Expedited Articles

L-N^6-(1-Iminoethyl)lysine: A Selective Inhibitor of Inducible Nitric Oxide Synthase

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L-N^6-(1-Iminoethyl)lysine (L-NIL) has been synthesized and is shown to be both a potent and selective inhibitor of mouse inducible nitric oxide synthase (miNOS). L-NIL has an IC_{50} of 3.3 μM for miNOS compared to an IC_{50} of 92 μM for rat brain constitutive NOS indicating that L-NIL is 28-fold more selective for inducible NOS. L-N^5-(1-Iminoethyl)ornithine (L-NIO), which differs from L-NIL by having one less methylene group, has very similar potency for inducible NOS, but lacks selectivity. DL-N^7-(1-Iminoethyl)homolysine was also synthesized and found to be substantially less potent than L-NIL or L-NIO, with intermediate selectivity for inducible NOS. These data suggest that L-NIL may be useful as a selective inhibitor of inducible NOS for determining the role of this enzyme in disease models.

Nitric oxide synthase (NOS) catalyzes the five-electron oxidation of L-arginine to L-citrulline and the free radical, nitric oxide.[1] Two major isoforms of NOS have been described: a constitutive NOS,[2-4] which is found predominantly in the vascular endothelium and in the brain, and an inducible NOS, which is present in activated macrophages.[5-7] The constitutive enzyme requires Ca^{2+}/calmodulin for activity and can be further subdivided into endothelial and neuronal isoforms. The endothelial enzyme generates nitric oxide which acts to lower blood pressure and inhibit platelet aggregation.[2] The nitric oxide produced by the neuronal enzyme is thought to function as a neurotransmitter to regulate neuronal transmission.[2] The major function of inducible NOS is to serve in host defense and it is this form that is implicated in the excessive production of nitric oxide that destroys functional tissue during chronic inflammatory processes.[2]

Structural analogs of L-arginine (Figure 1), including N^G-monomethyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA) have been shown to be inhibitors of the various forms of NOS.[2,8-11] Administration of L-NMA causes a marked and sustained increase in blood pressure, indicating the importance of nitric oxide synthesis by the vascular endothelium.[12] Differences in the arginine binding sites of the constitutive and inducible forms are suggested by the potent inhibition of constitutive NOS by L-NNA, a less potent inhibitor of inducible NOS.[11] Because of the importance of the constitutive forms in normal physiology, the highly selective inhibition of the inducible form is an essential characteristic of any agent likely to have clinical utility for the prevention and treatment of diseases mediated by excessive production of nitric oxide.[13]

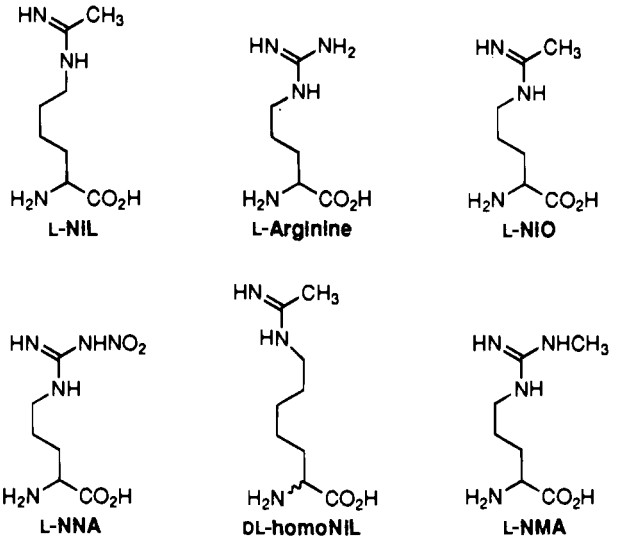

Figure 1. Structures of L-NIL, L-NIO, DL-homoNIL, L-arginine, L-NNA, and L-NMA.

We synthesized L-N^6-(1-iminoethyl)lysine (L-NIL), L-N^5-(1-iminoethyl)ornithine (L-NIO), and DL-N^7-(1-iminoethyl)homolysine (DL-homoNIL) (Figure 1) to determine their potency and selectivity as inhibitors of NOS. As shown in Figure 2, L-NIL produced a concentration-dependent inhibition of both the mouse inducible NOS (miNOS) and the rat brain constitutive NOS (rcNOS) and was considerably more potent for miNOS. The IC_{50} values determined for L-NIL with miNOS and rcNOS were 3.3 and 92 μM, respectively, indicating that L-NIL is 28-fold more selective for miNOS (Table 1). In addition, L-NIL had approximately 6-fold greater potency for miNOS than either L-NMA or L-NNA (Table 1). L-NIO has been reported to be an inhibitor of NOS in phagocytic cells,[14] rat brain,[15] and vascular systems[16] and was synthesized for comparison to L-NIL. As shown in Table 1, the IC_{50} values for L-NIL and L-NIO with miNOS are essentially the same, 3.3 and 2.2 μM

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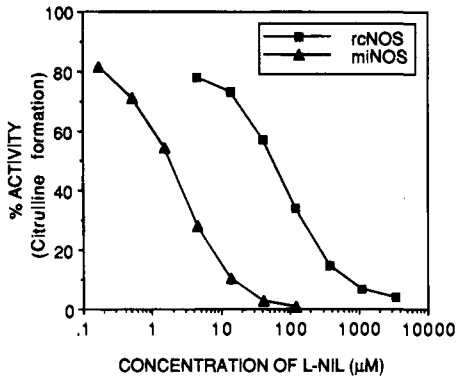

Figure 2. Effect of L-NIL concentration on the activity of mouse inducible NOS (miNOS) and rat brain constitutive NOS (rcNOS). NOS activity was determined by measuring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as described in the Experimental Section. The 100% activity values for miNOS and rcNOS were 401 and 180 pmol of citrulline generated per 15 min, respectively.

Table 1. Comparison of IC_{50} Values for Inhibition of Mouse Inducible NOS and Rat Brain Constitutive NOS

	IC_{50}[a] (μM)		selectivity[b]
	rcNOS	miNOS	rcNOS/miNOS
L-NIL	92	3.3	28
L-NIO	3.9	2.2	1.8
DL-homoNIL	808	73	11
D-NIL	2800	336	8
L-NMA	8.3	18	0.5
L-NNA	0.5	20	0.025

[a] IC_{50} values were determined with mouse inducible NOS (miNOS) and rat brain constitutive NOS (rcNOS) by testing each compound at eight concentrations. NOS activity was measured by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as described in the Experimental Section. [b] Selectivity is defined as the ratio of the IC_{50} rcNOS to IC_{50} miNOS.

respectively. However, when compared as inhibitors of the rcNOS, L-NIO had an IC_{50} of 3.9 μM and a selectivity ratio of only 1.8-fold. The structural difference of only one additional methylene for L-NIL compared to L-NIO afforded a dramatic increase in selectivity. DL-homoNIL was prepared to determine the effect of incorporation of an additional methylene group on potency and selectivity. As shown in Table 1, DL-homoNIL was substantially less potent than L-NIL and L-NIO as an inhibitor of miNOS and exhibited intermediate selectivity. D-NIL, the enantiomeric isomer of L-NIL, was a poor inhibitor of both inducible and constitutive NOS, indicating the importance of maintaining stereochemistry at the α-carbon, consistent with the observation that D-arginine is neither a substrate nor inhibitor of NOS.[13] The slight inhibition observed with D-NIL could be the result of a 1% contamination by the L-isomer. Since DL-homoNIL is an enantiomeric mixture and assuming the D-enantiomer is not an inhibitor, the IC_{50} reported for the mixture would be expected to be higher than for the L-enantiomer alone. However, the selectivity ratio should be the same. These results indicate that changes in the length of the amino acid side chain significantly affect potency and selectivity.

L-NIL promises to be a useful tool for evaluating the effect of a selective inhibitor of the inducible nitric oxide synthase in animal models of disease. This selective inhibitor may prove particularly useful in determining the role of nitric oxide production by inducible NOS in models of chronic inflammation (e.g., adjuvant arthritis) and autoimmune diseases (e.g., glomerulonephritis). We have found that L-NIL suppresses the increase in plasma nitrite/nitrate levels and joint inflammation associated with adjuvant arthritis.[17] Preliminary results suggest that L-NIL is an irreversible inhibitor of inducible NOS. Studies to elucidate the mechanism by which L-NIL inhibits NOS are in progress.

Experimental Section

L-[2,3-^{3}H]arginine was purchased from Dupont NEN (Boston, MA); (6*R*)-tetrahydro-L-biopterin was from Research Biochemicals, Inc. (Natick, MA); ethyl acetimidate was from Aldrich Chemical Co. (Milwaukee, WI); cupric carbonate was obtained from Fisher Chemicals (Pittsburgh, PA); L-NNA and L-NMA were from Sigma (St. Louis, MO); amino acids and other chemicals and reagents were obtained from either Sigma or Bachem California (Torrance, CA). Frozen rat cerebella were from Pel-Freez (Rogers, AR). Purification of synthesized compounds was performed using Dowex 50W cation-exchange resin purchased from Sigma. ^{1}H and ^{13}C NMR spectra were obtained at 300 MHz on a Varian VXR300 spectrometer in D_2O. Mass spectra were obtained on a VG Model 250 spectrometer, and high-resolution mass spectra were obtained with a Finnigan MAT 95. Elemental analyses were performed by Atlantic Microlabs, Inc. (Norcross, GA). Compounds were analyzed for purity by chromatography on a Zorbax NH2 column (4.6 × 250 mm) (MAC-MOD Analytical, Inc., Chads Ford, PA). Elution was accomplished using an isocratic gradient containing 10 mM KH_2PO_4:12.3% H_2O in acetonitrile (70:30) at a flow rate of 1 mL/min. Detection was by absorbance at 205 nm. Under these conditions L-NIO, L-NIL, D-NIL, and DL-homoNIL elute at 8.1, 7.7, 7.7, and 5.6 min, respectively, with corresponding purity of 96%, 94%, 91%, and 90%. NMR spectral data of L-NIL and L-NIO were consistent with reported values.[18,19]

Synthesis of L-NIO, L-NIL, D-NIL, and DL-homoNIL: General Procedure. L-NIO, L-NIL, D-NIL, and DL-homoNIL were prepared by modifications of literature procedures.[18,19] The α-Boc-amino acid as a 5% solution in water was stirred at pH 10 while ethyl acetimidate hydrochloride (1.3 equiv) was added portionwise with concomitant adjustment of pH to 9 by addition of 2.5 N NaOH. Thirty minutes after addition was complete the pH was adjusted to 7.5 with 2 N HCl. After 18 h at room temperature, the solution was poured onto a column packed with Dowex 50W cation-exchange resin. The column was washed with distilled water until neutral. The Boc-protected product was then eluted from the column with 10% aqueous pyridine. Concentration of the eluate in vacuo afforded a viscous oil. This oil was stirred with 4 M HCl in dioxane for 30 min. Removal of the solvent in vacuo afforded the N^{ω}-(1-iminoethyl) amino acid dihydrochloride salt.

L-N^5-(1-Iminoethyl)ornithine.[18] L-NIO was prepared from the α-Boc-L-ornithine to afford a 27% yield of the dihydrochloride salt as a very hygroscopic off-white solid, mp 65–70 °C. ^{1}H NMR (D_2O): δ 1.55–1.95 (m, 4H), 2.1 (s, 3H), 3.2 (t, 2H), 3.92 (t, 1H). ^{13}C NMR (D_2O): δ 171.42, 164.58, 52.23, 41.08, 26.77, 22.44, 18.23. High-resolution mass spectrum: calcd for $C_7H_{15}N_3O_2$ 174.1243, found 174.1237. Anal. ($C_7H_{15}N_3O_2$ + 1 H_2O) C, H, N.

L-N^6-(1-Iminoethyl)lysine.[19] L-NIL was prepared from the α-Boc-L-lysine to afford a 77% yield of the dihydrochloride salt as a colorless deliquescent solid. ^{1}H NMR (D_2O): δ 1.35–1.5 (m, 2H), 1.58 (p, 2H), 1.75–1.95 (m, 2H), 2.08 (s, 3H), 3.12 (t, 2H), 3.92 (t, 1H). ^{13}C NMR (D_2O): δ 171.69, 164.32, 52.47, 41.46, 29.00, 25.99, 21.35, 18.23. High-resolution mass spectrum: calcd for $C_8H_{17}N_3O_2$ 188.1400, found 188.1393. Anal. ($C_8H_{19}N_3O_2Cl_2$ + ½ H_2O) C, H, N.

D-N^6-(1-Iminoethyl)lysine. D-NIL was prepared from the α-Boc-D-lysine to afford an 85% yield of the dihydrochloride salt as a white hygroscopic foam. ^{1}H NMR (D_2O): δ 1.25–1.5 (m, 2H), 1.55 (p, 2H), 1.7–1.9 (m, 2H), 2.05 (s, 3H), 3.12 (t, 2H), 3.93 (t, 1H). Mass spectrum, 188 (MH^+). Anal. ($C_8H_{19}N_3O_2Cl_2$ + 1 H_2O) C, H, N.

DL-N^7-(1-Iminoethyl)homolysine. DL-homoNIL was prepared as in the general procedure from DL-homolysine[20]

utilizing Cu^{2+} as the α-amino acid protecting group. Elution from the Dowex column with 1 M ammonium hydroxide, followed by concentration in vacuo, afforded the free base as a colorless viscous oil. Acidification with acetic acid afforded a 42% yield of the diacetic acid salt as a colorless viscous oil. ^1H NMR (D_2O): δ 1.2–1.35 (m, 4H), 1.48–1.55 (m, 2H), 1.68–1.78 (m, 2H), 1.85 (s, 6H), 2.08 (s, 3H), 3.1 (t, 2H), 3.59 (t, 1H). ^{13}C NMR (D_2O): δ 180.70, 176.04, 165.74, 55.93, 43.14, 31.37, 27.54, 26.78, 24.98, 23.31, 19.49. High-resolution mass spectrum: calcd for $C_9H_{19}N_3O_2$ 202.1556, found 202.1565.

Assay of NOS Activity. NOS activity was measured by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]-citrulline.[3,21] Mouse inducible NOS (miNOS) was prepared from an extract of LPS-treated mouse RAW 264.7 cells and rat brain constitutive NOS (rcNOS) was prepared from an extract of rat cerebellum. Both preparations were partially purified by DEAE-Sepharose chromatography as previously described.[21] Enzyme (10 μL) was added to 40 μL of 50 mM Tris (pH 7.6) and the reaction initiated by the addition of 50 μL of a solution containing 50 mM Tris (pH 7.6), 2.0 mg/mL bovine serum albumin, 2.0 mM DTT, 4.0 mM $CaCl_2$, 20 μM FAD, 100 μM tetrahydrobiopterin, 2.0 mM NADPH, and 60 μM L-arginine containing 0.9 μCi of L-[2,3-^{3}H]arginine. For constitutive NOS, calmodulin was included at a final concentration of 40 nM. Following incubation at 37 °C for 15 min, the reaction was terminated by addition of 300 μL of cold buffer containing 10 mM EGTA, 100 mM HEPES (pH 5.5), and 1.0 mM L-citrulline. The [^{3}H]citrulline was separated by chromatography on Dowex 50W X-8 cation-exchange resin and radioactivity quantified with a liquid scintillation counter. All assays were performed at least in duplicate with standard deviations of 10% or less. Production of [^{3}H]citrulline was linear with time over the course of the assay.

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References

(1) Marletta, M. A. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **1993**, *268*, 12231–12234.
(2) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **1991**, *43*, 109–142.
(3) Bredt, D. S.; Snyder, S. H. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 682–685.
(4) Pollock, J. S.; Forstermann, U.; Mitchell, J. A.; Warner, T. D.; Schmidt, H. H. H. W.; Nakane, M.; Murad, F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10480–10484.
(5) Stuehr, D. J.; Marletta, M. A. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 7738–7742.
(6) Hevel, J. M.; White, K. A.; Marletta, M. A. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* **1991**, *266*, 22789–22791.
(7) Stuehr, D. J.; Cho, H. J.; Kwon, N. S.; Wiese, M. F.; Nathan, C. F. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7773–7777.
(8) Hibbs, J. B.; Taintor, R. R.; Vavrin, Z. Macrophage cytotoxicity: role for L-arginine deiminase activity and imino nitrogen oxidation to nitrite. *Science* **1987**, *235*, 473–476.
(9) Olken, N. M.; Marletta, M. A. N^G-Methyl-L-arginine functions as an alternate substrate and mechanism-based inhibitor of nitric oxide synthase. *Biochemistry* **1993**, *32*, 9677–9685.
(10) Feldman, P. L.; Griffith, O. W.; Hong, H.; Stuehr, D. J. Irreversible inactivation of macrophage and brain nitric oxide synthase by L-N^G-methylarginine requires NADPH-dependent hydroxylation. *J. Med. Chem.* **1993**, *36*, 491–496.
(11) Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. Selective Inhibition of constitutive nitric oxide synthase by N^G-nitroarginine. *Biochemistry* **1993**, *32*, 8512–8517.
(12) Rees, D. D.; Palmer, R. M. J.; Moncada, S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3375–3378.
(13) Marletta, M. A. Approaches toward selective inhibition of nitric oxide synthase. *J. Med. Chem.* **1994**, *37*, 1899–1907.
(14) McCall, T. B.; Feelisch, M.; Palmer, R. M. J.; Moncada, S. Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.* **1991**, *102*, 234–238.
(15) Knowles, R. G.; Palacios, M.; Palmer, R. M. J.; Moncada, S. Kinetic characteristics of nitric oxide synthase from rat brain. *Biochem. J.* **1990**, *269*, 207–210.
(16) Rees, D. D.; Palmer, R. M. J.; Schulz, R.; Hodson, H. F.; Moncada, S. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.* **1990**, *101*, 746–752.
(17) Connor, J. R.; Manning, P. T.; Settle, S. L.; Moore, W. M.; Jerome, G. M.; Webber, R. K.; Tjoeng, F. S.; Currie, M. G. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *Eur. J. Pharm.* in press.
(18) Scannell, J. P.; Ax, H. A.; Pruess, D. L.; Williams, T.; Demny, T. C.; Stempel, A. Antimetabolites produced by microorganisms. L-N^5-(1-iminoethyl)ornithine. *J. Antibiot.* **1972**, *25*, 179–184.
(19) Plapp, B. V.; Kim, J. C. Determination of ω-acetimidyllysine in proteins. *Anal. Biochem.* **1974**, *62*, 291–294.
(20) Payne, L. S.; Boger, J. Synthesis of alpha, omega – diamino acids: An efficient preparation of DL-homolysine and protected derivatives. *Synth. Commun.* **1985**, *15* (14) 1277–90.
(21) Misko, T. P.; Moore, W. M.; Kasten, T. P.; Nickols, G. A.; Corbett, J. A.; Tilton, R. G.; McDaniel, M. L.; Williamson, J. R.; Currie, M. G. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharm.* **1993**, *233*, 119–125.