Bioorganic & Medicinal Chemistry Letters 19 (2009) 1758–1762

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# 4,4-Difluorinated analogues of L-arginine and $N^G$-hydroxy-L-arginine as mechanistic probes for nitric oxide synthase

Nathaniel I. Martin [a], Joshua J. Woodward [b], Michael B. Winter [b], Michael A. Marletta [b,c,d,*]

[a] Department of Medicinal Chemistry and Chemical Biology, University of Utrecht, 3584 CA, The Netherlands
[b] Department of Chemistry, University of California, 556 Stanley Hall, Berkeley, CA 94720-3220, USA
[c] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA
[d] Division of Physical Biosciences, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

ARTICLE INFO

*Article history:*
Received 26 December 2008
Revised 21 January 2009
Accepted 22 January 2009
Available online 18 February 2009

*Keywords:*
Nitric oxide synthase
Fluorinated substrate analogues
Mechanistic probes

ABSTRACT

4,4-Difluoro-L-arginine and 4,4-difluoro-$N^G$-hydroxy-L-arginine were synthesized and shown to be substrates for the inducible isoform of nitric oxide synthase (iNOS). Binding of both fluorinated analogues to the NOS active site was also investigated using a spectral binding assay employing a heme domain construct of the inducible NOS isoform ($iNOS_{heme}$). 4,4-Difluoro-$N^G$-hydroxy-arginine was found to bind at the NOS active site in a unique manner consistent with a model involving ligation of the $Fe^{III}$ heme center by the oxygen atom of the $N^G$-hydroxy moiety.



The nitric oxide synthases (NOSs) carry out a complex series of redox reactions that convert L-arginine to NO, an important signaling molecule involved in a wide range of biological functions.[1–3] NOSs are comprised of both a reductase domain (binding NADPH, FAD, and FMN) and a catalytic oxygenase domain containing the heme prosthetic group. The NOS substrate L-arginine, and the essential redox cofactor (6*R*)-5,6,7,8 tetrahydro-L-biopterin ($H_4B$) also bind in the oxygenase domain with a specific orientation relative to the heme.[4] The three NOS isoforms identified in various mammalian cells (neuronal, inducible, and endothelial NOS) all catalyze the oxidation of L-arginine to L-citrulline and NO via the intermediate $N^G$-hydroxy-L-arginine (NHA) (Fig. 1).[2,3]

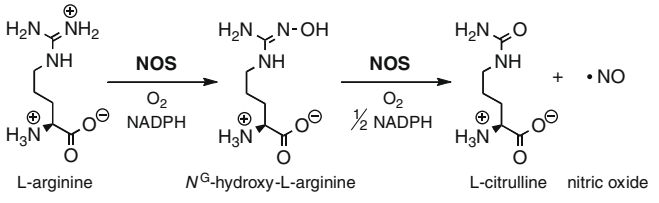



**Figure 1.** Two-step reaction catalyzed by NOS.

While physiological signaling roles for NO are well established, a thorough understanding of NO biosynthesis including the mechanistic details by which NOS performs its unique oxidative chemistry remains to be fully elucidated.[5,6] The first NOS reaction, in which L-arginine is oxidized to $N^G$-hydroxy-L-arginine, is often considered to proceed via an oxygen-rebound mechanism analogous to that of the cytochrome P450-type enzymes[7–9] (Silverman and co-workers have recently suggested an alternate mechanistic explanation for this step).[6] The second NOS reaction, however, is without enzymatic precedent. Requiring the net input of a single electron, NOS converts a $N^G$-hydroxyguanidine into a urea and NO. While the precise mechanism of the second NOS reaction remains unclear, a proposed[10,11] explanation involves nucleophilic addition of a $Fe^{III}$-peroxo species to $N^G$-hydroxy-L-arginine forming a tetrahedral intermediate. Collapse of this intermediate then leads to formation of L-citrulline and NO, along with one-electron reduction of the tetrahydro-L-biopterin cofactor, returning NOS to its resting state (Fig. 2). Experimental approaches to investigating the NOS reaction mechanism(s) have most often relied upon mutagenesis of the enzyme,[12–14] the use of structurally altered substrates,[6,15–20] and electron paramagnetic resonance (EPR) spectroscopy to identify radical reaction intermediates.[1,21,22] Given that fluorinated substrates have been successfully used to probe the mechanistic properties of numerous other enzymes[23–25] we were interested to see what effects substrate fluorination might have with NOS. While fluorinated analogues of L-lysine have been reported as moderately selective inhibitors for iNOS,[26] the effect of fluorination of the native substrates L-arginine and $N^G$-hydroxy-L-arginine (Fig. 3) on NOS catalysis has not been thoroughly investigated. In this regard, we recently reported the unique product distribution obtained when reacting a fluorinated substrate ana-

* Corresponding author. Tel.: +1 510 666 2763; fax: +1 510 666 2764.
*E-mail address:* marletta@berkeley.edu (M.A. Marletta).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.01.076

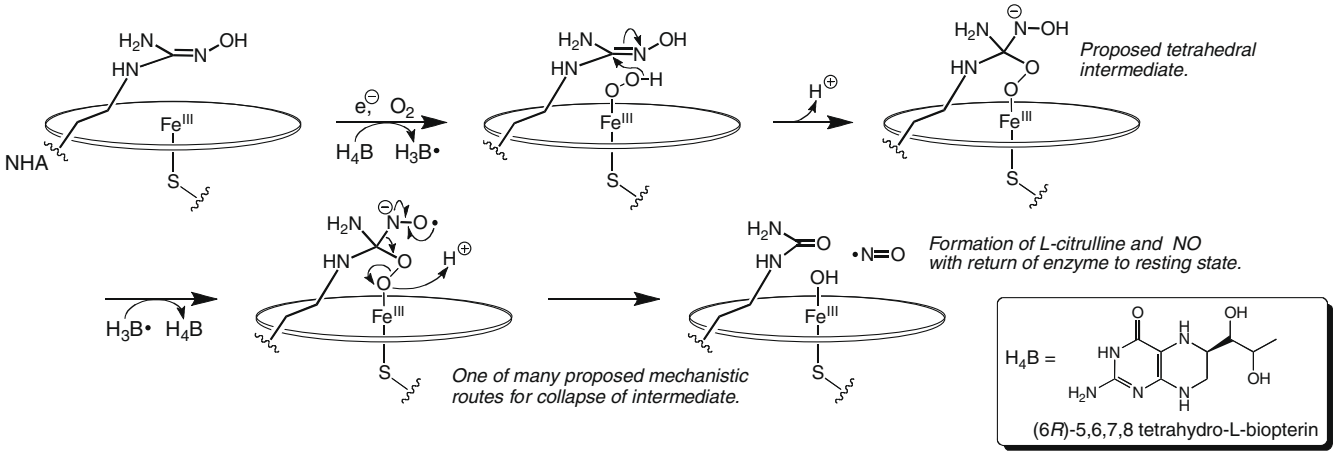



**Figure 2.** Proposed mechanism for the oxidation of $N^G$-hydroxy-L-arginine to L-citrulline and NO.

logue with the iNOS catalytic domain ($iNOS_{heme}$) in the presence of hydrogen peroxide.[14] We here further describe the syntheses of 4,4-difluoro-L-arginine **1** and 4,4-difluoro-$N^G$-hydroxy-L-arginine **2** and the effects observed in the interaction of both analogues with full-length iNOS.

The synthesis of 4,4-difluoro-L-arginine **1** (Scheme 1) was accomplished in a manner similar to that previously described by Kim and co-workers.[27] Incorporation of the $C_4$ difluoromethylene unit was accomplished via the zinc mediated addition of ethyl bromodifluoroacetate to the appropriately protected Garner aldehyde. Standard functional group manipulations were then employed to elaborate the amino acid side chain and install the guanidine moiety prior to global deprotection. 4,4-Difluoro-L-arginine **1** (12 linear steps overall yield 23%) was then tested as a substrate for iNOS. Incubation of full-length iNOS with **1**, $H_4B$, and NADPH, followed by a LC–MS-based amino acid product assay, showed that the difluorinated analogue was converted into the same pattern of products (the intermediate $N^G$-hydroxy-L-arginine and L-citrulline) as the native substrate L-arginine (data provided in the accompanying supplemental information section, see Supplemental Figure 1). While other NOS substrate analogues have previously been found to undergo alternate transformations when acted upon by NOS,[18,28,29] the difluoro analogue remains 'on path' in the enzyme reaction, an important consideration in establishing the validity of such compounds as representative mechanistic probes.

Once 4,4-difluoro-L-arginine had been confirmed as a mechanistically representative analogue of L-arginine, the $N^G$-hydroxylated intermediate analogue, 4,4-difluoro-$N^G$-hydroxy-L-arginine **2**, was pursued so as to permit independent study of the two discrete NOS reaction steps. The synthesis of the $N^G$-hydroxylated difluoro analogue, however, was significantly more challenging than that of the non-hydroxylated species and required the development of a new methodology for incorporation of the $N^G$-hydroxyguanidine moiety.[30] In the manner shown in Scheme 2, 4,4-difluoro-$N^G$-hydroxy-L-arginine was ultimately prepared in 16 linear steps with an overall yield of 23%.

With **1** and **2** in hand, the NOS activity for both fluorinated analogues was assessed using an indirect spectral assay making use of

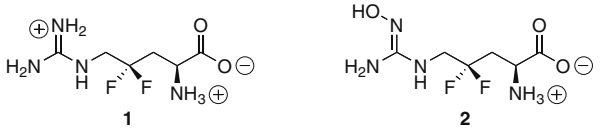
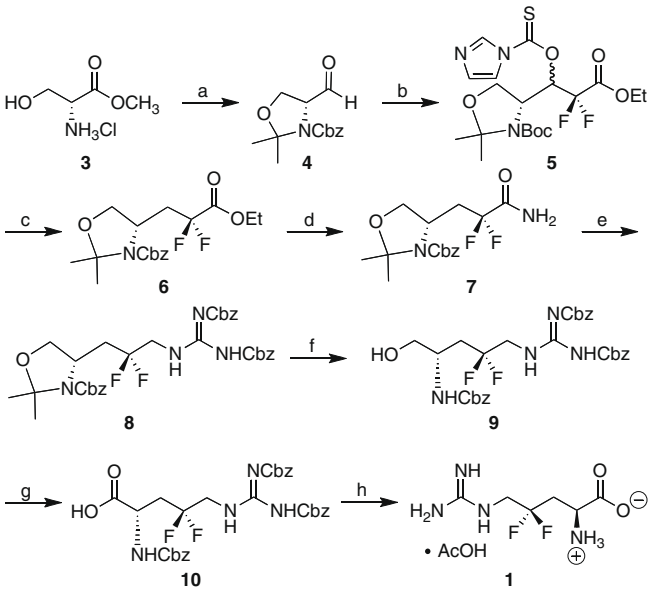


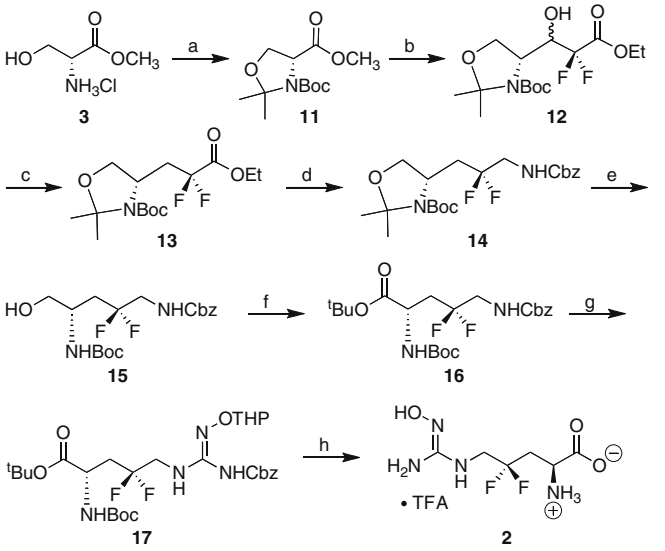
**Figure 3.** NOS substrate analogues 4,4-difluoro-L-arginine **1** and 4,4-difluoro-$N^G$-hydroxy-L-arginine **2**.



**Scheme 1.** Synthesis of 4,4-difluoro-L-arginine **1**. Reagents and conditions: (a) 1—CbzCl, $NaHCO_3$, $EtOAc/H_2O$; 2—2,2-dimethoxypropane, acetone, $BF_3{\cdot}Et_2O$; 3—DIBAL $Et_2O$, −78 °C, 74%, 3 steps; (b) 1—ethyl bromodifluoroacetate, Zn(s), THF; 2—thiocarbonyldiimidazole, THF, 40 °C, 48 h, 64%, 2 steps; (c) $Et_3SiH$, benzoyl peroxide, dioxane, 110 °C, 82%; (d) $NH_4OH$, MeOH, quantitative; (e) 1—Red-Al, toluene, 0 °C; 2—*N*,*N*′-di-Cbz-*N*″-triflylguanidine, $NEt_3$, $CH_2Cl_2$ 35 °C, 16 h, 81%, 2 steps; (f) 1,2-ethanedithiol, $BF_3{\cdot}Et_2O$, $CH_2Cl_2$ 82%; (g) $CrO_3$, $H_5IO_6$, $MeCN/H_2O$, 96%; (h) $H_2$, Pd/C, $MeOH/H_2O$ (1% AcOH), 93%.

the NO-induced oxidation of oxyhemoglobin to methemoglobin observable at 401 nm. Both 4,4-difluoro-L-arginine **1** and 4,4-difluoro-$N^G$-hydroxy-L-arginine **2** were found to be NO-forming substrates for iNOS. 4,4-Difluoro-L-arginine **1** was found to have a $K_m$ value of 814 ± 24 μM and a $k_{cat}$ value approximately 10% of that measured for L-arginine (Fig. 4A). 4,4-Difluoro-$N^G$-hydroxy-L-arginine **2** was determined to be a slightly better substrate for iNOS with a $K_m$ value of 328 ± 16 μM and a $k_{cat}$ value approximately 30% of that measured for $N^G$-hydroxy-L-arginine (Fig. 4B). The $K_m$ values for both fluorinated substrate analogues were found to be significantly higher than those measured for the native substrates L-arginine ($K_m$ = 8.6 ± 0.5 μM) and $N^G$-hydroxy-L-arginine ($K_m$ = 52 ± 3 μM).

The binding of both difluorinated analogues **1** and **2** to the iNOS active site was next investigated. Using a previously described



**Scheme 2.** Synthesis of 4,4-difluoro-$N^G$-hydroxy-L-arginine **2**. Reagents and conditions: (a) 1—$Boc_2O$, $NEt_3$, MeCN; 2—2,2-dimethoxypropane, acetone, $BF_3{\cdot}Et_2O$, 81%, 2 steps; (b) 1—DIBAL, $Et_2O$, −78 °C; 2—ethyl bromodifluoroacetate, Zn(s), THF, 69%, 2 steps; (c) 1—thiocarbonyldiimidazole, THF, 40 °C, 40 h; 2—$Et_3SiH$, benzoyl peroxide, dioxane, 110 °C, 2 steps, 60%; (d) 1—$NH_4OH$, MeOH; 2—Red-Al, toluene, 0 °C; 3—CbzCl, $NaHCO_3$, $H_2O$/dioxane, 90%, 3 steps; (e) *p*TsOH, MeOH, 50 °C, 85%; (f) 1—$NaClO_2$/NaOCl, TEMPO, MeCN/$NaHPO_4$ buffer; 2—O-*tert*-butyl-dicyclohexylisourea, $CH_2Cl_2$, 35 °C 16 h, 2 steps, 93%; (g) 1—$H_2$, Pd/C, EtOAc; 2—CbzNCS; 3—$H_2$ N-OTHP, EDCI, $NEt_3$, $CH_2Cl_2$, 96%, 3 steps[30]; (h) TFA/thioanisole, 5 h, quantitative.

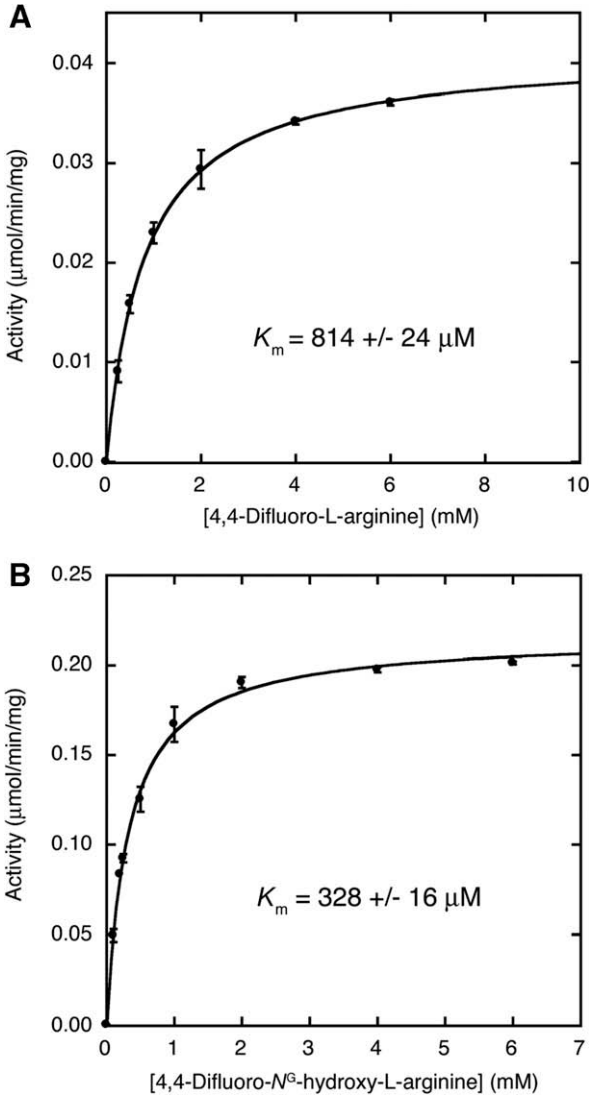



**Figure 4.** $K_m$ determination for: (A) 4,4-difluoro-L-arginine **1** and (B) 4-difluoro-$N^G$-hydroxy-L-arginine **2** (results of triplicate analysis).

spectral binding assay, the affinity of both **1** and **2** for $iNOS_{heme}$ was measured ($iNOS_{heme}$ binds heme, $H_4B$ and substrate(s) in the same fashion as the full-length enzyme).[1] As with the native substrates L-arginine and $N^G$-hydroxy-L-arginine, binding of 4,4-difluoro-L-arginine **1** to $H_4B$-bound $iNOS_{heme}$ induces a low- to high-spin state conversion at the heme center resulting from loss of water ($Fe^{III}$-aqua → $Fe^{III}$-unligated). This spin-state transition is accompanied by an observable decrease in absorbance at ~420 nm and an increase at ~396 nm in the UV–visible spectrum (Fig. 5A illustrates the spectral transition observed when $iNOS_{heme}$ is titrated with 4,4-difluoro-L-arginine **1**). As depicted in Figure 5B, the binding constant (spectral $K_d$) of **1** for $iNOS_{heme}$ is determined by applying the saturation binding equation ($\Delta\Delta Abs = (\Delta\Delta Absmax \times [compound])/(K_d + [\mathbf{1}])$). Like the iNOS activity-based $K_m$ value, the spectral $K_d$ measured for 4,4-difluoro-L-arginine **1** (3.1 ± 0.2 mM) is much higher than that for the non-fluorinated native substrate L-arginine (7.0 ± 0.7 µM). The results of the spectral binding analysis with $N^G$-hydroxy-4,4-difluoro-L-arginine **2**, however, were quite unexpected. Upon addition of **2** to $iNOS_{heme}$, a dramatic change in the UV–visible spectrum was observed leading to a split Soret absorbance with maxima at 431 and 368 nm (Fig. 6A). This spectral shift is much different than that seen with the corresponding non-fluorinated native substrate. Addition $N^G$-hydroxy-L-arginine to $iNOS_{heme}$ (measured spectral $K_d$ = 5.1 ± 0.4 µM) causes a low- to high-spin state change similar to that observed for both L-arginine and 4,4-difluoro-L-arginine **1**. Despite the unexpected change in the absorbance spectrum, a spectral $K_d$ value of 116 ± 4 µM for $N^G$-hydroxy-4,4-difluoro-L-arginine **2** was determined by plotting $\Delta\Delta Abs$ ($\Delta\Delta Abs = \Delta Abs_{431} - \Delta Abs_{395}$) as a function of the concentration of analogue added (Fig. 6B).

Interpretation of the spectral shift data obtained upon addition of $N^G$-hydroxy-4,4-difluoro-L-arginine **2** to $iNOS_{heme}$ is aided by previous reports describing comparable effects observed in the addition of non-amino acid $N^G$-hydroxyguanindines to NOS and microperoxidase 8 (MP8).[31,32] Mansuy and Stuehr found that certain $N^G$-hydroxyguanidines induce similar spectral shifts when added to $iNOS_{heme}$ but only in the absence of $H_4B$. Based on further EPR and resonance Raman findings, they proposed a model whereby $N^G$-hydroxyguanidines are able to directly ligate the iNOS $Fe^{III}$ heme center via the $N^G$-hydroxy oxygen atom. The resultant complexes are described as low-spin, 6-coordinate species with UV–visible absorbance maxima at 430 and 368 nm. They suggest that this mode of binding of $N^G$-hydroxyguanidines to $iNOS_{heme}$ is dependent upon the relative acidity of the –OH group in the $N^G$-hydroxy moiety.[32] Consistent with this proposal, difluorination at the $C_4$ position of $N^G$-hydroxy-L-arginine likely increases the acidity of the $N^G$-hydroxy moiety, weakening the O–H bond (possibly leading to deprotonation) allowing for oxygen ligation of the heme iron (Fig. 7).

The ability of certain *N*-hydroxyguanidines to ligate heme observed by Mansuy and Stuehr was reported to be also governed by steric effects, requiring $H_4B$ to be absent from the enzyme active site.[31] If, as our spectral data suggests, addition of analogue **2** to $H_4B$-bound $iNOS_{heme}$ leads to direct heme ligation, the observation would appear to be the first of its kind. In the case of difluorinated analogue **2**, $H_4B$ does not pose a steric impediment towards a ligation-based mode of binding. The proposed mode of NOS binding by analogue **2** is further supported by the work of Hoffman and

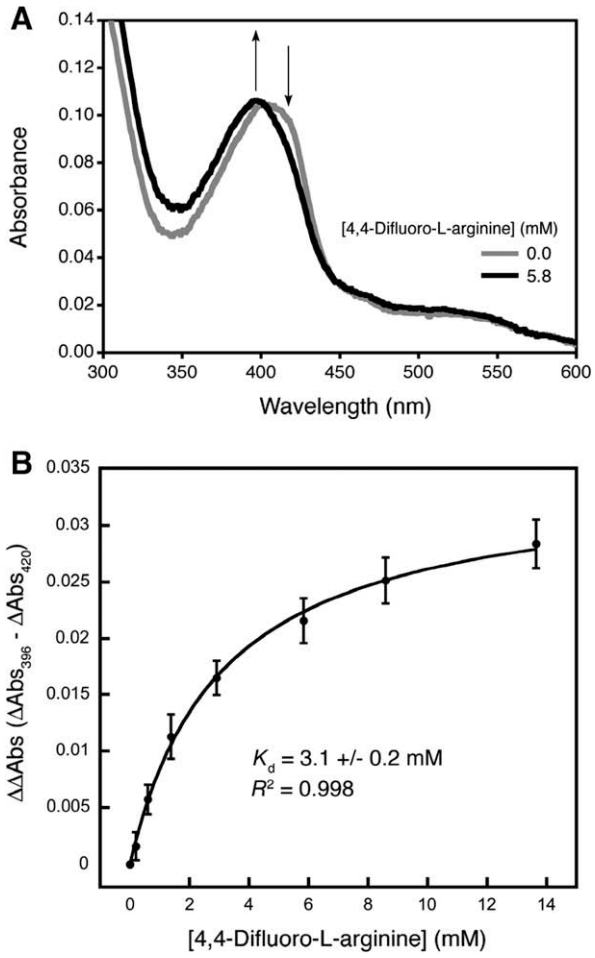



**Figure 5.** Spectral $K_d$ determination for 4,4-difluoro-L-arginine **1**. (A) Low- to high-spin state transition effected by addition of 4,4-difluoro-L-arginine **1** to iNOSheme. (B) Titration data used in determination of the spectral $K_d$ for 4,4-difluoro-L-arginine **1** (results of triplicate analysis).

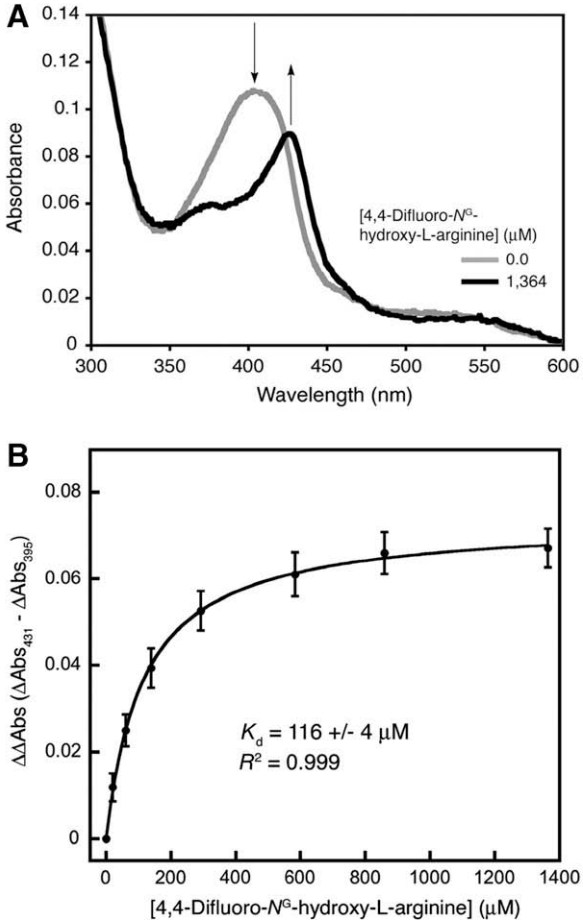



**Figure 6.** Spectral $K_d$ determination for $N^G$-hydroxy-4,4-difluoro-L-arginine. (A) Spectral transition effected by addition of $N^G$-hydroxy-4,4-difluoro-L-arginine **2** to $iNOS_{heme}$. (B) Titration data and fit used in determination of the spectral $K_d$ for $N^G$-hydroxy-4,4-difluoro-L-arginine **2** (results of triplicate analysis).

co-workers who described intermediates observed during the cryoreduction of a $Fe^{II}$-$O_2$-L-arginine NOS complex.[33] When the hydroxylation of L-arginine by NOS was carried out at low temperature (77 K), formation of a spectrally similar low-spin $Fe^{III}$ species was observed, proposed to be the oxygen-ligated complex.[33] This complex was stable up to 165 K and at higher temperatures reverted to the typically observed, high-spin, five-coordinate $Fe^{III}$ species. In contrast to the native substrate $N^G$-hydroxy-L-arginine, our observations would suggest that difluorinated analogue **2** is capable of stably coordinating the $Fe^{III}$ heme center at room temperature. Furthermore, if binding by **2** does involve ligation of the $Fe^{III}$ heme center, it is likely dynamic, as both analogues **1** and **2** support NO formation with full-length iNOS (i.e., the enzyme is still capable of binding and reducing $O_2$ in the presence of the fluorinated analogues). The unique NOS binding properties of difluorinated analogue **2** may provide for a useful tool in future mechanistic investigations.

In summary, we have successfully prepared and studied the $C_4$ difluorinated analogues of the NOS substrates L-arginine and $N^G$-hydroxy-L-arginine. While both analogues exhibit a reduced binding affinity for the enzyme, they were each shown to be NO-forming substrates and appear to follow the native reaction pathway. 4,4-difluoro-$N^G$-hydroxy-L-arginine also displays a mode of binding to iNOS previously unobserved with amino acid substrates at room temperature. This binding likely involves a direct ligation of the $Fe^{III}$ heme center in the enzyme and provides additional insight into reaction pathway followed during the NOS mediated

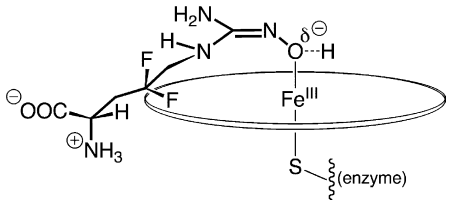



**Figure 7.** Proposed mode of binding of $N^G$-hydroxy-4,4-difluoro-L-arginine **2** to the active site H4B-bound $iNOS_{heme}$.

conversion of L-arginine to NO and L-citrulline. Difluorinated analogues **1** and **2** may also serve as useful molecular probes for future investigations into the NOS reaction mechanism using NMR and EPR spectroscopic methods.

## Acknowledgment

This work was supported by the Aldo DeBenedictis Fund, the Natural Sciences and Engineering Council of Canada, and the Alberta Heritage Foundation for Medical Research (postdoctoral fellowships to N.I.M.).

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.076.

## References and notes


1. Hurshman, A. R.; Marletta, M. A. *Biochemistry* **2002**, *41*, 3439.
2. Griffith, O. W.; Stuehr, D. J. *Annu. Rev. Physiol.* **1995**, *57*, 707.
3. Kerwin, J. F., Jr.; Lancaster, J. R., Jr.; Feldman, P. L. *J. Med. Chem.* **1995**, *38*, 4343.
4. Roman, L. J.; Martasek, P.; Masters, B. S. *Chem. Rev.* **2002**, *102*, 1179.
5. Groves, J. T.; Wang, C. C. *Curr. Opin. Chem. Biol.* **2000**, *4*, 687.
6. Zhu, Y.; Silverman, R. B. *Biochemistry* **2008**, *47*, 2231.
7. Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841.
8. Shaik, S.; Kumar, D.; de Visser, S. P.; Altun, A.; Thiel, W. *Chem. Rev.* **2005**, *105*, 2279.
9. Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. *Chem. Rev.* **2005**, *105*, 2253.
10. Pufahl, R. A.; Wishnok, J. S.; Marletta, M. A. *Biochemistry* **1995**, *34*, 1930.
11. Clague, M. J.; Wishnok, J. S.; Marletta, M. A. *Biochemistry* **1997**, *36*, 14465.
12. Wang, Z. Q.; Wei, C. C.; Santolini, J.; Panda, K.; Wang, Q.; Stuehr, D. J. *Biochemistry* **2005**, *44*, 4676.
13. Konas, D. W.; Takaya, N.; Sharma, M.; Stuehr, D. J. *Biochemistry* **2006**, *45*, 12596.
14. Woodward, J. J.; Chang, M. M.; Martin, N. I.; Marletta, M. A. *J. Am. Chem. Soc.* **2009**, *131*, 297.
15. Moreau, M.; Boucher, J. L.; Mattioli, T. A.; Stuehr, D. J.; Mansuy, D.; Santolini, J. *Biochemistry* **2006**, *45*, 3988.
16. Lefevre-Groboillot, D.; Boucher, J. L.; Mansuy, D.; Stuehr, D. J. *FEBS J.* **2006**, *273*, 180.
17. Moali, C.; Boucher, J. L.; Sari, M. A.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **1998**, *37*, 10453.
18. Luzzi, S. D.; Marletta, M. A. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3934.
19. Huang, H.; Hah, J. M.; Silverman, R. B. *J. Am. Chem. Soc.* **2001**, *123*, 2674.
20. Martin, N. I.; Woodward, J. J.; Winter, M. B.; Beeson, W. T.; Marletta, M. A. *J. Am. Chem. Soc.* **2007**, *129*, 12563.
21. Hurshman, A. R.; Krebs, C.; Edmondson, D. E.; Huynh, B. H.; Marletta, M. A. *Biochemistry* **1999**, *38*, 15689.
22. Wei, C. C.; Wang, Z. Q.; Durra, D.; Hemann, C.; Hille, R.; Garcin, E. D.; Getzoff, E. D.; Stuehr, D. J. *J. Biol. Chem.* **2005**, *280*, 8929.
23. Goldstein, J. A.; Cheung, Y. F.; Marletta, M. A.; Walsh, C. *Biochemistry* **1978**, *17*, 5567.
24. Filler, R.; Kobayashi, Y.; Yagupolskii, L. M. *Biomedical Aspects of Fluorine Chemistry*; Elsevier: Amsterdam, 1993.
25. Walsh, C. T. *Annu. Rev. Biochem.* **1984**, *53*, 493.
26. Hallinan, E. A.; Kramer, S. W.; Houdek, S. C.; Moore, W. M.; Jerome, G. M.; Spangler, D. P.; Stevens, A. M.; Shieh, H. S.; Manning, P. T.; Pitzele, B. S. *Org. Biomol. Chem.* **2003**, *1*, 3527.
27. Kim, S. K.; Qian, L. *Tetrahedron Lett.* **1993**, *34*, 7195.
28. Olken, N. M.; Marletta, M. A. *Biochemistry* **1993**, *32*, 9677.
29. Olken, N. M.; Marletta, M. A. *J. Med. Chem.* **1992**, *35*, 1137.
30. Martin, N. I.; Woodward, J. J.; Marletta, M. A. *Org. Lett.* **2006**, *8*, 4035.
31. Lefevre-Groboillot, D.; Frapart, Y.; Desbois, A.; Zimmermann, J. L.; Boucher, J. L.; Gorren, A. C.; Mayer, B.; Stuehr, D. J.; Mansuy, D. *Biochemistry* **2003**, *42*, 3858.
32. Lefevre-Groboillot, D.; Dijols, S.; Boucher, J. L.; Mahy, J. P.; Ricoux, R.; Desbois, A.; Zimmermann, J. L.; Mansuy, D. *Biochemistry* **2001**, *40*, 9909.
33. Davydov, R.; Ledbetter-Rogers, A.; Martasek, P.; Larukhin, M.; Sono, M.; Dawson, J. H.; Masters, B. S. S.; Hoffman, B. M. *Biochemistry* **2002**, *41*, 10375.