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Synthesis and Evaluation of Alternative Substrates for Arginase[1]

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Two novel carboxyl-containing arginase substrates, 4-guanidino-3-nitrobenzoic acid and 4-guanidino-2-nitrophenylacetic acid, have been synthesized and found to give enhanced catalysis and dramatically lower K_m values relative to 1-nitro-3-guanidinobenzene, a substrate designed for use in a chromophoric arginase assay. To more efficiently mimic the natural substrate, a series of sulfur analogs of L-arginine were synthesized and kinetically characterized. The parent compound, L-thioarginine, with the bridging guanidinium nitrogen of L-arginine replaced with sulfur, functions as efficiently as the natural substrate. The desamino analog shows extremely low turnover, while the k_{cat} of the descarboxy analog is only 75-fold lower than that of arginine. These results suggest that the bridging nitrogen of L-arginine is not important for either substrate binding or catalysis, while the α-carboxyl group facilitates substrate binding, and the α-amino group is necessary for efficient catalysis. Isothiourea homologs previously reported to be nitric oxide synthase inhibitors have been found to undergo a rapid non-enzymatic rearrangement to a species that is probably the true inhibitor.

Key Words: arginase; specificity; alternative substrates; thio analogs.

INTRODUCTION

Arginase is a ubiquitous enzyme that has been found in mammals (*1*), reptiles (*2*), plants (*3*), fungi (*4*), and bacteria (*5*). In mammals, arginase is found in liver (*1*) and also in nonheptatic tissues including red blood cells (*6*), lactating mammary gland, and kidney (*1*). Arginase is a trimeric manganese metalloenzyme that catalyzes the hydrolysis of L-arginine in the final step of the urea cycle, releasing urea and L-ornithine. In nonheptatic tissues, arginase plays a critical role in the biosynthesis of proline (*7*) and polyamines (*8*) by regulating the availability of L-ornithine. Arginase also regulates the biosynthesis of the cell-signaling molecule nitric oxide by affecting arginine availability, since both arginase and nitric oxide synthase share arginine as a common substrate (*9*). As a consequence of the reciprocal regulatory roles of arginase and nitric oxide synthase, arginase inhibition has therapeutic potential in treating nitric oxide-dependent smooth muscle disorders such as erectile dysfunction

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(*10*). A significant effort has been directed toward the evaluation of L-arginine analogs as possible arginase inhibitors (*11–15*).

A continuous chromophoric arginase assay was recently reported using 1-nitro-3-guanidinobenzene (NGB) as an alternative substrate (*16*). The k_{cat} with NGB is 5-orders of magnitude lower than that of L-arginine, but the K_m is almost identical. We recently reported a more sensitive spectrophotometric arginase assay using L-thioarginine as an alternative substrate (*17*). Thioarginine, in which the bridging guanidinium nitrogen is replaced with sulfur, functions as efficiently as arginine. Therefore a series of NGB and thioarginine analogs were synthesized and evaluated to probe the interactions of selected arginine moieties with rat liver arginase.

MATERIALS AND METHODS

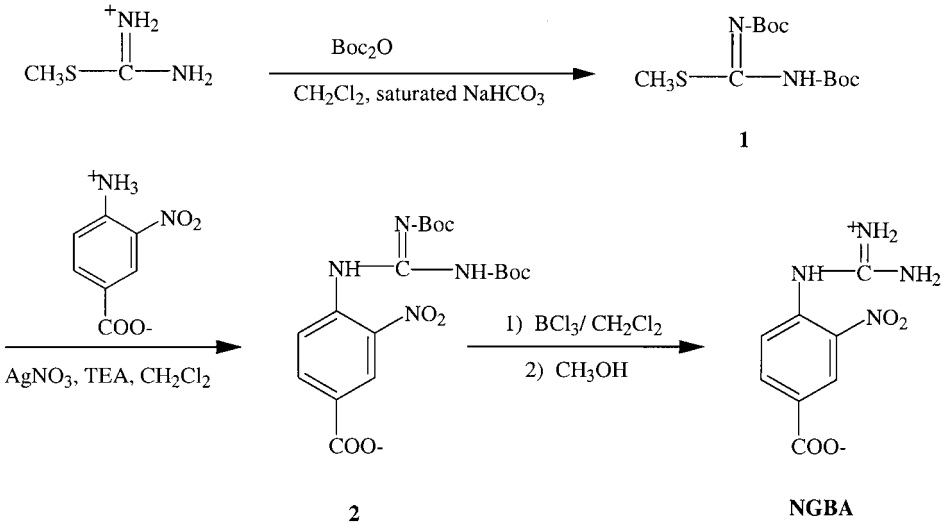
General Methods and Materials. ^{1}H- and ^{13}C-NMR spectra were obtained for all synthetic intermediates and products on a Varian VXRS400. Mass spectra (FAB) were obtained on Brucker Esquire-LC. Rat liver arginase, expressed and purified from *Escherichia coli*, was a gift from Dr. David Ash. *N*-Bromosuccinimide (NBS) was recrystallized from water and dried over P_2O_5 *in vacuo*. 2-(*S*)-*N*-(*t*-butyloxycarbonyl)-glutamic acid-*t*-butyl ester was purchased from Sigma. *S*-(3-aminopropyl)isothiourea hydrobromide and *S*-(2-aminoethyl)isothiourea hydrobromide were obtained from Alexis Biochemicals. All other reagents were purchased from either Aldrich or Lancaster. Synthetic reactions were conducted in oven-dried glassware under argon and all solvents were distilled prior to use. Flash column chromatography was performed on 230–400 mesh silica gel from Lancaster under positive argon pressure. Thin layer chromatography (TLC) was performed on Kodak 13181 plates with fluorescence indicator. Compounds were visualized by iodine chamber, UV light, or a ninhydrin dip (0.1% ninhydrin in 95% *n*-butanol, 4.5% water, 0.5% glacial acetic acid). Protein concentrations were determined by the method of Bradford (*18*), and free thiols were quantitated by Ellman's method (*19*).

Synthesis of 4-guanidino-3-nitrobenzoic acid (NGBA). NGBA was synthesized in three steps through the coupling of *N*-protected pseudothiourea with 4-amino-3-nitrobenzoate (Scheme 1).

N,N′-bis-t-boc-2-methyl-2-thiopseudourea **(1).** A modification of the Bergeron method (*20*) was used for preparation of **1** and **2**. Di-*t*-butyldicarbonate (Boc_2O) (23.2 g, 106.3 mmol) and 2-methyl-2-thiopseudourea (6.2 g, 44.5 mmol) were stirred for 5 days at rt in 100 ml of 1:1/CH_2Cl_2:saturated $NaHCO_3$. Extraction with dichloromethane (100 ml) and purification by flash column chromatography (15% hexanes in $CHCl_3$, then $CHCl_3$) gave **1** (10.0 g, 35.6 mmol) in 80% yield.

4-(N,N′-bis-t-boc-guanidino)-3-nitrobenoic acid **(2).** 4-amino-3-nitrobenzoic acid (1.8 g, 10.0 mmol), silver nitrate (1.8 g, 10.6 mmol), **1** (2.5 g, 10.0 mmol), and triethylamine (TEA) (1.5 ml, 10.8 mmol) were stirred at rt overnight in dichloromethane (250 ml). After filtration and chromatography a yellow solid (1.5 g, 4.0 mmol, 43% yield) was obtained using 10:1 hexanes:ethyl acetate. $R_f = 0.58$ (2:1; ethyl acetate:hexanes).

NGBA. A 10 mL solution of 1.0 M BCl_3 in dichloromethane was introduced dropwise at rt to a stirring solution of **2** (1.5 g, 4.0 mmol) in dichloromethane (40 ml), leading to the formation of a precipitate. The majority of BCl_3 and CH_2Cl_2 was



SCHEME 1. Synthesis of NGBA.

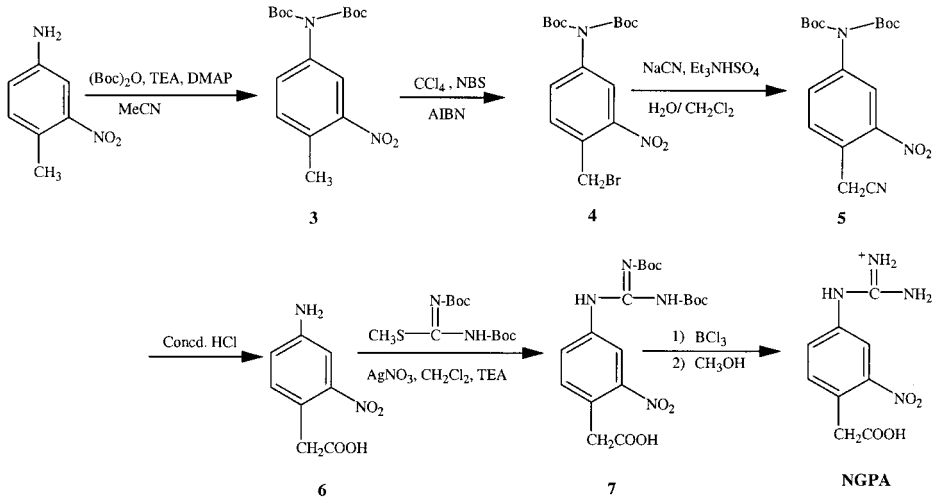
evaporated by a stream of argon and concentrated to a yellow solid which was treated with anhydrous methanol (10 ml). Excess methanol and the resulting $B(OCH_3)_3$ were removed *in vacuo*. Chromatography using 2:1 ethyl acetate:hexanes (R_f = 0.18) yielded NGBA (0.7g, 2.7mmol) in 67% yield. mp: 192–194°C; ^{1}H-NMR (DMSO): δ 6.99 (d, 1H, J = 9.2 Hz), 7.23 (s, broad, 1H), 7.75 (s, broad, 2H), 7.85 (dd, 1H, J = 8.4, 2.0 Hz), 7.93 (s, broad, 1H), 8.55 (d, 1H, J = 2.0 Hz). ^{13}C-NMR (DMSO): δ 173.37, 166.93, 148.40, 134.81, 130.32, 126.50, 121.96, 119.43; Elemental analysis, calculated (%): C: 36.87, H: 3.48, N: 21.50; found: C: 36.29, H: 3.80, N: 21.06; MS: (parent ion) $C_8H_9N_4O_4^+$; calculated: 225.2; found: 224.8.

Synthesis of 4-guanidino-2-nitrophenylacetic acid (NGPA). NGPA was synthesized by the oxidation of *t*-boc-protected 4-amino-2-nitrotoluene to the corresponding benzylacetic acid, followed by coupling to protected pseudoisothiourea (Scheme 2).

4-(N,N-bis-t-boc)-amino-2-nitrotoluene **(3).** 4-amino-2-nitrotoluene (2.8 g, 18.4 mmol), Boc_2O (9.0 g, 41.2 mmol), TEA (6.0 ml, 43.1 mmol) and 4-dimethylaminopyridine (DMAP) (0.12 g, 0.98 mmol) were dissolved in acetonitrile (80 ml), stirred at rt for 20 h, and purified by chromatography (10:1 hexanes:ethyl acetate, R_f = 0.41) producing **3** (4.3 g 12.2 mmol) in 65% yield.

(N,N-bis-t-boc)-4-amino-2-nitrophenylbromomethane **(4).** A mixture of **3** (4.3 g, 12.2 mmol), NBS (3.0 g, 17.0 mmol), and 2,2′-azobisisobutylnitrile (AIBN) (0.4 mg, 2.4 μmol) in CCl_4 (200 ml) were heated under reflux under a 100 W bulb. After 20 h, concentration and chromatography (10:1 petroleum ether:ethyl acetate, R_f = 0.56) gave **4** as a white crystalline solid (2.0 g, 4.6 mmol, 40% yield).

4-(N,N-di-t-boc)-amino-2-nitrophenylacetonitrile **(5).** Potassium cyanide (0.6 g, 9.2 mmol), **4** (2.0 g, 4.6 mmol), water (10 ml), dichloromethane (100 ml), and triethylammonium sulfate (0.3 g, 1.5 mmol) were stirred at rt overnight. Extraction



SCHEME 2. Synthesis of NGPA.

with dichloromethane and chromatography (4:1 hexanes:ethyl acetate, $R_f = 0.11$) afforded **5** as a pale yellow solid (1.4 g, 3.7 mmol) in 80% yield.

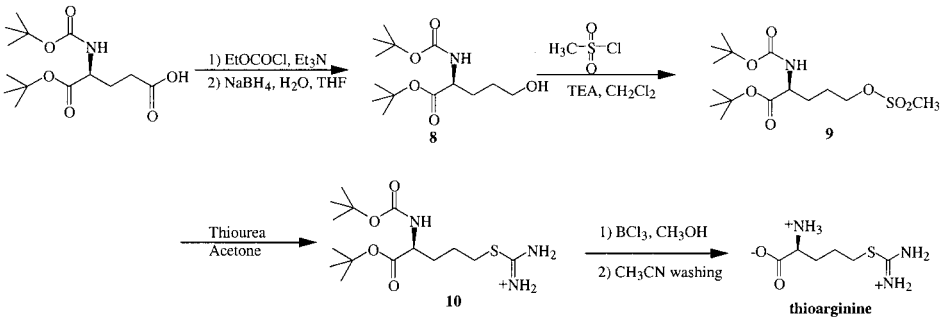
4-amino-2-nitrophenylacetic acid **(6).** Deprotection of the amino group was achieved by adding concentrated HCl (100 ml) to **5** (1.4 g, 3.7 mmol), heating under reflux for 20 h, then concentrating *in vacuo*. Chromatography (1:1 hexanes:ethyl acetate, then 3:3:1 hexanes:ethyl acetate:methanol, $R_f = 0.22$) afforded **6** (0.50 g, 2.6 mmol) in 70% yield.

4-(N,N′-t-boc-guanidino)-2-nitrophenylacetic acid **(7).** *N,N′*-bis-*t*-boc-2-methyl-2-thio-pseudourea (1.0 g, 4.0 mmol), **6** (0.5 g, 2.55 mmol), and $AgNO_3$ (1.2 g, 7.1 mmol) were stirred in anhydrous acetonitrile (100 ml) for 12 h, filtered, and then concentrated. After chromatography (4:1 hexanes:ethyl acetate, then 3:3:1 hexanes:ethyl acetate:methanol, $R_f = 0.45$), **7** (0.6 g, 1.4 mmol) was obtained as a white solid in 54% yield.

NGPA. Compound **7** (0.6 g, 1.4 mmol) was deprotected with 1.0 M BCl_3 in dichloromethane as described above for NGBA. NGPA (0.2 g, 0.73 mmol, 52%) was obtained as a white hygroscopic solid after chromatography with 1:1:1 hexanes:ethyl acetate:methanol, then methanol ($R_f = 0.18$); mp: 162–164°C; ^{1}H-NMR δ: 3.68 (s, 2H), 4.14 (s, broad, 1H), 7.26 (d, 1H, $J = 8.4$ Hz), 7.37 (d, 1H, $J = 8.4$ Hz), 7.58 (s, 1H), 7.98 (broad, 4H); ^{13}C-NMR δ: 174.35, 156.76, 150.49, 134.27, 130.78, 129.11, 128.21, 125.52, 119.60; elemental analysis, calculated (%): C: 39.36, H: 4.04, N: 20.40; found (%): C: 39.10, H: 4.14, N: 19.28; MS: (parent ion) $C_9H_{11}N_4O_4^+$; calculated: 239.2, found: 239.0.

*Synthesis of 2-amino-5-isothioureidovaleric acid (*L*-thioarginine).* L-Thioarginine was synthesized by coupling pseudothiourea to protected L-glutamic acid (Scheme 3).

2(S)-N-t-boc-5-hydroxy-t-butylpentanoate **(8).** Compound **8** was prepared by modification of a reported procedure (*11*). To a solution of 2-(*S*)-*N*-*t*-boc-glutamic acid α-*t*-butyl ester (6.6 g, 21.8 mmol) at −5°C in anhydrous THF (600 ml) was added



SCHEME 3. Synthesis of L-thioarginine.

TEA (24.0 ml, 237 mmol) and ethyl chloroformate (22.5 ml, 234 mmol), the mixture was stirred at −5°C for 20 min and then filtered. Water (25 ml) and then sodium borohydride (6.0 g, 158 mmol) were added to the filtrate and stirred overnight at rt. The supernatant was concentrated to a colorless oil and purified by column chromatography (2:1 hexanes:ethyl acetate) to afford **8** (5.0 g, 17.3 mmol) as a colorless oil in 80% yield.

2(S)-N-t-boc-5-mesyloxy-t-butylpentanoate **(9).** To a solution of **8** (10.0 g, 34.6 mmol), at 0°C was added methanesulfonyl chloride (6.0 g, 953 mmol) and TEA (9.0 g, 81.9 mmol) in CH_2Cl_2 (350 ml). The reaction mixture was stirred on ice for an additional 30 min and then at rt for 3 h. The mixture was washed successively with 4 M HCl (200 ml) and 50% saturated $NaHCO_3$ (200 ml). The organic layer was dried over Na_2SO_4, concentrated, and purified by chromatography (4:1 hexanes:ethyl acetate, $R_f = 0.55$), giving **9** (9.2 g, 25.0 mmol) in 73% yield.

2-(S)-N-t-boc-5-thioguanidino-t-butylpentanoate **(10).** Thiourea (4.0 g, 52.6 mmol) and **9** (9.2 g, 25.0 mmol) were dissolved in acetone (250 ml) and heated overnight under reflux. The concentrate was resuspended in CH_2Cl_2 (200 ml) and filtered. The filtrate was concentrated and purified by chromatography (2:1 hexanes:ethyl acetate, then 1:4:2 methanol:hexanes:ethyl acetate) yielding **10** (5.2 g, 11.7 mmol) in 47% yield.

*2-amino-5-isothioureidovaleric acid (*L*-thioarginine).* Compound **10** (5.2 g, 11.7 mmol) was deprotected as described above for NGBA. Chromatography (ethyl acetate, then methanol) afforded L-thioarginine (2.2 g, 9.6 mmol) as a white solid in 82% yield. ^{1}H-NMR (DMSO): δ 9.33 (s, 4H), 8.56 (s, 3H), 3.90 (t, 1H, $J = 6.4$ Hz), 3.20 (t, 2H, $J = 6.4$ Hz), 1.80 (m, 4H); ^{13}C-NMR (DMSO): 170.61, 169.92, 51.31, 29.33, 28.64, 24.49; Elemental analysis, calculated (%): C: 31.23, H: 6.11, N: 18.21, S: 13.89; found (%): C: 31.09, H: 6.00, N: 17.96, S: 13.83 (these values are corrected for a 7.6% ash content); MS: $C_6H_{14}N_3O_2S^+$: calculated: 192.26, found: 192.10.

Synthesis of 5-thioguanidinovaleric acid. 5-thioguanidinovaleric acid was prepared by modifications to the previously reported synthesis (*21*). Thiourea (0.6 g, 7.9 mmol) and 5-bromovaleric acid (3.0 g, 16.6 mmol) were heated under reflux overnight in acetone (150 ml). The white precipitate (5-thioguanidinovaleric acid) was washed with hot acetone (150 ml) and isolated in 90% yield (1.8 g, 7.0 mmol). mp; 138–140°C

^{1}H-NMR (DMSO): δ 8.99 (broad, s, 4H), 3.14 (t, 2H, J = 6.8 Hz), 1.58 (m, 4H), 2.24 (t, 2H, J = 6.8 Hz); ^{13}C-NMR (DMSO): δ 174.11, 169.85, 32.88, 29.82, 27.83, 23.21; MS: $C_6H_{13}N_2S^+$: calculated: 177.2; found: 177.0; Elemental analysis: $C_6H_{13}N_2SBr$; calculated (%): C: 28.03, H: 5.09, N: 10.89, S: 12.47; found (%): C: 28.21, H: 5.03, N: 10.69, S: 12.81.

Synthesis of S-(4-aminobutyl)isothiourea. This S-substituted isothiourea was synthesized by reacting protected 4-aminobutanol with thiourea (Scheme 4).

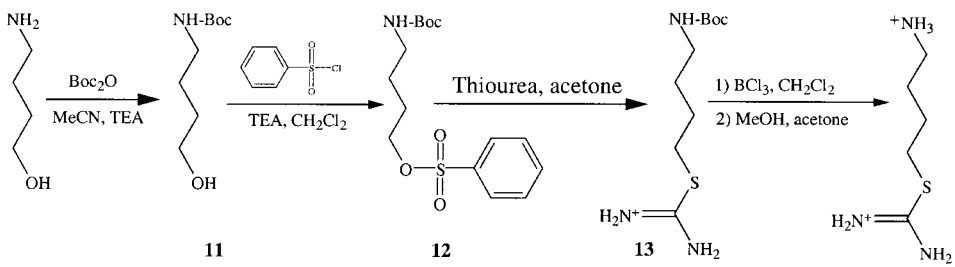
4-N-t-boc-amino-1-butanol **(11).** 4-amino-1-butanol (4.4 g, 49.4 mmol), Boc_2O (10.8 g, 49.5 mmol), and TEA (9.5 ml, 68.2 mmol) were stirred in acetonitrile (250 ml) overnight at rt and then concentrated. The colorless residue was dissolved in ethyl acetate (250 ml), washed successively with 200 ml each of saturated $NaHCO_3$, 3.0 M HCl, and water, and finally dried over Na_2SO_4. A pale yellow sticky liquid was obtained in 96% yield (9.0 g, 47.6 mmol).

4-N-t-boc-amino-1-butanoxyphenyl sulfoxide **(12).** To a solution of **11** (9.0 g, 47.6 mmol) in CH_2Cl_2 (300 ml) at 0°C was added benzenesulfonyl chloride (15.5 g, 87.5 mmol) and TEA (13.0 ml, 87.5 mmol) in CH_2Cl_2 (200 ml). This reaction was worked up as described for the synthesis of **9**. Chromatography (4:1 hexanes:ethyl acetate, R_f = 0.49; then 3:1 ethyl acetate: methanol) gave **12** (10.5 g, 31.8 mmol) in 67% yield.

S-(4-N-t-boc-aminobutyl)isothiourea **(13).** Thiourea (2.4 g, 31.6 mmol) and **12** (10.0 g, 30.4 mmol) were dissolved in acetone (250 ml) and heated under reflux for 30 h and then concentrated. The residue was resuspended in CH_2Cl_2 (200 ml) and filtered. The filtrate was concentrated and purified by chromatography (2:1 hexanes:ethyl acetate, then methanol) yielding **13** in 46% yield (5.7 g, 14.1 mmol).

S-(4-aminobutyl)isothiourea. To a solution of **13** (4.3 g, 10.6 mmol) in CH_2Cl_2 (150 ml) at rt was added 1.0 M of BCl_3 in CH_2Cl_2 (15 ml) dropwise. A white precipitate formed upon the addition of BCl_3. The reaction mixture was further stirred for 2 h. The precipitate was resuspended in 2% methanol/acetonitrile (50 ml), filtered, and washed with acetone to afford the product in 52% yield (1.2 g, 5.5 mmol); ^{1}H-NMR (DMSO): δ 9.31 (s, 4H), 8.18 (s, 3H), 3.19 (t, 2H, J = 6.4 Hz), 2.75 (q, 2H, J = 5.2 Hz), 1.63 (m, 4H); ^{13}C-NMR (DMSO): 170.02, 37.93, 29.42, 25.77, 25.65; Elemental analysis: $C_5H_{15}N_3SCl_2$; Calculated (%): C: 27.28, H: 6.87, N: 19.09; S: 14.56; Found (%): C: 27.47, H: 6.90, N: 18.96, S: 14.45.

Rearrangement of S-(2-aminoethyl)isothiourea. *S*-(2-aminoethyl)isothiourea (0.05 g, 0.18 mmol) was added to 25 ml of degassed 50 mM phosphate buffer, pH 7.4.



SCHEME 4. Synthesis of *S*-(4-aminobutyl)isothiourea.

The reaction mixture was stirred at rt under argon for 15 min and then concentrated to afford a white residue. The product was redissolved in CD_3OD and characterized by ^1H and ^{13}C NMR. ^1H-NMR (CD_3OD): δ 3.23 (t, 2H, J = 6.8 Hz), 2.56 (t, 2H, J = 6.8 Hz); ^{13}C NMR (CD_3OD): δ 158.73, 45.99, 24.44.

Arginase assay with chromophoric products. Methanolic stock solutions of NGBA and NGPA were prepared prior to use. Aliquots of arginase solution (24.6 μg) were added to cuvettes containing 50 mM bicine, pH 9.0, 100 μM $MnCl_2$, and 0.02–2.5 mM concentrations of NGBA or NGPA to a final volume of 1.0 ml. Reaction velocities were measured spectrophotometrically for the hydrolysis of NGBA (ΔE_{410} = 1,347 M^{-1} cm^{-1}) or NGPA (ΔE_{380} = 545 M^{-1} cm^{-1}). Control assays performed in the absence of arginase revealed no detectable hydrolysis of either NGBA or NGPA in the time scale used.

Coupled assay for thioarginine analogs. Stock solutions of selected L-arginine analogs in water (30 mM) and DTNB in methanol (10 mM) were prepared prior to use. Arginase (60 μl, 42 μg) and 10 mM DTNB (20 μl) were added to cuvettes containing 50 mM bicine, pH 9.0, with 0.3–6.0 mM concentrations of thioarginine analogs to a final volume of 1.0 ml. Reaction velocities were measured by observing the formation of 2-nitro-5-thiobenzoate (TNB) at 412 nm. Controls were run without arginase to assess the extent of nonenzymatic hydrolysis. Previous work (*17*) had shown that DTNB hydrolysis at higher pH is negligible for the duration of the assay. For the slowest substrates, particularly 5-thioguanidinovalerate, the nonenzymatic hydrolysis of DTNB was significant, so a fixed point assay was used for these substrates.

Fixed-point assay for thioarginine analogs. The reaction was initiated by adding arginase (0.41 mg) to a mixture containing 0.6 to 4.5 mM 5-thioguanidinovaleric acid in 50 mM Hepes, pH 8.0. At defined intervals aliquots of the reaction mixture were quenched with 1.0 M borate (pH 6.0) to terminate the reaction and adjust the pH to 7.0–7.5. Arginase was removed by ultrafiltration (Amicon) and the thiol concentration in the filtrate was quantitated with 10 μl of 10 mM DTNB in methanol. The change in absorbance was measured and product formation quantitated at 412 nm using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} (*19*).

RESULTS AND DISCUSSION

Synthesis of NGB Analogs. 1-Nitro-3-guanidinobenzene (NGB) has recently been reported as an alternative arginase substrate in a continuous spectrophotometric assay (*16*). Unfortunately, NGB is a poor substrate, with a very low k_{cat} value (0.09 min^{-1}) relative to L-arginine (15,000 min^{-1}). However, the K_m of NGB (1.6 mM) is indistinguishable from that of arginine; an unexpected observation considering the significant structural differences between these two substrates (Fig. 1). Arg21 of arginase was initially implicated in substrate binding *via* an electrostatic interaction with the carboxyl group of arginine (*22*). More detailed structural studies on the binding of the boronate-containing inhibitor-2-amino-6-boronohexanoic acid (*10*), and on the product complex with ornithine and urea (*23*), have identified Asn130 and Ser137 as hydrogen-bond donors to the substrate carboxyl group. Since NGB lacks this carboxyl group it probably binds to arginase primarily by its guanidinium group interacting with Glu277 similar to the way that the guanidinium group of arginine has been shown

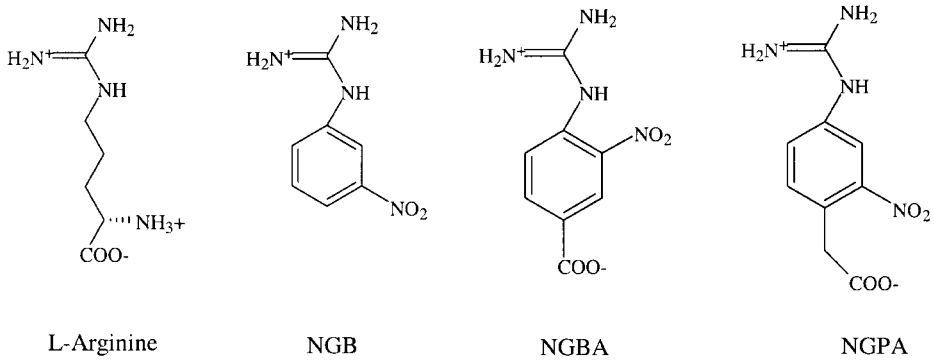


FIG. 1. Structural comparison of NGB analogs to L-arginine.

to bind (*23*). Several carboxyl-containing analogs of NGB have been synthesized to probe this enzyme-substrate interaction in an attempt to design improved alternative substrates of arginase. NGBA (Fig.1) was designed with a carboxyl group attached to the benzene ring, while NGPA was prepared with a methylene spacer between the benzene ring and the carboxyl group.

Both NGBA (Scheme 1) and NGPA (Scheme 2) were synthesized in good overall yield by the coupling of a *t*-boc-protected pseudothiourea to the corresponding nitroanilinic acid. Both products were obtained by deprotection with BCl_3 in dichloromethane. Although efficient removal of the protecting group was achieved, BCl_3 interacts to some extent with the unmasked guanidinium group. Thus, treatment of the crude products with methanol was essential to completely remove BCl_3 by forming trimethyl borate, which was removed *in vacuo* or by washing with acetone. Without this treatment, the residual BCl_3 forms boric acid, a known arginase inhibitor, under the assay conditions (*24*).

Kinetics of NGB Analogs. Enzyme-catalyzed hydrolysis of NGBA and NGPA releases chromophoric nitroaromatic acids whose formation can be observed spectrophotometrically. As predicted, the k_{cat} values for both analogs are enhanced relative to NGB, with a 40-fold increase observed for NGBA and a 60-fold increase for NGPA (Table 1). Although considerably enhanced, these values are still more than 1000-fold lower than that of arginine. Interestingly, the K_m values of NGBA and NGPA (7 and 10 μM, respectively) are significantly reduced and are now approximately 200-fold lower than those obtained for both NGB and for the physiological substrate arginine. Incorporation of a carboxyl group thus results in a 4-order of magnitude enhancement in k_{cat}/K_m for these substrates relative to NGB. These data support the hypothesis that selective placement of a carboxyl group on NGB contributes to tighter binding and enhanced catalysis by keeping the substrate in a more productive orientation.

Synthesis of thioarginine analogs. To further examine the interactions between arginine and arginase, a series of thio analogs of arginine were synthesized and tested for their ability to function as arginase substrates. Each analog contains a sulfur that replaces the bridging guanidinium nitrogen. L-Thioarginine differs from L-arginine

TABLE 1

Kinetic Parameters of Arginase Substrates

Substrates	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	Chromophore measured	Extinction coefficient (M^{-1} cm^{-1})
L-arginine[a]	15,000 ± 1,200	1.4 ± 0.3	1.8×10^5	none[b]	—
NGB[c]	0.09 ± 0.02	1.6 ± 0.2	0.94	1-amino-3-nitrobenzene	1,280
NGBA	3.5 ± 0.2	0.007 ± 0.002	8.3×10^3	4-amino-3-nitrobenzoate	1,350
NGPA	5.4 ± 0.2	0.010 ± 0.002	9.0×10^3	4-amino-2-nitro-phenylacetate	545
L-thioarginine	18,000 ± 1,700	0.5 ± 0.1	6.0×10^5	2-nitro-5-thiobenzoate	13,600
5-thioguanidino-valeric acid	0.8 ± 0.2	2.1 ± 0.6	6.3	2-nitro-5-thiobenzoate	13,600
S-(4-amino-butyl)isothiourea	200 ± 15	4.0 ± 0.5	8.3×10^2	2-nitro-5-thiobenzoate	13,600

[a] Kinetic parameters determined by Cavalli *et al.* (*31*).
[b] Reaction measured by the release of ^{14}C-labeled urea (*32*).
[c] Kinetic parameters determined by Baggio *et al.* (*16*).

only in the substitution of that bridging nitrogen by sulfur (Fig. 2). 5-Thioguanidinovaleric acid lacks the α-amino group and S-(4-aminobutyl)isothiourea is missing the α-carboxylic group. These compounds were prepared to test the role of the bridging nitrogen, the α-amino, and the α-carboxylic group in substrate recognition. Two isothiourea homologs, *S*-(3-aminopropyl)isothiourea and *S*-(2-aminoethyl)isothiourea, were also examined as potential arginase substrates to evaluate the relative importance of the separation between the functional ends of arginine in binding and catalysis.

Stability of the thioarginine analogs. While the arginase-catalyzed hydrolysis of the NGB analogs can be followed by direct observation of the chromophoric products, the enzyme-catalyzed hydrolyses of the selected thioarginine analogs were examined by a coupled assay using DTNB to quantitate the release of a thiol-containing product (*17*). Given the lack of specificity of this reaction it is essential that these analogs be stable under the assay conditions. Thioarginine and 5-guanidinothiovalerate both

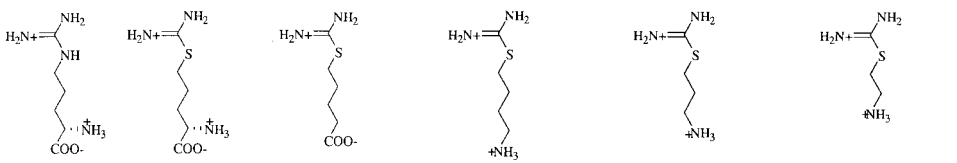


FIG. 2. Structural comparison of thioarginine analogs.

display only minimal production of thiol in the absence of arginase (Fig. 3). However, an appreciable nonenzymic rate of thiol release is observed in the DTNB-coupled assay with the thioisoureas. The rate of thiol production from *S*-(4-aminobutyl)isothiourea is 20-fold greater than that observed with 5-thioguanidinovaleric acid (Fig. 3), while the breakdown of the corresponding aminopropyl and aminoethyl homologs are so rapid that the reaction is complete before a rate can be measured.

Given the similarity of these structures to thioarginine it seems unlikely that thiol production results from hydrolysis of the thioguanidino bond. For these less stable isothioureas there is the possibility of an internal rearrangement in which attack of the amino group on the guanidine carbon leads to cleavage of the C–S bond and production of a thiol-containing product (Scheme 5). The thiols generated by this rearrangement react with DTNB in a manner that is indistinguishable from thiols produced by enzymatic hydrolysis. The nonenzymatically produced thiol-containing product was characterized by ^{1}H and ^{13}C NMR and is consistent with the rearrangment product shown in Scheme 5. There is no NMR evidence for the direct hydrolysis products, urea and 2-aminoethanethiol. When the rearrangement reaction is exposed to air a second set of proton NMR peaks, consistent with oxidation of the thiol rearrangement product, is observed. Conducting the reaction in an inert atmosphere dramatically decreases the amount of thiol oxidation product.

Since the rearrangement of the aminoethyl and aminopropyl isothioureas would proceed *via* five- or six-membered cyclic intermediates, respectively, the reaction

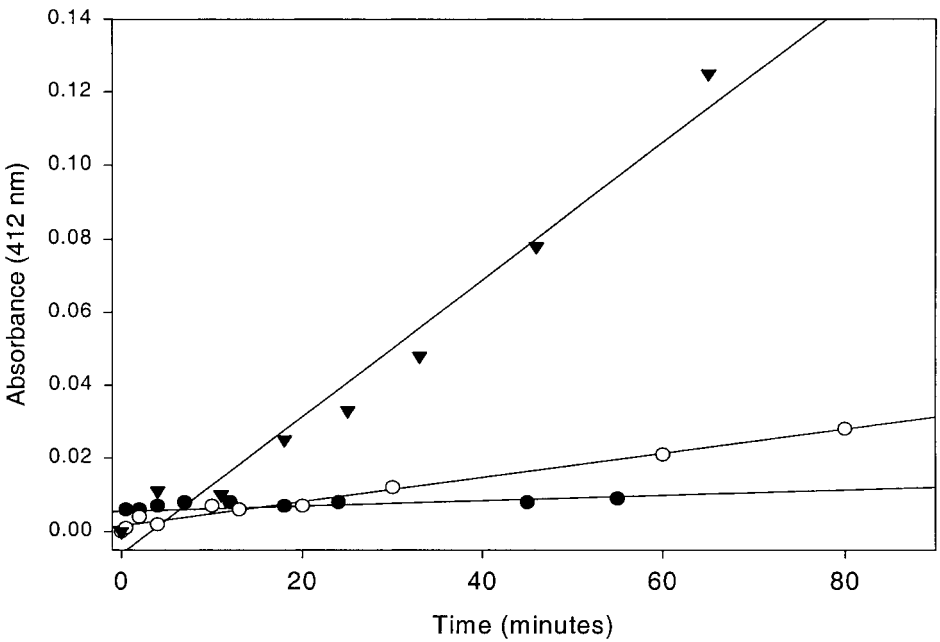
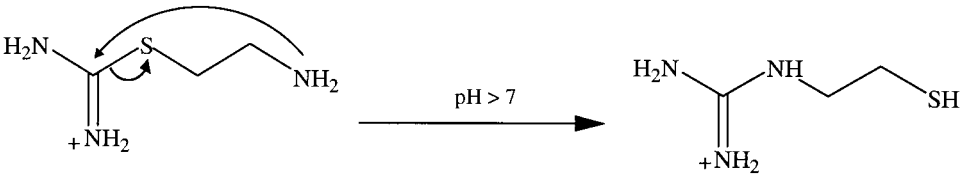


FIG. 3. Rates of nonenzymatic release of thiols. Samples were incubated in 50 mM Hepes, pH 8.0. Aliquots were removed at defined times, quenched by treatment with 1.0 M borate and then quantitated by the addition of DTNB. (•) 5-guanidinothiovaleric acid; (○) L-thioarginine; (▼) *S*-(4-aminobutyl)isothiourea.



SCHEME 5. Rearrangement of *S*-(3-aminoethyl)isothiourea under basic conditions.

with these compounds is expected to be more facile than that with the aminobutyl homolog which would require a seven-membered cyclic intermediate (*25*). The increased stability of thioarginine, which could also rearrange *via* a seven-membered cyclic intermediate, is due to the presence of the neighboring carboxyl group that moderates the nucleophilicity of the attacking amino group.

S-(3-aminopropyl)isothiourea and *S*-(2-aminoethyl)isothiourea have been reported to be potent inhibitors of nitric oxide synthases (*26*) and weak inhibitors of human type II arginase (*27*). Based on the observed rearrangements with these compounds it is likely that the resulting thiols, rather than the parent amines, are the actual inhibitory species.

Kinetics of thioarginine analogs. Enzymatic hydrolysis of the thioarginine analogs releases sulfhydryl-containing products that can be quantified with DTNB. Thioarginine and *S*-(4-aminobutyl)isothiourea were examined in a continuous coupled assay, with the enzyme concentration adjusted to compensate for the lower catalytic rate with the latter compound (Table 1). Under these conditions the rate of the nonenzymatic rearrangement of *S*-(4-aminobutyl)isothiourea is less than 10% of the arginase-catalyzed hydrolysis. Although significantly more stable, attempts to use 5-thioguanidinovaleric acid in this continuous assay were unsuccessful due to the extended durations required, even when assayed at higher enzyme concentrations. Buffer-catalyzed hydrolysis of DTNB during this time liberates the chromophore TNB, which interferes with the arginase-catalyzed reaction. Instead, the fixed-point assay described above was used to measure the enzyme-catalyzed hydrolysis of 5-thioguanidinovaleric acid. A linear increase in absorbance was observed over at least 80 min in the full-time course of this reaction and nonenzymatic hydrolysis under these conditions was less than 1% of the enzymatic rate.

Role of the bridging nitrogen in L*-arginine.* The mechanism of arginase catalysis has been proposed to involve formation of a tetrahedral intermediate after attack of the guanidinium carbon by a $(Mn^{+2})_2$-stabilized hydroxide ion, with subsequent expulsion of the bridging nitrogen to form urea and L-ornithine (*22*,*28*). Replacement of the bridging guanidinium nitrogen of arginine with sulfur gave an alternative substrate with kinetic properties essentially identical to those of the physiological substrate. Thus, the bridging nitrogen in arginine does not appear to play a significant role in either substrate binding or in catalysis. Since sulfur is typically a better leaving group than nitrogen, collapse of the tetrahedral intermediate should proceed more rapidly from the thio analog. The identical kinetics between thioarginine and arginine provide supporting evidence that C–N bond cleavage is not rate-limiting step in arginase catalysis.

Role of the α-carboxyl and α-amino groups. The active site of arginase provides a well-coordinated balance of binding interactions that take place at each end of the substrate. Examination of a series of boronate analogs of L-arginine demonstrated the critical relationship between α-amino to boron distance and IC_{50} values (*12*). The substrate carboxyl group makes important interactions with active site serine and asparagine residues that contribute to substrate binding (*10,23*). The arginase inhibitor, N^{ω}-hydroxy-L-arginine (NOHA) contains an *N*-hydroxyl group that displaces the metal-bridged hydroxide and moves the inhibitor closer to the enzyme-bound metal ions (*23*). This displacement disrupts interactions between the carboxyl end of the inhibitor and the enzyme active site binding groups. The affinity of the descarboxy analog of NOHA (Fig. 4) is 50-fold weaker than that of NOHA for arginase, and the corresponding descarboxy analog of *nor*-NOHA is a 300-fold less potent inhibitor (*23*). Despite the absence of a carboxyl moiety the alternative substrate NGB has a comparable K_m to the physiological substrate arginine. However, the addition of a carboxyl group to NGB results in a 200-fold decrease in K_m. This improved affinity is observed both for NGBA, which contains the same number of bonds between the bridging-nitrogen and the carboxyl group, and NGPA which is one methylene group longer than arginine.

The K_m of 5-thioguanidinovaleric acid, which lacks the α-amino group of thioarginine, is nearly the same as that of thioarginine and arginine. However, its k_{cat} of 0.8 min^{-1} is more than 20,000 times slower than that of thioarginine. Alternatively, *S*-(4-aminobutyl)isothiourea, in which the carboxyl group is replaced by an amine has a k_{cat} of 200 min^{-1}, 250-fold higher than that of 5-thioguanidinovaleric acid, suggesting that the α-amino group plays an essential role in catalysis. Interactions between the substrate amino group and binding groups on arginase (Asp183 and Glu186) may place the scissile guanidinium group in a more productive orientation relative to the metal-bridging hydroxide, resulting in more efficient catalysis.

Implications for inhibitor design. The substrate specificity of arginase is quite broad, accepting a range of structural alterations while retaining catalytic activity. However, the positioning and orientation between the metal-bridged hydroxide and the guanidinium carbon of the substrate is the one essential interaction that must be maintained. Boronate analogs such as 2-amino-6-boronohexanoic acid (*11*) and *S*-(2-boronoethyl)-L-cysteine (*29*) function as transition state analog inhibitors, either by binding of the protonated boronate to displace the metal-bridged hydroxide or binding of the unprotonated trigonal species followed by attack of the metal-hydroxide to generate the tetrahedral intermediate (*27*). N^{ω}-Hydroxy-*nor*-L-arginine (*nor*-NOHA), a very potent inhibitor of arginase (*30*), also functions by displacing the metal-bridging

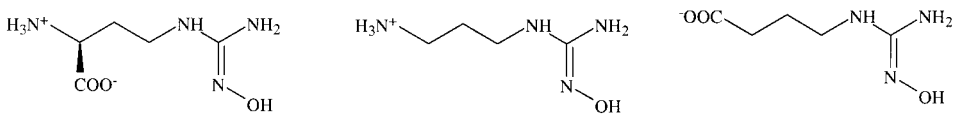


FIG. 4. Structures of *nor*-*N*-hydroxy-L-arginine analogs.

hydroxide ion of arginase with its N^{ω}-hydroxy group upon binding (*23*,*30*). An analog of *nor*-NOHA, descarboxy-*nor*-NOHA (Fig. 4), is almost 20-fold more potent than des-(α-amino)-*nor*-NOHA as an arginase inhibitor (*23*). Binding of the amino group of descarboxy-*nor*-NOHA places the *N*-hydroxyl moiety closer to the metal-bridging hydroxide ion than does des-(α-amino)-*nor*-NOHA, thus positioning the *N*-hydroxy group to replace the bridging hydroxide ion. This observation supports the essential role of the α-amino group in substrate orientation and catalysis.

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