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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

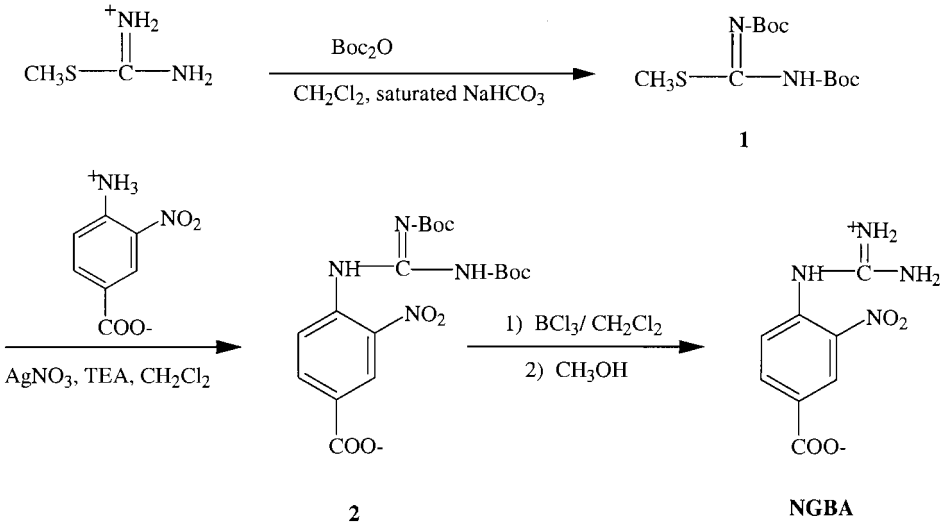
General Methods and Materials. ^{1}H- and ^{13}C-NMR spectra were obtained for all synthetic intermediates and products on a Varian VXRS400. Mass spectra (FAB) were obtained on Brucker Esquire-LC. Rat liver arginase, expressed and purified from *Escherichia coli*, was a gift from Dr. David Ash. *N*-Bromosuccinimide (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.

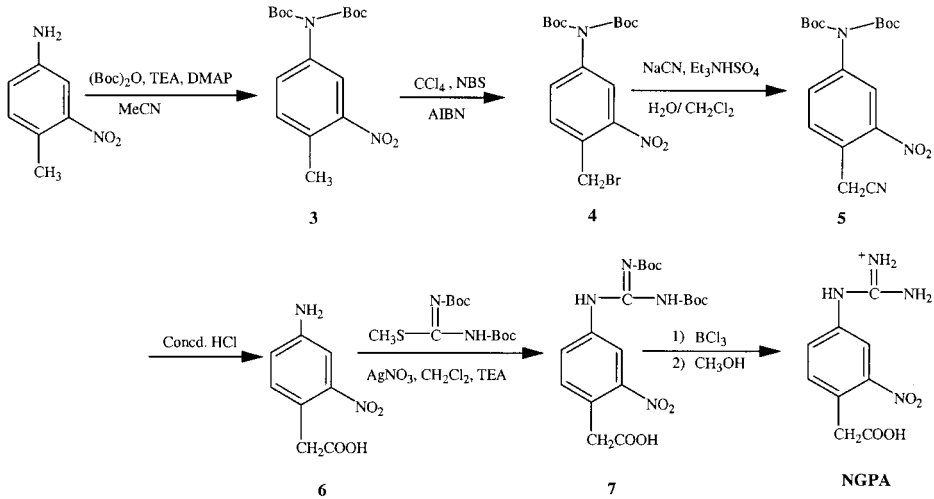
evaporated by a stream of argon and concentrated to a yellow solid which was treated with anhydrous methanol (10 ml). Excess methanol and the resulting $B(OCH_3)_3$ were removed *in vacuo*. Chromatography using 2:1 ethyl acetate:hexanes (R_f = 0.18) yielded NGBA (0.7g, 2.7mmol) in 67% yield. mp: 192–194°C; ^{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.

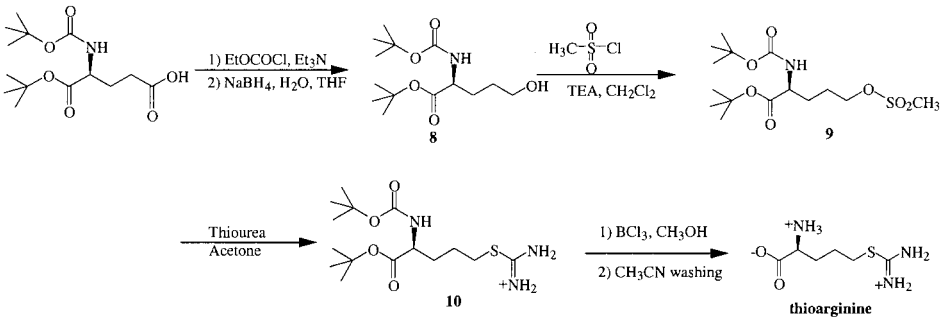
4-amino-2-nitrophenylacetic acid **(6).** Deprotection of the amino group was achieved by adding concentrated HCl (100 ml) to **5** (1.4 g, 3.7 mmol), heating under reflux for 20 h, then concentrating *in vacuo*. Chromatography (1:1 hexanes:ethyl acetate, then 3:3:1 hexanes:ethyl acetate:methanol, $R_f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

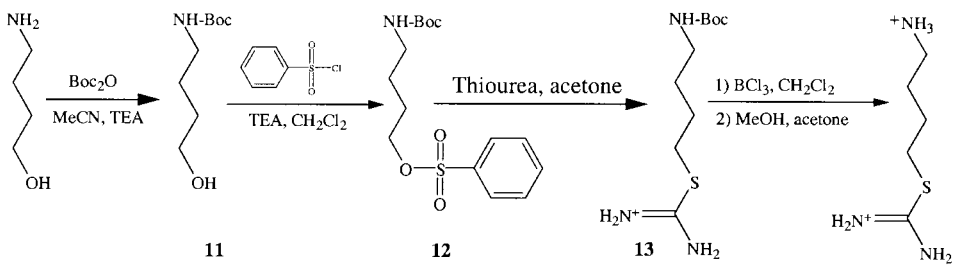
4-N-t-boc-amino-1-butanol **(11).** 4-amino-1-butanol (4.4 g, 49.4 mmol), Boc_2O (10.8 g, 49.5 mmol), and TEA (9.5 ml, 68.2 mmol) were stirred in acetonitrile (250 ml) overnight at rt and then concentrated. The colorless residue was dissolved in ethyl acetate (250 ml), washed successively with 200 ml 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