J. Med. Chem. 1994, *37*, 885-887

Synthesis of L-Thiocitrulline, L-Homothiocitrulline, and *S*-Methyl-L-thiocitrulline: A New Class of Potent Nitric Oxide Synthase Inhibitors

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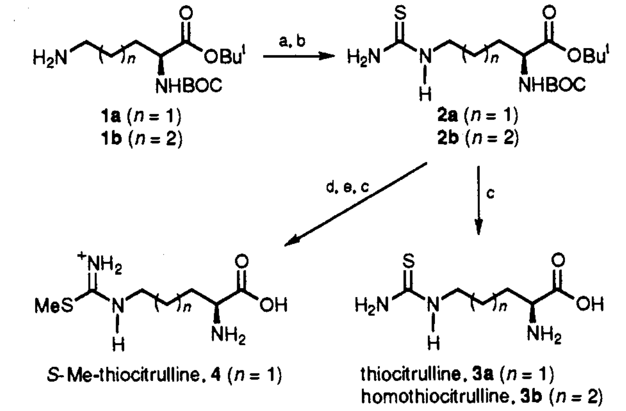
Received January 6, 1994[•]

Nitric oxide synthase catalyzes the NADPH- and O_2-dependent conversion of L-arginine to L-citrulline and nitric oxide. L-Thiocitrulline, L-homothiocitrulline, and *S*-methyl-L-thiocitrulline, novel citrulline analogs, have been synthesized and are shown to be potent inhibitors of both the constitutive brain and the inducible smooth muscle isoforms of nitric oxide synthase. Although many N^ω-monosubstituted arginine derivatives inhibit nitric oxide synthase, inhibitory citrulline derivatives have not previously been reported. *S*-Methyl-L-thiocitrulline is significantly more potent than N^ω-methyl-L-arginine, the prototypic nitric oxide synthase inhibitor.

Nitric oxide synthase (NOS) catalyzes the dioxygen and NADPH-dependent five electron oxidation of L-arginine to L-citrulline and nitric oxide (NO).[1] The fully active dimeric enzyme contains 1 equiv of FAD, FMN,[2] heme,[3-5] and tetrahydrobiopterin[6,7] per monomeric subunit. Both constitutive NOS (cNOS) and inducible NOS (iNOS) isoforms have been identified.[1] The cNOS are tightly regulated by Ca^{2+}/calmodulin and are best characterized from neuronal (brain) tissue (bNOS)[8] and from vascular endothelium (eNOS).[9] When synthesized in response to Ca^{2+} influx, bNOS-derived NO plays a poorly characterized role in neurotransmission whereas eNOS-derived NO (originally characterized as endothelium-derived relaxing factor, EDRF[10]) is a vasorelaxant important in normal blood pressure homeostasis.[11,12] Inducible NOS, in contrast to cNOS, is not regulated by Ca^{2+} and, once expressed in response to various cytokines or endotoxin, remains active for many hours.[1] In macrophages, where the iNOS concentration can be high, large amounts of NO are formed and constitute one aspect of the cytostatic/cytotoxic armamentarium of these cells.[13] When induced in vascular endothelial or smooth muscle cells, iNOS produces NO in amounts that cause severe vasodilation, loss of peripheral vascular resistance, and potentially lethal hypotension. When the inducing factor is bacterial endotoxin, the resulting condition is septic shock.[14] A similar NO-mediated hypotensive crisis may occur when various cytokines such as tumor necrosis factor, interleukin-1, or interleukin-2 are used therapeutically.[14,17]

Inhibitors of NOS such as N^ω-methyl-L-arginine (NMA) have been shown to normalize blood pressure in cytokine-induced and septic shock.[14,17] We have carried out studies probing the arginine/citrulline binding site of bNOS, eNOS, and iNOS with the view of identifying specific structural modifications to the substrate that will limit binding to a single isoform, provide exceptionally strong inhibition, or, preferably, both. Since the heme cofactor is the oxygen carrier for the oxidation of arginine to citrulline and NO, we considered the possibility of designing citrulline analogs containing groups positioned to interact directly with the heme iron. In view of the well-established affinity of heme iron for sulfur ligands,[18] we synthesized L-thiocitrulline and *S*-methyl-L-thiocitrulline as novel citrulline analogs in which the ureido oxygen is replaced by sulfur (Scheme 1) (see the Experimental Section for details). Because NOS is active toward L-homoarginine, we also synthesized L-homothiocitrulline starting from the corresponding L-lysine derivative (*n* = 2 in Scheme 1).

Scheme 1[a]



[a] (a) Thiophosgene, $CaCO_3$, $CHCl_3/H_2O$; (b) NH_3, MeOH, 0 °C; (c) 4 N HCl/dioxane; (d) MeI, CH_3CN; (e) $(BOC)_2O$, $NaHCO_3$, dioxane/H_2O.

When tested with isolated bNOS and iNOS, L-thiocitrulline and L-homothiocitrulline are potent inhibitors showing activity comparable to NMA, the prototypic NOS inhibitor (Table 1). *S*-Methyl-L-thiocitrulline is substantially more potent than L-thiocitrulline and NMA; in the studies shown, 10 μM *S*-methyl-L-thiocitrulline had an inhibitory activity comparable to that of 100 μM L-thiocitrulline or NMA (Table 1). Preliminary kinetic studies indicate that the K_i for *S*-methyl-L-thiocitrulline is substantially lower than those of the other inhibitors. The arginine/citrulline binding site of NOS shows complete stereoselectivity for L-enantiomers,[1] and consistent with the requirement for stereospecific binding, D-thiocitrulline and *S*-methyl-D-thiocitrulline are not active inhibitors when tested as shown in Table 1. In the presence of any of the inhibitors, product formation increased linearly with time, indicating that inhibition was not irreversible under the conditions used for these initial rate studies (i.e., 10.5-min incubations). Since NMA has been shown to yield citrulline and cause irreversible inhibition with longer incubation times,[19] the possibility that the novel inhibitors are either pseudosubstrates or irreversible inhibitors warrants consideration. In preliminary HPLC studies, we observed the formation of citrulline and diminution of the thiocitrulline peak size following incubation of thio-

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[•] Abstract published in *Advance ACS Abstracts*, March 1, 1994.

0022-2623/94/1837-0885$04.50/0

Table 1. Inhibition of bNOS and iNOS by Thiocitrulline and Related Compounds[a]

compound (μM)	% inhibition	
	bNOS	iNOS
L-thiocitrulline (100)	91	87
L-thiocitrulline (10)	50	34
D-thiocitrulline (100)	<1	<1
L-homothiocitrulline (100)	93	75
S-methyl-L-thiocitrulline (100)	>99	>99
S-methyl-L-thiocitrulline (10)	96	87
S-methyl-L-thiocitrulline (1)	73	29
S-methyl-D-thiocitrulline (10)	<1	<1
N^{ω}-methyl-L-arginine (100)	91	89
N^{ω}-methyl-L-arginine (10)	56	33

[a] Reaction mixtures for bNOS contained in a final volume of 200 μL: 20 mM Na HEPES buffer pH 7.5, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25 μM FAD, 25 μM FMN, 0.1 mM THB, 10 μg/mL calmodulin, 2 mM $CaCl_2$, 100 μg/mL BSA, 50 μM NADPH, and 20 μM L-[^{14}C]arginine (0.045 μCi). Reaction was initiated by adding 0.6 μg of rat brain nitric oxide synthase (prepared and provided by Kirk McMillan and Bettie Sue Masters, The University of Texas Health Science Center, San Antonio, TX[3]). Reaction mixtures for iNOS contained in a final volume of 200 μL: 20 mM Na HEPES buffer pH 7.5, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25 μM FAD, 25 μM FMN, 100 μM THB, 500 μM NADPH, and 20 μM L-[^{14}C]arginine (0.045 μCi). Reaction was initiated by adding 14 μg of a crude homogenate of cytokine-treated rat aortic smooth muscle cells (prepared and provided by S. S. Gross, Cornell University Medical College, New York, NY[25]). With either isoform, 50-μL portions of the reaction mixture were removed at 3.5, 7, and 10.5 min and added to 200 μL of 100 mM HEPES, pH 5.5, containing 5 mM EGTA. Those solutions were placed in boiling H_2O for 1 min, iced for 15 min, and then centrifuged. Portions (225 μL) of supernatants were loaded on to a 0.6- × 3-cm column of Dowex 50, and the [^{14}C]citrulline was eluted with 2 mL of H_2O. The entire eluant was submitted to liquid scintillation counting, and the amount of product formed was calculated based on the specific activity of the [^{14}C]arginine used. All reactions were carried out in at least duplicate, and replicates agreed within ±5%.

citrulline with bNOS in an arginine-free but otherwise complete reaction mixture. This observation indicates that L-thiocitrulline could indeed be a substrate for NOS. We are currently pursuing other aspects of this mechanism. With respect to *in vivo* activity, L-thiocitrulline and *S*-methyl-L-thiocitrulline were found to be strong pressor agents in anesthetized rats.[20]

Thiocitrulline, homothiocitrulline, and *S*-methylthiocitrulline represent a novel class of NOS inhibitors. They were designed with the view that the S atom would be bound to the enzyme active site in close proximity to heme iron; interaction between S and heme iron would be expected to increase the binding affinity of the inhibitors. Preliminary spectroscopic studies carried out with McMillan and Masters confirm this view for thiocitrulline (data not shown); such binding may involve the imino thiol tautomer of thiocitrulline. We also note that *S*-alkylpseudothiouronium salts are electrophilic and reactive with, for example, amines.[21] If the *S*-methylthiocitrulline side chain is protonated in the active site, covalent reaction with active-site lysyl side chains and perhaps other groups is possible. Studies to fully elucidate the mechanism(s) by which these novel derivatives inhibit NOS are in progress.

Experimental Section

Except where indicated otherwise, reagents were obtained from Aldrich Chemicals, Milwaukee, WI. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC 300-MHz spectrometer, and mass spectra were obtained on a Kratos MS80RFA AGC/MS spectrometer with a static FAB probe. After precolumn derivatization with o-phthalaldehyde, amino acids were analyzed for purity by reverse-phase HPLC on a Spherisorb ODS2 (C-18) column (5-μM particles; 0.46 × 25 cm; Phase Separations, Norwalk, CT) using 100 mM NaOAc (pH 7.2) in THF–MeOH–H_2O (0.5:0.95:9.0) as mobile phase A and MeOH as mobile phase B essentially as described;[22] detection was by fluorescence. Elution was effected using a linear gradient from 100% A to B 100% over 45 min at a flow rate of 1.7 mL/min. Under these conditions citrulline, thiocitrulline, homothiocitrulline, and *S*-methylthiocitrulline elute at 9.00, 11.07, 14.76, and 8.65 min, respectively. Purity of the novel amino acids was >99% in all cases.

Synthesis of Thiocitrulline and *S*-Methylthiocitrulline. N^{α}-(*tert*-Butyloxycarbonyl)-δ-thioureido-L-norvaline *tert*-Butyl Ester (2a).[23] N^{α}-(*tert*-Butyloxycarbonyl)-L-ornithine *tert*-butyl ester **1a**[24] (5.80 g, 20.14 mmol) was dissolved in 100 mL of chloroform and added to a solution of 5.70 g of calcium carbonate and 2.2 mL of thiophosgene (28.7 mmol) dissolved in 100 mL of water. The mixture was stirred vigorously overnight. The next day the reaction mixture was filtered, and the layers were allowed to separate. The aqueous layer was extracted with chloroform (2 × 50 mL), and the combined organic layers were dried ($MgSO_4$) and concentrated to an oil by evaporation at reduced pressure. The residue was taken up in dry methanol (200 mL) and cooled to 0 °C. Ammonia gas was passed through the solution for 20 min, and the solution was stirred for 3 h at 0 °C. Following reaction with ammonia, the solvent was evaporated under reduced pressure, and the residue was dissolved in 5 mL of ethyl acetate–hexane (4:1). That solution was chromatographed on a column of silica gel (3 × 25 cm) using the same ethyl acetate–hexane mixture as eluent. Fractions of approximately 5 mL were collected, and those containing product were identified by thin-layer chromatography on silica gel developed with ethyl acetate–hexane (4:1) ($R_f = 0.30$). Product-containing fractions (30 × 50 mL) were pooled and evaporated to dryness under reduced pressure to yield **2a** in 70% yield: ^{13}C NMR ($DCCl_3$) 183.4–180.4 (one carbon), 171.6, 155.9, 82.2–80.0, 53.6–52.5 (one carbon), 44.6–43.1 (one carbon), 30.4, 28.1, 27.8, 24.6.

N^{δ}-Thioureido-L-norvaline (3a). Compound **2a** (1.0 g, 2.89 mmol) was mixed with a solution of 4 N hydrogen chloride in dioxane and kept at room temperature for 24 h. A solid precipitate formed. The mixture was then diluted with ethyl ether (20 mL), and the entire solution was evaporated to dryness under reduced pressure. Methanol (10 mL) was added and evaporated at reduced pressure two to three times to provide N^{δ}-thioureido-L-norvaline (L-thiocitrulline, **3a**) as a white solid. The yield was 600 mg (91%): ^{1}H NMR (CD_3OD) δ 1.40–1.90 (m, 4H), 3.16 (t, 2H), 3.74 (t, 1H); ^{13}C NMR (CD_3OD) δ 171.48, 53.47, 44.53, 28.69, 25.40; IR (KBr, cm^{-1}) 1710, 1635, 1595, 1470, 1390, 1300; mass spectrum, 192 (MH^+).

D-Thiocitrulline was prepared as described for L-thiocitrulline except that N^{δ}-(benzyloxycarbonyl)-D-ornithine (Sigma Chemicals, Inc.) was used to prepare N^{α}-(*tert*-butyloxycarbonyl)-D-norvaline *tert*-butyl ester as starting material.[23] Starting with 5.0 g of N^{δ}-(benzyloxycarbonyl)-D-ornithine, the final yield of D-thiocitrulline was 600 mg (29%).

L-**Homothiocitrulline (3b)** was prepared as described for L-thiocitrulline, except that the starting material was compound **1b**, which was prepared from N^{ϵ}-(benzyloxycarbonyl)-L-lysine in a three-step procedure.[23] The intermediates and final product were characterized as follows: N^{ϵ}-(benzyloxycarbonyl)-L-lysine *tert*-butyl ester: ^{1}H NMR ($DCCl_3$) δ 1.42 (s, 9H), 1.42–1.66 (m, 6H), 3.14–3.27 (m, 3H), 5.04 (s and m, 3H), 7.30 (s, 5H). N^{ϵ}-(Benzyloxycarbonyl)-N^{α}-(*tert*-butyloxycarbonyl)-L-lysine *tert*-butyl ester: ^{1}H NMR ($DCCl_3$) δ 1.44 (s, 9H), 1.46 (s, 9H), 1.43–1.70 (m, 6H), 3.20 (m, 2H), 4.10 (m, 1H), 4.86 (m, 1H), 5.10 (s, 2H), 7.35 (s, 5H). N^{α}-(*tert*-Butyloxycarbonyl)-L-lysine *tert*-butyl ester (**1b**): ^{1}H NMR ($DCCl_3$) δ 1.44 (s, 9H), 1.46 (s, 9H), 1.45–1.80 (m, 6H), 3.00 (m, 2H), 4.10 (m, 1H). N^{α}-(*tert*-Butyloxycarbonyl)-ε-thioureido-L-norleucine *tert*-butyl ester (**2b**): ^{13}C NMR ($DCCl_3$) δ 183.0–180.0 (one carbon) 171.8, 155.6, 81.9, 79.8, 53.7, 44.7, 32.3, 28.2, 27.8, 22.5. N^{ϵ}-Thioureido-L-norleucine (L-homothiocitrulline, **3b**): ^{1}H NMR (D_2O) δ 1.38–1.42 (m, 4H), 1.74 (m, 2H), 3.10 (m, 2H), 3.87 (t, 1H).

Synthesis of *N^{δ}*-(*S*-methylisothioureido)-L-Norvaline (4). *N^{α}*-(*tert*-Butyloxycarbonyl)-*N^{δ}*-[*N*-(*tert*-butyloxycarbonyl)-*S*-methylisothioureido]-L-norvaline *tert*-Butyl Ester.[23] A solution of **2a** (2.35 g, 6.63 mmol) and iodomethane (1 mL, 16.8 mmol) in CH_3CN (15 mL) was stirred at 23 °C for 16 h. The solution was concentrated, and the residue was dissolved in dioxane (16 mL) and mixed with saturated aqueous $NaHCO_3$ (16 mL). To that solution was added di-*tert*-butyl pyrocarbonate (2.08 g, 9.54 mmol), and the reaction mixture was vigorously stirred at room temperature for 12 h. The dioxane was then removed by evaporation under reduced pressure, and the aqueous solution was extracted with ethyl acetate (3 × 75 mL). The organic layers were dried over $MgSO_4$ and evaporated under reduced pressure. The residue was chromatographed on silica gel using hexane–ethyl acetate (3:1) to provide the product as an oil (3.08 g, 70%). Although 4 can be prepared without forming the *N^{δ}*-(*N*-*tert*-butyloxycarbonyl) derivative, separation of the methylated product from starting material is facilitated by forming the di-Boc derivative: ^{13}C NMR ($DCCl_3$) δ 173.0, 171.1, 161.8, 155.0, 81.7, 79.3, 78.7, 53.0, 42.9, 29.7, 28.0, 27.9, 24.8, 13.2.

***N^{δ}*-(*S*-Methylisothioureido)-L-norvaline.** The above compound (0.470 g, 10.2 mmol) was dissolved in 4 N HCl/dioxane (3 mL), and the resulting solution was stirred at room temperature for 20 h. The reaction mixture was then diluted with ether (10 mL), and the solvents were evaporated under reduced pressure. This process was repeated two times. Methanol (10 mL) was then added and evaporated under reduced pressure to provide *N^{δ}*-(*S*-methylisothioureido)-L-norvaline (*S*-methyl-L-thiocitrulline, 4) as the hydrochloride salt (282 mg, 89.5%); ^1H NMR (D_2O) δ 1.5–1.9 (m, 4H), 2.36 (s, 3H), 3.23 (t, 2H), 3.88 (t, 1H); mass spectrum, 206 (MH^+).

Acknowledgment. We thank Mr. Kirk McMillan and Drs. Bettie Sue Masters and Steven S. Gross for the enzyme used to test the inhibitors. We thank Mr. Michael A. Hayward for excellent technical assistance.

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