Brief Articles

Reduced Amide Bond Peptidomimetics. (4S)-N-(4-Amino-5-[aminoalkyl]aminopentyl)-N-nitroguanidines, Potent and Highly Selective Inhibitors of Neuronal Nitric Oxide Synthase

Jung-Mi Hah,[†,‡] Linda J. Roman,[§,||] Pavel Martásek,[§,⊥] and Richard B. Silverman*,[†]

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208-3113, and Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284-7760

Selective inhibition of the isoforms of nitric oxide synthase (NOS) could be therapeutically useful in the treatment of certain disease states arising from the overproduction of nitric oxide. Recently, we reported nitroarginine-containing dipeptide amides (Huang, H; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Silverman, R. B. *J. Med. Chem.* **1999**, *42*, 3147.) and some peptidomimetic analogues (Huang, H; Martasek, P.; Roman, L. J.; Silverman, R.B. *J. Med Chem.* **2000**, *43*, 2938.) as potent and selective inhibitors of neuronal NOS (nNOS). Here, reduced amide bond pseudodipeptide analogues are synthesized and evaluated for their activity. The deletion of the carbonyl group from the amide bond either preserves or improves the potency for nNOS. Significantly, the selectivities for nNOS over eNOS (endothelial NOS), and iNOS (inducible NOS) are greatly increased in these series. The most potent nNOS inhibitor among these compounds is (4S)-N-(4-amino-5-[aminoethyl]aminopentyl)-N-nitroguanidine (**7**) (K_i = 120 nM), which also shows the highest selectivity over eNOS (greater than 2500-fold) and 320-fold selectivity over iNOS. The reduced amide bond is an excellent surrogate of the amide bond, and it will facilitate the design of new potent and selective inhibitors of nNOS.

Introduction

Nitric oxide (NO)[1] is synthesized enzymatically from arginine in numerous tissues and cell types by a family of enzymes collectively known as nitric oxide synthase (NOS, EC 1.14.13.39).[2] Three principal isoforms of this enzyme have been isolated and characterized,[3] each associated with different physiological functions: the immune response (inducible NOS or iNOS),[4] smooth muscle relaxation (endothelial NOS or eNOS),[5] and neuronal signaling (neuronal NOS or nNOS).[6] All of these isoforms utilize NADPH, FAD, FMN, (6R)-5,6,7,8-tetrahydrobiopterin, and heme as cofactors.

Overproduction of NO has been a factor in numerous disease states. NO overproduction by nNOS has been implicated in strokes,[7] migrane headaches,[8] and Alzheimer's disease[9] and with tolerance to and dependence on morphine.[10] iNOS-mediated overproduction of NO has been associated with development of colitis,[11] tissue damage and inflammation,[12] and rheumatoid arthritis.[13]

Animal studies and early clinical trials suggest that NOS inhibitors could be therapeutic in many of these disorders;[14] however, because of the importance of nitric oxide to physiological functioning, potent as well as isoform-selective inhibitors are essential. nNOS inhibition has been targeted for treatment of strokes[15] and iNOS inhibition for the treatment of septic shock[16] and arthritis.[17] Although there may be pathologies associated with overactivity of eNOS, blood pressure homeostasis is so critical that most investigators believe that therapeutically useful NOS inhibitors must not inhibit eNOS.

In our labs, excellent inhibitory potency and selectivity for nNOS over eNOS and iNOS have been achieved with certain nitroarginine dipeptide amides that have an amine-containing side chain (**1**−**3**).[18] The most potent nNOS inhibitor among these compounds is L-Arg^NO2^-L-Dbu-NH₂ (**1**) (K_i = 130 nM), which also shows excellent selectivity over eNOS (greater than 1500-fold) and 192-fold selectivity over iNOS. The change in carbon length of the side chain produced only a small effect on the potency of all of the isoforms of NOS. Peptidomimetic modifications[19] were made on compounds **1**−**3**. Incorporation of protecting groups at the N terminus of the dipeptide and masking of the NH− group of the peptide bond resulted in a dramatic loss in potency of nNOS, which demonstrates the importance of the α-amino group of the dipeptide and NH moiety of the peptide bond for binding at the active site. Surprisingly, removal of the carboxamide group (**4**−**6**) had only a minor effect on both potency and selectivity.

* To whom correspondence should be addressed. Phone: (847) 491-5653. Fax: (847) 491-7713. E-mail: Agman@chem.northwestern.edu. Address correspondence to this author at the Department of Chemistry.
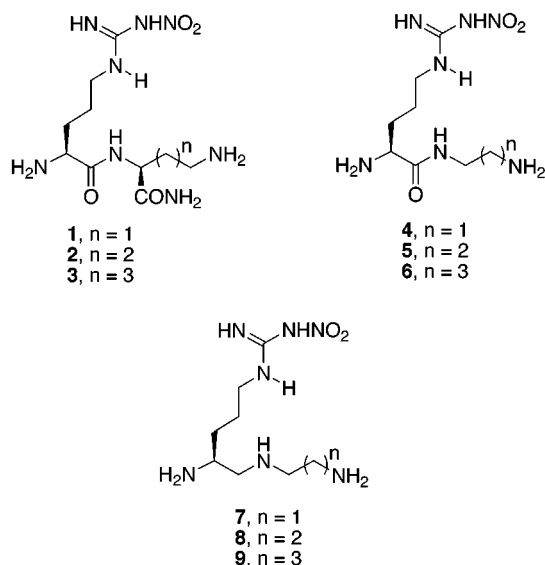† Northwestern University.
‡ Carried out all experiments in this study.
§ The University of Texas Health Science Center.
|| Developed the overexpression system for nNOS in *E. coli* and the purification of the enzyme.
⊥ Developed the eNOS overexpression system in *E. coli* and purification of eNOS and isolated the eNOS.

1, n = 1
2, n = 2
3, n = 3

4, n = 1
5, n = 2
6, n = 3

7, n = 1
8, n = 2
9, n = 3

Table 1. NOS Inhibition by the Reduced-Amide Bond Analogues **7**−**9** and N^{ω}-Nitroarginine-Containing Dipeptides (**1**−**6**)[a]

| compound | K_i (μM)[b] | | | selectivity[c] | |
	nNOS	iNOS	eNOS	eNOS/nNOS	iNOS/nNOS
1[d]	0.13	25	200	1538	192
2[d]	0.33	97	245	742	294
3[d]	0.45	104	141	313	231
4[e]	0.54	100	199	368	185
5[e]	0.46	118	213	463	256
6[e]	0.35	108	70	200	308
7	0.12	39	314	2617	325
8	0.29	73	524	1807	252
9	0.46	123	411	893	267

[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 at least 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 18. [e] Data taken from ref 19.
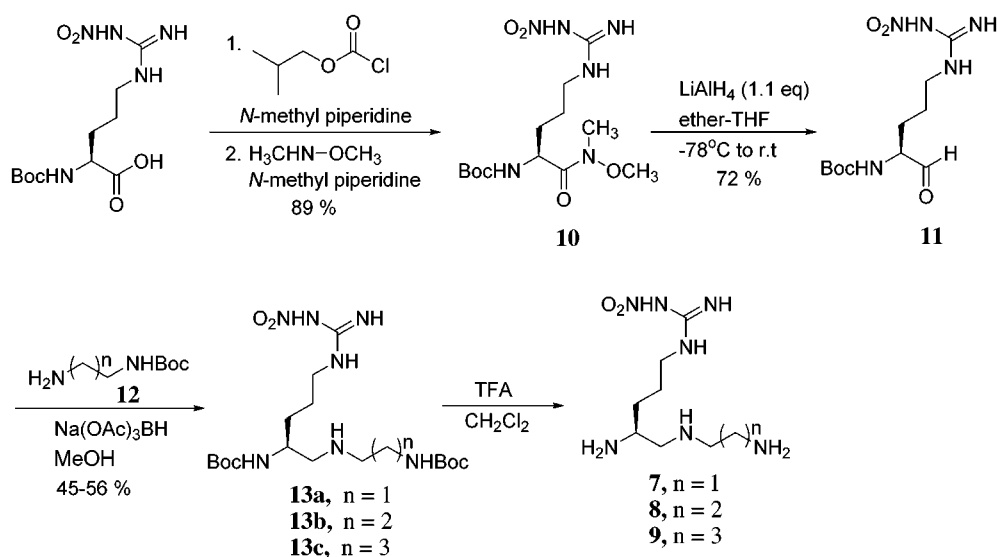
Further peptidomometic modifications are necessary for therapeutically useful compounds especially because peptides are generally not good drug candidates as a result of their poor bioavailability. Many bioisosteric replacements of the amide bond were considered; however, considering the results from the first peptidomimetic approach mentioned above,[19] the reduced amide bond (−CH$_2$−NH−) was chosen as the first target. Compounds **7**−**9** contain features important to good inhibitors of NOS. First, they contain several amine nitrogens, which have been found to be very important for interactions with the active site of the enzyme, and second, they do not contain amide bonds, so they should be stable toward endogenous peptidases in vivo.

Chemistry

The three reduced amide bond analogues (**7**−**9**) were synthesized according to the method in Scheme 1, which employs Weinreb amide **10**[20] as a key intermediate. The Weinreb amide was reduced to an aldehyde using lithium aluminum hydride according to a modified procedure of Goel et al.[21] The resulting N-Boc-nitro-L-argininal (**11**)[22] and mono-Boc-protected alkanediamines

(**12**)[23] were reductively coupled using sodium triacetoxyborohydride in dry methanol,[24] providing the reduced dipeptides **13**. After purification of these compounds, cleavage of the Boc groups was achieved with TFA. Compounds **7**−**9** were isolated as pale-yellow powders after lyophilization, and the elemental analyses showed that all of these compounds were triple trifluoroacetic acid dihydrate salts.

Results and Discussion

The K_i data for the reduced amide bond analogues (**7**−**9**) are given in Table 1 along with the data for the corresponding dipeptides (**1**−**3**)[18] and descarboxamide analogues (**4**−**6**).[19] Deletion of the carbonyl group of the amide bond either preserves or improves the potency toward nNOS. Compound **7** shows the best potency over nNOS (K_i = 120 nM) as well as the highest selectivity over eNOS (greater than 2500-fold) and iNOS (320-fold) in these series of compounds. The length of the amine side chain seems to have only a minor effect on the potency for all isoforms of NOS; **8** and **9** inhibit nNOS with K_i values of 290 and 460 nM, respectively. However, the shorter chain has better potency as well as selectivity.

Scheme 1

10

11

13a, n = 1
13b, n = 2
13c, n = 3

7, n = 1
8, n = 2
9, n = 3

When the data for **7**−**9** are compared with those for **4**−**6** to see the intrinsic effect of deletion of the amide carbonyl group, it is apparent that the potency on nNOS and iNOS is about the same or increased a little, but the potency with eNOS has greatly decreased. In particular, the large increase in selectivity for nNOS over eNOS by **7** comes from a 4.5-fold increase in potency for nNOS and a 1.5-fold decrease in potency for eNOS. For **8** and **9** this selectivity increase is driven more by large decreases in potency for eNOS (2.5-fold and almost 6-fold, respectively). Therefore, the selectivity for nNOS over eNOS is significantly increased for **7**−**9**, which implies that the carbonyl moiety of the amide bond might not be necessary for its activity toward nNOS and iNOS but that the rigid −CO−NH− group interacts better with the active site of eNOS than the reduced, flexible −CH$_2$−NH− group. The difference may also be the result of the nonbasic amide nitrogen becoming basic when reduced to the corresponding amine, which may not bind as well to eNOS.

In conclusion, the reduced amide bond analogues **7**−**9** show potencies similar to that of nNOS but greatly increased selectivities over eNOS and iNOS. Therefore, the reduced amide bond peptidomimetics are significant surrogates for the dipeptide inhibitors of nNOS, and this should facilitate further development of potent and selective inhibitors for NOS.

Experimental Section

General Methods. NOS assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Inova 500 MHz NMR spectrometer. Chemical shifts are reported as δ values in parts per million downfield from TMS (δ 0.00) as the internal standard in CDCl$_3$. For samples run in D$_2$O, the HOD resonance was arbitrarily set at 4.80 ppm. An Orion Research model 701 pH meter with a general combination electrode was used for pH measurements. Electrospray mass spectra were obtained on a Micromass Quattro II spectrometer. Elemental analyses were obtained by 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 column chromatography.

Reagents and Materials. Amino acids were purchased from Advanced ChemTech, Inc. NADPH, calmodulin, and human ferrous hemoglobin were obtained from Sigma Chemical Co. Tetrahydrobiopterin (H$_4$B) 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.

N^α-(***tert*-Butoxycarbonyl)-L-nitroarginine *N*-Methyl-*O*-methylcarboxamide (10).** This compound was prepared from 12.8 g (40.1 mmol) of N^α-(*tert*-butoxycarbonyl)-L-nitroarginine as described in ref 21 except that isobutyl chloroformate was used instead of methyl chloroformate. The residue was further evacuated on an oil pump to give a white solid product (12.9 g, 89%): ^{1}H NMR δ 5.65 (d, 1H, N−H, J = 9.0), 4.69 (t, 1H, J = 9.0), 3.79 and 3.73 (s, 3H), 3.25 and 3.10 (s, 3H), 3.64 (m, 1H), 3.32 (m, 1H), 1.79 (m, 2H), 1.63 (m, 2H), 1.47 (s, 9H).

N^α-(*tert*-Butoxycarbonyl)-L-nitroargininal (11).** This compound was prepared according to the method in ref 22. From 3.62 g of **10** (10 mmol), the white powder product 2.17 g (72%) was obtained, and it was stored in a deep freezer (−80 °C) prior to use. ^{1}H NMR showed that **11** is a mixture of the free aldehyde and cyclized hemiaminal.[22]

(4*S*)-4-*N*-*tert*-Butoxycarbonylamino-5-(2-[*N*-*tert*-butoxycarbonylaminoethyl]aminopentyl]-*N*′-nitroguanidine (13a). To a solution of **11** (303 mg, 1 mmol) in dry methanol, N^α-*tert*-butoxycarbonyl-1,2-ethanediamine (**12**, $n =$ 1; 184 μL, 1 mmol) and 3 Å molecular sieves were added, and the mixture was stirred at room temperature. After being stirred for 1 h, the reaction mixture was treated with sodium triacetoxyborohydride (334.6 mg, 1.5 mmol) and stirred overnight. The reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (EtOAc/MeOH = 1:1) to afford **13a** (201 mg, 45%) as a pale-yellow solid: ^{1}H NMR (500 MHz, CD$_3$OD) δ 3.67 (m, 1H), 3.33 (m, 2H), 3.04−3.20 (m, 2H), 2.62−2.73 (m, 4H), 1.52−1.75 (m, 4H), 1.45 (brs, 18H).

(4*S*)-4-*N*-*tert*-Butoxycarbonylamino-5-(2-[*N*-*tert*-butoxycarbonylaminopropyl]aminopentyl]-*N*′-nitroguanidine (13b). This compound (234 mg, 51%) was prepared as described above using N^α-*tert*-butoxycarbonyl-1,3-propanediamine (**12b**, n = 2): ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.90 (m, 1H), 3.69 (m, 1H), 3.18−3.21 (m, 4H), 2.77(m, 2H), 2.64 (m, 2H), 1.50−1.65 (m, 6H), 1.44 (brs, 18H).

(4*S*)-4-*N*-*tert*-Butoxycarbonylamino-5-(2-[*N*-*tert*-butoxycarbonylaminobutyl]aminopentyl]-*N*′-nitroguanidine (13c). This compound (265 mg, 56%) was prepared as described above using N^α-*tert*-butoxycarbonyl-1,4-butanediamine: ^{1}H NMR (500 MHz, CDCl$_3$) δ 4.67 (m, 1H), 3.71 (m, 1H), 3.14 (m, 4H), 2.72 (t, 2H, J = 6.0), 2.64 (m, 2H), 1.71 (m, 2H), 1.49−1.54 (m, 8H), 1.45 (brs, 18H).

(4*S*)-*N*-(4-Amino-5-[aminoethyl]aminopentyl)-*N*′-nitroguanidine (7). Compound **13a** (201 mg, 0.45 mmol) was treated with 10 mL of trifluoroacetic acid/CH$_2$Cl$_2$ (1:1 v/v) for 30 min. Excess TFA and solvent were removed by evaporation. The residue was dissolved in a small amount of water, and the mixture was washed with ether and lyophilized to give a pale-yellow foam (110 mg, 99%): ^{1}H NMR (500 MHz, D$_2$O) δ 3.63 (m, 1H), 3.32−3.39 (m, 6H), 3.24 (m, 2H), 1.69−1.78 (m, 4H). HRMS (M + 1) calcd for C$_8$H$_{21}$N$_7$O$_2$ 248.183, found 248.180. Anal. (C$_8$H$_{21}$N$_7$O$_2$·3TFA·2H$_2$O) C, H, N.

(4*S*)-*N*-(4-Amino-5-[aminopropyl]aminopentyl)-*N*′-nitroguanidine (8). This compound was prepared as described above using compound **13b**: ^{1}H NMR (500 MHz, D$_2$O) δ 3.62 (m, 1H), 3.32 (m, 2H), 3.24 (m, 2H), 3.13 (m, 2H), 3.01 (m, 2H), 2.03 (quin, 2H, J = 7.0), 1.69−1.78 (m, 4H). HRMS (M + 1) calcd for C$_9$H$_{23}$N$_7$O$_2$ 262.199, found 262.195. Anal. (C$_9$H$_{23}$N$_7$O$_2$·3TFA·2H$_2$O) C, H, N.

(4*S*)-*N*-(4-Amino-5-[aminobutyl]aminopentyl)-*N*′-nitroguanidine (9). This compound was prepared as described above using compound **13c**: ^{1}H NMR (500 MHz, D$_2$O) δ 3.75 (m, 1H), 3.62 (m, 2H), 3.32 (m, 2H), 3.24 (m, 2H), 2.94 (m, 2H), 1.67−1.82 (m, 8H). HRMS (M + 1) calcd for C$_{10}$H$_{25}$N$_7$O$_2$: 276.214, found 276.214. Anal. (C$_{10}$H$_{25}$N$_7$O$_2$·3TFA·2H$_2$O) C, H, N.

Enzyme and Assay. All of the NOS isoforms used were recombinant enzymes overexpressed in *E. coli* from different 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.[25] The rat nNOS was expressed[26] and purified as described.[27] The bovine eNOS was isolated as reported.[28] Nitric oxide formation from NOS was monitored by the hemoglobin capture assay as described.[29]

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[30] 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), 8.2 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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