Asymmetric Synthesis of Conformationally Restricted L-Arginine Analogues as Active Site Probes of Nitric Oxide Synthase

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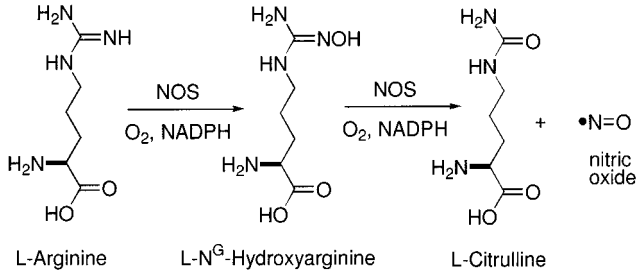
Received October 27, 1998

Using the catalytic asymmetric Sharpless carbamate aminohydroxylation, conformationally restricted L-arginine and L-homoarginine derivatives (**5**−**8**) were prepared in good enantiomeric excess to investigate the binding requirements of L-arginine-based compounds with nitric oxide synthase. The L-arginine derivatives (**5** and **6**) inhibited both the inducible and neuronal isoforms of nitric oxide synthase with little isoform selectivity (**5**, IC_{50} = 42 and 144 μM, **6**, 8 and 12 μM, respectively). The guanidine-containing compound (**5**) did not act as a nitric oxide producing substrate for nitric oxide synthase. The ability of these compounds to interact with the enzyme supports the idea that L-arginine-based inhibitors bind to the enzyme in a folded conformation. The L-homoarginine derivatives (**7** and **8**) did not interact with the enzyme as either substrates or inhibitors. The two-carbon L-arginine homologue (**9**), prepared from L-phenylalanine, demonstrated the greatest isoform selective inhibition of the compounds examined (IC_{50}(iNOS) = 19 and IC_{50}-(nNOS) = 147 μM, IC_{50}(nNOS)/IC_{50}i(NOS) = 7.7). These results suggest isoform selective inhibition may be related to the folded conformations required for binding of these higher L-arginine homologues.

Introduction

Nitric oxide (NO) plays various roles in a number of physiological processes.[1] The biochemical production of nitric oxide results from the stepwise oxidation of the terminal guanidino group of L-arginine to initially produce L-N^G-hydroxyarginine followed by a second oxidation to form nitric oxide and L-citrulline (Scheme 1).[2] The nitric oxide synthases (NOS) catalyze this conversion that requires 2 equiv of molecular oxygen and 1.5 equiv of reduced nicotine adenine dinucleotide phosphate (NADPH) as cosubstrates (Scheme 1).[3] Three distinct mammalian NOS isoforms exist: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed, and inducible NOS (iNOS).[3] The selective inhibition of the various NOS isoforms represents a potential therapeutic approach for disease states where an overproduction of nitric oxide from a particular isoform is implied.[4] A pictorial model of the NOS active site based upon experiments with various inhibitors and substrates includes an amino acid, guanidinium, and heme binding sites.[5,6,7] Recent X-ray crystallographic structures of the inducible NOS oxygenase domain with both inhibitors and substrate provide more detailed active site structural information regarding this isoform including the identification of a glutamic acid residue (Glu^{371}) as a critical binding point for both substrates and inhibitors.[8,9] Despite the discovery of a number of isoform specific inhibitors and the recent X-ray crystallographic studies, the structural basis of isoform selective NOS inhibition remains poorly understood.

Scheme 1



The X-ray crystallographic structure of the inducible NOS oxygenase domain with L-arginine indicates that L-arginine binds to this truncated isoform in a somewhat folded (not fully extended) conformation.[9] Results from a recent study with a group of conformationally restricted L-phenylalanine-derived inhibitors (including **1** and **2**), which display modest selective inhibition of the constitutive NOS isoforms, also suggest that L-arginine-derived inhibitors preferentially bind to the enzyme in a folded conformation.[10] One-carbon homologues of L-arginine also interact with nitric oxide synthase as L-homoarginine

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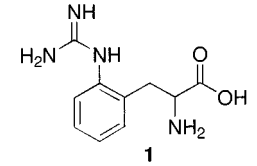
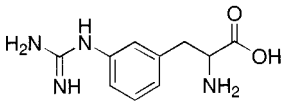
10.1021/jo982161f CCC: $18.00
Published on Web 04/29/1999

acts as an NO-producing substrate and N^G-L-(1-iminoethyl)lysine (**3**) preferentially inhibits the inducible isoform of NOS as compared to N^G-L-(1-iminoethyl)ornithine (**4**), which demonstrates little isoform selectivity.[11,12] L-Homoarginine and **3** must adopt a less than fully extended conformation to interact with the enzyme in a fashion similar to L-arginine or **4**, and these results suggest that differences between the folded conformations of homologous inhibitors may partially explain the structural basis of NOS isoform selective inhibition.



1



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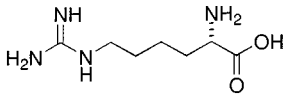
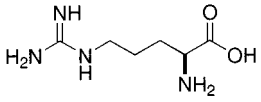
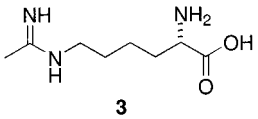


L-homoarginine



L-arginine
(fully extended)



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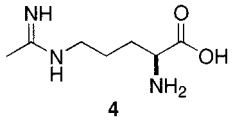
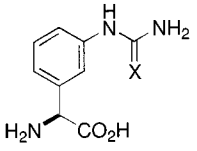
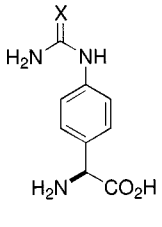


4

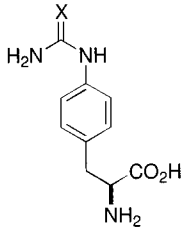
To further examine the effects of conformational restriction on the interaction of homologous substrates and inhibitors with NOS, we have prepared and biochemically evaluated derivatives **5**−**10** as probes of the NOS active site. The guanidine-containing derivatives (**5**, **7**, and **9**) represent constrained analogues of L-arginine, L-homoarginine, and a two-carbon homologue of L-arginine. The thiourea-containing derivatives (**6**, **8**, and **10**) represent conformationally restricted homologues of the known NOS inhibitor L-thiocitrulline.[13]



5, X = NH
6, X = S



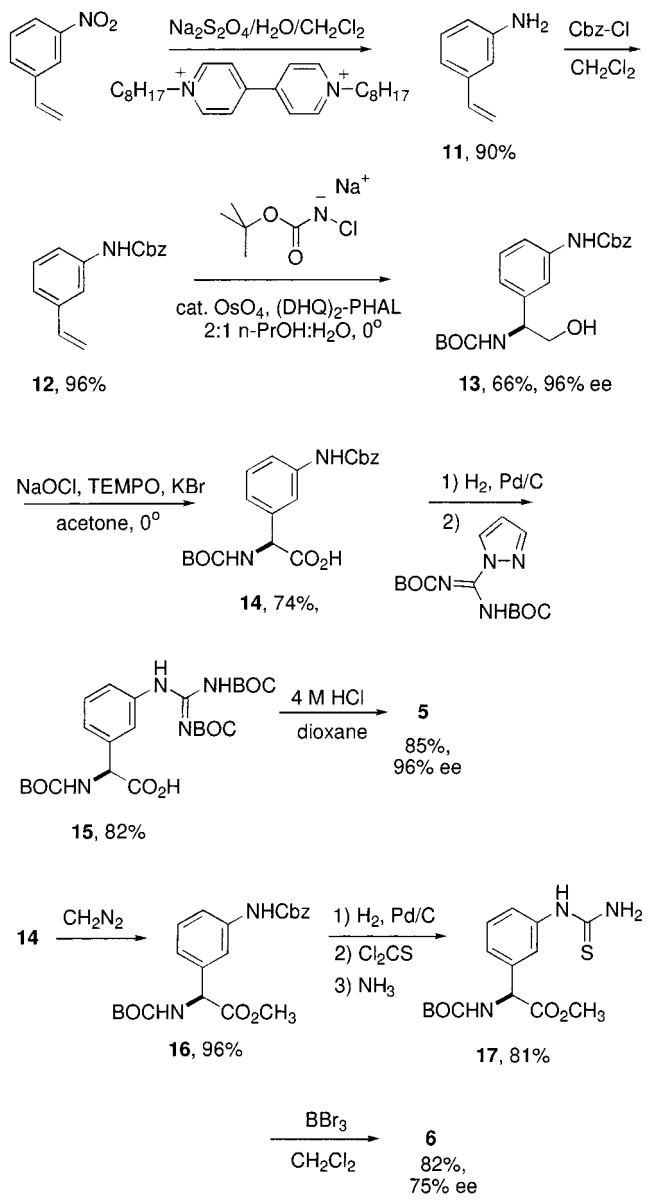
7, X = NH
8, X = S



9, X = NH
10, X = S

The Sharpless carbamate aminohydroxylation combined with an alcohol−carboxylic acid oxidation forms a newly described synthetic strategy for the preparation of enantiopure α-arylglycines from styrenes.[14] Use of the bis(dihydroquinyl)phthalazine ((DHQ)$_2$-PHAL) ligand establishes the *S*-configuration of the amino alcohol stereocenter and leads to the formation of natural L-amino acids. The Sharpless reaction introduces the nitrogen atom protected as either a BOC or Cbz carbamate, an attractive feature for further synthetic manipulations. The asymmetric preparation of the L-phenylglycines (**5**−**8**) using the Sharpless asymmetric aminohydroxylation demonstrates one of the first applications of this newly described methodology to the synthesis of biologically important compounds.[14]

Scheme 2



Results and Discussion

Compounds **5** and **6** were prepared from commercially available 3-nitrostyrene (Aldrich, Scheme 2). Selective nitro group reduction using sodium hydrosulfite and a phase- and electron-transfer catalyst to form amine (**11**) followed by Cbz protection produced **12** in good yield.[15] Gram-scale Sharpless asymmetric aminohydroxylation of **12** using the bis(dihydroquinyl)phthalazine ((DHQ)$_2$-PHAL) ligand yielded the BOC-protected amino alcohol (**13**) in 66% yield and 96% ee after removal of small

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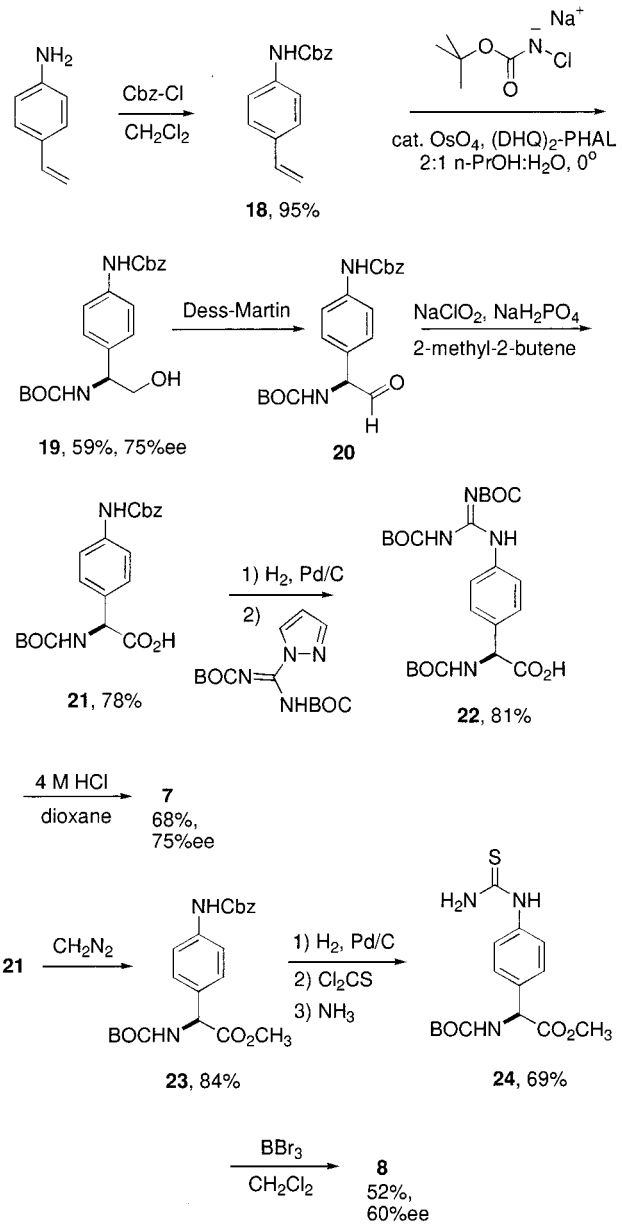
amounts of the unwanted regioisomer and diol by flash chromatography.[14] Oxidation of **13** with sodium hypochlorite, 2,2,6,6,-tetramethyl-1-piperidinyloxy free radical (TEMPO), and potassium bromide gave the carboxylic acid (**14**) in 74% yield (Scheme 2).[16] Hydrogenolysis of the Cbz group of **14** followed by condensation with *N*,*N*′-bis(BOC-1-guanyl)pyrazole in THF produced the protected guanidine (**15**) in 82% yield (Scheme 2).[17] Acidic deprotection of **15** formed the desired L-arginine derivative (**5**) in 85% yield and 96% ee (Scheme 2). Treatment of **14** with diazomethane in ether yielded the corresponding methyl ester (**16**, Scheme 2) in 97% yield. Hydrogenolysis of the Cbz group of **16** followed by condensation with thiophosgene and ammonia gave the thiourea (**17**) in 81% yield. Deprotection of **17** using boron tribromide (BBr_3) afforded the L-thiocitrulline derivative (**6**) in 82% yield and 75% ee (Scheme 2).[18]

Scheme 3 depicts the similar preparation of the L-homoarginine derivatives (**7** and **8**) from commercially available 4-aminostyrene (Aldrich). Cbz protection of the amine to give **18** followed by gram-scale Sharpless asymmetric aminohydroxylation using the ($(DHQ)_2$-PHAL) ligand formed the BOC-protected amino alcohol (**19**) in 59% yield and 75% ee (Scheme 3).[14] The TEMPO-catalyzed oxidation of **19** proved unsuccessful, and **19** was converted to the aldehyde (**20**) using the Dess−Martin periodinane. Difficulties in the oxidation of alcohols containing electron rich aromatic rings using this method have been noted, and the poor solubility of **19** in acetone, the reaction solvent, further complicated this oxidation.[16] Oxidation of **20** with sodium chlorite, sodium dihydrogen phosphate, and 2-methyl-2-butene formed the carboxylic acid (**21**) in 78% yield (Scheme 3).[19] Hydrogenolysis of the Cbz group of **21** followed by condensation with *N*,*N*′-bis(BOC-1-guanyl)pyrazole in THF produced the protected guanidine (**22**) in 81% yield (Scheme 3).[17] Acidic deprotection of **22** formed the desired L-homoarginine derivative (**7**) in 68% yield and 75% ee (Scheme 3). Treatment of **21** with diazomethane in ether yielded the corresponding methyl ester (**23**, Scheme 3) in 84% yield. Hydrogenolysis of the Cbz group of **23** followed by condensation with thiophosgene and ammonia gave the thiourea (**24**) in 69% yield and 60% ee. Deprotection of **24** using BBr_3 afforded the L-homothiocitrulline derivative (**8**) in 52% yield and 60% ee (Scheme 3).[18]

Compounds **9** and **10** were prepared from L-phenylalanine as outlined in Scheme 4. Nitration of L-phenylalanine yielded 4-nitro-L-phenylalanine monohydrate (**25**) in 53% yield (Scheme 4).[20] Sequential protection of the amino and carboxylic acid groups of **25** with BOC anhydride and *N*,*N*-DMF-di-*t*-Bu acetal produced **26** and **27** in 92 and 74% yields, respectively (Scheme 4). Catalytic hydrogenation of **27** formed amine (**28**) in 94% yield. Nitro group reduction of **26** followed by condensation with *N*,*N*′-bis(BOC-1-guanyl)pyrazole in THF produced the protected guanidine (**29**) in 93% yield (Scheme 4).[17] Acidic deprotection of **29** formed the desired L-arginine derivative (**9**) in 98% yield and 99% ee (Scheme 4). Treatment of **28** with thiophosgene and ammonia gave the thiourea (**30**) in 89% yield. Acidic deprotection of **30** afforded the derivative (**10**) in 98% yield and 99% ee (Scheme 4).

Scheme 3



These results indicate that carbamate-containing olefins act as suitable substrates for the Sharpless carbamate aminohydroxylation for the preparation of amino-substituted α-arylglycines and do not interfere with the catalytic cycle. While the regioselectivity of the aminohydroxylation of the meta- and para-substituted *N*-Cbz styrenes (**12** and **18**) compares well with the results of other reported styrenes,[14] the enantioselectivity of aminohydroxylation differed markedly (96% vs 75% ee) with the enantiomeric excess of **19** being improved to 81% by a single recrystallization. Both the regioselectivity and enantioselectivity of the Sharpless aminohydroxylation depend on the substrate structure, the asymmetric ligand, and the reaction solvent.[14] The influence of substituent position (ortho vs meta vs para) on the enantioselectivity of aminohydroxylation of substituted styrenes has not been reported, and at this time the

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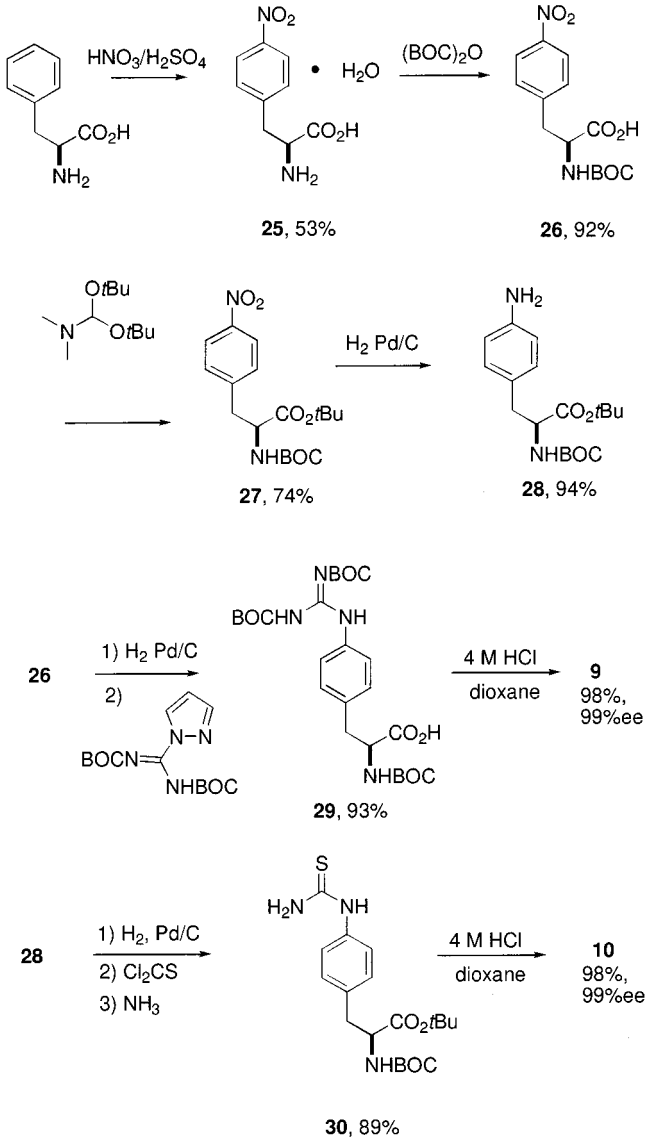
(20) Houghten, R. A.; Rapoport, H. *J. Med. Chem.* **1974**, *17*, 556−558.

Scheme 4



observed enantioselectivity difference in the reaction of **12** and **18** appears best explained by a structural difference between the substrates.

The conversion of carboxylic acids **14** and **21** to the guanidines **5** and **7** proceeded without the loss of enantiomeric excess, highlighting the utility of this methodology for the asymmetric preparation of enantiopure L-phenylglycines. Retention time analysis following chiral HPLC separation using a CROWNPAK CR-(+) column (Chiral Technologies, Inc., Exton, PA) indicated the major formation of the L-amino acid (*S*-configuration) for each of the final amino acid products (**5**−**10**). Using this column, natural and unnatural L-amino acids (*S*-configuration) consistently elute slower than D-amino acids (*R*-configuration), allowing for the assignment of absolute configuration.[21] These results support the predicted formation of the amino alcohols (**13** and **19**) with the *S*-configuration from the aminohydroxylation of **12** and **18** using the bis(dihydroquinyl)phthalazine ($(DHQ)_2$-PHAL) ligand.[14]

A loss of enantiomeric excess occurred during the preparation of the thioureas **6** and **8** (75 and 60% ee's, respectively). Chiral HPLC determination of the enantiomeric excess of the individual synthetic intermediates in these routes indicated that the loss of stereochemical integrity occurred during the thiourea-forming step. Treatment of the intermediate isothiocyanates formed by catalytic hydrogenation and thiophosgene condensation of **16** and **23** with excess ammonia resulted in complete racemization. Despite careful monitoring of this condensation by thin-layer chromatography to judge the reaction progress and the use of 1 equiv of ammonia, partial racemization occurred, suggesting that ammonia-mediated acid−base reactions contributed to the loss of stereochemical integrity of these compounds.

Table 1. Inducible and Neuronal NOS Inhibition and Selectivity by Compounds 5−10

	IC_{50} (μM)		selectivity
compd	iNOS	nNOS	IC_{50}(nNOS)/IC_{50}(iNOS)
5	42	144	3.4
6	8	12	1.5
7	>1000	>1000	
8	>1000	600	
9	19	147	7.7
10	>1000	>1000	

Preliminary biochemical evaluation in the presence of both inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS, a constitutive isoform) indicated that none of the synthetic guanidine-containing L-arginine derivatives (**5**, **7**, or **9**) supported nitrite synthesis. The identification of nitrite, the stable oxidative decomposition product of nitric oxide,[22] provides evidence for nitric oxide formation, and these results demonstrate the inability of these compounds to act as nitric oxide producing substrates. These results support the reported strict structural requirements for nitric oxide producing substrates of nitric oxide synthase.[5] A recently proposed mechanism suggests that proton donation from the guanidinium group of L-arginine plays an important role in nitric oxide formation from the nitric oxide synthase catalyzed oxidation of L-arginine and the decreased basicity of these aryl guanidines (pK_a = 10.7 for phenylguanidine vs pK_a = 12.5 for L-arginine)[23] may partially explain their inability to act as substrates.[9] Alternatively, the failure of these compounds to act as NO-producing substrates may be due to their decrease of the NOS heme midpoint reduction potential as demonstrated for a number of other NOS inhibitors.[24]

Using the radioactive L-citrulline assay to assay NOS activity,[25] the L-thiocitrulline derivative (**6**) demonstrated the greatest amount of inhibition of both the inducible and neuronal isoforms of NOS (IC_{50} = 8 and 12 μM, respectively, Table 1). In comparison, the K_i values for iNOS and nNOS inhibition by **1** have been determined as 2.60 and 0.37 μM, respectively, and the K_i value for iNOS inhibition by thiocitrulline has been determined as 9 μM.[10,13] The L-arginine derivative (**5**) also inhibited both isoforms of the enzyme (IC_{50} = 42 and 144 μM, respectively, Table 1). Both **5** and **6** demonstrated a slight

(21) *Application Guide for Chiral Column Selection*, 2nd ed.; Chiral Technologies Inc.: Exton, PA.

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selectivity for the inducible isoform of the enzyme (IC_{50}(nNOS)/IC_{50}(iNOS) = 3.4 and 1.5, respectively, Table 1). These results support previous evidence that L-arginine derivatives bind to the nitric oxide synthase active site in a folded conformation as **5** and **6** represent the only compounds in this study capable of adopting a folded conformation similar to that found in **1**. The ability of these compounds to assume a fully extended conformation by rotation about the bond between the α carbon and the aromatic ring may partially explain the decrease in activity compared to **1**.

Conformational restriction of L-homoarginine (compounds **7** and **8**) produced deleterious effects with regard to the inhibition of either isoform of the enzyme (Table 1). These results contrast the ability of **2**, a L-homoarginine analogue with more conformational flexibility between the guanidinium and amino acid groups, to modestly inhibit both inducible and neuronal NOS (K_i = 100 and 44 μM, respectively).[10] Other nonconformationally restricted L-homoarginine derivatives, such as **3** and L-homothiocitrulline, also demonstrate significant inhibition of NOS.[12,13a] While these other L-homoarginine analogues interact with the enzyme, these results clearly indicate that the orientation of the functional groups in **7** and **8** does not permit binding of these compounds to NOS, and this lack of activity prevents the evaluation of isoform selectivity.

The conformationally restricted two-carbon L-arginine homologue (**9**) inhibited both the inducible and neuronal isoforms of NOS (IC_{50} = 19 and 147 μM, Table 1) and displayed the greatest isoform selectivity of any of the compounds examined (IC_{50}(nNOS)/IC_{50}(iNOS) = 7.7, Table 1). These results indicate that longer L-arginine homologues can selectively interact with NOS isoforms by adopting a folded conformation. D,L-Homo-N^G-(1-iminoethyl)lysine, the one-carbon homologue of **3**, also preferentially inhibits inducible NOS compared to constitutive NOS (IC_{50} = 73 and 808 μM, respectively, IC_{50}(nNOS)/IC_{50}(iNOS) = 11).[12] These results suggest that differences in the folded conformations of L-arginine derivatives and L-arginine homologues partially explain the isoform selectivity observed by these longer homologues. The thiourea (**10**) did not demonstrate any interaction with the enzyme, possibly due to the loss of hydrogen-bonding interactions from the replacement of the guanidine group of **9** with the thiourea group of **10**.

To conclude, the catalytic asymmetric Sharpless aminohydroxylation provided a convenient route for the preparation of the α-arylglycines (**5**−**8**). The ability of the L-arginine derivatives **5** and **6** to inhibit both the inducible and neuronal isoforms of NOS provides further evidence that L-arginine-derived compounds bind to the active site in a folded conformation. The ability of the conformationally restricted two-carbon L-arginine homologue (**9**) to preferentially inhibit the inducible isoform suggests that the folded conformations adopted by homologous inhibitors partially controls isoform selective inhibition of NOS. However, the recent report of the K_m values of L-homoarginine with iNOS and nNOS (33 and 23 μM, respectively) weakens the proposal that chain length confers specificity.[25] The inability of **7** and **8** to interact with NOS also limits the confidence of any conclusions regarding the relationship of chain length and conformation on isoform selectivity. Modified substrates, such as **2**, **5**, **7**, and **9**, may bind to the NOS isoforms differently, and the active site deformability of each isoform should be considered in the design of future inhibitors. Further studies with other compounds combined with X-ray crystallographic data from the other NOS isoforms will provide useful information toward understanding the structural basis of NOS isoform selective inhibition.

Experimental Section

Chemistry: General. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F-254 precoated plates. Flash chromatography was performed on silica gel 60 (230−400 mesh). Proton and carbon NMR spectra were taken in commercial deuterated solvents on a Varian VXR 200 multinuclear spectrometer. Infrared spectra were obtained on a Perkin-Elmer 1600 Series FTIR spectrometer. Organic solvents were distilled from a drying agent prior to use. Commercially available reagents were used without further purification.

3-Aminostyrene (11). A solution of K_2CO_3 (14.86 g, 107.5 mmol) and $Na_2S_2O_4$ (16.85 g, 96.8 mmol) in water (75 mL) was added dropwise to a mixture of 3-nitrostyrene (3.00 mL, 21.5 mmol) and 1,1′-dioctyl-4,4′-bipyridinium bromide (0.58 g, 1.0 mmol) in CH_2Cl_2/H_2O (150 mL/20 mL) under an argon atmosphere. The reaction mixture was heated at 35 °C for 8 h with constant stirring. The organic layer was collected, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (1:4 EtOAc:hexanes) to afford **11** (2.31 g, 90%) as a yellow oil: R_f 0.35 (1:4 EtOAc:hexanes); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.13 (t, J = 8 Hz, 1H), 6.83 (d, J = 8 Hz, 1H), 6.73 (s, 1H), 6.62 (m, 2H), 5.70 (d, J = 18 Hz, 1H), 5.20 (d, J = 11 Hz, 1H), 3.85 (br. s, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 146.4, 138.1, 136.7, 128.9, 116.2, 114.3, 113.1, 112.2; IR ($CDCl_3$) 3462 cm^{-1}; LRMS (EI) m/z 120 (M + H)$^+$.

***N*-Benzyloxycarbonyl-3-aminostyrene (12).** To a solution of **11** (1.00 g, 8.4 mmol) and 2,6-lutidine (1.95 mL, 16.8 mmol) in CH_2Cl_2 (30 mL) at 0 °C was added benzyl chloroformate (1.80 mL, 12.6 mmol) over 10 min. The reaction was allowed to warm to room temperature and after completion as judged by thin-layer chromatography was washed with H_2O (20 mL). The organic layer was collected, dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (1:7 EtOAc:hexanes) to afford **12** (2.04 g, 96%) as an amber oil: R_f = 0.34 (1:7 EtOAc:hexanes); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.50 (s, 1H), 7.41 (m, 5H), 7.29 (d, J = 6 Hz, 2H), 7.17 (s, 1H), 6.90 (br. s, 1H), 6.71 (dd, J = 11, 18 Hz, 1H), 5.79 (d, J = 18 Hz, 1H), 5.29 (d, J = 11 Hz, 1H), 5.23 (s, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 153.4, 138.0, 137.9, 136.2, 135.7, 128.7, 128.2, 127.9, 127.8, 121.0, 118.0, 116.3, 114.0, 66.6; IR ($CDCl_3$) 3434, 1735, cm^{-1}; LRMS (FAB) m/z 253 (M$^+$), 276 (M + Na)$^+$. Anal. Calcd for $C_{16}H_{15}NO_2$: C, 75.86; H, 5.96; N, 5.52. Found: C, 75.87; H, 5.99; N, 5.46.

1(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-1-[3-*N*-(Benzyloxy)-carbonylaminophenyl]-2-hydroxyethylamine (13). A solution of NaOH (0.482 g, 12.1 mmol) in H_2O (30 mL) followed by *tert*-butyl hypochlorite (1.437 mL, 12.1 mmol) was added to a solution of *tert*-butyl carbamate (1.435 g, 12.3 mmol) in *n*-propyl alcohol (15 mL) and cooled to −5 °C. $(DHQ)_2$-PHAL (0.1846 g, 0.237 mmol) in *n*-propyl alcohol (15 mL) followed by **12** (1.00 g, 3.95 mmol) in *n*-propyl alcohol (30 mL) and OsO_4 (0.040 g, 0.158 mmol) in H_2O (1 mL) was added to this cooled mixture. The reaction mixture was stirred at −5 °C overnight and quenched with Na_2SO_3. The reaction mixture was extracted with EtOAc (3 × 50 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (2:3 EtOAc:hexanes) to afford **13** (1.00 g, 66%) as a white solid: mp = 113.0−113.5 °C. The enantiomeric excess (ee) of **13** was determined to be 96% by HPLC (CHIRALCEL OD-H, 0.4 cm ϕ × 25 cm, hexane:isopropyl alcohol, 65:35, 1.0 mL/min, UV

254 nm, t_R (*R*-enantiomer) 5.6 min, (*S*-enantiomer) 10.3 min); $R_f = 0.25$ (2:3 EtOAc:hexanes); $[\alpha]^{20}_D$ +31.7 ($c = 1.26$, EtOH); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.33 (m, 5H), 7.25 (m, 3H), 6.95 (m, 2H) 5.34 (d, $J = 6$ Hz, 1H), 5.14 (s, 2H), 4.65 (br. m, 1H), 3.73 (br. m, 2H), 2.63 (br. m, 1H), 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 156.0, 153.5, 140.6, 138.1, 135.9, 128.9, 128.3, 128.0, 121.3, 117.8, 116.9, 79.7, 66.6, 65.7, 56.4, 28.1; IR ($CDCl_3$) 3600, 3435, 1730, 1712 cm^{-1}; LRMS (FAB) *m*/*z* 387 $(M + H)^+$. Anal. Calcd for $C_{21}H_{26}N_2O_5$: C, 65.27; H, 6.78; N, 7.25. Found: C, 65.23; H, 6.81; N, 7.23.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[3-*N*-(Benzyloxy)carbonylaminophenyl]glycine (14) and Methyl (*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[3-*N*-(Benzyloxy)carbonyl aminophenyl]glycine (16). A 5% aqueous solution of $NaHCO_3$ (5 mL), KBr (0.012 g, 0.103 mmol) and TEMPO (0.182 g, 1.17 mmol) was added to a solution of **13** (0.400 g, 1.03 mmol) in acetone (5 mL) cooled to 0 °C. To this mixture was added an aqueous NaOCl solution (1.59 mL, 1.36 mmol, 5.25% Clorox Bleach) dropwise over 10 min. After 1 h, additional aqueous NaOCl solution (0.76 mL, 0.647 mmol) was added dropwise over 5 min. The reaction mixture was stirred for an additional hour, 20 mL of H_2O was added, and the aqueous mixture was extracted with EtOAc (3 × 50 mL). The combined organic layers were collected, dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (EtOAc) to afford **14** (0.3093 g, 74%) as a yellowish foam: $R_f = 0.22$ (EtOAc); $[\alpha]^{20}_D$ +68.3 ($c = 0.75$, EtOH).

A solution of CH_2N_2 (0.5 M, 12.5 mmol) in Et_2O (25 mL) was added dropwise to a cooled solution (0 °C) of **14** (1.00 g, 2.50 mmol) in Et_2O (10 mL). The reaction mixture was concentrated in vacuo, and the crude product was purified by flash chromatography (1:4 EtOAc:pentane) to afford **16** (1.001 g, 97%) as a white foam. The enantiomeric excess of **16** was determined to be 96% by HPLC (CHIRALPAK AD, 0.4 cm ϕ × 25 cm, hexane:isopropyl alcohol, 85:15, 1.25 mL/min, UV 254 nm, t_R (*R*-enantiomer) 11.5 min, (*S*-enantiomer) 14.5 min); $R_f = 0.22$ (1:4 EtOAc:pentane); $[\alpha]^{20}_D$ +90.4 ($c = 1.50$, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.48−7.27 (m, 10H), 7.00 (d, $J = 8$ Hz, 1H), 5.67 (d, $J = 7$ Hz, 1H), 5.22 (d, $J = 7$ Hz, 1H), 5.15 (s, 2H), 3.62 (s, 3H), 1.35 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.6, 154.7, 153.2, 138.7, 137.1, 135.9, 129.4, 128.4, 128.1, 121.9, 118.6, 117.1, 80.1, 66.7, 57.5, 52.6, 28.0; IR ($CDCl_3$) 3433, 1740, 1704 cm^{-1}; LRMS (FAB) *m*/*z* 421 $(M + Li)^+$. Anal. Calcd for $C_{22}H_{26}N_2O_6$: C, 63.75; H, 6.32; N, 6.75. Found: C, 63.70; H, 6.33; N, 6.84.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[3-*N, N′*-bis(*tert*-butyloxy)carbonyl(guanyl)aminophenyl]glycine (15). A mixture of **14** (0.700 g, 1.75 mmol) and 10% Pd−C (50 mg) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 12 h. The mixture was filtered through Celite to remove the catalyst, and the filtrate was concentrated under vacuum to give an oil. *N*,*N*′-Bis(Boc-1-guanyl)pyrazole (0.813 g, 2.63 mmol) in dry THF (10 mL) was added under argon and the mixture reacted for 3 days and concentrated in vacuo. The crude product was purified by flash chromatography (2:3: 0.0.025 EtOAc:pentane:AcOH) to afford **15** (0.732 g, 82%) as a white foam: R_f 0.28 (2:3:0.0.025 EtOAc:pentane:AcOH);^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.32 (br s, 1H), 7.77 (d, $J = 8$ Hz, 1H), 7.30 (m, 2H), 7.07 (d, $J = 8$ Hz, 1H), 5.53 (d, $J = 7$ Hz, 1H), 5.13 (d, $J = 7$ Hz, 1H), 1.48 (s, 9H), 1.46 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 172.6, 162.6, 154.8, 153.2, 153.0, 138.4, 137.5, 129.4, 123.5, 122.6, 120.7, 83.7, 80.2, 79.6, 56.5, 28.1, 27.9, 27.6; IR ($CDCl_3$) 3435, 3260, 1712 cm^{-1}; LRMS (FAB) *m*/*z* 523.3 $(M + H)^+$. Carboxylic acid (**15**) was further characterized by conversion to the methyl ester. A solution of CH_2N_2 (0.5 M, 5.00 mmol) in Et_2O (10 mL) was added dropwise to a cooled solution (0 °C) of **15** (0.100 g, 0.197 mmol) in Et_2O (10 mL). After addition, the reaction mixture was concentrated in vacuo and crude product was purified by flash chromatography (1:7 EtOAc:pentane) to afford the methyl ester of **15** (0.0988 g, 96%) as a white foam: $R_f = 0.23$ (1:7 EtOAc: pentane); $[\alpha]^{20}_D$ +83.5 ($c = 0.700$, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.30 (br. s, 1H), 7.71 (d, $J = 8$ Hz, 1H), 7.35−7.24 (m, 2H), 7.08 (d, $J = 8$ Hz, 1H), 5.54 (d, $J = 7$ Hz, 1H), 5.24 (d, $J = 7$ Hz, 1H), 3.68 (s, 3H), 1.50 (s, 9H), 1.47 (s, 9H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.3, 163.3, 154.7, 153.4, 153.1, 137.4, 137.1, 129.4, 123.5, 122.6, 120.7, 83.7, 80.0, 79.5, 57.3, 52.6, 28.1, 28.0, 27.9; IR ($CDCl_3$) 3440, 1718, 1704 cm^{-1}; LRMS (FAB) *m*/*z* 523.3 $(M + H)^+$. Anal. Calcd for $C_{25}H_{38}N_4O_8$: C, 57.46; H, 7.32; N, 10.72. Found: C, 57.41; H, 7.40; N, 10.61.

3-Guanyl-L-phenylglycine Hydrochloride (5). A solution of **15** (0.400 g, 0.787 mmol) in HCl/dioxane (4 M, 10 mL, Aldrich) was stirred at 23 °C for 12 h under an argon atmosphere. The mixture was concentrated in vacuo, dissolved in H_2O (10 mL), passed through a 6 mL Supelco LC-18 solid-phase extractor, and lyopholyzed to yield **5** (0.188 g, 85%) as a white solid. The enantiomeric excess of **5** was determined to be 96% by HPLC (CROWNPAK CR(+), 0.4 cm ϕ × 15 cm, aqueous $HClO_4$, pH = 1.5, 0.8 mL/min, UV 254 nm, t_R (*R*-enantiomer) 2.9 min, (*S*-enantiomer) 6.8 min); $[\alpha]^{20}_D$ +65.4 ($c = 0.43$, H_2O); ^{1}H NMR (D_2O, 200 MHz) δ 7.31 (m, 4H), 5.03 (s, 1H); ^{13}C NMR (D_2O, 50 MHz) δ 170.8, 156.4, 135.5, 133.8, 131.4, 127.5, 127.4, 125.6, 56.5; IR (film, 3M IR-card, polyethylene mesh) broad 3614−2350, 1732 cm^{-1}; LRMS (FAB) *m*/*z* 209.1 $(M + H)^+$; HRMS (FAB) calcd for $C_9H_{13}N_4O_2$ 209.1038, found 209.1307.

Methyl (*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[3-thioureaphenyl]glycine (17). A mixture of **16** (1.00 g, 2.41 mmol) and 10% Pd−C (50 mg) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 12 h. The mixture was filtered through Celite to remove the catalyst, and the filtrate was concentrated under vacuum to give an oil. This oil was dissolved in CH_2Cl_2 (20 mL) and added to a mixture of $CaCO_3$ (0.361 g, 3.62 mmol), H_2O (20 mL), CH_2Cl_2 (20 mL), and thiophosgene (0.220 mL, 2.89 mmol). This mixture was vigorously stirred at 23 °C, and after 1 h the organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated, yielding a yellow oil. A solution of NH_3 (2.0 M, 3.62 mmol) in EtOH (11.81 mL) was added to the crude isothiocyanate with vigorous stirring. The reaction was monitored closely by thin-layer chromatography and concentrated in vacuo, and the crude product was purified by flash chromatography (1:1 EtOAc:pentane) to afford **17** (0.661 g, 81%) as a white foam. The enantiomeric excess of **17** was determined to be 88% by HPLC (CHIRALPAK AD, 0.4 cm ϕ × 25 cm, hexane:EtOH:DEA, 85:15:0.1, 1.5 mL/min, UV 254 nm, t_R (*S*-enantiomer) 14.9 min, (*R*-enantiomer) 28.2 min); R_f = 0.26 (1:1 EtOAc:pentane); $[\alpha]^{20}_D$ +93.6 ($c = 0.30$, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.47 (br s, 1H) 7.43−7.16 (m, 4H), 6.33 (br s, 2H), 5.82 (d, $J = 6$ Hz, 1H), 5.31 (d, $J = 6$ Hz, 1H), 3.71 (s, 3H), 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 180.3, 171.0, 154.7, 138.3, 137.1, 130.0, 125.1, 124.1, 122.9, 80.1, 56.9, 52.7, 27.9; IR ($CDCl_3$) 3506, 3433, 1740, 1704 cm^{-1}; LRMS (FAB) *m*/*z* 340.1 $(M + H)^+$. Anal. Cacld for $C_{15}H_{21}N_3O_4S$: C, 53.08; H, 6.23; N, 12.38; S, 9.44. Found: C, 53.34; H, 6.41; N, 11.75; S, 9.01.

3-Thiourea-L-phenylglycine Trifluoroacetate (6). A solution of BBr_3 (1.0 M, 2.65 mmol) in CH_2Cl_2 (2.65 mL) was added to a cooled (−78 °C, acetone dry ice solution) solution of **17** (0.180 g, 0.531 mmol) in CH_2Cl_2 (20 mL) over 5 min with stirring. The reaction mixture was allowed to warm to 25 °C, stirred for 1 h, and quenched by the careful dropwise addition of water (30 mL). The aqueous phase was separated and the organic phase extracted with H_2O (3 × 20 mL). The combined aqueous layers were lyopholized to dryness, and the crude product (82% yield) was purified by preparative HPLC (Econosil-C-18 10 μ, 280 × 10 mm, aqueous 0.1% TFA, 4.0 mL/min, UV 254 nm, t_R 12.2 min.) to afford **6** (0.071 g, 29%) as a white solid. The enantiomeric excess of **6** was determined to be 75% by HPLC (CROWNPAK CR(+), 0.4 cm ϕ × 15 cm, aqueous $HClO_4$, pH = 1.5, 1.3 mL/min, UV 254 nm, t_R (*R*-enantiomer) 2.6 min, (*S*-enantiomer) 10.5 min); $[\alpha]^{20}_D$ +64.2 ($c = 0.35$, MeOH); ^{1}H NMR (CD_3OD, 200 MHz) δ 7.28 (m, 4H), 4.46 (s, 1H); ^{13}C NMR (D_2O, 50 MHz) δ 179.6, 172.9, 137.7, 135.7, 130.9, 127.0, 126.8, 125.4, 58.1; IR (film, 3M IR-card, polyethylene mesh) broad 3462−2583, 1733 cm^{-1}; LRMS (FAB) *m*/*z* 226.1 $(M + H)^+$; HRMS (FAB) calcd for $C_9H_{12}N_3O_2S$ 226.0650, found 226.0639.

***N*-Benzyloxycarbonyl-4-aminostyrene (18).** Benzyl chloroformate (5.46 mL, 38.3 mmol) was added to a solution of 4-aminostyrene (3.00 mL, 25.5 mmol) and 2,6-lutidine (5.91 mL, 51.1 mmol) at 0 °C over 10 min. The reaction mixture was allowed to warm to room temperature and after completion (judged by thin layered chromatography) was washed with H_2O (50 mL). The organic layer was collected, dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (1:10 EtOAc:pentane) to afford **18** (6.13 g, 95%) as a white solid: mp = 86.0−88.5 °C: R_f 0.42 (1:10 EtOAc:pentane); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.33 (m, 9H) 6.66 (m, 3H), 5.65 (d, J = 17 Hz, 1H), 5.18 (m, 3H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 153.3, 137.2, 135.9, 135.8, 132.6, 128.3, 128.1, 128.0, 126.6, 118.6, 112.4, 66.7; IR ($CDCl_3$) 3690, 1732 cm^{-1}; LRMS (FAB) m/z 253 (M^+). Anal. Calcd for $C_{16}H_{15}NO_2$: C, 75.87; H, 5.97; N, 5.53. Found: C, 75.77; H, 5.97; N, 5.51.

1(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-1-[4-*N*-(Benzyloxy)-carbonylaminophenyl]-2-hydroxyethylamine (19). A solution of NaOH (1.446 g, 36.2 mmol) in H_2O (60 mL) followed by *tert*-butyl hypochlorite (4.3131 mL, 36.2 mmol) was added to a solution of *tert*-butyl carbamate (4.307 g, 37.3 mmol) in *n*-propyl alcohol (30 mL) with stirring and was cooled to −5 °C. $(DHQ)_2$-PHAL (0.553 g, 0.710 mmol) in *n*-propyl alcohol (30 mL) followed by **18** (3.00 g, 11.8 mmol) in *n*-propyl alcohol (60 mL) and OsO_4 (0.120 g, 0.472 mmol) in H_2O (6 mL) was added to this solution. The reaction mixture was stirred at −5 °C overnight and quenched with Na_2SO_3. The reaction solution was extracted with EtOAc (3 × 50 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (2:3 EtOAc:pentane) to afford **19** (2.71 g, 59%) as a white solid: mp = 170.0−172.0 °C. The enantiomeric excess of **19** was determined to be 75% by HPLC (CHIRALCEL OD-H, 0.4 cm ϕ × 25 cm, hexane:isopropyl alcohol, 65:35, 1.5 mL/min, UV 254 nm, t_R (*S*-enantiomer) 3.6 min, (*R*-enantiomer) 9.1 min); R_f = 0.19 (2:3 EtOAc:pentane); $[\alpha]^{20}_D$ +46.19 (c = 1.05, acetone); ^{1}H NMR (acetone-d_6, 200 MHz) δ 8.73 (s, 1H), 7.40 (m, 9H), 6.25 (d, J = 6 Hz, 1H), 5.15 (s, 2H), 4.64 (br. m, 1H), 3.70 (d, J = 6 Hz, 2H), 2.90 (br. s, 1H), 1.36 (s, 9H); ^{13}C NMR (acetone-d_6, 50 MHz) δ 156.2, 154.3, 138.9, 137.8, 136.8, 129.2, 128.8, 128.7, 128.1, 118.9, 78.7, 66.7, 66.3, 57.3, 7.5; IR ($CDCl_3$) 3689−3600, 1700 cm^{-1}; LRMS (FAB) m/z 387 $(M + H)^+$. Anal. Calcd for $C_{21}H_{26}N_2O_5$: C, 65.27; H, 6.78; N, 7.25. Found: C, 65.26; H, 6.71; N, 7.24.

Aldehyde 20, (*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[3-*N*-(Benzyloxy)carbonylaminophenyl]glycine (21) and Methyl (*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-*N*-(Benzyloxy)carbonylaminophenyl]glycine (23). Dess−Martin periodinane reagent (0.330 g, 0.778 mmol) was added to a solution of **19** (0.150 g, 0.389 mmol) in CH_2Cl_2 (50 mL) at 0 °C with stirring. The reaction mixture was gradually warmed to room temperature, stirred for an additional 30 min, and quenched with a saturated aqueous solution of $NaHCO_3$ containing 1.5 g of $Na_2S_2O_3$ (25 mL). The organic layer was collected, and the aqueous layer was washed with CH_2Cl_2 (3 × 25 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (1:3 EtOAc:pentane) to afford **20** (0.1344 g, 90%) as a yellow oil: R_f = 0.21 (1:3 EtOAc:pentane).

A solution of $NaClO_2$ (Aldrich, 80%, 0.659 g, 5.83 mmol) and NaH_2PO_4 (0.626 g, 4.54 mmol) in H_2O (8 mL) was added dropwise to a solution of **20** (0.250 g, 0.648 mmol) and 2-methyl-2-butene (11.4 mL, 108 mmol) in *t*-BuOH (20 mL) at 25 °C. After stirring for 30 min, the reaction mixture was concentrated in vacuo, yielding a crude residue. The residue was diluted with H_2O (20 mL) and extracted with EtOAC (3 × 50 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo, and the crude product was purified by flash chromatography (EtOAc) to afford **21** (0.201 g, 78%) as a white foam: R_f = 0.33 (0.1% AcOH in EtOAc).

A solution of CH_2N_2 (0.5 M, 0.75 mmol) in Et_2O (1.5 mL) was added dropwise to a cooled solution (0 °C) of **21** (0.200 g, 0.500 mmol) in Et_2O (10 mL). The reaction mixture was concentrated in vacuo, and the crude product was purified by flash chromatography (1:3 EtOAc:pentane) to afford **23** (0.1735 g, 84%) as a white foam. The enantiomeric excess of **23** was determined to be 75% by HPLC (CHIRALPAK AD, 0.4 cm ϕ × 25 cm, hexane:isopropyl alcohol, 85:15, 1.5 mL/min, UV 254 nm, t_R (*R*-enantiomer) 20.5 min, (*S*-enantiomer) 35.8 min); R_f = 0.30 (1:3 EtOAc:pentane); $[\alpha]^{20}_D$ +72.0 (c = 1.70, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.32−7.20 (m, 10H), 6.87 (s, 1H), 5.52 (d, J = 7 Hz, 1H), 5.21 (d, J = 7 Hz, 1H), 5.15 (s, 2H), 3.65 (s, 3H), 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.6, 154.7, 153.3, 138.1, 135.8, 131.4, 128.4, 128.1, 126.1, 127.7, 118.8, 80.0, 66.8, 56.9, 52.5, 28.1; IR ($CDCl_3$) 3418, 1740, 1711 cm^{-1}; LRMS (FAB) m/z 415 $(M + H)^+$. Anal. Cacld for $C_{22}H_{26}N_2O_6$: C, 63.75; H, 6.32; N, 6.75. Found: C, 63.62; H, 6.35; N, 6.68.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-*N*, *N*′-bis(*tert*-butyloxy)carbonyl(guanyl)aminophenyl]glycine (22). A mixture of **21** (0.714 g, 1.78 mmol) and 10% Pd−C (50 mg) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 12 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated under vacuum to give an oil. *N*,*N*′-Bis(BOC-1-guanyl)pyrazole (1.103 g, 3.56 mmol) in dry THF (10 mL) was added to this oil under argon. After 4 days the reaction mixture was concentrated in vacuo and the crude product was purified by flash chromatography (1:1:0.5% EtOAc:pentane:AcOH) to afford **22** (0.738 g, 81%) as a white foam: R_f 0.33 (1:1:0.5% EtOAc:pentane:AcOH); $[\alpha]^{20}_D$ +69.3 (c = 1.50, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.30 (br s, 1H), 7.47 (d, J = 8 Hz, 2H), 7.30 (d, J = 8 Hz, 2H), 5.68 (d, J = 6 Hz, 1H), 5.13 (d, J = 6 Hz, 1H), 1.48 (s, 18H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 172.7, 162.7, 154.9, 153.3, 153.0, 136.2, 134.4, 127.5, 122.1, 83.6, 81.1, 79.6, 56.1, 28.1, 27.8, 27.7; IR ($CDCl_3$) 3434, 1713 cm^{-1}; LRMS (FAB) m/z 509.3 $(M + H)^+$. Carboxylic acid **22** was further characterized by conversion to the methyl ester as follows. A solution of CH_2N_2 (0.5 M, 5.00 mmol) in Et_2O (10 mL) was added dropwise to a cooled solution (0 °C) of **22** (0.150 g, 0.302 mmol) in Et_2O (10 mL). The reaction mixture was concentrated in vacuo, and the crude product was purified by flash chromatography (1:5 EtOAc:pentane) to afford the methyl ester of **22** (0.148 g, 94%) as a white foam: R_f = 0.33 (1:5 EtOAc:pentane); $[\alpha]^{20}_D$ +68.3 (c = 1.30, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.31 (br s, 1H), 7.55 (d, J = 8 Hz, 2H), 7.28 (d, J = 8 Hz, 2H), 5.52 (d, J = 7 Hz, 1H), 5.23 (d, J = 7 Hz, 1H), 3.68 (s, 3H), 1.50 (s, 9H), 1.47 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 171.3, 163.3, 154.5, 153.3, 153.1, 136.7, 133.0, 127.5, 122.4, 83.6, 79.9, 79.5, 56.9, 52.5, 28.1, 27.9 (2C); IR ($CDCl_3$) 3440, 1711 cm^{-1}; LRMS (FAB) m/z 523.3 $(M + H)^+$. Anal. Calcd for $C_{25}H_{38}N_4O_8$: C, 57.46; H, 7.32; N, 10.72. Found: C, 58.12; H, 7.47; N, 10.30.

4-Guanyl-L-phenylglycine Hydrochloride (7). A solution of **22** (0.276 g, 0.544 mmol) in HCl/dioxane (4 M, 10 mL, Aldrich) was stirred at 23 °C for 12 h under an argon atmosphere. The mixture was concentrated in vacuo, dissolved in H_2O (10 mL), passed through a 6 mL Supelco LC-18 solid-phase extractor, and lyopholyzed to yield **7** (0.162 g, 68%) as a white solid. The enantiomeric excess of **7** was determined to be 75% by HPLC (CROWNPAK CR(+), 0.4 cm ϕ × 15 cm, aqueous $HClO_4$, pH = 1.5, 0.8 mL/min, UV 254 nm, t_R (*R*-enantiomer) 2.7 min, (*S*-enantiomer) 9.3 min); $[\alpha]^{20}_D$ +71.2 (c = 1.62, MeOH); ^{1}H NMR (D_2O, 200 MHz) δ 7.38 (d, J = 6 Hz, 2H), 7.21 (d, J = 6 Hz, 2H), 5.06 (s, 1H); ^{13}C NMR (D_2O, 50 MHz) δ 170.5, 156.1, 136.2, 130.7, 129.9, 126.4, 56.0; IR (film, 3M IR-card, polyethylene mesh) broad 3614−2328, 3410, 1732 cm^{-1}; LRMS (FAB) m/z 209.1 $(M + H)^+$, 231.1 $(M + Na)^+$; HRMS (FAB) calcd for $C_9H_{12}N_4O_2Na$ 231.0857, found 231.0854.

Methyl (*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-thioureaphenyl]glycine (24). A mixture of **23** (0.133 g, 0.321 mmol) and 10% Pd−C (10 mg) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 12 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated under vacuum to give an oil. This oil was dissolved in CH_2Cl_2 (20 mL) and added to a mixture of $CaCO_3$ (0.0482 g, 0.482 mmol), H_2O (20 mL), CH_2Cl_2 (20 mL), and thiophosgene (0.0306 mL, 0.401 mmol). This mixture was vigorously stirred at 23 °C, and after 1 h the organic phase

was separated and the aqueous phase extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated, yielding a yellow oil. A solution of NH_3 (2.0 M, 0.241 mL) in EtOH (10.241 mL) was added to the crude isothiocyanate and stirred vigorously. Upon completion of reaction (determined by thin-layer chromatography), the solution was concentrated in vacuo and the crude product was purified by flash chromatography (2:1 EtOAc:pentane) to afford **24** (0.0751 g, 69%) as a white foam. The enantiomeric excess was determined to be 60% by HPLC (CHIRALPAK AD, 0.4 cm ϕ × 25 cm, hexane:EtOH:DEA, 85:15:0.1, 1.5 mL/min, UV 254 nm, t_R (*R*-enantiomer) 22.1 min, (*S*-enantiomer) 35.7 min); R_f 0.37 (2:1 EtOAc:pentane); $[\alpha]^{20}_D$ +67.7 (c = 0.75, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.49 (br. s, 1H) 7.41 (d, J = 8 Hz. 2H), 7.23 (d, J = 8 Hz, 2H), 6.24 (br s, 2H), 5.73 (d, J = 7 Hz, 1H), 5.30 (d, J = 7 Hz, 1H), 3.70 (s, 3H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 180.9, 171.0, 154.8, 136.7, 136.0, 128.7, 125.0, 80.5, 56.9, 52.9, 28.2; IR ($CDCl_3$) 3687, 1740, 1704 cm^{-1}; LRMS (FAB) m/z 340.1 (M + H)$^+$. Anal. Calcd for $C_{15}H_{21}N_3O_4S$: C, 53.08; H, 6.24; N, 12.38; S, 9.44. Found: C, 53.46; H, 6.52; N, 11.63; S, 9.20.

4-Thiourea-L-phenylglycine Trifluoroacetate (8). A solution of BBr_3 (1.0 M, 3.83 mmol) in CH_2Cl_2 (3.83 mL) was added to a cooled (−78 °C, acetone dry ice solution) solution of **24** (0.260 g, 0.766 mmol) in CH_2Cl_2 (20 mL) over 5 min with stirring. The reaction mixture was allowed to warm to 25 °C, stirred for 1 h, and quenched by the careful dropwise addition of water (30 mL). The aqueous phase was separated and the organic phase extracted with H_2O (3 × 20 mL). The combined aqueous layers were lyopholized to dryness, and the crude product was purified by preparative HPLC (Econosil-C-18 10 μ, 280 × 10 mm, aqueous 0.1% TFA, 4.0 mL/min, UV 254 nm, t_R 8.5 min) to afford **8** (0.183 g, 52%) as a white solid. The enantiomeric excess of **8** was determined to be 60% by HPLC (CROWNPAK CR(+), 0.4 cm ϕ × 15 cm, aqueous $HClO_4$, pH = 1.5, 1.5 mL/min, UV 254 nm, t_R (*R*-enantiomer) 2.1 min, (*S*-enantiomer) 12.1 min); $[\alpha]^{20}_D$ +40.0 (c = 0.200, 0.1% TFA); ^{1}H NMR (D_2O, 200 MHz) δ 7.38 (d, J = 9 Hz, 2H), 7.25 (d, J = 9 Hz, 2H), 4.99 (s, 1H); ^{13}C NMR (D_2O, 50 MHz) δ 180.0, 171.1, 138.6, 130.7, 129.5, 126.4, 56.4; IR (film, 3M IR-card, polyethylene mesh) broad 3644−2409, 1726 cm^{-1}; LRMS (FAB) m/z 248.1 (M + Na)$^+$, 232.1 (M + Li)$^+$; HRMS (FAB) calcd for $C_9H_{11}N_3O_2SNa$ 248.0469, found 248.0462.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-nitrophenyl]-alanine *tert*-Butyl Ester (27). A saturated aqueous solution of $NaHCO_3$ (10 mL) was added to a solution of 4-nitrophenylalanine monohydrate (**25**, 2.00 g, 8.77 mmol) in 1,4-dioxane/H_2O (30 mL/20 mL) at 0 °C. Di-*tert*-butyl dicarbonate (3.83 g, 17.5 mmol) was added to this mixture, and the mixture was allowed to warm to room temperature with stirring. After 24 h, the volume of the clear solution was reduced by $^1/_2$ in vacuo and acidified with 2 M $KHSO_4$ to pH 2−3 (using pH paper as an indicator). This solution was extracted with EtOAc (3 × 50 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (1:4 EtOAc:hexanes to EtOAC gradient) to afford **26** (2.50 g, 92%) as a white foam: R_f 0.15 (EtOAc); $[\alpha]^{20}_D$ +36.5 (c = 1.00, CH_2Cl_2).

N,*N*-Dimethylformamide di-*tert*-butyl acetal (4.63 mL, 19.4 mmol) was added dropwise over 20 min to a solution of **26** (1.50 g, 4.83 mmol) in dry toluene (20 mL) at 80 °C. The reaction mixture was stirred for 40 min at 80 °C and then cooled to room temperature. The mixture was concentrated in vacuo and the crude product purified by flash chromatography (1:7 EtOAc:pentane) to give **27** (1.30 g, 74%) as a white foam: R_f 0.35 (1:7 EtOAc:pentane); $[\alpha]^{20}_D$ +40.0 (c = 1.09, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.12 (d, J = 9 Hz, 2H), 7.32 (d, J = 9 Hz, 2H), 5.06 (d, J = 7 Hz, 1H), 4.45 (dt, J = 6, 7 Hz, 1H), 3.13 (ddd, J = 6, 4, 10 Hz, 2H), 1.38 (s, 18H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 169.9, 154.6, 146.4, 144.4, 130.0, 122.9, 82.0, 79.2, 54.2, 37.8, 27.8, 27.4; IR ($CDCl_3$) 3433, 1726 cm^{-1}; LRMS (FAB) m/z 367.2 (M + H)$^+$. Anal. Calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.64. Found: C, 58.76; H, 7.07; N, 7.56.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-aminophenyl]-alanine *tert*-Butyl Ester (28). A mixture of **27** (1.25 g, 3.41 mmol) and 10% Pd−C (0.100 g) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 1 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated under vacuum to give an oil. The crude product was purified by flash chromatography (1:2 EtOAc:pentane) to afford **28** (1.07 g, 94%) as an amber syrup: R_f = 0.31 (1:2 EtOAc:pentane); $[\alpha]^{20}_D$ +33.1 (c = 2.80, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.91 (d, J = 8 Hz, 2H), 6.57 (d, J = 8 Hz, 2H), 4.93 (d, J = 8 Hz, 1H), 4.34 (dt, J = 6, 8 Hz, 1H), 3.54 (broad, 2H), 2.90 (d, J = 6, 2H), 1.39 (s, 9H), 1.38 (s, 9H);^{13}C NMR ($CDCl_3$, 50 MHz) δ 170.8, 154.8, 145.1, 123.8, 125.2, 114.6, 81.2, 79.0, 54.7, 37.0, 27.9, 27.5; IR ($CDCl_3$) 3484, 1726 cm^{-1}; LRMS (FAB) m/z 337.2 (M + H)$^+$. Anal. Calcd for $C_{18}H_{28}N_2O_4$: C, 64.26; H, 8.38; N, 8.32. Found: C, 64.36; H, 8.45; N, 8.34.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-*N*, *N*′-bis(*tert*-butyloxy)carbonyl(guanyl)aminophenyl]alanine (29). A mixture of **26** (2.997 g, 9.67 mmol) and 10% Pd−C (0.100 g) in MeOH (20 mL) was shaken in a Parr hydrogenator under 60 psi of H_2 for 1 h. The mixture was filtered through Celite to remove the catalyst and the filtrate concentrated under vacuum to give an oil. *N*,*N*′-Bis(Boc-1-guanyl)pyrazole (5.98 g, 19.3 mmol) in dry THF (50 mL) was added to the oil under argon. After 2 days the solution was concentrated in vacuo and purified by flash chromatography (1:1:0.1 EtOAc:pentane:AcOH) to afford **29** (4.743 g, 93%) as a white foam: R_f 0.28 (1:1:0.1 EtOAc:pentane:AcOH); $[\alpha]^{20}_D$ +27.8 (c = 2.50, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.26 (broad, 1H), 8.94 (br s, 1H), 7.58 (s, 1H), 7.47 (d, J = 8 Hz, 2H), 7.11 (d, J = 8 Hz, 2H), 6.31 (br s, 2H), 5.00 (d, J = 7 Hz, 1H), 4.52 (dt, J = 5, 7 Hz, 1H), 3.08 (t, J = 5 Hz, 2H), 1.48 (s, 9H), 1.47 (s, 9H), 1.41 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 174.9, 163.1, 155.3, 153.5, 153.1, 135.2, 132.8, 129.8, 122.4, 83.6, 79.8, 79.6, 54.0, 37.0, 28.2, 27.9, 27.7; LRMS (FAB) m/z 523.3 (M + H)$^+$. Carboxylic acid **29** was further characterized by conversion to the methyl ester as follows. A solution of CH_2N_2 (0.5 M, 5.00 mmol) in Et_2O (10 mL) was added dropwise to a cooled solution (0 °C) of **29** (0.500 g, 0.958 mmol) in Et_2O (10 mL). The reaction mixture was concentrated in vacuo and the crude product purified by flash chromatography (1:4 EtOAc:pentane) to afford the methyl ester of **29** (0.5029 g, 98%) as a white foam: R_f = 0.24 (1:4 EtOAc:pentane); $[\alpha]^{20}_D$ +33.5 (c = 1.00, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.30 (s, 1H), 7.53 (d, J = 8 Hz, 2H), 7.05 (d, J = 8 Hz, 2H), 4.92 (d, J = 8 Hz, 1H), 4.52 (dt, J = 8, 6 Hz, 1H), 3.68 (s, 3H), 3.02 (d, J = 6 Hz, 2H) 1.50 (s, 9H), 1.48 (s, 9H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 172.1, 163.4, 155.0, 153.3, 153.1, 135.7, 132.1, 129.6, 122.0, 83.6, 79.8, 79.5, 54.2, 52.1, 37.4, 28.2, 28.0, 27.9; IR ($CDCl_3$) 3433, 1711 cm^{-1}; LRMS (FAB) m/z 537.3 (M + H)$^+$. Anal. Calcd for $C_{26}H_{40}N_4O_8$: C, 58.19; H, 7.51; N, 10.44. Found: C, 58.25; H, 7.50; N, 10.40.

4-Guanyl-L-phenylalanine Hydrochloride (9). A solution of **29** (2.00 g, 3.83 mmol) in HCl/dioxane (4 M, 20 mL, Aldrich) was stirred at 23 °C for 24 h under an argon atmosphere. The mixture was concentrated in vacuo, dissolved in H_2O (20 mL), passed through a 6 mL Supelco LC-18 solid-phase extractor, and lyopholyzed to yield **9** (1.102 g, 98%) as an off-white solid. The enantiomeric excess of **9** was determined to be >99% by HPLC (CROWNPAK CR(+), 0.4 cm ϕ × 15 cm, aqueous $HClO_4$, pH = 1.5, 0.8 mL/min, UV 254 nm, t_R (*S*-enantiomer) 6.9 min); $[\alpha]^{20}_D$ +2.05 (c = 2.00, H_2O); ^{1}H NMR (D_2O, 200 MHz) δ 8.00 (s, 1H), 7.23 (d, J = 8 Hz, 2H), 7.13 (d, J = 8 Hz, 2H), 4.19 (t, J = 6, 1H), 3.14 (m, 2H); ^{13}C NMR (D_2O, 50 MHz) δ 171.1, 156.0, 133.7, 133.6, 131.0, 126.2, 53.9, 35.0; IR (film, 3M IR-card, polyethylene mesh) broad 3614−2335, 1732 cm^{-1}; LRMS (FAB) m/z 223.1 (M + H)$^+$; HRMS (FAB) calcd for $C_{10}H_{15}N_4O_2$ 223.1195, found 223.1199.

(*S*)-*N*-[(*tert*-Butyloxy)carbonyl]-[4-thioureaphenyl]-alanine *tert*-Butyl Ester (30). A solution of **28** (1.00 g, 2.97 mmol) in CH_2Cl_2 (20 mL) was added to a mixture of $CaCO_3$ (0.594 g, 5.94 mmol), H_2O (20 mL), CH_2Cl_2 (20 mL), and thiophosgene (0.339 mL, 4.45 mmol). This mixture was vigorously stirred at 23 °C, and after 1 h the organic phase was

separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 25 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo, yielding a yellow oil. A solution of NH_3 (7.0 M, 29.7 mmol) in EtOH (14.24 mL) was added to the crude isothiocyanate and stirred vigorously. When the reaction was complete (judged by thin-layer chromatography), it was concentrated in vacuo and purified by flash chromatography (1:1 EtOAc:pentane) to afford **30** (1.04 g, 89%) as a white foam: $R_f = 0.38$ (1:1 EtOAc:pentane); $[\alpha]^{20}_D$ +40.4 (c = 1.00, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.57 (br s, 1H), 7.20 (d, 2H, J = 8 Hz), 7.12 (d, 2H, J = 8 Hz), 6.31 (br s, 2H), 5.24 (d, 1H, J = 8 Hz), 4.40 (dt, 1H, J = 6, J = 8), 2.99 (m, 2H), 1.38 (s, 9H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 179.9, 170.6, 154.7, 135.2, 134.8, 130.2, 123.9, 81.5, 79.0, 54.4, 37.1, 27.7, 27.3; IR ($CDCl_3$) 3506, 1711 cm^{-1}; LRMS (FAB) m/z 396.2 $(M + H)^+$. Anal. Calcd for $C_{19}H_{29}N_3O_4S$: C, 57.69; H, 7.39; N, 10.62; S, 8.10. Found: C, 57.93; H, 7.44; N, 10.44; S, 7.90.

4-Thiourea-L-phenylalanine Hydrochloride (10). A solution of **30** (1.00 g, 2.53 mmol) in HCl/dioxane (4 M, 15 mL, Aldrich) was stirred at 23 °C for 8 h under an argon atmosphere. The mixture was concentrated in vacuo, dissolved in H_2O (10 mL), passed through a 6 mL Supelco LC-18 solid-phase extractor, and lyopholyzed to yield **10** (0.777 g, 98%) as an off-white solid. The enantiomeric excess of **10** was determined to be >99% by HPLC (CROWNPAK CR(+), 0.4 cm ϕ × 15 cm, aqueous $HClO_4$, pH = 1.5, 0.8 mL/min, UV 254 nm, t_R (*S*-enantiomer) 9.6 min); $[\alpha]^{20}_D$ −5.68 (c = 1.09, H_2O); ^{1}H NMR (D_2O, 200 MHz) δ 7.10 (d, J = 7 Hz, 2H), 6.99 (d, J = 7 Hz, 2H), 4.11 (t, J = 5 Hz, 1H), 3.06 (m, 2H);^{13}C NMR (D_2O, 50 MHz) δ 178.9, 170.6, 136.2, 132.7, 130.6, 125.7, 53.8, 34.9; IR (film, 3M IR-card, polyethylene mesh) broad 3636−2401, 1733 cm^{-1}; LRMS (FAB) m/z 240.1 $(M + H)^+$; HRMS calcd for $C_{10}H_{14}N_3O_2S$ 240.0806, found 240.0797.

Biochemistry: General. Dithiothreitol, nicotine adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4B), L-arginine, lactate dehydrogenase, sodium pyruvate, and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma. Inducible nitric oxide synthase (mouse, 100 000 × g supernatant) was purchased from Cayman Chemical, Ann Arbor, MI. Neuronal NOS was isolated from rat cerebellum as previously described.[26]

Measurement of Nitrite Production by iNOS. Experiments to determine whether any of the synthetic analogues could be utilized by iNOS as substrates were performed using the Griess assay. Inducible NOS (25 nM) was incubated at 37 °C for 30 min with 1 mM dithiothreitol; 4 μM each of FAD, FMN, and H_4B; 1 mM each of NADPH and test compound in a 96-well plate with a final volume of 100 μL. Reactions were initiated with the addition of NADPH and were performed in triplicate. After depleting any remaining NADPH by incubation with lactate dehydrogenase (1000 unit/mL) and sodium pyruvate (50 mM) for 15 min at 37 °C, Griess reagent was added and the absorbance at 550 nm was measured with a microplate reader (Spectramax, Molecular Devices). Nitrite production was compared to controls containing either no substrate analogues or L-arginine (1 mM).

Radioactive L-Citrulline Assay for NOS Activity. A diluted solution of commercial iNOS or the cytosolic fraction of rat cerebellum containing nNOS (25 μL) was added to serial tubes containing 1 μM radiolabeled ^{14}C-arginine and $CaC1_2$ (1 mM) and NADPH (1 mM) and varying concentrations (1 × 10^{-6} to 1 × 10^{-3} M) of the compounds to be tested in a final volume of 200 μL. The reactions proceeded at 22 °C for 30 min and were terminated by addition of 2 mL of buffer containing 30 mM hydroxyethyl piperazine diethanesulfonic acid (HEPES) and 3 mM ethylenediamine tetraacetic acid (EDTA), pH 5.5. The reaction mixtures were applied to chromatography columns preapplied with 0.5 mL DOWEX AG50WX-8 ion-exchange resin (Na^+ form, pH 7.0). The columns were rinsed with water (2 × 1 mL) to ensure complete elution of citrulline. The eluant containing ^{14}C-citrulline was quantified by liquid scintillation spectroscopy with an efficiency of 90−94%. Reaction velocities of arginine conversion to an equimolar ratio of NO and ^{14}C-citrulline were calculated and data expressed as enzyme activity per milligram of protein (picomoles citrulline per milligram protein per minute). Inhibition of enzyme activity is expressed as percent inhibition of control (100%). All reactions were performed in duplicate or triplicate.

Acknowledgment. This work was supported by a grant (9630310N, S.B.K.) from the American Heart Association. The donors of the Petroleum Research Fund (PRF 32927-G1) and Wake Forest University (Cross Campus Collaborative Research Fund) are gratefully acknowledged for financial support. Mass spectrometry was performed by the Nebraska Center for Mass Spectrometry.

JO982161F

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