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Preparation and evaluation of new L-canavanine derivatives as nitric oxide synthase inhibitors

Xiaofeng Li, Robert N. Atkinson and S. Bruce King*

Department of Chemistry, Wake Forest University, Box 7486, Winston-Salem, NC 27109-7486, USA

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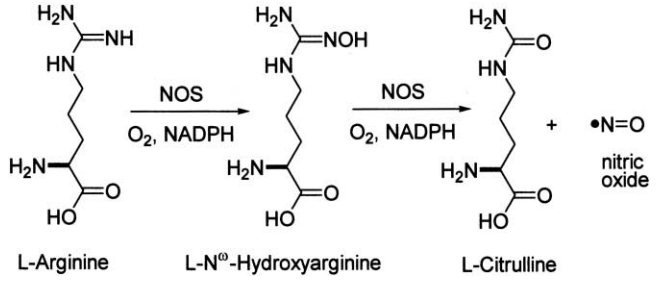
Abstract—New derivatives of the amino acid L-canavanine were prepared by total chemical synthesis and evaluated as inhibitors of inducible nitric oxide synthase (NOS). Replacement of the δ-methylene group of known NOS inhibitors with an oxygen atom produced dramatic effects as to the ability of these compounds to interact with the enzyme. These results indicate that the addition of an electron withdrawing group in the side chain of L-arginine-derived NOS inhibitors must be considered during inhibitor design depending on the expected mode of interaction with the enzyme.

1. Introduction

The nitric oxide synthases (NOS) catalyze the oxidation of the terminal guanidine group of L-arginine to nitric oxide (NO), a molecule that plays important roles in blood pressure control, neurotransmission, and the immune response (Scheme 1).[1] This conversion occurs in two steps, a two-electron oxidation of L-arginine to N^{ω}-L-hydroxyarginine followed by a three-electron oxidation of N^{ω}-L-hydroxyarginine to NO and L-citrulline (Scheme 1).[2] Each step requires molecular oxygen and reduced nicotine-adenine dinucleotide phosphate (NADPH) as co-substrates and (6*R*)-5, 6, 7, 8-tetrahydrobiopterin (H_4B), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphorin IX (heme) as cofactors.[1,3] Three distinct mammalian NOS isoforms exist: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed, and inducible NOS (iNOS).[3] All three isoforms require calmodulin for activity and exist as catalytically active homodimers of a monomer that contains an N-terminal oxygenase domain with L-arginine, H_4B, and heme binding sites and a C-terminal reductase domain with NADPH, FAD, FMN, and calmodulin binding sites.[3] The reductase domain delivers NADPH-derived electrons to the heme iron cofactor that directly participates in each oxidation shown in Scheme 1 by binding and activating oxygen.[3] Recent X-ray crystallographic structures of the iNOS and eNOS oxygenase domains with both inhibitors and substrates provide more detailed active site information.[4]

The selective inhibition of the various NOS isoforms represents a potential therapeutic approach for disease states where an overproduction of nitric oxide from a particular isoform is implied.[5] Many L-arginine derivatives non-selectively inhibit NO production from the various NOS isoforms.[6] However, N^{ω}-propyl-L-arginine (**1**) demonstrates a 3000-fold preference for inhibition of nNOS over iNOS and *N*-[3-(aminomethyl)benzyl]acetamidine (**2**) shows a



Scheme 1. NOS catalyzed oxidation of L-arginine to NO and L-citrulline.

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* Corresponding author. Tel.: +1-336-758-5774; fax: +1-336-758-4656; e-mail: kingsb@wfu.edu

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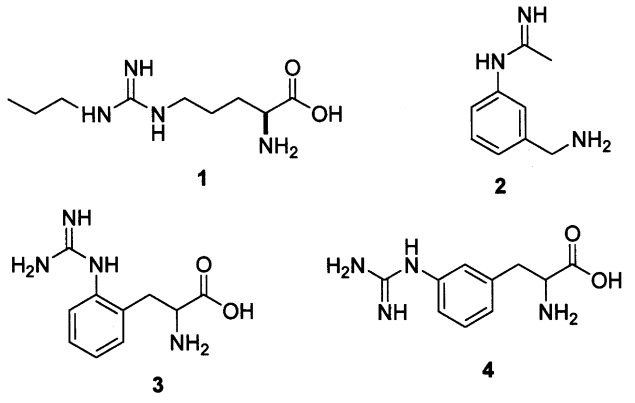
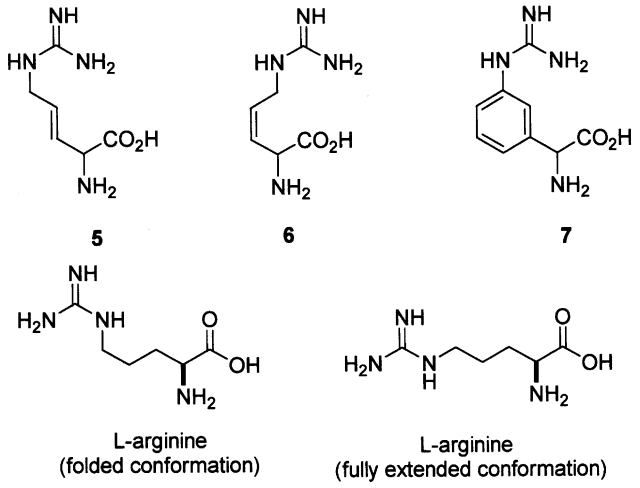
5000-fold preference for inhibition of iNOS over eNOS indicating the possibility of isoform selective NOS inhibition.[7,8] Despite the discovery of such compounds, the structural basis of isoform selective NOS inhibition remains poorly understood. X-ray crystallographic studies reveal a highly conserved active site structure between iNOS and eNOS with the only difference being replacement of Asp382 in iNOS by Asn368 in eNOS.[4a] As the side chains of these residues approach the substrate within hydrogen bonding distance, such a change from a negatively charged to a neutral group may be responsible for the observed selectivity of some NOS inhibitors.[4a]

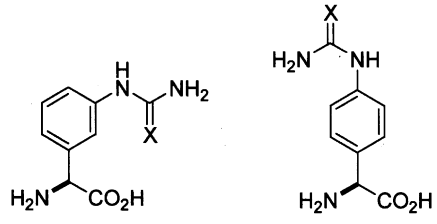
Conformationally restricted L-amino acid derivatives provide an alternative approach toward defining the optimal binding orientations of substrates and inhibitors in each of the NOS isoforms. Results from experiments with a group of conformationally restricted racemic phenylalanine-derivatives, such as **3** and **4**, suggest that L-arginine based inhibitors preferentially bind to the enzyme in a folded conformation where the guanidine group and the amino acid carboxylate group are not fully extended.[9] The X-ray crystallographic structure of the inducible NOS oxygenase domain with L-arginine ultimately demonstrated that L-arginine binds to this truncated isoform in such a conformation.[4c] These conformationally restricted compounds inhibited NOS catalyzed NO production with K_is between 0.25 and 100 μM for the different NOS isoforms.[9] Compounds **3** and **4** demonstrated only modest isoform selectivity with approximately a 10 and twofold preference for the constitutive isoforms over iNOS, respectively.[9]



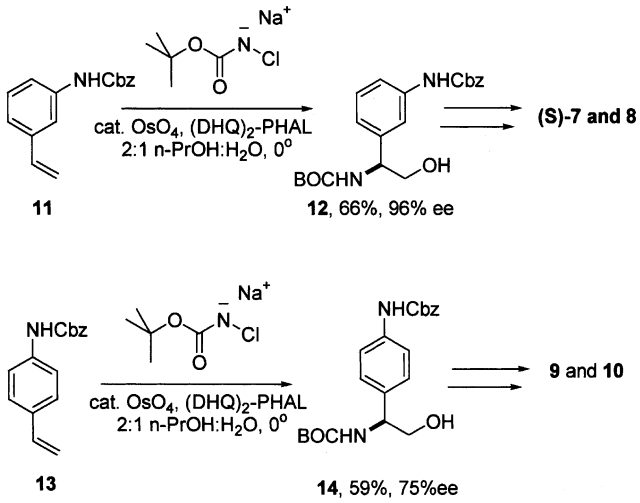
In a separate study, the racemic conformationally restricted arginine derivatives (**5**–**7**) acted as both NO producing substrates and inhibitors of NOS but demonstrated little isoform selectivity.[10] The *E*-alkene (**5**) non-selectively inhibited NOS catalyzed NO production (K_is between 25 and 50 μM) and served as a NO producing substrate with kinetic parameters comparable to L-arginine for nNOS and eNOS.[10] The *Z*-alkene (**6**) interacted poorly and non-selectively with the NOS isoforms as both a substrate and inhibitor (K_is between 6.5 and 13 mM).[10] These results suggest that **5**, like **3** and L-arginine, interacts with the enzyme in a conformation where the guanidine group and the amino acid carboxylate group are not fully extended.[9,10] Interestingly, the racemic phenylglycine derivative (**7**), which cannot assume a conformation identical to **3**, inhibited (K_is between 2 and 50 μM) NOS catalyzed NO production much better than **5** with a slight preference for nNOS, but was a much poorer NO producing substrate than **5**.[10] Such results suggest that **7** may bind to the active site differently than **5** (or L-arginine) in a manner that does not support oxidation.[10] These authors ultimately concluded that conformational restriction of L-arginine derivatives prevents the attainment of the appropriate conformations required for high isoform selectivity.[10]



During this same time period, we also prepared and evaluated a series of L-phenylglycines ((*S*)-**7**, **8**–**10**) to probe the active site structure of the NOS isoforms.[11] The guanidine containing derivatives ((*S*)-**7** and **9**) represent constrained analogs of L-arginine and L-homoarginine with (*S*)-**7** being the single enantiomer of **7** that possesses the stereochemistry of an L-amino acid. The thiourea-containing compounds (**8** and **10**) represent conformationally restricted derivatives of the known (but non-isoform selective) NOS inhibitor L-thiocitrulline.[12] These compounds were designed and prepared to determine whether differences between the conformations of homologous NOS substrates and inhibitors explained the structural basis of NOS isoform selectivity of L-arginine and homoarginine derived inhibitors.



The Sharpless carbamate aminohydroxylation combined with an alcohol to carboxylic acid oxidation forms a newly described synthetic strategy for the preparation of enantiopure α-arylglycines from styrenes.[13] Use of the bis(dihydroquinyl)phthalazine ($(DHQ)_2$-PHAL) ligand establishes the *S*-configuration of the amino alcohol stereocenter and leads to the formation of natural L-amino acids. The Sharpless reaction introduces the nitrogen atom protected as either a Boc or Cbz carbamate, an attractive feature for further synthetic manipulations. Gram-scale



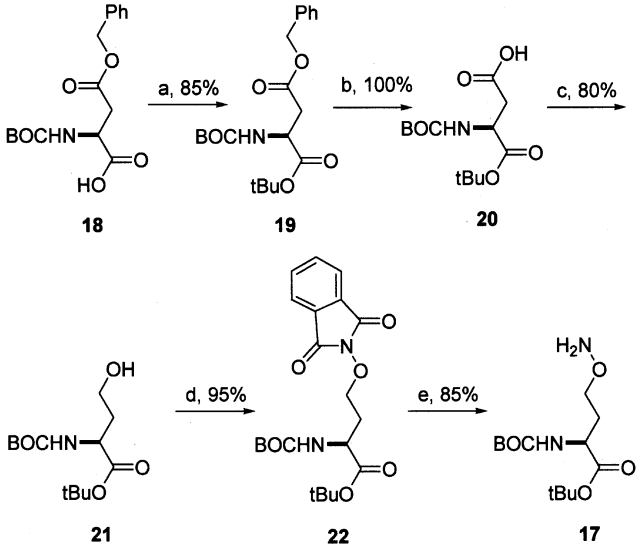
Scheme 2. Sharpless asymmetric aminohydroxylation of **11** and **13**.

Sharpless asymmetric aminohydroxylation of the Cbz-protected styrene (**11**) using the DHQ_2-PHAL ligand yielded the Boc protected amino alcohol (**12**) in 66% yield and 96% ee after removal of small amounts of the unwanted regioisomer and diol by flash chromatography (Scheme 2).[11] This amino alcohol was ultimately converted to the desired L-arginine and L-thiocitrulline derivatives ((*S*)-**7**–**8**) in 96 and 82% ee, respectively (Scheme 2). Similarly, the gram-scale Sharpless asymmetric aminohydroxylation of the Cbz-protected styrene (**13**) using the $(DHQ)_2$-PHAL ligand formed the BOC protected amino alcohol (**14**) in 59% yield and 75% ee (Scheme 2). This amino alcohol was converted to the desired L-homoarginine and L-homothiocitrulline derivatives (**9**–**10**) in 75 and 60% ee, respectively (Scheme 2).

The asymmetric preparation of the L-phenylglycines ((*S*)-**7**, **8**–**10**) using the Sharpless asymmetric aminohydroxylation demonstrates one of the first applications of this newly described methodology to the synthesis of biologically important compounds.[11] These results indicate that carbamate-containing olefins act as suitable substrates for the Sharpless carbamate aminohydroxylation for the preparation of amino substituted α-arylglycines and do not interfere with the catalytic cycle. The conversion of **12** and **14** to the guanidines (*S*)-**7** and **9** proceeded without the loss of enantiomeric excess highlighting the utility of this methodology for the asymmetric preparation of enantiopure L-phenylglycines. Retention time analysis following chiral HPLC separation of the final phenylglycines supported the predicted formation of the amino alcohols (**12** and **14**) with the *S*-configuration from the aminohydroxylation of **11** and **13** using the $(DHQ)_2$-PHAL ligand. A loss of enantiomeric excess occurred during the preparation of the thioureas **8** and **10** and chiral HPLC determination of the enantiomeric excess of the individual synthetic intermediates in these routes indicated that the loss of stereochemical integrity occurred during the thiourea-forming step.

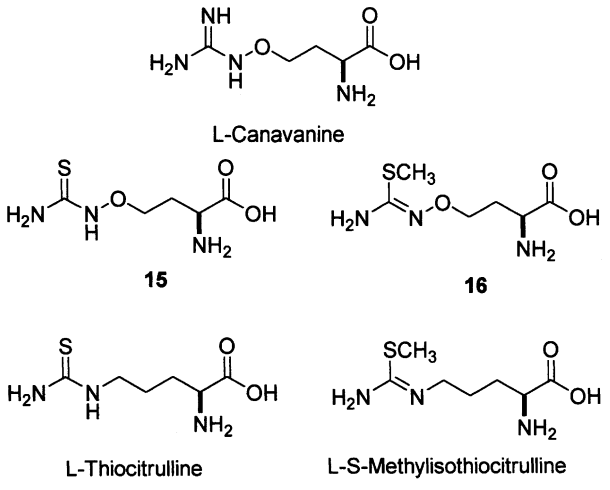
Compound (*S*)-**7**, similar to racemic **7**,[10] inhibited both iNOS and nNOS catalyzed production of NO with IC_{50}s in the micromolar range (42 and 144 μM, respectively).[11] The difference in inhibitory potency from our experiments to those with racemic **7** may be due to differences in the source and preparation of the enzymes, the method of assay (oxyhemoglobin vs radioactive L-citrulline) or our choice to determine IC_{50} values rather than K_is.[10,11] Interestingly, our results indicate that (*S*)-**7** prefers iNOS over nNOS compared to racemic **7**, which shows a preference for nNOS over iNOS.[10,11] A possible explanation for this trend may be that (*R*)-**7** selectively inhibits nNOS relative to iNOS. Such a proposal would be consistent with previous experiments that suggest **7** binds to the enzyme differently than L-arginine.[10] An alternative binding mode for **7** compared to L-arginine would not necessarily require the stereochemistry of the α-amino acid carbon to possess the natural *S* configuration for optimal binding and the opposite configuration could impart some isoform selectivity. While previous results demonstrate that racemic **7** can support NOS catalyzed NO synthesis, our work showed that (*S*)-**7** did not support iNOS catalyzed NO production, as measured by the Griess assay for nitrite, a stable oxidative decomposition product of NO.[10,11,14] Our inability to observe NOS catalyzed NO production from (*S*)-**7** probably results from the relatively small concentration (1 mM) of this alternative substrate used in our experiments.[10,11]

The L-thiocitrulline derivative (**8**) demonstrated potent but non-selective inhibition of both the inducible and neuronal isoforms of NOS (IC_{50}=8 and 12 μM, respectively).[11] Conformational restriction of L-homoarginine in an extended conformation (compounds **9** and **10**) produced deleterious effects with regards to the inhibition of both iNOS and nNOS (IC_{50}s>600 μM). These results contrast the ability of **4**, a L-homoarginine analog with more conformational flexibility between the guanidine and amino acid groups, to modestly inhibit both inducible and neuronal NOS (K_i=100 and 44 μM, respectively).[9] While other non-conformationally restricted L-homoarginine derivatives, such as N^{ω}-L-(1-iminoethyl) lysine and L-homothiocitrulline, also demonstrate significant inhibition of NOS,[12,15] these results clearly indicate that the extended orientation of the functional groups in **9** and **10** does not permit binding of these compounds to NOS and this lack of activity prevents the evaluation of isoform selectivity. As (*S*)-**7** and **8** represent the only compounds in our study



Scheme 3. *Reagents and conditions*: (a) *t*-butyl alcohol, DCC, DMAP, CH_2Cl_2, 2 h; (b) 50 psi H_2, Pd/C, 2 h; (c) BH_3/THF, −15 to 25°C, 4 h; (d) *N*-hydroxyphthalimide, triphenylphosphine, DEAD, 30 min; (e) NH_2NH_2/EtOH, 30 min.

capable of adopting a folded conformation similar to **3**, the results with (*S*)-**7**, **8**–**10** further support all previous evidence indicating that L-arginine (and L-homoarginine) derivatives bind to the NOS active site in a folded conformation. Taken together, our results agree with previous work that conformational restriction of L-arginine-derived inhibitors of NOS does not produce highly isoform selective inhibitors.[10] In light of the structural similarities revealed by the X-ray crystallographic studies of both iNOS and eNOS,[4] such a conclusion now appears expected. Recent alternative approaches toward isoform selective NOS inhibitors include the design of unique tetrahydrobiopterin antagonists or compounds capable of preventing NOS monomer dimerization.[16,17]

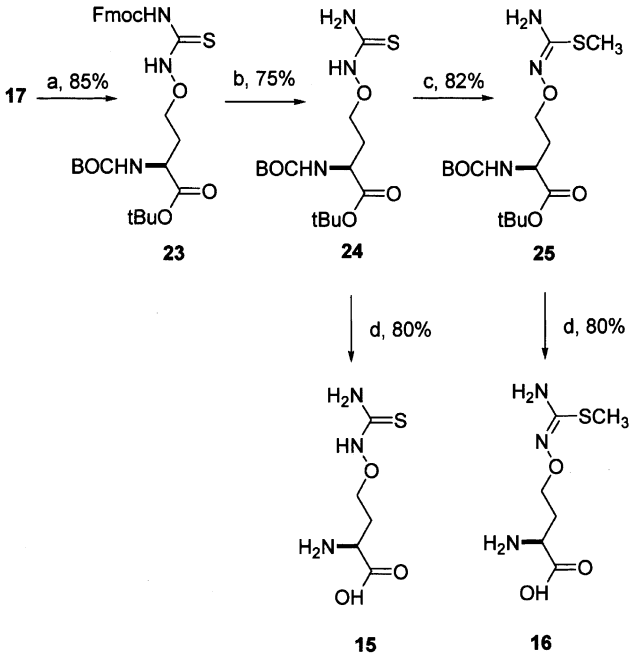


At this stage, we began to examine potential NOS inhibitors derived from the unique amino acid, L-canavanine. L-Canavanine, produced by over 500 species of leguminous plants, represents an L-arginine analog in which the δ-methylene group is replaced by oxygen. L-Canavanine inhibits NOS catalyzed NO production (K_i=11.1 μM at pH 7.4) but does not act as a NO producing substrate of NOS.[18] The aminooxy group dramatically influences L-canavanine's physical and chemical properties decreasing the p*K*a of the guanidinine group to 7.[19] To examine how replacement of the δ-methylene group by oxygen would alter the ability of known NOS inhibitors to interact with the enzyme, compounds **15** and **16** were prepared. Compounds **15** and **16** represent the L-canavanine analogs of the known NOS inhibitors L-thiocitulline and *S*-methylisothiocitrulline.[12,20]

2. Results and discussion

The L-canaline derivative (**17**) served as the common synthetic intermediate for the preparation of both **15** and **16** and Scheme 3 illustrates the preparation of **17** from commercially available *N*-(*tert*-butoxycarbonyl)-L-aspartic acid 4-benzyl ester (**18**, Scheme 3). Protection of **18** as the *t*-butyl ester using *t*-butanol, DMAP, and DCC afforded the diester **19** (Scheme 3). Catalytic hydrogenolysis of the benzyl group of **19** gave the carboxylic acid **20** in good yield and of sufficient purity for use in subsequent reactions (Scheme 3). The BH_3/THF complex selectively reduced the carboxylic acid group of **20** to the primary alcohol **21** (Scheme 3).[21] Treatment of **21** with *N*-hydroxyphthalimide under Mitsunobu conditions produced compound **22** and removal of the phthalimide group with hydrazine gave the protected L-canaline **17** (Scheme 3).[22]

Scheme 4 depicts the conversion of **17** into the desired L-canavanine derivatives **15** and **16**. Addition of fluorenylmethyloxycarbonyl isothiocyanate (Fmoc-NCS) to a solution of **17** produced the Fmoc-protected thiourea **23** (Scheme 4).[23] Removal of the Fmoc-group from **23** under basic conditions gave thiourea **24** and methylation of **24** with iodomethane yielded isothiourea **25** (Scheme 4). Acid deprotection of **24** produced the L-canavanine derivative of the known NOS inhibitor L-thiocitrulline (**15**) in 80% yield (Scheme 4). A similar deprotection of the *S*-methylisothiourea (**25**) gave the L-canavanine derivative of the



Scheme 4. *Reagents and conditions*: (a) Fmoc-NCS/CH_2Cl_2; (b) piperidine/CH_2Cl_2; (c) CH_3I/CH_3CN; (d) 4 M HCl/dioxane.

known NOS inhibitor *S*-methylisothiocitrulline (**16**, Scheme 4).

The synthetic L-canavanine derivatives (**15**–**16**, 1 mM) were initially evaluated, as inhibitors of iNOS catalyzed NO production from L-arginine (1 mM) using the Griess assay. This assay provides a rapid and economical method for the determination of nitrite, the stable oxidative decomposition product of nitric oxide (NO).[24] Compared to control NO production with L-arginine alone (100%, Lane 1, Fig. 1), L-thiocitrulline (Lane 4, Fig. 1) reduced nitrite production to 13% of control under these conditions. The thiourea derivative (**15**) did not significantly reduce nitrite production in this assay (88% of control, Lane 2, Fig. 1). The *S*-methylisothiourea analog of L-canavanine (**16**, Lane 3, Fig. 1) demonstrated a greater ability to inhibit iNOS mediated nitrite production (9% of control) than L-thiocitrulline, identifying **16** as a potentially potent inhibitor of NOS.

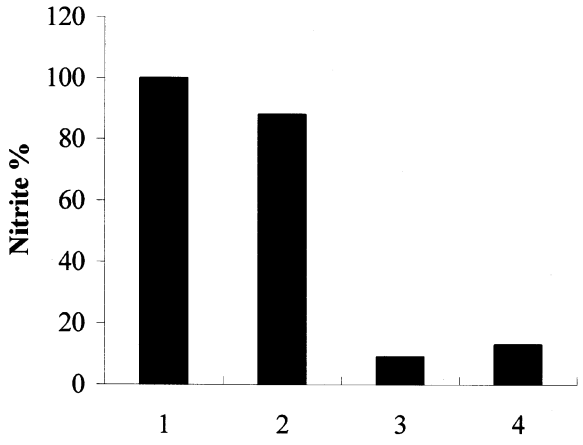


Figure 1. Inhibition of iNOS catalyzed nitrite production by **15**, **16**, and L-thiocitrulline. Lane 1: control, L-arginine (1 mM), Lane 2: L-arginine (1 mM) +**15** (1 mM), Lane 3: L-arginine (1 mM)+**16** (1 mM), Lane 4: L-arginine (1 mM)+L-thiocitrulline (1 mM).

Based upon the promising Griess assay results with **16**, this compound was further evaluated as an inhibitor of NOS using the continuous oxyhemoglobin assay.[25] Determination of the initial rate of NO production at various concentrations of L-arginine and **16** followed by Lineweaver–Burk kinetic analysis revealed **16** competitively inhibited iNOS (Fig. 2). Replotting the apparent K_m values of these experiments vs. the concentration of **16**, provided a K_i of 1.34 μM for **16**. Pre-incubation of **16** with iNOS did not show time-dependent loss of enzyme activity indicating **16** does not irreversibly inactivate the enzyme.

Combining the known chemical properties of L-canavanine with previous results regarding the interaction of both L-thiocitrulline and *S*-methylisothiocitrulline with NOS may explain the difference in the ability of **15** and **16** to interact with iNOS. Replacement of L-arginine's δ-methylene group with the electronegative oxygen atom to yield L-canavanine greatly reduces the electron density of L-canavanine's guanidine group as evidenced by the dramatic decrease in pKa (~7 vs ~12.5 for L-arginine).[19] Optical difference spectrophotometric experiments and X-ray crystallographic studies indicate that the thiourea group of L-thiocitrulline acts as an electron-donating sixth axial ligand of the iron heme group of NOS.[4d,26] Such an interaction decreases electron flux through NOS by reducing the reduction potential of the heme iron.[12b] Replacement of L-thiocitrulline's δ-methylene group with an electronegative oxygen atom to yield **15** would be expected to greatly reduce the electron density of the thiourea group of **15** and reduce its ability to interact with the heme iron group of

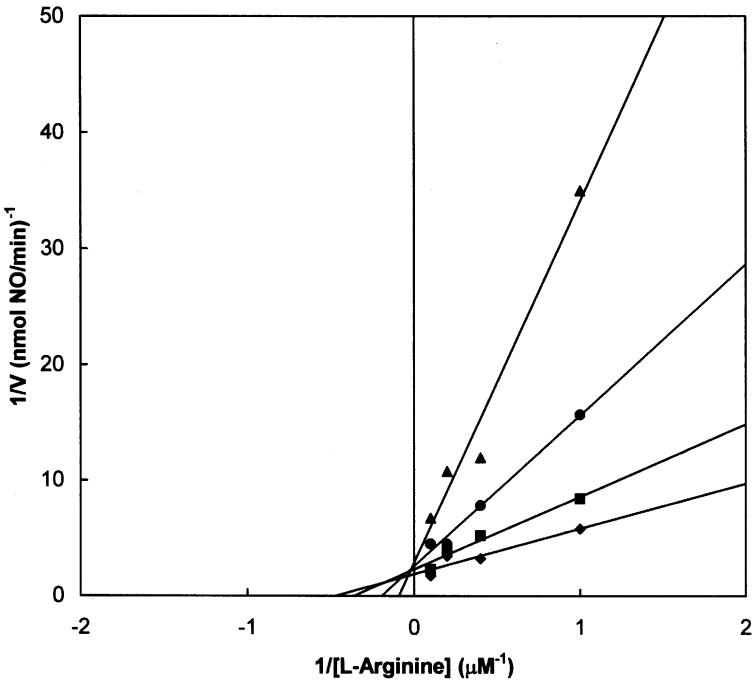


Figure 2. Lineweaver–Burk kinetic analysis of **16**. ◆ **16** (0 μM), ■ **16** (2.5 μM), ● **16** (5 μM), ▲ **16** (10 μM).

NOS. The inability of **15** to interact well with iNOS supports this idea and all other evidence that indicates that L-thiocitrulline interacts with NOS as an electron donor through its thiourea group. Compound **15** with its reduced electron donating ability behaves more like L-citrulline, the reaction product and a poor NOS inhibitor that contains the relatively weakly electron donating urea group, than L-thiocitrulline.[26]

In contrast, similar optical difference spectrophotometric experiments indicate that *S*-methylisothiourea interacts with the heme iron group similarly to L-arginine but differently than L-thiocitrulline suggesting that *S*-methylisothiourea binds near but not directly to the heme iron group.[20,27] As *S*-methylisothiocitrulline does not interact with NOS by electron donation to the iron heme group, replacement of *S*-methylisothiocitrulline's δ-methylene group with an electronegative oxygen atom to yield **16** would not be expected to greatly alter the ability of **16** to interact with NOS. By competitively inhibiting iNOS with a K_i=1.34 μM, compound **16** demonstrates activity very similar to *S*-methylthiocitrulline (K_i=2.2 μM, iNOS).[20] Such results indicate that the electron donating ability of the isothiourea group of inhibitors like **16** or *S*-methylisothiocitrulline is not important for interaction with the enzyme and that these compounds do not inhibit the enzyme by direct binding to the heme iron group. Taken together, the results with **15** and **16** show that the substitution of the δ-methylene group of known L-arginine-derived NOS inhibitors with an oxygen atom can dramatically alter activity depending on how the compound interacts with NOS. Experiments to further characterize the interactions of **15** and **16** as well as to synthesize new compounds containing other electronically modified side chains are planned.

3. Conclusions

Our group has maintained an interest in the synthesis and evaluation of small molecules designed to interact with the NOS. The conformationally restricted L-phenylglycines (*S*)-**7**, and **8**–**10** were prepared using the Sharpless asymmetric aminohydroxylation in an effort to define NOS isoform selective conformations. Results with these compounds support all previous evidence that L-arginine derived inhibitors of NOS bind to the enzyme in a folded conformation. Given the similarity of the active site structure between NOS isoforms, the conformational restriction of L-arginine-derived compounds does not appear to be the most productive method for discovering isoform selective inhibitors. Compounds **15** and **16**, derivatives of L-canavanine, were prepared to evaluate the consequences of substitution of an oxygen atom for the δ-methylene group in the side chain of known NOS inhibitors. Results with these unique compounds demonstrate that such a substitution can dramatically alter activity depending on the mode of interaction with NOS and such information should be incorporated into the rational design of other NOS inhibitors.

4. Experimental

4.1. Chemistry—general

Melting points were determined with a Mel-Temp apparatus and are uncorrected. Optical rotations were determined with a Rudolph Autopol IV automatic polarimeter. Analytical thin layer chromatography (TLC) was performed on glass plates coated with silica gel 60 F-254 (E. Merck). Flash column chromatography was performed on Mallinckrodt silica gel 60 (230–400 mesh). Organic solvents were

distilled from a drying agent prior to use. Commercially available reagents were used without further purification. Proton and Carbon-13 NMR spectra were obtained on a Bruker Avance 300 multinuclear spectrometer. Chemical shifts were reported in δ scale in parts per million from Me_4Si.

4.1.1. *N*-(*tert*-Butoxycarbonyl)-L-*tert*-butyl-aspartic acid 4-benzyl ester (19). *t*-Butanol (1.7 mL, 18.4 mmol), 1, 3-dicyclohexylcarbodiimide (DCC, 3.7 g, 18.0 mmol) and 4-dimethylaminopyridine (DMAP, 0.19 g, 1.5 mmol) were added to a solution of *N*-(*tert*-butoxycarbonyl)-L-aspartic acid 4-benzyl ester (5 g, 15.5 mmol) in dichloromethane (CH_2Cl_2, 10 mL). This solution was stirred at room temperature for 2 h and then filtered, concentrated and subjected to column chromatography (pentane/EtOAc, 10:1) to give **19** (5 g, 85%): α=2.35° (EtOAc, c=1.95); mp=65.0°C; TLC R_f=0.3 (pentane/EtOAc, 10:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (s, 5H), 5.47 (d, 1H, J=8.2 Hz), 5.14 (d, 2H, J=5.7 Hz), 4.47 (m, 1H), 3.05 (dd, 1H, J=4.5, 6.8 Hz), 2.84 (dd, 1H, J=4.5, 6.8 Hz), 1.45 (s, 9H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.18, 170.28, 155.79, 135.96, 128.94, 128.71, 128.66, 82.69, 80.21, 67.00, 50.96, 37.46, 28.46, 28.20; LRMS (ESI) m/z 378 ($M-H^+$).

4.1.2. *N*-(*tert*-Butoxycarbonyl)-L-*tert*-butyl-aspartic acid (20). A mixture of **19** (5 g, 13.2 mmol) and 10% Pd/C (1 g) in EtOH was shaken in a Parr Hydrogenator under hydrogen (50 psi) for 1 h. The catalyst was filtered and the filtrate concentrated to give **20** (3.8 g, 100%): α=−2.73° (EtOAc, c=0.44); mp=104.8°C; TLC R_f=0.5 (pentane/EtOAc, 1:1.5); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (bs, 1 H), 5.51 (d, 1H, J=8.2 Hz), 4.43 (m, 1H), 2.98 (dd, 1H, J=4.0, 7.1 Hz), 2.78 (dd, 1H, J=4.0, 7.1 Hz), 1.45 (s, 9H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.05, 170.28, 155.96, 82.83, 80.46, 50.74, 37.20, 28.64, 28.18; LRMS (ESI) m/z 288 ($M-H^+$).

4.1.3. *tert*-Butyl L-2-(*tert*-butoxycarbonyl)amino-4-hydroxy-butyrate (21). Borane (BH_3)–THF complex (1 M, 6 mL) was added dropwise to a solution of **20** (1.7 g, 5.9 mmol) in anhydrous THF (4 mL) under argon at −15°C in an ice-salt bath. Hydrogen evolution occurred during the addition of BH_3–THF. The mixture was stirred for 1 h, allowed to warm to room temperature, and quenched with saturated $NaHCO_3$. The liquid solution was extracted with EtOAc (3×10 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated to give an oil that was purified by column chromatography (pentane/EtOAc, 2:1) to give **21** (1.3 g, 80%): α=−0.34° (EtOAc, c=3.56); TLC R_f=0.34 (pentane/EtOAc, 1.5:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.32 (d, 1H, J=6.9 Hz), 3.62 (m, 2H), 2.06 (m, 1H), 1.50 (m, 1H), 1.40 (s, 9H), 1.38 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.23, 156.80, 82.46, 80.49, 58.54, 51.26, 36.66, 28.50, 28.21; LRMS (ESI) m/z 298 ($M+Na^+$).

4.1.4. *tert*-Butyl L-2-(*tert*-butoxycarbonyl)amino-4-phthalimidooxy-butyrate (22). Diethyl azodicarboxylate (DEAD, 0.72 mL, 4.6 mmol) was added to a solution of **21** (1.16 g, 4.2 mmol), *N*-hydroxyphthalimide (0.75 g, 4.6 mmol) and triphenylphosphine (1.22 g, 4.6 mmol) in CH_2Cl_2 (10 mL) at 0°C. The solution immediately turned red and the color quickly disappeared. The reaction appeared complete within 5 min as judged by TLC. The solution was concentrated and purified by column chromatography to give **22** (1.68 g, 95%): α=−0.35° (EtOAc, c=2.30); mp=115.9°C; TLC R_f=0.33 (pentane/EtOAC, 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (s, 4H), 5.49 (d, 1H, J=7.7 Hz), 4.30 (m, 1H), 4.24 (t, 3H, J=6.6 Hz), 2.20 (m, 2H), 1.40 (s, 9H), 1.38 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.30, 163.82, 155.90, 134.89, 129.31, 123.97, 82.54, 80.10, 75.50, 51.86, 31.28, 28.71, 28.33; LRMS (ESI) m/z 419 ($M-H^+$).

4.1.5. *tert*-Butyl L-2-(*tert*-butoxycarbonyl)amino-4-aminoxy-butyrate (17). Hydrazine (43. μL, 1.37 mmol) was slowly added to a solution of **21** (0.56 g, 1.33 mmol) in EtOH (2 mL). A white precipitate formed and the mixture was stirred until the reaction was complete as judged by TLC. The mixture was filtered and the filtrate concentrated and purified by column chromatography (pentane/EtOAc, 2:1) to afford compound **17** (0.33 g, 85%): α=−0.32° (EtOAc, c=2.14); TLC R_f=0.35 (pentane/EtOAc, 1.2:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.19 (d, 1H, J=7.5 Hz), 4.30–4.90 (bs, 2H), 4.20 (m, 1H), 3.69 (t, 2H, J=5.5 Hz), 1.95 (m, 2H), 1.40 (s, 9H), 1.38 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.05, 155.77, 82.20, 79.99, 72.35, 52.12, 31.74, 28.69, 28.34; Anal. Calcd for $C_{13}H_{26}N_2O_5$: C, 53.78; H, 9.05; N; 9.65. Found: C, 53.71; H, 9.05; N, 9.71; LRMS (ESI) m/z 291 ($M+H^+$).

4.1.6. *tert*-Butyl L-2-(*tert*-butoxycarbonyl)amino-4-(fluorenyl-methyloxycarbonyl)thioureio-oxy)-buryrate (23). Fmoc-NCS (0.56 g, 2 mmol) was added to a solution of **17** (0.43 g, 1.48 mmol) in dry dichloromethane (5 mL). The mixture was stirred for 30 min and concentrated under reduced pressure to give an oil that was purified by column chromatography (pentane/EtOAc 4:1) to afford **23** (0.72 g, 85%): α=−0.28° (EtOAc, c=2.53); TLC R_f=0.35 (pentane/EtOAc, 2.5:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.8 (s, 1H), 8.32 (s, 1H), 7.78 (d, 2H, J=7.5 Hz), 7.57 (d, 2H, J=7.5 Hz), 7.44 (t, 2H, J=7.4 Hz), 7.34 (t, 2H, J=7.4 Hz), 5.52 (d, 1H, J=7.5 Hz), 4.49 (d, 2H, J=6.5 Hz), 4.40 (m, 1H), 4.18 (m, 3H), 2.10 (m, 2H), 1.45 (s, 9H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.39, 171.70, 155.95, 152.28, 143.72, 143.20, 141.72, 128.49, 128.28, 127.66, 125.40, 125.19, 120.61, 120.44, 82.75, 80.20, 72.84, 69.01, 51.79, 46.92, 31.26, 28.71, 28.34; Anal. Calcd for $C_{29}H_{37}N_3O_7S$: C, 60.93; H, 6.52; N, 7.35; S, 5.61. Found: C, 60.90; H, 6.55; N, 7.31; S, 5.62; LRMS (ESI) m/z 571 ($M-H^+$).

4.1.7. *tert*-Butyl L-2-(*tert*-butoxycarbonyl)amino-4-(thioureidooxy)-butyrate (24). A solution of 20% piperidine in methanol (3.4 mL) was added to a solution of **23** (1.08 g, 1.9 mmol) in dry dichloromethane (10 mL). After 10 h, the reaction was judged complete by TLC and the solution concentrated and purified by column chromatography (pentane/EtOAc 3:1) to yield **24** (0.50 g, 75%): α=−2.54° (EtOAc, c=1.18); TLC R_f=0.35 (pentane/EtOAc, 1.2:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.47 (s, 1H), 7.38 (bs, 1H), 7.28 (bs, 1H), 6.11 (d, 1H, J=6.8 Hz), 4.15 (m, 1H), 4.12 (t, 2H, J=5.3 Hz), 2.21 (m, 2H), 1.40 (s, 9H), 1.38 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.78, 172.65, 157.12, 82.35, 80.03, 73.17, 52.65, 31.87, 28.93, 28.49; Anal. Calcd for

$C_{14}H_{27}N_3O_5S$: C, 48.12; H, 7.79; N, 12.02; S, 9.18. Found: C, 48.39; H, 7.78; N, 11.97; S, 9.20; LRMS (ESI) *m*/*z* 348 ($M-H^+$).

4.1.8. *tert*-Butyl L-2-(*tert*-butoxycarbonyl)amino-4-(*S*-methylthioureidooxy)-butyrate (25). Iodomethane (0.14 mL, 2.2 mmol) was added slowly to a mixture of **24** (0.4 g, 1.1 mmol) and $NaHCO_3$ (0.5 g) in dry CH_3CN (2 mL) at 0°C. The reaction was allowed to warm to room temperature and the progress was monitored by TLC. After 20 h, the solution was filtered and the filtrate concentrated and purified by column chromatography (pentane/EtOAc 3:1) to yield **25** (0.34 g, 82%): $\alpha=-0.70°$ (EtOAc, $c=2.28$); TLC R_f=0.4 (pentane/EtOAc, 2:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.14 (d, 1H, J=8.0 Hz), 4.92 (bs, 2H), 4.25 (q, 2H, J=5.6 Hz), 4.01 (t, J=5.9 Hz, 2H), 2.01 (m, 2H), 1.38 (d, J=7.5 Hz, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.49, 155.81, 153.01, 82.51, 80.01, 69.73, 52.01, 32.25, 28.71, 28.39, 14.18; Anal. Calcd for $C_{15}H_{29}N_3O_5S$: C, 49.57; H, 8.04; N, 11.56; S, 8.82. Found: C, 49.56; H, 8.02; N, 11.59; S, 8.90; LRMS (ESI) *m*/*z* 386 ($M+Na^+$).

4.1.9. L-2-Amino-4-(thioureidooxy) butanoic acid (15). A solution of **24** (100 mg, 0.29 mmol) in 4N HCl/dioxane (4 M, 2 mL) was stirred for 4 h under an argon atmosphere. The mixture was concentrated and the product dissolved in distilled water (5 mL). The solution was passed through a 6 mL Supelco LC-18 solid phase extraction tube and lyophilyzed to yield **15** (44 mg, 80%): ^{1}H NMR (300 MHz, D_2O) δ 4.11 (m, 3H), 2.28 (t, 2H, J=5.5 Hz); ^{13}C NMR (75 MHz, D_2O) δ 178.49, 171.83, 72.20, 50.84, 28.73; LRMS (ESI) *m*/*z* 194 ($M+H^+$); Anal. Calcd for $C_5H_{11}N_3SO_3\cdot(2H_2O+HCl)$: C, 22.60; H, 6.07; N, 15.81; S, 12.01. Found: C, 22.35; H, 7.60; N, 16.00; S, 11.94.

4.1.10. L-2-Amino-4-(*S*-methylthioureidooxy) butanoic acid (16). A solution of **25** (100 mg, 0.28 mmol) in 4N HCl/dioxane (4 M, 2 mL) was stirred for 4 h under an argon atmosphere. The mixture was concentrated and the product dissolved in distilled water (5 mL). The solution was passed through a 6 mL Supelco LC-18 solid phase extraction tube and lyophilyzed to yield **16** (53 mg, 85%): ^{1}H NMR (300 MHz, D_2O) δ 4.10 (m, 3H), 2.44 (s, 3H), 1.38 (t, 2H, J=5.5 Hz); ^{13}C NMR (75 MHz, D_2O) δ 172.69, 163.56, 72.59, 51.32, 28.94, 13.32; LRMS (ESI) *m*/*z* 208 ($M+H^+$); Anal. Calcd for $C_6H_{13}N_3SO_3\cdot(3H_2O+HCl)$: C, 24.12; H, 7.08; N, 14.06; S, 10.73. Found: C, 24.95; H, 7.71; N, 14.99; S, 10.70.

4.2. Biochemistry—general

Dithiothreitol (DTT), nicotine adenine dinucleotide phosphate (NADPH), FAD, FMN, (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4B), L-arginine, lactate dehydrogenase, sodium pyruvate, and *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma. Inducible NOS was a generous gift from Dr. Dennis Stuehr (The Cleveland Clinic Foundation).

4.3. Griess assay of nitrite production by iNOS

Initial experiments to determine whether any of the synthetic compounds could inhibit iNOS catalyzed NO production from L-arginine were performed using the Griess assay. Specifically, inducible NOS (5 nM) was incubated at 37°C for 30 min with 1 mM dithiothreitol; 4 μM each of FAD, FMN, and H_4B; 1 mM each of NADPH, L-arginine and test compound (1 mM) in a 96-well plate with a final volume of 100 μL. Reactions were initiated with the addition of NADPH and were performed in triplicate. After depleting any remaining NADPH by incubation with lactate dehydrogenase (1000 unit/mL) and sodium pyruvate (50 mM) for 15 min at 37°C, Griess reagent A (40 μL) was added followed by Griess reagent B (40 μL). The absorbance at 550 nm was measured with a microplate reader (Spectramax, Molecular Devices). Nitrite production was compared to controls containing only L-arginine (1 mM).

4.4. Inhibition of iNOS by oxyhemoglobin assay[25]

Aliquots (5 μL) of each of the following agents were added to sample wells in a 96-well plate: NADPH (20 mM), L-arginine, DTT (20 mM), and H_4B (80 μM). The sample volume was adjusted to 80 μL by further addition of HEPES buffer (pH=7.5) and the desired inhibitor. Formation of NO was initiated by addition of iNOS (20 μL, 1 μM). The microplates were mixed by gentle shaking at 37°C. The rate of change in absorbance at 405 nm, reflecting ferroheme oxidation, was continually measured in all samples at 15 s intervals for 15 min using a microplate reader. In samples where linearity is maintained throughout the duration of the assay, all points are used to calculate the slope. The maximum increase in ΔOD per min is calculated by considering only the linear phase.

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