Supporting Information

Methylated N^{ω}-Hydroxy-L-arginine Analogs as Mechanistic Probes for the Second Step of the Nitric Oxide Synthase-Catalyzed Reaction

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Contents

Page S2 **Synthesis**

Page S8 **Michaelis-Menten plots for substrates**

Page S10 **K_s determination**

Page S13 **K_i determination**

Page S14 **^{14}C experiments**

Page S17 **Crystal structure information**

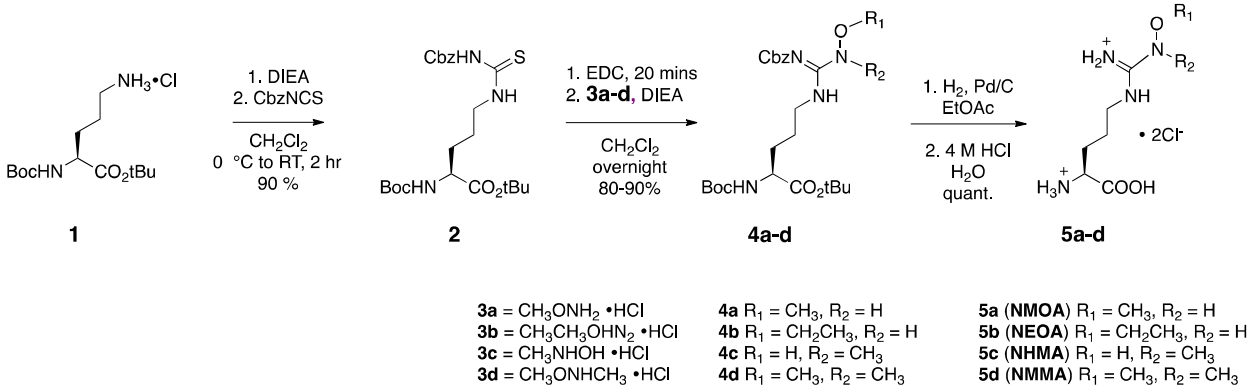
Page S19 **NMR spectra of final products**

Page S26 **References**

Synthesis

NMOA, NEOA, NHMA and NMMA were synthesized through nucleophilic addition of appropriate amines (**3a-d**) to a protected ornithine thiourea (**2**, Scheme S1).[1,2] MHA was synthesized according to Clement and coworkers.[3]

Scheme S1. Synthesis of NHA Analogs NMOA, NEOA, NHMA, NMMA.



General Synthetic Materials and Methods

CbzNCS was prepared according to the procedure of Martin *et al*. [1] *N*-Boc-L-ornithine *tert*-butylester hydrochloride (**1**) was purchased from Bachem, methoxylamine-[^{14}C]-hydrochloride (34 mCi/mmol) was purchased from American Radiolabeled Chemicals. All other reagents were commercially available (Sigma Aldrich) and were used without further purification. Analytical thin layer chromatography was visualized by ultra violet light and/or ninhydrin stain. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O on a 500 or 125 MHz spectrometer, respectively. Chemical shifts are reported as δ values in parts per million with the $CDCl_3$ and D_2O peaks set at 7.26 and 4.80 ppm, respectively for ^{1}H spectra. ^{13}C spectra were referenced to $CDCl_3$ at δ 77.0 ppm High-resolution mass spectra were obtained using an Agilent 6210 ToF-LC/MS mass spectrometer; analyses were performed in the positive ion ESI mode.

N^{ω}-Benzyloxycarbonyl-N^{α}-*tert*-butyloxycarbonyl-L-thiocitrulline *tert*-Butyl Ester (2)

N^{α}-*tert*-Butyloxycarbonyl-L-ornithine *tert*-butyl ester hydrochloride (**1**, 400 mg 1.4 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL). DIEA (0.28 mL, 1.6 mmol) was added to obtain the free base. The flask was cooled to 0 °C and 4.0 mL of a 0.5 M solution of CbzNCS in CH_2Cl_2 was added dropwise over 30 minutes. The mixture was further stirred for 2 hours while warming to room temperature. The reaction was washed with 1 % HCl (25 mL), H_2O (25 mL) and brine (25 mL). The organic phase was dried over Na_2SO_4 and concentrated. The crude material was purified by column chromatography (hexanes:EtOAc 4:1, R_f = 0.45) to obtain a yellow oil (540 mg, 90 %). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.45 [s, 9 H, $C(CH_3)_3$], 1.47 [s, 9 H, $C(CH_3)_3$], 1.70 -1.85 (m, 4 H, β,γ-CH_2), 3.66 (m, 2 H, NCH_2) , 4.22 (m, 1 H, α-CH), 5.10 (m, 1 H, NH), 5.12 (s, 2 H, C*H*$_2$Ph), 7.38 (m, 5 H, ArH), 8.11 (br s, 1 H, NHCbz), 9.67 (br s, 1 H NHBoc). ^{13}C NMR (500 MHz, $CDCl_3$): δ = 24.2 (γ-CH_2), 28.1 [C(*C*H_3) $_3$], 28.4 [C(*C*H_3) $_3$], 30.3 (β-CH_2), 45.2 (NCH_2), 53.6 (α-CH_2), 68.2 (*C*H_2Ph), 79.8 [*C*$(CH_3)_3$], 82.2 [*C*$(CH_3)_3$], 128.4, 128.6, 128.8 (ArCH), 134.6 (ArC), 152.5 (thiourea-C), 155.4 (CO-Boc), 171.5 (*C*O_2-*t*-Bu), 179.0 (CO-Cbz).

General Method for Preparation of Protected NHA Analogs (4a-d)[1,2]

To a solution of EDC (1.5 eqv) and thiourea (**2**) (0.5 mmol) in anhydrous CH_2Cl_2 (5 mL), was added a mixture of DIEA (1.5 eqv) and the amine hydrochloride (**3a-d,** 3.0 eqv) in 5 mL of CH_2Cl_2. The reactions were stirred overnight at room temperature. The organic phase was washed with 1 % HCl (10 mL), H_2O (10 mL) and brine (10 mL). The organic phase was dried over Na_2SO_4 and concentrated. The resulting oils were purified by column chromatography.

N^{α}-*tert*-butylcarbonyl-N^{ω}-Benzyloxycarbonyl-$N^{\omega'}$-methoxy-L-arginine *tert*-Butyl Ester (4a)

Amine (**3a**) = methoxyamine hydrochloride; eluent: hexanes:EtOAc (4:1); R_f = 0.3; yield 93%, colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.46 [s, 9H, $C(CH_3)_3$], 1.48 [s, 9H, $C(CH_3)_3$], 1.72 -1.87 (m, 4H, β,γ-CH_2), 3.13 (m, 2H, NCH_2), 3.68 (s, 3H, OCH_3) 4.21 (m, 1H, α-CH), 5.16 (m, 1H, NH), 5.17 (s, 2H, CH_2Ph), 6.29 (m, 1H NH), 7.40 (m, 5H, ArH), 7.95 (br s, 1 H NHBoc). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 24.9 (γ-CH_2), 28.1 [C(CH_3)$_3$], 28.4 [C(CH_3)$_3$], 30.2 (β-CH_2), 40.6 (NCH_2), 53.8 (α-CH_2), 61.4 (OCH_3), 67.7 (OCH_2Ph), 79.6 [$C(CH_3)_3$], 81.9 [$C(CH_3)_3$], 128.4, 128.6, 128.7 (ArCH), 135.1 (ArC), 148.2, 152.9 (guanidine-C, CO-Cbz), 155.4 (CO-Boc), 171.8 (CO_2-*t*-Bu).

N^{α}-*tert*-butylcarbonyl-N^{ω}-Benzyloxycarbonyl-$N^{\omega'}$-ethoxy-L-arginine *tert*-Butyl Ester (4b)

Amine (**3b**) = ethoxyamine hydrochloride; eluent: hexanes:EtOAc (4:1); R_f = 0.27; yield 85%, colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.47 [s, 9H, $C(CH_3)_3$], 1.49 [s, 9H, $C(CH_3)_3$], 1.70 -1.90 (m, 4H, β,γ-CH_2), 3.12 (m, 2H, NCH_2), 3.27 (t, *J* = 6 Hz, 2H, OCH_2), 3.98 (q, *J* = 6.5 Hz, 3H, OCH_2CH_3), 4.21 (m, 1H, α-CH), 5.16 (m, 1H, NH), 5.17 (s, 2H, CH_2Ph), 6.29 (m, 1H NH), 7.40 (m, 5H, ArH), 7.95 (br s, 1H, NHBoc). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 12.6 (OCH_2CH_3), 23.8 (γ-CH_2), 28.1 [C(CH_3)$_3$], 28.4 [C(CH_3)$_3$], 30.3 (β-CH_2), 40.3 (NCH_2), 53.8 (α-CH_2), 67.8 (OCH_2Ph), 72.9 (OCH_2), 79.6 [$C(CH_3)_3$], 81.9 [$C(CH_3)_3$], 128.4, 128.6, 128.7 (ArCH), 135.1 (ArC), 148.3, 153.2 (guanidine-C, CO-Cbz), 156.7 (CO-Boc), 171.6 (CO_2-*t*-Bu).

N^{α}-*tert*-butylcarbonyl-N^{ω}-Benzyloxycarbonyl-$N^{\omega'}$-hydroxy-$N^{\omega'}$-methyl-L-arginine *tert*-Butyl Ester (4c)

Amine (**3c**) = *N*-methylhydroxylamine hydrochloride; eluent: hexanes:EtOAc (4:1); R_f = 0.22; yield 80%, colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.42 [s, 9H, $C(CH_3)_3$], 1.46 [s, 9H,

$C(CH_3)_3$], 1.65 -1.80 (m, 4H, β,γ-CH_2), 3.45 (m, 2H, NCH_2), 3.48 (s, 3H, NCH_3) 4.11 (m, 1H, α-CH), 5.20 (m, 1H, NH), 5.25 (s, 2H, CH_2Ph), 7.40 (m, 5H, ArH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 25.1 (γ-CH_2), 28.1 [C(CH_3) $_3$], 28.4 [C(CH_3) $_3$], 30.2 (β-CH_2), 40.7 (NCH_2), 42.1 (NCH_3), 53.4 (α-CH_2), 67.8 (OCH_2Ph), 80.2 [$C(CH_3)_3$], 82.3 [$C(CH_3)_3$], 128.4, 128.6, 128.7 (ArCH), 135.1 (ArC), 148.0, 152.8 (guanidine-C, CO-Cbz), 156.4 (CO-Boc), 171.8 (CO_2-*t*-Bu).

N^{α}-*tert*-butylcarbonyl-N^{ω}-Benzyloxycarbonyl-$N^{\omega'}$-methoxy-$N^{\omega'}$-methyl-L-arginine *tert*-Butyl Ester (4d)

Amine (**3d**) = *N,O*-dimethylhydroxylamine hydrochloride; eluent: 5% MeOH in CH_2Cl_2; R_f = 0.28; yield 95%, colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.44 [s, 9H, $C(CH_3)_3$], 1.46 [s, 9H, $C(CH_3)_3$], 1.65 -1.80 (m, 4H, β,γ-CH_2), 3.12 (s, 3H, NCH_3) 3.30 (m, 2H, NCH_2), 3.66 (s, 3H, OCH_3) 4.16 (m, 1H, α-CH), 5.14 (m, 1H, NH), 5.10 (s, 2H, CH_2Ph), 7.32 (m, 5H, ArH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 25.3 (γ-CH_2), 28.0 [C(CH_3) $_3$], 28.3 [C(CH_3) $_3$], 30.3 (β-CH_2), 38.5 (NCH_2), 42.6 (NCH_3), 53.5 (α-CH_2), 60.8 (OCH_3), 66.8 (OCH_2Ph), 79.7 [$C(CH_3)_3$], 82.1 [$C(CH_3)_3$], 127.6, 127.8, 128.2 (ArCH), 137.6 (ArC), 152.8, 155.4 (guanidine-C, CO-Cbz), 160.4 (CO-Boc), 171.6 (CO_2-*t*-Bu).

General Procedure for NHA Analogs (5a-d)

To protected NHA analogs (**4a-d**) dissolved in 20 mL MeOH was added Pd/C (10 mol %). The flask was sealed then purged and flushed three times with H_2. The reaction was stirred under H_2 atmosphere for two hours. The reaction was then filtered through a pad of Celite, rinsing thoroughly with MeOH. Solvent was removed in vacuo and the intermediates were redissolved

in 4 M aqueous HCl. Intermediates were stirred for two hours at room temperature and solvent and HCl was removed in vacuo.

N^{ω}-Methoxy-L-arginine (5a)

^{1}H NMR (500 MHz, D_2O): δ = 1.65 -1.75 (m, 2H, γ-CH_2), 1.85-1.95 (m, 2H, β-CH_2), 3.25 (t, *J* = 7 Hz, 2H, NCH_2), 3.69 (s, 2H, OCH_3), 3.99 (t, *J* = 6 Hz, 1 H, α-CH).

^{13}C NMR (125 MHz, D_2O): δ = 23.6 (γ-CH_2), 26.9 (β-CH_2), 40.2 (NCH_2), 52.8 (α-CH_2), 64.5 (OCH_3), 157.4 (guanidine-C), 172.3 (COOH).

HRMS (ESI): *m/z* $[M+H]^+$ calculated 205.1295, found 205.1299.

N^{ω}-Ethoxy-L-arginine (5b)

^{1}H NMR (500 MHz, D_2O): δ = 1.20 (t, *J* = 7 Hz, 3H, CH_3), 1.60 -1.75 (m, 2H, γ-CH_2), 1.85-1.95 (m, 2H, β-CH_2), 3.25 (t, *J* = 7 Hz, 2H, NCH_2), 3.85 (t, *J* = 6 Hz, 1H, α-CH), 3.92 (q, *J* = 7 Hz, 2H, OCH_2).

^{13}C NMR (125 MHz, D_2O): δ = 12.4 (CH_3), 23.6 (γ-CH_2), 27.1 (β-CH_2), 40.2 (NCH_2), 53.4 (α-CH_2), 72.9 (OCH_2), 157.6 (guanidine-C), 173.2 (COOH).

HRMS (ESI): *m/z* $[M+H]^+$ calculated 219.1452, found 219.1461.

N^{ω}-Hydroxy-N^{ω}-methyl-L-arginine (5c)

^{1}H NMR (500 MHz, D_2O): δ = 1.65 -1.95 (m, 4H, β,γ-CH_2), 3.23 (s, 3H, NCH_3), 3.27 (t, *J* = 7 Hz, 2H, NCH_2), 4.03 (t, *J* = 6.5 Hz, 1H, α-CH).

^{13}C NMR (125 MHz, D_2O): δ = 23.6 (γ-CH_2), 26.7 (β-CH_2), 39.4 (NCH_3), 40.7 (NCH_2), 52.6 (α-CH_2), 157.7 (guanidine-C), 171.9 (COOH).

HRMS (ESI): *m/z* $[M+H]^+$ calculated 205.1295, found 205.1297.

N^{ω}-Methoxy-N^{ω}-methyl-L-arginine (5d)

^{1}H NMR (500 MHz, D_2O): δ = 1.65 -1.85 (m, 4H, β,γ-CH_2), 3.25 (s, 3H, NCH_3), 3.30 (t, *J* = 6 Hz, 2H, NCH_2), 3.67 (s, 3H, OCH_3), 4.98 (t, 1H, α-CH).

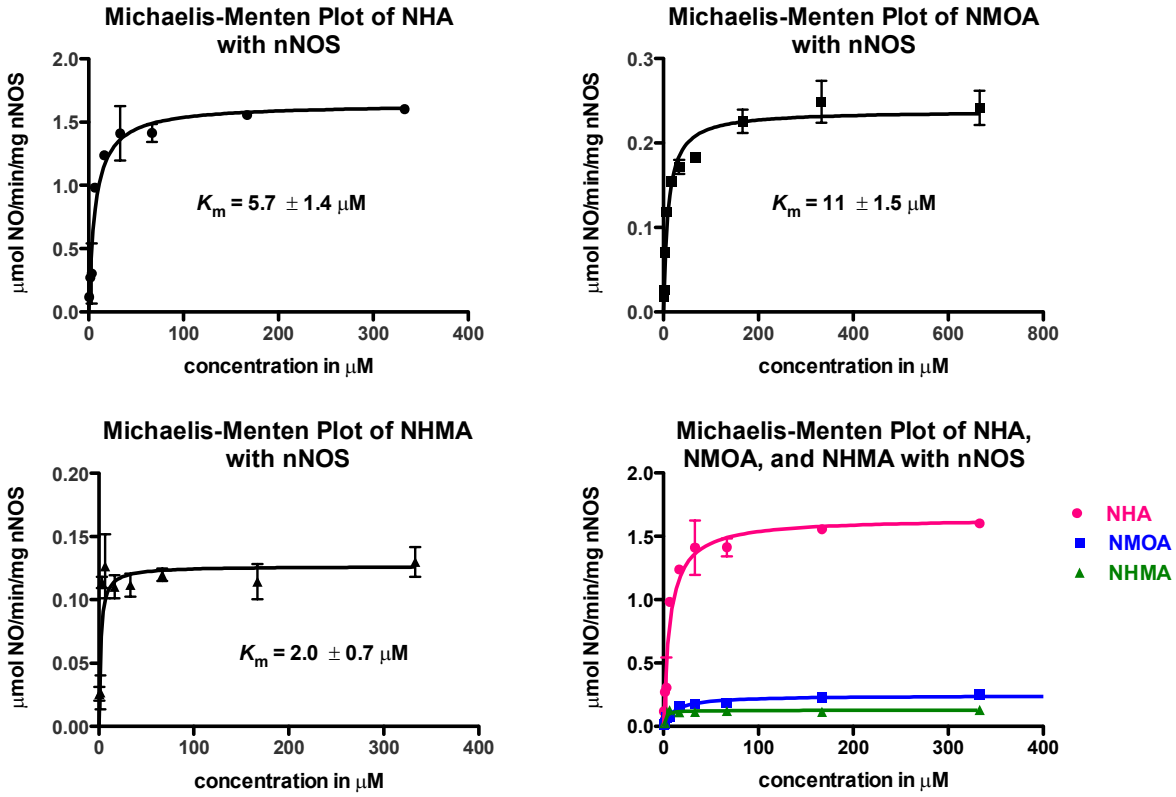
^{13}C NMR (125 MHz, D_2O): δ = 26.4 (γ-CH_2), 29.9 (β-CH_2), 33.1 (NCH_3), 42.9 (NCH_2), 55.8 (α-CH_2), 67.5 (OCH_3), 159.8 (guanidine-C), 171.5 (COOH).

HRMS (ESI): *m/z* $[M+H]^+$ calculated 219.1452, found 219.1453.

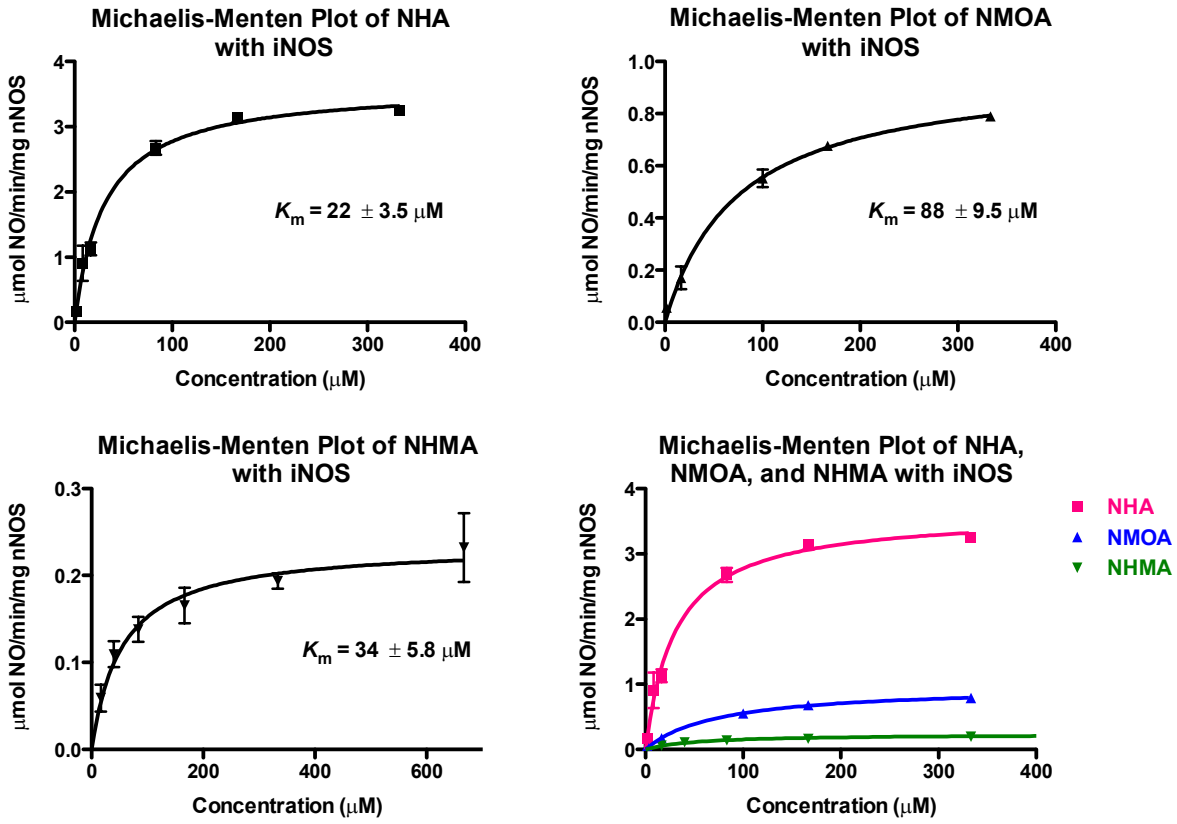
Determination of Michaelis-Menten kinetics for NHA, NMOA and NHMA

Figure S1. Michaelis-Menten curves for NHA, NMOA, and NHMA with (A) nNOS and (B) iNOS. NO production was measured using the hemoglobin capture assay. Each concentration was tested in triplicate.

A.



B.


Michaelis-Menten Plot of NHA with iNOS
μmol NO/min/mg nNOS
Concentration (μM)
K_m = 22 ± 3.5 μM
Michaelis-Menten Plot of NMOA with iNOS
μmol NO/min/mg nNOS
Concentration (μM)
K_m = 88 ± 9.5 μM
Michaelis-Menten Plot of NHMA with iNOS
μmol NO/min/mg nNOS
Concentration (μM)
K_m = 34 ± 5.8 μM
Michaelis-Menten Plot of NHA, NMOA, and NHMA with iNOS
μmol NO/min/mg nNOS
Concentration (μM)
NHA
NMOA
NHMA

Spectral Binding Assays: K_s determination

Figure S2. iNOS-imidazole. **A.** iNOS with imidazole titrations, difference spectra. **B.** Michaelis-Menten plots of difference spectra of iNOS with imidazole titrations, K_s = 120 - 150 μM, over multiple experiments.

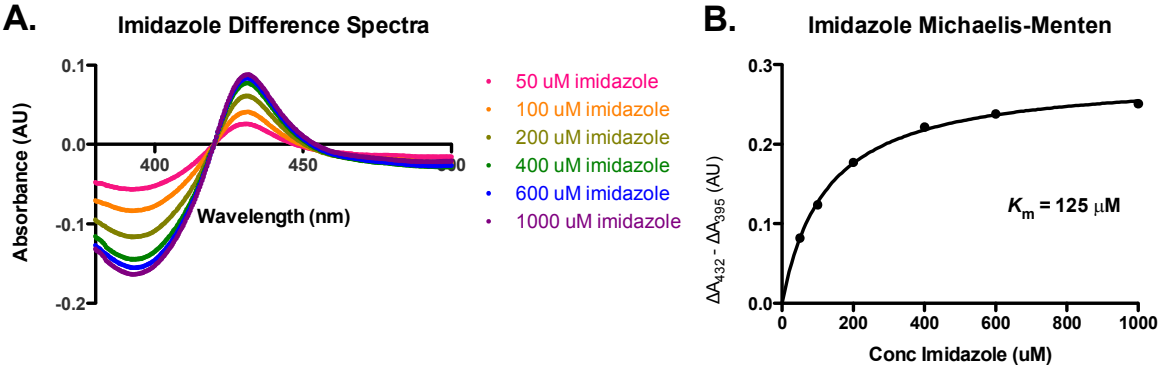


Figure S3. iNOS-NHA. **A.** iNOS plus 300 μM imidazole with NHA titrations, difference spectra. **B.** Michaelis-Menten plots of difference spectra of iNOS plus 300 μM imidazole with NHA titrations, $K_{s\ apparent}$ = 82 μM, $K_{s\ actual}$ = 29 μM.

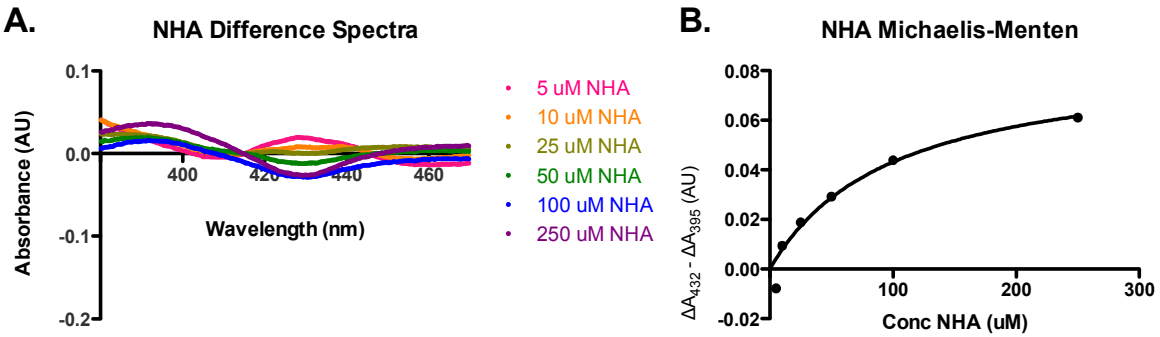


Figure S4. iNOS- NMOA. **A.** iNOS plus 300 μM imidazole with NMOA titrations, difference spectra. **B.** Michaelis-Menten plots of difference spectra of iNOS plus 300 μM imidazole with NMOA titrations, $K_{s\ apparent}$ = 350 μM, $K_{s\ actual}$ = 122 μM.

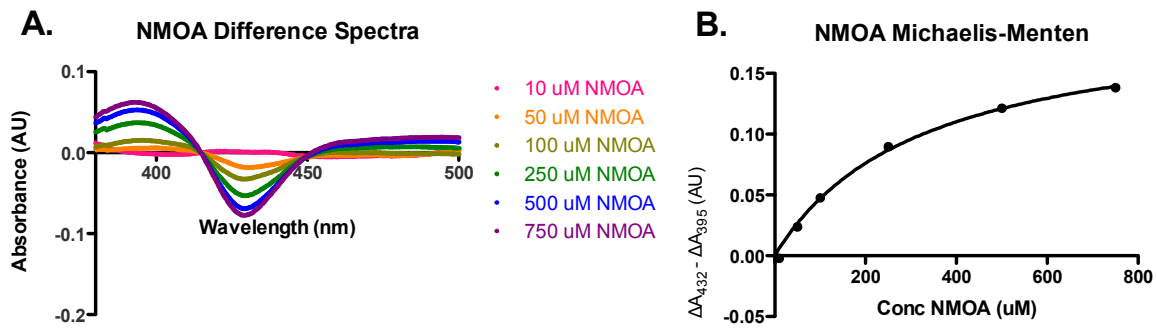


Figure S5. iNOS-NHMA. **A.** iNOS plus 300 μM imidazole with NHMA titrations, difference spectra. **B.** Michaelis-Menten plots of difference spectra of iNOS plus 300 μM imidazole with NHMA titrations, $K_{s\ apparent}$ = 95 μM, $K_{s\ actual}$ = 34 μM.

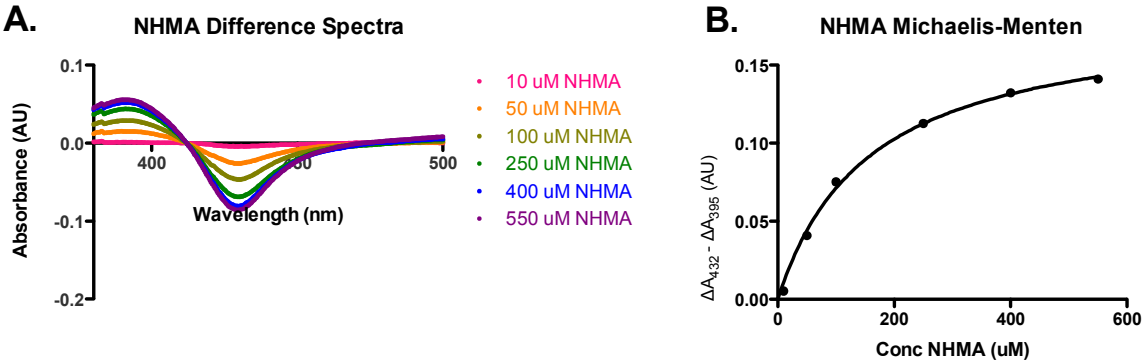


Figure S6. iNOS-NMMA. **A.** iNOS plus 200 μM imidazole with NMMA titrations, difference spectra. **B.** Michaelis-Menten plots of difference spectra of iNOS plus 200 μM imidazole with NMMA titrations, $K_{s\ apparent}$ = 187 μM, $K_{s\ actual}$ = 70 μM.

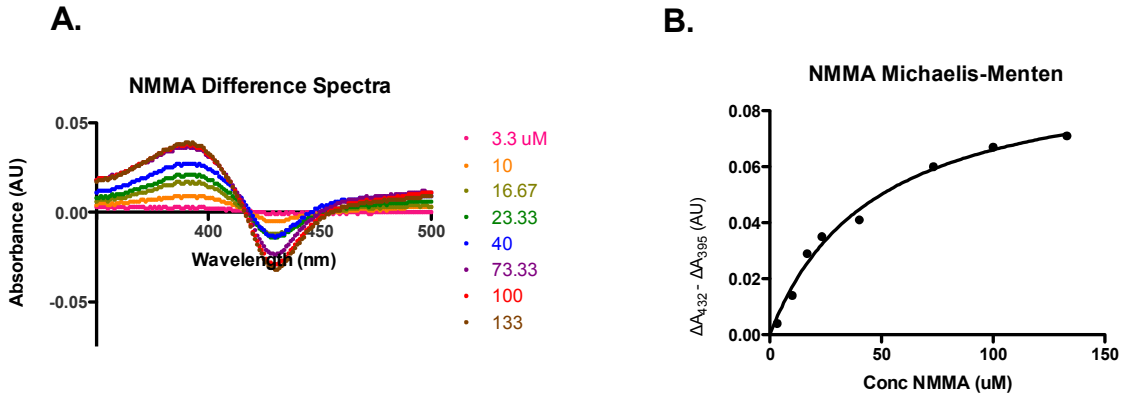


Figure S7. iNOS MHA **A.** iNOS plus 100 μM imidazole with MHA titrations, difference spectra. **B.** Michaelis-Menten plots of difference spectra of iNOS plus 100 μM imidazole with MHA titrations, K_s predicted to be greater than 10 mM.

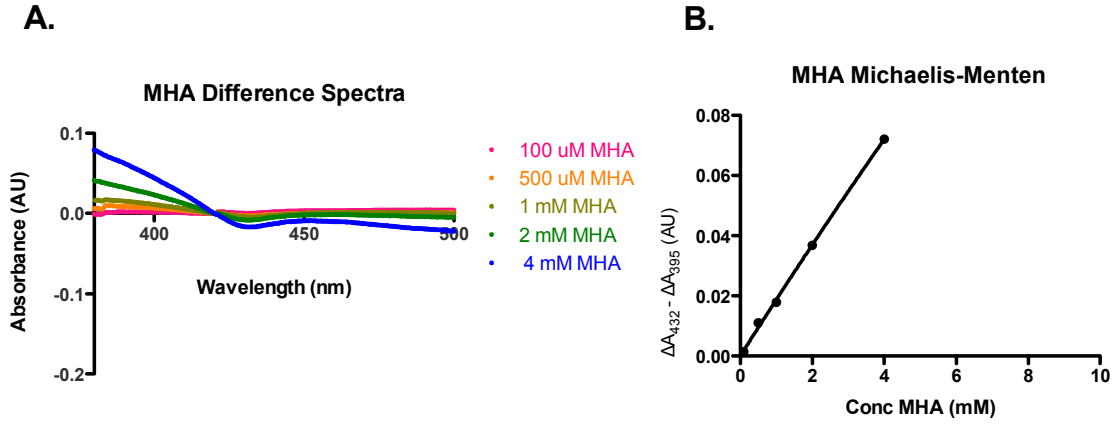
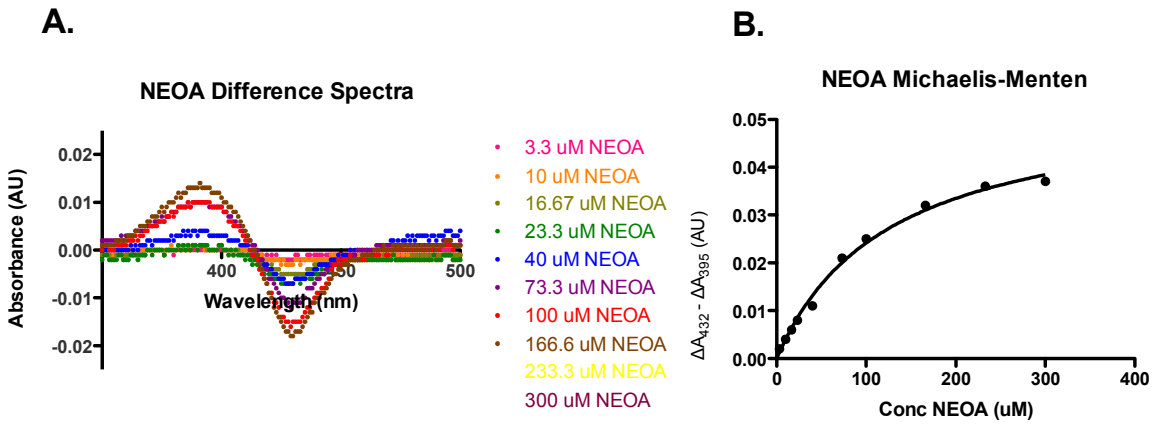


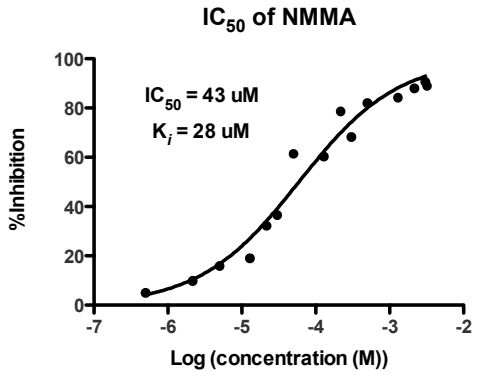
Figure S8. iNOS-NEOA. **A.** iNOS plus 200 μM imidazole with NEOA titrations, difference spectra **B.** Michaelis-Menten plots of difference spectra of iNOS plus 200 μM imidazole with NEOA titrations, $K_{s\ apparent}$ = 320 μM, $K_{s\ actual}$ = 120 μM.



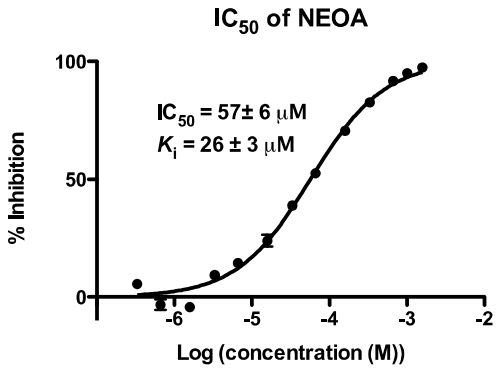
Determination of Inhibition Constants (K_i)

Figure S9. Dose-response curves for (A) NMMA and (B) NEOA with iNOS. NO production was measured using the hemoglobin capture assay; from the IC_{50} value, K_i was calculated using the Cheng-Prushoff relationship, $K_i = IC_{50}/(1 + [S]/K_m)$, where K_m of 8.3 μM was used for murine iNOS.[4] (C) Inactivation evaluation. NAHA = N^{ω}-allyl-N^{ω}-hydroxy-L-arginine, a known NOS inactivator [5] was used as a control. No significant difference was observed between control and NMOA, NHMA, NEOA, NMMA, or MHA at 12 and 24 h.

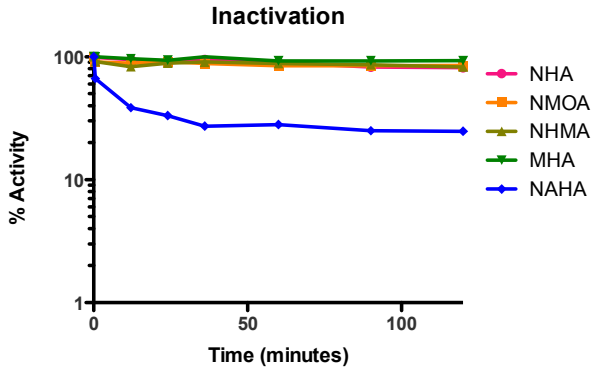
A.



B.



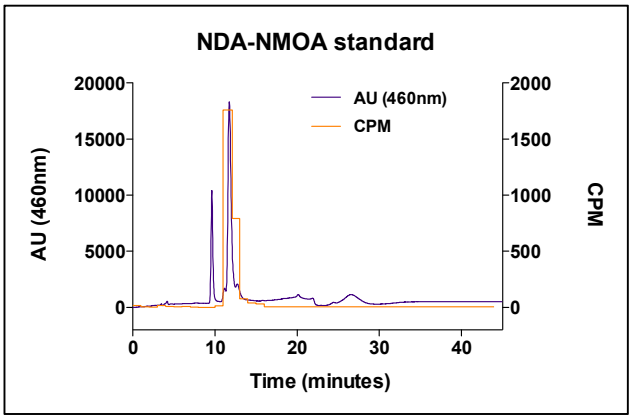
C.



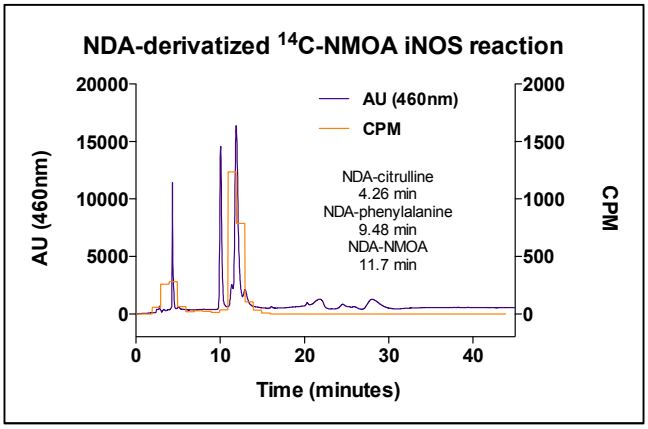
^{14}C-NMOA experiments

Figure S10. HPLC Chromatographs of iNOS-[^{14}C]-NMOA Reactions Analyzed at 460 nm and by ^{14}C-Scintillation Counting.

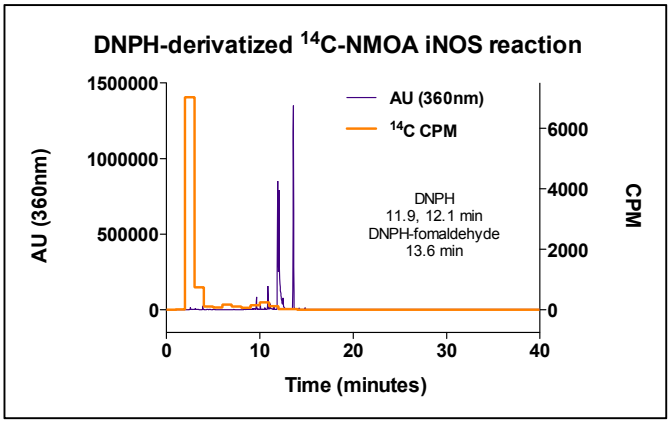
(A) A control NDA-derivatization of [^{14}C]-NMOA stock solution was separated by HPLC with detection at 460 nm and one minute fractions were collected and ^{14}C counted. These spectra show that all ^{14}C elutes at 11.7 minutes, indicating there are no ^{14}C-containing contaminants eluting elsewhere from the column.



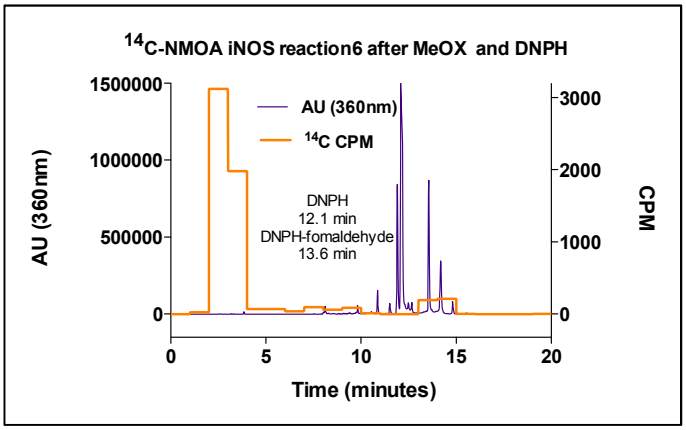
(B) NDA-derivatization of the [^{14}C]-NMOA iNOS reaction was separated by HPLC, fraction-collected and scintillation-counted. These spectra show early fractions (2-4 minutes) contain ^{14}C. This low retention time, of what is presumably a one-carbon metabolite from the iNOS-[^{14}C]-NMOA reaction, appropriately suggests it is higly polar. Remaining ^{14}C elutes at 11.7 minutes indicating it is unreacted NMOA. Turnover for this specific reaction analyzed was measured to be 19% by absorbance and 16% by ^{14}C detection. The peak at 4.2 minutes that absorbs at 460 nm is NDA-citrulline, confirmed by retention time as well as by mass spectrometry (see below and in main text).



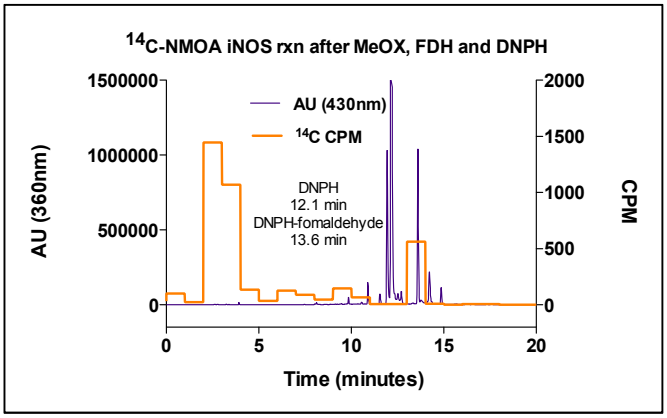
(C) The [^{14}C]-NMOA-iNOS reaction DNPH-derivatized and analyzed by HPLC separation with 360 nm detection and fraction collected for scintillation counting. In this case, nearly all ^{14}C elutes very early. No significant ^{14}C eluted at 13.6 minutes, the retention time DNPH-formaldehyde standards, indicating the one carbon metabolite of these reactions is not formaldehyde. There is, surprisingly, a large DNPH-formaldehyde peak that is detected at 360 nm that elutes at 13.6 minutes, as well as some unreacted DNPH eluting at 11.9 and 12.1 minutes (as expected). Upon further examination of all iNOS-substrate reactions, all reactions, even the substrate-free iNOS reaction, has a significant level (between 600-1000 μM) of background DNPH-formaldehyde detected by absorbance at 360 nm. The source of this background DNPH-formaldehyde has not been found but seems to be from the NOS enzyme itself. The mass of this peak from iNOS reactions has been confirmed to be DNPH-formaldehyde by mass spectrometry analysis (see below).



(D) DNPH-derivatized [^{14}C]-NMOA-iNOS reactions incubated with MeOX. A DNPH-formaldehyde peak is present at 13.6 minutes by spectral detection at 360 nm, consisting of background formaldehyde as well as some [^{14}C]-formaldehyde. The ^{14}C eluting between 13 and 15 minutes corresponds to 4.6 % of the total ^{14}C, but the [^{14}C]-NMOA-iNOS reaction had 25 % turnover by measurement of NDA-citrulline. Incomplete conversion to [^{14}C]-formaldehyde is expected because MeOX also catalyzes formaldehyde into formate, which would have a very low retention time.



(E) DNPH-derivatized [^{14}C]-NMOA-iNOS reactions incubated with MeOX and FDH. From a total of 25% turnover (by measurement of NDA-citrulline) ^{14}C can be accounted for as DNPH-formaldehyde (7.8 %), CO_2 (12 %) and the missing 5.2% remains undetectable as formate.



Crystal Structures

Figure S11. The Fo – Fc omit electron density map contoured at 2.5 σ for the substrate analogues (and active site water) bound in rat nNOS: (A) NMOA; (B) NEOA; (C) NHMA; (D) NMMA; (E) MHA.

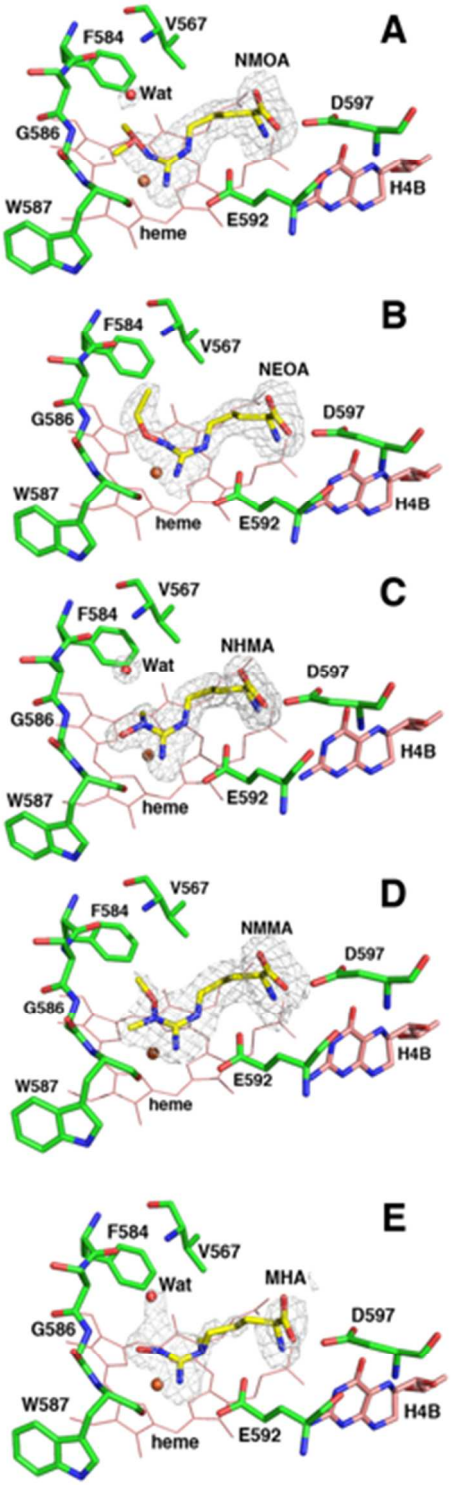


Figure S12. Crystal structure of tBuOArg in the nNOS active site. As expected, for bearing such a bulky substituent, the active site water is displaced.

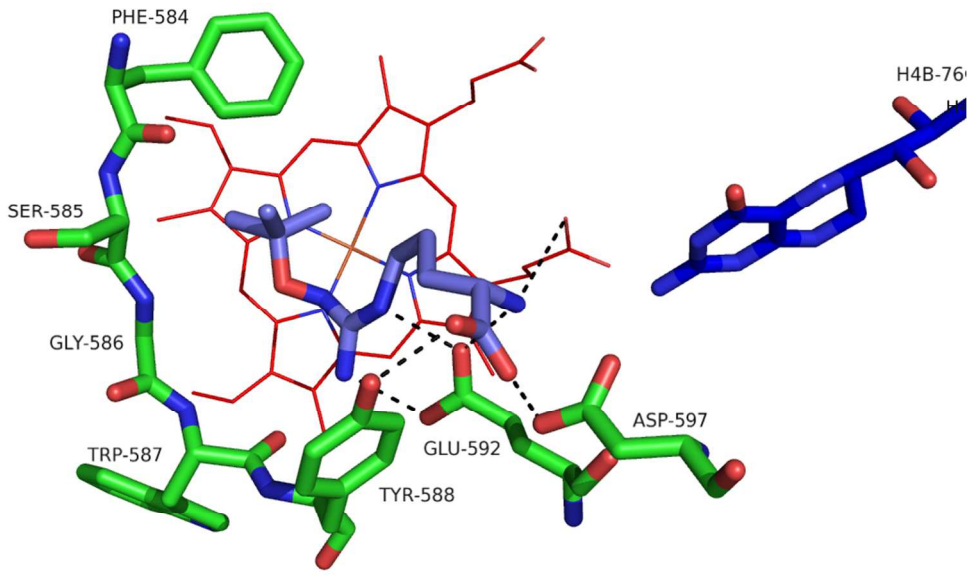
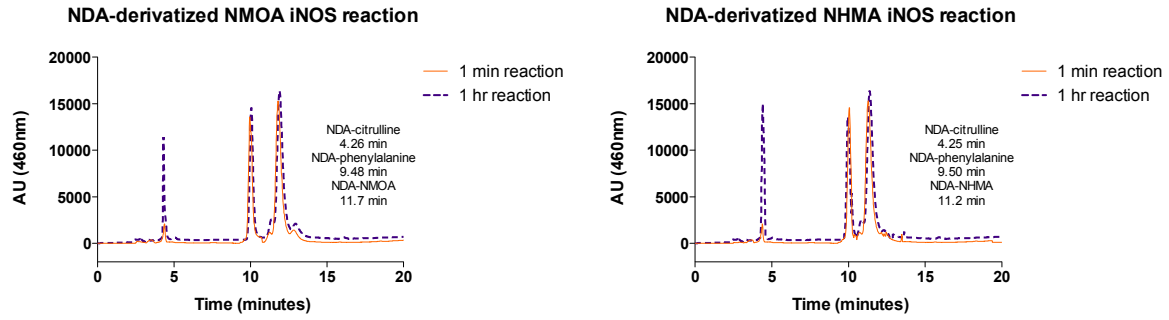
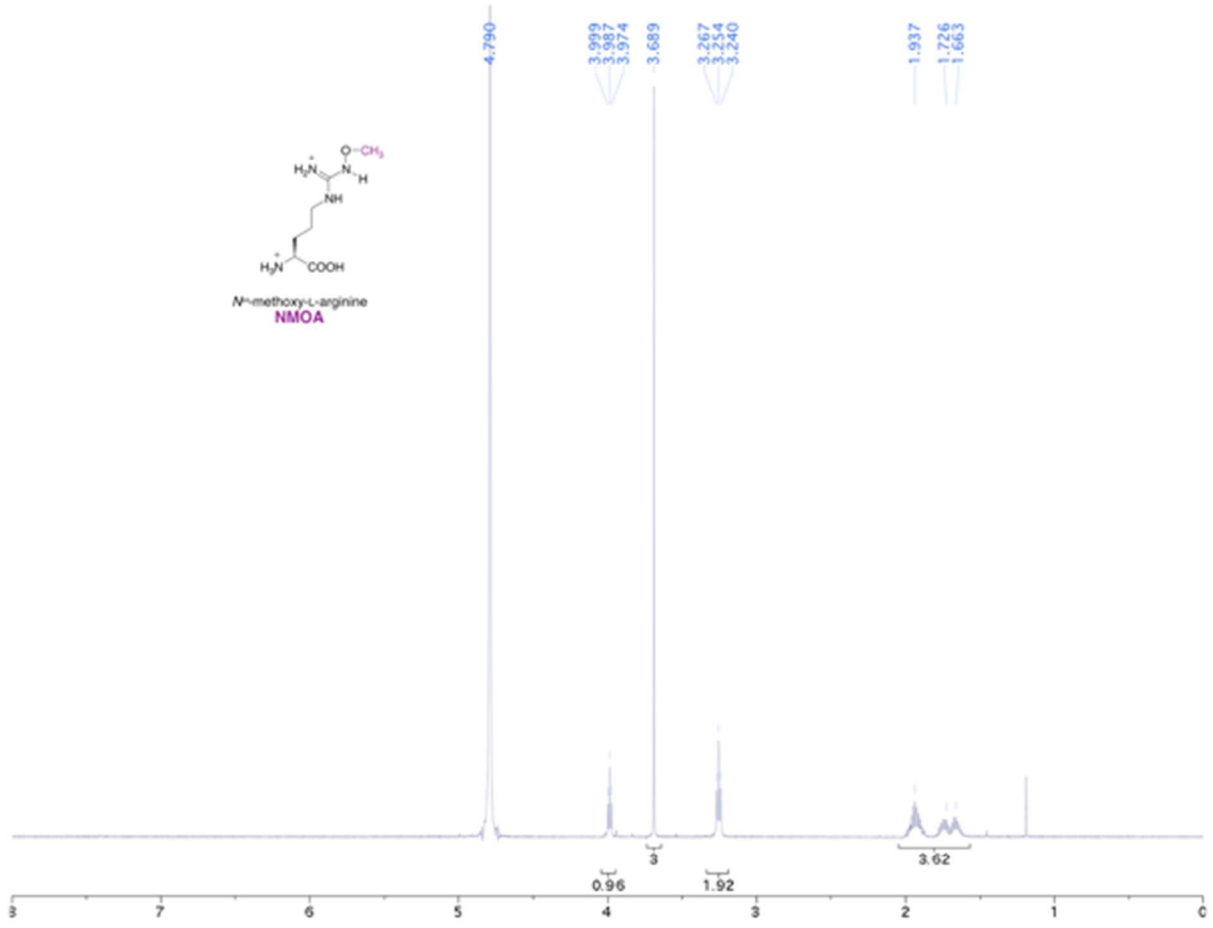
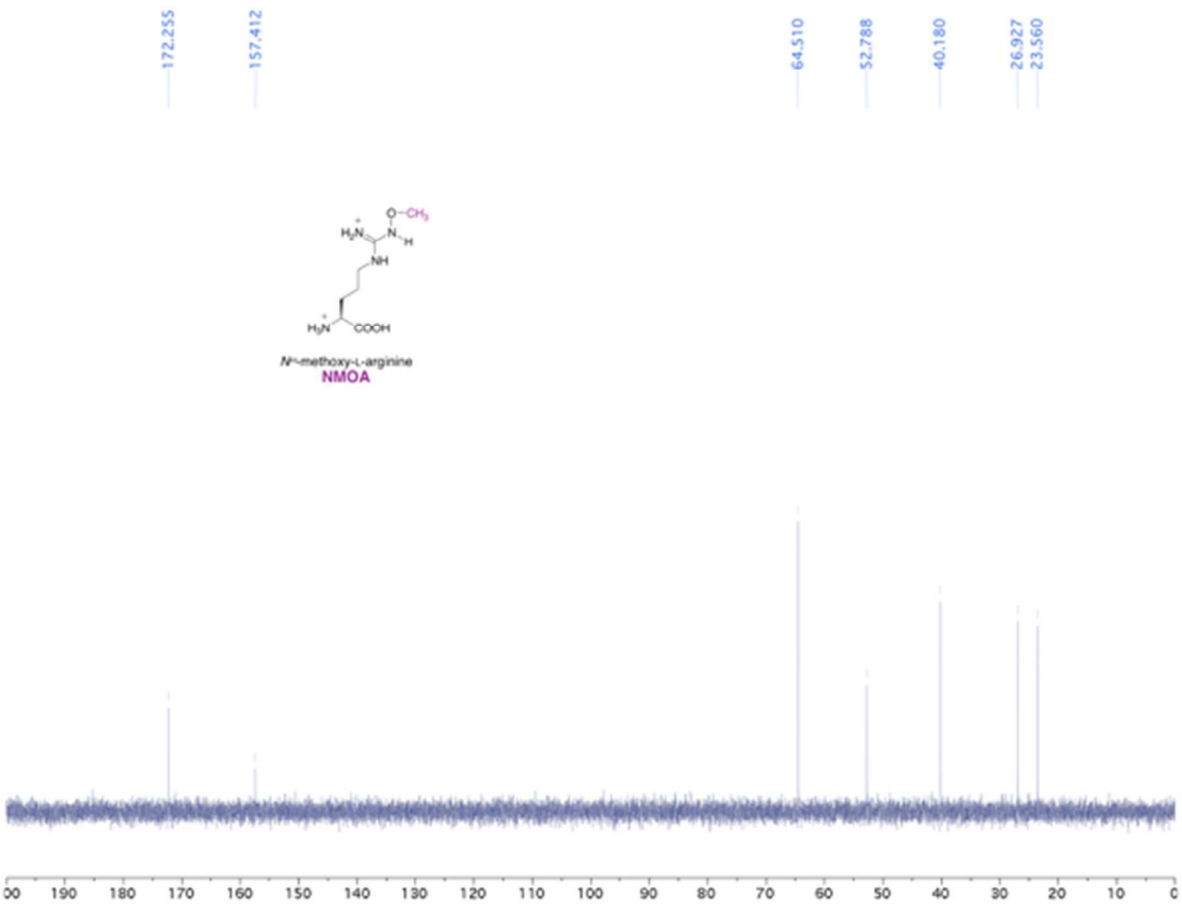


Figure S13. HPLC chromatographs of NDA derivatized products of iNOS-NMOA (A) and iNOS-NHMA (B) reactions (citrulline), including an initial timepoint (1 min) and a timepoint after 1 h of reaction time. Phenylalanine is used as an internal standard.

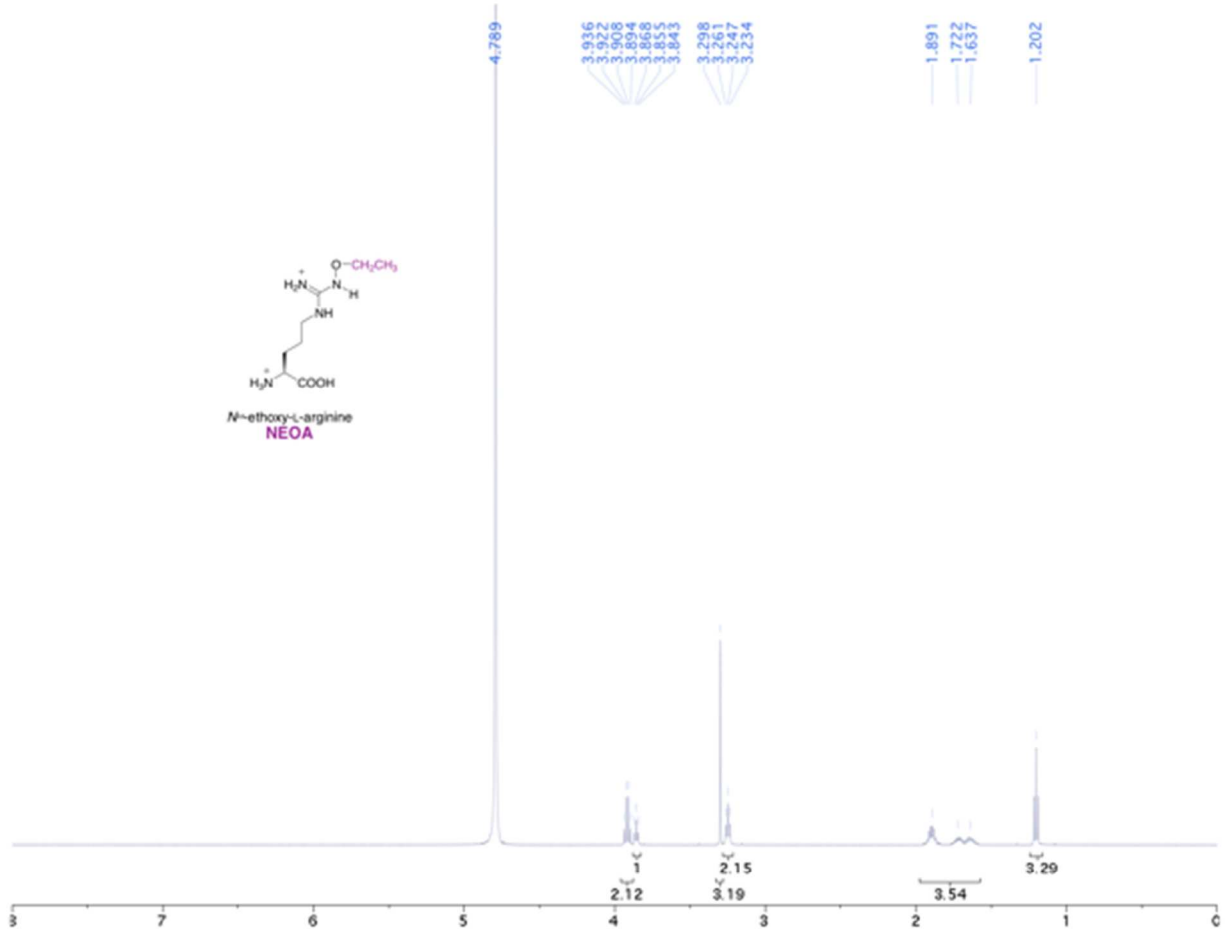


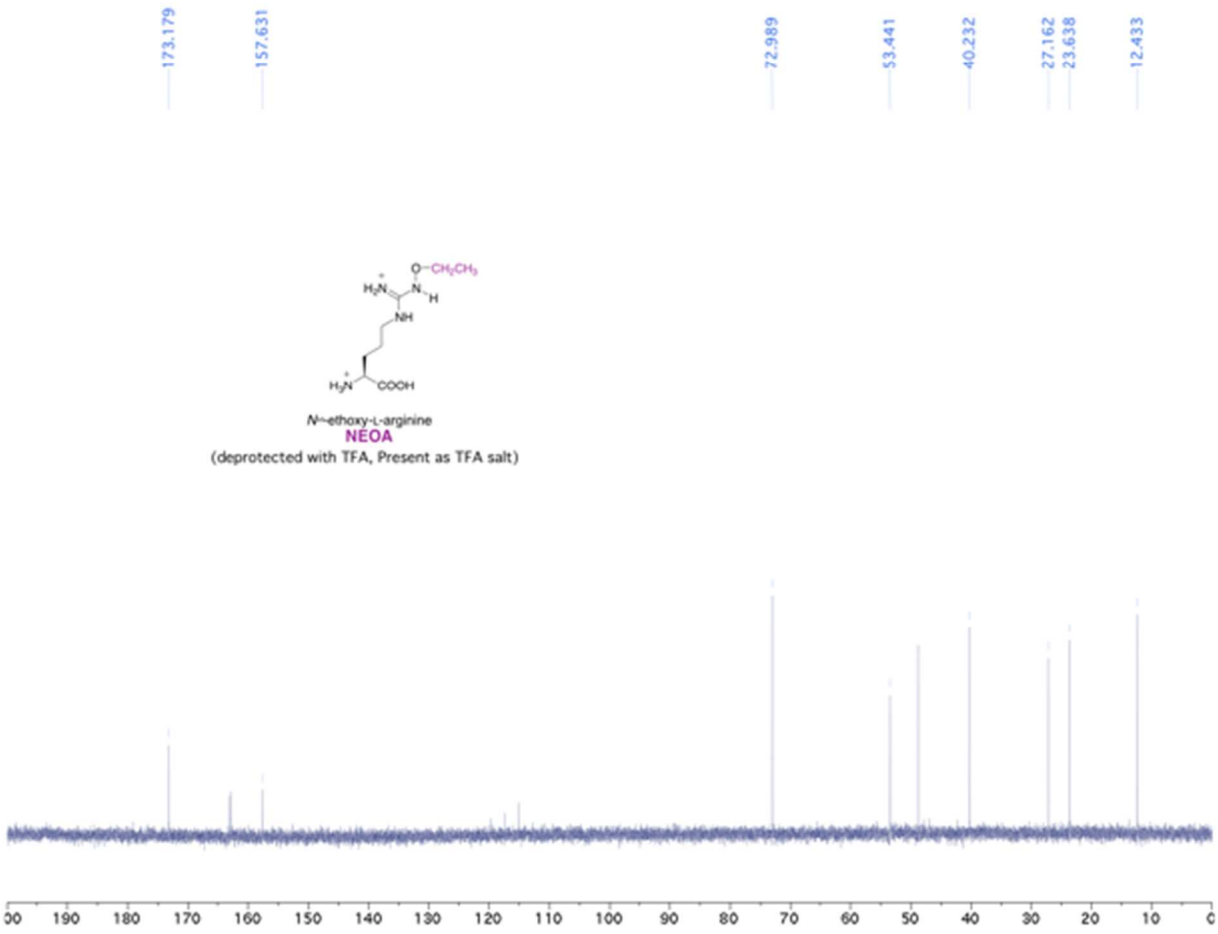
NMR Spectra of Final Products
N^{ω}-Methoxy-L-arginine (5a), D_2O



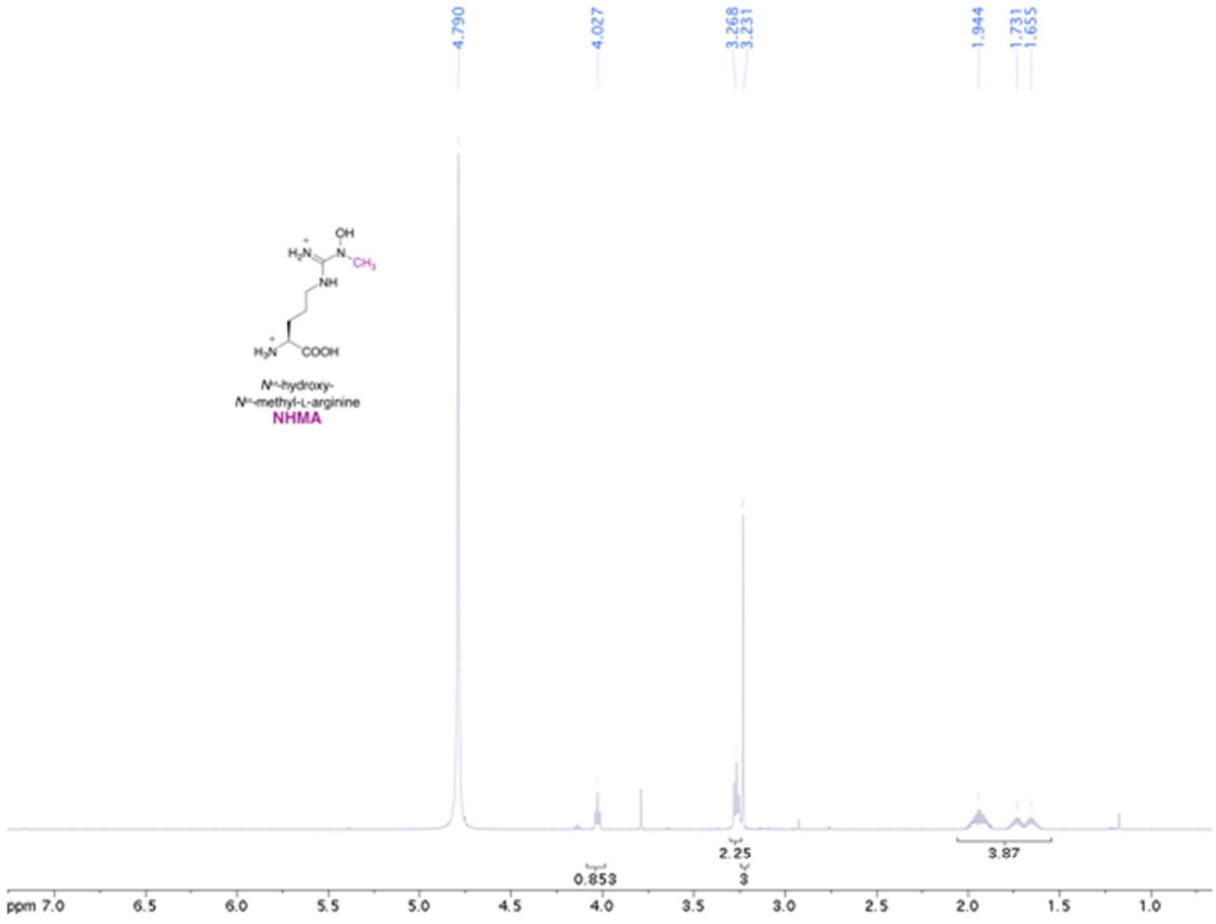

172.255
157.412
64.510
52.788
40.180
26.927
23.560
N^{ω}-methoxy-L-arginine
NMOA
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10

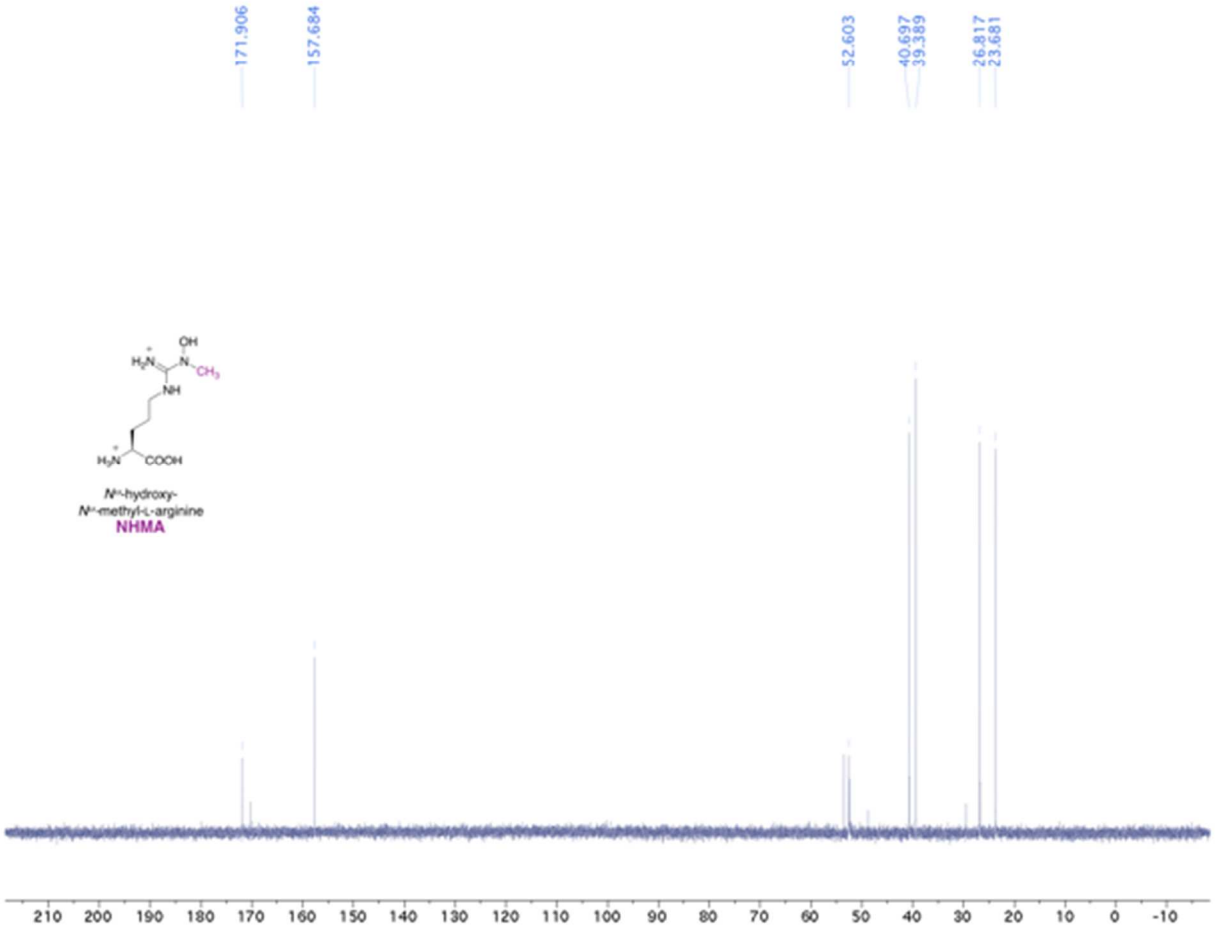
N^{ω}-Ethoxy-L-arginine (5b), D_2O



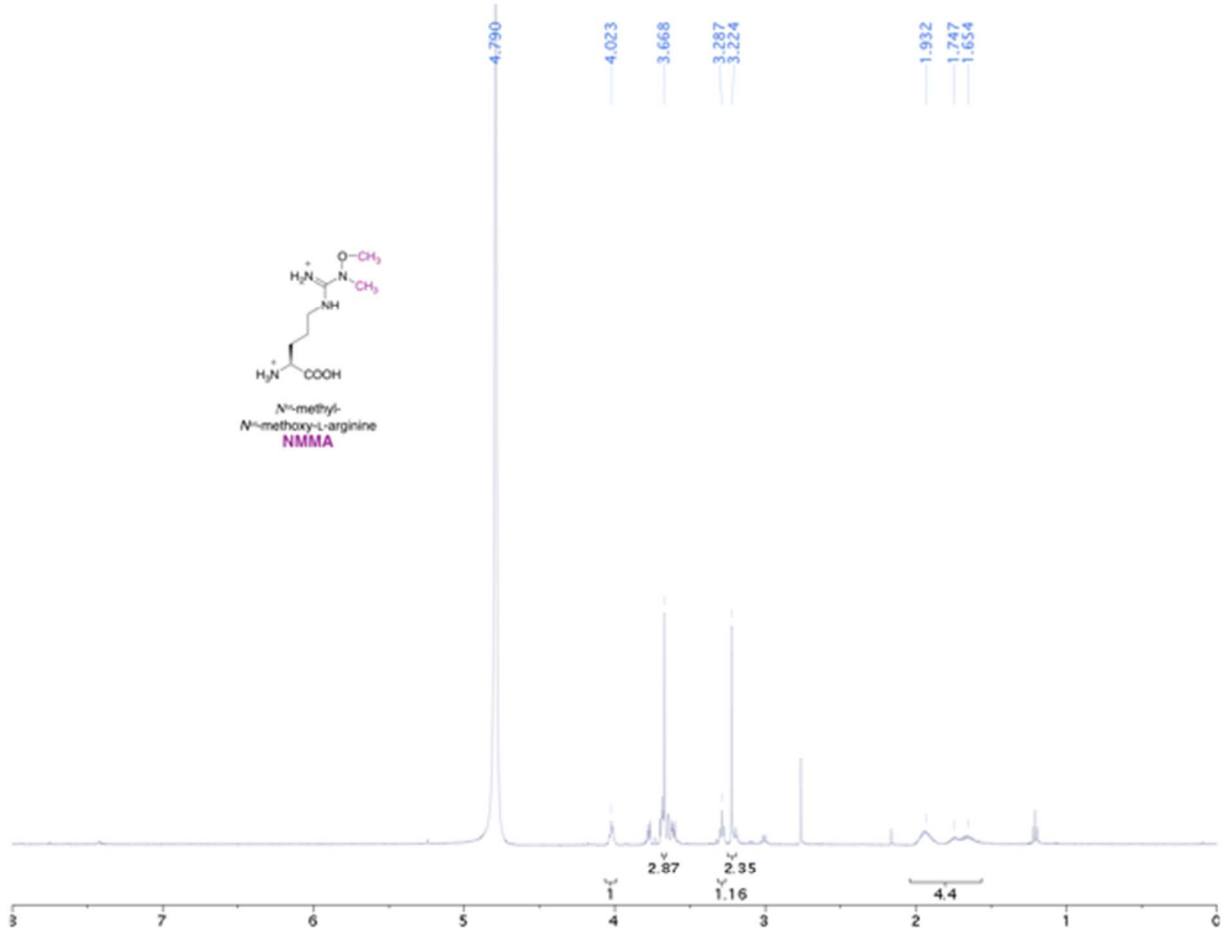

173.179
157.631
72.989
53.441
40.232
27.162
23.638
12.433
O–CH2CH3
H2N
N
H
NH
H2N
COOH
N-ethoxy-L-arginine
NEOA
(deprotected with TFA, Present as TFA salt)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10

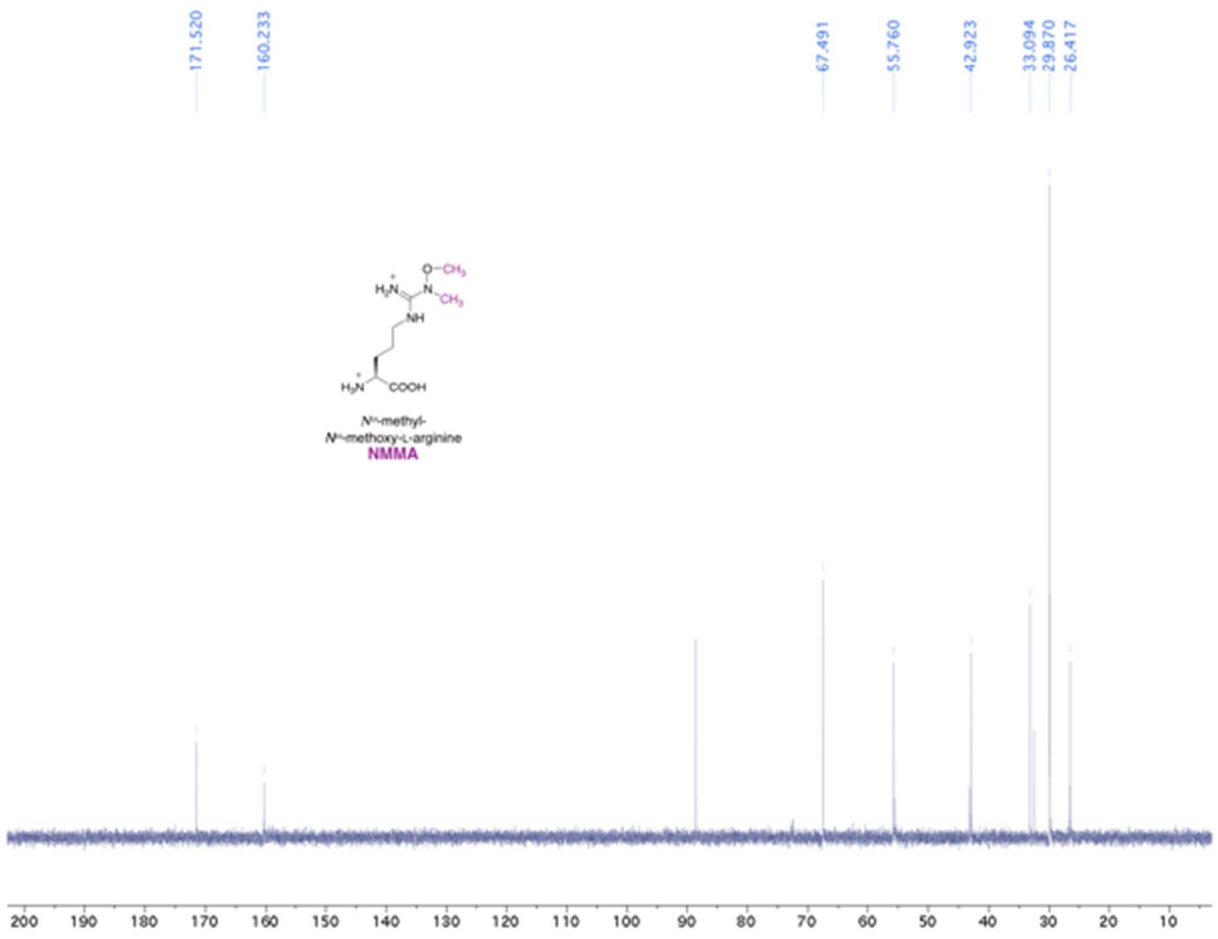
N^{ω}-Hydroxy-N^{ω}-methyl-L-arginine (5c), D_2O




171.906
157.684
52.603
40.697
39.389
26.817
23.681
OH
H_2N
N
CH_3
NH
H_2N
COOH
N^{ω}-hydroxy-
N^{ω}-methyl-L-arginine
NHMA
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10

N^{ω}-Methoxy-N^{ω}-methyl-L-arginine (5d), D_2O





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