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GUANIDINE-SUBSTITUTED IMIDAZOLES AS INHIBITORS OF NITRIC OXIDE SYNTHASE

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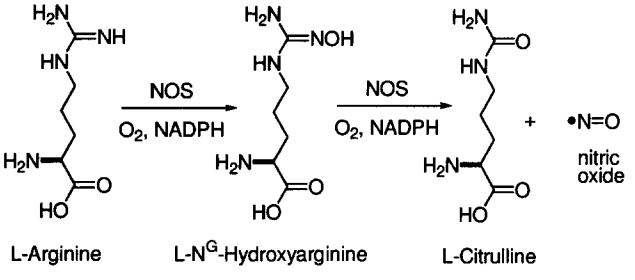
Abstract: Guanidine-substituted imidazoles were prepared and evaluated as inhibitors of the three isoforms of nitric oxide synthase. These results identify a new 2-substituted imidazole as an isoform selective inhibitor and illustrate the possible importance of the L-arginine side chain in selective isoform recognition.

The nitric oxide synthases (NOS) catalyze the oxidation of the terminal guanidino group of L-arginine to nitric oxide (NO), a molecule that plays important roles in blood pressure control, neurotransmission, and the immune response (Scheme 1).[1] This conversion occurs in two steps, a two-electron oxidation of L-arginine to L-N^G-hydroxyarginine followed by a three-electron oxidation of L-N-hydroxyarginine to NO and L-citrulline (Scheme 1).[2] Each step requires molecular oxygen and reduced nicotine-adenine-dinucleotide phosphate (NADPH) as co-substrates and (6*R*)-5, 6, 7, 8-tetrahydrobiopterin (H_4B), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and iron protoporphorin IX (heme) as cofactors.[3] The heme iron cofactor binds oxygen, becomes activated through reduction, and directly participates in each oxidation.[1,3] Three distinct mammalian NOS isoforms exist: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed, and inducible NOS (iNOS).[3] X-ray crystallographic structures of both the inducible and endothelial NOS oxygenase domains provide active site structural information.[4]

Scheme 1



With respect to L-arginine, imidazole inhibits the inducible and neuronal isoforms of NOS non-competitively and competitively inhibits the endothelial isoform.[5,6] Imidazole also inhibits all three isoforms non-competitively with respect to H_4B.[5] Optical difference spectrophotometric experiments indicate direct binding of imidazole to the heme group of the enzyme.[7] Coordination of imidazole as a sixth ligand to the heme iron prevents oxygen binding and subsequently blocks product formation.[5,7] X-ray crystallographic studies with the inducible oxygenase domain and imidazole reveal two molecules of imidazole bound in the active site.[4c] One imidazole molecule binds directly to the heme iron as a sixth ligand and the other hydrogen

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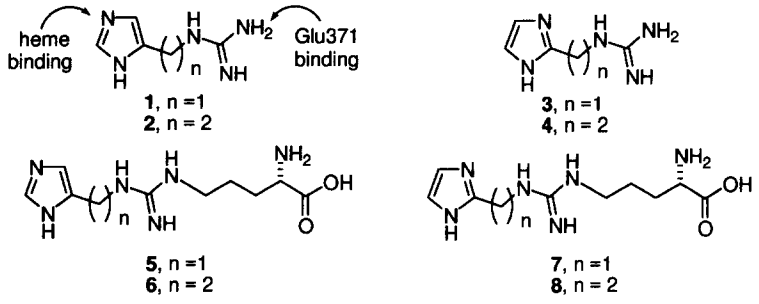
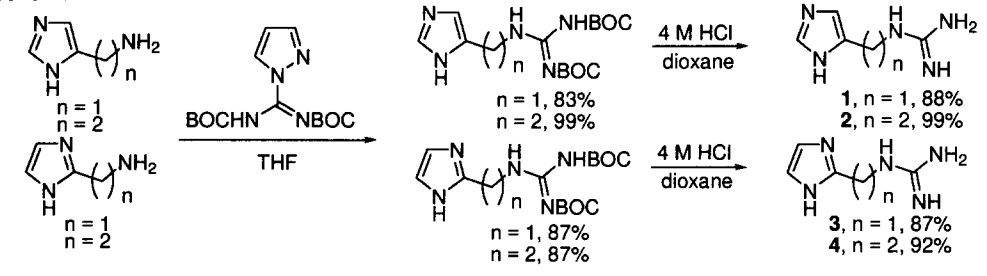
bonds to Glu371, an important residue that also hydrogen bonds to the guanidinium group of L-arginine.[4b]



Compounds capable of simultaneous binding to the heme group and Glu371 may provide superior interaction with NOS and we wish to present results regarding the guanidine-substituted imidazoles (**1-8**). These compounds contain groups capable of heme (imidazole ring) and Glu371 binding (guanidine group) separated by various distances and arranged differently about the imidazole ring. Compounds **5-8** include the L-arginine backbone as a potentially added point of interaction.

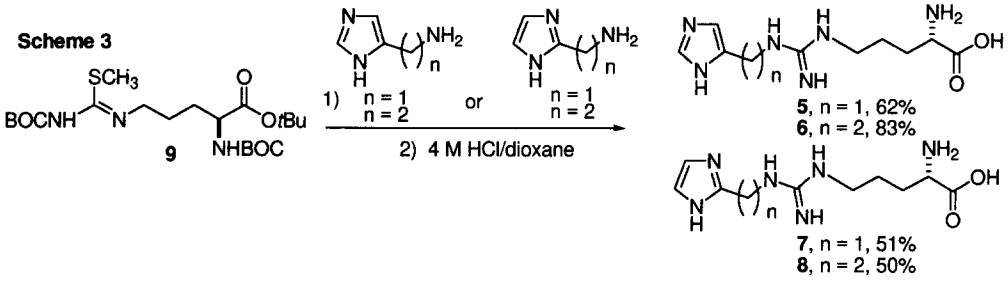
Condensation of *N*, *N'*-bis-BOC-1-guanylpyrazole with the appropriate amino-containing imidazoles followed by acidic deprotection produced compounds **1-4** in good yields (Scheme 2).[8] Commercially available histamine was used for the synthesis of **2** and the amino imidazoles required for the syntheses of **1**, **3**, and **4** were prepared by literature procedures.[9]

Scheme 2



Condensation of the same amines with the BOC protected S-methylisothiocitrulline derivative (**9**,[10] 40 °C, 72 h) followed by acidic deprotection generated the L-arginine derivatives (**5-8**) in good yields for two steps (Scheme 3).

Scheme 3



Compounds **1-8** were evaluated as inhibitors of purified mouse inducible, rat neuronal, and bovine endothelial nitric oxide synthase using the radioactive ^{14}C-L-citrulline assay.[11] Table 1 summarizes the IC_{50} values for the compounds that demonstrated greater than 50% inhibition of iNOS, eNOS, or nNOS catalyzed ^{14}C-L-citrulline production at 1 mM inhibitor concentration in the presence of 50 μM L-arginine. Compounds **1**, **2**, **4**, and **5** did not exhibit this level of inhibition with any NOS isoform. Compounds **1-8** were also evaluated as nitric oxide producing substrates of the same NOS isoforms using the Griess assay for nitrite production.[11] None of these compounds supported nitrite synthesis in the presence of any NOS isoform demonstrating the inability of these compounds to act as NO producing substrates of NOS.

Table 1. Inhibition of NOS isoforms by guanidine-substituted imidazoles (**3**, **6**, **7**, and **8**).

	IC_{50} (μM)		
Compound	iNOS	eNOS	nNOS
3	138	-	-
6	-	1085	-
7	-	347	1586
8	-	629	1494

The 2-substituted imidazole with a single methylene group separating the ring and the guanidine group (**3**) demonstrated selective inhibition of iNOS mediated L-citrulline production. In comparison to **3**, imidazole inhibits iNOS with an IC_{50} of 40 μM, but 2-phenylimidazole only weakly inhibits iNOS (IC_{50} > 1 mM).[5a,c] These results identify **3** as a potential lead for the development of new iNOS selective inhibitors and indicate that the nature of the substituent attached to the 2-position of the imidazole ring (-Ph vs. -$CH_2NHC(=NH)NH_2$) influences interactions with the enzyme. If the imidazole rings of **3** and 2-phenylimidazole interact with the heme to a similar extent, then the enhanced activity of **3** compared to 2-phenylimidazole suggests the interaction of the guanidine group of **3** with the active site Glu371 residue. The activity of **3** appears structurally sensitive as chain elongation (Compound **4**) or side chain movement to the 4-position of the imidazole ring (Compounds **1-2**) abolishes NOS inhibition. The poor activity of **1**, **2**, and **4** indicates that neither the imidazole or guanidine groups of these compounds interact well with the enzyme. While X-ray crystallographic studies clearly indicate the ability of the active site to simultaneously accommodate two molecules of imidazole,[4b] the lack of activity of these compounds most likely arises from the improper orientation of the potential binding groups of these imidazole derivatives and subsequent active site steric crowding.

Interestingly, the L-arginine-derived 2-substituted imidazole with a single methylene group separating the ring and guanidine group (**7**) did not inhibit iNOS but displayed inhibition of both constitutive isoforms

(especially eNOS). Compared to other alkyl substituted L-arginine derivatives, compound **7** only weakly inhibits the NO synthases.[12] The isoform selectivity shift of **7** compared to **3** suggests that the amino acid portion of **7** (in addition to the guanidine group) participates in enzyme binding. These results also suggest that active site structural differences in the amino acid binding region of the different NOS isoforms may play an important role in determining selective inhibition. Separation of the imidazole ring and L-arginine portion by two methylene groups (**8**) or placement of the L-arginine portion of these molecules in the 4-position of the imidazole ring (**5-6**) diminished activity, again most likely due to active site steric limitations resulting from the improper alignment of binding groups. While the presented results do not clearly identify a compound capable of binding simultaneously to the heme and guanidine binding sites of NOS, they reveal a new isoform selective inhibitor and illustrate the possible importance of the L-arginine side chain in selective isoform recognition. Further synthetic studies of new imidazole containing molecules based upon the recent X-ray studies should provide further information regarding the isoform selective inhibition of the NO synthases.

General Procedure for the preparation of 1-4. A solution of the amino substituted imidazole and *N, N'*-bis-Boc-1-guanylpyrazole in dry THF was stirred for 12 hours and concentrated in vacuo to produce a crude product that was purified by flash chromatography (20:1:1 EtOAc:MeOH:TEA). Treatment of these white solids with HCl/dioxane (4 M, Aldrich) at 23 °C for 12 h under an argon atmosphere produced a solid after concentration in vacuo. This crude material was dissolved in H_2O, passed through a 6 mL Supelco LC-18 solid phase extractor, lyopholyzed, recrystallized from EtOH to yield the hydrochloride salts of compounds **1-4**. For **1**: mp = 216-218 °C; ^{1}H-NMR (D_2O, 200 MHz) δ 8.69 (s, 1H), 7.46 (s, 1H), 4.56 (s, 2 H); ^{13}C-NMR (D_2O, 50 MHz) δ 156.9, 134.4, 128.5, 117.2, 35.6; LRMS (FAB) *m/z* 140.1 $(M+H)^+$, HRMS (FAB) calcd for $C_5H_{10}N_5$ 140.0936, found 140.0930. Anal. Calcd for $C_5H_{11}Cl_2N_5$: C, 28.31; H, 5.22; N, 33.02. Found: C, 28.47; H, 5.28; N, 33.00. For **2**: mp = 217.0 - 219.0 °C; ^{1}H-NMR (D_2O, 200 MHz) δ 8.51 (s, 1H), 7.21 (s, 1H), 3.40 (t, J = 7 Hz, 2H), 2.91 (t, J = 7 Hz, 2H); ^{13}C-NMR (D_2O, 50 MHz) δ 156.7, 133.4, 129.9, 116.7, 39.9, 23.8; LRMS (FAB) *m/z* 154.1 $(M+H)^+$, HRMS (FAB) calcd for $C_6H_{12}N_5$ 154.1092, found 154.1087. Anal. Calcd for $C_6H_{13}Cl_2N_5$: C, 31.87; H, 5.79; N, 30.97. Found: C, 31.96; H, 5.86; N, 30.88. For **3**: ^{1}H-NMR (D_2O, 200 MHz) δ 7.37 (s, 2H), 4.82 (s, 2 H); ^{13}C-NMR (D_2O, 50 MHz) δ 157.3, 142.5, 119.5, 37.1; LRMS (FAB) *m/z* 140.1 $(M+H)^+$, HRMS (FAB) calcd for $C_5H_{10}N_5$ 140.0936, found 140.0931. Anal. Calcd for $C_5H_{11}Cl_2N_5$: C, 28.31; H, 5.22; N, 33.02. Found: C, 28.42; H, 5.27; N, 32.97. For **4**: ^{1}H-NMR (D_2O, 200 MHz) δ 7.26 (s, 2H), 3.51 (t, J = 7 Hz, 2 H), 3.15 (t, J = 7 Hz, 2 H); ^{13}C-NMR (D_2O, 50 MHz) δ 156.9, 143.9, 119.1, 38.9, 25.4; LRMS (FAB) *m/z* 154.1 $(M+H)^+$, HRMS (FAB) calcd for $C_6H_{12}N_5$ 154.1093, found 154.1099. Anal. Calcd for $C_6H_{13}Cl_2N_5$: C, 31.87; H, 5.80; N, 30.97. Found: C, 32.13; H, 5.86; N, 30.86.

General Procedure for the preparation of 5-8. A solution of **9** and the amino substituted imidazole in MeOH was stirred at 40 °C for 2-3 days and concentrated in vacuo to produce a crude product that was purified by flash chromatography (25:1 EtOAc:MeOH). Treatment of this material with HCl/dioxane (4 M, Aldrich) at 23 °C for 12 h under an argon atmosphere produced a solid after concentration in vacuo. This crude material was dissolved in H_2O, passed through a 6 mL Supelco LC-18 solid phase extractor, and lyopholyzed to yield the hydrochloride salts of compounds **5-8**. For **5**: $[\alpha]^{20}_D$ + 14.33 (c = 1.13, MeOH); ^{1}H-NMR (D_2O, 200 MHz) δ 8.55 (s, 1H), 7.30 (s, 1H), 4.43 (s, 2H), 3.94 (t, J = 6 Hz, 1H), 3.13 (t, J = 6 Hz, 2H), 1.82 (m, 2H), 1.59 (m, 2H); ^{13}C-NMR (D_2O, 50 MHz) δ 171.4, 155.5, 134.3, 128.4, 117.1, 52.4, 40.6, 35.5, 26.8, 23.7; LRMS (FAB) *m/z* 255.2 $(M+H)^+$, HRMS (FAB) calcd for $C_{10}H_{19}N_6O_2$ 255.1569, found 255.1561. Anal. Calcd for $C_{10}H_{21}Cl_3N_6O_2$ + 2.1 H_2O: C, 29.92; H, 6.33; Cl, 26.48; N, 20.93. Found: C, 29.95; H, 6.04; Cl, 26.78; N, 20.54. For **6**: $[\alpha]^{20}_D$ + 14.71 (c = 1.40, MeOH); ^{1}H-NMR (D_2O, 200 MHz) δ 8.41 (s, 1H), 7.11 (s, 1H), 3.91 (t, J = 6 Hz, 1H), 3.31 (t, J = 6 Hz, 2H), 3.01 (t, J = 6 Hz, 2H), 2.79 (t, J = 6 Hz, 2H), 1.75 (m, 2H), 1.49 (m, 2H); ^{13}C-NMR (D_2O, 50 MHz) δ 171.3, 155.4, 133.3, 129.8, 116.6, 52.3, 40.4, 39.9, 26.7, 23.8; LRMS (FAB) *m/z* 269.1 $(M+H)^+$, HRMS (FAB) calcd for $C_{11}H_{21}N_6O_2$ 269.1726, found 269.1723. Anal. Calcd for $C_{11}H_{23}Cl_3N_6O_2$ + 1 H_2O: C, 33.38; H, 6.36; Cl, 26.87; N, 21.23. Found: C, 33.38; H, 6.49; Cl, 27.09; N, 20.35. For **7**: $[\alpha]^{20}_D$ + 16.34 (c = 1.26, MeOH); ^{1}H-NMR (D_2O, 200 MHz) δ 7.27 (s, 2H), 4.73 (s, 2H), 3.94 (t, J = 6 Hz, 1H), 3.16 (t, J = 6 Hz, 2H), 1.80 (m, 2H), 1.62 (m, 2H); ^{13}C-NMR (D_2O, 50 MHz) δ 171.8, 156.2, 142.7, 119.5, 52.6, 40.9, 37.2, 27.0, 23.7; LRMS (FAB) *m/z* 255.2 $(M+H)^+$, HRMS (FAB) calcd for $C_{10}H_{19}N_6O_2$ 255.1569, found 255.1578. Anal. Calcd for $C_{10}H_{21}Cl_3N_6O_2$ + 2.1 H_2O: C, 29.92; H, 6.33; Cl, 26.48; N, 20.93. Found: C, 30.35; H, 6.26; Cl, 26.88; N, 20.44. For **8**: $[\alpha]^{20}_D$ + 13.62 (c = 2.59, MeOH); ^{1}H-NMR (D_2O, 200 MHz) δ 7.23 (s, 2H), 3.94 (t, J = 6 Hz, 1H), 3.51 (t, J = 6 Hz, 2H), 3.13 (m, 4H), 1.81 (m, 2H), 1.57 (m, 2H); ^{13}C-NMR (D_2O, 50 MHz) δ 171.6, 155.5, 143.8, 119.1, 52.6, 40.5, 38.9, 26.9, 25.5, 23.9; LRMS (FAB) *m/z* 269.2 $(M+H)^+$, HRMS (FAB) calcd for $C_{11}H_{21}N_6O_2$ 269.1726, found 269.1715. Anal. Calcd for $C_{11}H_{23}Cl_3N_6O_2$ + 2 H_2O: C, 31.93; H, 6.57; Cl, 25.71; N, 20.31. Found: C, 32.18; H, 6.76; Cl, 26.12; N, 19.98.

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