The New α-Amino Acid N^{ω}-Hydroxy-nor-L-arginine: a High-Affinity Inhibitor of Arginase Well Adapted To Bind to Its Manganese Cluster

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Mammalian arginases specifically require a Mn(II)−Mn(II) cluster for their catalytic activity, the hydrolysis of L-arginine (L-arg) to L-ornithine and urea.[1] Quite recently, the crystal structure of rat liver arginase has shown that the two Mn ions are bridged by two carboxylate side chains of aspartates from the protein and a water molecule (or hydroxide ion).[2] It has been proposed that L-arg binds in close proximity of the Mn-cluster and that the transition state of the reaction is a tetrahedral species resulting from nucleophilic attack of the metal-bridging hydroxide at the guanidinium carbon of L-arg (Figure 1).[2,3] Very few data are presently available about the accessibility of the Mn-cluster to molecules different from L-arg, and borate was the only compound described so far to significantly modify the EPR spectrum of arginase.[4] Inhibitors often are interesting tools to explore enzyme active sites. For arginase, for a long time the best known inhibitor was L-valine, which exhibits a modest K_i value (in the millimolar range).[5] More recently, N^{ω}-hydroxy-L-arginine (NOHA)[6] (Scheme 1), an intermediate in the biosynthesis of NO from L-arginine, and some N^{ω}-hydroxy-L-α-aminoacids[7] have been shown to act as much more potent inhibitors of arginases with K_i values in the 20−50 μM range. A model has been proposed for the interaction of those inhibitors with arginase, which postulated that they could be of the transition state analog type, the N−OH group being able to replace the hydroxo ligand of the arginase Mn-cluster.[6a,7] On the basis of this model, it appeared to us that N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) should be a very good arginase inhibitor, well suited to interact with the Mn cluster *via* its N−OH function (better than NOHA, Figure 1).

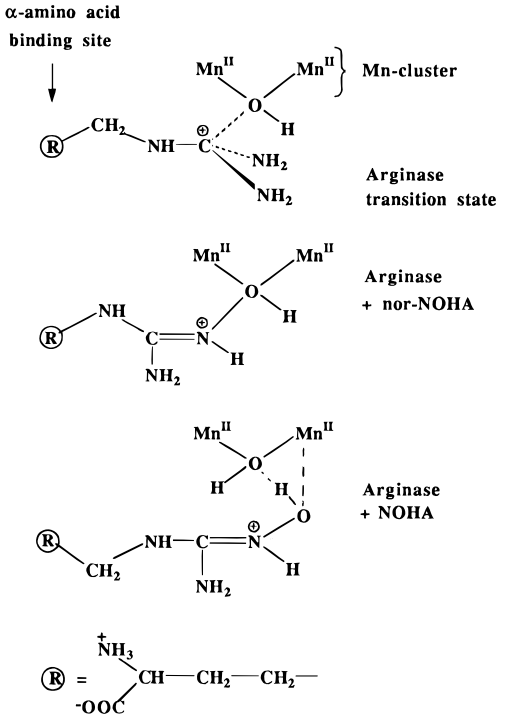
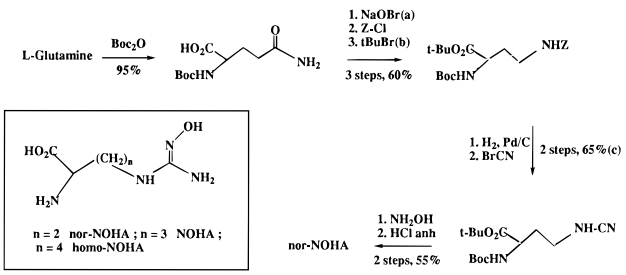


Figure 1. Schematic view of the postulated arginase transition state and a proposed model for interaction of arginase with nor-HOHA and NOHA.

Scheme 1. Synthesis of N^{ω}-Hydroxy-nor-L-arginine (nor-NOHA) and Formula of NOHA and homo-NOHA[a]



[a] Boc: −COOtBu; Z = $COOCH_2Ph$; (a) according to the general procedure of ref 10; (b) conditions as in ref 11; (c) conditions of those steps and the following ones as described for the synthesis of NOHA.[8d]

This paper reports preliminary results about (i) the first synthesis of N^{ω}-hydroxy-nor-L-arginine and its homolog N^{ω}-hydroxy-homo-L-arginine, (ii) a comparison of the inhibitory effects of NOHA, homo-NOHA, and nor-NOHA toward arginases, and (iii) EPR studies of the interaction of these compounds with purified rat liver arginase. They show that nor-NOHA not only is the best inhibitor (K_i = 0.5 μM) but also specifically modifies the arginase EPR spectrum.

N^{ω}-hydroxy-homo-L-arginine (homo-NOHA) (Scheme 1)[9] was synthesized from L-lysine following a procedure previously described for the synthesis of NOHA.[8] Nor-NOHA[9] was obtained from L-glutamine from reactions shown in Scheme 1 (15% overall yield). The key step was the decarboxylation of N^{α}-Boc-L-glutamine by sodium hypobromite to intermediate N^{α}-Boc-nor-L-ornithine which was readily protected as a N^{δ}-

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(1) Reczkowski, R. S.; Ash, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 10992−10994.
(2) Kanyo, Z. F.; Scolnick, L. R.; Ash, D. E.; Christianson, D. W. *Nature* **1996**, *383*, 554−557.
(3) Reczkowski, R. S.; Ash, D. E. *Arch. Biochem. Biophys.* **1994**, *312*, 31−37.
(4) Khangulov, S. V.; Pessiki, P. J.; Barynin, V. V.; Ash, D. E.; Dismukes, G. C. *Biochemistry* **1995**, *34*, 2015−2025.
(5) Hunter, A.; Downs, C. E. *J. Biol. Chem.* **1946**, *157*, 427−446.
(6) (a) Daghigh, F.; Fukuto, J. M.; Ash, D. E. *Biochem. Biophys. Res. Commun.* **1994**, *202*, 174−180. (b) Boucher, J. L.; Custot, J.; Vadon, S.; Delaforge, M.; Lepoivre, M.; Tenu, J. P.; Yapo, A.; Mansuy, D. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1614−1621.
(7) Custot, J.; Boucher, J. L.; Vadon, S.; Guedes, C.; Dijols, S.; Delaforge, M.; Mansuy, D. *J. Biol. Inorg. Chem.* **1996**, *1*, 73−82.
(8) (a) Feldman, P. L. *Tetrahedron Lett.* **1991**, *32*, 875−878. (b) Wallace, G. C.; Fukuto, J. M. *J. Med. Chem.* **1991**, *34*, 1746−1748. (c) Bailey, D. M.; DeGrazia, C. G.; Lape, H. E.; Frering, R.; Fort, D.; Skulan, T. *J. Med. Chem.* **1973**, *16*, 151−156. (d) Vadon, S.; Custot, J.; Boucher, J. L.; Mansuy, D. *J. Chem. Soc., Perkin Trans. 1* **1996**, 645−648.
(9) Both homo- and nor-NOHA were characterized by ^1H and ^{13}C NMR spectroscopies: $\delta^{13}C(D_2O)$ 24.3, 30.3, 32, 43.5, 55.9, 161.6, 175.3 and 31.8, 40.1, 53.2, 161.5, 174.2, respectively. Mass spectrometry *m/z* (ES): 205.23 and 177.18 ($M + H^+$), respectively.
(10) Rudinger, J.; Poduska, K.; Zaoral, M. *Coll. Czechoslov. Chem. Commun.* **1960**, *25*, 2022−2028.
(11) Chevallet, P.; Garrouste, P.; Malawska, B.; Martinez, J. *Tetrahedron Lett.* **1993**, *34*, 7409−7412.

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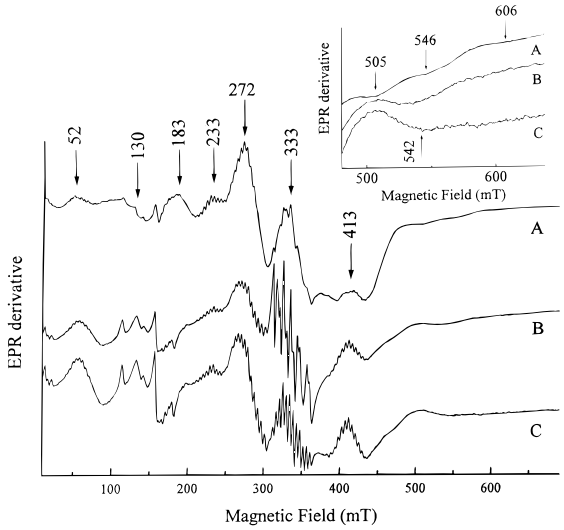

Figure 2. Effects of nor-NOHA on the EPR spectrum of rat liver arginase: (A) 1 mM arginase in 50 mM Tris-HCl buffer, pH 7.4, at 15 K (microwave frequency 9.42 GHz); (B**)** spectrum after addition of 2 mM nor-NOHA; (C) spectrum of sample B after ultrafiltration through a Bio-spin column.

benzyloxycarbonyl (Boc) derivative before esterification to the corresponding *tert*-butyl ester.

nor-NOHA was found to be much more potent than NOHA and homo-NOHA to inhibit the rat liver arginase-dependent hydrolysis of L-arg (respective IC_{50} of 2 ± 0.5, 40 ± 10, and 3000 ± 500 μM at pH 7.4).[12] nor-NOHA is also a much better inhibitor of the arginase activity of mouse macrophages than NOHA (IC_{50} of 50 ± 10 and 450 ± 50 μM, respectively). Lineweaver−Burk plots of data from incubations of purified rat liver arginase with L-arg and either nor-NOHA or NOHA, at pH 7.4, clearly indicated that the two latter compounds were competitive inhibitors with respect to L-arg ($K_i = 0.5 \pm 0.1$ and 10 ± 2 μM, respectively). Experiments based on preincubations of arginase with nor-NOHA showed that this compound is, as NOHA,[6] a reversible inhibitor. Finally, in double-inhibition experiments performed with arginase, NOHA, and nor-NOHA, plots of 1/activity as a function of nor-NOHA and NOHA concentrations led to parallel curves, with a ratio of about 20 between the two K_i values (data not shown). All these results indicate that both compounds are reversible, competitive inhibitors of arginase, with nor-NOHA having a much better affinity.

Stepwise additions of nor-NOHA to purified rat liver arginase led to a progressive change of the EPR spectrum of this metalloprotein (Figure 2). Almost all EPR features previously reported for native arginase[4] were changed; for instance, the signal set at 272 mT markedly decreased and that at 183 mT disappeared, while the intensity of the 413 mT signal was enhanced. Other changes concern troughs at high-field related to the zero-field splitting structure and observed between 460 and 700 mT. Three troughs at 505, 546, and 606 mT found for native arginase were replaced by a new trough centered at 542 mT. Clear changes were already obtained after addition of 1 equiv of nor-NOHA per Mn-dimer (1 mM); further additions (up to 2 equiv) only slightly modified the spectrum except for an increase of a six-line signal centered at 335 mT with a 90 G hyperfine coupling constant (Figure 2B). The latter signal could be due to loosely bound Mn ions (or Mn ions not coupled as in native arginase)[13] as it disappeared after ultrafiltration of the incubate. Moreover, the EPR spectrum of the recovered sample was almost identical to the one obtained after addition of 2 equiv of nor-NOHA to arginase, except for the region around 335 mT which now showed a signal with at least 11 lines and hyperfine constants of 45 G that was masked by the six-line (90 G) signal due to loosely bound Mn (Figure 2C). These data suggest that nor-NOHA stoichiometrically interacts with the Mn-cluster of arginase and leads to a marked change of its EPR spectrum which is indicative of a change in the Mn···Mn distance[4] and could be due to the replacement of the arginase μ-OH ligand by its N−OH moiety. Upon addition of excess nor-NOHA, nonspecific demetallation of the active site could occur. Similar experiments performed with homo-NOHA failed to induce any EPR spectral change on arginase, whereas preliminary studies performed with NOHA only led to a marked increase of the signal centered at 335 mT with a 90 G hyperfine coupling constant. However, the other spectral changes observed after addition of 1−2 equiv of nor-NOHA to arginase (Figure 2) were not observed with either NOHA or homo-NOHA.

The aforementioned results show that the length of the chain linking the amino acid and *N*-hydroxyguanidine functions of NOHA is very important for recognition by arginase. Addition of a CH_2 moiety (homo-NOHA) led to a dramatic decrease of this recognition (75-fold increase of IC_{50}), whereas removal of one CH_2 group markedly improved it (20-fold decrease of K_i). This particularly high affinity of nor-NOHA for arginase appears to be correlated to its specific interaction with the Mn-cluster of the enzyme active site, which occurs with a 1:1 stoichiometry, as suggested by EPR data. Additional experiments are required in order to determine the molecular nature of the interaction of nor-NOHA with arginase. A possible explanation for the specific effects of this molecule is that it acts, as previously assumed (Figure 1), by replacement of the H_2O (or OH) bridging ligand of the Mn-cluster by its *N*-hydroxy function. Molecular models suggest that it would be better than NOHA to play this role (Figure 1). The new α-amino acid, nor-NOHA, is the most potent inhibitor of arginase (both from rat liver or mouse macrophages) reported so far. Its use for better understanding the physiological roles of arginases *in vitro* and *in vivo* is underway.

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(12) Rat liver arginase was purified as described in ref 1 and its activity measured as reported in ref 7.

(13) Ash, D. E.; Schramm, V. L. *J. Biol. Chem.* **1982**, *257*, 9261−9264.