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Evaluation of New L-Thiocitrulline Derivatives as Inhibitors of Nitric Oxide Synthase

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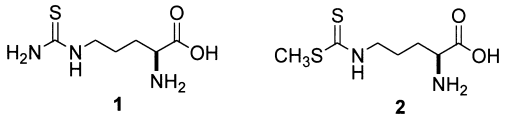
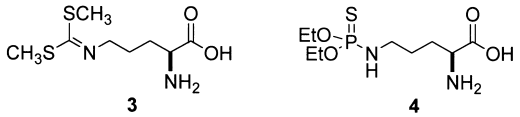
Abstract—New derivatives of L-thiocitrulline were prepared and assayed as inhibitors of the three isoforms of nitric oxide synthase. These compounds demonstrated weak inhibitory activity against the NOS isoforms and these results directly support a recently described model of the L-arginine binding site of NOS.

The nitric oxide synthases (NOS) catalyze the oxidation of the terminal guanidino 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^G-L-hydroxyarginine followed by a three-electron oxidation of N^G-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 provide detailed active site structural information.[4]

With respect to L-arginine, L-thiocitrulline (**1**) competitively inhibits the nitric oxide synthases with reported K_i values 0.06–2 and 3.6–9 μM for nNOS and iNOS, respectively.[5,6] L-Thiocitrulline decreases the electron flux through NOS and NADPH oxidase activity of NOS by reducing the reduction potential of the heme iron.[6] Optical difference spectrophotometric experiments indicate the direct binding of L-thiocitrulline to the iron heme group of the enzyme most likely through the sulfur atom.[5] X-ray crystallographic studies with the inducible oxygenase domain reveal that L-thiocitrulline binds with its sulfur atom directly positioned above the heme iron.[4d] These studies also show that L-thiocitrulline binds in a similar conformation as L-arginine with stabilization provided by hydrogen bonds between the carboxylate group of Glu371 and the thiourea group of L-thiocitrulline.[4d] Other thiourea containing compounds, including thiourea, interact with NOS in a similar fashion.[7] These results combined with the ability of thioureas to act as stabilizing ligands of iron in various oxidation states prompted our examination of new L-thiocitrulline derivatives.[8] We wish to report the synthesis and evaluation of the xanthamate (**2**), its *S*-methyl derivative (**3**) and the thiophosphoramidate (**4**) as NOS inhibitors.



1 **2**



3 **4**

Scheme 2 depicts the preparation of the proposed inhibitors **2**–**4**. Treatment of a previously described L-ornithine

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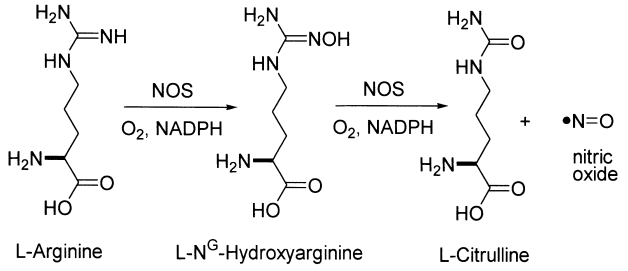
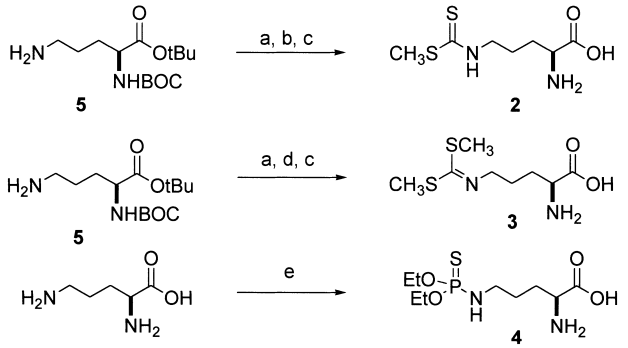


Scheme 1. NOS catalyzed oxidation of L-arginine to NO and L-citrulline.



Scheme 2. Reagents and conditions: (a) CS_2; (b) 1 equiv CH_3I; (c) 4.0 M HCl/dioxane; (d) 2 equiv CH_3I; (e) $(EtO)_2P(=S)Cl$.

derivative (**5**) with carbon disulfide, followed by one equivalent of methyl iodide and acidic deprotection yielded the xanthamate (**2**) (Scheme 2).[9] An identical procedure using two equivalents of methyl iodide gave the *S*-methyl compound (**3**) (Scheme 2). Treatment of L-ornithine with diethyl thiophosphinoyl chloride under Schotten–Baumann conditions directly produced the thiophosphoramidate (**4**) (Scheme 2).

Compounds **2**–**4** were evaluated as inhibitors of purified mouse inducible, rat neuronal, and bovine endothelial nitric oxide synthase using the radioactive ^{14}C-L-citrulline assay.[7a] Table 1 summarizes the per cent inhibition of iNOS, eNOS, and nNOS catalyzed ^{14}C-L-citrulline production at 1 mM inhibitor concentration in the presence of 50 μM L-arginine. Under these conditions, L-thiocitrulline effectively blocked greater than 99% of NOS catalyzed ^{14}C-L-ctirulline formation (Table 1). Compounds **2** and **3** inhibited iNOS catalyzed ^{14}C-L-citrulline formation by approximately 50% at these high inhibitor concentrations (Table 1). Compounds **2** and **3** were only about half as potent in blocking ^{14}C-L-ctirulline synthesis catalyzed by the constitutive isoforms (Table 1).

Table 1. Percent inhibition of NOS isoforms by compounds **1**–**4**

Compounds	iNOS[a]	nNOS[a]	eNOS[a]
1	>99	>99	>99
2	54	27	25
3	56	39	46
4	< 10		

[a]Values are means of three experiments.

The thiophosphoramidate (**4**) failed to inhibit ^{14}C-L-citrulline formation by iNOS and was not assayed against the other isoforms (Table 1).

Recent mapping studies of the L-arginine binding site of NOS using synthetic L-arginine derivatives provide an explanation of the poor interaction of **2**–**4** with NOS.[10] This work indicates that the reactive heme binding site can accommodate groups that extend 4–5 Å from the guanidinium group but the non-reactive guanidinium binding site is relatively intolerant of larger substitutions.[10] Based upon these results, the large thiomethyl groups of **2** and **3** will not fit into the non-reactive guanidinium binding site. Placement of the thiocarbonyl group of **2** into this binding site and the thiomethyl in the reactive heme binding site also will decrease interaction according to this model.[10] While the thiophosphoramidate (**4**) mimics the tetrahedral geometry of a proposed intermediate in the conversion of *N*-hydroxy-L-arginine to NO and L-citrulline,[1] the large size of this group apparently does not allow interaction at the L-arginine binding site. In conclusion, these structurally unique L-thiocitrulline derivatives (**2**–**4**) do not interact well with NOS and these results directly support the recently described steric limitations to NOS binding.[10] These results indicate that the simple incorporation of an iron binding group into L-arginine is not sufficient to produce a potent NOS inhibitor and the previously defined steric limitations must be taken into account in the design of new L-arginine based inhibitors of NOS.[10]

Experimental

Xanthamate (2)

Carbon disulfide (0.225 g, 2.95 mmol) was added dropwise to a stirred solution of *N*-α-Boc-L-ornithine-*t*-butyl ester (**5**, 0.340 g, 1.18 mmol) and potassium phosphate (0.501 g, 2.36 mmol) in acetone (15 mL) at 0 °C. After 1 h, methyl iodide (0.168 g, 1.18 mmol) was added dropwise and stirred for an additional 18 h. The crude product was purified by flash chromatography (pentane:EtOAc, 10:1) and concentrated in vacuo to yield (0.217 g, 49%) of a clear yellow oil. R_f 0.62 (pentane:EtOAc, 4:1); $[\alpha]_D^{20.0}$ +16.51 (*c* 0.945, CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.78–7.64 (b s, 1H), 5.24–5.09 (m, 1H), 4.19–4.10 (m, 1H), 3.88–3.40 (m, 2H), 2.61 (s, 3H), 1.90–1.62 (m, 4H), 1.45 (s, 9H), 1.43 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.8, 171.3, 155.4, 82.1, 79.7, 53.2, 46.5, 30.4, 28.1, 27.8, 23.7, 17.9. Anal. calcd for $C_{16}H_{30}N_2S_2O_4$: C, 50.76; H, 7.99; N, 7.40; found: C, 52.02; H, 8.11; N, 6.95; LRMS (FAB) $(M+H)^+$ *m*/*z* 379. A solution of 4.0 M HCl in dioxane (10.0 mL) was added to this oil (0.189 g, 0.499 mmol) under argon and stirred for 24 h. The solution was concentrated in vacuo, dissolved in water (10.0 mL) and filtered through a Supleco LC-18 filter. The resulting solution was lyopholized to give **2**: 0.110 g (85%). $[\alpha]_D^{20.0}$ +8.78 (*c* 0.581, MeOH); ^{1}H NMR (200 MHz, D_2O) δ 3.88 (t, *J* = 6.2 Hz, 1H), 3.65 (t, *J* = 5.9 Hz, 2H), 2.39 (s, 3H), 1.87–1.46 (m, 4H); ^{13}C NMR (50 MHz, D_2O) δ 199.9, 172.4, 53.5, 46.3, 27.6, 23.7, 17.5; LRMS (FAB) $(M+H)^+$ *m*/*z* 223.

Thiomethyl derivative (3)

Carbon disulfide (0.290 g, 3.81 mmol) was added dropwise to a stirred solution of *N*-α-Boc-L-ornithine-*t*-butyl ester (**5**, 0.44 g, 1.53 mmol) and potassium phosphate (0.648 g, 3.05 mmol) in acetone (15 mL) at 0 °C. After 1 h, methyl iodide (0.325 g, 2.29 mmol) was added dropwise and stirred for an additional 18 h. The crude product was purified by flash chromatography (pentane:EtOAc, 20:1) and concentrated in vacuo to yield (0.274 g, 48%) of a clear yellow oil. R_f 0.20 (pentane:EtOAc, 20:1); $[\alpha]_D^{20.0}$ −11.19° (*c* 0.590, MeOH); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.19–5.12 (m, 1H), 4.24–4.13 (m, 1H), 3.36 (t, J = 5.8 Hz, 2H), 2.51 (s, 3H), 2.34 (s, 3H), 1.92–1.64 (m, 4H), 1.44 (s, 9H), 1.42 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.7, 157.5, 155.1, 81.1, 79.0, 53.6, 30.2, 28.1, 27.7, 26.1, 14.2. Anal. calcd for $C_{17}H_{34}N_2S_2O_4$: C, 51.74; H, 8.68; N, 7.10; found: C, 52.79; H, 8.24; N, 6.88; LRMS (FAB) $(M+H)^+$ m/z 393. A solution of 4.0 M HCl in dioxane (10.0 mL) was added to this oil (0.252 g, 0.666 mmol) under argon and stirred for 24 h. The solution was concentrated in vacuo, dissolved in water (10.0 mL) and filtered through a Supleco LC-18 filter. The resulting solution was lyopholized to produce **3**: 0.164 g (91%). $[\alpha]_D^{20.0}$ +18.41 (*c* 0.277, MeOH); ^{1}H NMR (200 MHz, D_2O) δ 3.86 (t, J = 6.6 Hz, 1H), 3.61 (t, J = 6.7 Hz, 2H), 2.64 (s, 3H), 2.59 (s, 3H), 1.92–1.63 (m, 4H); ^{13}C NMR (50 MHz, D_2O) δ 193.7, 171.6, 48.0, 27.0, 23.0, 15.9, 15.5; HRMS (FAB) calcd for $C_8H_{17}N_2O_2S_2$ 237.0732, found 237.0725.

Thiophosphoramidate (4)

Diethyl thiophosphinoyl chloride (1.20 mL, 5.24 mmol) was added to a solution of L-ornithine (1.77 g, 10.49 mmol) in 1 N NaOH:EtOH (4:1, 21 mL). After 2 h, a white solid precipitated and the EtOH was removed in vacuo. This aqueous solution was extracted with EtOAc (3×20 mL), the organic layers dried over $MgSO_4$ and concentrated in vacuo. The resulting solid was recrystallized from EtOAc:diethyl ether (3:1) to afford **4** (0.698 g, 47%) as a white crystalline solid: $[\alpha]_D^{20.0}$ +14.83 (*c* 0.017, MeOH); ^{1}H NMR (200 MHz, D_2O) δ 4.04–3.83 (m, 5H), 2.99–2.88 (m, 2H), 1.95–1.85 (m, 2H), 1.61–1.43 (m, 2H), 1.21 (t, J = 7.1 Hz, 6H); ^{13}C NMR (50 MHz, D_2O) δ 172.9, 64.2 (d, J = 5.1 Hz), 53.5, 40.9, 27.7, 26.6, 15.6, 15.5; ^{31}P NMR (122 MHz, D_2O) δ 73.2. Anal. calcd for $C_9H_{22}N_2O_4PS$–HCl: C, 33.70; H, 6.91; N, 8.73; found: C, 32.90; H, 6.86; N, 9.03; LRMS (FAB) $(M+H)^+$ m/z 285.

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