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# Synthesis and Evaluation of *trans* 3,4-Cyclopropyl L-Arginine Analogues as Isoform Selective Inhibitors of Nitric Oxide Synthase

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**Abstract**—Four optically pure conformationally restricted L-arginine analogues *syn*- **1** and *anti*- **2** *trans*-3,4-cyclopropyl L-arginine, and *syn*- **3** and *anti-trans*-3,4-cyclopropyl *N*-(1-iminoethyl) L-ornithine **4** were synthesized. These compounds were tested as potential inhibitors against the three isoforms of nitric oxide synthase (NOS). Compound **1** was determined to be a poor substrate of NOS, while compound **2** was determined to be a poor mixed type inhibitor and did not exhibit any isoform selectivity. S*yn*- **3** and *anti-trans*-3,4-cyclopropyl *N*-(1-iminoethyl) L-ornithine **4** were found to be competitive inhibitors of NOS. These compounds were time dependent inhibitors of inducible NOS (iNOS), but not of neuronal NOS (nNOS) or endothelial NOS (eNOS). Compound **3** was 10- to 100-fold more potent an inhibitor than **4**, exhibited a 5-fold increase in nNOS/iNOS and eNOS/iNOS selectivity over **4**, and displayed tight binding characteristics against iNOS. These results indicate that the relative configuration of the cyclopropyl ring in the L-arginine analogues significantly affects their inhibitory potential and NOS isoform selectivity.


## Introduction

Nitric oxide (NO) is involved in the regulation of diverse physiological processes such as smooth muscle contractility, platelet reactivity, central and peripheral neurotransmission.[1,2] At high concentrations NO functions as a defensive cytotoxin against tumor cells and pathogens.[3] While crucial for many physiological functions, inappropriate excess of this mediator has been associated with a number of pathologies including diabetes, rheumatoid arthritis, carcinogenesis, septic shock, multiple sclerosis, transplant rejection and stroke.[4]

NO is produced in different mammalian tissues by three distinct nitric oxide synthase isoforms (NOS; EC 1.14.13.39). These enzymes are homodimeric proteins that catalyze a five-electron oxidation of L-arginine to yield NO and L-citrulline. Two of the three NOS isoforms, endothelial (eNOS) and neuronal (nNOS), are constitutively expressed enzymes, while the third, inducible NOS (iNOS) is transcriptionally regulated. Once expressed, iNOS is active, essentially unregulated, and produces NO at potentially cytotoxic levels, resulting in local tissue damage. The selective inhibition of iNOS has thus been an area of active research.

A number of conformationally restricted α-amino acid NOS inhibitors have been reported in the literature,[5–8] though none has shown dramatically improved potency or selectivity. These efforts have involved the introduction of unsaturation or aromaticity into the amino acid backbone, or a saturated ring within the amino acid or guanidinium functionality. The introduction of a cyclopropyl ring into the amino acid backbone has not been examined.

Some cyclopropyl amino acids have been synthesized or isolated from natural sources, but not 3,4-cyclopropyl arginine derivatives.[9–11] A series of 3,4-cyclopropyl analogues of arginine were designed in order to evaluate what effect the subtle difference in cyclopropane conformation might have on inhibitory activity and selectivity. The cyclopropyl methylene may introduce some steric bulk that could interact favorably and differentially

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with the binding pockets of the NOS isoforms. Such selectivity has been observed with cyclopropyl glutamate isomers with NMDA and neuronal glutamate receptors.[12,13]

In order to evaluate their potency and selectivity for human NOS isoform inhibition, the optically pure *syn* **1** and *anti* **2** isomers of *trans*-3,4-cyclopropyl L-arginine and *syn* **3** and *anti-trans*-3,4-cyclopropyl *N*-(1-iminoethyl) L-ornithine **4** were synthesized.

## Results and Discussion

Both guanidine and acetamidine side chains were desired for direct comparison, in order assess the effect on potency and selectivity of the amino acid backbone structure versus the side-chain reactive functionality.

Our group has recently reported the stereoselective synthesis of L-cyclopropyl glutamates **6**/**7**[14] as well as the *syn* and *anti* isomers of *trans*-cyclopropyl arginine **1**/**2**.[15] These were accessed via the 1,3-dipolar addition of diazomethane to a chiral dehydroglutamate derivative **5**, followed by photolysis of the resultant pyrazoline (Scheme 1). This approach exploits the 4-methyl-2,6,7-trioxabicyclo-[2.2.2] *ortho* (OBO) ester function, used to protect the carboxylic acid of amino acids. The OBO protection scheme prevents epimerization at the α-carbon of serine allowing the direct oxidation of the hydroxyl side chain to the corresponding aldehyde without loss of chirality. This serine aldehyde equivalent has been used to obtain a variety of non-natural α-amino acids.[16–21]

This methodology was applied to the synthesis of *syn*- and *anti-trans*-3,4-cyclopropyl *N*-(1-iminoethyl) L-ornithine **3** and **4**, via the Cbz protected *E*-3,4-L-di-dehydrogluatamate OBO derivative **5**. Cyclopropanation and purification of each diastereomer **6** and **7** by crystallization, followed by DIBAL reduction of each to **8** and **9** was performed as previously described.[14,15]

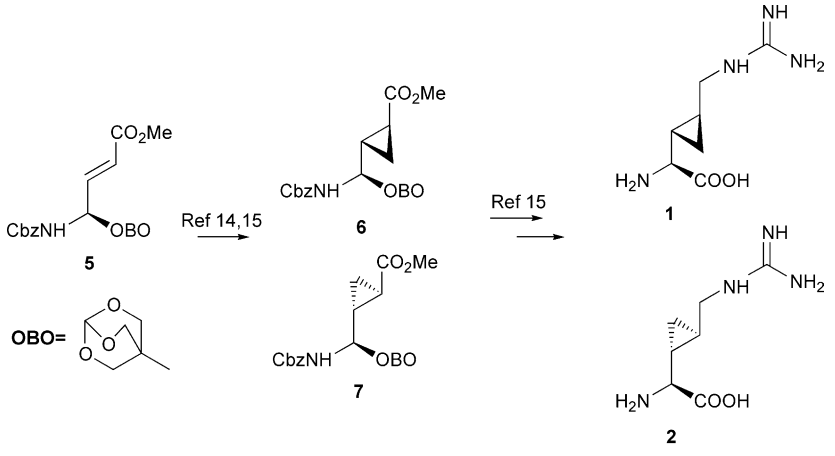
Conversion to the azides **10**, **11** was achieved by Mitsunobu reaction of the corresponding alcohol with hydrazoic acid (Scheme 2). Isolated yields following flash chromatography were excellent (85–93%). Staudinger reduction of each azide to the corresponding amine **12**/**13** also proceeded in excellent yields, providing the protected diamino acid substrates.

Shearer et al.[22] has reported the highly reactive *S*-2-naphthylmethyl thioacetamidate hydrobromide **14** as an acetamidation reagent. Simply stirring each of the protected cyclopropyl amines **12**/**13** with **14** produces the corresponding acetamidine HBr salt that was isolated by extraction. The aqueous phase was lyophilized and the product immediately deprotected under standard conditions. Acid hydrolysis of the OBO ester was performed using very mild and brief conditions to minimize decomposition to an acetamide by-product. Cation exchange chromatography provided the free *syn* **3** and *anti* 3,4-cyclopropyl-*N*-iminoethyl-L-ornithine **4** that were assayed for activity against the NOS isoforms, along with the arginine analogues **1** and **2**.

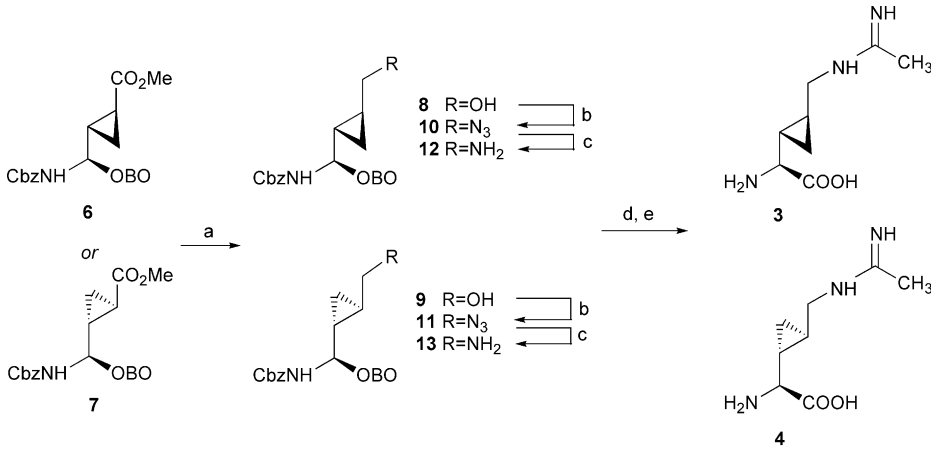
Arginine analogue **1** did not inhibit any of the NOS isozymes, but was found to be a substrate, with $K_m$ values greater than those found for L-arginine ($8.5 \pm 1$ μM vs $2.8 \pm 1.6$ μM for nNOS, respectively). Compound **2** was found to be a mixed inhibitor of the NOS isoforms and not a substrate. It was not isoform selective, nor did it exhibit any time dependent inhibition characteristics. Further investigations were not performed on compound **2** due to its poor inhibitory properties and its lack of isozyme selectivity.

Compound **3** was found to be a competitive inhibitor of all NOS isozymes. It strongly inhibited iNOS in a time dependent manner, but its pre-incubation with nNOS and eNOS did not result in increase inhibition as evidenced by the $IC_{50\text{-PI}}/[E]$ (pre-incubated $IC_{50}$) values given in Table 1. Compound **3** exhibited nNOS/iNOS, eNOS/iNOS, and eNOS/nNOS selectivities of 109, 59, and 1, respectively (Table 1).

Compound **4** was found to be a weak competitive inhibitor of the cNOS isoforms and a slow binding inhibitor of iNOS with an $IC_{50\text{-PI}}/[E]$ value of $383 \pm 35$ compared to over 4500 for the cNOS isoforms (Table 1). Com-




**Scheme 1.** Access to *syn*- and *anti-trans*-(3,4)-cyclopropyl L-arginine.




**Scheme 2.** a) DIBAL-H, $CH_2Cl_2$, −78°C; b) DEAD, $Ph_3P$, $HN_3$, $CH_2Cl_2$; c) $Ph_3P$, THF, then $H_2O$, reflux; d) *S*-2-naphthylmethyl thioacetamidate hydrobromide, EtOH; e) i) $H_2$, Pd/C; ii) 0.1% TFA/$H_2O$; iii) $Cs_2CO_3/H_2O$; iv) Dowex 50W X4-100.

**Table 1.** Inhibition kinetics of iNOS, nNOS and eNOS by compounds **3**, **4** and 1400 W

| Inhibitors | iNOS | | nNOS | | eNOS | | Selectivity[a] | |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}/[E]$ | $IC_{50\text{-}PI}$[b]$/[E]$ | $IC_{50}/[E]$ | $IC_{50\text{-}PI}$[b]$/[E]$ | $IC_{50}/[E]$ | $IC_{50\text{-}PI}$[b]$/[E]$ | nNOS/iNOS | eNOS/iNOS |
| **3** | 825±115 | 3.0±0.3 | 584±261 | 331±13 | 305±57 | 178±75 | 109 | 59 |
| **4** | 22405±609 | 383±26 | 15065±3396 | 7259±1368 | 6071±834 | 4905±620 | 19 | 13 |
| 1400 W[c] | N.D. | 2.4±0.4 | N.D. | 53±9 | N.D. | 916±170 | 22 | 382 |

N.D., not determined. 95% Confidence intervals are shown.
[a]Selectivity is defined as the ratio of the $IC_{50\text{-}PI}/[E]$ (eNOS) or $IC_{50\text{-}PI}/[E]$ (eNOS) to $IC_{50\text{-}PI}/[E]$ (iNOS).
[b]Assayed after 15 min of preincubation (PI) with enzyme, as described in the Experimental. Enzyme concentrations were 28.7 nM for iNOS and nNOS, and 100 nM for eNOS.
[c]Data obtained from Montgomery et al.[23]

pound **4** exhibited nNOS/iNOS, eNOS/iNOS, and eNOS/nNOS selectivities of 19, 13, and 1, respectively (Table 1).

The alteration of inhibitory potency and isoform selectivity of the diastereomers **3** and **4** is of particular interest. Compound **3** was found to be a 10- to 100-fold more potent inhibitor of the NOS isozymes than compound **4** (Table 1). The iNOS isoform selectivities of compound **3** improved by a factor of 5 over the corresponding selectivities for compound **4**. No significant change in eNOS/nNOS selectivity was observed between compounds **3** and **4**. Compound **3** exhibited a tight binding nature with iNOS as evidenced by the $IC_{50\text{-}PI}/[E]$ ratio that is less than 10, whereas compound **4** did not.[24]

A comparison of the inhibitory effects of compound **3** and the potent iNOS selective slow tight binding inhibitor *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W)[25] reveals several interesting similarities.[23,25] 1400 W and compound **3** exhibited $IC_{50\text{-}PI}$ values of similar order of magnitude for all NOS isoforms. Both inhibitors are slow binding inhibitors of iNOS, but not of the cNOS enzymes.[25] The nNOS/iNOS selectivity of compound **3** was 5-fold better than that for 1400 W, but the eNOS/iNOS and eNOS/nNOS selectivities of 1400W are 6 and 32 times greater than that for compound **3**. In fact 1400 W selectively inhibits nNOS over eNOS whereas compound **3** inhibits eNOS and nNOS to the same extent.

The incorporation of constraining elements into arginine analogues,[5,7,26] L-*N*-iminoethylornithine analogues[6] or homocysteines[8] have presumably prevented these inhibitors from adopting the correct binding orientation to fit within the enzyme active site. It is noteworthy that these studies were performed on racemic mixtures of enantiomers. In contrast, *syn-trans*-3,4-cyclopropyl L-arginine **3**, was found to be a classical competitive inhibitor of cNOS enzymes and a competitive slow tight binding inhibitor of iNOS. The presence of a cyclopropyl group places the binding groups in different orientations and these geometries provide some selectivity between the isoforms. Whether the cyclopropyl methylene group interacts with hydrophobic residues in the active site of each or any of the isoforms awaits structural studies of the inhibitor bound enzymes. Our results provide the basis for a novel class of NOS inhibitors composed of conformationally restricted compounds.

## Experimental

### Materials

All reagents were purchased from Aldrich Canada, Ltd. and used directly unless otherwise stated. $CH_2Cl_2$ was distilled from $CaH_2$ and THF from Na/benzophenone. $H_2O$ was deionized and distilled. All reactions were performed under argon atmosphere in oven-dried glass-

ware. TLC was performed using Merk aluminum backed silica gel 60 254, and visualized using 5% $(NH_4)_6MoO_{24}$/0.2% $Ce(SO_4)_2$/5% $H_2SO_4$. Chromatography was performed using silica gel (230–400 mesh). NMR spectra were recorded in $D_2O$ or $CDCl_3$ pre-filtered through basic alumina to remove traces of acid. Optical rotations were measured on a Perkin–Elmer 241 digital polarimeter. Elemental analyses were performed by MHW Laboratories, Phoenix, AZ. HR-FAB was performed by Tim Jones at Brock University, Ontario. HR-QTOF was performed by Amanda Doherty-Kirby at University of Western Ontario.

**Procedure A: conversion of alcohols to $N_3$: Cbz-L-*trans* β,γ-(*anti*)-cyclopropyl-pentahomoserine($N_3$)-OBO ester, 10.** Cbz-L-β,γ-(*syn*)-cyclopropyl-pentahomoserine-OBO ester **8** (400 mg, 1.1 mmol) was dissolved in dry $CH_2Cl_2$ (15 mL) under argon and $Ph_3P$ (511 mg, 1.94 mmol) was added. This solution was cooled to 0 °C, and DEAD (0.31 mL, 1.94 mmol) was added dropwise over 20 min and stirred an additional 5 min. Fresh $HN_3$[27,28] (0.77 M solution in benzene, 5.9 mL, 4.52 mmol) was then added dropwise over 1 h while maintaining the reaction mixture at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 16 h. The solvent was removed in vacuo, trapping the excess $HN_3$ with a NaOH trap. Purification by column chromatography (silica, 1:1 EtOAc:Hex with 0.2% triethylamine) provided 363 mg of azide **10** (85% yield) as a clear oil.

$[\alpha]_D^{25}$ −7.8 ($c = 0.9$, EtOAc); TLC (1:1 EtOAc/Hex) $R_f = 0.59$; $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.27 (m, 5H), 5.06 (m, 1H + 2H), 3.88 (s, 6H), 3.51 (app t, $J = 9.0$ Hz, 1H), 3.10 (dd, $J = 6.4$, 12.8 Hz, 1H), 2.87 (dd, $J = 7.7$, 12.8 Hz, 1H), 1.13 (m, 1H, 0.99 (m, 1H)), 0.78 (s, 3H), 0.64 (m, 1H), 0.47 (m, 1H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 156.5, 136.7, 128.5, 128.1, 108.5, 72.8, 66.9, 57.0, 54.9, 30.7, 18.1, 14.4, 13.9, 9.4. Anal. calcd for $C_{19}H_{24}O_5N_4$: C, 58.75; H, 6.23; N, 14.42. Found: C, 58.65; H, 6.22; N, 14.46.

**Cbz-L-*trans* β,γ-(*anti*)-cyclopropyl-pentahomoserine($N_3$)-OBO ester, 11.** As described in procedure A, Cbz-L-β,γ-(*anti*)-cyclopropyl-pentahomoserine-OBO ester **9** (146 mg, 0.402 mmol) provided 145 mg of azide product **11** (93% yield) as a clear oil.

$[\alpha]_D^{25}$ −22.1 ($c = 0.95$, EtOAc); TLC (1:1 EtOAc/Hex) $R_f = 0.59$); $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.33 (m, 5H), 5.08 (m, 1H), 4.89 (m, 1H), 3.89 (s, 6H), 3.40 (app br t, $J = 9.3$ Hz, 1H), 3.13–3.02 (m, 2H), 1.19 (m, 1H), 0.97 (m, 1H), 0.78 (s, 3H), 0.70 (m, 1H), 0.37 (m, 1H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 136.6, 128.6, 128.1, 108.8, 72.8, 67.0, 57.7, 55.0, 30.7, 18.2, 17.0, 14.5, 7.5. Anal. calcd for $C_{19}H_{24}O_5N_4$: C, 58.75; H, 6.23; N, 14.42. Found: C, 58.68; H, 6.23; N, 14.21.

**Procedure B: Staudinger reduction of $N_3$ to $NH_2$.** In a typical procedure, azide was dissolved in dry $CH_2Cl_2$ and $Ph_3P$ (4 molar equiv) was added. The solution was stirred at room temperature for 24 h under argon, and then $H_2O$ (~15 molar equiv) was added. This mixture was heated at reflux for 5 h, allowed to cool and the solvent evaporated in vacuo. Column chromatography (silica, 3:1 $CHCl_3$/MeOH with 0.2% $Et_3N$) provided the amine as a clear oil in yields ranging 85–93%.

**Cbz-L-β,γ-(*syn*)-cyclopropyl-ornithine-OBO ester, 12.** $[\alpha]_D^{25}$ −10.3 ($c = 1.0$, EtOAc); $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.32 (m, 5H), 5.11 (d, $J = 12.0$ Hz, 1H), 5.04 (d, $J = 12.0$ Hz, 1H), 5.0 (d, $J = 9.1$ Hz, 1H), 3.88 (s, 6H), 3.54 (m, 1H), 2.65 (dd, $J = 6.1$, 12.2 Hz, 1H), 2.28 (dd, $J = 8.0$, 12.7 Hz, 1H), 0.89 (m, 1H), 0.83 (m, 1H), 0.78 (s, 3H), 0.52 (m, 1H), 0.38 (m, 1H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 156.5, 136.7, 128.5, 128.1, 108.8, 72.7, 66.7, 56.9, 46.1, 30.7, 18.1, 17.9, 14.4, 9.3. HR-MS (QTOF) expected for $C_{19}H_{27}N_2O_5$: 363.1920. Found: 363.1922.

**Cbz-L-β,γ-(*anti*)-cyclopropyl-ornithine-OBO ester, 13.** $[\alpha]_D^{25}$ −18.3 ($c = 1.03$, EtOAc); $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.32 (m, 5H), 5.05 (m, 2H + 1H), 3.86 (s, 6H), 3.35 (app t, $J = 9.3$ Hz, 1H), 2.69 (dd, $J = 5.7$, 13.0, 1H), 2.27 (dd, $J = 8.1$, 13.0, 1H), 1.71 (br s), 0.94 (m, 1H), 0.77 (m, 1H), 0.77 (s, 3H), 0.54 (m, 1H), 0.23 (m, 1H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 136.6, 128.5, 128.1, 108.8, 72.8, 66.9, 58.1, 46.3, 30.7, 21.9, 18.5, 14.4, 7.5. Anal. calcd for $C_{19}H_{26}O_5N_2$: C, 62.97; H, 7.26; N, 7.73. Found: C, 62.74; H, 7.22; N, 7.61.

**Procedure C: *trans* β,γ-(*syn*)-cyclopropyl-iminoethyl L-ornithine, 3.** Cbz-L-β,γ-(*syn*)-cyclopropyl-ornithine-OBO ester **12** (635 mg, 0.17 mmol) was dissolved in EtOH (2 mL) at 0 °C, to which *S*-2-naphthylmethyl thioacetamidate hydrobromide **14** (51 mg, 0.17 mmol) was added portionwise with stirring. This mixture was stirred a total of 6 h, and then the solvent evaporated to provide a pale yellow oil. The residue was suspended in distilled $H_2O$ (5 mL) and $Et_2O$ (5 mL), and extracted with additional $H_2O$ (2×5 mL). The aqueous fractions were combined and lyophilized to provide 79 mg of white powder. This fully protected amino acid was dissolved in 1:1 EtOH/EtOAc with an equal mass of 10% (w/w) Pd/C. The suspension was stirred vigorously under a pure $H_2$ atmosphere for 16 h. The mixture was filtered and the solvent evaporated. The clear oil was dissolved in 0.1% TFA in $CH_2Cl_2$ and the solvent immediately evaporated in vacuo. The clear oil was suspended in 10% $Cs_2CO_3$ and stirred for 16 h, then lyophilized. The residue was dissolved in minimal purified $H_2O$, acidified with double distilled 6 N HCl to pH 4 then loaded onto Dowex 50W X-4-100 cation exchange resin. After washing with pure $H_2O$, the product was eluted with 0.5 N $NH_4OH$. Fractions were combined and lyophilized to provide 26 mg of the desired amino acid (82%).

Mp 187–194 °C dec.; $[\alpha]_D^{25}$ +48.9 ($c = 1.15$, $H_2O$); $^1$H NMR ($D_2O$, 300 MHz) δ 3.01 (dd, $J = 7.2$, 13.9 Hz, 1H), 2.95 (dd, $J = 7.4$, 14.1 Hz, 1H), 2.66 (d, $J = 8.6$ Hz, 1H), 2.03 (s, 3H), 0.93 (m, 1H), 0.81 (m, 1H), 0.58 (m, 1H), 0.42 (m, 1H); $^{13}$C NMR ($D_2O$, 75 MHz) δ 180.4, 164.4, 58.6, 45.7, 18.3, 21.4, 14.7, 8.4. HR-MS (Q-TOF) expected for $C_8H_{16}N_3O_2$: 186.1243. Found: 186.1241.

***trans* β,γ-(Anti)-cyclopropyl-iminoethyl L-ornithine, 4.** Procedure C was applied to Cbz-L-β,γ-(*anti*)-cyclopropyl-ornithine-OBO ester **13** (80 mg, 0.22 mmol) and provided 28 mg of the desired amino acid (70%).

Mp 192–197 °C dec.; $[\alpha]_D^{25}$ +19.0 ($c = 0.5$, $H_2O$); $^1H$ NMR ($D_2O$, 300 MHz) δ 3.09 (dd, $J = 7.0$, 13.9 Hz, 1H), 2.98 (dd, $J = 7.6$, 14.0 Hz, 1H), 2.90 (d, $J = 9.8$ Hz, 1H), 2.05 (s, 3H), 1.20 (m, 1H), 0.93 (m, 1H), 0.64 (m, 2H); $^{13}C$ NMR ($D_2O$, 75 MHz) δ 175.8, 164.5, 58.5, 45.3, 19.3, 18.4, 15.6, 9.0; HR-MS (Q-TOF) expected for $C_8H_{16}N_3O_2$: 186.1243. Found: 186.1249.

### Protein expression and purification

Human iNOS enzyme carrying a deletion of the first 70 amino acids and an amino terminal polyhistidine tail was co-expressed with calmodulin in *E. coli* and purified using metal chelating chromatography followed by 2′,5′-ADP column chromatography.[23] The expression and protein purification of bovine eNOS and rat nNOS enzymes in *E. coli* was performed as previously reported.[29]

### Enzyme kinetics

The activity of NOS was determined using the hemoglobin capture assay[30,31] that yield results consistent with the direct radioactive assay that monitors the formation of L-[$^3$H]-citrulline from L-[$^3$H]-arginine.[29] Quadruplicate reactions were initiated by the addition of L-arginine and monitored on a 96-well plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA) at 26 °C. Nitric oxide-mediated oxidation of oxyhemoglobin was monitored at 401 nm (ε = 0.1034 OD/nanomole).[30] Experiments were conducted in 100 μL volumes that contained 50 mM Tris–HCl, pH 7.5, 10–100 nM of NOS isozyme, 2.5–50 uM L-arginine, 500 μM NADPH, 5.0 μM $H_4B$, 1.0 μM CaM, 1.0 mM $CaCl_2$, 16 μM DTT, 1.0 μM FAD, 1.0 μM FMN, 100 units/mL superoxide dismutase (SOD), 50 units/mL catalase, 10.0 μM bovine oxyhemoglobin, and 0.2 mg/mL bovine serum albumin. Various concentrations of the optically pure conformationally restricted synthesized cyclopropane based L-arginine analogues were added to the reaction mixtures to test for NOS inhibition. To determine if inhibitors displayed time dependent inhibition $IC_{50}$ values were calculated by preincubating enzyme in the presence of varying amounts of cyclopropane based L-arginine analogues, 500 μM NADPH, 10.0 μM FAD, 10.0 μM FMN, 50.0 μM $H_4B$, 157.0 μM DTT, 100 units/mL SOD and 50 units/mL catalase for 15 min at 26 °C.[25] Blank wells that did not contain enzyme demonstrated similar behaviour to wells with a sufficiently high concentration of inhibitor to fully inhibit NOS activity. Blank wells were run in parallel with reaction mixtures and kinetic data was corrected for the loss of signal in the blank.

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