Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1759–1766

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Screening of Conformationally Restricted and Conformationally Free *N*-(Tertiary aminoalkyl)dithiocarbamic Acids and Esters as Inhibitors of Neuronal Nitric Oxide Synthase

Mark B. Sassaman,[a,*] John Giovanelli,[b] Virendar K. Sood[c] and William C. Eckelman[a]

[a]*Positron Emission Tomography Department, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, MD 20892-1180, USA*

[b]*Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, MD 20892-4096, USA*

[c]*Department of Radiology, George Washington University Medical Center, Washington, DC 20037, USA*

Received 6 February 1998; accepted 1 May 1998

Abstract—*N*-(Tertiary aminoalkyl)dithiocarbamic acids and esters were synthesized and evaluated for their ability to inhibit neuronal nitric oxide synthase. Preliminary results show these compounds are able to act at the binding site for L-arginine and the conformationally restricted esters may have a second site of activity involving the cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin. Published by Elsevier Science Ltd.

Introduction

Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to L-citrulline and the biologically active free radical, nitric oxide, in a two step, five electron process, involving NADPH and molecular oxygen. The enzyme exists in two constitutive and one inducible isoform, which bear a high degree of structural homology to one another and to cytochrome P-450 reductase, in regions associated with the binding of the cofactors FMN, FAD, and NADPH. NOS contains an iron protoporphyrin IX (heme) prosthetic group bound to the protein by axial coordination of the heme iron to a cysteine thiolate and a vacant second axial position is available to bind molecular oxygen. The protein is thus organized into a reductase and an oxygenase domain, the latter being associated with the binding site for the natural substrate, L-arginine. In addition, NOS shows dependence on the presence of Ca^{2+}/calmodulin, the binding region of calmodulin acting as a hinge between the two domains; and on the cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4). These properties have been reviewed by Griffith and Stuehr.[1]

The role of BH_4 is unclear, however, a growing body of evidence supports several complimentary functions including stabilization of the catalytically active NOS homodimer,[2] enhancement of the binding of L-arginine to the enzyme,[3] facilitating the coupling of reductive oxygen activation to the oxygenation of L-arginine,[3,4] protecting the enzyme from product inhibition,[5] and involvement in the overall odd-electron redox process.[6–8] BH_4 resides in the oxygenase domain[2,9] in close proximity to the heme prosthetic group; binding of BH_4 to NOS may, like heme, involve a cysteine residue.[10]

The majority of NOS inhibitors are modified arginines, other guanidines, or bioisosteric equivalents, such as amidines, *S*-alkylisothioureas, and iminoazaheterocycles.[11,12] L-Thiocitrulline,[13] obtained by replacement of L-citrulline's ureido oxygen with sulfur, results in potent inhibition of the enzyme. EPR studies[14] have

Key words: Inhibitors; nitric oxide synthase; tetrahydrobiopterin; dithiocarbamic acid; dithiocarbamic ester.

*Corresponding author. Tel: 301 435 2229; Fax: 301 402 3521.

0968-0896/98/$—see front matter. Published by Elsevier Science Ltd.
PII: S0968-0896(98)00132-1

shown that L-thiocitrulline may act as a sixth axial ligand at the heme cofactor by sulfur-iron coordination. These studies also show that L-arginine perturbs the heme cofactor, but does not act as a sixth ligand.

Several other sulfur-containing compounds are known to inactivate cytochrome P-450.[15] The interaction of cytochrome P-450 with 1-octanethiol shows a characteristic (UV/VIS) spectral shift attributed to iron-thiolate ligation, as does the interaction of BH_4 deficient NOS with dithiothreitol.[16] Pyrrolidine dithiocarbamate (PDTC) inhibits the bacterial lipopolysaccharide (LPS)-induced synthesis of both NOS and BH_4.[17] While PDTC[18] and diethylcarbamate[19] suppress the induction of NOS synthesis by blocking the activation of nuclear factor kB (which, in turn, is stimulated by LPS), the mechanism appears to involve binding of intracellular iron.[20]

These observations led us to explore the possibility that other sulfur compounds, particularly functionalized dithiocarbamates, might inhibit NOS by a mechanism involving heme, BH_4 or both. We began our study with the synthesis of a number of *N*-substituted dithiocarbamic acids and *S*-methyl esters. Each contained a four or five carbon linker (chain or ring), terminating in hydroxyl, carboxyl or tertiary amino group. Biological activity was assessed by competitive binding and functional studies (see below). Only those compounds containing the tertiary amino moiety showed activity. The synthesis and results of preliminary screening of the *N*-(Tertiary aminoalkyl)dithiocarbamates are reported in this paper.

Results and Discussion

While salts of dithiocarbamic acids are stable, the parent acids ordinarily are not, decomposing spontaneously into amines and carbon disulfide. However, when a tertiary amine is present in the same molecule, dithiocarbamic acids, like amino acids, form stable zwitterions. Preparation of the requisite five-carbon diamino compounds, dithiocarbamic acids and esters is described below. They may be categorized in two groups: linear compounds, which are free to rotate; and those derived from 2-aminomethylpyrrolidines, which are conformationally restricted and possess a chiral center at C-2.

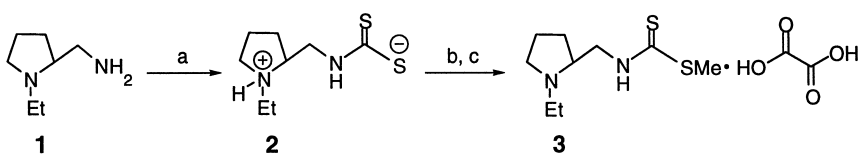
Synthesis

The general method of preparing the parent zwitterionic dithiocarbamic acids is by the reaction of carbon disulfide with tertiary (aminoalkyl)amines. Thus, the reaction of 2-aminomethyl-1-ethylpyrrolidine (**1**) with carbon disulfide in methanol gave *N*-(1-ethylpyrrolidin-2-yl)methyldithiocarbamic acid (**2**) in 90% yield. This was then alkylated with methyl iodide and treated with oxalic acid to obtain a 61% yield of *S*-methyl *N*-(1-ethylpyrrolidin-2-yl)methyldithiocarbamate (**3**) as its oxalate salt (see Scheme 1).

In order to prepare optically active analogues, L-prolineamide (**4**) was reductively methylated with paraformaldehyde and H_2 over 10% palladium on carbon (Scheme 2) to obtain (2*S*)-1-methylpyrrolidine-2-carboxamide (**5**). Recrystallization from acetone afforded an 83% yield as white needles. Reduction of **5** with $LiAlH_4$, gave the chiral diamine (2*S*)-2-aminomethyl-1-methylpyrrolidine (**6**) in 65% yield after purification by vacuum distillation. Compound **6** was treated with carbon disulfide, methyl iodide, and oxalic acid, in the same manner as above, to obtain the optically active dithiocarbamic acid **7** in 95% yield and ester oxalate **8** in 70% yield. The noncyclic conformationally mobile analogues were prepared according to Scheme 3. Alkylation of dimethylamine with 5-bromovaleronitrile (**9**) gave 5-dimethylaminopentanenitrile (**10**), in 98% yield after distillation. Compound **10** was hydrogenated over Raney nickel to obtain 5-dimethylaminopentaneamine (**11**) in 70% yield and 95% purity after distillation, the remaining 5% being unreacted nitrile. Treatment of diamine **11** with carbon disulfide gave a 93% yield of dithiocarbamic acid **12**, which was methylated and treated with oxalic acid to obtain a 70% yield of **13**.

Biological evaluation

Compounds **2**, **3**, **7**, **8**, **12**, and **13** were assayed for biological activity by two methods: inhibition of [^{3}H]-L-N^G-nitroarginine ([^{3}H]-NOARG) binding, using rat brain membranes as the source of neuronal NOS (nNOS), and by functional studies, that is, inhibition of



1 **2** **3**

Scheme 1. Reagents: (a) CS_2, MeOH; (b) Me_4NOH, MeI, MeOH; (c) oxalic acid, acetone-Et_2O.

the conversion of [^{3}H]-L-arginine ([^{3}H]-ARG) to [^{3}H]-L-citrulline ([^{3}H]-CIT), which utilized purified enzyme.

Competitive binding

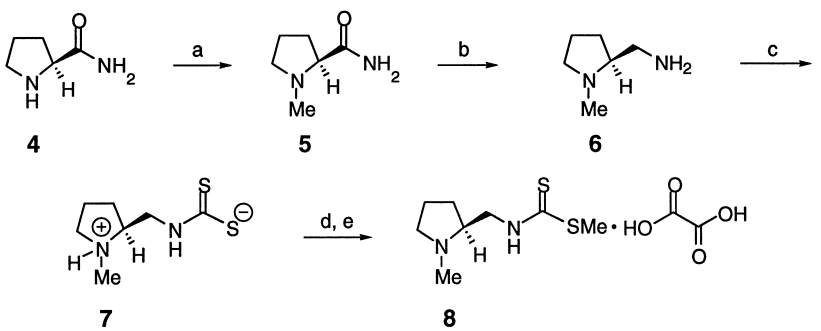
Competitive binding studies were performed by NOVASCREEN®, according to a published procedure.[21] Briefly, reactions were carried out in 50 mM TRIS-HCl (pH 7.4) for 60 min at 25 °C in the presence of 5 nM [^{3}H]-NOARG and 1 μM test compound. Reactions were terminated by rapid vacuum filtration onto glass fiber filters. Radioactivity trapped onto the filters was determined and compared to control values in order to ascertain interactions of test compounds with the enzyme.

The results of the competive binding studies are listed in Table 1 under the heading "Nitroarginine binding". All the compounds showed weak to moderate inhibition of [^{3}H]NOARG binding at a concentration of 1 μM, ranging from 3.7% for the conformationally restricted compound **2** to 20.2% for its *S*-methyl ester, compound **3**. The chiral center in the structurally related optically active dithiocarbamic acid **7**, which showed 9.8% inhibition, and its *S*-methyl ester **8**, which showed 15% inhibition, had no dramatic effect on binding. The conformationally free dithiocarbamic acid **12** and its *S*-methyl ester **13**, which gave 5.2% and 14% inhibition, respectively, showed very similar characteristics to the two sets of conformationally restricted compounds, differing by only a few percentage points. In each case, the *S*-methyl esters showed stronger inhibition than the zwitterionic parents.

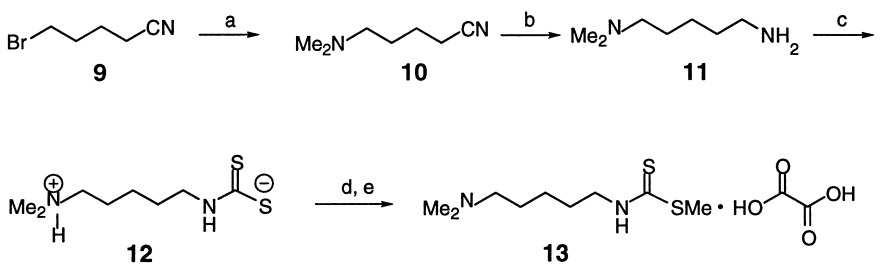
Functional studies

Purification of nNOS. Rat brain nNOS was purified from transfected human kidney 293 cells,[22] by modification of the procedure reported by McMillan and coworkers.[23] All buffers, with the exception of that used in the final gel filtration step, contained 0.5 mM L-arginine, 5 mM BH_4, and 0.5 mM dithiothreitol. The preparation had greater than 95% purity when evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and typically had a specific activity of 250–400 nmol min^{-1} mg^{-1}. The BH_4 content of the purified nNOS was approximately 0.5 mol per mol subunit as determined by high performance liquid chromatography of the oxidized sample.[24] Protein was determined by the bicinchonic acid method[25] with the use of a bovine serum albumin standard.

Determination of nNOS inhibition. nNOS activity was determined by monitoring the conversion of [^{3}H]-ARG



Scheme 2. Reagents: (a) Ch_2O, H_2, MeOH, 10% Pd/C; (b) $LiAlH_4$, THF, Et_2O; (c) CS_2, THF; (d) MeNOH, MeI; (e) oxalic acid, acetone-Et_2O.



Scheme 3. Reagents: Me_2NH, MeOH; (b) H_2, MeOH satd w/NH_3, RaNi; (c) CS_2, THF; (d) NaOH, MeI; (e) oxalic acid, acetone-Et_2O.

Table 1. Effects of dithiocarbamates on the inhibition of binding of nitroarginine and inhibition of nitric oxide synthase activity determined with or without added (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4)

Dithiocarbamate	Nitroarginine binding % competitive inhibition	Nitric oxide synthase activity	
		Minus BH_4 (% inhibition)	Plus BH_4 (% inhibition)
2	3.7	39 [10, $n=8$]	33 [14, $n=8$]
3	20.2	59 [7, $n=10$]	21 [6, $n=10$]
7	9.8	24 [8, $n=6$]	22 [8, $n=6$]
8	15	40 [4, $n=7$]	29 [16, $n=7$]
12	5.2	68 [4, $n=7$]	64 [7, $n=5$]
13	14	3 [6, $n=6$]	2 [4, $n=4$]

Values for percent inhibition are means with standard deviations and number of determinations, respectively, shown in parentheses.

to [^{3}H]-CIT[26] over a 10 min period at 25 °C. Reaction mixtures were prepared containing 5 μM [^{3}H]-ARG, 0.67 mM NADPH, 0.88 μM calmodulin, 0.9 mM $CaCl_2$, 0.1 mM FAD and FMN, 0.6 mM EDTA, 1 mg mL^{-1} catalase, and 33.3 mM HEPES buffer (pH 7.4). BH_4 (10 μM) and dithiocarbamate derivatives (25 μM) or L-thiocitrulline (1 μM) were added as required (see Table 1 and text). Immediately prior to starting the reaction with NOS, a stock solution of enzyme was diluted 100-fold with a solution containing 50 mM Tris–HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mM EDTA, 100 mM NaCl, 1 mg mL^{-1} bovine serum albumin and the protease inhibitors: 4-(2-aminoethyl)benzenesulfonyl fluoride (0.1 mM) leupeptin (0.5 μM), and pepstatin (0.5 μM). The reaction was initiated by adding 5 μL (approximately 0.15 μg) of this dilute nNOS solution to obtain a final volume of 200 μL for the reaction mixture. After the incubation period, reactions were terminated by the addition of 0.8 mL of a solution containing 16.7 mM sodium HEPES (pH 5.5), 1.67 mM EDTA, 8.1 mM L-arginine and 8.1 mM L-citrulline. [^{3}H]-L-Arginine was trapped on a column of Dowex 50 Na^+, and [^{3}H]-L-citrulline in the effluent quantified by liquid scintillation.

Typically, each experiment consisted of 18 separate reaction mixtures designed to measure baseline enzyme activity (in triplicate), activities in the presence of two dithiocarbamate derivatives (each in duplicate) and activities in the presence of the NOS inhibitor L-thiocitrulline (in duplicate). As mentioned in the previous section, purification yields nNOS that is stoichiometrically deficient in BH_4, but still contains approximately 0.5 mol of endogenous, irreversibly bound cofactor per mol of nNOS subunit. Each determination was, therefore, measured in the deficient state obtained by purification and with the addition of BH_4 ("Minus BH_4" and "Plus BH_4" in Table 1). In the absence of inhibitor, nNOS activity was stimulated approximately 25% by the addition of BH_4. Determinations of baseline activities were distributed at the beginning, middle and end of each experiment to detect any progressive loss of enzyme activity during the course of the run. The results of any experiment in which the baseline controls varied by more than 10% of the initial value were discarded.

Control experiments on the inhibition of nNOS activity by L-thiocitrulline (1 μM) showed no effect when exogenous BH_4 was added, giving a mean value of 43% inhibition [12, $n=28$] in the absence of BH_4, and 45% inhibition [14, $n=26$] in the presence of BH_4 (data not in Table 1).

The results of the functional studies are listed in Table 1 under the heading "Nitric oxide synthase activity". The linear, conformationally free dithiocarbamic acid **12**, showed the strongest inhibition, with mean values of 68% in the absence of added BH_4 and 64% with added BH_4. In contrast, its *S*-methyl ester **13** had negligible ability to inhibit the conversion of [^{3}H]-ARG to [^{3}H]-CIT, showing only 2% inhibition with added BH_4 and 3% in its absence.

The racemic, conformationally restricted dithiocarbamic acid **2** and optically active **7**, performed equally well with or without BH_4 and did not differ significantly from one another in their ability to inhibit the enzyme. Their mean values for inhibition [**2**: 39% (minus BH_4), 33% (plus BH_4); **7**: 24% (minus BH_4), 22% (plus BH_4)] suggest a slight increase in potency for the racemate.

As in the case of the competitive binding studies, the two conformationally restricted *S*-methyl esters, **3** and **8**, were somewhat better at inhibition than their parent acids in the absence of added BH_4. This behavior was markedly different than for the conformationally free ester **13**, described earlier. One of the most interesting and unexpected observations was the effect that adding BH_4 had on the conformationally restricted esters. In both cases, their ability to inhibit nNOS activity was reduced upon the addition of BH_4, mean values falling from 59 to 21% for **3** and from 40 to 29% for **8**. A one-tailed paired *t*-test on the raw data showed statistical

relevance, giving $p < 0.01$ for **3** with and without BH_4 and $p < 0.03$ for **8** with and without BH_4.

Conclusions

The *N*-(tertiary aminoalkyl)dithiocarbamic acids and esters in this study appear to have two modes of inhibition, one involving the site for substrate binding and one involving the BH_4 cofactor.

As mentioned earlier, L-thiocitrulline exerts its inhibition by coordinating to the heme cofactor at or near the binding site for L-arginine. Similarly, the strong inhibition of NOS by NOARG is caused by binding to the same site as the substrate.[27,28] Since all the compounds were able to inhibit NOARG binding to some extent, interaction with the L-arginine binding site is implied. The anticipated manner of interaction is between the heme iron and one or both sulfurs of the dithiocarbamates, but we have no direct evidence of this.

The results of the functional studies, inhibition of the conversion of [^{3}H]ARG to [^{3}H]CIT, suggest a second mode of inhibition. While none of the zwitterionic dithiocarbamic acids or L-thiocitrulline controls were affected by the addition of BH_4, both conformationally restricted *S*-methyl esters (**3** and **8**) showed a substantial loss of inhibitory activity when the cofactor was added. It is likely, therefore, that a second mode of inhibition involves the binding site for BH_4 or interaction with the cofactor itself. Dual modes of inhibition of NOS involving the binding sites for L-arginine and for BH_4 are not without precedent. 1-(2-Trifluoromethylphenyl)imidazole was recently reported to exert its inhibition by a similar process;[29] and 7-nitroindazole appears to bind at a distinct site which allosterically interacts with the binding domains for both L-arginine and BH_4.[30]

The divergence of behavior of the linear, conformationally free dithiocarbamic acid–ester pair, compounds **12** and **13**, is dramatic. While acid **12**, which possesses the highest mean values for inhibition, is similar in its inhibitory effect to the conformationally restricted acids **2** and **7**, and is likewise unaffected by the addition of BH_4, its ester (**13**) shows only minimal ability to inhibit the enzyme. The inhibition of [^{3}H]-NOARG binding by **13** is comparable to that of the conformationally restricted esters **3** and **8**. Therefore, the relatively poor inhibition of enzyme activity shown by **13**, may imply a size limitation involving the BH_4 binding site, the conformationally restricted, more compact pyrrolidines having more accessibility.

It is difficult to draw meaningful conclusions concerning structure activity relationships without additional data. However, a trend is evident from this study. The six *N*-(tertiary aminoalklyl)dithiocarbamic acids and *S*-methyl esters show an ability to inhibit nitric oxide synthase activity at the active site of the enzyme by virtue of their capacity to competitively inhibit the binding of [^{3}H]-L-nitroarginine. Functional studies, with and without the addition of BH_4, show the conformationally restricted *S*-methyl esters can also inhibit enzyme activity at a second site, involving the BH_4 cofactor.

Experimental

Biology

Materials used in the functional studies described earlier were obtained from the following sources: beef liver catalase and bovine serum albumin (fraction V, protease free) from Boehringer Mannheim (Indianapolis, IN, USA); high purity bovine brain calmodulin from Calbiochem (San Diego, CA, USA); (6*R*)-5,6,7,8-tetrahydro-L-biopterin from Schircks Laboratories (Jona, Switzerland); [2,3,4,5-^{3}H]-L-arginine from Amersham Corp. (Arlington Heights, IL, USA); FAD from Sigma Chemical Corp. (St. Louis, MO, USA); FMN from Fluka Chemical Corp. (Ronkonkoma, NY, USA); L-thiocitrulline from Alexis Corp. (San Diego, CA, USA); and analytical grade Dowex 50 (AG 50W-X8, 200–400) mesh from Bio-Rad Laboratories (Hercules, CA, USA).

Chemistry

THF and Et_2O used for reaction solvents were freshly distilled from sodium/benzophenone ketyl. All other chemical reagents and solvents were purchased from Aldrich and used without further purification. ^{13}C and ^{1}H NMR data were obtained at 50 and 200 MHz, respectively. Chemical shifts are reported in ppm (δ) downfield of tetramethylsilane and coupling constants are given in Hertz. Mass spectra were obtained by GC/MS analysis, using a 12.0 meter HP-5 capillary (5% diphenyl/95% dimethylpolysiloxane) or by direct injection probe (DIP) for zwitterionic compounds. Microanalyses were performed by Galbraith Laboratories.

***N*-(1-Ethylpyrrolidin-2-yl)methyldithiocarbamic acid (2).** To a rapidly stirring solution of 1-(aminomethyl)-1-ethylpyrrolidine[31] (**1**) (1.28 g, 10.0 mmol) in 10 mL MeOH was added carbon disulfide (1.2 mL, 20 mmol). An exothermic reaction gave a heavy pale yellow precipitate within several seconds. After cooling to room temperature, 10 mL Et_2O was added, the precipitate collected, rinsed with Et_2O and air-dried to obtain 1.85 g (90%) of the zwitterionic title compound as a pale yellow powder, mp 156–157 °C (d); ^{1}H NMR (MeOH-d_4 + NaOD/D_2O) δ 1.15 (t, $J = 7.2$, 3H), 1.59–2.04 (m, 4H), 2.16–2.40 (m, 2H), 2.72–3.19 (m, 3H), 3.40 (d, $J = 7.1$ of d, $J = 13.4$, 1H), 3.92 (d, $J = 4.0$, of d, $J = 13.4$, 1H), 5.78

(s, 1H); ^{13}C NMR (MeOH-d_4 + NaOD/D_2O) δ 13.9, 23.4, 29.5, 49.6, 51.3, 54.4, 63.5, 214.0; EIMS (DIP) *m/z* 205 (MH^+), 98 (base). Anal. calcd for $C_8H_{16}N_2S_2$: C, 47.02; H, 7.89; N, 13.71; found: C, 47.38; H, 8.11; N, 13.55.

***S*-Methyl *N*-(1-ethylpyrrolidin-2-yl)methyldithiocarbamate oxalate (3).** Compound **2** (716 mg, 3.50 mmol) and tetramethylammonium hydroxide pentahydrate (634 mg, 3.50 mmol) were combined in 14 mL MeOH to give a bright yellow solution. Methyl iodide (240 mL, 3.8 mmol) was added all at once via syringe, instantaneously producing a white precipitate of tetramethylammonium iodide. After stirring 10 min, the mixture was diluted with 14 mL Et_2O, the precipitate removed by filtration and rinsed with 25 mL Et_2O, and the combined filtrate and rinse evaporated under reduced pressure. The residue was redissolved in 20 mL Et_2O, washed with H_2O (4×10 mL), brine (1×10 mL) and dried over Na_2SO_4/K_2CO_3. This was filtered and allowed to slowly drip into a solution of oxalic acid (315 mg, 3.50 mmol) in 20 mL acetone. The resulting mixture was warmed slightly to dispel cloudiness and allowed to stand at room temperature several hours to obtain 661 mg (61%) of the product as a crystalline solid. For analytical purposes, a small portion was recrystallized from boiling acetone, mp 111–113 °C (d); ^{1}H NMR (DMSO-d_6) δ 1.21 (pseudo t, 3H), 1.71–2.19 (m, 4H), 2.54 (s, 3H overlapping solvent signal), 2.94–3.35 (m, 4H), 3.47–4.09 (m, 4H), 8.96 (bs, 2H), 10.50 (bs, 1H); ^{13}C NMR (DMSO-d_6) δ 10.3, 17.4, 21.7, 27.6, 46.1, 48.6, 52.8, 63.4, 165.0, 199.1; EIMS (free base) *m/z* 170 ($M^+ - HSCH_3$), 98 (base). Anal. calcd for $C_{11}H_{20}N_2O_4S_2$: C, 42.84; H, 6.54; N, 9.08; found: C, 42.86; H, 6.66; N, 9.03.

(2*S*)-1-Methylpyrrolidine-2-carboxamide[32] (5). L-Prolineamide (**4**) (1015 mg, 8.89 mmol) and paraformaldehyde (293 mg, 9.76 mmol) were dissolved in 18 mL MeOH. To this was added 10% Pd/C (96 mg, 0.090 mmol) and the mixture hydrogenated 14 h at 60 psig. The catalyst was removed by filtration (Whatman #5 filter paper), rinsed with 10 mL MeOH, and the filtrate evaporated under reduced pressure to obtain a white powder. Recrystallization from a minimal volume of boiling acetone followed by chilling to dry ice temperature gave 945 mg (83%) of the title compound as white needles, mp 140–141 °C; ^{1}H NMR (DMSO-d_6) δ 1.60–1.77 (m, 3H), 1.97–2.26 (m [containing 2.26 (s)] 5H), 2.49–2.62 (m, 1H), 2.95–3.04 (m, 1H), 7.00 (bs, 1H), 7.11 (bs, 1H); ^{13}C NMR (DMSO-d_6) δ 23.4, 30.3, 41.2, 56.2, 69.1, 175.6; EIMS *m/z* 128 (M^+), 84 (base); $[\alpha]^{21}_D$ −0.83 (*c* 0.75, MeOH). Anal. calcd for $C_6H_{12}N_2O$: C, 56.22; H, 9.44; N, 21.86; found: C, 56.08; H, 9.29; N, 21.50.

(2*S*)-2-Aminomethyl-1-methylpyrrolidine (6). A 1.0 M commercial solution of $LiAlH_4$ (20 mL, 20 mmol) in THF, was added to 20 mL dry Et_2O in a 250 mL 3-neck flask equipped with condenser, magnetic stir-bar, addition funnel and inlet for argon. Compound **5** (1.28 g, 10.0 mmol) in 50 mL THF was added dropwise over 30 min. On completion, the slightly cloudy reaction solution was stirred at room temperature 60 min, then at reflux 18 h. The resulting opaque milky-white solution was cooled to room temperature and quenched by the careful addition of 2 mL satd Na_2SO_4. Inorganic salts were removed by filtration and rinsed liberally with Et_2O. Solvent was removed under reduced pressure and the residue distilled at 55–57 °C (20 mbar) to obtain 0.74 g (65%) of the title compound. ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 2H), 1.54–1.99 (m, 4H), 2.12–2.28 (m, 2H), 2.32 (s, 3H), 2.63–2.81 (m, 2H), 3.01–3.10 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 22.2, 27.9, 40.5, 43.7, 57.2, 67.2; EIMS *m/z* 115 (MH^+), 114 (M^+), 84 (base); $[\alpha]^{21}_D$ −2.04 (*c* 2.6, MeOH). For microanalysis, the dihydrochloride was prepared by adding excess ethereal HCl to a methanolic solution of free base. Anal. calcd for $C_6H_{16}ClN_2$: C, 38.51; H, 8.62; N, 14.93; found: C, 38.53; H, 8.62; N, 14.89.

***N*-[(2*S*)-1-Methylpyrrolidin-2-yl]methyldithiocarbamic acid (7).** To a solution of compound **6** (509 mg, 4.43 mmol) in 4.5 mL MeOH was added carbon disulfide (0.5 mL, 8.3 mmol) via syringe. Precipitation of the product began within several seconds. After stirring 1 min, 4.5 mL THF was added and the zwitterionic product collected, rinsed with Et_2O and air-dried to obtain 804 mg (95%) as a white salt, mp 165–166 °C (d); ^{1}H NMR (MeOH-d_4 + NaOD/D_2O) δ 1.65–2.19 (m, 4H), 2.22–2.32 (m, 1H), 2.42 (s, 3H), 2.62 (m, 1H), 3.09 (m, 1H), 3.39–3.52 (m, 2H), 3.98 (d, J = 3.9 of d, J = 13.4, 1H), 6.43 (s, 1H); ^{13}C NMR (MeOH-d_4 + NaOD/D_2O) δ 23.3, 29.7, 41.4, 50.9, 58.1, 65.3, 214.5, EIMS (DIP) *m/z* 190 (M^+), 84 (base); $[\alpha]^{21}_D$ −1.62 (*c* 2.0, 50% MeOH/50% 1 N NaOH). Anal. calcd for $C_7H_{14}N_2S_2$: C, 44.18; H, 7.41; N, 14.72; found: C, 44.08; H, 7.45; N, 14.55.

***S*-Methyl *N*-[(2*S*)-1-methylpyrrolidin-2-yl]methyldithiocarbamate oxalate (8).** Following the same procedure as for compound **3**, 717 mg (70%) product was obtained, in three crops, as a white powder, mp 100–101 °C; ^{1}H NMR (DMSO-d_6) δ 1.70–2.18 (2 overlapping m's, 4H), 2.54 (s, 3H), 2.78 (s, 3H), 2.93–3.06 (m, 1H), 3.46–3.61 (m, 2H), 3.80–3.90 (m, 1H), 4.04–4.13 (m, 1H), 10.49 (bs, 1H), 11.30 (bs, 1H); ^{13}C NMR (DMSO-d_6) δ 17.7, 21.7, 28.0, 39.6, 46.1, 55.9, 65.2, 165.5, 199.2; $[\alpha]^{23}_D$ + 0.18 (*c* 1.5,MeOH). Anal. calcd for $C_{10}H_{18}N_2O_4S_2$: C, 40.80; H, 6.16; N, 9.52; found: C, 41.18; H, 6.41; N, 9.74.

5-Dimethylaminopentanenitrile[33] (10). 5-Bromovaleronitrile (1.62 g, 10.0 mmol) and 40% aq dimethylamine

(3.8 mL, 30 mmol) were dissolved in 20 mL THF and refluxed 120 min under argon. After cooling to room temperature, K_2CO_3 (2.76 g, 20.0 mmol) was added, followed by 5 mL H_2O. Stirring 10–15 min gave two clear phases, which were separated and the aqueous portion extracted with Et_2O (2×20 mL). The combined organic portions were dried over Na_2SO_4/K_2CO_3, the volatiles removed under reduced pressure, and the residue distilled from anhydrous K_2CO_3 at 50–51 °C (0.8 torr) to obtain 1.24 g (98%) of the title compound as a colorless liquid; ^{1}H NMR ($CDCl_3$) δ 1.43 (bm, 4H), 1.98–2.18 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 16.4, 22.8, 26.0, 44.9, 58.0, 119.1; EIMS *m*/*z* 127 (MH^+), 58 (base).

5-Dimethylaminopentaneamine[33] (11). In a 60 mL Parr bottle, compound **10** (1.63 g, 12.9 mmol) was dissolved in 20 mL MeOH and a 50% aqueous slurry of W-2 Raney nickel (580 mg, 4.9 mmol) added. The mixture was packed in dry ice and saturated with anhydrous NH_3 by introducing a slow stream of bubbles over 15 min. After warming to room temperature, the bottle was sealed and hydrogenated 18 h at 65 psig. Catalyst was removed by filtration and the filtrate evaporated under reduced pressure using added acetonitrile to remove residual water as an azeotrope. Distillation of the residue at 51–52 °C (0.6 torr) gave 1.18 g (70%) product as a clear, colorless liquid. Analysis by GC/MS showed 95% purity, the remaining 5% being unreacted starting material. ^{1}H NMR ($CDCl_3$) δ 1.25–1.55 (m, 6H), 1.70 (bs, 2H), 2.21–2.29 (overlapping s and t, 8H), 2.69 (pseudo t, 2H); ^{13}C NMR ($CDCl_3$) δ 24.8, 27.5, 33.6, 42.1, 45.4, 59.8; EIMS *m*/*z* 130 (M^+), 58 (base).

***N*-(5-Dimethylaminopentan-1-yl)dithiocarbamic acid (12).** Compound **5** (1.10 g, 8.45 mmol) was dissolved in 8.5 mL dry THF and carbon disulfide (1.0 mL, 17 mmol) was added all at once. The resulting exothermic reaction immediately produced a white precipitate, which was triturated with a small volume of hot MeOH, then collected, rinsed with Et_2O and air-dried to obtain 1.63 g (93%) of the zwitterionic product, mp 157–159 °C (d); ^{1}H NMR (MeOH-d_4 + NaOD/D_2O) δ 1.37–1.74 (m, 6H), 2.24–2.36 (overlapping m and s, 9H), 3.58 (t, J = 7.1, 2H), 6.17 (s, 1H); ^{13}C NMR (MeOH-d_4 + NaOD/D_2O) δ 25.8, 27.8, 29.4, 45.5, 48.7, 60.5, 213.4, EIMS (DIP) *m*/*z* 206 (M^+), 58 (base). Anal. calcd for $C_8H_{18}N_2S_2$: C, 46.56; H, 8.79; N, 13.57; found: C, 46.60; H, 9.04; N, 13.43.

***S*-Methyl *N*-(5-dimethylaminopentan-1-yl)dithiocarbamate (13).** Compound **12** (206 mg, 1.00 mmol) was suspended in 1 mL MeOH and neutralized with 1 mL 1 N NaOH. Methyl iodide (65 mL, 1.0 mmol) was added all at once (via syringe) to the resulting solution. After stirring 2 h at room temperature, the reaction mixture was diluted with 5–6 mL Et_2O and separated. The aqueous portion was extracted with Et_2O (2×5 mL) and the combined organic portions dried over Na_2SO_4/K_2CO_3. This was filtered and allowed to drip into a solution of oxalic acid (90 mg, 1.00 mmol) in 2 mL acetone and the resulting solution stored at −20 °C overnight. The product, 217 mg (70%), was obtained as a white salt, mp 100–101 °C; ^{1}H NMR [@ 60 °C (DMSO-d_6)] δ 1.27–1.38 (m, 2H), 1.54–1.68 (m, 4H), 2.51 (s, 3H), 2.67 (s, 6H), 2.87–2.95 (m, 2H), 3.58–3.61 (m, 2H), 9.99 (bs, 1H), 10.90 (vbs, 2H); ^{13}C NMR [@ 60 °C (DMSO-d_6)] δ 17.1, 23.2, 23.3, 26.8, 42.0, 46.2, 56.4, 165.0, 197.1; EIMS (free base) *m*/*z* 172 ($M^+ - HSCH_3$), 58 (base). Anal. calcd for $C_{11}H_{22}N_2O_4S_2$: C, 42.56; H, 7.14; N, 9.02; found: C, 42.90; H, 7.30; N, 8.98.

Acknowledgements

The authors gratefully acknowledge the NIMH/NOVASCREEN® Psychotherapeutic Drug Discovery and Development Program (Contract #NO1MH20003) for the in vitro assays of [^{3}H]-NOARG binding and Mr. K. Park for assisting in the NOS inhibition assays.

References and Notes

1. Griffith, O. W.; Stuehr, D. J. *Ann. Rev. Physiol.* **1995**, *57*, 707.

2. Stuehr, D. J. *Ann. Rev. Pharmacol. Tox.* **1997**, *37*, 339.

3. Mayer, B.; Werner, E. R. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1995**, *351*, 453.

4. Gorren, A. C. F.; List, B. M.; Schrammel, A.; Pitters, E.; Hemmens, B.; Werner, E. R.; Schmidt, K.; Mayer, B. *Biochemistry* **1996**, *35*, 16735.

5. Griscavage, J. M.; Fukuto, J. M.; Komori, Y.; Ignarro, L. J. *J. Biol. Chem.* **1994**, *269*, 21644.

6. Werner, E. R.; Pitters, E.; Schmidt, K.; Wachter, H.; Werner-Felmayer, G.; Mayer, B. *Biochem. J.* **1996**, *320*, 193.

7. Pfeiffer, S.; Gorren, A. C. F.; Pitters, E.; Schmidt, K.; Werner, E. R.; Mayer, B. *Biochem. J.* **1997**, *328*, 349.

8. Presta, A.; Siddhanta, U.; Wu, C.; Sennequier, N.; Huang, L.; Abu-Soud, H. M.; Ersurum, S.; Stuehr, D. J. *Biochemistry* **1998**, *37*, 298.

9. Masters, B. S. S.; McMillan, K.; Sheta, E. A.; Nishimura, J. S.; Roman, L. J.; Martasek, P. *FASEB J.* **1996**, *10*, 552.

10. Chen, P.-F.; Tsai, A. L.; Wu, K. K. *Biochem. Biophys. Res. Commun.* **1995**, *215*, 1119.

11. Southan, G. J.; Szabó, C. *Biochem. Pharmacol.* **1996**, *51*, 383.

12. Moore, W. M.; Webber, R. K.; Fok, K. F.; Jerome, G. M.; Connor, J. R.; Manning, P. T.; Wyatt, P. S.; Misko, T. P.; Tjoeng, F. S.; Currie, M. G. *J. Med. Chem.* **1996**, *39*, 669.

13. Frey, C.; Narayanan, K.; McMillan, K.; Spack, L.; Gross, S. G.; Masters, B. S.; Griffith, O. W. *J. Biol. Chem.* **1994**, *269*, 26083.

14. Salerno, J. C.; Frey, C.; McMillan, K.; Williams, R. F.; Masters, B. S. S.; Griffith, O. W. *J. Biol. Chem.* **1995**, *270*, 27423.

15. De Matteis, F. *Mol. Pharmacol.* **1974**, *10*, 849.

16. Gorren, A. C. P.; Schrammel, A.; Schmidt, K.; Mayer, B. *Biochemistry* **1997**, *36*, 4360.

17. Hattori, Y.; Nakanishi, N.; Kasai, K.; Shimoda, S. I.; Gross, S. S. *Eur. J. Pharmacol.* **1996**, *296*, 107.

18. Sherman, M. P.; Aeberhard, E. E.; Wong, V. Z.; Griscavage, J. M.; Ignarro, L. J. *Biochem. Biophys. Res. Commun.* **1993**, *191*, 1302.

19. Múlsch, A.; Schray-Utz, B.; Mordvintec, P. I.; Hauschildt, S.; Busse, R. *FEBS Lett.* **1993**, *321*, 215.

20. Schreck, R.; Meier, B.; Männel, D. N.; Dröge, W.; Baeuerle, P. A. *J. Exp. Med.* **1992**, *175*, 1181.

21. Michel, A. D.; Phul, R. K.; Stewart, T. L.; Humphrey, P. P. A. *Br. J. Pharmacol.* **1993**, *109*, 287.

22. Bredt, D. S.; Hwang, P. M.; Glatt, C. E.; Lowenstein, C.; Reed, R. R.; Snyder, S. H. *Nature* **1991**, *351*, 714.

23. McMillan, K.; Bredt, D. S.; Hirsch, D. J.; Snyder, S. H.; Clark, J. E.; Masters, B. S. S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11141.

24. Fukushima, T.; Nixon, J. C. *Anal. Biochem.* **1980**, *102*, 176.

25. For details of this method, refer to *Pierce BCA Application Note #11. The Sorensen Method: Use of BCA for determination of protein and reducing agents. Use of BCA to locate enzyme conjugates*. Product #23225, Pierce, P.O. Box 117, Rockford, IL 61105–9976.

26. Bredt, D. S.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 682.

27. Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. *Biochemistry* **1993**, *32*, 8512.

28. Klatt, P.; Schmidt, K.; Brunner, F.; Mayer, B. *J. Biol. Chem.* **1994**, *269*, 1674.

29. Handy, R. L. C.; Moore, P. K. *Life Sci.* **1997**, *60*, PL389.

30. Mayer, B. In *Nitric Oxide in the Nervous System*; Vincent, S. R., Ed.; Academic: New York, 1995; pp 21–42.

31. Reitsema, R. H. *J. Am. Chem. Soc.* **1949**, *71*, 2041.

32. For the racemic compound, see: Renshaw, R. R.; Cass, W. E. *J. Am. Chem. Soc.* **1939**, *61*, 1195.

33. Stewart, J. M. *J. Am. Chem. Soc.* **1954**, *76*, 3228.