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N-Hydroxyl Derivatives of Guanidine Based Drugs as Enzymatic NO Donors

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Abstract—Recent research suggests that NO may play a role in the physiological effects of some guanidine-containing drugs. In this report, three guanidine-containing drugs (guanadrel, guanoxan, and guanethidine) together with their *N*-hydroxyl derivatives were synthesized and their NO-releasing abilities catalyzed by nitric oxide synthases (NOSs) and horseradish peroxidase were evaluated. The guanidine containing compounds could not release NO in the presence of NOS or peroxidase. The corresponding *N*-hydroxyl compounds exhibited weak NO-releasing ability under the catalyzed of NOS and good NO-releasing ability under the oxidation by horseradish peroxidase in the presence of H_2O_2. These compounds also displayed vasodilatory activity. © 2001 Elsevier Science Ltd. All rights reserved.

Nitric oxide (NO) is a small gaseous molecule that serves as a mediator of many physiological events.[1,2] The synthesis of NO in vivo is catalyzed by neuronal, inducible, and endothelial isoforms of nitric oxide synthase (NOS) which use L-arginine as the substrate.[3–6] So far, only a few compounds have been clearly shown to act as NOS substrates with the formation of NO.[7–10] Those substrates include L-arginine, hydroxy-L-arginine, homo-L-arginine, and *N*-hydroxy-homo-L-arginine. Other L-arginine analogues, such as canavanine,[11] ε-guanidino-carproic acid,[11] agmatine,[9] *N*-hydroxy-agmatine,[12] and L-tyrosyl-L-arginine,[13] have been proposed to be NOS substrates because they caused endothelium-dependent vasorelaxation or accumulation of nitrite in cell cultures. However, only some of these results have been confirmed with purified NOSs and the precise nature of the transformations of these compounds is not yet known.[14]

Since all the known substrates of NOS have the functional guanidine groups, it would be helpful to search other guanidine containing compounds as substrates of NOSs which could be used as NO donor drugs in vivo. In fact, numerous natural and non-natural guanidine-containing compounds have had a significant impact on the medicinal chemistry.[15,16] Moreover, many of them have shown unprecedented activities ranging from antimicrobial, antiviral, antifungal, to neurotoxic, making these compounds and their derivatives clear targets for drug design and discovery.[17,18] Guandrel **1** and guanethidine **3** are two established anti-hypertensive drugs, while guanoxan **2** is used as a cardiovascular drug. Recent research suggested that NO might play a role in their physiological effects.[19–21] We hypothesized that if the bioactivities of these drugs were related to NO releasing catalyzed by some enzymes like NOS in vivo, their metabolic intermediates should be corresponding *N*-hydroxyguanidine compounds. Those *N*-hydroxyguanidine derivatives should be similar or even better NO donors than the parent compounds, because some non-physiological *N*-hydroxyguanidines have been demonstrated to produce NO by the catalysis of NOS or other enzyme such as cytochrome P450.[10,22] In this study, the enzymatic controlled NO-releasing abilities of three drugs (**1**–**3**) and their *N*-hydroxyl derivatives (**4**–**6**) by NOS and horseradish peroxidase were evaluated. Furthermore, the vasodilatory efficacy of **4**–**6** was measured (Fig. 1).

The procedure to synthesize compounds **1** and **4** is shown in Scheme 1. Compound **10** was obtained from 3-amino-1,2-propanediol **7** by Goodman's procedure.[23]

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We improved the yield of **9** by modifying the procedure. Instead of mixing **8** and two and a half equivalents of cyclohexanone in a mixture of methanol at one time,[23] we first dissolved **8** and one and a half equivalents of cyclohexanone in methanol and toluene. The methanol was removed after the mixture was refluxing for 5 h. Then an additional one equivalent of cyclohexanone was added, and the mixture was refluxed for another 7 h. The yield of **9** was increased to 80%. Compound **4**[24] was prepared by the reaction of cyanamide **11**[24] with hydroxyamine. Compound **1**[24] was obtained from the reaction of **11** with ammonium chloride in aqueous ammonia.

Guanethidine **3** is commercially available. The synthetic routes for **2**, **5**, and **6** are illustrated in Schemes 2 and 3. Compound **14** was prepared from *ortho*-diphenol **12**.[31] Compounds **5**[24] and **6**[24] were obtained from amines **15**[24] and **18**[24] by a similar procedure for the synthesis of **4**. Guanoxan **2**[24] was synthesized through compound **15** using Goodman's reagent.[25]

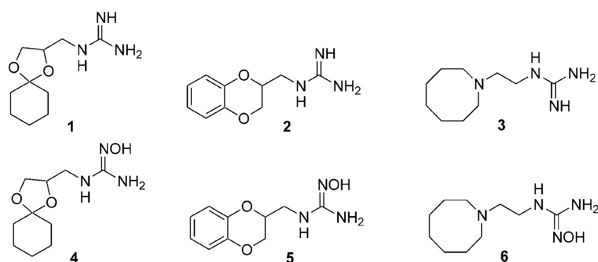
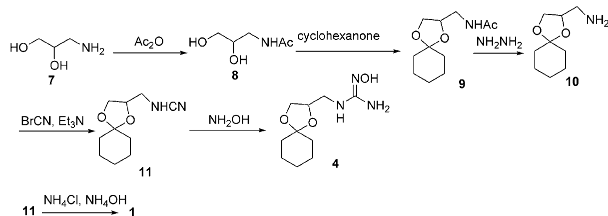
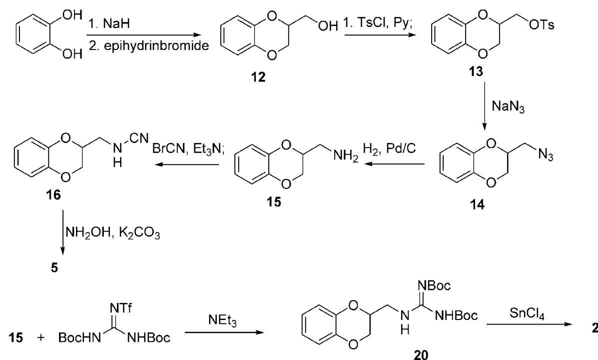


Figure 1. The guanidine-containing drugs: guanadrel **1**, guanoxan **2**, guanethidine **3**, and their hydroxyguanidine derivatives (**4**–**6**).



Scheme 1.



Scheme 2.

Biological Evaluation

The NO releasing abilities of compounds **1**–**6** were measured through the formation of nitrite (NO_2^-) in the solution, which was followed upon incubation of each compound with nNOS or iNOS under the usual conditions for NOS activity measurements,[26] with L-arginine as a control. The results are summarized in Table 1. The three *N*-hydroxy compounds **4**–**6** led to weak formation of nitrite. Their NO releasing abilities were much worse than L-arginine. Compound **4** was the best among these three compounds. However, its NO releasing ability was only about 8 and 11% of L-arginine for nNOS and iNOS, respectively. The abilities of **5** and **6** were less than 7% of L-arginine. For compounds **1**, **2**, and **3**, no detectable nitrite could be detected in the system. These data indicated that hydroxyguanidine-containing drugs might be weak substrates of NOS, whereas guanidine-containing drugs were not substrates.

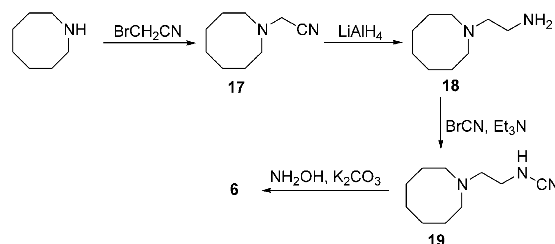
Since compound **4** was the best NO donor in this series, the effect of incubation condition on the NO release of **4** with NOS was studied next. The results are shown in Table 2. The data obtained clearly indicated that the formation of NO_2^- from the *N*-hydroxyguanidine was an enzymatic process, as it required the presence of active NOS and other cofactors such as NADPH and

Table 1. NO release abilities of guanidine drugs compared with L-arginine[26]

Substrate	$[NO_2^-]$/with nNOS (μM)	$[NO_2^-]$/with iNOS (μM)
L-Arg	14.5	14
1	<0.1	<0.1
2	<0.1	<0.1
3	<0.1	<0.1
4	1.2	1.6
5	0.8	0.9
6	0.3	0.2

Table 2. Effect of incubation conditions on the formation of NO_2^- from the oxidation of compounds **4** in the presence of nNOS, NADPH and O_2

Conditions	% Residual activity
Complete system	100
− nNOS	<2
− NADPH	18
− BH_4	30
+ NO_2-L-Arg	50



Scheme 3.

BH_4. The oxidation of **4** to NO should occur in the active site of NOS since it was strongly inhibited by classical inhibitor of NOS like *N*-nitro-L-arginine.

The second step involved in the NOS-catalyzed oxidation of L-arginine is an oxidative cleavage of the C=N–OH bond of *N*-hydroxyarginine with the formation of citrulline and NO. Besides NOS, cytochromes P450 have also been found to catalyze the oxidative cleavage of the C=N–OH bond not only of *N*-hydroxyarginine but also of many other compounds such as ketoximes, amidoximes.[27] In this study, we found compounds **4–6** could be effectively catalyzed by peroxidase with the formation of NO. Horseradish peroxidase has been shown to be an effective oxidzing enzyme for cupferron and other compounds with the hydroxynitrosamine moiety.[28] This peroxidase affects a one-electron oxidation of the substrate and is regenerated by transferring two electrons to a molecule of H_2O_2. Compounds **4–6** together with NHA and L-arginine were incubated with horseradish peroxidase and H_2O_2 in our study. Twenty minutes later, the concentration of NO_2^- generated in the solution was determined by Griess method.[29] The results were shown in Table 3.

As we expected, incubation of the L-arginine with peroxidase and H_2O_2 did not result in the generation of nitrite. The results for other guanidine-containing drugs (**1–3**) were the same as for L-arginine. However, incubation of corresponding *N*-hydroxyguanidines with the peroxidase and H_2O_2 produced different amount of nitrite. Compound **4** was the best NO donor among these tested compounds, it produced 65 μM nitrite in 20 min, followed by **5** (60 μM), NHA (12 μM), and **6** (10 μM). The different value of nitrite generated from these compounds may reflect the different binding affinity to the enzyme. In the control experiments, incubation of these compounds along with H_2O_2 did not produce any nitrite. These data clearly show that *N*-hydroxyguanidine derivatives could be considered as potential NO donors under the catalysis of peroxidase.

Table 3. Nitrite concentration in the reaction of substrate with peroxidase for 20 min (substrate: 600 μM, peroxidase: 80 μg/mL, H_2O_2: 600 μM)[30]

Substrate	$[NO_2^-]$ μM
L-Arg	0
NHA	12
4	65
5	60
6	10

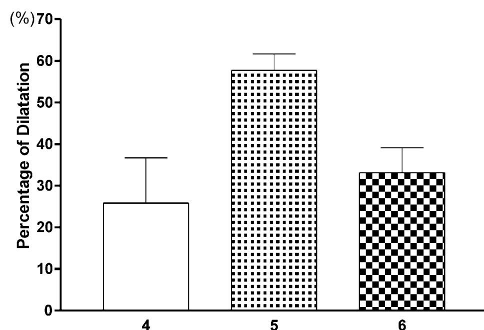


Figure 2. Vasodilatory efficacy of compounds **4–6**.

In a previous study,[21] it was shown that some guanidine-containing drugs like guanethidine evoked vasodilatation in isolated guinea pig mesenteric artery. In this study, the vasodilatory efficacy of three *N*-hydroxyguanidine-containing drugs (**4–6**) was measured. In precontracted, endothelium-free guinea pig mesenteric artery, which was sympathectomized by preincubation with guanethidine (30 μM) for 30 min, we measured the vasodilatation produced by **4–6** (30 μM) using computer-assisted video-microscopy.[21,32] The vasodilatation produced by each of the compounds was compared to that produced by sodium nitroprusside, which produced 100% relaxation of the blood vessels. All of them produced vasodilation in the range from 26.8 to 57.7% (Fig. 2). Compound **5** is the most effective dilator. This result confirms that hydroxyguanidine-containing compounds produce vasodilatation by through a mechanism that involves periarterial sensory nerves.

In summary, the results reported here show that NOS could catalyze the oxidation of some *N*-hydroxy guanidine-containing drugs with the formation of NO. But their NO generation ability was much worse than L-arginine. It indicates that highly specific structural features are required for NOS substrates. Our results also show that *N*-hydroxyguanidine compounds are substrates for horseradish peroxidase and thereby are potential NO donors in the existence of this enzyme. Such enzymatic controlled NO generating compounds could have potential applications in a variety of biomedical fields. The vasodilatory efficacy of them has been demonstrated in this study.

Acknowledgements

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References and Notes

1. Ignarro, L.; Murad, F. *Nitric Oxide: Biochemistry, Molecular Biology, and Therapeutic Implications*; Academic: San Diego, CA, 1995.
2. Lancaster, J. *Nitric Oxide: Principles and Actions*; Academic: San Diego, CA, 1996.
3. Knowles, R. G.; Moncada, S. *Biochem. J.* **1994**, *298*, 249.
4. Masters, B. S.; McMillan, K.; Sheta, E. A.; Nishimura, J. S.; Roman, L. J.; Martasek, P. *FASEB J.* **1996**, *10*, 552.
5. Klatt, P.; Schmidt, K.; Uray, G.; Mayer, B. *J. Biol. Chem.* **1993**, *268*, 14781.
6. Abu-Soud, H. M.; Presta, A.; Mayer, B.; Stuehr, D. J. *Biochemistry* **1997**, *36*, 10811.
7. Stuehr, D. J.; Griffith, O. W. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1992**, *65*, 287.
8. Moali, C.; Boucher, J. L.; Sari, M. A.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **1998**, *37*, 10453.

9. Grant, S. K.; Green, B. G.; Stiffey-Wilusz, J.; Durette, P. L.; Shah, S. K.; Kozarich, J. W. *Biochemistry* **1998**, *37*, 4174.
10. Renodon-Corniere, A.; Boucher, J. L.; Dijols, S.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **1999**, *38*, 4663.
11. Thomas, G.; Ramwell, P. W. *J. Pharm. Exp. Ther.* **1991**, *259*, 490.
12. Ishikawa, T.; Misonou, T.; Ikeno, M.; Sato, K.; Sakamaki, T. *Biochem. Biophys. Res. Commun.* **1995**, *214*, 145.
13. Arima, T.; Kitamura, Y.; Nishiya, T.; Takagi, H.; Nomura, Y. *Neurosci. Lett.* **1996**, *212*, 1.
14. Arima, T.; Kitamura, Y.; Nishiya, T.; Taniguchi, T.; Takagi, H.; Nomura, Y. *Neurochem. Int.* **1997**, *30*, 605.
15. Davidoff, F. *N. Engl. J. Med.* **1973**, *289*, 141.
16. Durant, G. J.; Roe, A. M.; Green, A. L. *Prog. Med. Chem.* **1970**, *7*, 124.
17. Berlinck, R. G. S. *Nat. Prod. Rep.* **1996**, *13*, 377.
18. Berlinck, R. G. S. *Nat. Prod. Rep.* **1999**, *16*, 339.
19. Hogikyan, R. V.; Supiano, M. A. *Am. J. Physiol.* **1994**, *266*, E717.
20. Park, J. I.; Shin, C. Y.; Lee, Y. W.; Huh, I. H.; Sohn, U. D. *J. Pharm. Pharmcol.* **2000**, *52*, 409.
21. Zheng, Z. L.; Shimamura, K.; Anthony, T. L.; Kreulen, D. L. *Neurosci. Lett.* **2000**, *288*, 231.
22. Clement, B.; Boucher, J. L.; Mansuy, D.; Harsdorf, A. *Biochem. Pharm* **1999**, *58*, 439.
23. Baker, T. J.; Goodman, M. *Synthesis* **1999**, 1423.
24. Compound **4**: ^{1}H NMR (400 MHz, CD_3OD) δ 4.17–4.13 (m, 1H), 4.00 (dd, J=8.4, 6.4 Hz, 1H) 3.64 (dd, J=8.0, 6.4 Hz 1H), 3.31–3.27 (m, 1H), 3.22–3.18 (m, 1H), 1.61–1.40 (m, 10H); ^{13}C NMR (100 MHz, CD_3OD) δ 161.0, 109.7, 75.0, 66.3, 42.2, 36.4, 34.7, 25.1, 23.9, 23.7; EI-MS 230 (M^++1), 229 (M^+). Compound **1**: ^{1}H NMR (500 MHz, CD_3OD) δ 4.64 (bs, 5H), 4.28–4.25 (m, 1H), 4.09 (dd, J=8.5, 6.5 Hz, 1H), 3.68 (dd, J=8.5, 6.0 Hz, 1H), 3.45 (dd, J=14.5, 3.5 Hz, 1H), 3.25 (dd, J=14.5, 6.0 Hz, 1H), 1.64–1.41 (m, 10H); HR-MS calcd for $C_{10}H_{19}N_3O_2$ 213.1477, found 213.1480. Compound **2**: ^{1}H NMR (400 MHz, CD_3OD) δ 6.81–6.89 (m, 4H), 4.30–4.36 (m, 2H), 4.00 (dd, J=12.4, 7.2 Hz, 1H), 3.58 (dd, J=14.4, 4.0 Hz, 1H), 3.48 (dd, J=14.4, 7.2 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.9, 143.2, 142.6, 121.6, 121.5, 117.2, 117.0, 71.7, 65.2, 41.7. Compound **5**: ^{1}H NMR (400 MHz, CD_3OD) δ 6.81–6.88 (m, 4H), 4.31–4.35 (m, 2H), 3.99 (dd, J=12, 8 Hz, 1H), 3.50–3.59 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.6, 143.3, 142.7, 121.6, 121.5, 117.2, 117.0, 71.7, 65.3, 41.4. EI-MS 224 (M^++1). Compound **6**: ^{1}H NMR (400 MHz, CD_3OD) δ 3.07 (t, J=6 Hz, 2H), 2.58–2.61 (m, 6H), 1.59–1.66 (m, 10H); ^{13}C NMR (100 MHz, CD_3OD) δ 157.9, 57.1, 54.0, 39.4, 27.8, 27.3, 26.2. EI-MS 215 (M^++1). Compound **11**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.17–4.12 (m, 1H), 3.96 (dd, J=9.0, 6.5 Hz, 1H), 3.66–3.63 (m, 1H), 3.08 (dd, J=13.5, 4.5 Hz, 1H), 3.01 (dd, J=13.5, 6.0 Hz, 1H), 1.56–1.30 (m, 10H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 116.2, 110.8, 73.8, 66.1, 48.9, 36.7, 34.7, 25.2, 24.2, 23.9; HR-MS calcd for $C_{10}H_{16}N_2O_2$ 196.1212, found 196.1211. Compound **14**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86–6.90 (m, 4H), 4.32–4.37 (m, 2H), 4.07 (dd, J=12, 6.8 Hz, 1H), 3.43–3.59 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.1, 142.6, 122.2, 122.0, 117.7, 117.5, 72.2, 65.5, 50.9. HR-MS calcd for $C_9H_9N_3O_2$ 191.0695, found 191.0694. Compound **15**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.80–6.88 (m, 4H), 4.24 (dd, J=11.1, 2.1 Hz, 1H), 4.08–4.12 (m, 1H), 3.98 (dd, J=11.1, 7.5 Hz, 1H), 2.94–2.96, (m, 2H), 1.49 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.4, 121.8, 121.6, 117.5, 117.3, 74.7, 66.3, 42.8. HR-MS calcd for $C_9H_{11}NO_2$ 165.0790, found 165.0789. Compound **18**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.64 (t, J=5.6 Hz, 2H), 2.42–2.47 (m, 4H), 1.79 (s, 2H), 1.49–1.58 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 62.3, 54.8, 40.4, 28.4, 27.8, 26.3. Compund **19**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.11 (t, J=5.6 Hz, 2H), 2.58 (t, J=5.6 Hz, 2H), 2.51 (t, J=5.2 Hz, 4H), 1.54 (b, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 117.0, 57.8, 54.6, 44.5, 28.1, 27.8, 26.2. CIMS 182 (M^++1).
25. Baker, T. J.; Goodman, M. *Synthesis* **1999**, 1423.
26. The enzymatic reaction was carried out in 50 mM HEPES buffer (pH 7.4). Incubated mixtures containing 1 mM substrate, 100 μM NADPH, 4 μM FAD, 4 μM FMN, 6 μM BH_4, 400 μM DTT, 100 units SOD, 50 units Catalase and 0.5 unit of NOS in a 500 μL final volume were shaken at 37 °C for the indicated time. For nNOS, 1 mM $CaCl_2$ and 10 μg/mL CaM were also added. For iNOS, 1 mM magnesium acetate was added. Reactions were quenched with 100 μL of ethanol and the mixtures allowed to stay at room temperature for at least 1 h before addition of the Griess reagent [150 μL of 1% sulfanilamide in 1 N HCl and 150 μL of 0.1% *N*-(1-naphthyl)ethylenediamine in 1 N HCl]. Absorbances were measured at 548 nm. Calibration curves were made from identical incubated mixtures without NOS and containing various concentration of $NaNO_2$ to properly determine the amounts of NO_2^- formed in the enzymatic reactions.
27. Jousserandot, A.; Boucher, J. J.; Henry, Y.; Niklaus, B.; Clement, B.; Mansuy, D. *Biochemistry* **1998**, *37*, 17179.
28. George, M. V.; Kierstead, R. W.; Wright, G. F. *Can. J. Chem.* **1959**, *37*, 679.
29. Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **1982**, *126*, 131.
30. This enzymatic reaction was carried out in PBS buffer (pH 7.4) with 1 mM EDTA. Incubated mixtures containing 0.6 mM substrate, 0.6 mM H_2O_2, and 80 μg/mL horseradish peroxidase in a 500 μL final volume were shaken at 25 °C for 20 min. The concentration of NO_2^- generated in the system was determined by addition of the Griess reagent. Absorbances were measured at 548 nm. Calibration curves were made from identical incubated mixtures without the enzyme and containing various concentration of $NaNO_2$ to properly determine the amounts of NO_2^- formed in the enzymatic reaction.
31. Marciniak, G.; Delgado, A.; Leclerc, G.; Velly, J.; Decker, J.; Schwartz, J. *J. Med. Chem.* **1989**, *32*, 1402.
32. Galligan, J. J.; Hess, M. C.; Miller, S. B.; Fink, G. D. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 478.