Synthesis and Biological Characterization of L-N^6-(1-Iminoethyl)lysine 5-Tetrazole-amide, a Prodrug of a Selective iNOS Inhibitor

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Received September 5, 2001

The 5-tetrazole amide of L-N^6-(1-iminoethyl)lysine (L-NIL), L-N^6-(1-iminoethyl)lysine 5-tetrazole amide (**1**), has been prepared and evaluated. In contrast to L-NIL, **1** is a stable, nonhygroscopic, crystalline solid. Unlike L-NIL, **1** has minimal inhibitory activity in vitro on human inducible nitric oxide synthase (iNOS). However, it is rapidly converted in vivo to L-NIL and produces dose-dependent inhibition of iNOS in acute and chronic models of inflammation in the rodent with efficacy comparable to L-NIL. In addition, both **1** and L-NIL exhibit significant and comparable in vivo selectivity for the inhibition of iNOS vs endothelial NOS. Doses approximately 80-fold greater than those that inhibited inflammation do not elevate systemic blood pressure. In summary, both the physical properties and the pharmacological profile of **1** make it an ideal molecule for preclinical and clinical studies on the role of selective iNOS inhibitors in mediating inflammatory disease processes.

Introduction

Nitric oxide (NO) is involved in the regulation of many physiological processes, as well as in the pathophysiology of a number of diseases. It is synthesized enzymatically from L-arginine via a five electron transfer process in numerous tissues and cell types by three distinct isoforms of the enzyme, nitric oxide synthase (NOS). Two of these isoforms are expressed in a constitutive manner, predominantly in the vascular endothelium (eNOS, type III NOS) and in the nervous system (nNOS, type I NOS). Under normal physiological conditions, these constitutive forms of NOS generate low, transient levels of NO (picomolar to nanomolar concentrations) in response to increases in intracellular calcium concentrations. These low levels of NO act to regulate blood pressure, platelet adhesion, gastrointestinal motility, bronchomotor tone, and neurotransmission.[1] The expression of the third isoform (iNOS, type II NOS) is induced by endotoxin and/or cytokines and generates high, sustained levels of NO (up to micromolar concentrations). These elevated levels of NO and resulting NO-derived metabolites cause cellular cytotoxicity and tissue damage and are thought to contribute to the pathophysiology of a number of human diseases.[2]

NO, or more likely peroxynitrite, a product of the reaction of NO and superoxide, has also been found to directly activate both the constitutive and inducible cyclooxygenase enzymes (COX-1 and COX-2, respectively) by serving as a substrate for the peroxidase activities of these enzymes[3,4] resulting in an increase in local prostaglandin production. This enhanced production of local prostaglandins may subsequently augment the inflammation and tissue damage elicited by the cytotoxic actions of NO. This observation is of particular importance with regard to iNOS and COX-2, since the expression of these two inducible proteins appears to be regulated by the same factors produced during chronic inflammation.[5] The activation of COX-2 by NO generated from iNOS may increase and/or sustain inflammation during both acute and chronic inflammatory conditions.

Selective iNOS inhibitors, including L-NIL,[6] have been shown to suppress the increase in plasma nitrites and/or paw swelling associated with the overproduction of NO in animal models of acute and chronic inflammation including nonlethal endotoxemia,[6b] carrageenan-induced paw edema,[7,8] and adjuvant-induced arthritis.[9,10] Importantly, L-NIL was found to be both orally active and produce marked efficacy at doses that did not produce an elevation in systemic blood pressure, demonstrating in vivo selectivity.[8,9] This suggests that selective iNOS inhibitors may have therapeutic potential for treatment of diseases mediated by the overproduction of NO.

The pharmaceutical properties of L-NIL are suboptimal for carrying out chronic studies, particularly preclinical studies in large animals as well as clinical studies. Therefore, an attempt was made to identify a prodrug form of L-NIL that possessed the necessary physical properties required for more in depth characterization. Compound **1**, a tetrazole-amide of L-NIL, was synthesized and found to be a stable crystalline solid that exhibited comparable biological activity to L-NIL. The synthesis and the preliminary biological characterization of this prodrug (**1**) are described.

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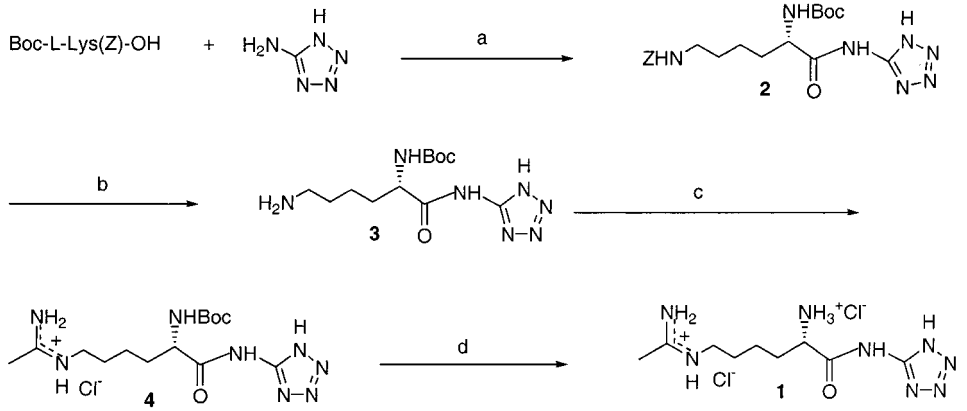
10.1021/jm010420e CCC: $22.00
Published on Web 03/13/2002

Scheme 1[a]



[a] Key: (a) BOP, NMM, DMF, room temperature, 20 h, 31%. (b) H_2, 5% Pd/C, EtOH, HOAc, 5 psi, room temperature, 9 h, quantitative. (c) Methyl acetimidate hydrochloride, TEA, DMF or ethyl acetimidate HCl, EtOH, NaOH, o.n., room temperature. (d) 4 N HCl/dioxane, HOAc, 2 h, 91% from step 2.

Synthesis

As shown in Scheme 1, the synthetic sequence required acylation of 5-amino-tetrazole with N^{α}-Boc-N^{ϵ}-Z-L-Lys-OH. Coupling of 5-aminotetrazole to protected lysine was more difficult than expected. The mixed anhydride coupling method using iso-butyl chloroformate or a carbodiimide-mediated acylation with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and N-hydroxybenzotriazole resulted in **2** in poor yields. Benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) proved to be the most effective coupling agent, with yields of approximately 60% on a small scale and 30% on a large scale. The poor nucleophilicity of 5-aminotetrazole probably accounted for the poor coupling yield. The product of the coupling was subsequently digested in dichloromethane to give pure, crystalline product with a melting point of 196.6 °C. Alternatively, the reaction mixture could be poured onto cold water. With stirring, a solid formed that could be filtered and washed with H_2O to provide **2**. Removal of the carbobenzoxy group using Pd/C was quantitative. Recrystallization from ethyl acetate gave a crystalline acetate salt **3** with a melting point of 205.5 °C. Amidine formation was uneventful, but immediate protonation of the amidine was necessary to avoid decomposition to the ϵ amide. The Boc group was removed under anhydrous acid conditions using HCl in dioxane. The final product **1** was purified by washing with HOAc and Et_2O followed by recrystallization from methanol and acetone yielding a white solid with a melting point of 168.2 °C. The tetrazole amide was stable under normal reaction conditions.

Biological Assays

As shown in Table 1, **1** in comparison to L-NIL produced essentially no inhibition of the human NOS isoforms. The IC_{50} values for human recombinant iNOS, eNOS, and nNOS were greater than 1850 μM, indicating that the prodrug exhibited little to no iNOS inhibitory activity. However, when evaluated in vivo for efficacy following oral administration in a nonlethal endotoxemia model in the rat, **1** gave dose-dependent inhibition of elevated plasma nitrites, similar to that which occurred with L-NIL[6a] (Figure 1). The shift (not statistically significant) between the two dose−response curves observed in Figure 1 could be attributed to the higher molecular weight of **1** due to the aminotetrazole moiety. The mean ED_{50} for **1** is 0.6 vs 0.5 mg/kg for L-NIL. Compound **1** was also assessed in an acute model of inflammation, the rat carrageenan-induced paw edema model, and was found to reduce paw swelling in a dose-dependent manner (ED_{50} between 10 and 30 mg/kg, Figure 2). This is in good agreement with data reported for L-NIL (50% reduction in paw swelling at approximately 30 mg/kg) found 3 h after administration of compound.[7] For further characterization, the efficacy of **1** was assessed in a chronic model of inflammation, rat adjuvant-induced arthritis. As shown in Figure 3, **1** gave dose-dependent inhibition of paw swelling/joint inflammation, producing complete inhibition between

Table 1. Comparison of IC_{50} Values for Inhibition of Human NOS Isoforms (IC_{50} − μM)[a]

compd	iNOS	eNOS	nNOS
L-NIL	5.9	138	35
1	>1850	2420	>1850

[a] IC_{50} values were determined with recombinant human inducible, endothelial, and neuronal NOS isoforms as described in the Experimental Section.

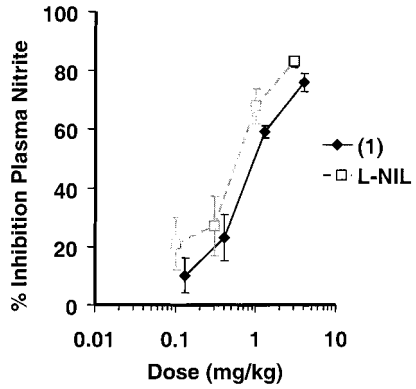


Figure 1. Efficacy of L-N^6-(1-iminoethyl)lysine 5-tetrazole amide (**1**) and L-NIL in the nonlethal rat endotoxin model. Values are means ± SEM for $n = 6$ animals/dose and represent the percent inhibition in plasma nitrite levels of animals treated with endotoxin alone as compared to saline-treated controls. Data shown is from a representative experiment. Mean values from multiple experiments gave an ED_{50} of 0.6 mg/kg for **1** and 0.5 mg/kg for L-NIL.

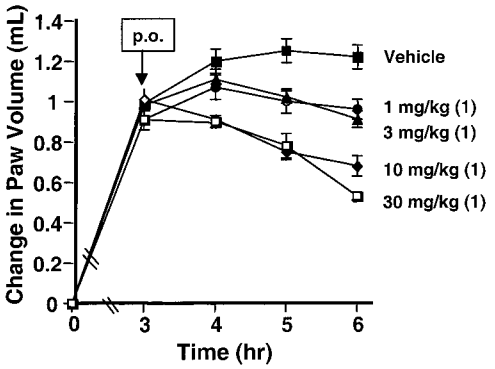


Figure 2. Efficacy of L-N^6-(1-iminoethyl)lysine 5-tetrazole amide (**1**) in the carrageenan-induced model of acute paw inflammation. Compound was administered by gavage 3 h after carrageenan injection. Edema was measured by the change in paw volume. Values are means ± SEM for $n = 5$ animals/dose.

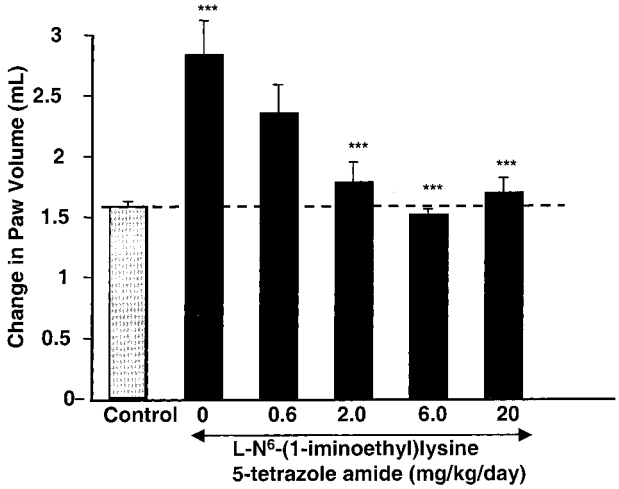


Figure 3. Efficacy of L-N^6-(1-iminoethyl)lysine 5-tetrazole amide (**1**) in the adjuvant-induced arthritis model on changes in hind paw volume 21 days following adjuvant administration. Doses expressed are milligrams per kilogram per day. Compound **1** was administered at doses of 0.3, 1, 3, or 10 mg/kg/dose twice daily throughout the course of the study starting at the time of adjuvant administration. Values are means ± SEM for $n = 7-10$ animals/dose.

6 and 20 mg/kg/day. This value agrees well with the literature value reported for L-NIL (maximal inhibition at 20 mg/kg/day).[10] In addition, similar efficacy was observed with **1** and L-NIL when evaluating plasma or paw exudate nitrite/nitrate levels (data not shown). Finally, the in vivo selectivity for iNOS vs eNOS was determined by assessing the effect on systemic blood pressure following oral administration of both compounds in the rat. No increase in blood pressure occurred following the oral administration of 40 mg/kg of either **1** or L-NIL, while a modest increase occurred (~10%) following the administration of 50 mg/kg of either compound, demonstrating substantial in vivo selectivity in the rat. Importantly, ex vivo studies demonstrate that **1** is rapidly metabolized in whole blood of rat, dog, and monkey to generate L-NIL and aminotetrazole (data not shown). In vivo studies in the rat indicate that **1** is rapidly metabolized to L-NIL following iv administration and L-NIL is rapidly available in the systemic circulation after oral administration of **1** (data not shown). In our initial in vivo studies, no toxicity has been associated with the aminotetrazole portion of the prodrug (data not shown), in agreement with data in the literature.[11]

Conclusions

Compound **1** was synthesized and shown to be a stable, nonhygroscopic, highly water soluble crystalline solid, clearly distinct from L-NIL, which rapidly deliquesces on exposure to air. Importantly, these physical properties of **1** are preferable for use in chronic preclinical studies, particularly in larger animals such as the dog and primate, as well as for use in clinical studies.

While **1** is a poor inhibitor of the human recombinant NOS isoforms in enzyme assays in comparison to L-NIL (Table 1), it is readily converted in vivo to generate L-NIL and aminotetrazole. The pharmacological properties of **1** have been assessed and compared to L-NIL in vivo in several models of acute and chronic inflammation. Both compounds are potent inhibitors of iNOS in a rodent nonlethal endotoxin model, exhibiting dose-dependent inhibition of nitric oxide production following the systemic induction of iNOS. Treatment with either **1** or L-NIL[7,8] reduced paw inflammation following carageenan administration in a dose-dependent fashion and with similar efficacy. In a chronic inflammatory model, rat adjuvant-induced arthritis, iNOS expression is elevated in the inflamed, swollen joint tissue. In this model, the prophylactic treatment with either **1** or L-NIL[9,10] reduced the paw swelling with similar efficacy as in the acute models.

In addition to characterizing their pharmacological activity, these compounds were also examined for effects on blood pressure (regulated by eNOS) in order to provide an indication of their functional selectivity in vivo. Neither compound produced an increase in blood pressure at doses up to approximately 80-fold of the ED_{50} (~0.5 mg/kg) in the rat nonlethal endotoxin model, demonstrating in vivo selectivity for the inhibition of iNOS vs eNOS. In summary, we have found that **1** possesses significantly improved physical properties as compared to L-NIL, while maintaining its favorable pharmacological properties. Finally, we are further exploring the potential of the prodrug, **1**, in both preclinical and clinical settings.

Experimental Section

All solvents and reagents were used without further purification unless otherwise noted. Thin layer chromatograms were run on 0.25 mm EM precoated plates of silica gel 60 F254. Visualization was achieved by exposure to $Ca(ClO)_2$ followed by spraying with a 0.5% KI 0.5% potato starch solution. High-performance liquid chromatograms (HPLC) were obtained from YMC AQ C-18 reverse phase columns. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained from either General Electric QE-300 or Bruker-400 MHz Ultrashield spectrometers with tetramethylsilane or 3-(trimethylsilyl)-propionic-*2,2,3,3-d*$_4$ acid, sodium salt as an internal standard. ^{13}C NMR were obtained from a Bruker-400 MHz Ultrashield spectrometer at 125.8 MHz with tetramethylsilane or 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt as an internal standard. Melting points are obtained by differential scanning calorimetry.

Phenylmethyl (5*S*)-5-[[(1,1-Dimethylethoxy)carbonyl]-amino]-6-oxo-6-(1H-tetrazol-5-ylamino)hexyl]carbamate (2). A solution of N$^\alpha$-Boc-N$^\epsilon$-Z-L-Lys-OH (100 g, 0.263 mol), 5-aminotetrazole hydrate (27.1 g, 0.263 mol), 4-methylmorpholine (NMM, 78.9 g, 0.780 mol), and BOP (116.4 g, 0.263 mol) in dimethyl formamide (DMF, 400 mL) was stirred at

room temperature under nitrogen for 20 h. About 350 mL of DMF was removed under high vacuum. The resulting syrup was added slowly to 2 L of mechanically stirred ice-water and stirred for 90 min. A tan solid was filtered, washed with cold H_2O, and dried at 60 °C yielding 100 g of **2**. The solid was recrystallized from MeOH:EtOAc (1:1) yielding 36.0 g of a white solid (31% of theory). ^{1}H NMR (DMSO-d_6): δ 7.21−7.40 (m, 5H), 5.00 (s, 2H), 3.95−4.18 (m, 1H), 2.90−3.08 (m, 2H), 1.58−1.70 (m, 2H), 1.40 (s, 9H), 1.20−1.32 (m, 4H). Anal. ($C_{20}H_{29}N_7O_5$) C, H, N.

1,1-Dimethylethyl [(1*S*)-5-Amino-1-[(1H-tetrazol-5-ylamino)carbonyl]pentyl]carbamate Monoacetate (3). The benzyloxycarbonyl group of amide **2** (37 g, 0.0827 mol) was removed in the presence of 4% Pd/C in EtOH:HOAc (9:1) at 5 psi and room temperature for 9 h. The thick orange oil was digested with EtOAc. When solidification was complete, a white solid was filtered, washed with EtOAc, and dried in a vacuum oven at 35 °C to give 31.9 g of **3** (quantitative).

1,1-Dimethylethyl [(1*S*)-5-[(1-Iminoethyl)amino]-1-[(1H-tetrazol-5-ylamino)carbonyl]pentyl]carbamate Monoacetate (4). To a stirring suspension of **3** (31.8 g, 0.0827 mol) and triethylamine (TEA, 25.8 g, 0.255 mol) in DMF (300 mL) was added solid methyl acetimidate hydrochloride (6.57 g, 0.060 mol) in one portion. Addition of methyl acetimidate hydrochloride was repeated two times at 1 h intervals. After the mixture was stirred at room temperature for 16 h, the NEt_3·HC1 was removed by filtration and washed with a small amount of DMF. The filtrate was acidified with glacial HOAc (20 mL) and concentrated under high vacuum to an orange oil. The oil was dissolved in 50% aqueous HOAc (200 mL) and concentrated under high vacuum to give 65 g of an oil. The crude product was used in the next step without further purification.

(2*S*)-2-Amino-6-[(1-iminoethyl)amino]-N-(1H-tetrazol-5-yl) Hexanamide, Hydrate, Dihydrochloride (1). Crude amidine (94 g, estimated as 0.107 mol) was dissolved in glacial HOAc (800 mL) with magnetic stirring, and the solution was treated with 4 N HC1 in dioxane (80 mL, 0.32 mol). The mixture was stirred at room temperature for 2 h as an oil formed on the sides of the flask. The acetic acid was decanted from product, and the oil was triturated with HOAc (300 mL) followed by anhydrous Et_2O (500 mL). The oil was then triturated in MeOH to obtain a crystalline solid, which was then diluted with acetone. The solid was filtered, washed with acetone, and dried in a vacuum oven at 35 °C to give 29.5 g of tan powder.

The MeOH/acetone filtrate from above was concentrated and diluted with acetone to give a second crop of 6.7 g. On the basis of the starting material in step 2, the yield is 34.12 g (91%). The product was washed with HOAc, followed by Et_2O, and then recrystallized from MeOH:acetone (15:85). ^{1}H NMR (D_2O): δ 4.38 (t, 1H, J = 6.0 Hz), 3.29 (t, 2H, J = 8.0 Hz), 2.21 (s, 3H), 2.06−2.17 (m, 2H), 1.70−1.78 (m, 2H), 1.50−1.59 (m, 2H). ^{13}C NMR (D_2O): δ 171.40, 167.62, 152.86, 56.38, 44.51, 32.96, 29.20, 24.15, 21.26. Anal. ($C_9H_{18}N_8O$·2HCl·1.25H_2O) C, H, N, Cl.

Bioassay. 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 with recombinant human NOS isoforms, as previously described.[6b] ADP−Sepharose affinity purified iNOS[12] and nNOS[13] and DEAE purified eNOS[6b] were used to determine IC_{50} values by testing each compound at eight concentrations in the presence of a final arginine concentration of 30 μM.

Nonlethal Endotoxin Model. The in vivo efficacy of **1** and L-NIL was determined following oral administration in the rat following the systemic induction of iNOS by endotoxin administration, as previously described.[6b] Compounds were administered 30 min prior to endotoxin administration, and plasma nitrite/nitrate levels were measured 5 h following endotoxin administration.

Carrageenan-Induced Paw Edema. The efficacy of **1** in the carrageenan-induced rat model of acute paw inflammation was determined as previously described.[7] Compound **1** was administered orally 3 h following the intraplantar administration of carrageenan at which time near maximal edema had developed. Edema was measured by the increase in paw volume determined using a plethysmometer. Paws were measured at the start of the experiment and served as their own baseline controls.

Adjuvant-Induced Arthritis. Adjuvant-induced arthritis was induced by the intradermal injection of *Mycobacterium butyricum* in Lewis rats as previously reported.[9] Compound **1** was administered orally by gavage, twice daily, beginning on the day of adjuvant administration. Twenty-one days following adjuvant administration, each hindpaw was assessed for swelling using a plethysmometer.

Determination of Mean Arterial Pressure in the Rat. Male Wistar rats (Harlan Sprague Dawley, Inc., Indianapolis IN) weighing 250−350 g were anesthetized, and the femoral artery was cannulated. The rats were placed in restraining cages, and the cannula was connected to a Cobe disposable pressure transducer (Cobe Laboratories, Inc., Lakewood CO). Following recovery from anesthesia, hemodynamic parameters were measured using a Gould Transducer Signal Conditioner and TA Recording System (Gould Instrument Systems Inc., Valley View, OH). Baseline data were collected for approximately 30 min prior to the administration of a single dose of either vehicle (water) or compound dissolved in water. Compounds were administered orally by gavage. Mean arterial pressure and heart rate were recorded for 4 h following compound administration (n = 3−4 rats for compound-treated groups, 2−3 rats for vehicle-treated groups).

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JM010420E