Journal of Medicinal Chemistry

Volume 44, Number 20 September 27, 2001

Letters

Oxidation of N^{ω}-Hydroxyarginine Analogues by NO-Synthase: The Simple, Non Amino Acid *N*-Butyl *N*′-Hydroxyguanidine Is Almost as Efficient an NO Precursor as N^{ω}-Hydroxyarginine

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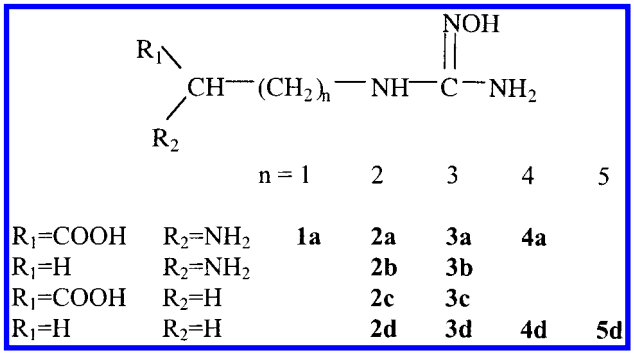
Received June 19, 2001

Introduction. Nitric oxide (NO) is a key inter- and intracellular messenger molecule involved in the maintenance of vascular tone, neuronal signaling, and host response to infection.[1,2] The biosynthesis of NO is catalyzed by constitutively expressed neuronal and endothelial nitric oxide synthases (NOS I and NOS III, respectively) and by inducible NOS (NOS II) that is expressed in macrophages following induction by inflammatory mediators.[2−5] All three NOSs produce NO and L-citrulline from the oxidation of L-arginine (L-arg) by NADPH and O_2 with formation of N^{ω}-hydroxy-L-arginine (NOHA) as an intermediate.[6,7]

Because of the great importance of either an excess of NO or a deficit of NO in many physiopathological situations,[2−5] many groups have worked during these last years to find selective NOS inhibitors and substrates. Whereas a great number of strong NOS inhibitors have been described,[8] only a few NOS substrates have been reported so far. Most of them are α-amino acids closely related to L-arg or NOHA, such as homo-L-arg and homo-NOHA,[9,10] or E-dehydro-L-arg,[11] as small changes of the L-arg or NOHA structure completely abolish the NOS-dependent formation of NO.[9−14] However, it has been recently reported[15] that some *N*-aryl *N*′-hydroxyguanidines, such as *N*-*p*-chlorophenyl *N*′-hydroxyguanidine, are oxidized by NOS II with concomitant formation of the corresponding *N*-aryl urea and NO in a 1:1 molar ratio.

Scheme 1



To better understand the structural factors that are important for a substrate to be oxidized by NOS with formation of NO, and to find new selective and efficient NO donors after in situ oxidation by NOS, we have synthesized a series of NOHA analogues and studied their NOS-catalyzed oxidation. We here report that, although the removal of the α-NH_2 or the α-COOH groups of NOHA leads to a dramatic decrease of NO formation, removal of both groups leads to a simple non α-amino acid compound, *N*-butyl *N*′-hydroxyguanidine, which acts as a substrate almost as efficient as NOHA itself for NO production. Our results also show that it is so far the best NOS substrate among a series of *N*-alkyl *N*′-hydroxyguanidines.

Chemistry. A series of *N*-substituted *N*′-hydroxyguanidines (Scheme 1) related to NOHA, **3a**, and its previously described analogues,[10] dinor-NOHA, **1a**, nor-NOHA, **2a**, and homo-NOHA, **4a**, have been synthesized, by using the general procedure previously reported for the preparation of *N*-hydroxyguanidines starting from the corresponding amines[16] (Scheme 2).

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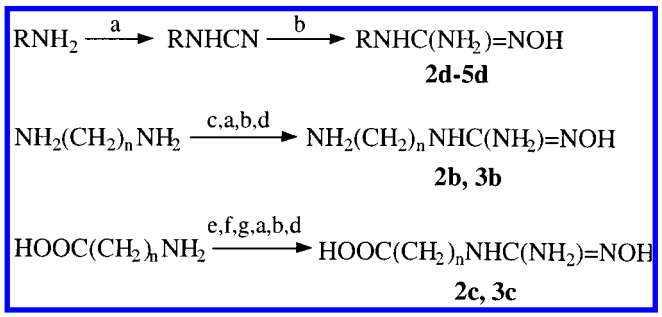
10.1021/jm0155446 CCC: $20.00
Published on Web 09/11/2001

Scheme 2[a]



[a] Reagents and conditions: (a) BrCN, CH_3CO_2Na, CH_3OH, 0 °C; (b) NH_2OH, HCl, EtOH, reflux; (c) $(tBuOCO)_2O$, $NaHCO_3$, dioxane, H_2O, 20 °C; (d) HCl in dioxane, 20 °C; (e) $PhCH_2OCOCl$, NaOH, 20 °C; (f) tBuBr, $(CH_3)_2NCOCH_3$, triethylbenzylammonium chloride, 55 °C; (g) H_2, Pd/C, CH_3OH, 20 °C.

In the particular case of compounds **2b**, **2c**, **3b**, and **3c**, protection of the ω-NH_2 or ω-COOH function was necessary (see Scheme 2 for the conditions). All the new compounds were fully characterized by ^1H NMR spectroscopy and by mass spectrometry and/or elemental analysis. They were found to be pure from TLC and ^1H NMR (>95% from comparison with an internal standard). Their detailed synthesis and characteristics will be published elsewhere.

Results and Discussion. Formation of NO upon oxidation of the various *N*-hydroxyguanidines shown in Scheme 1 by recombinant, purified NOS II, in the presence of NADPH and O_2, was followed by the classical hemoglobin assay[17a,b] that is based on the conversion of oxyhemoglobin to methemoglobin by NO. Although NOHA itself, **3a**, was very efficiently oxidized by NOS II with formation of 1920 nmol of NO min^{-1} mg $protein^{-1}$, its analogue lacking the α-COOH function, *N*-hydroxyagmatine, **3b**,[18] was much less active, its rate of oxidation into NO being only 8% of that found for NOHA (Table 1). The NOHA analogue lacking the α-NH_2 function, **3c**, was even less active, in agreement with the great importance of the presence of both α-COOH and α-NH_2 functions of L-arg or NOHA for their oxidation by NOS with NO formation.[9,12] However, quite surprisingly, oxidation by NOS II of the NOHA analogue **3d**, in which both the α-NH_2 and α-COOH functions of NOHA have been removed, led to a rate of NO formation very close to that found for NOHA (1300 nmol min^{-1} mg $protein^{-1}$, 68% of that found for NOHA). Nor-NOHA itself, as dinor-NOHA, **1a**, is not a substrate for NOSs,[10] presumably because of its shorter chain length that does not permit its α-amino acid moiety to correctly interact with the α-amino acid recognition site of NOS.[10] Its descarboxy and desamino analogues **2b** and **2c** also failed to produce NO upon oxidation by NOS II (Table 1). However, removal of both the α-NH_2 and α-COOH functions of nor-NOHA led to compound **2d** that becomes a reasonable substrate of NOS II, producing NO in a rate of 15% of that found for NOHA. In a similar manner, the desamino-, descarboxy-analogue of homo-NOHA, **4d**, was found active for NO formation, with a rate only 2 times lower than that found for homo-NOHA.

These results indicate that simple, non amino acid *N*-alkyl *N*′-hydroxyguanidines **2d**, **3d**, and **4d** are efficient NOS II substrates for NO formation, the most active compound being the *N*-butyl derivative **3d**.

Table 1. Formation of NO from Oxidation of Compounds **1**−**5** by NOSs[a]

compd	NOS I initial rates (% relative to NOHA)	NOS II initial rates (% relative to NOHA)	NOS III initial rates (% relative to NOHA)
NOHA, **3a**	100	100	100
3b	2 ± 1	8 ± 2	<0.5
3c	<0.5	1 ± 1	2 ± 1
3d	65 ± 10	68 ± 10	41 ± 5
nor-NOHA, **2a**	<0.5	<0.5	<0.5
2b	<0.5	<0.5	<0.5
2c	<0.5	<0.5	<0.5
2d	36 ± 5	15 ± 3	41 ± 5
1a	<0.5	<0.5	<0.5
4a	82 ± 10	55 ± 10	70 ± 10
4d	34 ± 8	25 ± 5	<0.5
5d	2 ± 1	1 ± 1	<0.5

[a] NO formation was detected spectrophotometrically by following the transformation of hemoglobin Fe^{II}−O_2 to hemoglobin Fe^{III} as described previously.[17a,b] Incubations were performed at 37 °C in 150 μL quartz cuvettes containing 1 mM NADPH, 100 μM tetrahydrobiopterin (BH_4), 100 U/mL superoxide dismutase (SOD), 100 U/mL catalase, 12−15 μM hemoglobin Fe^{II}-O_2, 5 mM dithiothreitol, and 1 mM of the tested compound in 50 mM HEPES buffer pH 7.4. In the case of NOS I and III, 1 mM $CaCl_2$, and 10 μg/mL calmodulin were also present. Purified, recombinant NOSs[17c−e] were added to sample cuvette, and the same volume of buffer was added to the reference cuvette. NOS concentrations were 5−50 nM. Initial rates were expressed as a percentage of those found for NOHA, which were 430, 1920, and 260 nmol NO min^{-1} mg $protein^{-1}$, respectively.

Compounds **4d** and **2d** which have a *N*-pentyl and *N*-propyl substituent, respectively, are 3- to 4-times less active, whereas the *N*-hexyl derivative **5d** is almost inactive (Table 1).

Similar results were obtained for the oxidations of compounds **2**−**5** by the constitutive NOSs, NOS I and NOS III (Table 1). The three NOSs were almost inactive in producing NO from oxidation of the desamino- and descarboxy-derivatives of NOHA and nor-NOHA. Conversely, they were remarkably active for NO formation from oxidation of compounds **2d**, **3d**, and **4d** (except for NOS III in the case of **4d**). Compound **3d** was clearly the best substrate for the three NOSs with rates of NO formation equal to 65 ± 10, 68 ± 10, and 41 ± 5% of those observed for NOHA in the case of NOS I, II, and III, respectively. Moreover, the three NOSs were all found to be almost unable to produce NO from oxidation of the *n*-hexyl derivative **5d**.

It thus seems that two classes of *N*-hydroxyguanidines may act as efficient substrates of NOSs: (1) α-amino acid compounds such as NOHA, and (2) simpler, non amino acid *N*-alkyl *N*′-hydroxyguanidines such as **3d**. The former class contains NOHA and its close analogue homo-NOHA;[10] their *N*-hydroxyguanidine moiety binds to a conserved glutamate residue of NOS and their α-amino acid moiety binds to several residues of the NOS active site.[19,20] Even small changes in the NOHA structure, such as the removal of the α-NH_2 or α-COOH function or of a CH_2 group, as in nor-NOHA, lead to compounds that are very bad NOS substrates (Table 1). The latter class involves *N*-substituted *N*′-hydroxyguanidines bearing hydrophobic and relatively small substituents such as propyl, butyl, or pentyl groups. It is likely that the corresponding compounds also bind to the conserved NOS glutamate via their *N*-hydroxyguanidine moiety. They also should bind to another part of the NOS active site, which is different from the polar α-amino acid binding site, and could be the hydrophobic

Table 2. Kinetic Constants for NO Formation from Oxidation of Some *N*-Hydroxyguanidines by NOS II[a]

compd	K_m[b]	k_{cat}[b]	k_{cat}/K_m[b]
NOHA, **3a**	40 ± 10	480 ± 60	12
3d	55 ± 10	320 ± 50	5.8
4a	146 ± 20	410 ± 50	2.8
4d[c]	310 ± 50	280 ± 50	0.9
$pClC_6H_4NOHG$[d]	500 ± 50	100 ± 20	0.2

[a] Activities were measured as indicated in Table 1. [b] K_m in μM, k_{cat} in min^{-1}, k_{cat}/K_m in μM^{-1} min^{-1}. [c] Data for homo-NOHA from ref 10. [d] Data for *N*−*p*-chlorophenyl *N*′-hydroxyguanidine from ref 15.

cavity close to the heme,[21] which is also found in the complex of NOS III with *N*-*p*-chlorophenyl *N*′-hydroxyguanidine.[22] Interaction of the hydrophobic chain of substrates with the 338-valine residue of this cavity could play a crucial role in determining substrate affinity.[21] *N*-hydroxyguanidines bearing an α-amino acid function at an appropriate position, NOHA and homo-NOHA, bind well to the α-amino acid NOS binding site, whereas nor-NOHA and desamino- or descarboxy-NOHA exhibit structures that neither permit their efficient binding to the α-amino acid binding site nor permit binding to the hydrophobic cavity. The alkyl group of *N*-alkyl *N*′-hydroxyguanidines should prefer to bind to the small NOS hydrophobic cavity, provided that it is not too long (<6 carbons).

More detailed kinetic studies were performed on the NOS II-catalyzed oxidations of the most active compounds **3d** and **4d** by comparison with NOHA and homo-NOHA. Formations of NO (from the hemoglobin assay)[17] and of NO_2^- (using the Griess assay),[23] the stable end product of NO under aerobic conditions, from oxidation of **3d** or **4d** exhibited characteristics very similar to those of NOS II-catalyzed oxidation of NOHA (data not shown): (1) they absolutely required the presence of NOS II containing BH_4, NADPH, and O_2, (2) they were strongly inhibited by classical NOS inhibitors such as N^{ω}-nitro-L-arginine and *S*-ethyl-*iso*-thiourea,[8] and (3) they were not significantly inhibited by superoxide dismutase and catalase, indicating that they were not due to oxidations by $O_2^{\bullet -}$ or H_2O_2 derived from the oxidase function of NOS.[24] NOS II-catalyzed oxidation of compounds **3d** and **4d** exhibited classical saturation kinetics and Lineweaver−Burk plots. Table 2 shows that the kcat values calculated for **3d** and **4d** were only slightly lower than the one found for NOHA (320 and 280 instead of 480 min^{-1}, respectively). However, whereas the K_m value observed for **3d** was only slightly greater than that found for NOHA, the K_m value calculated for **4d** was 8 times higher. Thus, the catalytic efficiency of NOS II-dependent oxidation of **3d** was only 2 times lower than that of NOHA (k_{cat}/K_m = 5.8 instead of 12 μM^{-1} min^{-1}), whereas those calculated for the oxidations of **4d** and of the previously reported substrate *N*-*p*-chlorophenyl *N*′-hydroxyguanidine[15] were very much lower (k_{cat}/K_m of 0.9 and 0.2 μM^{-1} min^{-1}).

Another parameter that is important for the evaluation of the quality of a substrate for NOS is the level of coupling between the electron transfer from NADPH and the transfer of an oxygen atom from O_2 to the substrate. This level is optimal in NOS II-catalyzed oxidation of NOHA, with consumption of 0.5 mol of NADPH per mol of NO produced, as expected for the three-electron oxidation of NOHA to citrulline and NO,

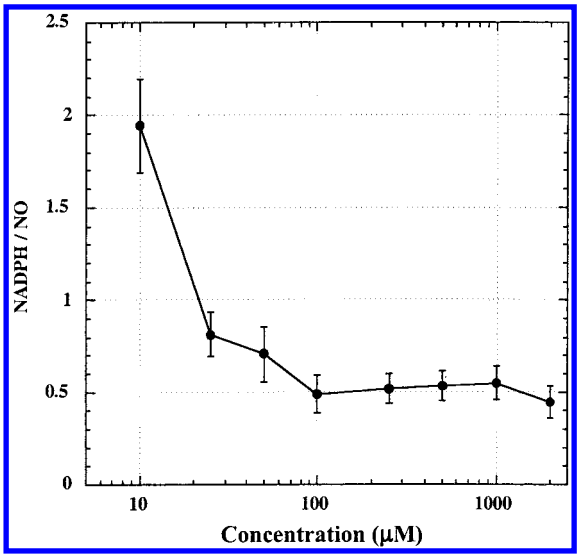


Figure 1. NADPH consumption for NO formation during oxidation of compound **3d** by NOS II as a function of **3d** concentration. Rates of NADPH consumption were measured by following the decrease in absorbance at 340 nm, under conditions identical to those described in Table 1, except for 0.2 mM NADPH. Values for the ratio moles of NADPH consumed/moles of NO formed are means ± SD from three experiments.

which requires the consumption of one electron and 1 mol of O_2[6,10] (eq 1). Figure 1 shows that NOS II-

$$RNHC(NH_2){=}NOH + O_2 + 1e^- (0.5\ NADPH) + H^+ \rightarrow RNHCONH_2 + {}^{\bullet}NO + H_2O \quad (1)$$

catalyzed oxidation of **3d** to NO consumed 0.5 mol of NADPH per mole of NO produced at saturating **3d** concentrations. Interestingly, this level of 0.5 mol of NADPH consumed per mole of NO is already almost reached at 50 μM **3d**. By comparison, formation of 1 mol of NO upon oxidation of *N*-*p*-chlorophenyl *N*′-hydroxyguanidine consumes 2 mol of NADPH when using a substrate concentration of 500 μM (K_m value, Table 2).[15]

Conclusion. Our results identify a new class of structurally simple, NOS-mediated NO donors that is based on non α-amino acid *N*-alkyl, *N*′-hydroxyguanidines. NOS II-catalyzed oxidations of NOHA and **3d** exhibit strikingly similar efficiencies, as shown by their very close k_{cat}/K_m values (12 and 5.8 μM^{-1} min^{-1}, Table 2) and the lack of any decoupling between electron transfer and oxygen atom transfer (consumption of only 0.5 mol of NADPH) in both cases. These results open the way toward the research of new NO donors based on selective substrates of each class of NOS.

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

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JM0155446