Synthesis and Evaluation of Peptidomimetics as Selective Inhibitors and Active Site Probes of Nitric Oxide Synthases

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Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrilline and nitric oxide (NO). Selective inhibition of the isoforms of NOS could have great therapeutic potential in the treatment of certain disease states arising from pathologically elevated synthesis of NO. Recently, we reported dipeptide amides containing a basic amine side chain as potent and selective inhibitors of neuronal NOS (Huang, H.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *J. Med. Chem.* **1999**, *42*, 3147). The most potent nNOS inhibitor among these compounds is L-Arg^{NO_2}-L-Dbu-NH_2 (**1**) (K_i = 130 nM), which also exhibits the highest selectivity over eNOS (>1500-fold) with excellent selectivity over iNOS (190-fold). Here we describe the design and synthesis of a series of peptidomimetic analogues of this dipeptide as potential selective inhibitors of nNOS. The biochemical evaluation of these compounds also revealed the binding requirements of the dipeptide inhibitors with NOS. Incorporation of protecting groups at the N-terminus of the dipeptide amide **1** (compounds **4** and **5**) resulted in dramatic decreases in the inhibitory potency of nNOS. Masking the NH group of the peptide bond (peptoids **6**−**8** and *N*-methylated compounds **9**−**11**) also gave much poorer nNOS inhibitors than **1**. Both of the results demonstrate the importance of the α-amine of the dipeptide and the NH moiety of the peptide bond for binding at the active site. Modifications at the C-terminus of the peptide included converting the amide to the methyl ester (**12**), *tert*-butyl ester (**13**), and carboxylic acid (**14**) and also descarboxamide analogues (**15**−**17**), which revealed less restricted binding requirements for the C-terminus of the dipeptide. Further optimization should be possible when we learn more about the binding requirements at the active sites of NOSs.

Introduction

Nitric oxide (NO) has been of great interest to medicinal chemists since it was discovered as an important biological second messenger over a decade ago.[1] A family of enzymes, the nitric oxide synthases (NOS, E.C. 1.14.13.39), catalyzes the stepwise oxidation of L-arginine to L-citrulline and nitric oxide.[2] To date, three structurally distinct NOS isoforms have been identified.[3] One constitutively expressed isoform is located in neuronal tissue[4] (nNOS) and is involved in neurotransmission and long-term potentiation.[5] The other constitutive isoform is endothelial NOS (eNOS), which is involved in the regulation of smooth muscle relaxation and vascular tone.[6] NO produced by the inducible isoform (iNOS) in activated macrophage cells plays a key role in normal immune responses by functioning as a cytotoxic agent.[7] The isoforms of NOS share only approximately 50% primary sequence homology, which suggests that they may differ from each other in regulatory aspects;[8] however, there is very high sequence identity across species. The catalytic reaction of NOS requires 2 equiv of molecular oxygen and 1.5 equiv of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cosubstrates.[9] All isoforms of NOS contain an N-terminal oxygenase domain with binding sites for L-arginine, cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4B) and heme, and a linked C-terminal reductase domain with NADPH, FAD, FMN, and calmodulin binding sites.[9]

Overproduction of NO has been implicated in a wide variety of disease states including septic shock, inflammatory, and neurodegenerative diseases; selective inhibitors of the isoforms of NOS could have great therapeutic potential in the treatment of these diseases.[10,11] Prototypical NOS inhibitors were mostly analogues of the natural substrate, L-arginine, including N^{ω}-methyl-L-arginine (L-NMA),[12] N^{ω}-nitro-L-arginine (L-NNA),[13] and N^{δ}-(iminoethyl)-L-ornithine (L-NIO).[14] Most of these amino acid-based inhibitors are irreversible inactivators of NOS with minimal selectivity among the isoforms. Modifications of the guanidine moiety of L-arginine led to some potent NOS inhibitors, such as *S*-alkyl-L-thiocitrulline,[15] amidines,[16] guanidines,[17] and isothioureas.[18] Apart from the L-arginine binding site of NOS, other binding sites have been targeted when designing the inhibitors of NOS: various indazoles or imidazoles inhibit NOS acting as ligands to the heme prosthetic group,[19] and 4-aminopteridine derivatives bind specifically at the H_4B binding site of NOS.[20]

We have previously reported a library of dipeptide amides containing nitroarginine as inhibitors of nNOS.[21]

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§ Carried out all of the experiments in this study.

Developed the eNOS overexpression system in *E. coli*, purified the eNOS, and isolated the eNOS.

|| Developed the overexpression system for nNOS in *E. coli* and purified the enzyme.

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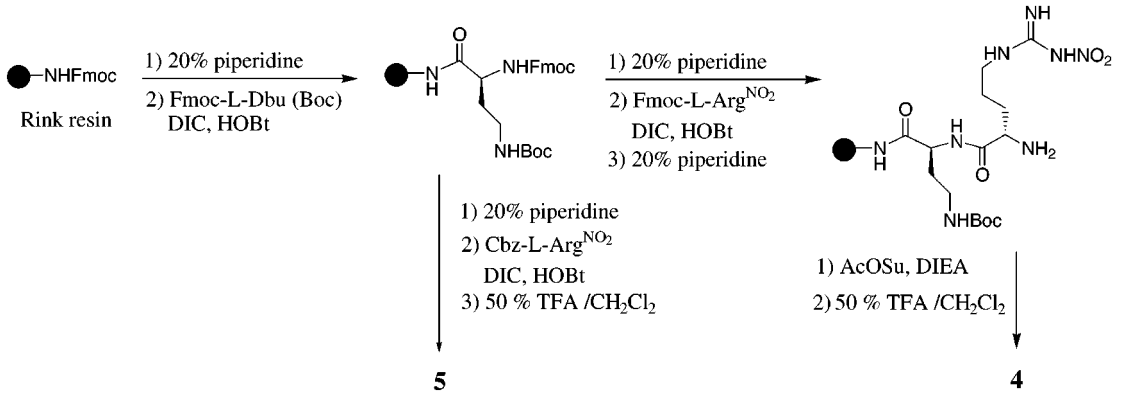
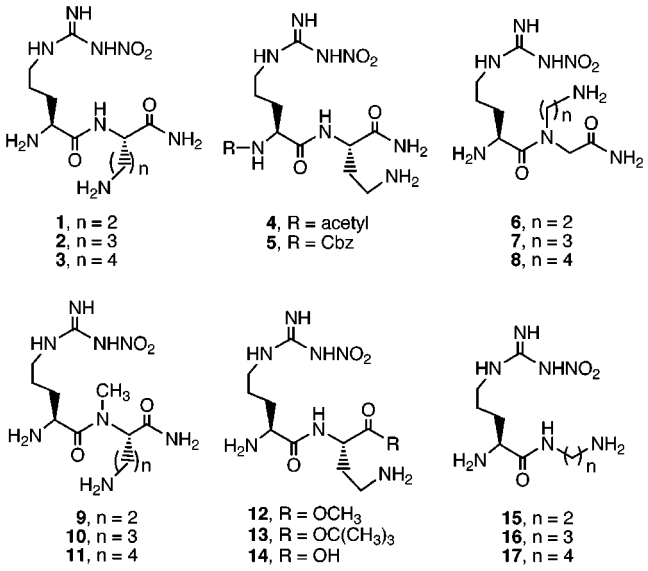
Scheme 1



Excellent inhibitory potency and selectivity for nNOS over eNOS and iNOS are achieved with the dipeptide amides that have an amine-containing side chain. The most potent nNOS inhibitor among these compounds is the dipeptide amide containing L-2,4-diaminobutyric acid (Dbu), L-Arg^{NO_2}-L-Dbu-NH_2 (**1**) (K_i = 130 nM), which also exhibits the highest selectivity over eNOS (>1500-fold) with a 192-fold selectivity over iNOS. The length of the amine side chain seems to have only a minor effect on the potency for all isoforms of NOS: L-Arg^{NO_2}-L-Orn-NH_2 (**2**) and L-Arg^{NO_2}-L-Lys-NH_2 (**3**) inhibit nNOS with K_i values of 330 and 450 nM, respectively. Unlike nitroarginine, none of these dipeptides exhibited time-dependent inhibition of any of the isoforms. To investigate the SAR studies and improve the potential bioavailability of the dipeptide inhibitors, a series of peptidomimetic analogues were synthesized and evaluated in the present study. Protecting the α-amino group of the dipeptide amide **1** with an acetyl and Cbz (benzyloxycarbonyl) group gives compounds **4** and **5**. Peptoid analogues (**6**−**8**) and *N*-methylated compounds (**9**−**11**) mask the NH group of the peptide bond. Compounds **12**−**14** are the methyl ester, *tert*-butyl ester, and carboxylic acid of the dipeptide **1**, respectively. Removal of the C-terminal carboxamide moiety results in descarboxamide analogues **15**−**17**. (See Chart 1 for structures of compounds **1**−**17**.) These modifications of the original potent dipeptide inhibitors also reveal the binding requirements of the dipeptide with NOS.

Chart 1



Chemistry

The two α-amino-protected dipeptide amides (**4**, **5**) were synthesized on solid phase as shown in Scheme 1. Initially, acetylation of L-Arg^{NO_2} was carried out prior to the peptide coupling. However, it was realized later that the acetyl-protected amino acid epimerized easily during the peptide coupling reaction. Acetylation of the dipeptide on the Rink resin worked well.

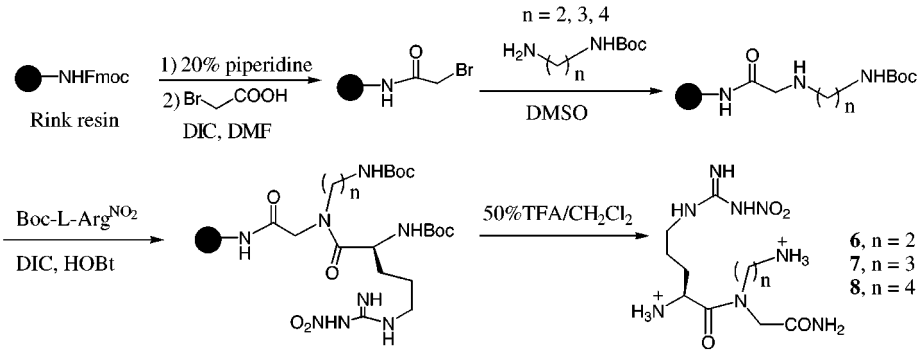
A peptoid differs from a peptide as a result of the transfer of the side chain of the amino acid from the α-carbon to the nitrogen of the peptide bond. Peptoids represent a new class of imino acid residues that are not found in nature and have shown good proteolytic stability.[22] The syntheses of the peptoid analogues **6**−**8** were carried out on solid phase, as shown in Scheme 2. The only difficulty with this synthesis was the formation of the monoprotected diamines, which were prepared by carefully adjusting the pH of the reaction mixture.

N-Methylated diepeptides (**9**−**11**) were synthesized on a solid support as shown in Scheme 3. The α-amino group of the amino acids (Dbu, Orn, and Lys) was protected and activated by 2-nitrophenylsulfonyl chloride, followed by methylation using the Mitsunobu reaction.[23] The second amino acid (L-Arg^{NO_2}) was coupled to the resin after removal of the 2-nitrophenylsulfonyl group.

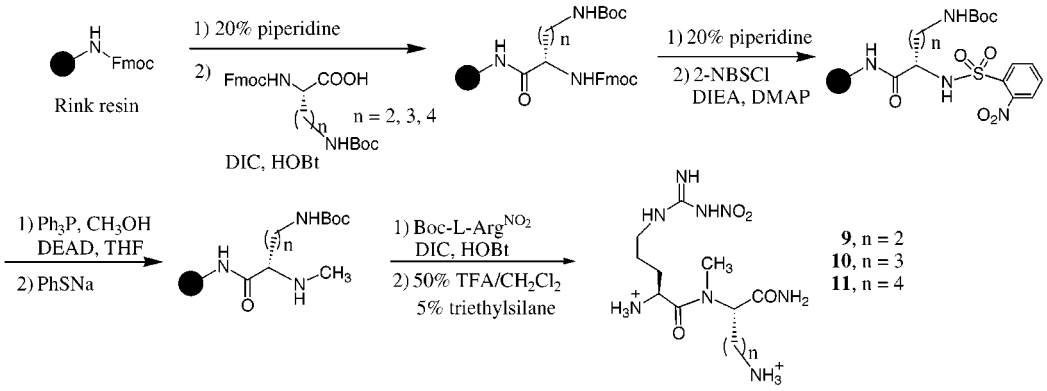
The dipeptide methyl ester, L-Arg^{NO_2}-L-Dbu-OCH_3 (**12**), was obtained by the coupling of Boc-L-Arg^{NO_2} and L-Dbu(Boc)-OCH_3, which was prepared by the methylation of commercially available Fmoc-L-Dbu(Boc)-OH followed by removal of the Fmoc group. The synthetic route of L-Arg^{NO_2}-L-Dbu *tert*-butyl ester (**13**) is shown in Scheme 4; the key step is the amide degradation of N^{α}-Cbz-L-Gln *tert*-butyl ester to N^{α}-Cbz-L-Dbu *tert*-butyl ester. The N^{α}-Cbz group was removed by carefully monitoring the reaction, because the N^{δ}-Fmoc group also can be cleaved during catalytic hydrogenation, although in a slower reaction. The dipeptide carboxylic acid **14** was obtained from the dipeptide *tert*-butyl ester **13** by acid deprotection (Scheme 4).

The synthesis of compounds **15**−**17** was carried out on Wang resin (Scheme 5). The resin was activated by *p*-nitrophenyl chloroformate followed by coupling with L-Arg^{NO_2} methyl ester. Monoprotected diamines were coupled to the resin after the hydrolysis of the methyl

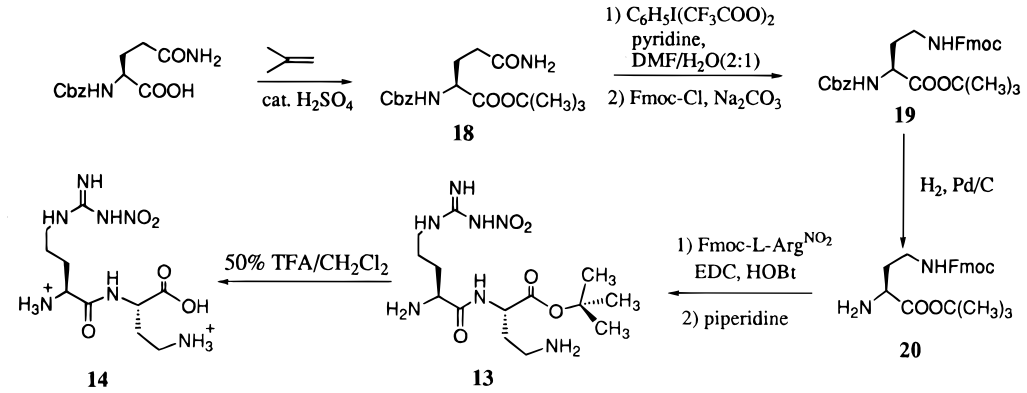
Scheme 2



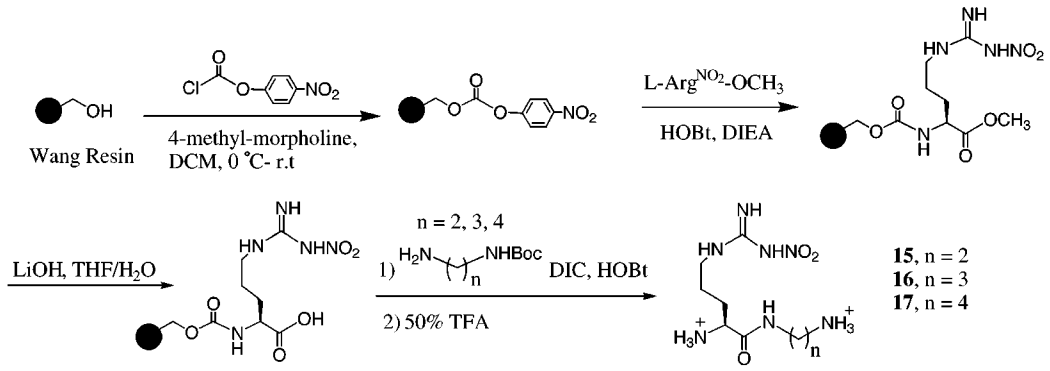
Scheme 3



Scheme 4



Scheme 5



ester. Compounds **15**−**17** were also synthesized in a solution-phase reaction, which gave pure compounds after chromatography. All of the compounds synthesized on solid phase were >80% pure by HPLC. They were purified using prep-HPLC prior to the enzyme assay.

Results and Discussion

The K_i data for these peptidomimetic compounds are given in Table 1 along with the data for the three potent and selective dipeptide amides inhibitors (**1**−**3**). None

Table 1. NOS Inhibition by the N^{ω}-Nitroarginine-Containing Dipeptides, Dipeptide Esters, and Peptidomimetics[a]

	K_i (μM)[b]			selectivity[c]	
compound	nNOS	iNOS	eNOS	eNOS/ nNOS	iNOS/ nNOS
1, L-Arg^{NO_2}-L-Dbu-NH_2[d]	0.13	25	200	1538	192
2, L-Arg^{NO_2}-L-Orn-NH_2[d]	0.33	97	245	742	294
3, L-Arg^{NO_2}-L-Lys-NH_2[d]	0.45	104	141	313	231
4, Ac-L-Arg^{NO_2}-L-Dbu-NH_2	30.4	2600	263	9	86
5, Cbz-L-Arg^{NO_2}-L-Dbu-NH_2	52	1600	827	16	31
6, peptoid-Dbu	19	760	55.5	3	40
7, peptoid-Orn	30.6	1000	108	3.5	33
8, peptoid-Lys	47	1100	197	4	23
9, L-Arg^{NO_2}-(*N*-CH_3)-L-Dbu-NH_2	2.4	340	95	40	142
10, L-Arg^{NO_2}-(*N*-CH_3)-L-Orn-NH_2	4.6	1300	623	135	283
11, L-Arg^{NO_2}-(*N*-CH_3)-L-Lys-NH_2	7.3	1900	631	86	260
12, L-Arg^{NO_2}-L-Dbu-OCH_3	0.58	27	463	798	46
13, L-Arg^{NO_2}-L-Dbu-$OC(CH_3)_3$	10.2	413	288	28	40
14, L-Arg^{NO_2}-L-Dbu-OH	28.4	1210	98	3.5	43
15, L-Arg^{NO_2}-NH-$(CH_2)_2$-NH_2	0.54	100	199	368	185
16, L-Arg^{NO_2}-NH-$(CH_2)_3$-NH_2	0.46	118	213	463	256
17, L-Arg^{NO_2}-NH-$(CH_2)_4$-NH_2	0.35	108	70	200	308

[a] The enzymes used for the K_i measurements are bovine brain nNOS, recombinant murine iNOS, and recombinant bovine eNOS. [b] The K_i values represent duplicate measurements; standard deviations of ±8−12% were observed. [c] The ratio of K_i(eNOS or iNOS) to K_i(nNOS); all are nNOS-selective. [d] Data taken from ref 21.

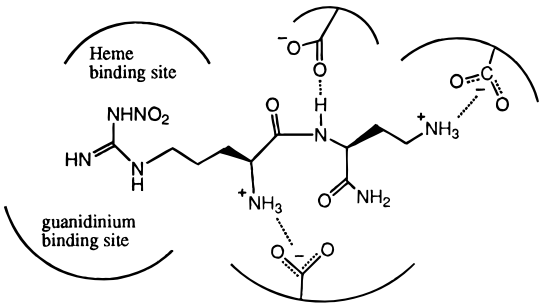


Figure 1. Hypothetical model of the dipeptide amide inhibitor binding at the active site of nitric oxide synthase.

of these dipeptide analogues shows slow, tight binding inhibitory pattern like L-nitroarginine. Incorporation of the acetyl and Cbz protecting groups into the N-terminus of the dipeptide amide L-Arg^{NO_2}-L-Dbu-NH_2 (**1**) results in dramatic decreases in potency of nNOS and iNOS inhibition while having minimal influence on eNOS inhibition. Even though these two protecting groups differ greatly in size, there is little difference in the inhibition of the isoforms of NOS by compounds **4** and **5**. This result suggests that a charged N-terminus may be significant for the interaction of the dipeptide inhibitors with nNOS and iNOS, but not with eNOS, and that there is not much steric crowding at the N-terminus. Our study on the D-forms of the dipeptide amide inhibitors[21,24] indicates that there are two important ionic interactions between the two amino residues of the dipeptide (the α-amino group of the N-terminal amino acid and the ω-amino group of Dbu) and the NOS active site (Figure 1), which is consistent with the findings here. The requirement for this charged N-terminus for binding at the active sites of nNOS and iNOS appears to be more demanding than that of eNOS, which is beneficial for the future design of isoform selective NOS inhibitors.

A peptide is changed to a peptoid by moving the side chain of the amino acid from the α-carbon to the nitrogen of the peptide bond. This makes the peptide bond less susceptible to esterases, but it totally changes the spatial geometry of the peptide. The peptoid analogues of the dipeptide inhibitors (**6**−**8**) were found to be poorer inhibitors of nNOS and iNOS than were the corresponding dipeptides (**1**−**3**), while having little difference toward eNOS inhibition. This results in the dramatic decreases of isoform selectivity of nNOS over eNOS (from 1538-fold to 3−4-fold). The length of the amine side chain has only a small effect on the inhibitory potency of the isoforms of NOS: shorter is slightly better. The spatial change of the peptoids moves the amino group of the side chain away from the possible hydrogen bond or ionic donor of the enzyme (see Figure 1), which may explain the decreases in inhibitory potency.

It is also possible that the potency decreases of the peptoid analogues result from the masking of the NH group of the peptide bond, which may be a crucial hydrogen bond donor. Simple *N*-methylation of the peptide bond of the dipeptide inhibitors supports this hypothesis. The inhibitory potency of the N-methylated dipeptide amides (**9**−**11**) drops about 20-fold for both nNOS and iNOS, compared with the corresponding dipeptide amides (**1**−**3**), but the potency changes much less toward eNOS. This suggests that the NH group of the peptide bond may be involved in hydrogen bonding to nNOS and iNOS, but not so much to eNOS, and that this interaction is not as strong as the ionic interactions of the two amino groups with the active site. A comparison between **9**−**11** and the peptoid analogues **6**−**8** shows an increase in potency of **9**−**11** toward nNOS, comparable inhibitory activities toward iNOS, but decreased potency of **9**−**11** against eNOS. Again, there is an increase in potency toward eNOS by peptide bond *N*-alkylation. However, a geometric change caused by *N*-alkylation cannot be ruled out as the reason for the potency decreases with nNOS and iNOS. Although there is not much difference in the inhibition of the NOS isozymes by these *N*-alkylated dipeptide amides, the one with the shortest amine side chain is a little more potent than the other two.

To investigate the importance of the C-terminal carboxamide moiety of the dipeptide inhibitors for binding, several modifications were made. A methyl ester has a size and charge similar to those of the carboxamide, and the dipeptide methyl ester **12** is also a potent inhibitor of nNOS with only a 4-fold decrease in potency as compared to the corresponding dipeptide amide **1**. The inhibitory potency remains the same for iNOS and decreases 2-fold for eNOS, which results in an nNOS/eNOS selectivity (800-fold) only one-half of that with the carboxamide. To increase the stability of the ester toward possible esterase hydrolysis, the *tert*-butyl ester **13** was synthesized. This compound is 78 times less potent an inhibitor of nNOS and 17-fold less potent toward iNOS but comparable to the corresponding carboxamide in inhibitory properties toward eNOS. Steric hindrance of the *tert*-butyl group is most likely the reason for such dramatic potency decreases. To investigate the importance of an electrostatic effect at the C-terminus, the corresponding dipeptide (carboxylic acid, **14**) was synthesized. This compound is a much poorer inhibitor of both nNOS (218-fold) and iNOS (48-

fold) but is a slightly better inhibitor of eNOS than **1**. One explanation could be a repulsive ionic interaction between the carboxylic acid moiety of the dipeptide and the active site. The cause is probably not an intramolecular hydrogen bond between the carboxylic acid and the amino side chain of the dipeptide, because that very different geometry should have had an effect on eNOS as well.

Finally, we wondered if the carboxamide group was necessary at all. The descarboxamide analogues, L-Arg^{NO_2}-$NH(CH_2)_nNH_2$ ($n = 2-4$, **15**−**17**), were designed and synthesized. These compounds retain the amino side chain of the potent dipeptides but have more flexibility. The K_i data of these compounds in Table 1 show that they also are potent and selective inhibitors of nNOS with potencies and selectivities similar to those of dipeptide inhibitors **1**−**3**. Interestingly, nNOS potency increases slightly with the length of the amine chain, which is opposite that observed in **1**−**3**. It is apparent that the C-terminal carboxamide moiety of the dipeptide inhibitors is not essential for the active site binding.

In conclusion, peptidomimetic approaches have been applied to the potent and selective dipeptide amide inhibitors of nNOS **1**−**3**. Incorporation of protecting groups at the N-terminus of the dipeptide amide **1** (compounds **4** and **5**) and masking of the NH group of the peptide bond (peptoids **6**−**8** and N-methylated compounds **9**−**11**) result in dramatic decreases in the inhibitory potency of nNOS as compared to **1**, which demonstrates the importance of the α-amino group of the dipeptide and the NH moiety of the peptide bond for binding at the active site. Conversion of the carboxamide to the methyl ester (**12**) resulted in a small decrease in potency and selectivity, but conversion to either the *tert*-butyl ester (**13**) or carboxylic acid (**14**) resulted in a dramatic loss of both potency and selectivity. The descarboxamide analogues (**15**−**17**) had only a small effect on both potency and selectivity. The ability to delete the carboxamide group opens up a variety of important peptidomimetic approaches that can be taken.

Experimental Section

Materials. All amino acids and coupling reagents were purchased from Advanced ChemTech, Inc. NADPH, calmodulin, and human ferrous hemoglobin were obtained from Sigma Chemical Co. Tetrahydrobiopterin (H_4B) was purchased from Alexis Biochemicals. HEPES, DTT, and conventional organic solvents were purchased from Fisher Scientific. All other chemicals were purchased from Aldrich, unless otherwise stated.

Analytical Methods. The dipeptides and peptidomimetics were purified on an Alltech Hyperprep PEP HPLC column (250 × 22 mm) using a gradient of 100% solvent A (0.1% TFA in H_2O) to 60% of solvent B (0.08% TFA in CH_3CN) over 30 min at a flow rate of 7.5 mL/min. Optical spectra and enzyme assays were performed on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. ^{1}H NMR spectra were recorded on a Varian VXR-300 spectrometer in the solvent indicated. Chemical shifts are reported as δ values in parts per million relative to TMS in $CDCl_3$ or to DSS in D_2O. Electrospray mass spectra were performed on a Micromass Quattro II spectrometer. Elemental analyses were obtained from Oneida Research Services, Inc., Whiteboro, NY. Thin-layer chromatography was carried out on E. Merck precoated silica gel 60 F_{254} plates. Amino acids were visualized with a ninhydrin spray reagent or a UV/vis lamp. E. Merck silica gel 60 (230−400 mesh) was used for flash chromatography.

General Procedure for Solid-Phase Peptide and Peptidomimetic Synthesis. Rink resin (300 mg, 0.8 mmol/g) was swelled in 2 mL of DMF. The Fmoc group was removed by treatment of the resin with 20% piperidine in DMF (4 mL) for 30 min, followed by successive washings with DMF (3 times), methanol (2 times), and vacuum-drying. The Fmoc protected C-terminal amino acid (3 equiv) was coupled to the resin using diisopropylcarbodiimide (DIC; 3 equiv) and HOBt (3 equiv) as the coupling reagents. The mixture was agitated for 5 h at room temperature. The resin was washed successively with DMF, methanol, and DMF, followed by Fmoc deprotection and washing. The second amino acid was coupled to the resin in the same way. After Fmoc deprotection, washing, and drying, the dipeptide was cleaved from the resin using TFA/CH_2Cl_2 (1:1 v/v) for 1 h. The resin was removed by filtration, and the filtrate was concentrated to dryness. The oily residue was dissolved in a small amount of water, which was washed with ether, and lyophilized.

***N*-(*O*-Acetyl)hydroxysuccinimide (AcOSu).** To a stirred CH_2Cl_2 solution containing acetic acid (2 g, 33.3 mmol) were added *N*-hydroxysuccinimide (3.8 g, 33.3 mmol) and DCC (7.5 g, 36.6 mmol). The milky solution was stirred overnight. The urea was filtered off and washed with CH_2Cl_2. The combined filterates were concentrated under vacuum. The product was crystallized from ethanol as a white powder (4 g, 76% yield): ^{1}H NMR ($CDCl_3$) δ 2.86 (brs, 4H), 2.37 (s, 3H).

N^α-Acetyl-L-Arg^{NO_2}-L-Dbu-NH_2 (4). This dipeptide amide was synthesized as described in the general procedure. Following the peptide coupling N-acetylation was conducted for 2 days according to the procedure of Pacofsky et al:[25] ^{1}H NMR (D_2O) δ 4.48 (dd, 1H), 4.29 (dd, 1H), 3.32 (t, 2H), 3.09 (m, 2H), 2.17−2.28 (m, 1H), 2.03−2.17 (m, 1H), 2.05 (s, 3H), 1.65−1.91 (m, 4H); HRMS (M + 1) calcd for $C_{12}H_{24}N_8O_5$ 361.1943, found 316.1926. Anal. ($C_{12}H_{24}N_8O_5$·1.5TFA) C, H, N.

N^α-Cbz-L-Arg^{NO_2}-L-Dbu-NH_2 (5). This dipeptide amide was synthesized as described in the general procedure using commercially available N^α-Cbz-L-Arg^{NO_2}: ^{1}H NMR (D_2O) δ 7.35 (m, 5H), 5.16 (s, 2H), 4.50 (dd, 1H), 4.35 (dd, 1H), 3.35 (t, 2H), 3.19 (m, 2H), 2.20−2.31 (m, 1H), 2.07−2.20 (m, 1H), 1.55−1.71 (m, 2H), 1.71−1.91 (m, 2H); HRMS (M + 1) calcd for $C_{18}H_{28}N_8O_6$ 453.2205, found 453.2212. Anal. ($C_{18}H_{28}N_8O_6$·1TFA·0.5H_2O) C, H, N.

N^α-(*tert*-Butoxycarbonyl)alkanediamines. The monoprotected alkanediamines were prepared according to the procedure of Videnov et al.[26] with some modifications. The diamines (80 mmol) were dissolved in water (100 mL) in the presence of phenolphthalein. Concentrated HCl was added until the red color disappeared. The colorless solution was titrated with 1 N NaOH until the red color stayed. In the case of 1,2-ethanediamine, the color of the solution was pale pink. To each mixture was added a solution of $(Boc)_2O$ (4.4 g, 20 mmol) in 2-propanol (100 mL). The reactions with 1,2-ethanediamine and 1,3-propanediamine were stirred at room temperature for 2 days, while the reaction with 1,4-butanediamine was stirred for 5 days. The reactions were monitored by TLC (*n*-butanol:acetic acid:water 4:1:1). Water and 2-propanol were removed by evaporation. The NaCl was precipitated by ethanol, filtered, and washed. The filtrate was concentrated in a vacuum, and the residue was dissolved in 1 N NaOH solution. The aqueous solution was extracted with ethyl acetate (EtOAc) twice. The organic layer was extracted twice with 10% citric acid solution. The combined aqueous solution was basified with 1 N NaOH again and extracted with EtOAc several times. Yellowish oily products were obtained after evaporation of the solvent and used in solid-phase synthesis without further purification. N^α-Boc-1,2-ethanediamine (1.8 g, 56% yield): ^{1}H NMR ($CDCl_3$) δ 4.95 (brs, 1H), 3.19 (dt, 2H), 2.81 (t, 2H), 1.48 (s, 9H), 1.34 (s, 2H). N^α-Boc-1,3-propanediamine (1.5 g, 43% yield): ^{1}H NMR ($CDCl_3$) δ 4.94 (brs, 1H), 3.22 (dt, 2H), 2.79 (t, 2H), 1.63 (tt, 2H), 1.56 (brs, 2H), 1.48 (s, 9H). N^α-Boc-1,4-butanediamine (0.3 g, 8% yield): ^{1}H NMR ($CDCl_3$) δ 5.12 (brs, 1H), 2.95 (dt, 2H), 2.54 (t, 2H), 1.17−1.45 (m, 13H).

Peptoid Analogues 6−8. The procedure for solid-phase synthesis of the peptoid analogues was similar to the general procedure mentioned above with two exceptions: each coupling step was conducted overnight; 2-bromoacetic acid, N^{α}-Boc-alkanediamines, and Boc-L-Arg^{NO_2} were in 5 equiv excess.

Peptoid-Dbu (6): ^{1}H NMR (D_2O) δ 4.60 (t, 1H), 4.00−4.30 (m, 2H), 3.90 (m, 1H), 3.45 (m, 1H), 3.22 (m, 4H), 1.75−1.98 (m, 2H), 1.50−1.75 (m, 2H); HRMS (M + 1) calcd for $C_{10}H_{22}N_8O_4$ 319.1837, found 319.1823. Anal. ($C_{10}H_{22}N_8O_4$·2TFA·0.5H_2O) C, H, N.

Peptoid-Orn (7): ^{1}H NMR (D_2O) δ 4.57 (t, 1H), 3.95−4.23 (m, 2H), 3.45 (m, 2H), 3.23 (m, 2H), 2.92 (m, 2H), 1.75−2.00 (m, 4H), 1.45−1.75 (m, 2H); HRMS (M + 1) calcd for $C_{11}H_{24}N_8O_4$ 333.1993, found 333.1975. Anal. ($C_{11}H_{24}N_8O_4$·2TFA·0.5H_2O) C, H, N.

Peptoid-Lys (8): ^{1}H NMR (D_2O) δ 4.55 (t, 1H), 3.91−4.20 (m, 2H), 3.44 (m, 1H), 3.30 (m, 3H), 2.98 (m, 2H), 1.83−2.03 (m, 2H), 1.54−1.80 (m, 6H); HRMS (M + 1) calcd for $C_{12}H_{26}N_8O_4$ 347.2150, found 347.2183. Anal. ($C_{12}H_{26}N_8O_4$·2TFA·0.5H_2O) C, H, N.

***N*-Methylated Dipeptide Amides 9−11.** Fmoc-L-Dbu(Boc)-OH, Fmoc-L-Orn(Boc)-OH, and Fmoc-L-Lys(Boc)-OH were coupled to the Rink resin as described in the general procedure. N-Methylation was carried out according to the procedure of Yang et al.[23]

L-Arg^{NO_2}-(*N*-CH_3)-L-Dbu-NH_2 (9): ^{1}H NMR (D_2O) δ 4.95 (dd, 1H), 4.54 (t, 1H), 3.75 (t, 2H), 2.99 (s, 3H), 2.94 (m, 2H), 2.20 (m, 1H), 2.08 (m, 1H), 1.89 (m, 2H), 1.67 (m, 2H); HRMS (M + 1) calcd for $C_{11}H_{24}N_8O_4$ 333.1993, found 333.1977. Anal. ($C_{11}H_{24}N_8O_4$·2TFA·H_2O) C, H, N.

L-Arg^{NO_2}-(*N*-CH_3)-L-Orn-NH_2 (10): ^{1}H NMR (D_2O) δ 4.89 (dd, 1H), 4.54 (t, 1H), 3.29 (t, 2H), 3.02 (s, 3H), 2.97 (m, 2H), 1.91 (m, 4H), 1.64 (m, 4H); HRMS (M + 1) calcd for $C_{12}H_{26}N_8O_4$ 347.2150, found 347.2183. Anal. ($C_{12}H_{26}N_8O_4$·2TFA·0.5H_2O) C, H, N.

L-Arg^{NO_2}-(*N*-CH_3)-L-Lys-NH_2 (11): ^{1}H NMR (D_2O) δ 4.87 (dd, 1H), 4.53 (t, 1H), 3.31 (t, 2H), 3.04 (s, 3H), 2.96 (m, 2H), 1.91 (m, 4H), 1.69 (m, 4H), 1.35 (m, 2H); HRMS (M + 1) calcd for $C_{13}H_{28}N_8O_4$ 361.2307, found 361.2305. Anal. ($C_{13}H_{28}N_8O_4$·2TFA·H_2O) C, H, N.

L-Arg^{NO_2}-L-Dbu Methyl Ester (12). An ether solution of CH_2N_2 was dropped slowly into a stirred solution of Fmoc-L-Dbu(Boc)-OH (380 mg, 0.86 mmol) in ether. The reaction was stirred for 10 min more and stopped by evaporation of the ether. The oily product, Fmoc-L-Dbu(Boc)-OCH_3 (390 mg, quantitative yield), was used in the next step without purification. After removal of the Fmoc group by catalytic hydrogenation, L-Dbu(Boc)-OCH_3 was purified chromatographically (EtOAc:CH_3OH:NH_4OH 20:1:0.1), giving L-Dbu(Boc)-OCH_3 (190 mg, 97% yield): ^{1}H NMR ($CDCl_3$) δ 5.24 (brs, 1H), 3.67 (s, 3H), 3.45 (dd, 1H), 3.29 (m, 1H), 3.19 (m, 1H), 1.90 (m, 1H), 1.63 (m, 1H), 1.38 (s, 9H). Boc-L-Arg^{NO_2}-OH (295 mg, 0.92 mmol) and L-Dbu(Boc)-OCH_3 (190 mg, 0.84 mmol) were coupled to form the dipeptide, Boc-L-Arg^{NO_2}-L-Dbu(Boc)-OCH_3 (350 mg, 80% yield), according to the procedure of Silverman et al.:[27] ^{1}H NMR ($CDCl_3$) δ 7.55 (brs, 1H), 5.48 (brs, 1H), 4.87 (brs, 1H), 4.64 (m. 1H), 4.32 (brs, 1H), 3.73 (s, 3H), 3.22−3.55 (m, 3H), 3.19 (m, 1H), 1.50−2.19 (m, 6H), 1.41 (s, 18H). l-Arg^{NO_2}-L-Dbu methyl ester (**12**) was obtained after deprotection of the Boc group using TFA/CH_2Cl_2 (1:1 v/v) (104 mg, 96% yield): ^{1}H NMR (D_2O) δ 4.38 (dd, 1H), 3.98 (t, 1H), 3.68 (s, 3H), 3.20 (t, 2H), 2.98 (t, 2H), 2.20 (m, 1H), 1.99 (m, 1H), 1.80 (m, 2H), 1.62 (m, 2H); HRMS (M + 1) calcd for $C_{11}H_{23}N_7O_5$ 334.1833, found 334.1834. Anal. ($C_{11}H_{23}N_7O_5$·2TFA·H_2O) C, H, N.

***N*$^{\alpha}$-(Benzoxycarbonyl)-L-glutamine *tert*-Butyl Ester (18).** This compound was synthesized according to the procedure of Anderson et al.[27] To a suspension of Cbz-L-Gln (5 g, 17.8 mmol) in CH_2Cl_2 (10 mL) in a pressure bottle was added concentrated sulfuric acid (100 μL). While the bottle was cooled in liquid nitrogen, precooled isobutylene liquid (5.51 g, 98 mmol) was added to the mixture. The pressure bottle was sealed and kept at room temperature for 6 days. The bottle was cooled again before the lid was opened. After the mixture was warmed to room temperature, a solution of 1 N $NaCO_3$ (100 mL) was added carefully. The organic layer was separated, and washed with water, then with 1 N $NaCO_3$ solution. The solvent was evaporated, resulting in a transparent oily product (1.5 g, 25% yield): ^{1}H NMR ($CDCl_3$) δ 7.30 (m, 5H), 6.21 (brs, 1H), 5.97 (brs, 1H), 5.83 (brs, 1H), 5.08 (s, 2H), 4.20 (m. 1H), 2.25 (m, 2H), 2.05−2.20 (m, 1H), 1.82−1.97 (m, 1H), 1.43 (s, 9H).

2-*N*-(Benzoxycarbonyl)-4-*N*-(9-fluorenylmethyloxycarbonyl)-L-2,4-diaminobutyric Acid *tert*-Butyl Ester (19). The amide moiety of **18** (1.5 g 4.5 mmol) was converted into an amine and was protected with an Fmoc group in a one-pot reaction according to the procedure of Kazmierski.[28] The product was purified by flash chromatography (hexanes:EtOAc 3:1) and was obtained as a white solid in a 46% yield (1.31 g): ^{1}H NMR ($CDCl_3$) δ 7.77 (d, 2H), 7.61 (d, 2H), 7.35 (m, 9H), 5.56 (brs, 1H), 5.14 (s, 2H), 4.37 (m, 2H), 4.24 (t, 1H), 3.52 (m, 1H), 3.10 (m, 1H), 2.12 (m, 1H), 1.70 (m, 1H), 1.45 (s, 9H).

4-*N*-(9-Fluorenylmethyloxycarbonyl)-L-2,4-diaminobutyric Acid *tert*-Butyl Ester (20). Compound **19** (1.31 g, 2 mmol) was dissolved in methanol (20 mL) and was hydrogenated with a 5% Pd/C catalyst until the Cbz group was removed. The catalyst was filtered through a Celite pellet, and the methanol was evaporated in a vacuum. To avoid cleavage of the Fmoc group, which usually reacts much slower under the same conditions, TLC (hexanes:EtOAc:NH_4OH 1:1:0.1) was taken every 0.5 h to monitor the progress of the reaction until compound **19** was gone and most of the product was compound **20**: ^{1}H NMR ($CDCl_3$) δ 7.78 (d, 2H), 7.59 (d, 2H), 7.39 (t, 2H), 7.30 (t, 2H), 5.62 (brs, 1H), 4.40 (d, 2H), 4.37 (m, 2H), 4.24 (t, 1H), 3.52 (m, 1H), 3.10 (m, 1H), 2.12 (m, 1H), 1.70 (m, 1H), 1.45 (s, 9H). This yellow oily compound (0.77 g, 95% yield) was used in the next step without further purification.

L-Arg^{NO_2}-L-Dbu *tert*-Butyl Ester (13). Fmoc-L-Arg^{NO_2}-OH (864 mg, 1.96 mmol) and L-Dbu(Fmoc) *tert*-butyl ester (**20**; 770 mg, 1.78 mmol) were coupled to form Fmoc-L-Arg^{NO_2}-L-Dbu(Fmoc) *tert*-butyl ester as described.[29] The Fmoc-L-Arg^{NO_2}-L-Dbu(Fmoc) *tert*-butyl ester (600 mg, 40% yield) was purified by flash chromatography (1% methanol in EtOAc): ^{1}H NMR ($CDCl_3$) δ 7.74 (m, 4H), 7.55 (m, 4H), 7.39 (m, 4H), 7.28 (m, 4H), 6.02 (brs, 1H), 5.44 (brs, 1H), 4.49 (brs, 1H), 4.30 (m, 4H), 4.12 (brs, 1H), 3.05−3.40 (m, 4H), 2.01 (m, 1H), 1.83 (m, 1H), 1.65 (m, 4H), 1.40 (s, 9H). The Fmoc-protected dipeptide (600 mg, 0.78 mmol)) was treated with 20% piperidine in DMF (10 mL). After being stirred at room temperature for a 0.5 h, the solution was dried under high vacuum (at least 8 h, to make sure all of the piperidine and DMF were evaporated). The residue was dissolved in water and washed with ether twice. The aqueous layer was acidified to pH 4 using 10% HCl and lyophilized to dryness (170 mg, 50% yield): ^{1}H NMR (D_2O) δ 4.51 (m, 1H), 4.09 (t, 1H), 3.34 (m, 2H), 3.10 (t, 2H), 2.27 (m, 1H), 2.07 (m, 1H), 1.91 (m, 2H), 1.72 (m, 2H), 1.44 (s, 9H); HRMS (M + 1) calcd for $C_{14}H_{29}N_7O_5$ 376.2303, found 376.2325. Anal. ($C_{14}H_{29}N_7O_5$·2TFA·H_2O) C, H, N.

L-Arg^{NO_2}-L-Dbu-OH (14). Compound **13** (100 mg, 0.23 mmol) was treated with TFA (4 mL) for 1 h. The solution was concentrated to dryness. The oily residue was dissolved in a small amount of water, which was washed with ether and lyophilized to a white foam (80 mg, 92% yield): ^{1}H NMR (D_2O) δ 4.53 (dd, 1H), 4.06 (t, 1H), 3.27 (m, 2H), 3.07 (t, 2H), 2.26 (m, 1H), 2.07 (m, 1H), 1.92 (m, 2H), 1.70 (m, 2H); HRMS (M + 1) calcd for $C_{10}H_{21}N_7O_5$ 320.1677, found 320.1684. Anal. ($C_{10}H_{21}N_7O_5$·2TFA·H_2O) C, H, N.

L-Arg^{NO_2}-NH$(CH_2)_n$$NH_2$ (n = 2−4, 15−17). To an ice-cooled mixture of Wang resin (2.4 g, 0.76 mmol/g, 100−200 mesh) and 4-nitrophenyl chloroformate (1.10 g, 3 equiv) in CH_2Cl_2 (15 mL) was added 4-methylmorpholine (602 μL, 3 equiv). The mixture was stirred overnight from 0 °C to room temperature. The resin was drained, washed successively with CH_2Cl_2, methanol, water, and methanol and dried in vacuum. The resin was treated with L-Arg^{NO_2} methyl ester (3 equiv), HOBt (3 equiv), and diisopropylethylamine (DIEA; 5 equiv) in DMF/CH_2Cl_2 (1:1, 15 mL) overnight at room temperature. After removal of the excess reagents, the resin was washed thor-

oughly with DMF, CH_2Cl_2, and methanol and dried. The resin was treated with lithium hydroxide (5 equiv) in THF/H_2O (5:1 v/v, 15 mL) for 3 h at room temperature followed by washing with THF, H_2O, and methanol and then dried in vacuum. The resin was divided into three portions, each of which was coupled with a different N^{α}-Boc-alkanediamine using a 4 equiv excess of coupling reagents. After washing and drying, the resin was treated with TFA/CH_2Cl_2 (1:1 v/v) for 30 min. The products were purified by HPLC. These compounds were also synthesized by solution phase according to the peptide synthesis procedure as described.[29]

L-Arg^{NO_2}-NH(CH$_2$)$_2$NH$_2$ (15): ^{1}H NMR (D_2O) δ 4.08 (t, 1H), 3.32 (m, 4H), 3.15 (m, 2H), 1.98 (m, 2H), 1.64−1.88 (m, 2H); HRMS (M + 1) calcd for $C_8H_{19}N_7O_3$ 262.1622, found 262.1639. Anal. ($C_8H_{19}N_7O_3$·2.5TFA) C, H, N.

L-Arg^{NO_2}-NH(CH$_2$)$_3$NH$_2$ (16): ^{1}H NMR (D_2O) δ 3.98 (t, 1H), 3.30 (m, 4H), 2.97 (t, 2H), 1.32−1.96 (m, 4H), 1.60−1.72 (m, 2H); HRMS (M + 1) calcd for $C_9H_{21}N_7O_3$ 276.1779, found 276.1786. Anal. ($C_9H_{21}N_7O_3$·2TFA·0.8H_2O) C, H, N.

L-Arg^{NO_2}-NH(CH$_2$)$_4$NH$_2$ (17): ^{1}H NMR (D_2O) δ 3.95 (t, 1H), 3.28 (m, 4H), 2.98 (m, 2H), 1.90 (m, 2H), 1.50−1.73 (m, 6H); HRMS (M + 1) calcd for $C_{10}H_{23}N_7O_3$ 290.1935, found 290.1926. Anal. ($C_{10}H_{23}N_7O_3$·2TFA·0.5H_2O) C, H, N.

Enzyme and Assay. All of the NOS isoforms used are recombinant enzymes overexpressed in *E. coli* from difference sources; there is very high sequence identity for the isoforms from different sources. The murine macrophage iNOS was expressed and isolated according to the procedure of Hevel et al.[30] The rat nNOS was expressed[31] and purified as described.[32] The bovine eNOS was isolated as reported.[33] Nitric oxide formation from NOS was monitored by the hemoglobin capture assay as described previously.[34]

Determination of K_i Values. The apparent K_i values were obtained by measuring percent inhibition in the presence of 10 μM L-arginine with at least three concentrations of inhibitor. The parameters of the following inhibition equation[35] were fitted to the initial velocity data: % inhibition = 100[I]/[[I] + K_i(1 + [S]/K_m)]. K_m values for L-arginine were 1.3 μM (nNOS), 8.3 μM (iNOS), and 1.7 μM (eNOS). The selectivity of an inhibitor was defined as the ratio of the respective K_i values.

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Supporting Information Available: Analytical data for compounds **4**−**17**. This material is available free of charge via the Internet at http://pubs.acs.org.

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