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CONFORMATIONALLY RESTRICTED ARGININE ANALOGUES AS INHIBITORS OF HUMAN NITRIC OXIDE SYNTHASE

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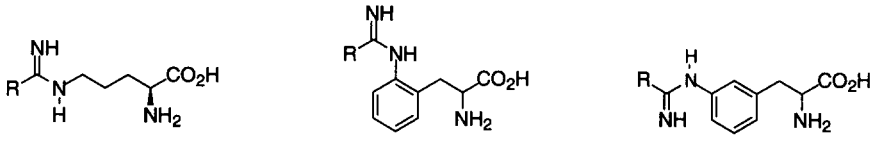
Abstract: Conformationally restricted analogues of the endogenous NOS substrate L-arginine and the arginine based NOS inhibitors N^G-methyl-L-arginine (L-NMA) and N^δ-iminoethyl-L-ornithine (L-NIO) were synthesized for evaluation as inhibitors of human NOS. Incorporation of a phenyl ring into the C4-C5 backbone chain provided 2-aminophenylalanine analogues which retained potent NOS inhibition. Structurally related analogues of 3-aminophenylalanine were significantly weaker inhibitors. © 1997 Elsevier Science Ltd.

Nitric oxide (NO) exhibits diverse roles in both normal and pathological physiologies.[1] The production of NO results from the oxidation of L-arginine to L-citrulline in a NADPH and O_2-dependent process catalyzed by the enzyme nitric oxide synthase (NOS). Three structurally distinct NOS isoforms have been identified.[2] Constitutive isoforms have been distinguished in the vascular endothelium (eNOS) where NO synthesis is involved in the regulation of blood pressure homeostasis; and in neuronal tissue (nNOS) where NO serves as an important intracellular second messenger involved in neurotransmission. The inducible isoform (iNOS) is found in macrophages producing NO which plays a key role in normal immune responses by functioning as a cytotoxic agent.

Nitric oxide has been postulated as a contributor to the etiology of various diseases including septic shock, inflammatory arthritis and neurodegenerative diseases.[3] The use of NOS inhibitors to regulate the synthesis of NO, therefore, has potential therapeutic value. Prototypical NOS inhibitors are analogues of the endogenous substrate L-arginine. These include the guanidine and amidine derivatives N^G-methyl-L-arginine (L-NMA) and N^δ-iminoethyl-L-ornithine (L-NIO) shown in Figure 1. These inhibitors exhibit low isoform selectivity. We have previously shown that sufficient active site diversity exists between the three isoforms such that the design of selective inhibitors is achievable.[4] In an effort to better understand the preferred binding orientation of amino acid analogues within the arginine binding site, we designed a series of conformationally restricted arginine based analogues that incorporate an aromatic ring as a rigidifying backbone spacer. Guanidine and amidine analogues **1-6** were synthesized for comparative evaluation with L-arginine and the known inhibitors L-NMA and L-NIO (Figure 1).



R = NH_2 **L-Arginine**
R = $NHCH_3$ **L-NMA**
R = CH_3 **L-NIO**

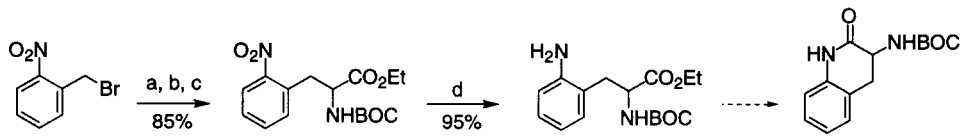
1 R = NH_2
2 R = $NHCH_3$
3 R = CH_3

4 R = NH_2
5 R = $NHCH_3$
6 R = CH_3

Figure 1. L-Arginine and Arginine Based NOS Inhibitors

Initially, we required the synthesis of suitably protected amino acid intermediates that could be functionalized to our desired targets. The synthesis of substituted amino acids as described by O'Donnell proved to be an efficient protocol as illustrated in Scheme I.[5] Alkylation of 2-nitrobenzyl bromide **7** with the anion of ethyl N-(diphenylmethylene)glycinate followed by imine hydrolysis and N-BOC protection of the crude amine gave protected α-amino ester **8** in 85% overall yield. Catalytic hydrogenation of the nitro group provided the requisite intermediate 2-aminophenylalaninate **9** in 95% yield. The propensity of compound **9** to readily undergo intramolecular cyclization to lactam **10** required the hydrogenolysis of **8** to be performed immediately prior to utilization of this intermediate. In fact, the use of compound **9** proved quite difficult due to rapid lactamization under numerous reaction conditions.

Scheme I [a]



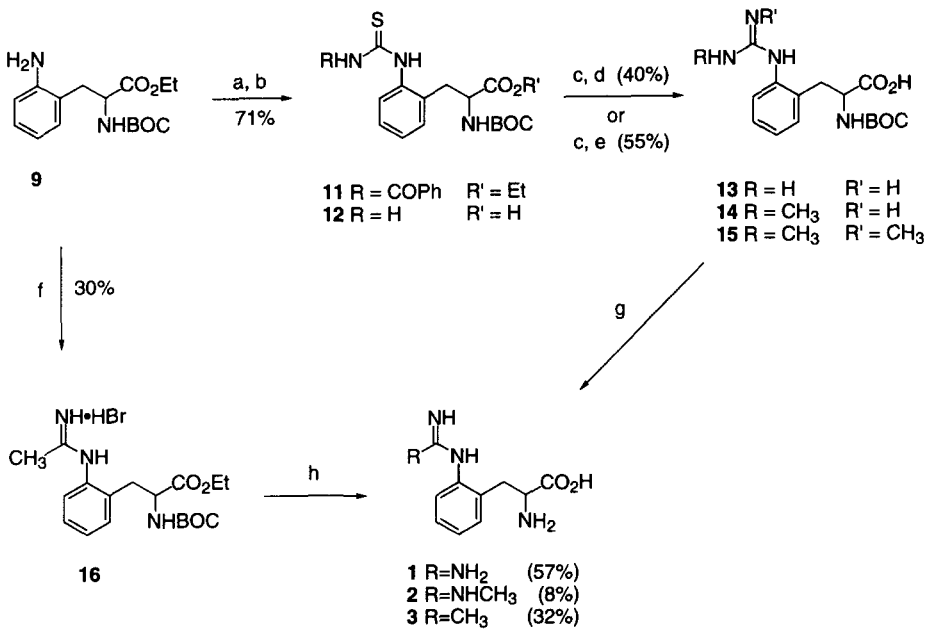
7 **8** **9** **10**

[a] (a) $Ph_2C{=}NCH_2CO_2Et$, LDA, THF, HMPA, -78°C; (b) 1N Aq. HCl, Et_2O, rt; (c) BOC_2O, CH_2Cl_2; (d) H_2, 10% Pd/C, EtOH.

The conformationally restricted targets **1-3** were synthesized as outlined in Scheme II. Reaction of amino ester **9** with benzoyl isothiocyanate in THF at 0°C and warming slowly to room temperature overnight gave N-benzoyl isothiourea **11** in 82% yield. The efficient reaction of amine **9** with isothiocyanates was surprising given the major difficulties generally encountered in attempted manipulations of the amine functionality. Removal of the benzoyl group with aqueous NaOH proceeded with concommitant ester

saponification to furnish thiourea **12** in 86% yield. Sulfur alkylation of **12** with iodomethane and subsequent treatment of the resulting crude isothiourea hydroiodide with excess methanolic ammonia in a sealed reaction vessel at 80°C gave guanidine **13** in 40% yield. In a similiar process, S-alkyl displacement of the crude isothiourea with excess ethanolic methylamine led to an inseparable mixture of N^G-monomethyl and N^G, $N^{G'}$-dimethyl guanidines **14** and **15**.[6] Removal of the N-BOC protecting groups from these intermediates and final purification by reverse phase HPLC provided the desired guanidine targets **1** and **2**.[7]

Scheme II [a]



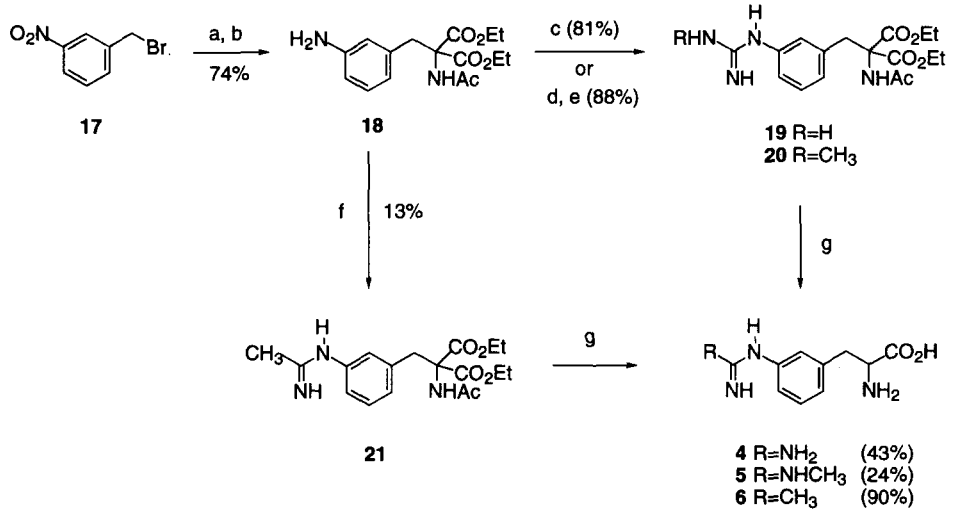
[a] (a) PhCONCS, THF, 0°C to rt; (b) NaOH, THF-H_2O, reflux; (c) CH_3I, acetone, reflux; (d) NH_3, MeOH 80°C; (e) CH_3NH_2, EtOH, 80°C; (f) $CH_3C(=NH•HBr)SCH_2Ph$, EtOH, 0°C to rt; (g) 4N HCl, dioxane, rt; (h) 6N HCl, reflux.

Attempts to convert 2-aminophenylalaninate **9** to the acetamidine intermediate **16** with O-ethyl acetimidate were unsuccessful due to exclusive lactam formation. The successful conversion of amine **9** to acetamidine **16** demanded a reagent sufficiently reactive to preclude intramolecular cyclization to lactam **10**. We felt the enhanced reactivity of a thioimidate reagent would facilitate amidine formation particularly with weakly nucleophilic aromatic amines. S-Benzyl thioimidate was found to successfully fulfill our synthetic requirements.[8] Treatment of amine **9** with S-benzyl thioimidate hydrobromide in EtOH at 0°C and then stirring at room temperature for 18h gave the hydrobromide salt of acetamidine **16** in 30% yield. The undesired

competing cyclization product **10** was also isolated in 30% yield. Simultaneous removal of the N-BOC protecting group and ester hydrolysis with refluxing 6N aqueous HCl and purification by HPLC gave a 32% yield of amidine **3**.[7]

The conformationally restricted targets **4-6** were synthesized using alternative synthetic strategies as depicted in Scheme III. Alkylation of 3-nitrobenzyl bromide **17** with diethyl acetamidomalonate followed by hydrogenation of the nitro group provided the key intermediate **18** in 74% overall yield. The reaction of **18** with aminoiminomethanesulfonic acid[9] followed by hydrolysis and decarboxylation with refluxing 6N aqueous HCl yielded guanidine target **4**.[7] Alternatively, N-methylguanidine **20** was efficiently prepared in 88% yield by conversion of **18** to the corresponding cyanamide derivative with cyanogen bromide and subsequent thermal addition of methylamine. Exposure to refluxing aqueous acid gave substituted guanidine target **5**.[7] The amidine intermediate **21** was synthesized from **18** and O-ethyl acetimidate in a very sluggish low yielding process. Final treatment with refluxing 6N aqueous HCl afforded the final amidine target **6**.[7]

Scheme III [a]

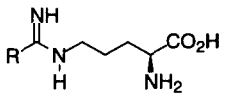
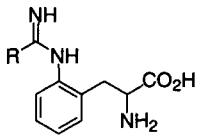
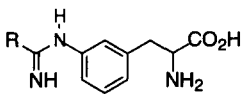


[a] (a) $AcHNCH(CO_2Et)_2$, KOtBu, EtOH, reflux; (b) H_2, 10% Pd/C, MeOH; (c) $H_2NC(=NH)SO_3H$, MeOH, rt to 45°C; (d) CNBr, NaOAc, MeOH, 0°C to rt; (e) CH_3NH_2, EtOH, 70°C; (f) $CH_3CH_2OC(=NH)CH_3$, Et_2O-THF rt; (g) 6N HCl, reflux.

The inhibition of the three human NOS isoforms was determined by monitoring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as previously described.[10] Results for comparison of the conformationally restricted analogues with the acyclic arginine based NOS inhibitors are presented in Table 1. All of the synthesized conformationally restricted compounds were prepared as racemic mixtures. The stereospecific binding requirement for the L-enantiomer of both substrates and arginine based inhibitors at the NOS arginine

binding site has been demonstrated. The inhibition potency of the pure L-enantiomers of **1-6**, therefore, could be greater than the racemates assuming the enantiomeric D-isomers lack any appreciable inhibitory properties. If this is in fact true, the selectivity ratios should remain unchanged.

Table 1. Inhibition of Human NOS by Arginine Based Inhibitors and Conformationally Restricted Analogues

			K_i, (μM) [a]		
STRUCTURE	**COMPOUND**	**R**	**iNOS**	**eNOS**	**nNOS**
	L-Arginine	NH_2	2.20 (b)	0.90 (b)	1.60 (b)
	L-NMA	$NHCH_3$	0.86	0.40	0.84
	L-NIO	CH_3	0.34	0.81	0.23
	**1**	NH_2	2.60	0.25	0.37
	2	$NHCH_3$	0.60	0.27	0.41
	3	CH_3	1.90	1.60	1.30
	**4**	NH_2	100	46	44
	5	$NHCH_3$	96	8.8	8.4
	6	CH_3	82	39	29

[a] Inhibition constants were obtained by measuring percent inhibition at a single time point with at least three concentrations of inhibitor. Values had a standard deviation of ≤ 10% (n=3). [b] Value represents K_m for L-arginine in μM concentration units.

Guanidine analogue **1** is a constrained analogue of the natural substrate L-arginine and is a potent NOS inhibitor. The observed isoform inhibition potencies are similiar to the respective K_m values for L-arginine as indicated in Table 1. Since the measured binding constant for L-arginine is approximately the same as its K_m constant, one can directly compare the K_m values for L-arginine with the K_i values for restricted analogue **1**.[11] Thus, in this comparison, the introduction of the phenyl ring resulted in no more than a four-fold effect on binding potency. Restricted N^G-methylguanidine analogue **2** is a submicromolar inhibitor of all three human NOS isoforms exhibiting potency comparable to L-NMA. Compound **2** inhibits NOS-mediated relaxation of rat aortic rings in whole tissue preparations.[12] Both eNOS (IC_{50}=2.76 μM) and iNOS (IC_{50}=4.46 μM) were inhibited in a dose-dependent manner. The restriction of the L-arginine backbone chain did not significantly alter its transport into cells. The observed lack of selectivity in the whole tissue and purified enzyme assays are in good agreement. Amidine analogue **3** is also an inhibitor of all three isoforms. Although the inhibition

potency of this analogue is diminished relative to L-NIO, the magnitude of decrease is only slight. The analogous meta-substituted derivatives **4-6** are substantially weaker NOS inhibitors. Interestingly, the restricted N^G-methylguanidine analogue **5** shows selectivity for both constitutive isoforms over iNOS in contrast to the nonselective N^G-methylguanidine inhibitors L-NMA and constrained analogue **2**.

Placement of the phenyl spacer in analogues **1-6** reduces the flexibility of the backbone chain limiting the range of available binding conformations. The ortho relationship of the amino acid group and the modified guanidine functionality on the aromatic ring prevents the constrained analogues **1-3** from achieving a fully extended conformation. These structural constraints provided analogues which retained potent inhibitory properties. In contrast, the phenyl spacer in the weaker inhibitors **4-6** limits the ability of these analogues to bind in a conformation that mimics analogues **1-3**. These results, while clearly not definitive, suggest that arginine based inhibitors preferentially bind in an orientation where the arginine alkyl chain is in a folded conformation that allows the appropriate spatial alignment of the amino acid and terminal guanidine moieties within the NOS active site for potent binding affinity. The design and evaluation of additional constrained analogues to further define enzyme-inhibitor interactions essential for potency and selectivity are required.

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