Letters

Isoform-Selective Substrates of Nitric Oxide Synthase

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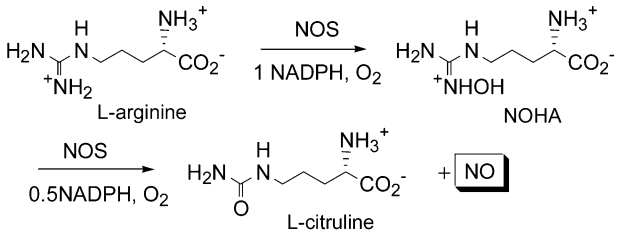
Abstract: Because of the double-edged nature of NO, the development of isoform-selective NOS substrates is a highly desirable goal. Given the striking similarity in the heme active sites of the three NOS isoforms, it presents an challenging problem. Several *N*-aryl-*N*′-hydroxyguanidines have recently been shown as substrates that are selective for iNOS over nNOS. Here, we report the first success that **3** is a good substrate for nNOS (70% activity of NOHA, $K_m \approx 40 \pm 6\ \mu$M) over iNOS.

Introduction. NO is involved in a number of physiological and pathophysiological processes[1] such as smooth muscle relaxation,[2] inhibition of platelet aggregation, macrophage cytotoxicity, inflammatory diseases,[3,4] iron metabolism, neurotransmission, and neurotoxicities.[5,6] The endogenous synthesis of NO is catalyzed by three isoforms of NO synthase (NOS) (iNOS, nNOS, and eNOS), using L-arginine as substrates via a two-step reaction (Scheme 1).[7] In the first step, an intermediate, *N*-hydroxyarginine (NOHA), is formed by the incorporation of an oxygen atom into the guanidine function of L-arginine. In the second step, NOHA is further oxidized to form L-citrulline and NO.

Three different NOS isoforms are expressed in different tissues and are highly regulated transcriptionally or posttranscriptionally (Scheme 1).[1,8] Because of the double-edged nature of NO in both basic physiological functions and various pathological conditions,[9] the development of isoform-selective NOS inhibitors and substrates is a highly desirable goal. Many compounds have been found to be potent, selective NOS inhibitors.[10,11] However, only several α-amino acids closely related to L-Arg or NOHA, such as homo-L-Arg, homo-NOHA, and *E*-dehydro-L-Arg are shown to be NOS substrates.[12] The very limited number of substrates for NOSs suggests that the α-amino acid portion and highly specific structural features are required for substrates of NOS.[13]

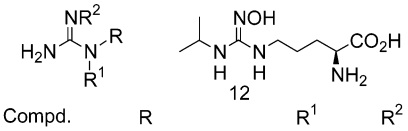
Very recently, some *N*-alkyl/aryl substituted hydroxyguanidine compounds were found to be novel NOS substrates that act as substrates for the second step of the NOS catalytic cycle and that "short-circuit" the first oxidation step.[12−14] Among them, *N*-butyl-*N*′-hydroxyguanidine **1** and *N*-isopropyl-*N*′-hydroxyguanidine **2**

Scheme 1. NO Formation and Differences in Three Isoforms



NOS	Locations	Major biological functions
nNOS	brain, spinal cord, peripheral	Neuromediator
iNOS	macrophages, other tissues	Host defender, cytotoxic
eNOS	endotheliun	Vasodilator tone modulator

Chart 1



Compd.	R	R^1	R^2
1	*n*-butyl	H	OH
2	*i*-propyl	H	OH
3	cyclopropyl	H	OH
4	-$CH_2CH_2C{\equiv}CH$	H	OH
5	*E*-$CH_2CH{=}CHCH_3$	H	OH
6	cyclopropylmethyl	H	OH
7	*n*-butyl	H	-OCH_2Ph
8	*n*-butyl	H	-OCH_3
9	*n*-butyl	CH_3	OH
10	-$CH_2CH_2CH_2CH_2$-		OH
11	-$CH_2CH_2OCH_2CH_2$-		OH
13	cyclopropylmethyl	H	H
14	2-methyl-cyclopropylmethyl	H	H
15	*E*-$CH_2CH{=}CHCH_3$	H	H
16	*n*-butyl	H	H

(Chart 1) are the most active substrates for all three NOS isoforms. Crystal structures show that **1** has the same binding mode as that of NOHA while **2** showed a novel binding mode that shed light on substrate binding and the catalytic mechanism.[15]

In an effort to better understand the structure−activity relationship and to find new isoform-selective NOS substrates, we have synthesized a series of compounds based on known structure−activity relationships. Compound **3** is so far the first selective substrate for nNOS over iNOS. A new mechanism is proposed that could also aid in further understanding NOS-selective substrates.

Chemistry. A series of N-substituted *N*′-hydroxyguanidines developed from NOHA, **1**, and **2** have been synthesized by using the general procedures previously reported for the preparation of hydroxyguanidines starting from the corresponding amines (Scheme 2).[13,16] N-substituted guanidines were synthesized according to ref 17 (Scheme 2). Compound **12** was synthesized according

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Scheme 2

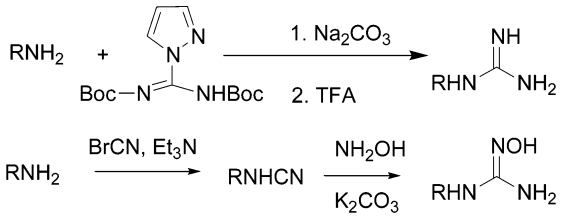

Table 1. NO Formation from Compounds **1**−**16** by NOS[a]

	NO formation, %		
compd	iNOS	nNOS	eNOS
NOHA	100	100	100
L-Arg	50	72	38
1[12]	42	64[b]	20
2[12]	15	70[c]	38
3	<0.5	70 ± 8[d]	26 ± 4
4	28 ± 8	37 ± 4	<0.5
5	<0.5	5[e]	<0.5
6	<0.5	<0.5	<0.5
7	<0.5	<0.5	<0.5
8	<0.5	<0.5	<0.5
9	<0.5	<0.5	<0.5
10	<0.5	<0.5	<0.5
11	<0.5	<0.5	<0.5
12	<0.5	<0.5	<0.5
13	<0.5	<0.5	<0.5
14	<0.5	<0.5	<0.5
15	<0.5	<0.5	<0.5
16	6% to Arg[18]	<0.5	<0.5

[a] The initial rate of NO synthesis was determined at 37 °C using spectrophotometric oxyhemoglobin assay for NO. Briefly, 0.5−1 unit of enzyme, 5 μM BH_4, and 2−5 mM DTT were added to a prewarmed cuvette that contained 50 mM HEPES (pH 7.4), supplemented with 15 μM oxyhemoglobin, 100 units/mL SOD, 100 units/mL catalase, 200 μM NADPH, 4 μM FAD, 4 μM FMN, 5 μM BH_4, and 0.5 mM substrate at the desired concentration, to give a final volume of 0.9 mL. In the case of nNOS and eNOS, 1 mM $CaCl_2$ and 10 μg/mL CaM were present. For iNOS, 1 mM magnesium acetate was added. The reference cuvette had the same composition except that 50 mM HEPES, 5 μM BH_4, and 2−5 mM DTT were added instead of NOS-containing solutions. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an increase in absorbance at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1}. The rates were expressed as a percentage of those found for NOHA (mean value ± SD from at least three experiments). [b] $K_m \approx 67$ μM. [c] $K_m \approx 56$ μM. [d] $K_m \approx 40 \pm 6$ μM. [e] The highest value from three experiments.

to ref 15. All new compounds were fully characterized by ^1H and ^{13}C NMR and high-resolution mass spectrometry.

Results and Discussion. The activity of these *N*′-hydroxyguanidine compounds as substrates of NOS was evaluated by a hemoglobin assay that is based on the conversion of oxyhemoglobin to methemoglobin by NO.[12,13] For evaluation, NOHA was used as a control indicating 100% activity. The results are summarized in Table 1.

According to the crystal structure of nNOS complexed with **1**,[15] the hydroxyguanidino moiety is anchored by multiple H-bonds and is oriented in a manner similar to what is observed for NOHA at the NOS active site (Figure 1A). Such orientation allows the *n*-butyl group to be accommodated in the space surrounded by Gln478 and Val567 of nNOS. According to the structure−activity-relationship assay,[12,13] the alkyl group size should be between three and five carbons for good substrates. Large alkyl groups cannot fit into the space

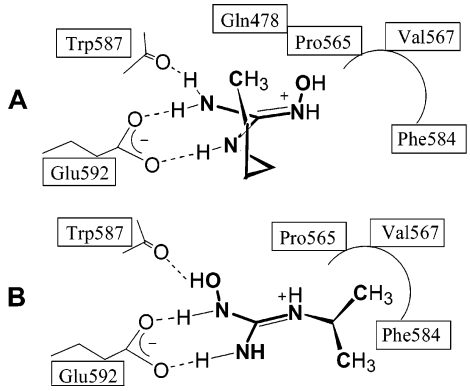

Figure 1. Two different orientations of **1** (A) and **2** (B) at the NOS substrate-binding site. The hydrophobic patch is marked by an arc.

owing to steric crowding by surrounding protein side chains, while smaller alkyl groups cannot be held tightly by the enzyme. A double or triple bond (**4** and **5**) that introduced rigidity of the butyl group also reduced activity (nNOS: 37% and 5% to NOHA, respectively). The terminal branched alkyl group, such as **6**, *tert*-butyl, isobutyl, and 3-methylbutyl, reduced the activity dramatically (nNOS: <0.5%, <0.5%, 2%, and 6% to NOHA, respectively).[13] The structure−activity relationship for iNOS and eNOS is similar to that of nNOS.[12,13]

Substitution on the hydroxyl group of **1** (giving **7** and **8**) abolished its NO formation activity for all three NOS isoforms. Silverman and co-workers also reported two NOHA derivatives with the hydrogen of the hydroxyl group substituted by allyl or *tert*-butyl groups. The K_m values of the two NOHA derivatives for iNOS were 100 times higher than that of NOHA.[19] Of the plain guanidine compounds, only *N*-(3,3,3-trifluoropropyl)guanidine was reported to have 35% NO formation activity compared to Arg for iNOS.[18] All of the others tested were very week substrates or nonsubstrates. It is obvious that the hydroxyl group is important for guanidine-derived compounds to be NOS substrates.

Unsymmetric N-disubstituted **9**−**11** were not substrates. Symmetric *N*,*N*′-disubstituted **12** was also not a substrate. *N*-Methyl-substituted NOHA is even an NOS inhibitor.[20] Therefore, we conclude that the N-monosubstituted hydroxyguanidine moiety is the key structure for NOS substrates.

A very exciting discovery is that **3** exhibits 70% NOHA activity for nNOS but no activity for iNOS. This is the first highly active substrate selective for nNOS over iNOS. According to the crystal structure of nNOS complexed with **2**,[15] the binding mode of **2** (Figure 1B) is totally different from that of NOHA and **1** (Figure 1A). The hydroxyguanidine moiety rotates 120°, and the isopropyl-attached N atom takes the place of the OH-substituted terminal N atom. The isopropyl group fits in a small hydrophobic pocket formed by Pro565, Val567, and Phe584. Given the structural similarity between **2** and **3**, **3** is very likely bound to the active site in mode B as well. The molecular modeling results also support mode B binding (Table 2).

Data in Table 2 indicate that all the good substrates have high affinity in mode A except for **3**. Compound **2** has high affinity in both modes. The crystal structure cannot fully exclude the possibility that **2** adopts mode

Scheme 3

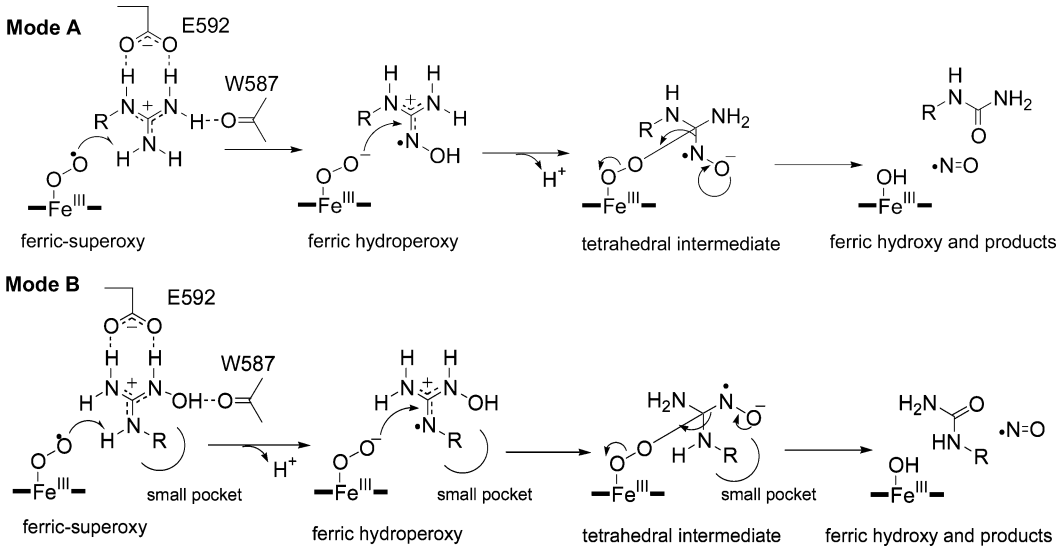

Table 2. FlexiDock Binding Affinity of *N*-Alkyl-*N*′-hydroxyguanidines with nNOS[a]

compd	mode A	mode B	nNOS activity, %
1	−74.12		64
2	−74.24	−62.68	70
3	−46.72	−65.58	70
4	−71.48		33
N-*sec*-butyl	−60.18		12
N-(3-methylbutyl)	−51.48		6
N-isobutyl	−47.47		2

[a] FlexiDock on Sybyl 6.7 is used to calculate the binding affinity. All of the docking scores have units of energy (kcal/mol). These values are considered to be relative docking energies and do not represent the actual binding energies. However, for a series of compounds, the docking energies are expected to have the same rank-order correlation as the true binding energies.

A. According to the calculations, **3** prefers binding mode B. This is very likely the catalytically active binding mode because the low calculated binding affinity in mode A is inconsistent with the observed high activity and affinity ($K_m \approx 40 \pm 6$ μM for this compound; $K_m \approx 13 \pm 2$ μM for NOHA[21]).

Since all the known mechanisms are based on mode A (Scheme 3),[15,19,22−27] we propose a new mechanism to accommodate the novel binding mode B. The OH group of hydroxyguanidine in mode B is located too far away from the heme iron and lends support to the mechanism wherein the N atom is the source of hydrogen atom supplied to the ferric superoxy species.[15] The binding mode B can be enforced only if a small N-substituted alkyl group, such as isopropyl and cyclopropyl, fits into the small hydrophobic pocket next to the substrate-binding site. Any smaller or larger *N*-alkyl group on hydroxyguanidine will lead to the preferred binding mode A. It is very likely that binding mode B contributes to the high NOS selectivity observed for **3**.

Conclusion. Given the striking similarity in dimeric quaternary structure and especially in the heme active sites of the three NOS isoforms,[28,29] the discovery of isoform-selective substrates presents an especially challenging problem. We have reported *N*-aryl-*N*′-hydroxyguanidines as substrates selective for iNOS over nNOS.[13] Here, we showed the first reverse case that **3** is a good substrate selective for nNOS over iNOS. These results open the way toward a better understanding of structure−function relationships of isoform-selective NOS substrates and the mechanism of NO synthase.

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Supporting Information Available: Experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

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