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Synthesis and Evaluation of Dipeptide Amides Containing N^{ω}-Nitroarginine and D-2,4-Diaminobutyric Acids as Inhibitors of Neuronal Nitric Oxide Synthase

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Selective inhibition of the isoforms of nitric oxide synthase (NOS) could be beneficial in the treatment of certain disease states arising from the overproduction of nitric oxide by NOS. Recently, we reported dipeptide amides containing a basic amine side chain as potent and selective inhibitors of neuronal NOS (Huang, H. *et al.* (1999) *J. Med. Chem.*, 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). The D,D-dipeptide, D-Lys-D-Arg^{NO_2}-NH_2 (3) also shows high potency and selectivity. Here the dipeptide amides containing Arg^{NO_2} and D-Dbu (9-12) were synthesized and evaluated. They are all modest inhibitors of nNOS, but poor inhibitors of eNOS and iNOS. D-Dbu-D-Arg^{NO_2}-NH_2 (12) exhibits decreased inhibitory potency as compared with 3. A hypothesis regarding the binding at the active site of nNOS is proposed to explain the potency differences between the L- and D-form dipeptide amides.

Keywords: Dipeptide amide, Nitric oxide synthase, Nitroarginine, D-2,4-Diaminobutyric acid, Enzyme inhibition

INTRODUCTION

Nitric oxide (NO) plays an important role in mediating a number of physiological functions including neuronal signaling, vascular tone, and the immune response.[1] Nitric oxide synthases (NOS, EC 1.14.13.39) are responsible for the production of NO and L-citrulline from L-arginine in a NADPH- and O_2-dependent process.[2] At least three isoforms of NOS exist, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), and each has distinct physiological roles.[3] nNOS and eNOS are constitutive isoforms, which are regulated by the calmodulin and Ca^{2+} concentrations, while iNOS is independent of calmodulin and Ca^{2+}. Overproduction of NO has been implicated in a wide variety of diseases; selective inhibitors of

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†Carried out all of the experiments in this study.
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¶Developed the overexpression system for nNOS in *E. coli* and the purification of the enzyme.

the isoforms of NOS could be therapeutically useful in the treatment of these diseases.[4,5]

Most of the NOS inhibitors fall into two categories: amino-acid-based inhibitors that are analogues of the natural substrate, L-arginine, and non-amino-acid-based inhibitors.[6,7] Most of the first generation compounds, including N^{ω}-methyl-L-arginine[8] and N^{ω}-nitro-L-arginine,[9] are time-dependent inactivators of all or one isoform of NOS, but exhibit minimal inhibitory selectivity among the isoforms. Some of the non-amino-acid-based compounds, for example, aminoguanidine,[10] *N*-(3-(aminomethyl)benzyl) acetamidine,[11] and 2-iminopyrrolidines,[12] however, are potent and selective inhibitors of NOS. Recently, we reported a library of dipeptide amides containing nitroarginine as inhibitors of nNOS.[13] Excellent inhibitory potency and selectivity for nNOS over eNOS and iNOS is achieved with the dipeptide amides containing an amine-containing side chain. The most potent nNOS inhibitor among these compounds is the dipeptide amide containing 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 (Table I). Even though it has two methylene units in the side chain longer than Dbu, the L-Lys-containing dipeptide amide, L-Arg^{NO_2}-L-Lys-NH_2 (**2**), also inhibits nNOS with good potency ($K_i = 450$ nM) and selectivity (300-fold and 230-fold selective for nNOS over eNOS and iNOS, respectively). Interestingly, the D-lysine containing dipeptide amide, D-Lys-D-Arg^{NO_2}-NH_2 (**3**) exhibits similar inhibitory potency ($K_i = 890$ nM for nNOS) with even higher selectivity over iNOS (1000-fold). Because (**2**) and (**3**) are *retro-inverso* dipeptides,[14] it was rationalized that they bind to the same binding site of nNOS, but flipped 180° relative to each other (Figure 1 and Figure 2A). Recent ENDOR spectroscopic results support the hypothesis that the guanidino moiety of both *retro-inverso* dipeptides bind in the heme binding site similar to that of the substrate arginine.[15] None of the dipeptide amides in our previous library contained D-Dbu because that amino acid is not commercially available. We now report the synthesis of D-Dbu, its incorporation into dipeptide amides with Arg^{NO_2}, and the evaluation of the Arg^{NO_2}- and D-Dbu-containing dipeptide amides as inhibitors of nNOS.

MATERIALS AND METHODS

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 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 DSS in D_2O. Electro spray 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.

2-[N-Benzoxycarbonyl)amino]-D-homoserine, (**4**). D-Homoserine (1 g, 8.4 mmol) was dissolved in 10 mL of 1 M Na_2CO_3 solution in an ice-bath. To the stirred mixture was added dropwise 1.2 mL of benzyl chloroformate (8.4 mmol). The solution was stirred at 0 °C for 30 min then at room temperature for 2 h. Concentrated HCl was used to acidify the mixture, then the white precipitate was extracted twice with EtOAc. The organic layer was washed with 10% Na_2CO_3 solution. The basic aqueous layer was acidified again and extracted with EtOAc twice. The EtOAc layer was washed with water and saturated NaCl and was dried over $MgSO_4$. After evaporation of the solvent, the product (1.9 g) was obtained in a 90% yield as a white solid. ^{1}H-NMR (CD_3OD) δ 7.36 (m, 5H), 5.72 (d, 1H), 5.14 (s, 2H), 3.95 (m, 1H), 3.23 (m, 2H), 2.16 (m, 1H), 1.75 (m, 1H).

2-[N-Benzoxycarbonyl)amino]-D-homoserine methyl ester, (**5**). Diazomethane was prepared freshly according to the procedure which came with the Diazald Kit glassware purchased from Aldrich. Excess diazomethane was added dropwise to a stirred soluton of (**4**) (1.9 g, 7.5 mmol) in ether until the yellow color persisted. After being stirred for an additional 20 min, the solvent was evaporated, leaving the product as a yellow oil (1.95 g, 97%). ^{1}H-NMR ($CDCl_3$) δ 7.36 (m, 5H), 5.72 (d, 1H), 5.14 (s, 2H), 4.55 (m, 1H), 3.78 (s, 3H), 3.70 (m, 2H), 2.90 (br.s, 1H), 2.16 (m, 1H), 1.75 (m, 1H).

2-[N-Benzoxycarbonyl)amino]-4-[N-(tert-butoxycarbonyl)-N′-ethyl oxamate)amino]-D-diaminobutylic acid methyl ester, (**6**). *N*-Boc Ethyl oxamate was prepared according to the known procedures.[16] The mixture of (**5**) (1.95 g, 7.3 mmol), *N*-Boc ethyl oxamate (2 g, 9.2 mmol), DEAD (1.38 mL, 8.8 mmol) and PPh_3 (2.31 g, 8.8 mmol) in THF was stirred at room temperature overnight. The solvent was evaporated, and the residue was applied to a silica gel column. The product was eluted from the column using 2:1 hexanes: EtOAc; after evaporation of the solvent the product was obtained as a white solid in a 95% yield (3.3 g). ^{1}H-NMR ($CDCl_3$) δ 7.36 (m, 5H), 5.60 (d, 1H), 5.14 (s, 2H), 4.46 (m, 1H), 4.33 (q, 2H), 3.76 (m, 5H), 2.11 (m, 2H), 1.52 (s, 9H), 1.35 (t, 3H).

2-[N-Benzoxycarbonyl)amino]-4-[N-(tert-butoxycarbonyl)amino]-D-diaminobutylic acid, (**7**). To a solution of (**6**) (3.6 g, 7.73 mmol) in 15 mL THF, was added a solution of LiOH (4.6 M in H_2O, 10 mL). After being stirred for 3 h at room temperature the mixture was diluted with water and then extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated under vacuum to give the product as a white solid (2.4 g) in an 88% yield. ^{1}H-NMR (DMSO-d_6) δ 7.60 (d, 1H), 7.35 (m, 5H), 6.84 (m, 1H), 5.03 (s, 2H), 3.97 (m, 1H), 2.98 (m, 2H), 1.85 (m, 1H), 1.67 (m, 1H), 1.36 (s, 9H).

2-[N-(9-Fluorenylmethyloxycarbonyl)amino]-4-[N-(tert-butoxycarbony)amino]-D-diaminobutylic acid, (**8**). Compound (**7**) (2.4 g, 6.8 mmol) was dissolved in methanol (20 mL) and hydrogenated with a 5% Pd/C catalyst. The catalyst was filtered through a Celite pellet, and the methanol was evaporated in vacuum to give 1.46 g (98%) of N^{δ}-Boc-D-Dbu as a yellow oil. ^{1}H-NMR (DMSO-d_6) δ 7.00 (m, 1H), 3.15 (t, 2H), 3.03 (m, 2H), 1.84 (m, 1H), 1.65 (m, 1H), 1.35 (s, 9H). N^{δ}-Boc-D-Dbu (1.46 g, 6.7 mmol) was dissolved in a mixture of 10% aqueous Na_2CO_3 solution (20 mL) and dioxane (20 mL). To the stirred mixture was added dropwise a solution of 9-fluorenylmethyl chloroformate (1.73 g, 6.7 mmol) in 10 mL dioxane with ice-bath cooling. The solution was stirred at 0 °C for 1 h then at room temperature for 2 h, then was poured into 100 mL of cold water. The mixture was extracted twice with ether, cooled in an ice bath and acidified with 10% citric acid. The white precipitate was extracted twice with EtOAc. The combined extracts were washed with water and saturated NaCl, and then dried over $MgSO_4$. After evaporation of the solvent, the product was obtained in a 90% yield (2.7 g) as a white solid. ^{1}H-NMR ($CDCl_3$) δ 7.79 (d, 2H), 7.61 (d, 2H), 7.28–7.45 (m, 4H), 4.43 (m 2H), 4.24 (t, 1H), 3.20 (m, 2H), 2.20 (m, 2H), 1.49 (s, 9H). HRMS (M + 1) Calcd. for $C_{24}H_{28}N_2O_6$ 441.2020, Found 441.2035. $[\alpha]^{22.0\,°C} = +13.0$ (c = 1, methanol).

General Procedure for Solid-phase Peptide 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 washing with DMF (3 times), methanol, DMF, and vacuum drying. The Fmoc protected C-terminal amino acid (3 eq) was coupled to the resin using DIC (3 equiv), 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 a same way. After Fmoc deprotection, washing and drying, the dipeptide amide 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. The crude dipeptides were purified by prep-HPLC.

L-Arg^{NO_2}*-D-Dbu-NH*$_2$, (**9**). ^{1}H-NMR (D_2O) δ 4.32 (dd, 1H), 3.98 (t, 1H), 3.22 (brs, 2H), 2.97 (t, 2H), 2.10 (m, 1H), 1.99 (m, 1H), 1.78–1.92 (m, 2H), 1.50–1.68 (m, 2H). HRMS (M + 1) Calcd. for $C_{10}H_{22}N_8O_4$ 319.1837, Found 319.1801. Anal; Calcd. for $C_{10}H_{22}N_8O_4 \bullet 2$ TFA $\bullet$ 0.5 H_2O: C 30.25; H 4.68; N 20.17. Found: C 30.54; H 4.50; N 20.26%.

D-Arg^{NO_2}*-D-Dbu-NH*$_2$, (**10**). ^{1}H-NMR (D_2O) δ 4.38 (dd, 1H), 3.99 (t, 1H), 3.20 (t, 2H), 2.99 (q, 2H), 2.08 (m, 1H), 1.99 (m, 1H), 1.82–1.90 (m, 2H), 1.52–1.66 (m, 2H). HRMS (M + 1) Calcd. for $C_{10}H_{22}N_8O_4$ 319.1837, Found 319.1830. Anal; Calcd. for $C_{10}H_{22}N_8O_4 \bullet 2$ TFA $\bullet$ H_2O: C 29.79; H 4.60; N 19.86. Found: C 29.60; H 4.48; N 20.13%.

D-Dbu-L-Arg^{NO_2}*-NH*$_2$, (**11**). ^{1}H-NMR (D_2O) δ 4.23 (dd, 1H), 4.03 (t, 1H), 3.20 (m, 2H), 3.01 (m, 2H), 2.17 (m, 2H), 1.52–1.83 (m, 4H). HRMS (M + 1) Calcd. for $C_{10}H_{22}N_8O_4$ 319.1837, Found 319.1855. Anal; Calcd. for $C_{10}H_{22}N_8O_4 \bullet 2.8$ TFA: C 29.38; H 3.92; N 17.58. Found: C 29.41; H 4.07; N 17.62%.

D-Dbu-D-Arg^{NO_2}*-NH*$_2$, (**12**). ^{1}H-NMR (D2O) δ 4.24 (dd, 1H), 4.03 (t, 1H), 3.18 (m, 2H), 3.02 (m, 2H), 2.15 (m, 2H), 1.52–1.80 (m, 4H). HRMS (M + 1) Calcd. for $C_{10}H_{22}N_8O_4$ 319.1837, Found 319.1840. Anal; Calcd. for $C_{10}H_{22}N_8O_4 \bullet$ 2TFA: C 30.78; H 4.43; N 20.51. Found: C 30.71; H 4.47; N 20.36%.

Enzymes and Assay

All of the NOS isoforms used were recombinant enzymes overexpressed in *Escherichia coli* from different sources. The murine macrophage iNOS was expressed and isolated according to the procedures of Hevel *et al.*[17] The rat neuronal NOS was expressed[18] and purified as described.[19] The bovine endothelial NOS was isolated and purified as reported.[20] Nitric oxide formation from NOS was monitored by the hemoglobin capture assay as described previously.[21]

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 and by assuming competitive inhibition. The parameters of the following inhibition equation[22] were fitted to the initial velocity data:

$$\% \text{ Inhibition} = 100[I]/[[I] + K_i(1 + [S]/K_m)] \quad (1)$$

K_m values for L-arginine were 1.3 μM (nNOS), 1.7 μM (eNOS), and 8.3 μM (iNOS). The selectivity of an inhibitor was defined as the ratio of the respective K_i values.

RESULTS AND DISCUSSION

Syntheses of the dipeptide amides (9–12)

The protected amino acid, Fmoc-D-Dbu (Boc)-OH (**8**) was synthesized from D-homoserine according to the route shown in Scheme 1.

TABLE I Nitric oxide synthase inhibition by the N^{ω}-nitroarginine and D-Dbu containing dipeptide amides[a]

Compound	K_i (μM)[b]			Selectivity[c]	
	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-Lys-NH_2[d]	0.45	104	141	313	231
3 D-Lys-D-Arg^{NO_2}-NH_2[d]	0.89	910	30	34	1022
9 L-Arg^{NO_2}-D-Dbu-NH_2	2.4	460	586	244	192
10 D-Arg^{NO_2}-D-Dbu-NH_2	12.8	4500	213	17	352
11 D-Dbu-L-Arg^{NO_2}-NH_2	9.2	4500	63	7	489
12 D-Dbu-D-Arg^{NO_2}-NH_2	5.7	891	104	18	156

[a]The enzymes used for the K_i measurements are recombinant rat nNOS, recombinant murine iNOS, and recombinant bovine eNOS. [b]The K_i values represent duplicate measurements (±8–12%). [c]The ratio of K_i (eNOS or iNOS) to K_i (nNOS); all are nNOS selective. [d]Data taken from reference 13.

The key step is the Mitsunobu reaction, which converts the hydroxyl group of D-homoserine into the protected amino group using *N*-Boc ethyl oxamate.[23] The oxamate group was removed with LiOH, yielding the *N*-Boc protected amine (**7**). Exchanging the α-amino protecting group from Cbz to Fmoc gave the desired compound, which is the starting material for the solid-phase dipeptide synthesis. The corresponding D-amino acid is optically pure, as determined by its optical rotation compared with the L-amino acid, which is commercially available.

Dipeptide amides (**9–12**) were synthesized manually on solid-phase using Rink resin as the solid support. Each of the dipeptide amides was >90% pure after cleavage from the resin. Further purification by prep-HPLC was carried out prior to the enzyme assay.

Kinetic data for the dipeptide amides (9–12)

All of the compounds tested were rapid reversible inhibitors of the three NOS isoforms. The K_i data listed in Table I illustrate that the D-Dbu containing dipeptide amides (**9–12**) are poorer inhibitors of nNOS and iNOS than the L-Dbu containing compound (**1**), while having minimal influence on eNOS inhibition. This results in the dramatic decreases of isoform selectivity of nNOS over eNOS.

L-Arg^{NO_2}-D-Dbu-NH_2 (**9**) has a 20-fold decreased inhibitory potency toward nNOS and iNOS compared with its diastereomer, L-Arg^{NO_2}-L-Dbu-NH_2, which suggests that the chirality of the *C*-terminal amino acid is critical to its binding at the nNOS and iNOS active sites. Previously, we proposed a hypothetical model for the binding of the dipeptide amides at the active site of nNOS (Figure 1);[13] because there is no crystallographic structure of nNOS available, this is purely speculative. In this model 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 terminal amino group of the Lys or Dbu side chain) and the enzyme active site. Because the two interactions occur at different amino acids (Arg^{NO_2} and Lys), there is relatively greater freedom for both binding interactions. This may explain why there is only a three-fold potency difference between L-Arg^{NO_2}-L-Lys-NH_2 (**2**) and L-Arg^{NO_2}-L-Dbu-NH_2 (**1**). The D-Lys containing dipeptide amide, D-Lys-D-Arg^{NO_2}-NH_2 (**3**),

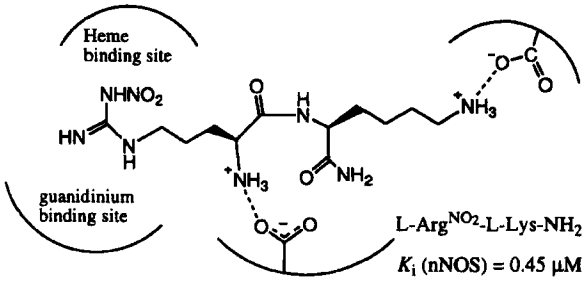


FIGURE 1 A hypothetical model for the binding of L-Arg^{NO_2}-L-Lys-NH_2 at the active site of nNOS.

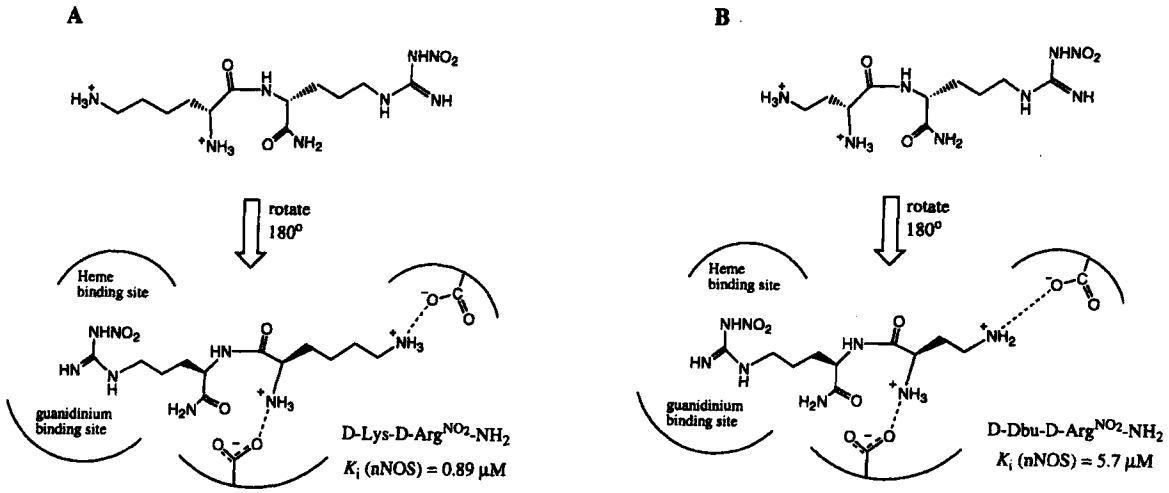
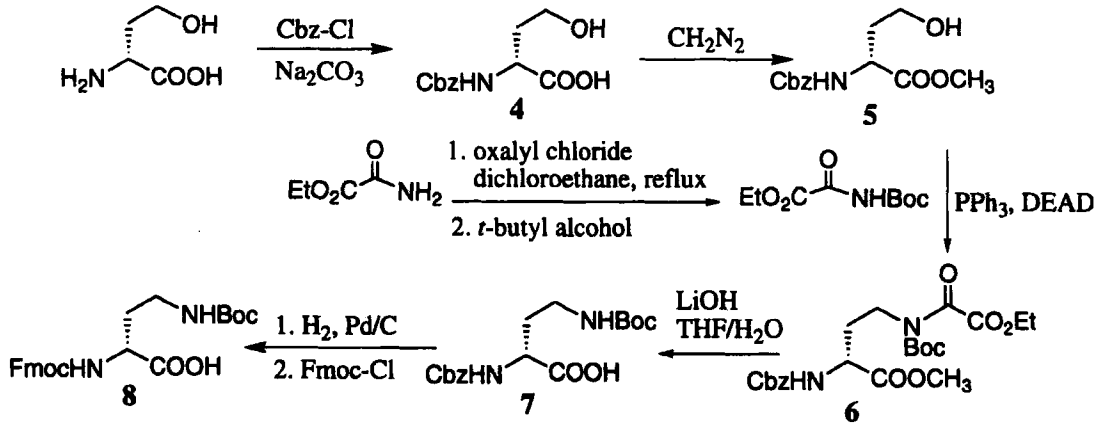


FIGURE 2 A hypothetical model for the binding of (A) D-Lys-D-Arg^{NO_2}-NH$_2$ and (B) D-Dbu-D-Arg^{NO_2}-NH$_2$ at the active site of nNOS.

exhibits only a 2-fold decrease in the potency of nNOS inhibition and more selectivity of nNOS binding over iNOS, as compared to the L-form analogue, L-Arg^{NO_2}-L-Lys-NH$_2$ (**2**). D-Dbu-D-Arg^{NO_2}-NH$_2$ (**12**) was expected to act in a similar way compared to (**1**); however, it is a 44-fold less potent nNOS inhibitor relative to (**1**). The dipeptide amides (**2**) and (**3**) may have similar nNOS inhibitory potencies because the *retro-inverso* dipeptide[14] appears to flip over 180° to assume an L-form-like configuration for binding (Figure 2A).[15a] When the compound flips over, the two interactions with the enzyme would occur with the two amino groups of the same amino acid (in this case, Lys). If that is the case, then the length of the amine side chain becomes crucial for the two interactions with the active site. D-Lys has a longer side chain than D-Dbu, and, therefore, there is less restriction for the two interactions to occur simultaneously. D-Dbu, having two methylenes less than lysine in the side chain, may have difficulty accommodating both interactions simultaneously (Figure 2B). This would explain why there is a dramatic



SCHEME 1 Synthesis of Fmoc-D-Dbu (Boc)-OH (**8**).

decrease in potency by D-Dbu-D-Arg^{NO_2}-NH_2 (**12**) relative to its *retro-inverso* analogue (**1**).

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