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SYNTHESIS OF BORONIC ACID ANALOGS OF L-ARGININE AS ALTERNATE SUBSTRATES OR INHIBITORS OF NITRIC OXIDE SYNTHASE

Caroline Lebarbier, † François Carreaux,*† Bertrand Carboni,† Jean Luc Boucher*‡

†*Synthèse et electrosynthèse organiques, UMR CNRS , Université de Rennes 1, Campus de Beaulieu, F-35042 Rennes Cedex, France*

‡*Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques. Université Paris V, URA CNRS 400, 45 rue des Saints Pères, 75270, Paris Cedex 06, France*

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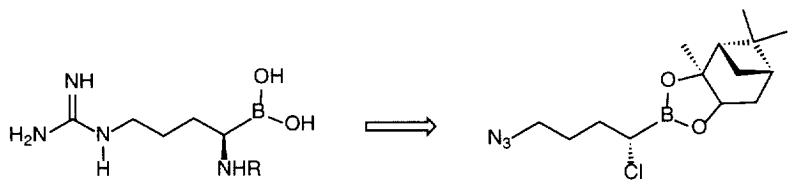
Abstract - The asymmetric synthesis of an unprotected α-amino boronic acid analog of L-arginine **1a** (boroarg-OH. 2 HCl) and its N^{α}-acetyl derivative **1b** (Ac-boroarg-OH. HCl) is described. These compounds were evaluated as substrates and inhibitors of recombinant nitric oxide synthases (NOS). Boroarg-OH **1a** selectively inhibited inducible NOS (IC_{50} = 50 μM) compared to the neuronal isoform (IC_{50} = 300 μM).

Nitric oxide, NO, has become in the past few years an exciting entity to study in a variety of biological systems.[1,2] NO displays potent activities in the cardiovascular system as well as in the central and peripheral nervous systems and is an important cytotoxic agent produced by activated macrophages. NO and its coproduct L-citrulline (Cit) are formed by the enzymatic oxidation of one of the two equivalent terminal guanidino nitrogens of L-arginine (Arg) by nitric oxide synthase (NOS). Two NOS isoforms are constitutively expressed in cells such as neurons (nNOS) and endothelium (eNOS) and are regulated by Ca^{++} levels, whereas expression of an inducible isoform (iNOS) is induced by inflammatory stimuli.[1] The search for selective modulation of NO biosynthesis appears as a key therapeutic challenge in neurodegenerative diseases, atherosclerosis or endotoxemia.[3]

Based on the mechanism proposed for NO biosynthesis, we became interested in synthesizing Arg-based analogs which could either inhibit or act as alternative subtrates for NOS. Boronic acid analogs of α-amino acids represent an interesting class of enzymes inhibitors. Main results were hitherto reported in the field of serine and threonine proteases[4] where the mechanism of inhibition appears to involve the coordination of the active site nucleophilic serine or threonine on the boron atom. For other classes of enzymes, the inhibition process is less clear.[5] In connection with our ongoing program related to aminoboronic acids,[6] we synthesized the unprotected (R)-boroarginine dihydrochloride **1a** and its N^{α}- acetamido derivative **1b** as potential substrates or inhibitors of nitric oxide synthase (Figure 1).



1a. 2HCl R= H
1b. HCl R= $COCH_3$

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Figure 1

Fax: (33) 2 99 28 69 55; e-mail: françois.carreaux@univ-rennes1.fr
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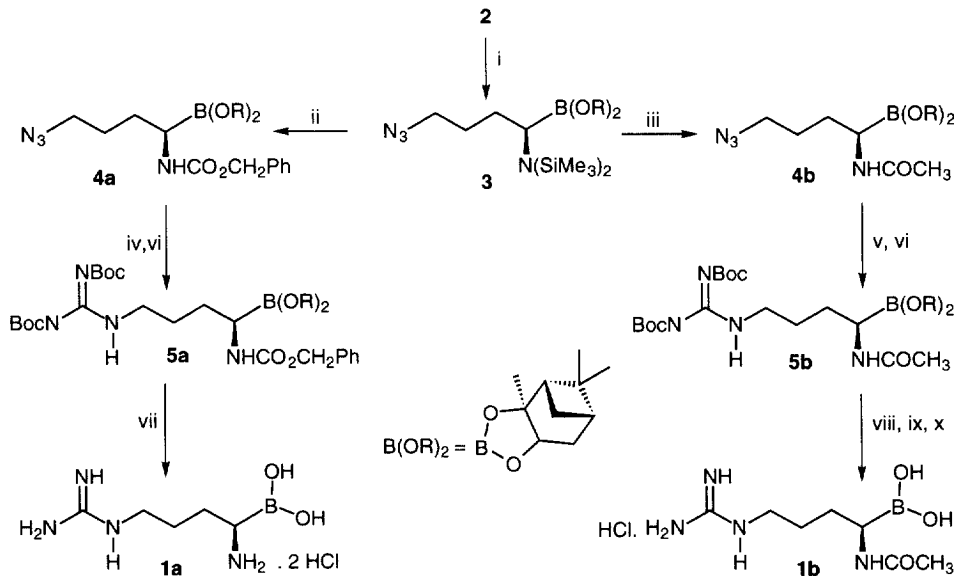
The general synthetic sequence is based on the asymmetric synthetic methodology developed by Matteson and coworkers.[7] α-Chloro boronic ester **2** was prepared in a 93 % de from allyl bromide by a previously described procedure.[6] To introduce the amino function α to the boron, we decided to realize the displacement of chloride using lithium hexamethyldisilazane (Scheme 1). Desilylation was performed with a single equivalent of methanol followed by treatment with benzylchloroformate to afford the N-benzyloxycarbonyl derivative **4a**. Selective reduction of the azido function of **4a** over PtO_2 was performed in 70% yield. After several attempts, the best yield of N-(*tert*-butoxycarbonyl) guanidine **5a** was obtained using N,N'-di-(*tert*-butoxycarbonylthiourea) with the presence of an external base and mercury chloride.[8] The acetamido derivative **4b** was obtained by cleavage of the silyl protecting groups of the amine **3** using acetic acid and acetic anhydride.[9] In parallel way, **5b** was obtained in 60% yield from **4b**. Treatment of **5a** by aqueous hydrochloric acid afforded unprotected boroarginine **1a** in 15 % overall yield starting from **2**. It is noteworthy that the great majority of amino boronic acids were isolated and tested as their N-acyl derivatives or (and) as their boronic esters. (*R*)-1-amino-4-guanidinobutyl boronic acid **1a** is unstable in aqueous solution at room temperature for long periods (> 6h) and is tested the same day.[10] Removal of the *tert*-butoxycarbonyl group of **5b** with trifluoroacetic acid followed by cleavage of pinanediol using phenyl boronic acid in a two phase ether/water system afforded boroarginine **1b**.[11] **1b** is much more stable in aqueous solution than **1a** and was obtained in 86% overall yield from **4b**.



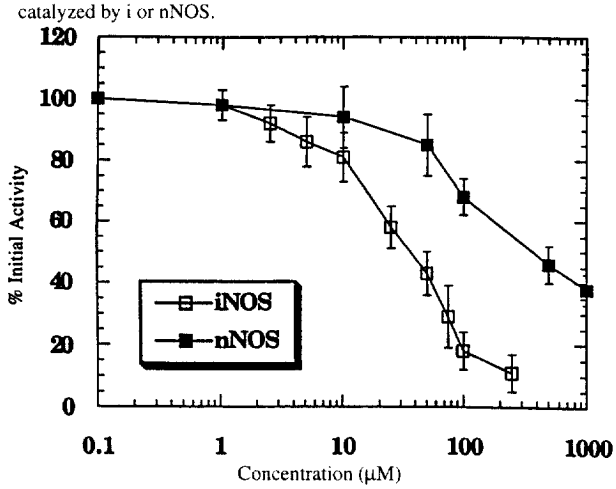
Reagents and conditions : (i) HMDS, BuLi, -78°C (ii) a) CH_3OH, -78°C, 1h30. b) $PhCH_2OCOCl$, -78°C to r.t. (70% from **2**) (iii) a) AcOH, -78°C. b) Ac_2O, -78°C, to r.t. (75% from **2**) (iv) H_2, PtO_2 (70%) (v) H_2, $Pd(OH)_2/C$, HCl (85%) (vi) $(BocHN)_2CS$, $HgCl_2$, Et_3N, DMF, 0°C to 25°C. (vii) HCl 6N, reflux. (viii) CF_3CO_2H, CH_2Cl_2, 1h, 25°C. (ix) $PhB(OH)_2$, ether/water, 25°C, 24h. (x) Dowex 50X8, HCl.

Scheme 1

The abilities of the boro-L-arginines **1a** and **1b** to generate NO and to inhibit [^{3}H]L-cit formation from [^{3}H]L-arg were investigated according to established methods,[12] in the presence of purified recombinant neuronal[13] and inducible NOSs.[14]

N$^\alpha$-acetyl derivative **1b** did not lead to any significant NO formation and poorly inhibited L-cit formation catalyzed by neuronal and inducible NOS (half inhibitory concentration, IC_{50}, higher than 500 μM). However, unprotected α-amino acid boro-L-arginine **1a** inhibited L-cit formation catalyzed by recombinant neuronal and inducible NOS with IC_{50} values of 300 ± 50 μM and 50 ± 15 μM respectively (Figure 2).

Figure 2: Effect of boro-L-arg **1a** on the formation of L-citrulline catalyzed by i or nNOS.



Concentration-depend inhibitory effects of **1a** on [^{14}C] L-citrulline formation from [^{14}C] L-arginine were followed as described previously,[12] using Dowex 50X8 resin (Na^+ form) to separate L-citrulline from unreacted L-arginine. Results show mean ± SEM of percentages of inhibition (n=4). Specific activities for purified recombinant i and nNOS were 850 ± 80 and 600 ± 100 nmoles. min^{-1}. mg protein^{-1}, respectively.

Inhibition of inducible NOS by **1a** was competitive with substrate L-arginine and reversible. Stepwise additions of **1a** to the oxygenase domain of inducible NOS[15, 16] led to the appearance of a Type I differential UV/Visible spectra, with a Ks value of 200 ± 40 μM. This result suggests that the guanidino group of **1a** could interact near the active site of NOS. However, following the formation of MetHb Fe(III) from Hb Fe(II)O_2 as a sensitive assay for NO production,[12] incubations of 500 μM **1a** with inducible NOS led to less than 5% of the NO detected when 100 μM L-arginine was used as substrate under identical conditions.

Compounds known to strongly inhibit NOSs bear a free L-α-amino acid group and a guanidino function separated by three CH_2 groups. Accordingly, N$^\alpha$-acetyl derivative **1b** is a very weak NOS inhibitor. Usual NOSs inhibitors (N$^\omega$-Me- , N$^\omega$-NH_2- and N$^\omega$-NO_2-L-arginine) show limited selectivity toward the three NOSs isoforms. Boro-L-arginine **1a** is a poor inhibitor of neuronal NOS, but is a relatively good competitive inhibitor of the inducible isoform (selectivity of **1a** is about 6 for iNOS versus nNOS). NOSs display a very strong substrate specificity with only arg, N$^\omega$-OH-arg, homo-arg (one CH_2 group longer than L-arginine) and N$^\omega$-Me-arg being transformed to NO by NOSs.[1,2] Compounds which differ from arg by a shorter chain-length (L-2-amino-4-guanidino-butanoic acid), by substitution of the α-NH_2 by an OH group (argininic acid) or by the

absence of the α-COOH group (agmatine) interact by their guanidino-group near the active site of NOSs and are reversible inhibitors. However, they are not substrate for NO formation.[17] Substitution of the α-COOH group of arg by a $B(OH)_2$ group results in compound **1a**, which interacts at the guanidino-binding site, but is unable to form NO when incubated with iNOS (in the limit of the detection method). These results demonstrate the strict substrate specificity of NOSs.

The synthetic methodology described herein allows the asymmetric preparation of the unprotected boro-L-arginine **1a**. The same methodology will produce N^{ω}-substituted (N^{ω}-Me- and N^{ω}-NO_2-) boro-L-arginines which could have increased potency.

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10. Similar instability of unprotected α-amino boronic acids have been already reported[5].
11. **5a**: $[\alpha]_D^{20}$= + 6.7°; $[\alpha]_{365}^{20}$= +21.5° (c = 1.31, CH_2Cl_2). ^1H NMR (200MHz, $CDCl_3$) δ = 8.28 (brs, 1H, NH), 7.36-7.32 (m, 5H, H_{arom}), 5.17-5.08 (m, 3H, NHCO, CH_2Ph), 4.31 (m, 1H, H-2), 3.39 (m, 2H, H-4'), 3.27 (m, 1H, H-1'), 2.40-1.66(m, 10H, H-2', H-3', H-3, H-4, H-6, H-7), 1.51 (s, 3H, H-10), 1.49 (s, 18H, $(CH_3)_3C$), 1.31 (s, 3H, H-9), 0.83 (s, 3H, H-8). FAB MS calcd for $C_{33}H_{52}BN_4O_8$ $(M+H)^+$ 643.3878, found 643.3897. **5b**: $[\alpha]_D^{20}$= -57.1°; $[\alpha]_{365}^{20}$= -208° (c = 1.05, CH_2Cl_2). ^1H NMR (200MHz, $CDCl_3$) δ = 8.95 (brs, 1H, NH), 8.55 (m, 1H, NH), 4.17 (dd, 1H, *J* = 1,6 and 6.8 Hz, H-2), 3.32 (m, 2H, H-4'), 2.70 (m, 1H, H-1'), 2.12 (s, 3H, $COCH_3$), 2.17-1.66 (m, 10H, H-2', H-3', H-3, H-4, H-6, H-7), 1.49 (s, 9H, $(CH_3)_3C$), 1.46 (s, 9H, $(CH_3)_3C$), 1.31 (s, 3H, H-10), 1.26 (s, 3H, H-9), 0.85 (s, 3H, H-8). ^{11}B NMR (96 MHz, $CDCl_3$) δ = 18.0. Anal. Calcd. for $C_{27}H_{47}BN_4O_7$ (550.5): C, 58.90; H, 8.60; N, 20.34; found: C, 58.73; H, 8.47; N, 20.58. **1a**: ^1H NMR (200MHz, D_2O) δ = 3.25 (t, *J* =6.4 Hz, 2H, H-4), 2.89 (m, 1H, H-1), 1.83-1.79 (m, 4H, H-2, H-3). ^{13}C NMR (50.3MHz, D_2O) δ = 160.4, 62.9, 41.1, 26.5, 25.6. ^{11}B NMR (96 MHz, D_2O) δ = 29.3. **1b**: $[\alpha]_D^{24}$= -32.2°;$[\alpha]_{365}^{24}$= -116.3°; (c = 0.66, H_2O). ^1H NMR (200MHz, D_2O) δ = 2.92 (t, *J* =8 Hz, 2H, H-4), 2.18 (t, *J* =7Hz, 1H, H-1), 1.84 (s, 3H, H-6), 1.34-1.17 (m, 4H, H-2, H-3). ^{13}C NMR (50.3MHz, D_2O) δ = 176.5, 165, 41.5, 27.5, 26.7, 16.6. ^{11}B NMR (96 MHz, D_2O) δ = 11.0. FAB MS: m/z=487.21 $(M+H)^+$ for ester of **1b** with matrix metanitrobenzylic alcohol.
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