Supporting Information

Materials. Conventional organic solvents were purchased from Fisher Scientific. All other chemicals were purchased from Aldrich, unless otherwise stated.

Analytical methods. ^{1}H NMR spectra were recorded on a Varian VXR-300 spectrometer in the solvent indicated. Chemical shifts are reported as δ values in parts per million relative to TMS in $CDCl_3$ or to DSS in D_2O. Electrospray mass spectra were performed on a Micromass Quattro II spectrometer. Thin-layer chromatography was carried out on E. Merck precoated silica gel 60 F_{254} plates. Amino acids were visualized with a ninhydrin spray reagent or a UV/vis lamp. E. Merck silica gel 60 (230-400 mesh) was used for flash chromatography.

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-L-ornithine *tert*-butyl ester.** An ice-cold mixture of *N*$^{\alpha}$-Boc-*N*$^{\delta}$-Cbz-L-ornithine (2.03 g, 5.54 mmol) and dicyclohexylcarbodiimide (1.26 g, 6.1 mmol) in dry CH_2Cl_2 (40 mL) was added slowly to a cold solution of CH_2Cl_2 (20 mL) containing *tert*-butanol (1.64 g, 22 mmol) and 4-dimethylaminopyridine (50 mg). After being stirred for 2 h at 0 °C, the reaction was warmed up to room temperature and allowed to proceed for an additional 44 h. The mixture was filtered, and the filtrate was concentrated. To the resulting oil, cold anhydrous diethyl ether (30 mL) was added, and the precipitate was removed by filtration. After removal of the solvent, the oil was applied to a silica gel column, and *N*$^{\alpha}$-Boc-*N*$^{\delta}$-Cbz-L-ornithine *tert*-butyl ester was eluted with hexanes: EtOAc (10:3) as a colorless oil (0.53 g, 23% yield). This compound was dissolved in methanol (50 mL) to which the catalyst (10% Pd on charcoal) was added with caution. Hydrogen gas was applied to the sealed reaction system overnight. The solution was filtered through Celite, and the filtrate was concentrated to afford 0.38 g (96%) of product as a colorless oil; ^{1}H NMR ($CDCl_3$) δ 5.44 (brs, 1H), 4.12 (t, 1H), 3.07 (t, 2H), 1.60-1.88 (m, 4H), 1.46 (s, 9H), 1.42 (s, 9H).

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\delta}$-cyano-L-ornithine *tert*-butyl ester.** An ethereal solution (5 mL) containing cyanogen bromide (154 mg, 1.45 mmol) was added to an ice-cold solution of *N*$^{\alpha}$-

(*tert*-butoxycarbonyl)-L-ornithine *tert*-butyl ester (0.38 g, 1.32 mmol) and triethylamine (200 µL, 1.45 mmol) in anhydrous ether (10 mL) over a period of 10 min. After being stirred for 3 h at 0 °C, the mixture was filtered and concentrated. The product was purified by chromatography (hexanes: EtOAc, 2:1) as a yellowish oil (0.17 g; 41%). This compound was stored in an ethanol solution for stability reason; ^{1}H NMR ($CDCl_3$) δ 5.16 (brs, 1H), 4.59 (brs, 1H), 4.12 (t, 1H), 3.06 (m, 2H), 1.80 (m, 1H), 1.63 (m, 3H), 1.42 (s, 9H), 1.39 (s, 9H).

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\omega}$-(*tert*-butoxy)-L-arginine *tert*-butyl ester.** In a modification of the literature method,[1] *N*$^{\alpha}$-(*tert*-butoxycarbonyl)-*N*$^{\delta}$-cyano-L-ornithine *tert*-butyl ester (70 mg, 0.23 mmol) and *O*-(*tert*-butyl)hydroxylamine (100 mg, 0.75 mmol) were dissolved in anhydrous dioxane (5 mL) to which Na_2CO_3 (200 mg, 1.89 mmol) was added. After being refluxed for 5 h, the solvent was evaporated, and the residue was chromatographed on silica gel eluted with 10:1 EtOAC: (pyridine: acetic acid: water 10:3:5). About 56 mg (65%) of product was obtained as a yellow oil; ^{1}H NMR ($CDCl_3$) δ 5.15 (d, 1H), 4.18 (brs, 1H), 3.11 (m, 2H), 1.82 (m, 1H), 1.62(m, 3H), 1.46 (s, 9H), 1.42 (s, 9H), 1.25 (s, 9H).

***N*$^{\omega}$-*tert*-Butoxy-L-arginine.** The diprotected *N*$^{\alpha}$-(*tert*-butoxycarbonyl)-*N*$^{\omega}$-(*tert*-butoxy)-L-arginine *tert*-butyl ester (500 mg) was treated with 50% TFA in CH_2Cl_2 (5 mL) for 2 h at room temperature. The solvent was evaporated, and the resulting oil was applied to a Dowex 50 cation exchange (H^+ form) column. A gradient solution of NH_4OH (0.1 N-0.3 N) was used to elute the product from the column. The water was removed by evaporation and lyophilization. About 50 mg (18%) of product was obtained as a white foamy solid; ^{1}H NMR (D_2O) δ 4.07 (t, 1H), 3.28 (t, 2H), 1.95 (m, 2H), 1.72 (m, 2H), 1.23 (s, 9H); HRMS (M+1); HRMS: Calcd for $C_{10}H_{22}N_4O_3$ 247.1715, Found 247.1714. HPLC showed the product to be >99% pure.

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\omega}$-*O*-(3-Methyl-2-butenyl)hydroxy-L-arginine *tert*-butyl ester.** This compound (250 mg, 38%) was synthesized in a similar way as for *N*$^{\alpha}$-(*tert*-butoxycarbonyl)-*N*$^{\omega}$-(*tert*-butoxy)-L-arginine *tert*-butyl ester; ^{1}H NMR ($CDCl_3$) δ 5.41 (t, 1H),

5.20 (brs, 1H), 4.32 (d, 2H), 4.18 (m, 1H), 3.20 (m, 2H), 1.52-1.88 (m, 4H), 1.75 (s, 3H), 1.68 (s, 3H), 1.45 (s, 9H), 1.41 (s, 9H).

***N*$^{\omega}$-*O*-(3-Methyl-2-butenyl)hydroxy-L-arginine**. *N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\omega}$-*O*-(3-Methyl-2-butenyl)hydroxy-L-arginine *tert*-butyl ester (79 mg, 0.19 mmol) was dissolved in CH_2Cl_2 (407 μL), to which was added TFA (200 μL) and triethylsilane (90 μL). The reaction was allowed to stir at room temperature for 3 h. The solvent was evaporated under vacuum. The residue was dissolved in 5 mL of 0.1 M NH_4HCO_3 solution (pH 9.0, adjusted with NH_4OH). The aqueous solution was washed with EtOAc twice and concentrated to a smaller volume. Then it was loaded onto a Dowex 50W×8 cation exchange column (2 mL, NH_4^+ form) which was pre-equilibrated with 10 mM NH_4HCO_3 (pH 9.0) buffer. Three buffer solutions were used to elute the compound in gradients: 20 mM NH_4HCO_3 (pH 7.5), 50 mM NH_4HCO_3 (pH 8.0), 100 mM NH_4HCO_3 (pH 8.5). The fractions containing the desired compound (*n*-butanol:acetic acid:water 5:1:1, R_f 0.2) were pooled. The NH_4HCO_3 buffer was removed first by evaporation then lyophilization; ^{1}H NMR (D_2O) δ 5.38 (t, 1H), 4.24 (d, 2H), 3.63 (t, 1H), 3.05 (t, 2H), 1.82 (m, 2H), 1.74 (s, 3H), 1.68 (s, 3H), 1.59 (m, 2H); HRMS: Calcd for $C_{11}H_{22}N_4O_3$ 258.1692, Found 258.1691. HPLC showed the product to be >97% pure.

HPLC of *N*$^{\omega}$-*tert*-Butoxy-L-arginine (7) and *N*$^{\omega}$-*O*-(3-methyl-2-butenyl) hydroxyl-L-arginine (8) (Figures 2 and 3). A solution of **7** (or **8**, 6 mM, 20 μL) was derivatized with *o*-phthalaldehyde reagent (40 μL, OPA)[2] and injected onto a Hypersil ODS C_{18} column (Agilent, 5 μm, 4.0 x 250 mm), eluting at 1 mL/min. A Beckman System Gold 125P solvent module was used to control the gradient elution. The mobile phase was a gradient from 100% buffer A (50 mM NaOAc, pH 5.9) and 0% buffer B (80% of methanol, 20% of buffer A) to 0% buffer A and 100% buffer B over 30 min and then to100% buffer A and 0% buffer B over 15 min. Sample elution was detected by absorbance at 340 nm. The stability of compound **7** (or **8**) in the enzyme reaction medium was checked using the same RP-HPLC system. The incubation mixture

(without iNOS) containing compound **7** (or **8)** (0.91 mM), NADPH (0.56 mM), tetrahydrobiopterin (15 μM), and dithiothreitol (100 μM), were diluted to a total volume of 112 μL with HEPES buffer (100 mM, pH 7.5). It was incubated for 10 min at 30 °C, OPA (200 μL) was added, and it was injected onto the HPLC column and analyzed as described above.

LC-ES Mass Spectra of OPA-derivatized 7 and 8 (Figures 4 and 5). OPA-derivatized **7** (or **8**) was injected onto a Hypersil BDS C_{18} column (Hewlett Packard, 5 μm, 2.0 x 250 mm), and eluted using a HPLC (Hewlett Packard Series 1100) at a rate of 0.15 mL/min. The mobile phase was a gradient from 90% buffer A (95% 0.1 M NH_4OAc, pH 7.2, 5% methanol) and 10% solvent B (methanol) to 0% buffer A and 100% solvent B over 30 min and then to 90% buffer A and 10% solvent B over 10 min. The column outlet was attached to a Micromass Quattro II mass spectrometer using positive mode ionization. Source parameters: capillary voltage 3.18 kV; counter electrode 0.45 kV; cone voltage 32 V; temperature 140 °C; unit resolution scan 100-500 at 1s/scan.

Production of L-Citrulline during incubation of iNOS with 7 and 8 (Figure 6 and 7). Samples of iNOS (222 μg in 200 μL total volume) were incubated with compound **7** (or **8**) for 8 h at 30 °C: **7** (or **8**, 6mM), NADPH (0.44 mM), tetrahydrobiopterin (20 μM), and dithiothreitol (100 μM), were diluted to a total volume of 200 μL with HEPES buffer (100 mM, pH 7.5). The enzyme reaction was stopped by addition of acetone (200 μL) at 0 °C; sonication (5 min) and centrifugation (5 min) were used to precipitate the protein. OPA reagent (40 μL) was added to this incubation mixture (20 μL). After 10 min LC-MS ES was carried out with the OPA-derivatized sample (10 μL) as described above.

Substrate Activities of Compound 7 and 8:

A. Nitric Oxide (NO) formation (Figure 1 in paper). The generation of nitric oxide by iNOS was measured by the rapid oxidation of oxyHb to metHb by nitric oxide. The assay mixture,

which contained iNOS (0.3 μM), **7** (or **8**, 0.1-20 mM), NADPH (0.15 mM), tetrahydrobiopterin (10 μM), dithiothreitol (100 μM), and oxyhemoglobin (7.5 μM), was diluted to a total volume of 600 μL with HEPES buffer (100 mM, pH 7.5). The relative rate of nitric oxide formation was determined by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm for 1 min on a Perkin-Elmer Lamda 10 UV/vis spectrophotometer. All assays were performed at 30 °C.

B. Citrulline Formation (Figure 1 in supporting information). A preincubation mixture containing iNOS (0.37 nmol, 55.6 μg), **7** (or **8**, 0.1-20 mM), NADPH (0.15 mM), tetrahydrobiopterin (10 μM), and dithiothreitol (100 μM), diluted to a total volume of 200 μL with HEPES buffer (100 mM, pH 7.5), was incubated at 30 °C for 2 min. The reaction was stopped by addition of acetone (200 μL) at 0 °C; sonication (5 min) and centrifugation (5 min) were used to precipitate the protein. This incubation mixture (20 μL) was filtered and the compounds were OPA(40 μL)-derivatized. A sample (10 μL) was injected onto a Hypersil ODS C_{18} column (Agilent, 5 μm, 4.0 x 250 mm), eluting at 1 mL/min. A Beckman System Gold 125P solvent module was used to control the gradient elution. The mobile phase was a gradient from 90% buffer A (95% 0.1 M NaOAc, pH 7.2, 5 % methanol) and 10% solvent B (methanol) to 0% buffer A and 100% solvent B over 30 min and then to 90% buffer A and 10% solvent B over 10 min. The amount of citrulline formation at each concentration of **7** (or **8**) was quantified by comparing the areas of the peaks with an OPA-derivatized L-citrulline (Sigma) standard curve.

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[1] Wallace, G. C.; Fukuto, J. M. *J. Med. Chem.* **1991**, *34*, 1746-1748.
[2] Roth, M. *Anal. Chem*. **1971***, 43*, 880-882.