reagent; no inactivation resulted. The propargyl group is, apparently, important to the inactivation because *N*-methylguanidine is a reversible inhibitor of all three isoforms of NOS.[15] The detailed mechanism of inactivation of NOS by **3** is under investigation.

**Figure 1.** Time-dependent Inhibition of Neuronal Nitric Oxide Synthase by **3**

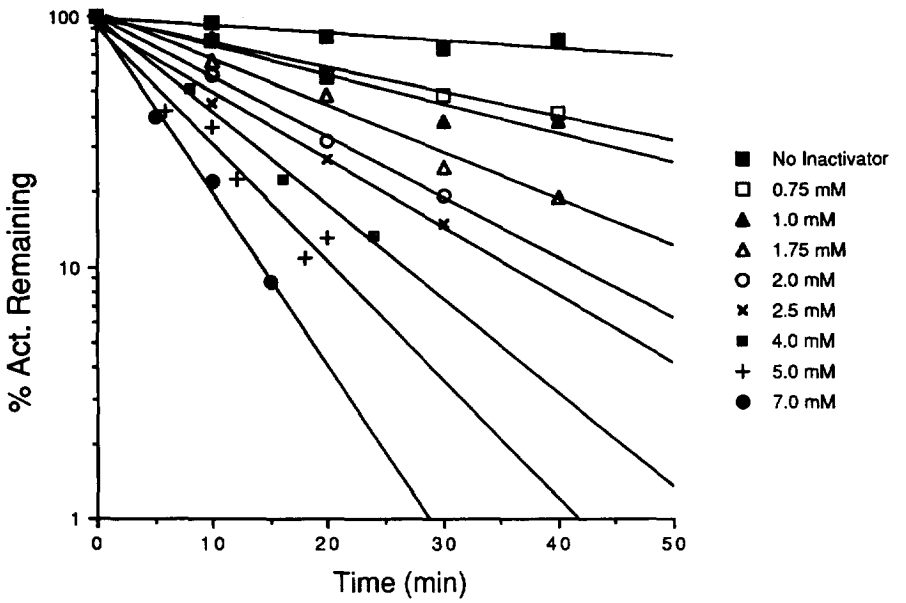



**Figure 2.** Kitz and Wilson[14] Replot of the Data from Figure 1

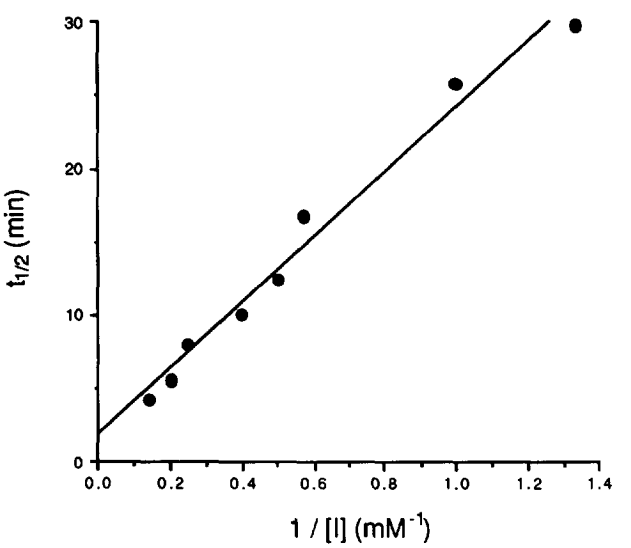


**Figure 3.** Effect of $NADP^+$ on the Inhibition of Neuronal Nitric Oxide Synthase by **3**. $NADP^+$ was substituted for NADPH during incubation of **3** (3 mM) with nNOS and cofactors. (●), no inactivator; (■), NADPH; (❑), $NADP^+$.

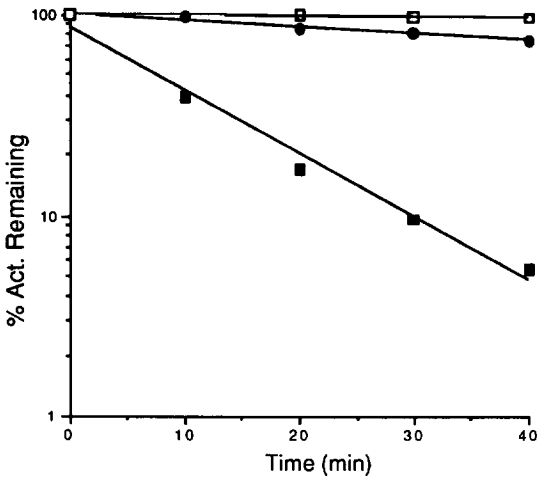



**Figure 4.** Protection Against Inactivation by Substrate. (■), no inactivator; (●), 3 mM **3**; (▲), 3 mM **3** plus 1.2 mM *L*-arginine; (Δ), 3 mM **3** plus 1.2 mM *D*-arginine.

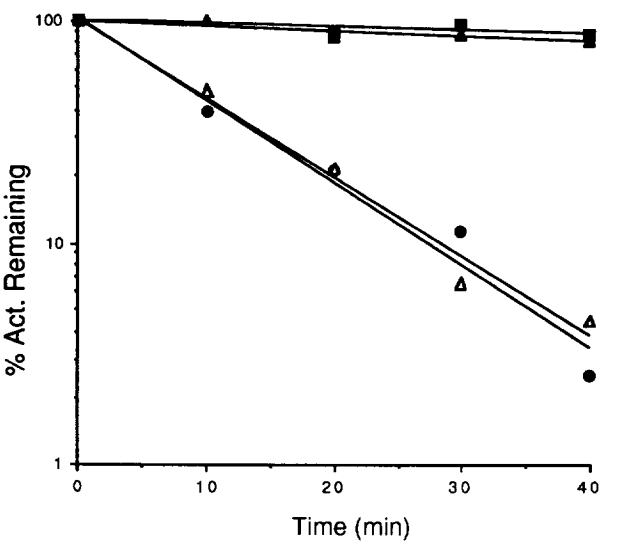
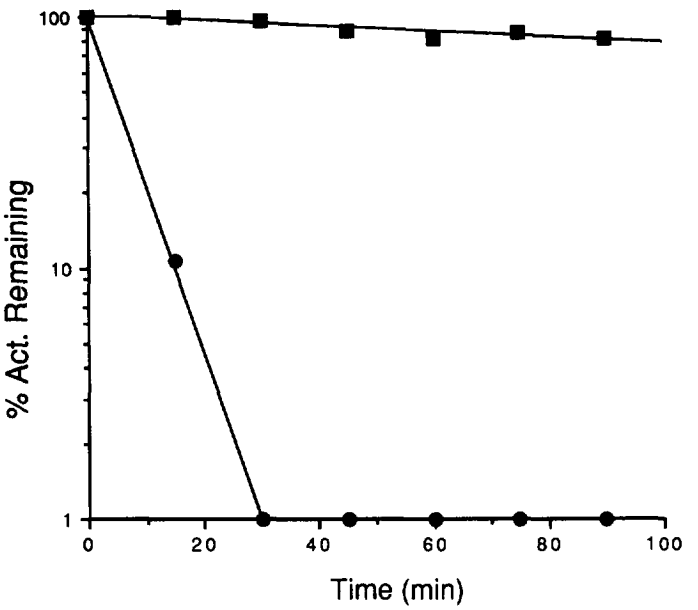


**Figure 5.** Irreversible Inhibition. The same experiment as shown in Figure 1 was carried out using 7 mM **3** (●); (■), no inactivator. After complete inactivation (at 35 min), *L*-arginine was added to a concentration of 1.2 mM, and aliquots were assayed with time.




**Acknowledgments**

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10. To propargylamine (0.52 mL, 7.5 mmol), 1-*H*-pyrazole-1-carboxamidine hydrochloride (0.7424 g, 5 mmol), and freshly distilled diisopropylethylamine (0.875 mL, 5 mmol) was added 8.8 mL of DMF to produce a solution that was 2 *M* in reactants. The mixture was stirred at room temperature under nitrogen with additional propargylamine (0.34 mL, 5 mmol) added to replace what was lost by evaporation during the reaction, until TLC analysis indicated that all of the 1-*H*-pyrazole-1-carboxamidine hydrochloride had been consumed (144 h). Ethyl ether was added, and the resulting pale yellow powder was washed twice with ether and dried in vacuo. The powder was recrystallized from ethanol/ethyl acetate to give white needles of propargylguanidine hydrochloride (0.4056 g, 60 %); mp 115-116 °C; $^1$H NMR (DMSO) δ 2.52 (1 H), 4.04 (d, 2 H), 7.55 (br s, 5 H); $^1$H NMR (D2O/DSS) δ 2.74 (t, 1 H), 4.03 (d, 2 H); $^{13}$C NMR ($D_2O$) δ 30.4, 73.5, 77.5, 156.3; EI-HRMS ($C_4H_7N_3$) calcd 97.0640, found 97.0632. Anal. Calcd for $C_4H_8N_3Cl$: C, 35.97; H, 6.04; N, 31.46. Found: C, 35.89; H, 5.86; N, 31.26.

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