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L-Arginine analogs as alternate substrates for nitric oxide synthase

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Abstract—The L-arginine analogs, N^{δ}-methyl-L-arginine (δMA) and L-canavanine, were used to probe the role of the N^{δ} nitrogen of L-arginine in the reaction catalyzed by nitric oxide synthase (NOS). δMA was synthesized and found to be a partial alternate substrate and a weak, reversible inhibitor of NOS with a K_i equal to 1.4 mM. δMA undergoes hydroxylation; however, it is not converted further, hence it functions as a partial substrate. L-Canavanine was converted to an L-homoserine presumably via initial hydroxylation and decomposition. The mechanism of this reaction and products of this reaction were not probed further.

1. Introduction

Nitric oxide synthases (NOS,[1] EC 1.14.13.39) catalyze the biosynthesis of ·NO and L-citrulline from the amino acid L-arginine (Fig. 1).[1–3] The overall reaction occurs in two definable steps and overall involves a five-electron oxidation of L-arginine requiring NADPH and O_2 as cosubstrates in each step. All NOS isoforms are homodimeric and bind an equivalent each of FAD, FMN,[4–6] and protoporphyrin IX heme.[7–9] One bound tetrahydrobiopterin (H_4B) per monomer is also required for full activity.[5,6,10]

The roles of the heme and H_4B are not completely understood. The first step of the reaction is the hydroxylation of L-arginine forming the intermediate N^{G}-hydroxy-L-arginine (NHA).[11–13] The three-electron oxidation of NHA forming L-citrulline and ·NO has been proposed to involve the nucleophilic attack of a heme ferric-peroxide species.[14] Previous CO inhibition studies have suggested a catalytic role for the heme in both steps of the reaction.[7,15]

The H_4B cofactor has been shown to affect enzyme dimerization,[16–18] affinity for substrate,[19,20] heme spin state equilibrium,[21,22] and heme midpoint potential.[23] In addition, H_4B is essential for both steps of NOS catalysis and has been proposed to play a role in

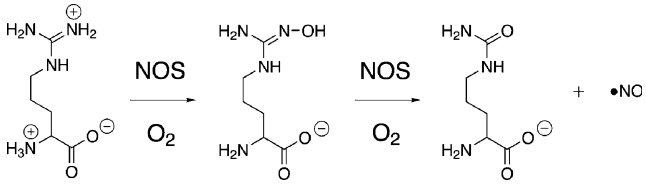

Figure 1. Reaction catalyzed by NOS.

electron transfer.[24,25] Direct evidence for the presence of a pterin radical ($H_3B^{\cdot}$) in the heme domain of iNOS has been obtained by rapid freeze-quench EPR in reactions with L-arginine,[26] as well as with NHA.[27]

L-Indospicine (Fig. 2), a natural product from the legume *Indigofera spicata*, lacks a N^{δ} nitrogen and has previously been shown to be a weak inhibitor of NOS.[28] Crystal structures of the iNOS heme domain with L-arginine bound show the N^{δ} involved in a hydrogen bond to a glutamate.[29] This same glutamate is also hydrogen bonded to one of the two guanidino nitrogens. Feldman et al.[28] have suggested that poor affinity of L-indospicine is due to the lack of a N^{δ} hydrogen bond. L-Canavanine (Fig. 2), an amino acid found in certain leguminous plants, has an oxygen atom adjacent to the N^{δ} nitrogen. This L-arginine analog has been reported to inhibit NOS with an apparent K_i of 0.22 mM.[30] These results indicate that the N^{δ} position is important for catalysis and that substitutions around this position also influence the chemistry taking place. We decided to probe the mechanistic role of the N^{δ} nitrogen of

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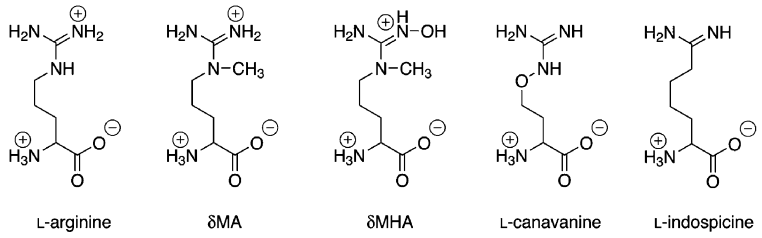


Figure 2. Structures of L-arginine and mechanistic probes.

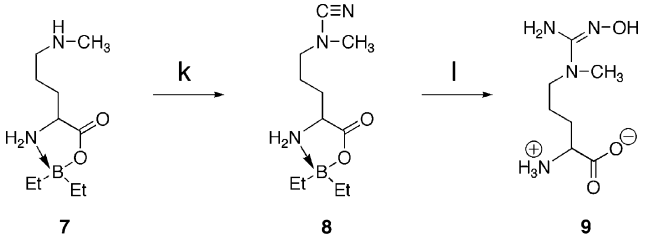
L-arginine through the synthesis and study of L-arginine analogs that would test the role of the N^{δ} nitrogen in the reaction. N^{δ}-methyl-L-arginine (dMA) (Fig. 2) would disrupt the hydrogen bond to the glutamate and would block chemistry from occurring at the N^{δ} position including the abstraction of a hydrogen atom. L-Canavanine, on the other hand, has an electron-withdrawing oxygen atom adjacent to the N^{δ} nitrogen. We have used analytical HPLC and LCMS product assays to demonstrate that δMA and L-canavanine are alternate substrates for NOS. The possible mechanistic implications of these results are discussed.

2. Results

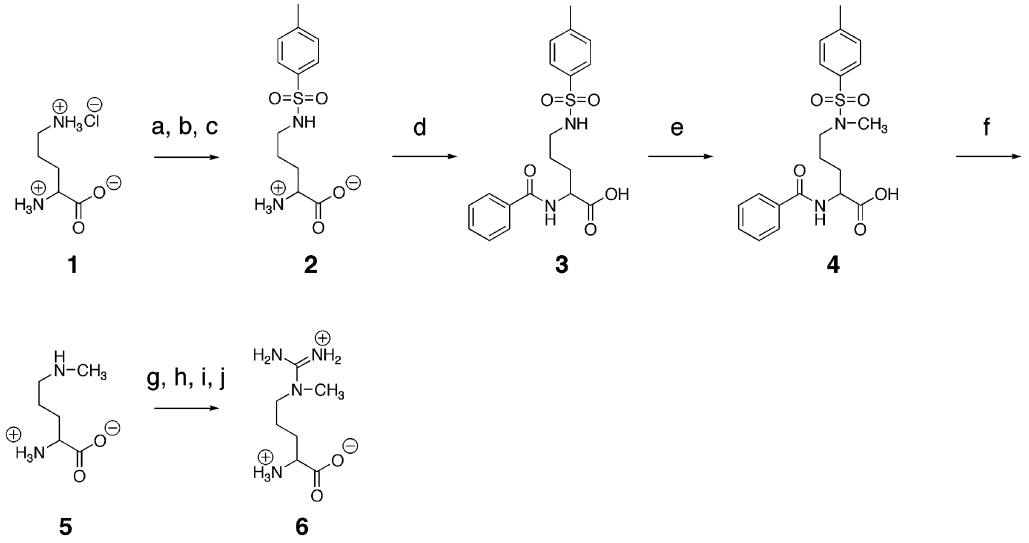
2.1. Synthesis

The synthesis of **6** (δMA) (Scheme 1) began with commercially available L-ornithine, which was first protected as the copper chelate and tosylated using the methods of Erlanger[31] to give **2**. Following the procedure detailed by Benotoin,[32] **2** was deprotected with H_2S and the α-amino group protected using benzoyl chloride, which yielded **3**. The δ nitrogen was methylated using methyl iodide and the protecting groups were removed by heating in hydrobromic acid, which gave **5**. The *B*,*B*-diethylboroxazolidone protecting group was added by boiling with triethylborane in dimethoxyethane (DME) according to Garcia et al.[33] The guanidine group was appended using *N*,*N*′-bis-*tert*-butoxycarbonylthiourea in a procedure detailed by Poss et al.[34] giving **6**. Removal of the *B*,*B*-diethylboroxazolidone group was accomplished using the methods of Nefkens and Zwanenburg.[35]

Compound **8** (δMHA) was prepared from **5** in two steps (Scheme 2). Treating **5** with cyanogen bromide in DME afforded **7**. Formation of the hydroxyguanidine and removal of the *B*,*B*-diethylboroxazolidone protecting group were achieved in one step using hydroxylamine hydrochloride giving **8**.



Scheme 2. Synthesis of N^{G}-hydroxy-N^{δ}-methyl-L-arginine. Reagents: (k) CNBr, Et_3N, DME; (l) $NH_2OH \cdot HCl$, Et_3N, MeOH.



Scheme 1. Synthesis of N^{δ}-methyl-L-arginine. Reagents and conditions: (a) L-Ornithine · HCl, $CuCO_3 \cdot Cu(OH)_2$, reflux; (b) *p*-TsCl, 2 M NaOH; (c) H_2S, 2 M HCl; (d) benzoyl chloride, 1 M NaOH; (e) MeI, 2 M NaOH; (f) 48% HBr, reflux; (g) 1 M $Et_3B \cdot THF$, DME; (h) *N*,*N*′-bis-*tert*-butoxycarbonylthiourea, $HgCl_2$, Et_3N, DMF; (i) 1.5 M HCl, 100 °C; (j) TFA.

2.2. HPLC analysis of alternate substrates

δMA was converted by iNOS to a single amino acid product analyzed by naphthalene-2,3-dicarboxaldehyde (NDA) derivatization and HPLC. The retention time of this new amino acid peak was 6.5 min (Fig. 3). This peak co-eluted with the NDA derivative of authentic δMHA (data not shown). Oxidation of L-canavanine by iNOS also gave rise to a new peak in the HPLC chromatogram (Fig. 4). This peak had a retention time of 11.0 min and co-eluted with the NDA derivative of authentic L-homoserine. In both the δMA and L-canavanine reactions, no product was detected when either NADPH or iNOS was omitted from the reaction mixture.

2.3. LCMS product identification

The mass spectrum of the product of δMA oxidation by iNOS (Fig. 5) shows a peak with a *m*/*z* of 378.2 corresponding to the NDA derivative of δMHA. Another peak is visible with a *m*/*z* of 334.2 arising from neutral loss of CO_2 (M − 44) from this derivative. The mass spectrum of the new peak in the HPLC chromatogram of L-canavanine oxidation by iNOS contains a peak with a *m*/*z* of 293.2 (Fig. 6) corresponding to the NDA derivative of L-homoserine. The mass spectrum also contains a peak with a *m*/*z* of 249.2 corresponding to neutral loss of CO_2 (M−44) from this derivative.

2.4. δMA inhibition studies

δMA was expected to be an alternate substrate of iNOS, possibly very slow compared to L-arginine as we observed with NMA[36]. Slow, alternate substrates can also be described as inhibitors, if turnover is relatively slow. Since δMA did not produce ·NO and the HPLC assay described above showed conversion to δMHA but no further reaction, inhibition by δMA was assessed. Lineweaver–Burk analysis of δMA inhibition of iNOS showed that δMA is a competitive inhibitor of iNOS (data not shown). A K_m/V_{max} versus [δMA] re-plot of data derived from Michaelis–Menten plots of competitive iNOS inhibition by δMA versus L-arginine (Fig. 7) gave a K_i for δMA equal to 1.4 mM.

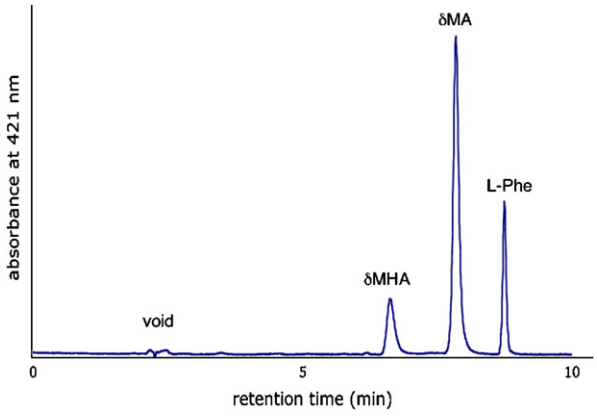


Figure 3. HPLC chromatogram of δMA oxidation by iNOS.

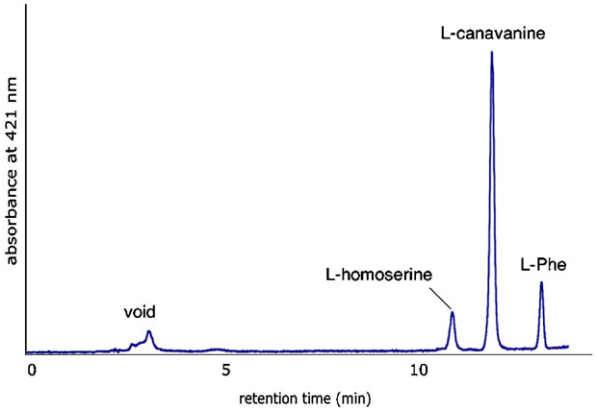


Figure 4. HPLC chromatogram of L-canavanine oxidation by iNOS.

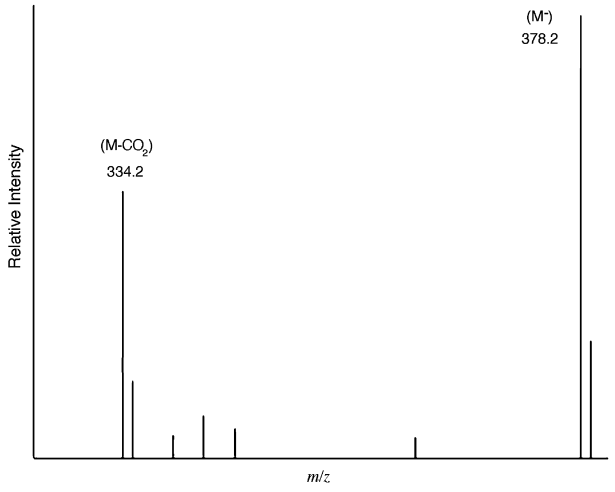


Figure 5. MS spectrum of $t = 6.5$ min unknown arising from oxidation of δMA by iNOS.

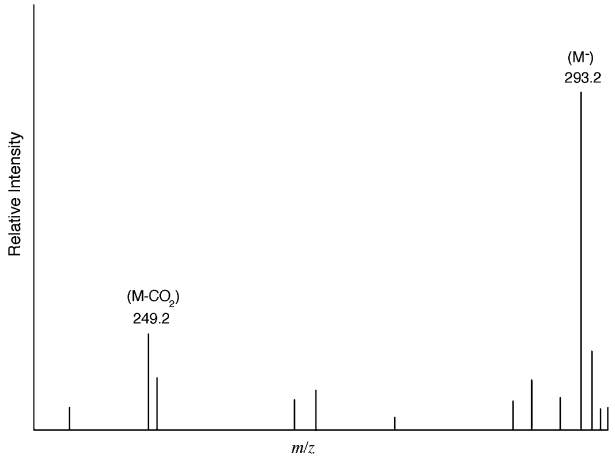


Figure 6. MS spectrum of $t = 11.0$ min unknown arising from oxidation of L-canavanine by iNOS.

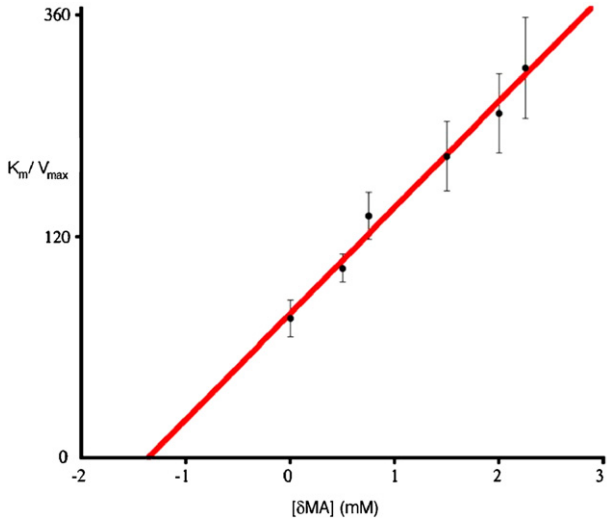


Figure 7. K/V versus [δMA] re-plot used in determining K_i for δMA.

3. Discussion

Feldman et al.[28] attributed the poor affinity of L-indospicine to the loss of one critical hydrogen bond between the N^{δ} position and the iNOS active site residue Glu 371. δMA (K_i = 1.4 mM) is a slightly more potent inhibitor than L-indospicine (K_i > 3 mM) and it lacks the same critical hydrogen bond donor. This increase in potency, while not great, suggests that other determinants are, in part, responsible for the poor binding properties of L-indospicine and δMA. δMA contains an intact, presumably planar guanidine, group for the delocalization of positive charge, while the lack of a N^{δ} nitrogen in L-indospicine prohibits planarity around the N^{δ} position. This may cause misalignment of the amidine group with the additional hydrogen bond acceptors, namely Glu 371 and the backbone carbonyl of Trp 366. In total, this would result in the loss of not one but three hydrogen bonds. The ability to assume a planar geometry is clearly very important for productive binding.

δMA could be an important mechanistic tool since it is a substrate for step 1 in the NOS reaction (L-arginine conversion to NHA) but not step 2 (NHA conversion to citrulline and ·NO). Previous work has suggested that H_4B plays a similar role in catalysis with either L-arginine or NHA as a substrate. The pterin has been proposed to directly reduce a heme-bound oxygen atom, generating the active oxidant and a pterin radical.[37–40] This is illustrated in Figure 8 for step 2 of the NOS reaction with NHA (A) and δMA (B). The regeneration of the pterin cofactor in the two separable steps of the reaction is different. The hydroxylation of L-arginine forms $H_3B^{\bullet}$ that is reduced by one electron provided by the reductase domain. The oxidation of NHA again forms $H_3B^{\bullet}$ (Fig. 8A, reaction 4). The reduction of this radical has been proposed to occur by a one-electron oxidation of the substrate NHA (Fig. 8A, reaction 5).[41] It is possible that the one-electron substrate oxidation step that reduces the $H_3B^{\bullet}$ formed in the second step occurs at the N^{δ} position. If oxidation at the N^{δ} position is indeed the case, then δMA would be unable to go forward (Fig. 8B, reaction 5) and would be expected to undergo hydroxylation but not conversion to ·NO in the second step of the reaction. However, no turnover of δMA to δMHA was observed. Based on past results with pterin-free NOS[21] and the peroxide shunt,[14] δMHA should be converted to NO^- and the amino acid products δ-methyl-citrulline and possibly δ-methyl-*N*-cyano-ornithine. The lack of turnover suggests that the current view of the mechanism, as shown in Figure 8, is not correct or perhaps steric issues described below come into play.

An electron-donating N^{δ} methyl group would decrease the electrophilicity of the carbon atom of the hydroxyguanidine group. The methyl group may make this carbon less susceptible to the proposed nucleophilic attack

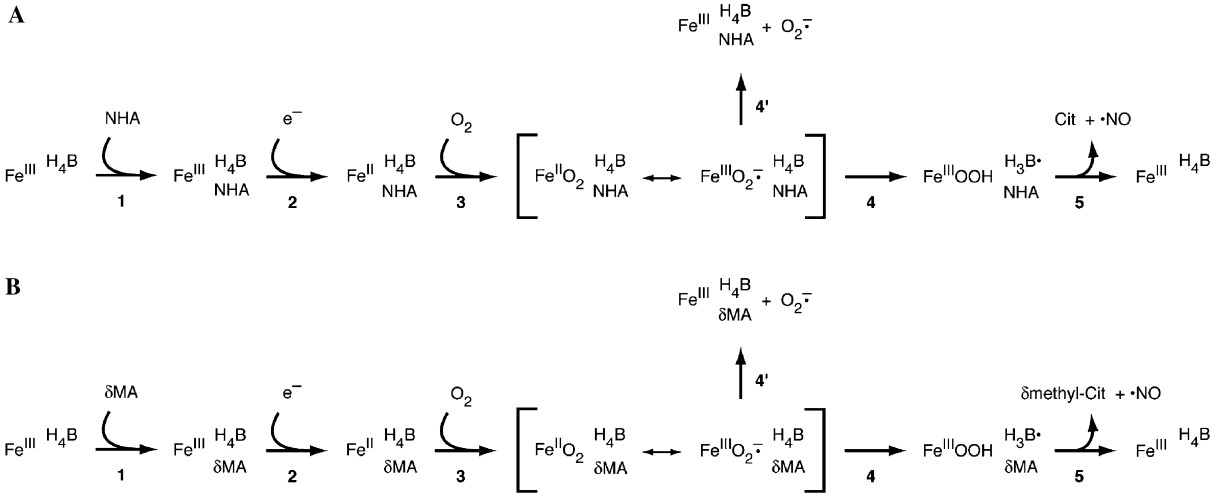


Figure 8. NOS reaction scheme for the second step in the reaction. (A) The conversion of NHA to citrulline and ·NO. (B) The same reaction scheme for δMA.

by the heme ferric-peroxide species. Also, it cannot be ruled out that the steric bulk imposed by the N^{δ} methyl group could cause misalignment of the hydroxy-guanidine of δMHA rendering it unable to undergo nucleophilic attack by the ferric-peroxy species.

L-Canavanine, a known inhibitor of NOS,[30] we find here functions as an alternate substrate for the NOS reaction, as it was shown to be converted to L-homoserine by the enzyme. The enzymatic product of L-canavanine oxidation is speculated to be N^{G}-hydroxy-L-canavanine, which would then decompose into L-homoserine in aqueous solution. We were unable to determine whether L-canavanine participates in the second step of the reaction or decomposes after participating in the first step making the L-canavanine results difficult to interpret. Significantly, NO was not formed with L-canavanine.

In summary, we have probed further how small changes in substrate influence activity. Remarkably, the simple addition of a methyl group to the δ nitrogen of L-arginine leads to a substrate that is specific for step 1 of the NOS reaction.

4. Experimental

4.1. General

Chemicals used in synthetic methods were of reagent grade quality and were obtained from Sigma–Aldrich. Methyl iodide was purchased from Fisher. Anhydrous DMF was purchased from Sigma–Aldrich. Anhydrous DME and triethylamine were distilled under nitrogen from sodium benzophenone ketyl. Microgranular cellulose powder was purchased from Whatman. All HPLC solvents were of HPLC grade. The melting points were uncorrected. NMR spectra were recorded on a Bruker AMX-300, AMX-400, AM-400, or AV-400 spectrometer. NMR spectra in D_2O used $CDCl_3$ (sealed capillary), or (Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TPS) as an internal standard. FAB mass spectra were recorded either on a Micromass ZAB2-EQ or on a VG70 mass spectrometer equipped with a FAB ion source. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin was from Schircks Laboratories (Jona, Switzerland). Pefabloc SC hydrochloride was purchased from Roche diagnostics. The 2′,5′-ADP Sepharose 4B resin and the S200 16/60 gel filtration column were from Pharmacia-LKB Biotechnology Inc. The EmulsiFlex-C5 high pressure homogenizer was from Avestin, Inc. DEAE Biogel A and AG50W-X8 (H^+) 200–400 mesh cation exchange resin were purchased from Bio-Rad. Centrifugal filtration units (Ultrafree-15, Biomax-50K NMWL membrane) were purchased from Millipore.

4.1.1. N^{δ}-*p*-Toluenesulfonyl-L-ornithine (2). $CuCO_3 \cdot Cu(OH)_2$ (7 g, 0.07 mol) was added to a solution of L-ornithine · HCl (**1**) (10.1 g, 0.06 mol) in refluxing water (30 mL) over a period of 15 min. The blue solution was then cooled for 1 h and filtered. To this filtrate were added *p*-toluenesulfonyl chloride (7.75 g, 0.04 mol) in Et_2O (30 mL) and 2 M NaOH (65 mL) with vigorous stirring. After 40 min, a solution of *p*-toluenesulfonyl chloride (7.75 g, 0.04 mol), Et_2O (35 mL), and 2 M NaOH (15 mL) was added and stirred for 3 h. The light blue precipitate was filtered and washed with both EtOH and Et_2O. The filtered compound was dissolved in 2 M HCl (200 mL) and H_2S was bubbled through the solution for 2.5 h. To the resulting black solution was added activated charcoal (4 g) and the mixture was filtered through Celite. The filtrate was adjusted to pH 6 with NaOH and left overnight. The precipitated solid was collected using vacuum filtration, yielding **2** (9.57 g, 56%) as a gray solid; mp 212–213 °C; ^{1}H NMR (300 MHz, D_2O) δ (ppm): 7.76 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 4.06 (t, J = 6.5 Hz, 1H), 2.96 (t, J = 6.6 Hz, 2H), 1.92 (m, 2 H), 1.60 (m, 2H); ^{13}C NMR (400 MHz, D_2O) δ 170.7, 144.3, 134.5, 129.6, 126.2, 51.9, 41.4, 26.4, 24.0, 20.3; FABMS calcd for $C_{12}H_{19}N_2O_4S$ ($[M+H]^+$), found m/z 287.

4.1.2. N^{α}-Benzoyl-N^{δ}-*p*-toluenesulfonyl-L-ornithine (3). A solution of **2** (9.57 g, 0.033 mol) in 1 M NaOH (53 mL) was stirred at 0 °C, while a solution of benzoyl chloride (4.28 mL, 0.037 mol) in 2 M NaOH (20 mL) was added over 30 min. The solution was brought to room temperature, stirred for 2 h, and then acidified to pH 3 with HCl. EtOAc (350 mL) and the precipitated solid was filtered yielding **3** (3.95 g, 30%); mp 184 °C; ^{1}H NMR (300 MHz, DMSO) δ (ppm): 8.54 (d, J = 7.5 Hz, 1H), 7.86 (d, J = 7.2 Hz, 2H), 7.65 (d, J = 8.1 Hz, 2H), 7.50 (m, 1H), 7.49 (d, J = 7.5, 2H), 4.28 (m, 1H), 2.73 (q, 2H), 2.35 (s, 3H), 1.62–1.91 (m, 2H), 1.36–1.57 (m, 2H); ^{13}C NMR (400 MHz, DMSO) δ 173.6, 166.6, 142.5, 137.6, 134.0, 131.4, 129.6, 128.3, 127.5, 126.5, 52.3, 42.2, 27.8, 26.1, 20.9; FABMS calcd for $C_{19}H_{23}N_2O_5S$ ($[M+H]^+$), found m/z 391. Anal. Calcd (%) for $C_{19}H_{22}N_2O_5S$: C 58.45, H 5.68, N 7.17. Found: C 57.96, H 5.90, N 6.92.

4.1.3. N^{α}-Benzoyl-N^{δ}-methyl-N^{δ}-*p*-toluenesulfonyl-L-ornithine (4). In a pressure bottle with a stir bar was added a solution of **3** (4.109 g, 0.011 mol) in 2 N NaOH (47 mL). MeI (0.65 mL, 0.010 mol) was added and the solution was heated to 70 °C for 1 h. The solution was cooled to room temperature and 0.65 mL of MeI was again added, and the solution was brought to 70 °C for 1 h and then cooled to room temperature. This cycle was repeated one additional time with MeI (0.65 mL) before the solution was cooled to room temperature. The solution was cooled to 0 °C and acidified to pH 2 with 6 M HCl. The precipitated product was extracted with ethyl acetate (3 × 75 mL) and the combined EtOAc layers were dried ($MgSO_4$) and evaporated to dryness. Recrystallization from EtOH/H_2O gave **4** (3.7 g, 86%); mp 163 °C; ^{1}H NMR (300 MHz, DMSO) δ (ppm): 8.62 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 6.9 Hz, 2H), 7.64 (d, J = 8.4 Hz, 2H), 7.51 (1H, m), 7.50 (d, J = 7.2 Hz, 2H), 7.39 (d, J = 8.1 Hz, 2H), 4.38 (1H, m), 2.94 (2H, m), 2.61 (3H, s), 2.37 (3H, s), 1.80 (2H, m), 1.56 (2H, m); ^{13}C NMR (400 MHz, DMSO) δ 173.6, 166.5, 143.1, 133.9, 131.3, 129.8, 128.2, 127.4, 127.1, 52.3, 49.2, 34.4, 27.7, 23.8, 21.0; FABMS calcd for $C_{20}H_{25}N_2O_5S$ ($[M+H]^+$), found m/z 405. Anal. Calcd

(%) for $C_{20}H_{24}N_2O_5S$: C 59.39, H 5.98, N 6.93. Found: C 59.39, H 6.08, N 6.84.

4.1.4. N^{δ}-Methyl-L-ornithine (5). A solution of **4** (2.66 g, 0.007 mol) in 48% HBr (21 mL) was heated to reflux for 2 h. The solution was cooled to room temperature and filtered. The filtrate was evaporated to give a viscous red liquid, which was dissolved in water (25 mL) and applied to a 26 mL of AG50W-X8 (H^+) cation exchange resin. The column was washed with water until the pH of the eluent was neutral and then eluted with 2 N NH_4OH. Tubes containing (**5**) were combined and evaporated to give a brown oil, which was dissolved in water (15 mL). The pH was adjusted to 5.8 using HCl and the solution was boiled for 30 s with activated charcoal. The charcoal was filtered off and the filtrate was evaporated to dryness giving **5** (804 mg, 83%) as a solid; mp 217 °C; ^{1}H NMR (400 MHz, D_2O/TPS) δ (ppm): 3.32 (t, 1H), 2.96 (t, 2H), 2.62 (s, 3H), 1.67 (4H, m); ^{13}C NMR (400 MHz, D_2O/TPS) δ 183.9, 58.0, 51.7, 35.5, 33.7, 25.1; FABMS calcd for $C_6H_{15}N_2O_2$ ($[M+H]^+$), found *m*/*z* 147.

4.1.5. N^{δ}-Methyl-L-arginine (δMA) (6). Compound **5** was dissolved in water and the pH was adjusted to 3 using concentrated HCl. The solution was evaporated to dryness to yield 995 mg of **5** as the HCl salt. The solid was dried overnight on a vacuum line and ground with glass stirring rod until a dry, finely divided powder was obtained. To a suspension of the HCl salt of **5** (650 mg, 0.004 mol) in dry DME (5.6 mL) was added 1 M triethylborane in THF (4.5 mL, 0.0045 mol). The mixture was heated to reflux under argon for 48 h. The resulting suspension was filtered, rinsed twice with petroleum ether, and dried. The white compound (381 mg, 0.002 mol) was dissolved in anhydrous DMF (3.15 mL) to which *N*,*N*′-bis-*tert*-butoxycarbonylthiourea (492 mg, 1.78 mmol) and triethylamine (819 μL, 5.9 mmol) had been added. The reaction was cooled to 0 °C with an ice bath and $HgCl_2$ (532 mg, 0.002 mol) was added. After 1.5 h of reaction, EtOAc (25 mL) was added and the mixture was filtered through Celite to remove mercury salts. The EtOAc layer was washed twice with water and once with brine and then dried ($MgSO_4$). Evaporation of the EtOAc gave a yellow oil, which was applied to a silica gel column, and eluted with EtOAc. Ninhydrin positive fractions were combined and evaporated. Hydrolysis of the boroxazolidone was effected by the addition of 1.5 M HCl (10 mL) and heating the acidic solution at 100 °C for 1 h. Evaporation of the solvent resulted in a clear oil to which was added TFA (10 mL). This solution was stirred for 2 h before being loaded onto AG50WX-8 (H^+), washed with 5 column volumes of water, and eluted with 1 M NH_4OH. Ninhydrin positive tubes were combined and evaporated to give a clear oil. The oil was dissolved in water and the pH was adjusted to 2.4 with acetic acid and evaporated to give **6** (218 mg, 48%) as a white crystalline solid; mp 195 °C; ^{1}H NMR (400 MHz, D_2O/TPS) δ (ppm): 4.12 (t, J = 6.0 Hz, 1H), 3.43 (t, 2H, J = 8.4 Hz), 3.05 (s, 3H), 1.91 (4H, m); ^{13}C NMR (400 MHz, D_2O/TPS) δ 172.20, 156.76, 52.97, 49.71, 36.12, 26.95, 22.48; HRMS calcd for $C_7H_{17}N_4O_2^+$: 189.1352. Found: 189.1352. Anal. Calcd (%) for $C_9H_{21}N_4O_{4.5}$: C 42.01, H 8.23, N 21.78; Found: C 42.36, H 8.21, N 21.74.

4.1.6. N^{δ}-Methyl-N-cyano-L-ornithine *B*,*B*-boroxazolidone (8). To a solution of **7** (419 mg, 2.85 mol) in dry DME (17 mL) was added dry triethylamine (412 μL, 2.98 mmol). A solution of CNBr (622 mg, 5.87 mmol) in dry DME (10 mL) was added and the solution was stirred under argon for 3 h. The reaction mixture was concentrated, applied to a silica gel column, and eluted with EtOAc to give **7** (89 mg, 25%) as a white solid; mp 143–145 °C; ^{1}H NMR (400 MHz, DMSO) δ (ppm): 6.52 (t, J = 8.0 Hz, 1H), 5.60 (t, J = 8.0 Hz), 3.51 (m, 1H), 2.98 (t, J = 6.8 Hz, 2H), 2.81 (s, 3H), 1.81–1.88 (m, 1H), 1.65–1.72 (m, 2H), 1.14–1.57 (m, 1H), 0.68–0.73 (m, 6H), 0.15–0.28 (m, 4H); ^{13}C NMR (400 MHz, DMSO) δ 173.83, 118.56, 54.05, 51.66, 38.20, 27.49, 23.73, 12.79, 12.06, 8.93; FABMS calcd for $C_{11}H_{22}BN_3O_2$ ($^{18+}$) found *m*/*z* 240; Anal. Calcd (%) for $C_{11}H_{22}BN_3O_2$: C 55.25, H 9.27, N 17.57. Found: C 54.91, H 9.54, N 17.86.

4.1.7. N^{G}-Hydroxy-N^{δ}-methyl-L-arginine (δMHA) (9). To a round-bottomed flask containing **8** (25 mg, 0.104 mmol) was added triethylamine (73 μL, 0.523 mmol). To this flask was added MeOH (750 μL), followed immediately by $NH_2OH \cdot HCl$ (33 mg, 0.475 mmol). The reaction was stirred for 2 h and then concentrated on a rotary evaporator, loaded onto a 4×17 cm microgranular cellulose column, and eluted with 5:3 MeCN: 0.1% $TFA_{(aq)}$ to give **9** (13 mg, 86%) as a clear oil: ^{1}H NMR (400 MHz, D_2O/TPS) δ (ppm): 4.12 (t, J = 8.0 Hz, 1H), 3.41 (t, J = 8.0 Hz, 2H), 3.02 (s, 3H), 1.85 (m, 4H); ^{13}C NMR (400 MHz, D_2O/TPS) δ 171.8, 158.7, 52.7, 49.6, 35.7, 26.7, 22.1; HRMS calcd for $C_7H_{17}N_4O_3^+$: 205.1295. Found: 205.1301.

4.1.8. Expression and purification of inducible nitric oxide synthase. Expression and purification of iNOS were carried out, as described previously,[6,21] with minor modifications that follow. All buffers used in the lysis, purification, and concentration steps contained 10 μM H_4B. Cell pellets from 3 L culture were resuspended in lysis buffer containing 50 mM Hepes (pH 7.4), 10% glycerol, 1 mM Pefabloc SC, 10 μg/mL benzamidine, 5 μg/mL leupeptin, and 1 μg/mL each of pepstatin, chymostatin, and antipain. The resuspended cells were lysed with a high-pressure homogenizer. Centrifugation for 1 h at 42,000 rpm yielded a supernatant that was immediately purified using 2′,5′-ADP-Sepharose affinity chromatography and DEAE Bio-Gel A anion exchange chromatography, as previously described.[6] The eluate was concentrated using a centrifugal filtration device and loaded onto a gel filtration column (S200 16/60), which was equilibrated with 100 mM Hepes (pH 7.4), 100 mM NaCl, 10 μM H_4B, and 10% glycerol. Fractions containing iNOS were pooled, concentrated, and frozen in aliquots at −80 °C. iNOS purified in this manner is >95% pure by SDS–PAGE with Coomassie staining and had a specific activity of ~122 μmol ·NO/h/mg measured by the oxyhemoglobin assay (see below).[42] Protein concentration was determined using the Bradford protein assay using bovine serum albumin as a standard.

4.1.9. Enzyme reactions. To determine if δMA and L-canavanine were substrates for iNOS, enzyme reaction mixtures were evaluated for amino acid products by HPLC. Total reaction volumes were 500 μL and contained 240 μM NADPH, 11 μM H_4B, 100 μM DTT, 50 μM L-phenylalanine (as an internal standard), and 120 μM of either δMA or L-canavanine in 15 mM Hepes. After initiating the reaction with enzyme, δMA reactions contained 250 nM iNOS and were incubated at 37 °C for 4 h, while L-canavanine reactions contained 500 nM iNOS and were incubated at 37 °C for 2 h.

4.1.10. Analytical reverse-phase HPLC. Amino acids from enzyme reaction mixtures were derivatized with NDA, as previously described,[41,43] and separated by reverse-phase HPLC at 40 °C using a Nova-Pak C_{18} column (150 × 3.9 mm, 4 μm, Waters) with an Alltima C_{18}-LL guard column (5 μm, Alltech), using a Hewlett-Packard 1090 series II HPLC with a diode array detector. The derivatization conditions were as follows: 21 μL sample, 9 μL of 50 mM NaCN in 0.1 M potassium borate (pH 9.5), and 3 μL of 10 mM NDA in methanol were allowed to react for 15 min at room temperature immediately before 25 μL of the reaction was injected onto the column and eluted at 0.5mL/min. Elution conditions for δMA reactions were modified from a previously reported procedure.[41] Solvent A consisted of 5 mM ammonium acetate to which 20% methanol (v/v) had been added and solvent B was acetonitrile. The column was equilibrated in 20% solvent B prior to each 25 μL injection of derivatized sample. Elution conditions were 20% solvent B for 4 min, followed by a linear gradient of 20–65% solvent B over 4.5 min, 65–100% solvent B over 1.5 min, and 100% solvent B for 3 min, followed by a return to 20% solvent B over 2 min. Retention times: void volume, 2.3 min; δMA, 7.9 min; and L-phenylalanine, 8.6 min. For separation of amino acids in enzyme reaction mixtures containing L-canavanine, solvent A consisted of 10 mM ammonium acetate and solvent B was methanol. The column was equilibrated in 20% solvent B, prior to each 25 μL injection of derivatized sample. Elution conditions were 20% solvent B for 3 min, followed by a linear gradient of 20–100% solvent B over 12 min, and 100% B for 2 min, followed by a return to 20% solvent B for over 3 min. Retention times: void volume, 3.3 min; L-canavanine, 12.0 min; and L-phenylalanine, 13.3 min.

4.1.11. LCMS analysis of amino acids. Mass spectrometry data for NDA derivatives of amino acid products of enzyme reactions mixtures were obtained on an Agilent 1100 LCMS system. The same respective sets of elution conditions listed above for analytical HPLC were used to separate amino acids from the δMA and L-canavanine enzyme reaction mixtures.

4.1.12. Initial velocity measurements. ·NO synthesized by iNOS can be measured indirectly by observing the rapid oxidation of oxyhemoglobin by ·NO which produces methemoglobin and NO_3^-, as described previously.[42] All initial velocity measurements were recorded at 37 °C. The reference cuvette contained 6 μM oxyhemoglobin in 100 mM Hepes (pH 7.4). The sample cuvette contained 100 μM NADPH, 12 μM H_4B, 100 μM DTT, 15 nM iNOS, varying concentrations of L-arginine as specified in each experiment below, and 6 μM oxyhemoglobin in 100 mM Hepes (pH 7.4). The formation of methemoglobin was followed by monitoring the increase in absorbance at 401 nm.

4.1.13. δMA reversible inhibition kinetics. The oxyhemoglobin assay was utilized to determine the type of inhibition exhibited by δMA on iNOS. Assay reactions contained L-arginine (2, 4, 10, 60, 100, 500, or 1000 μM), δMA (0, 0.5, 0.75, 1.5, 2.0, and 2.25 mM), 100 μM NADPH, 12 μM H_4B, 100 μM DTT, and 15 nM iNOS in 100 mM Hepes (pH 7.4). A [δMA] versus (K/V) re-plot of data derived from Michaelis–Menten plots of iNOS inhibition by δMA versus L-arginine was constructed for determination of a K_i for δMA.

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