N-Aryl *N'*-Hydroxyguanidines, A New Class of NO-Donors after Selective Oxidation by Nitric Oxide Synthases: Structure–Activity Relationship

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The formation of nitric oxide (NO) was followed during the oxidation of 37 *N*-hydroxyguanidines or related derivatives, including 18 new *N*-aryl *N'*-hydroxyguanidines, by recombinant inducible nitric oxide synthase (NOS II). Several *N*-aryl *N'*-hydroxyguanidines bearing a relatively small, electron-donating para subtituent, such as H, F, Cl, CH_3, OH, OCH_3, and NH_2, led to NO formation rates between 8 and 41% of that of NO formation from the natural NOS substrate, N^ω-hydroxy-L-arginine (NOHA). The characteristics of these reactions were very similar to those previously reported for the oxidation of NOHA by NOS: (i) the strict requirement of NOS containing (6*R*)-5,6,7,8-tetrahydro-L-biopterin, reduced nicotinamide adenine dinucleotide phosphate, and O_2 for the oxidation to occur, (ii) the formation of NO and the corresponding urea in a 1:1 molar ratio, and (iii) a strong inhibitory effect of the classical NOS inhibitors such as N^ω-nitro-L-arginine and *S*-ethyl-*iso*-thiourea. Structure–activity relationship studies showed that two structural factors are crucial for NO formation from compounds containing a C=NOH function. The first one is the presence of a monosubstituted *N*-hydroxyguanidine function, since disubstituted *N*-hydroxyguanidines, amidoximes, ketoximes, and aldoximes failed to produce NO. The second one is the presence of a *N*-phenyl ring bearing a relatively small, not electron-withdrawing para substituent that could favorably interact with a hydrophobic cavity close to the NOS catalytic site. The k_{cat} value for NOS II-catalyzed oxidation of *N*-*para*-fluorophenyl *N'*-hydroxyguanidine was 80% of that found for NOHA, and its k_{cat}/K_m value was only 9-fold lower than that of NOHA. Interestingly, the K_m value found for NOS II-catalyzed oxidation of *N*-(3-thienyl) *N'*-hydroxyguanidine was 25 μM, almost identical to that of NOHA. Recombinant NOS I and NOS III also oxidize several *N*-aryl *N'*-hydroxyguanidines with the formation of NO, with a clearly different substrate specificity. The best substrates of the studied series for NOS I and NOS III were *N*-(*para*-hydroxyphenyl) and *N*-(*meta*-aminophenyl) *N'*-hydroxyguanidine, respectively. Among the studied compounds, the *para*-chlorophenyl and *para*-methylphenyl derivatives were selective substrates of NOS II. These results open the way toward a new class of selective NO donors after in situ oxidation by each NOS family.

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] In mammals, the synthesis of NO is catalyzed by constitutively expressed neuronal and endothelial nitric oxide synthases (NOS I and NOS III, respectively), which are controlled by the Ca^{2+}-dependent binding of the regulatory protein calmodulin (CaM). The inducible NOS (NOS II) is expressed in macrophages following induction by inflammatory mediators and binds CaM at basal levels of Ca^{2+}.[3,4] All three NOSs produce NO and L-citrulline from the oxidation of L-arginine (L-Arg), in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, with intermediate formation of N^ω-hydroxy-L-arginine (NOHA) (eq 1).[5–7]

R = HO₂C-CH(NH₂)-(CH₂)₃-
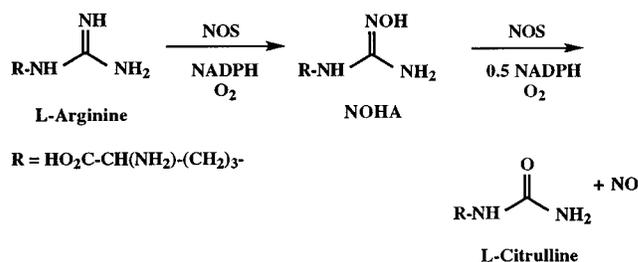
All NOSs are constituted by two domains, an NH_2-terminal oxygenase domain and a CO_2H-terminal reductase domain, which are separated by the CaM binding region. Heme (iron protoporphyrin IX) and (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4) are the essential prosthetic groups found in the oxygenase domain, which also contains the substrate binding site. Flavin adenine dinucleotide and flavin mononucleotide are found in the reductase domain, which also bears an NADPH binding site.[8–10] NOSs display high substrate specificity, and only very few compounds have been clearly shown to act as NOS substrates with the formation of NO. The

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analogues of L-Arg and NOHA with a longer side chain, homo-L-Arg and homo-NOHA, are less efficiently transformed than their parent compounds, whereas shorter side chain analogues, nor-L-Arg and nor-NOHA, are not transformed.[11−13] The introduction of a C=C double bond in the L-Arg side chain gives *E*-dehydro-L-Arg, which is a poor substrate for NOSs.[14] In a similar manner, rigidification of the L-Arg side chain by introduction of a phenyl ring abolishes NO formation.[15,16] Modifications of the α-amino acid function of L-Arg give compounds such as N^{α}-acetyl-L-Arg, L-Arg methyl ester, and D-Arg that are not substrates of NOS.[11] The introduction of substituents on the guanidino function of L-Arg leads to potent inhibitors such as N^{ω}-nitro-L-arginine (NO₂-L-Arg) and N^{ω}-amino-L-arginine[1,9,11,17] whereas N^{ω}-methyl-L-arginine, which is also a widely used inhibitor of NOSs, is oxidized by NOSs with a very limited formation of NO.[18,19] Replacement of the guanidino function of L-Arg with an acetamidino function results in N^{δ}-iminoethyl-L-ornithine, a potent NOS inhibitor.[17,20] L-Indospicine and N^{ω}-hydroxyindospicine are compounds in which the δ-NH group of L-Arg and NOHA has been replaced with a CH₂ group. Both compounds fail to act as substrates for NOSs and do not interact with the NOS active site.[21,22]

The very limited number of substrates known so far for NOSs suggests that highly specific structural features are required for NO generation by these enzymes. However, some simple guanidines and alkyl-*iso*-thioureas, such as aminoguanidine, *S*-ethyl-*iso*-thiourea (SEITU), and *N*-phenyl *S*-methyl-*iso*-thiourea are strong inhibitors of NOSs.[17,23−26] Spectroscopic studies have demonstrated that some of these simple molecules are bound in the active site of NOS, in close proximity to the heme.[27−30] These results suggest that many compounds bearing a positively charged group, but not the α-amino acid function of L-Arg, can interact with the NOSs' active site. We thus started with the idea that some simple guanidines or *N*-hydroxyguanidines could be oxidized by NOSs with the formation of NO. In fact, we have reported preliminary results showing that *N*-(4-chlorophenyl) *N'*-hydroxyguanidine (eq 2, X = *para*-Cl) is efficiently oxidized by NOS II with concomitant formation of NO and the corresponding *N*-aryl urea.[31]
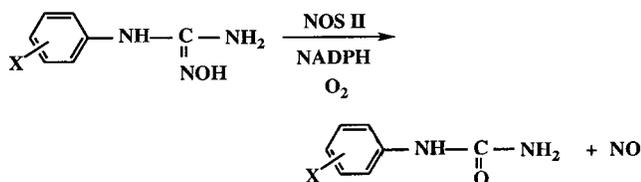
This new reaction of NOS II required NADPH and the presence of BH₄ and was inhibited by the addition of L-Arg analogues (NO₂-L-Arg and L-thiocitrulline (TC)) and by the heme ligand imidazole but was not inhibited by superoxide dismutase (SOD). This reaction thus clearly differed from the P450 and BH₄-free NOS II-catalyzed oxidation of *N*-hydroxyguanidines,[32,33] which is dependent on $O_2^{\bullet-}$ derived from the oxidase function of NOS[34−36] and strongly inhibited by SOD.[32,33] These results clearly indicated that this new reaction proceeded at the active site of NOS II,[31,33] in a manner similar to what is occurring in the second step of NO

synthesis catalyzed by NOSs, the oxidation of NOHA to L-citrulline, and NO.

In an effort to find new substrates of NOSs that lead to NO formation after selective oxidation by these enzymes and to determine the structural factors required for a compound to act as a substrate of NOSs, we have synthesized a series of *N*-aryl *N'*-hydroxyguanidines and studied their oxidation by purified recombinant NOS II. Furthermore, related compounds bearing an amidoxime, ketoxime, or aldoxime function instead of the *N*-hydroxyguanidine function were also synthesized and tested as potential NOS II substrates. Finally, these compounds were tested as substrates of NOS I, NOS II, and NOS III in a comparative manner. Our results show the importance of a monosubstituted *N*-hydroxyguanidine function for NO formation. They also show that several *N*-aryl *N'*-hydroxyguanidines are efficiently oxidized by NOS I, II, and III with the formation of NO and suggest that new, selective substrates should be found for each NOS and could constitute a new class of NO donors of pharmacological interest.

Results

Chemistry. A series of *N*-aryl *N'*-hydroxyguanidines, **1−23**, that involve either a phenyl ring bearing various substituents or an heterocyclic aryl ring were prepared by a classical procedure starting from substituted anilines with the intermediate formation of the corresponding arylcyanamides (eq 3).

This two-step procedure allowed us to prepare compounds **1−11**, **14−17**, and **23** from commercially available anilines or from 3-aminothiophene, which was obtained by a previously reported method that is mentioned in the Experimental Section.

In the case of *N*-aryl *N'*-hydroxyguanidines bearing an NH₂ function, **12**, **13**, and **18**, the starting compound was the corresponding nitroarene (Figure 1-1). After protection of its NH₂ function by the formation of a *tert*-butyloxycarbonyl (Boc) derivative, the nitroarene was reduced (H₂, Pd/C) to the corresponding aniline. A reaction with BrCN, then with NH₂OH, and deprotection of the NH₂ function by HCl in dioxane eventually led to the expected *N*-aryl *N'*-hydroxyguanidines **12**, **13**, and **18**. The heterocyclic *N*-hydroxyguanidine **22** was prepared by following the same strategy from 6-nitroindazole after similar protection of its NH function (Figure 1-2).

Three *N*-hydroxyguanidines bearing a COOH function were also used in this study. Two of them, **19** and **21**, were obtained by previously reported methods that are mentioned in the Experimental Section. The third one, **20**, was synthesized by following a strategy similar to that of eq 3 applied to the *tert*-butyl ester obtained by the reaction of 4-nitrophenol with *tert*-butyl bromoacetate (Figure 1-3).

For the sake of comparison, two *N*-alkyl *N'*-hydroxyguanidines, **24** and **25**, were also prepared by using the procedure of eq 3 (with alkyl instead of aryl). Moreover, to study the influence of another *N*-alkyl or *N*-aryl
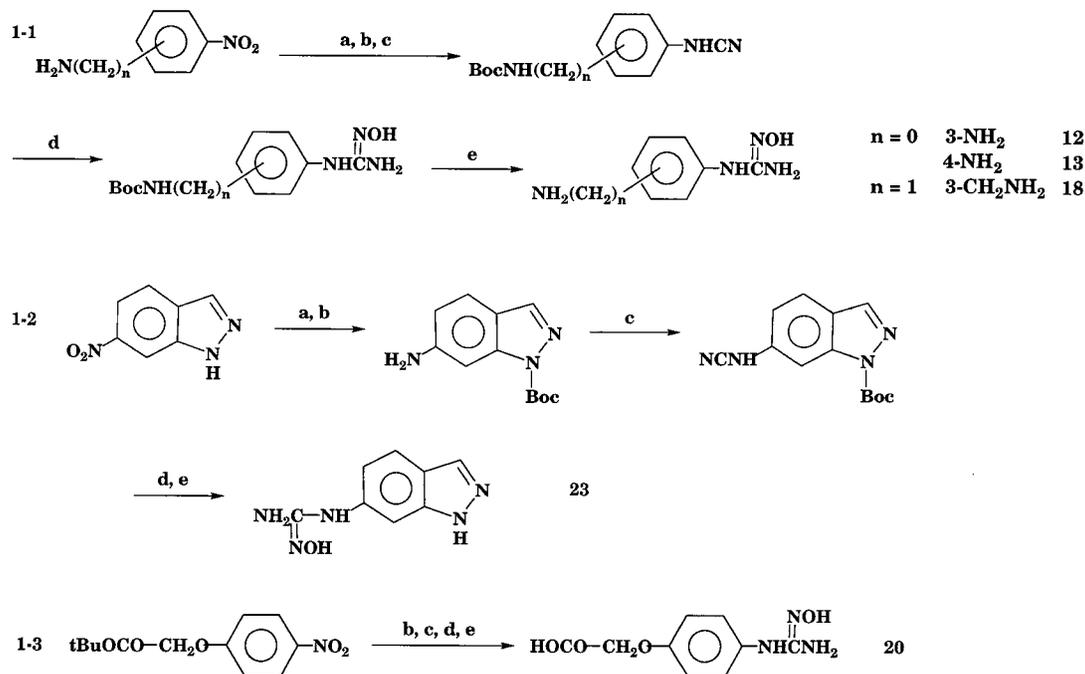
Figure 1. Synthetic routes followed for the preparation of *N*-aryl *N*-hydroxyguanidines bearing NH$_2$ (1 and 2) or COOH (3) functions. Key: (a) (*t*-BuOCO)$_2$O; (b) H$_2$, Pd/C; (c) BrCN; (d) NH$_2$OH, HCl; (e) HCl in anhydrous dioxane.

substituent on the hydroxyguanidine function, two disubstituted *N*-hydroxyguanidines bearing their substituents either on the same nitrogen, **26**, or on two different nitrogen atoms, **27**, were also synthesized. Finally, several aryl compounds bearing a C=NOH function, such as amidoximes, ketoximes, and aldoximes, **28**–**37**, were prepared in order to determine the structural factors necessary for a compound to be oxidized by NOS with NO formation. A detailed report of the synthesis and characteristics of the new *N*-hydroxyguanidines is included in the Experimental Section.

NOS II-Catalyzed Oxidation of Various *N*-Aryl *N*-Hydroxyguanidines, 1–23, by NADPH and O$_2$. The formation of NO during oxidation of *N*-hydroxyguanidines by NADPH and O$_2$ in the presence of purified, recombinant NOS II was measured by the classical spectrophotometric assay following the conversion of oxyhemoglobin to methemoglobin.[37,38] The assay was always done in the presence of SOD and catalase; thus, the observed formation of methemoglobin was not due to any possible oxidation of the *N*-hydroxyguanidines by O$_2$°$^-$ or H$_2$O$_2$ derived from the oxidase function of NOS II.[33-35]

NOS II oxidized not only *N*-(4-chlorophenyl) *N*-hydroxyguanidine, **5**, as previously reported,[31] but also several *N*-aryl *N*-hydroxyguanidines, with a rate of NO formation that may be up to 41% of that found for the endogenous substrate, NOHA (Table 1). In fact, only five compounds among the 23 *N*-aryl *N*-hydroxyguanidines that have been tested led to NO formation with rates higher than 10% of that found for NOHA. These compounds are *N*-phenyl *N*-hydroxyguanidine itself, **1**, and four of its para-substituted derivatives, **2**, **5**, **7**, and **9**, that bear F, Cl, CH$_3$, and OH para substituents, respectively. In a more general manner, the introduction of a given substituent in the para position of the phenyl ring of **1** led to better activities of NO formation than

the introduction of the same substituent in ortho or meta positions. Table 1 shows that this is true for the Cl (compare **3**–**5**), CH$_3$O (see **10** and **11**), and NH$_2$ (see **12** and **13**) substituents. If one now compares the activities observed for various para-substituted compounds, it clearly appears that both electronic and steric factors are important. NO formation rates are highly dependent on the size of the para substituent of the phenyl ring. The largest rates were observed with the smallest possible substituents H and F (20 and 41% as compared to NOHA); they were smaller with medium size substituents such as Cl, CH$_3$, OH, and NH$_2$ (between 8 and 17%) and close to zero with bigger substituents such as C(CH$_3$)$_3$, CH$_2$CO$_2$H, or OCH$_2$CO$_2$H. When comparing the rates observed in the case of substituents of similar size, it appears that electron-donating substituents led to higher rates than electron-withdrawing substituents. Thus, compounds **14** and **16** bearing 4-NO$_2$ and 4-CF$_3$ substituents led to very low rates of NO formation, close to the detection limit, whereas compounds **7** and **13** bearing 4-CH$_3$ and 4-NH$_2$ substituents led to activities 17 and 8% of those found for NOHA.

Table 1 also shows that *N*-hydroxyguanidines bearing a heterocyclic aryl substituent, **22** and **23**, are also oxidized by NOS II with the formation of NO, and the activity observed with the 3-thienyl derivative is 9% of that found with NOHA. Insertion of one or two CH$_2$ groups between the phenyl ring and the *N*-hydroxyguanidine function of **1** led to compound **24**, which acts as a substrate of NOS almost as well as **1**, and to compound **25**, which is a poorer substrate (Table 2). However, changes at the level of the *N*-hydroxyguanidine function itself led to compounds that did not act as substrates of NOS II with NO formation. This is the case of *N*-hydroxyguanidines bearing two substituents, either on two different nitrogen atoms, such as **27**, or on the same nitrogen atom, such as **26**. This is also the

Table 1. NO Formation from the Oxidation of Monosubstituted *N*-Aryl *N*-Hydroxyguanidines R−NH−C(=N−OH)−NH$_2$ Catalyzed by NOS II

compd	R	activity[a] (% NOHA)[b]
1	C$_6$H$_5$−	20 ± 3
2	4-F−C$_6$H$_4$	41 ± 6
3	2-Cl−C$_6$H$_4$−	5 ± 1
4	3-Cl−C$_6$H$_4$−	5 ± 1
5	4-Cl−C$_6$H$_4$−	13 ± 3
6	4-Br−C$_6$H$_4$−	7.5 ± 2
7	4-CH$_3$−C$_6$H$_4$−	17.5 ± 4
8	4-*tert*-butyl−C$_6$H$_4$−	<0.2
9	4-OH−C$_6$H$_4$−	16.5 ± 3
10	2-CH$_3$O−C$_6$H$_4$−	5 ± 2
11	4-CH$_3$O−C$_6$H$_4$−	8 ± 2
12	3-NH$_2$−C$_6$H$_4$−	3.5 ± 1
13	4-NH$_2$−C$_6$H$_4$−	8 ± 2
14	4-NO$_2$−C$_6$H$_4$−	2 ± 1
15	3-CF$_3$−C$_6$H$_4$−	2.5 ± 1
16	4-CF$_3$−C$_6$H$_4$−	0.5 ± 0.2
17	3-CH$_2$OH−C$_6$H$_4$−	6 ± 1
18	3-CH$_2$NH$_2$−C$_6$H$_4$−	1.5 ± 0.5
19	4-(CH$_2$CO$_2$H)−C$_6$H$_4$−	<0.2
20	4-(O−CH$_2$−CO$_2$H)−C$_6$H$_4$−	<0.2
21	4-[CH−(CH$_3$)−CO$_2$H]−C$_6$H$_4$−	<0.2
22	6-indazolyl	2.5 ± 0.5
23	3-thienyl	9.5 ± 2

[a] NO formation was detected spectrophotometrically by following the transformation of oxyhemoglobin to methemoglobin under standard conditions. Incubations were performed at 37 °C in 150 μL quartz cuvettes containing 1 mM NADPH, 100 μM BH$_4$, 100 U/mL SOD, 100 U/mL catalase, 12−15 μM oxyhemoglobin, 5 mM DTT, and 500 μM of the tested compounds in 50 mM HEPES buffer, pH 7.4. NOS II was added to the sample cuvette, and the same volume of buffer was added to the reference cuvette. NOS II concentration was 20−30 nM for all compounds, except for NOHA (5−6 nM). 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 34 mM^{-1} cm^{-1}.[37,38] In the case of compound **14**, NO-mediated consumption of oxyhemoglobin was followed at 577 nm and quantitated using an extinction coefficient of 14.5 mM^{-1} cm^{-1}.[63] Values are means ± SD from three to eight experiments. [b] NO formation from NOHA was 425 ± 100 nmol (nmol NOS)$^{-1}$ min^{-1} under those conditions.

case of *N*-aryl acetamidoximes **28** and **29**, in which the terminal NH$_2$ group of **5** and **11** has been replaced with a CH$_3$ group, and of benzamidoximes **30**−**32**, which derive from **1**, **5**, and **11**, respectively, by deletion of their internal NH group. Neither aryl ketoximes **34** and **35** nor aryl aldoximes **36** and **37** were able to produce NO upon oxidation by NOS II. Finally, compound **33** in which a −N=N− function was inserted between the aryl and the −C(=NOH)−NH$_2$ groups of amidoxime **31** also failed to act as a substrate of NOS II.

Characteristics of the NOS II-Catalyzed Oxidations of *N*-Aryl *N*-Hydroxyguanidines 1−23. Oxidations of compounds **1**, **2**, **5**, **7**, **9**, and **11** catalyzed by NOS II led to the highest levels of NO formation and were thereafter more deeply investigated. Analysis of the reaction mixtures by high-performance liquid chromatography (HPLC) revealed the formation of the corresponding *N*-aryl ureas that were identified by their UV spectra and HPLC retention times, which were identical to those of authentic compounds. Minor amounts of a second metabolite that was identified to be the corresponding *N*-aryl cyanamide were also observed. Under the conditions of the hemoglobin assay for NO formation, it was checked that hemoglobin alone did not react with *N*-aryl *N*-hydroxyguanidines and that for-

Table 2. Formation of NO from the Oxidation by NOS II of Various Compounds Related to *N*-Aryl *N*-Hydroxyguanidines and Corresponding to the General Formula R$_1$−C(=N− OH)−R$_2$

compd	R$_1$	R$_2$	activity[a] (% NOHA)[b]
	N-Hydroxyguanidines		
24	C$_6$H$_5$−CH$_2$−NH−	−NH$_2$	15 ± 4
25	C$_6$H$_5$−CH$_2$−CH$_2$−NH−	−NH$_2$	6 ± 2
26	tetrahydro*iso*quinolenyl−	−NH$_2$	<0.2
27	C$_6$H$_5$−NH−	−NH−C$_6$H$_5$	<0.2
	Acetamidoximes		
28	4-Cl−C$_6$H$_4$−NH−	−CH$_3$	<0.2
29	4-CH$_3$O−C$_6$H$_4$−NH−	−CH$_3$	<0.2
	Benzamidoximes		
30	C$_6$H$_5$−	−NH$_2$	<0.2
31	4-Cl−C$_6$H$_4$−	−NH$_2$	<0.2
32	4-CH$_3$O−C$_6$H$_4$−	−NH$_2$	<0.2
33	4-Cl−C$_6$H$_4$−N=N−	−NH$_2$	<0.2
	Ketoximes/Aldoximes		
34	4-Cl−C$_6$H$_4$−	−CH$_3$	<0.2
35	4-CH$_3$O−C$_6$H$_4$−	−CH$_3$	<0.2
36	4-Cl−C$_6$H$_4$−	−H	<0.2
37	4-CH$_3$O−C$_6$H$_4$−	−H	<0.2

[a] Conditions as in Table 1. Values are means ± SD from three to five experiments. [b] NO formation from NOHA was 425 ± 100 nmol (nmol NOS)$^{-1}$ min^{-1} under those conditions.

mation of NO did not occur in the presence of all of the assay components except NOS II (data not shown). Similar incubations were performed in the absence of oxyhemoglobin and studied by HPLC for the formation of ureas and cyanamides. These experiments showed that the presence of oxyhemoglobin had no effect on the formation of *N*-aryl ureas and cyanamides. The formations of *N*-aryl ureas and NO from compounds **1**, **2**, **5**, **7**, **9**, and **11** were clearly enzymatic, as they required the presence of active NOS II, BH$_4$, and NADPH, and occurred in the active site of NOS II, as they were strongly inhibited by classical NOS inhibitors, such as NO$_2$-L-Arg, TC, and SEITU (data not shown). Oxidation of all of these substrates by NOS II led to NO and the corresponding *N*-aryl urea in a 1:1 molar ratio (data not shown). These results clearly showed that oxidations of *N*-aryl *N*-hydroxyguanidines **1**, **2**, **5**, **7**, **9**, and **11** proceeded in the active site of NOS II in a manner very similar to the oxidation of its natural substrate, NOHA.

Kinetic Parameters for the NOS II-Dependent Oxidations of *N*-Aryl *N*-Hydroxyguanidines 1, 2, 5, 7, 9, 11, and 23. To more deeply characterize these new reactions, we measured the kinetic parameters K_m and V_m for the NOS II-dependent oxidations of the aforementioned *N*-aryl *N*-hydroxyguanidines using the hemoglobin assay and/or the HPLC detection of the *N*-aryl ureas. The formation of NO and *N*-aryl ureas proceeded linearly as a function of time for about 4 min and of the NOS II concentration in the 5−200 nM range (data not shown). Under these conditions, formations of NO and *N*-aryl ureas were saturable reactions. Conversely, the formation of the minor products, *N*-aryl cyanamides, was not, even at the highest concentrations of substrate used (up to 5 mM, data not shown), and this side reaction was not further investigated.

Table 3 shows that the NOS II-catalyzed oxidations of most of the studied *N*-aryl *N*-hydroxyguanidines exhibited K_m values 7−65-fold larger than the K_m value of NOHA. Their k_{cat} values were very much closer to

Table 3. Kinetic Constants Measured for the Oxidation of NOHA and of Some *N*-Aryl *N*-Hydroxyguanidines by NADPH and O_2 in the Presence of NOS II

compd	aryl	K_m (μM)[a]	k_{cat} (min^{-1})[a]	k_{cat}/K_m (% NOHA)[b]
NOHA		40 ± 10	425 ± 75	10.60 (100.0)
1	C$_6$H$_5-$	270 ± 90	130 ± 15	0.48 (4.5)
2	4-F$-$C$_6$H$_4-$	300 ± 40	350 ± 80	1.17 (11.0)
5	4-Cl$-$C$_6$H$_4-$	500 ± 50	98 ± 15	0.20 (2.0)
7	4-CH$_3-$C$_6$H$_4-$	1100 ± 300	295 ± 50	0.27 (2.5)
9	4-OH$-$C$_6$H$_4-$	425 ± 60	110 ± 15	0.26 (2.5)
11	4-CH$_3$O$-$C$_6$H$_4-$	2600 ± 600	145 ± 50	0.06 (0.05)
23	3-thienyl$-$	25 ± 15	40 ± 10	1.60 (15.1)

[a] Kinetic constants for NO formation were measured following the transformation of oxyhemoglobin to methemoglobin under the conditions described in Table 1, except that the concentrations of the tested compounds were varied from 5 μM to 2.5 mM. NOS II concentrations were adjusted to get less than 30% conversion of oxyhemoglobin. In the case of compounds **1**, **2**, **5**, **7**, **9**, and **11**, the formation of the corresponding ureas was also quantitated after HPLC analyses of similar incubations performed in the absence of oxyhemoglobin. Values are means $\pm$ SD from three to four experiments. [b] In min^{-1} μM^{-1}.

Table 4. Formation of NO from the Oxidation of *N*-Aryl *N*-Hydroxyguanidines R$-$NH$-$C(=N$-$OH)$-$NH$_2$ Catalyzed by NOS I or NOS III in the Presence of NADPH and O_2

compd	R	NOS I[a]	NOS III[a]
1	H	1.5 ± 0.5	<0.2
2	4-F$-$C$_6$H$_4-$	4.5 ± 1	<0.2
5	4-Cl$-$C$_6$H$_4-$	<0.2	<0.2
7	4-CH$_3-$C$_6$H$_4-$	<0.2	<0.2
9	4-OH$-$C$_6$H$_4-$	8.5 ± 1	<0.2
11	4-CH$_3$O$-$C$_6$H$_4-$	3.5 ± 1	<0.2
12	3-NH$_2-$C$_6$H$_4-$	2.5 ± 1	6.5 ± 2
13	4-NH$_2-$C$_6$H$_4-$	4.5 ± 1	<0.2
17	3-CH$_2$OH$-$C$_6$H$_4-$	<0.2	<0.2

[a] NO formation was detected spectrophotometrically following the transformation of oxyhemoglobin to methemoglobin under the conditions described in Table 1 (500 μM substrate), except that 1 mM CaCl$_2$ and 10 μg/mL CaM were added to the incubation mixtures. Results are expressed as percent of the activity measured in the presence of 500 μM NOHA and are means $\pm$ SD from three to five experiments. Rates of NO formation from the oxidation of NOHA catalyzed by NOS I and NOS III were 130 $\pm$ 20 and 65 $\pm$ 20 nmol (nmol NOS)$^{-1}$ min^{-1}, respectively.

that found for NOHA oxidation; the value measured for compound **2** was as high as 82% of the k_{cat} found for NOHA. Quite interestingly, compound **23**, which involves a thiophene ring, had an opposite behavior as its oxidation exhibited a K_m value very close to that of NOHA, but it had a k_{cat} value almost 10 times lower. Anyway, it is quite remarkable that two of these simple *N*-aryl *N*-hydroxyguanidines, **2** and **23**, which do not bear an α-amino acid function, are oxidized by NOS II with a catalytic efficiency, k_{cat}/K_m, only 9 and 6 times lower than that of NOHA.

Comparison of the Oxidations of *N*-Aryl *N*-Hydroxyguanidines by NOS I, II, and III. Oxidations of *N*-hydroxyguanidines **1**–**27** by purified, recombinant NOS I and III containing BH$_4$, in the presence of NADPH and O_2, were similarly studied with the detection of NO using the hemoglobin assay as described above. NOS I only formed significant amounts of NO from a limited number of *N*-aryl *N*-hydroxyguanidines (Table 4). The best activity (8.5% of that found for NOS I-catalyzed oxidation of NOHA) was observed with compound **9**, which bears a *para*-hydroxyphenyl substituent. Compounds **1**, **2**, and **11**–**13** led to NO with rates between 2 and 5% of that found for NOHA. The

oxidation of these compounds by NOS I exhibited characteristics almost identical to those of the corresponding oxidations catalyzed by NOS II, i.e., they required the presence of the enzyme containing BH$_4$, were inhibited by NO$_2$-L-Arg and SEITU, and were not inhibited by the addition of SOD and catalase (data not shown). Moreover, they also led to the corresponding *N*-aryl urea and NO in stoichiometric amounts. An even smaller number of *N*-aryl *N*-hydroxyguanidines were oxidized by NOS III with the formation of NO. Actually, compound **12** that bears a *meta*-aminophenyl substituent was the only derivative oxidized by NOS III with the formation of NO at a reasonable rate (6.5% of the activity found with NOHA).

Discussion

The aforementioned results demonstrate that several *N*-aryl *N*-hydroxyguanidines are oxidized by NOSs in the presence of NADPH and O_2 with formation of the corresponding *N*-aryl urea and NO (eq 4).
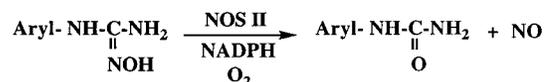
The characteristics of these reactions are very similar to those of the oxidation of NOHA by NOS. This includes (i) the strict requirement of NOS containing BH$_4$, NADPH, and O_2 for the oxidation to occur; (ii) the formation of NO and the corresponding urea in a 1:1 molar ratio; and (iii) a strong inhibitory effect of classical NOS inhibitors such as NO$_2$-L-Arg and SEITU.

When considering all of the compounds, **1**–**37**, that have been tested, it appears that the presence of a monosubstituted *N*-hydroxyguanidine function is absolutely required for a compound to be a substrate of NOS with NO formation. Related compounds involving an *N,N*- or *N,N'*-disubstituted hydroxyguanidine, amidoxime, ketoxime, or aldoxime function do not produce NO upon oxidation by NOS II (Table 2). Actually, the best substrates found for NOS II are *N*-aryl *N*-hydroxyguanidines involving a relatively small and sufficiently electron-rich para substituent. Thus, it is clear that both steric and electronic factors are important for NOS II-dependent oxidation of *N*-aryl *N*-hydroxyguanidines with NO formation. The best NO precursor is the compound bearing a *para*-fluorophenyl substituent, and it is remarkable that one can reach 41% of the activity of NO formation from NOHA with a nonamino acid compound as simple as **2**. In fact, during the preparation of this paper, we found that other nonamino acid, simple *N*-alkyl *N*-hydroxyguanidines were oxidized by NOSs with an even better efficacy.[39]

An analysis of the kinetic parameters determined for the oxidation of a series of *N*-aryl *N*-hydroxyguanidines bearing small, electron-donating para substituent **1**, **2**, **5** **7**, **9**, and **11** (Table 3) was performed in order to find quantitative structure−activity relationships (QSAR).[40] K_m and k_{cat} values were thus correlated with many classical descriptors for geometric, electronic, and hydrophobic properties of the para substituents, such as the Hansch constant Π, the Hammett constants σ, σ^-, or σ^+, or the dipole moment.[41] The k_{cat} values did not lead to any simple correlation with any of the descriptors used, and the K_m values did not correlate with any
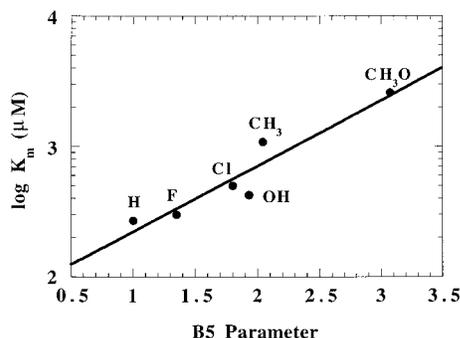
Figure 2. Relationship between the K_m values found for NO formation upon oxidation of several *N-para*-substituted phenyl *N*-hydroxyguanidines by NOS II and the maximal width of the para substituent (B5 parameter according to Verloop et al.).[42] The K_m values are those indicated in Table 3.

of the descriptors for electronic or hydrophobic properties, taken either one by one or by pairs. However, a straightforward relationship was found between K_m and a geometric descriptor. A classical method for describing the geometry of nonspherical chemical groups is the one developped by Verloop et al.,[41,42] which uses STERIMOL parameters L, B1, and B5 that are related to the length, the minimal width, and the maximal width of the substituent, respectively. As shown in Figure 2, there is a clear linear relationship between the log K_m values found for the *N*-para-substituted phenyl *N*-hydroxyguanidines **1**, **2**, **5**, **7**, **9**, and **11** and the B5 parameter (maximal width) of the substituent. This importance of the width of the substituent for recognition by NOS II would explain why compound **1** leads to the lowest K_m value whereas **11** leads to such a high K_m value.

The X-ray structures recently published for NOS oxygenase domains,[28−30,43] and, more particularly, the structure of the complex of compound **5** with the NOS III oxygenase domain,[44] allow one to explain, at least in part, the aforementioned data. NOHA is bound in the NOS active site via two major interactions: (i) strong hydrogen bonds between its δ-NH and its ω-NH₂ groups with highly conserved glutamate and tryptophane NOS residues and (ii) a network of hydrogen bonds between its α-NH₂ and its α-COOH functions with the α-amino acid binding site of NOS.[43] In the case of compound **5**, the first interaction between the *N*-hydroxyguanidine function and the glutamate and tryptophane NOS residues is kept.[44] The second interaction does not occur with the α-amino acid binding site of NOS but with a small hydrophobic cavity that is located in close proximity to one of the heme propionates and involves valine and phenylalanine residues.[44,45] It is thus understandable that oxidation of molecules containing a C=NOH function by NOS with the formation of NO requires the presence of a *N*-hydroxyguanidine moiety for an efficient anchoring in the NOS catalytic site, just above the heme, via hydrogen bonds with the conserved glutamate and tryptophane residues. Amidoximes and ketoximes that lack one of the anchoring δ-NH and ω-NH₂ moieties are not substrates of NOSs. Conversely, *N*-aryl *N*-hydroxyguanidines should be strongly anchored via their hydroxyguanidine moiety, provided that steric repulsions do not occur between substituents of the substrate and some amino acid NOS residues. Binding of the ω-NH₂

of NOHA or **5** with NOS-conserved glutamate and tryptophane residues is occurring in a highly hindered site.[43−45] This should explain why the *N,N*-disubstituted *N′*-hydroxyguanidine **27** does not act as a NOS substrate (Table 2).

As far as *N*-aryl *N*-hydroxyguanidines are concerned, more or less favorable interactions between the aryl substituent and the NOS hydrophobic cavity involving the conserved valine and phenylalanine residues should play a role in determining the efficiency of NOS-catalyzed oxidation.[44,45] The small size of this cavity would explain the correlation between the width of the para substituent and the log K_m value shown in Figure 2. A favorable interaction of the substrate aryl ring with the NOS valine residue that is the closest one seems only to occur with para-substituted phenyl rings bearing small enough substituents such as H or F, and, to a lesser extent, CH₃, Cl, OH, NH₂, or OCH₃. Preliminary molecular modeling studies (Lefevre-Groboillot, D.; Boucher, J. L.; Attias, R.; Mansuy, D. Manuscript in preparation.) indicate that the presence of more bulky para substituents, such as *tert*-butyl or O−CH₂−CO₂H, and of meta substituents, even small in that case, disfavors binding in this cavity mainly because of steric restrictions with the valine residue and one of the heme propionate groups. Interaction between the aryl group and the NOS hydrophobic cavity also appears less favorable in the case of ortho-substituted aryl substituents because of steric constraints with one heme propionate of the active site and one *meso*-carbon of the heme. However, this does not mean that the position and size of the aryl substituents and their interactions with the aforementioned NOS hydrophobic cavity completely control NO formation. Electronic effects also are important as compounds bearing strong electron-withdrawing para substituents, such as CF₃ or NO₂, are very bad substrates of NOS II (Table 1). Moreover, one cannot presently exclude that some *N*-aryl *N*-hydroxyguanidines, such as those bearing a polar *N*-aryl substituent, could bind to NOS differently and interact with other NOS amino acid residues.

NOS I, II, and III exhibit very different specificities toward *N*-aryl *N* hydroxyguanidines. In a general manner, NOS II efficiently catalyzes the oxidation with formation of NO, of a much larger variety of such substrates than NOS I and III. Moreover, the best *N*-aryl *N*-hydroxyguanidine substrates for NOS I, II, and III are different; compound **9** bearing a *para*-OH substituent gave the highest NO formation rate in the case of NOS I, whereas compounds **2** and **12**, with *para*-F and *meta*-NH₂ substituents, gave the highest rates of NO formation in the case of NOS II and III, respectively. When examining the selectivity of the oxidation of these compounds by the different NOS isoforms, our data show that *para*-Cl- and *para*-CH₃-substituted compounds, **5** and **7**, are completely selective substrates for NOS II, whereas the *meta*-NH₂-substituted compound, **12**, is not selective for NOS III but is so far the only *N*-aryl *N*-hydroxyguanidine efficiently oxidized by NOS III to NO. Further studies are currently being performed in order to better understand the substrate specificities of NOSs toward *N*-hydroxyguanidines and to find highly selective NO donors after oxidation by each class of NOS.

Experimental Section

Chemicals and reagents of the highest grade commercially available were obtained from Aldrich, Fluka, or Janssen and used without further purification. Chemical reactions were monitored by thin-layer chromatography using Merck precoated silica gel 60F$_{254}$ (0.25 mm thickness) plates. Millipore SA silica gel 60 (35−70 MY) was utilized for flash chromatography. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ARX 250 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane with peak multiplicities abbreviated as singlet, s; broad singlet, br s; doublet, d; triplet, t; and multiplet, m. Coupling constants (J) are reported in Hertz. IR spectra were recorded on a Perkin-Elmer 783 spectrometer and wavenumbers are reported in cm^{-1}. Melting points were determined on a Kofler apparatus and are uncorrected. Mass spectra were recorded at Ecole Normale Superieure, Paris, on a RiberMag system with fast atom bombardment (FAB), electron impact (EI), high-resolution mass spectroscopy (HRMS), and chemical ionization using methane (CI) capabilities. Elemental analyses were performed at Service de Microanalyze, Pierre and Marie Curie University, Paris.

N^ω-Hydroxy-L-arginine was prepared by a modification of the procedure of Wallace and Fukuto using N^δ-(benzyloxycarbonyl)-L-ornithine as a starting material.[46]

Synthesis of Compounds 1−26. General Procedures. N-Hydroxyguanidines **1−26** were obtained, as well as small amounts of the corresponding N-aryl ureas, by the addition of hydroxylamine hydrochloride to intermediate cyanamides in anhydrous ethanol, following a general method previously described.[47,48] The routes for preparing these compounds are indicated in eq 3 and Figure 1. Before describing the characteristics of the final N-hydroxyguanidines themselves, we first report (i) the general protocol for the preparation of cyanamides RNHCN from the corresponding amines RNH$_2$, (ii) a more detailed description of the synthesis of some cyanamides derived from not commercially available anilines that were prepared from nitroarenes bearing NH$_2$, NH, or COOH functions after protection of these functions (corresponding to compounds **12**, **13**, **18**, **20**, and **23**, Figure 1), and (iii) the general procedure for Boc or *tert*-butyl ester deprotection.

General Protocol for Synthesis of Cyanamides.[46,47] A solution of 10 mmol of the aryl- or alkylamine in 15 mL of methanol was added dropwise at 0 °C to a solution of 12 mmol BrCN in 15 mL of methanol containing 30 mmol anhydrous sodium acetate. The reaction mixture was stirred for 2 h at 0 °C and 2−20 h at room temperature. The solvent was removed under vacuum, and the crude product was extracted with CH$_2$Cl$_2$. The organic phase was washed with brine, dried, and evaporated. Crude cyanamides were purified by flash chromatography over SiO$_2$ using CH$_2$Cl$_2$ as the solvent and/or recrystallized from CH$_2$Cl$_2$/cyclohexane mixtures. In the following, we will only describe the detailed preparation of the cyanamides for which the amine precursor was not commercially available.

4-(N-*Tert*-butyloxycarbonylamino)phenylcyanamide. A solution of 3.5 g (14.3 mmol) of di-*tert*-butylpyrocarbonate in 10 mL of CH$_2$Cl$_2$ was added dropwise to a mixture of 2.0 g (14.5 mmol) of 4-nitroaniline, 2 mL (14.5 mmol) of triethylamine, and 0.89 g (7.25 mmol) of 4-(N,N-dimethylamino)pyridine in 30 mL of anhydrous CH$_2$Cl$_2$. The mixture was stirred at room temperature for 3 h and washed with brine. The organic phase was dried over MgSO$_4$ and evaporated, and the crude product was purified by flash chromatography (SiO$_2$, ethyl acetate/cyclohexane 1/3) giving 2.05 g (62%) of N-(tert-butyloxycarbonyl)-4-nitroaniline. The protected 4-nitroaniline (0.70 g, mmol) was dissolved into 25 mL of ethyl acetate containing 70 mg Pd 10%/C and stirred for 4 h at room temperature under a hydrogen atmosphere. The reaction mixture was filtered over Celite and evaporated under vacuum, and the crude product was recrystallized in diethyl ether/cyclohexane giving 0.50 g (82%) of 4-(N-*tert*-butyloxycarbonylamino)aniline as an off-white solid; mp 105−106 °C. ^{1}H NMR (CDCl$_3$): δ 7.11 (d, 2H, J = 8.4), 6.62 (d, 2H, J = 8.4),

6.22 (br s, 1H), 3.51 (br s, 2H), 1.48 (s, 9H). Treatment of this protected aniline with BrCN under the conditions of the general procedure afforded 4-(N-*tert*-butyloxycarbonylamino)phenylcyanamide as a white solid in 85% yield; mp 151−152 °C. ^{1}H NMR (CDCl$_3$): δ 7.29 (d, 2H, J = 8.4), 6.89 (d, 2H, J = 8.4), 6.42 (br s, 1H), 6.03 (br s, 1H), 1.50 (s, 9H). IR (KBr): 3350−3290, 2210, 830.

3-(N-*Tert*-butyloxycarbonylamino)phenylcyanamide. N-(*Tert*-butyloxycarbonyl) 3-nitroaniline was similarly obtained in a 62% yield from 3-nitroaniline. The protected 3-nitroaniline was reduced under a hydrogen atmosphere in the presence of Pd 10% Pd/C and gave 3-(N-*tert*-butyloxycarbonylamino)aniline as a pale yellow solid in 90% yield; mp 110−111 °C. ^{1}H NMR (CDCl$_3$): δ 7.01 (t, 1H, J = 8.0), 6.95 (br s, 1H), 6.52 (d, 1H), 6.34 (m, 2H), 3.64 (br s, 2H), 1.49 (s, 9H). Treatment of this protected aniline with BrCN following the general procedure afforded 3-(N-*tert*-butyloxycarbonylamino)phenylcyanamide as an oil in 96% yield. ^{1}H NMR (CDCl$_3$): δ 7.20 (t, 1H, J = 8.4), 6.84 (d, 1H, J = 8.4), 6.70 (d, 1H, J = 8.4), 6.56 (s, 1H), 1.50 (s, 9H). IR (KBr): 3300−3180, 2215.

1-(N-*Tert*-butyloxycarbonyl)-6-cyanamidino-indazole. 1-(N-*Tert*-butyloxycarbonyl)-6-nitroindazole was obtained in an 81% yield from 6-nitroindazole and reduced under a hydrogen atmosphere in the presence of Pd 10%/C to give 1-(N-*tert*-butyloxycarbonyl)-6-aminoindazole as an off-white solid in 86% yield; mp 171−172 °C. ^{1}H NMR (CDCl$_3$): δ 7.95 (s, 1H), 7.44 (m, 2H), 6.65 (d, 1H, J = 8.4), 3.99 (br s, 2H), 1.69 (s, 9H). Treatment with BrCN under the conditions of the general procedure afforded 1-(N-*tert*-butyloxycarbonyl)-6-cyanamidino-indazole as a pale yellow solid in 52% yield; mp 167−170 °C (dec). ^{1}H NMR (CDCl$_3$): δ 8.11 (s, 1H), 7.88 (s, 1H), 7.68 (d, 1H, J = 8.6), 7.01 (d, 1H, J = 8.6), 6.48 (br s, 1H), 1.71 (s, 9H). IR (KBr): 3200, 2220.

3-(N-*Tert*-butyloxycarbonylaminomethyl)phenylcyanamide. N-(*Tert*-butyloxycarbonylaminomethyl)-3-nitroaniline was obtained from 3-(aminomethyl)nitrobenzene. The protected amine was reduced under a hydrogen atmosphere in the presence of Pd 10%/C and treated with BrCN following the general procedure to afford 3-(N-*tert*-butyloxycarbonylaminomethyl)phenylcyanamide as a beige solid in 80% overall yield; mp 120−121 °C. ^{1}H NMR (CDCl$_3$): δ 7.25 (t, 1H, J = 8.4), 6.96 (d, 1H, J = 8.4), 6.87 (m, 2H), 6.54 (br s, 1H), 4.95 (br s, 1H), 4.27 (m, 2H), 1.45 (s, 9H). IR (KBr): 3330, 3160, 2215.

(4-Cyanamidinophenoxy)acetic Acid *Tert*-butyl Ester. *Tert*-butyl bromoacetate (5.2 mmol) was added to a solution of 4-nitrophenol (5.0 mmol) in 10 mL of anhydrous N,N-dimethylformamide containing 5.2 mmol Na$_2$CO$_3$, and the mixture was heated for 1.5 h at 70 °C. N,N-Dimethylformamide was evaporated, and the residue was dissolved in CH$_2$Cl$_2$. The organic phase was washed with water, dried over MgSO$_4$, and evaporated. Recrystallization from CH$_2$Cl$_2$/cyclohexane gave (4-nitrophenoxy)acetic acid *tert*-butyl ester as an off-white compound in 91% yield; mp 84−85 °C. ^{1}H NMR (CDCl$_3$): δ 8.19 (d, 2H, J = 9.2), 6.93 (d, 2H, J = 9.2), 4.59 (s, 2H), 1.47 (s, 9H). Reduction of (4-nitrophenoxy)acetic acid *tert*-butyl ester under a hydrogen atmosphere in the presence of Pd 10%/C and treatment with BrCN following the general procedures afforded (4-cyanamidinophenoxy)acetic acid *tert*-butyl ester as an off-white solid in 80% overall yield; mp 90−91 °C. ^{1}H NMR (CDCl$_3$): δ 6.88 (m, 4H), 5.80 (br s, 1H), 4.47 (s, 2H), 1.47 (s, 9H). IR (KBr): 3180, 2210.

General Protocol for the Formation of N-Hydroxyguanidines from the Corresponding Cyanamides.[46−48] A 0.2 M solution of the cyanamide in anhydrous ethanol containing 1.1 equiv of hydroxylamine hydrochloride was heated under reflux for 1−15 h. The solvent was evaporated, and the residue was dissolved in water. The byproduct urea was filtered off or extracted with ethyl acetate. The aqueous phase was brought to pH 8−9 by the addition of a saturated solution of NaHCO$_3$ and extracted with ethyl acetate. The organic layer was dried over MgSO$_4$ and evaporated. The crude N-hydroxyguanidines were either recrystallized in diethyl ether/pentane mixtures (free bases) or redissolved in minimum amounts of

methanol and precipitated after the addition of a solution of anhydrous HCl, HBr, or acetic acid in diethyl ether.

General Protocol for Boc or Tert-butyl Ester Deprotection. A solution of the Boc-protected amine (corresponding to **12**, **13**, **18**, or **23**) or of the *tert*-butyl ester (corresponding to **20**), in dioxane, was treated with anhydrous HCl (4 M) in dioxane at room temperature for 12 h.

***N*-Phenyl *N*-Hydroxyguanidine Hydrochloride (1).** Compound **1** was obtained as a beige solid in 63% yield from phenylcyanamide; mp 128−129 °C. ^{1}H NMR (dimethyl sulfoxide (DMSO)-*d*$_6$): δ 10.87 (br s, 1H), 10.01 (br s, 1H), 9.93 (br s, 1H), 7.78 (br s, 2H), 7.42 (m, 2H), 7.23 (m, 3H). IR (KBr): 3380, 3340, 3200, 765, 700. MS (CI): 152 (MH$^+$). Anal. (C$_7$H$_9$N$_3$O, HCl) C, H, N.

***N*-(4-Fluorophenyl) *N*-Hydroxyguanidine Hydrochloride (2).** Compound **2** was prepared as a white solid in 67% yield from (4-fluorophenyl)cyanamide; mp 131−132 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 10.83 (br s, 1H), 10.18 (br s, 1H), 9.87 (br s, 1H), 7.84 (br s, 2H), 7.25 (m, 4H). IR (KBr): 3380, 3330, 830. MS (EI): 169 (M$^+$). Anal. (C$_7$H$_8$N$_3$OF, HCl) C, H, N.

***N*-(2-Chlorophenyl) *N*-Hydroxyguanidine Hydrochloride (3).** Compound **3** was prepared as a colorless oil in 68% yield from (2-chlorophenyl)cyanamide.[49] ^{1}H NMR (DMSO-*d*$_6$): δ 10.78 (br s, 1H), 10.05 (br s, 1H), 9.72 (br s, 1H), 7.84 (br s, 2H), 7.59 (d, 2H, *J* = 8.5), 7.40 (m, 3H). IR (film): 3450, 3120, 760, 730;.MS (CI): 186 and 188 (MH$^+$). HRMS calcd for C$_7$H$_8$N$_3$O,[37] Cl 187.0320 and for C$_7$H$_8$N$_3$O,[35] Cl 185.0359; found, 187.0328 and 185.0356.

***N*-(3-Chlorophenyl) *N*-Hydroxyguanidine Hydrochloride (4).** Compound **4** was prepared as a white solid in 51% yield from (3-chlorophenyl)cyanamide; mp 164 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 11.06 (br s, 1H), 10.18 (br s, 1H), 10.04 (br s, 1H), 8.06 (br s, 2H), 7.42 (m, 1H), 7.29 (m, 2H), 7.17 (m, 1H). IR (KBr): 3380, 3240, 3120, 780. MS (CI): 186 and 188 (MH$^+$). Anal. (C$_7$H$_8$N$_3$OCl, HCl) C, H, N.

***N*-(4-Chlorophenyl) *N*-Hydroxyguanidine (5).**[47] Compound **5** was obtained as a white solid in 45% yield from (4-chlorophenyl)cyanamide; mp 129 °C (literature: 129−131 °C).[47]

***N*-(4-Bromophenyl) *N*-Hydroxyguanidine (6).** Compound **6** was obtained as a white solid in 65% yield from (4-bromophenyl)cyanamide; mp 162 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 8.54 (s, 1H), 7.83 (s, 1H), 7.33 (m, 4H), 5.14 (br s, 2H). IR (KBr): 3470, 3340, 820. MS (EI): 231 and 229 (M$^+$). Anal. (C$_7$H$_8$N$_3$OBr) C, H, N.

***N*-(4-Tolyl) *N*-Hydroxyguanidine (7).** Compound **7** was prepared as a white solid in 32% yield from 4-tolylcyanamide; mp 140−142 °C. ^{1}H NMR (CD$_3$OD): δ 7.09 (d, 2H, *J* = 8.4), 7.01 (d, 2H, *J* = 8.4), 2.24 (s, 3H). IR (KBr): 3470, 3360, 3020, 810. MS (EI): 165 (M$^+$). Anal. (C$_8$H$_{11}$N$_3$O) C, H, N.

***N*-(4-*Tert*-butyl)phenyl *N*-Hydroxyguanidine (8).** Compound **8** was prepared as a white solid in 43% yield from (4-*tert*-butylphenyl)cyanamide; mp 128−130 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 8.31 (br s, 1H), 7.47 (br s, 1H), 7.26 (m, 4H), 5.05 (br s, 2H), 1.28 (s, 9H). IR (KBr): 3480, 3250, 820. MS (CI): 208 (MH$^+$). Anal. (C$_{11}$H$_{17}$N$_3$O) C, H, N.

***N*-(4-Hydroxyphenyl) *N*-Hydroxyguanidine Hydrochloride (9).** Compound **9** was prepared as a beige solid in 27% yield from (4-hydroxyphenyl)cyanamide; mp 85−88 °C (dec). ^{1}H NMR (DMSO-*d*$_6$): δ 10.56 (br s, 1H), 9.92 (br s, 1H), 9.70 (br s, 1H), 9.57 (br s, 1H), 7.61 (br s, 2H), 7.00 (d, 2H, *J* = 8.5), 6.82 (d, 2H, *J* = 8.5). ^{13}C NMR (D$_2$O): δ 159.62 (C=N), 131.71, 129.22 (CH), 128.47, 119.12 (CH). IR (KBr): 3420, 3340, 3260, 3120, 830. MS (CI): 168 (MH$^+$). HRMS calcd for C$_7$H$_9$N$_3$O$_2$, 167.0695; found, 167.0700.

***N*-(2-Methoxyphenyl) *N*-Hydroxyguanidine Hydrochloride (10).** Compound **10** was prepared as an off-white solid in 50% yield from (2-methoxyphenyl)cyanamide; mp 122−123 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 10.64 (br s, 1H), 9.97 (br s, 1H), 9.37 (br s, 1H), 7.66 (br s, 2H), 7.33 (t, 1H, *J* = 8.5), 7.15 (m, 2H), 6.98 (t, 1H, *J* = 8.5), 3.80 (s, 3H). IR (KBr): 3460, 3060, 760. MS (CI): 182 (MH$^+$). Anal. (C$_8$H$_{11}$N$_3$O$_2$, HCl, 0.2H$_2$O) C, H, N.

***N*-(4-Methoxyphenyl) *N*-Hydroxyguanidine Hydrochloride (11).** Compound **11** was prepared as a white solid in 51% yield from (4-methoxyphenyl)cyanamide; mp 128−131 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 10.64 (br s, 1H), 9.96 (br s, 1H), 9.67 (br s, 1H), 7.67 (br s, 2H), 7.15 (d, 2H, *J* = 8.5), 6.98 (d, 2H, *J* = 8.5), 3.75 (s, 3H). IR (KBr): 3420, 3320, 830. MS (FAB): 182.22 (MH$^+$). Anal. (C$_8$H$_{11}$N$_3$O$_2$, HCl) C, H, N.

***N*-(3-Aminophenyl) *N*-Hydroxyguanidine Dihydrobromide (12).** Compound **12** was prepared as a beige solid in 64% yield from 3-(*N-tert*-butyloxycarbonylamino)phenylcyanamide; mp 192−194 °C (dec). ^{1}H NMR (DMSO-*d*$_6$): δ 10.84 (br s, 1H), 10.03 (br s, 1H), 9.77 (s, 1H), 8.07 (br s, 2H), 7.33 (m, 1H), 6.88 (m, 3H). IR (KBr): 3380, 3200. MS (FAB): 167.2 (MH$^+$). Anal. (C$_7$H$_{10}$N$_4$O, 2HBr) C, H, N.

***N*-(4-Aminophenyl) *N*-Hydroxyguanidine Dihydrochloride (13).** Compound **13** was prepared as an off-white solid in 74% yield from 4-(*N-tert*-butyloxycarbonylamino)phenylcyanamide; mp >200 °C (dec). ^{1}H NMR (DMSO-*d*$_6$): δ 10.90 (br s, 1H), 10.10 (br s, 1H), 9.94 (br s, 3H), 7.93 (br s, 2H), 7.25 (m, 4H), 4.10 (br s, 3H). IR (KBr): 3320, 3040, 1660, 830. MS (CI): 167 (MH$^+$). Anal. (C$_7$H$_{10}$N$_4$O, 2HCl, 0.1MeOH) C, H, N.

***N*-(4-Nitrophenyl) *N*-Hydroxyguanidine (14).** Compound **14** was prepared as a yellow solid in 42% yield from (4-nitrophenyl)cyanamide;[50] mp 174 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 8.80 (br s, 1H), 8.66 (br s, 1H), 8.07 (d, 2H, *J* = 9.2), 7.39 (d, 2H, *J* = 9.2), 5.34 (br s, 2H). IR (KBr): 3400, 3350, 3050, 830. MS (CI): 197 (MH$^+$). Anal. (C$_7$H$_8$N$_4$O$_3$) C, H, N.

***N*-(3-Trifluoromethyl)phenyl *N*-Hydroxyguanidine (15).** Compound **15** was prepared as an off-white solid in 43% yield from (3-trifluoromethyl)phenylcyanamide; mp 92 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 8.55 (s, 1H), 8.03 (s, 1H), 7.88 (s, 1H), 7.35 (m, 2H), 7.05 (s, 1H), 5.15 (s, 2H). IR (KBr): 3460, 3350, 850, 760. MS (CI): 220 (MH$^+$). Anal. (C$_8$H$_8$N$_3$OF$_3$) C, H, N.

***N*-(4-Trifluoromethyl)phenyl *N*-Hydroxyguanidine (16).** Compound **16** was prepared as an off-white solid in 44% yield from (4-trifluoromethyl)phenylcyanamide; mp 142 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 8.56 (br s, 1H), 8.12 (br s, 1H), 7.44 (m, 4H), 5.19 (br s, 2H). IR (KBr): 3510, 3400, 3150, 830. MS (CI): 220 (MH$^+$). Anal. (C$_8$H$_8$N$_3$OF$_3$) C, H, N.

***N*-(3-Hydroxymethylphenyl) *N*-Hydroxyguanidine (17).** Compound **17** was prepared as an oil in 68% yield from (3-hydroxymethyl)phenylcyanamide. ^{1}H NMR (DMSO-*d*$_6$): δ 8.32 (br s, 1H), 7.51 (br s, 2H), 7.34 (s, 1H), 7.15−7.04 (m, 2H), 6.69 (d, 1H, *J* = 7.1), 5.03 (br s, 3H), 4.37 (s, 2H). IR (film): 3400, 2940, 1040, 860, 750. MS (FAB): 181.13 (M$^+$). HRMS calcd for C$_8$H$_{11}$N$_3$O$_2$, 181.1292; found, 181.1308.

***N*-(3-Aminomethylphenyl) *N*-Hydroxyguanidine Dihydrochloride (18).** Compound **18** was prepared as an oil in 53% yield from 3-(*N-tert*-butyloxycarbonyl aminomethyl)phenylcyanamide. ^{1}H NMR (DMSO-*d*$_6$): δ 10.94 (br s, 1H), 10.15 (br s, 2H), 8.53 (br s, 3H), 7.98 (br s, 2H), 7.47−7.33 (m, 3H), 7.20 (m, 1H), 4.01 (s, 2H). IR (film): 3470, 3170, 850, 760. MS (CI): 181 (MH$^+$). HRMS calcd for C$_8$H$_{13}$N$_4$O, 181.1089; found, 181.1092.

4-(*N*-Hydroxyguanidino)phenyl-acetic Acid Hydrochloride (19).[51] This compound was obtained as a white solid in 30% overall yield from 4-aminophenyl-acetic acid as previously described; mp 160 °C (literature: 160 °C).[51]

4-(*N*-Hydroxyguanidino)phenoxy-acetic Acid Hydrochloride (20). Treatment of (4-cyanamidinophenoxy)-acetic acid *tert*-butyl ester with hydroxylamine hydrochloride under the general conditions afforded 4-(*N*-hydroxyguanidinophenoxy)-acetic acid *tert*-butyl ester as an oil in 51% yield. ^{1}H NMR (DMSO-*d*$_6$): δ 8.21 (br s, 1H), 7.33 (br s, 1H), 7.20 (d, 2H, *J* = 9.0), 6.71 (d, 2H, *J* = 9.0), 4.96 (br s, 2H), 4.50 (s, 2H), 1.41 (s, 9H). Treatment of this ester with anhydrous HCl in dioxane gave **20** as an off-white solid in a quantitative yield; mp 157 °C. ^{1}H NMR (DMSO-*d*$_6$): δ 13.03 (br s, 1H), 10.67 (br s, 1H), 9.98 (br s, 1H), 9.68 (br s, 1H), 7.14 (d, 2H, *J* = 8.9), 6.96 (d, 2H, *J* = 8.9), 4.67 (s, 2H). IR (KBr): 3450, 3330, 830. MS (CI): 226 (MH$^+$). Anal. (C$_9$H$_{11}$N$_3$O$_4$, HCl) C, H, N.

2-[4-(*N*-Hydroxyguanidino)phenyl] Propionic Acid Hydrochloride (21).[51] This compound was obtained as white

crystals in 25% overall yield from 2-(4-nitrophenyl) propionic acid as previously described;[51] mp 54 °C (literature: 54 °C).[51]

N-(6-Indazolyl) N-Hydroxyguanidine Dihydrochloride (22). This compound was prepared as a white solid in 49% yield from 1-(N-tert-butyloxycarbonyl)-6-cyanamidino-indazole after reaction with hydroxylamine hydrochloride and treatment with anhydrous HCl in dioxane; mp 215−216 °C (dec). ^{1}H NMR (DMSO-d_6): δ 10.90 (br s, 1H), 10.05 (br s, 1H), 8.15 (s, 1H), 7.95 (br s, 2H), 7.78 (d, 1H, $J = 8.5$), 7.36 (s, 1H), 6.95 (d, 1H, $J = 8.5$). IR (KBr): 3280, 3100, 850, 780. MS (FAB): 192.1 (MH$^+$). Anal. (C$_8$H$_9$N$_5$O, 2HCl): C, H; N: Calcd, 26.52; found, 25.97.

N-(3-Thienyl) N-Hydroxyguanidine Hydrochloride (23). 3-Aminothiophene was prepared by the hydrolysis of 2-carboxylic acid 3-aminothiophene methyl ester and decarboxylation in the presence of oxalic acid, following a previously described protocol.[52] Addition of cyanogen bromide following the general protocol gave 3-thienyl cyanamide that was immediately used in the next step without further purification in order to minimize polymerization. Reaction of 3-thienylcyanamide with hydroxylamine hydrochloride following the conditions of the general method gave the expected **23** as a brown powder in 9% overall yield; mp 141 °C. ^{1}H NMR (DMSO-d_6): δ 10.82 (br s, 1H), 10.07 (s, 1H), 9.92 (s, 1H), 7.98 (br s, 2H), 7.73 (s, 1H), 7.34 (s, 1H), 7.00 (s, 1H). ^{13}C NMR (D$_2$O): δ 160.9 (C=N), 134.05, 130.2 (CH), 127.64 (CH), 123.6 (CH). IR (KBr): 3390, 3120, 790. MS (CI): 158 (MH$^+$), 142 (MH$^+$-16). HRMS calcd for C$_5$H$_8$N$_3$OS, 158.0388; found, 158.0390.

N-Benzyl N-Hydroxyguanidine Hydrochloride (24). Compound **24** was obtained as a white solid in 21% yield from benzylcyanamide; mp 158−159 °C. ^{1}H NMR (DMSO-d_6): δ 10.52 (br s, 1H), 9.85 (br s, 1H), 8.36 (br s, 1H), 7.77 (br s, 2H), 7.33 (m, 5H), 4.40 (s, 2H). IR (KBr): 3150, 750, 700. MS (FAB): 166.2 (MH$^+$). HRMS calcd for C$_8$H$_{12}$N$_3$O, 166.0980; found, 166.0985. Anal. (C$_8$H$_{11}$N$_3$O, HCl, 0.2H$_2$O) C, H, N.

N-(2-Phenylethyl) N-Hydroxyguanidine Acetate (25). Compound **25** was obtained as a white solid in 46% yield from 2-phenylethyl-cyanamide; mp 94−96 °C (dec). ^{1}H NMR (DMSO-d_6): δ 7.25 (m, 5H), 5.80 (br s, 3H), 3.15 (m, 2H), 2.73 (t, 2H, $J = 6.7$), 1.87 (s, 3H). IR (KBr): 3150, 750, 700. MS (FAB): 180.2 (MH$^+$). HRMS calcd for C$_9$H$_{14}$N$_3$O, 180.1137; found, 180.1137. Anal. (C$_9$H$_{13}$N$_3$O, HCl) C, H, N.

2-Hydroxyamidino-1,2,3,4-tetrahydro*iso*quinoline (26).[53] Compound **26** was obtained as a white solid in 52% yield from N-cyano-1,2,3,4-tetrahydro*iso*quinoline; mp 120 °C. ^{1}H NMR (DMSO-d_6): δ 8.32 (br s, 1H), 7.17 (m, 4H), 5.29 (br s, 2H), 4.24 (s, 2H), 3.41 (t, 2H, $J = 5.8$), 2.83 (t, 2H, $J = 5.8$). IR (KBr): 3460, 3300, 2820, 750, 700. MS (EI): 191 (M$^+$). Anal. (C$_{10}$H$_{13}$N$_3$O) C, H, N.

N,N-Diphenyl N′-Hydroxyguanidine (27). Compound **27** was obtained as a white solid in 59% yield upon reaction of hydroxylamine hydrochloride with diphenylcarbodiimide,[54] following a previously described protocol;[55] mp 150−151 °C (literature: 151 °C).[55]

Acetamidoximes **28** and **29** were prepared by heating for 3 days under reflux the corresponding anilines in anhydrous ethanol in the presence of an excess of ethyl N-hydroxyace-timidate according to a previously described protocol.[56]

N-(4-Chlorophenyl)acetamidoxime (28). Compound **28** was obtained as an off-white solid in 26% yield from 4-chloroaniline; mp 132−133 °C. ^{1}H NMR (DMSO-d_6): mixture of E (70%) and Z (30%) isomers δ 9.56 (br s, 0.3H), 9.21 (br s, 0.7H), 8.16 (br s, 0.7H), 8.00 (br s, 0.3H), 7.50 (d, 1.4H, $J = 8.8$), 7.22 (m, 2H), 7.09 (d, 0.6H, $J = 8.8$), 1.95 (s, 2.1H), 1.85 (s, 0.9H). IR (KBr): 3380, 3200, 830. MS (EI): 186 and 184 (M$^+$). Anal. (C$_8$H$_9$N$_2$OCl) C, H, N.

N-(4-Methoxyphenyl)acetamidoxime (29). Compound **29** was obtained as a beige solid in 28% yield from 4-meth-oxyaniline; mp 146−148 °C. ^{1}H NMR (DMSO-d_6): mixture of E (30%) and Z (70%) isomers δ 9.34 (br s, 0.7H), 8.99 (br s, 0.3H), 7.82 (br s, 0.3H), 7.64 (br s, 0.7H), 7.46 (d, 0.6H, $J = 6.9$), 7.08 (d, 1.4H, $J = 6.8$), 6.91 (d, 1.4H, $J = 6.8$), 6.82 (d, 0.6H, $J = 6.9$), 3.77 (s, 2.1H), 3.73 (s, 0.9H), 1.99 (s, 0.9H),

1.78 (s, 2.1H). IR (KBr): 3390, 3250, 840. MS (EI): 180 (M$^+$). Anal. (C$_9$H$_{12}$N$_2$O$_2$) C, H, N.

Benzamidoximes **30−32** were prepared by refluxing anhydrous methanolic solutions of hydroxylamine hydrochloride with the corresponding nitrile in the presence of sodium carbonate as previously described.[57]

Benzamidoxime (30). Compound **30** was obtained as a white solid in 75% yield from benzonitrile; mp 76 °C (literature: 76 °C).[57]

4-Chlorobenzamidoxime (31). Compound **31** was obtained as a white solid in 85% yield from 4-chlorobenzonitrile; mp 133−134 °C (literature: 134−135 °C).[57]

4-Methoxybenzamidoxime (32). Compound **32** was obtained as a white solid in 65% yield from 4-methoxybenzoni-trile; mp 121 °C (literature: 122−123 °C).[57]

N-(4-Chlorophenylazo)formamidoxime (33). This compound was prepared as a yellow solid in 35% yield following a previously described protocol.[58] The reaction of 4-chloroaniline with sodium nitrite in hydrochloric acid was followed by the addition of potassium cyanide and the immediate reaction of intermediate N-(4-chlorophenylazo)nitrile with hydroxylamine hydrochloride in the presence of sodium hydroxide; mp 218 °C (literature: 214 °C).[58]

Ketoximes **34** and **35** and aldoximes **36** and **37** were prepared following conventional methods by reacting hydroxylamine hydrochloride with the corresponding acetophenone or benzaldehyde in aqueous ethanol.[59]

4-Chloroacetophenone Oxime (34). Compound **34** was obtained as a white solid in 75% yield from 4-chloroacetophe-none; mp 94 °C (literature: 95 °C).[59]

4-Methoxyacetophenone Oxime (35). Compound **35** was obtained as a white solid in 65% yield from 4-methoxyace-tophenone; mp 86 °C (literature: 87 °C).[59]

4-Chlorobenzaldoxime (36). Compound **36** was obtained as a white solid in 68% yield from 4-chlorobenzaldehyde; mp 109 °C (literature: 107 °C).[59]

4-Methoxybenzaldoxime (37). Compound **37** was obtained as a white solid in 78% yield from 4-methoxybenzal-dehyde; mp 131 °C (literature: 132 °C).[59]

Biology. BH$_4$ was purchased from Alexis (Coger, Paris, France). NADPH came from Boehringer Mannheim Biochemicals. L-Arg, L-citrulline, NO$_2$-L-Arg, SEITU, bovine erythrocyte SOD, bovine liver catalase, bovine hemoglobin, bovine serum albumin, and porcine brain CaM were purchased from Sigma. Recombinant NOS I was isolated and purified from the yeast *Saccharomyces cerevisiae* transformed with a plasmid containing rat brain NOS I as previously described.[12] Recombinant NOS II and III were expressed in *Escherichia coli* and purified as described previously.[60,61] Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard and the Bradford reagent from Bio-Rad.[62] They were estimated to be more than 90% pure by sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

Hemoglobin Assay and Measurement of NO$_2^-$ and NO$_3^-$ Formation. The initial rate of NO synthesis was determined at 37 °C using the classical spectrophotometric oxyhemoglobin assay for NO.[37,38] Briefly, 5−10 μL aliquots containing NOS were added to a prewarmed cuvette that contained 50 mM HEPES (N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid) buffer, pH 7.4, supplemented with 5 mM dithiothreitol (DTT), 10−15 μM oxyhemoglobin, 100 units/mL SOD, 100 units/mL catalase, 1 mM NADPH, 100 μM BH$_4$, and substrate at the desired concentration to give a final volume of 150 μL. The reference cuvette had the same composition, except that 50 mM HEPES was added instead of NOS-containing solutions. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an increase in the absorbance at 401 nm and quantitated using an extinction coefficient of 34 mM^{-1} cm^{-1}.[37,38] In the cases of compounds **14** and **33**, which strongly absorb in the 400 nm range, the NO-mediated consumption of oxyhemoglobin was monitored over time as a decrease in the absorbance at 577 nm and quantitated using an extinction coefficient of 14.5 mM^{-1} cm^{-1}.[63] Measurements of NO$_2^-$ and NO$_3^-$ were per-

formed following a described colorimetric method using the Griess reagent [sulfanilamide 1% in 0.5 N HCl and *N*-(1-naphthyl)ethylenediamine 0.1% in 0.5 N HCl].[64] When nitrate determination was necessary, reduction of nitrate to nitrite was performed in the presence of *Aspergillus niger* nitrate reductase and a NADPH-regenerating system, following a previously described protocol.[65]

Identification of *N*-Aryl Ureas Derived from Compounds 1, 2, 5, 7, 9, and 11. In the last step of the preparation of the *N*-aryl *N'*-hydroxyguanidines **1**–**23**, the addition of NH$_2$OH, HCl to the cyanamide precursor in anhydrous ethanol, small amounts of the corresponding *N*-aryl ureas were formed. These ureas were easily separated from the *N'*-hydroxyguanidines, which are much more polar and water soluble, as described previously.[32] The *N*-aryl ureas corresponding to **1**, **2**, **5**, **7**, **9**, and **11** exhibited characteristics identical to those of commercially available ureas (deriving from **1**, **2**, **5**, **7**, and **11**) or of the previously described *N*-aryl urea derived from **9**.[66] Those *N*-aryl ureas were used as authentic samples in the HPLC analyses of the reaction mixtures coming from the reactions of **1**, **2**, **5**, **7**, **9**, and **11** with NOS II.

Reverse-Phase HPLC Identification and Quantitation of Metabolites from NOS II-Dependent Oxidation of *N*-Aryl *N'*-Hydroxyguanidines.[31] Separations of metabolites from *N*-aryl *N'*-hydroxyguanidines were performed at 25 °C on a 250 mm × 4.6 mm Nucleosil ODS 5 mm column (SFCC-Shandon, France) as previously described.[31] The flow rate was 1 mL/min, and the mobile phases were gradients between solvent A (water containing 5 mM phosphoric acid, pH 2.6) and solvent B (acetonitrile) following the general program: 0 min, 10% B; 5 min, linear gradient to 40% B in 15 min; 20 min, linear gradient to 100% B in 10 min; 30 min, linear gradient to 10% B in 5 min followed by 15 min reequilibration. The absorbance was monitored at 240 nm. Calibration curves were made from identical mixtures containing various concentrations of *N*-aryl *N'*-hydroxyguanidine, *N*-aryl urea, *N*-aryl cyanamide, and all the required cofactors but without NOS II.

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