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1*H*-Pyrazole-1-carboxamidines: New Inhibitors of Nitric Oxide Synthase

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Abstract—1*H*-Pyrazole-1-carboxamidines were prepared as potential inhibitors of the three isozymes of nitric oxide synthase. All of the compounds were found to be competitive inhibitors of all three isoforms. The most selective compound prepared was 1*H*-pyrazole-*N*-(3-aminomethylanilino)-1-carboxamidine (**14**), which is 100-fold selective for nNOS over eNOS with a K_i value of 2 μM.

Introduction

A family of enzymes known as nitric oxide synthase (NOS) catalyze the oxidation of L-arginine to L-citrulline and nitric oxide (NO), a molecule that plays an important role in the regulation of blood pressure, neurotransmission, and the immune response.[1] Three isoforms of nitric oxide synthase have been identified in different tissues. Neuronal (nNOS) and endothelial (eNOS) nitric oxide synthase are expressed constitutively and are Ca^{++} ion- and calmodulin-dependent for activity; inducible macrophage (iNOS) nitric oxide synthase is expressed by inflammatory stimuli and is independent of calmodulin. The normal biological functions regulated by NO are attributed to those NOS isoforms. However, an excess or deficiency of nitric oxide produced by NOS isoforms has been implicated in a variety of diseases, such as septic shock,[2] inflammatory arthritis,[3] schizophrenia,[4] Alzheimer's disease,[5] impotence,[6] and susceptibility to infection.[7] Therefore, selective inhibition of NOS isoforms would be beneficial in cases when an excess of NO is produced as well as to define the roles of each NOS isoform in biological systems.

A wide variety of inhibitors of NOS are known,[8] and some of the earliest inhibitors are N^{ω}-substituted-L-arginines, such as methylarginine[9] and nitroarginine.[10] We recently reported that N^{ω}-propyl-L-arginine was a competitive inhibitor and also an inactivator of nNOS.[11] This compound was found to have about a 3000-fold selectivity for nNOS over iNOS and 150-fold selectivity of nNOS versus eNOS, unusually large selectivities compared to other analogues of L-arginine.[12] In an effort to design selective inhibitors of NOS based on the other known L-arginine-derived inhibitiors of NOS, a library of N^{ω}-nitroarginine-containing dipeptides (or dipeptide analogues) was synthesized by varying the chirality and the order of amino acids. Several of the compounds from this library showed selectivities higher than 1000-fold in favor of one or two isozymes over the others.[13] These studies suggested that selective inhibition of NOS via substrate modification is an achieveable goal.

In our continued efforts to examine other compounds for inhibition of NOS, a series of 1*H*-pyrazole-1-carboxamidines was synthesized, and the inhibition by each of these compounds with the three isozymes of NOS was investigated.

Chemistry

Treatment of alkylcyanamides **1a**–**g** with pyrazole in 4 N HCl/dioxane produced a series of 1*H*-pyrazole-1-carboxamidines **2a**–**g**,[14] as shown in Scheme 1. To determine the effect of methylation of the pyrazole ring in **2** on enzyme binding, 3-methyl-, 4-methyl-, and 3,5-dimethylpyrazole (**3a**–**c**, respectively) were treated with cyanamide

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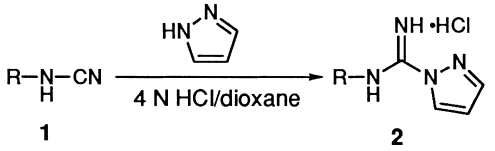
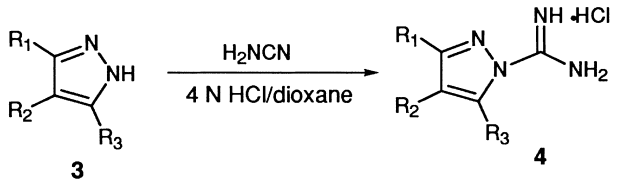


1 2

a, R = H
b, R = propyl
c, R = butyl
d, R = pentyl
e, R = cyclopropyl
f, R = cyclopropylmethyl
g, R = isobutyl

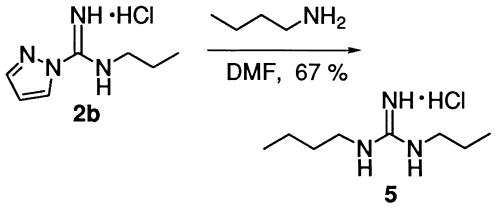
Scheme 1.



3 4

a, $R_1 = CH_3$, $R_2 = H$, $R_3 = H$
b, $R_1 = H$, $R_2 = CH_3$, $R_3 = H$
c, $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$

Scheme 2.



2b 5

Scheme 3.

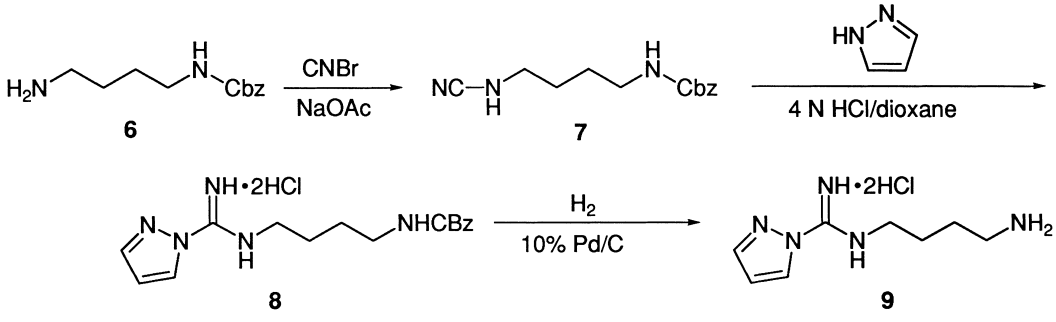
in acidic media to afford **4a**–**c**, respectively (Scheme 2). *N*-Butyl-*N*′-propylguanidine (**5**) was prepared in a 67% yield by condensation of guanylating agent **2b** with excess *n*-butylamine, as described in Scheme 3; this compound was made to determine if the pyrazole ring was important to binding.

1*H*-Pyrazole-*N*-(4-aminobutyl)-1-carboxamidine (**9**) was synthesized from mono *N*-Cbz-protected 1,4-diaminobutane (**6**) in three steps as outlined in Scheme 4. Treatment of **6** with cyanogen bromide and sodium acetate in methanol afforded cyanamide **7** in 73% yield. Subsequent condensation of **7** with pyrazole in 4 N HCl/dioxane gave **8** followed by hydrogenolysis of the *N*-Cbz protecting group provided the desired **9** in 82% yield for the last two steps.

Scheme 5 illustrates the synthetic route to 1*H*-pyrazole-*N*-(3-aminomethylanilino)-1-carboxamidine (**14**). Commercially available 3-nitrobenzylamine (**10**) was treated with acetyl chloride in the presence of triethylamine to afford *N*-acetyl-3-nitrobenzylamine in an 83% yield; reduction of the nitro group under catalytic hydrogenation conditions (H_2, 10% Pd/C) provided the desired aniline analogue **13** (92%). In a similar manner as described above, **11** was converted to cyanamide **12** (78%), which was subsequently condensed with pyrazole in 4 N HCl/dioxane to give rise to carboxamidine **13** (61%). Deprotection of the *N*-acetyl group of **13** was achieved in refluxing 6 N HCl for 30 min to give **14** in a 72% yield.

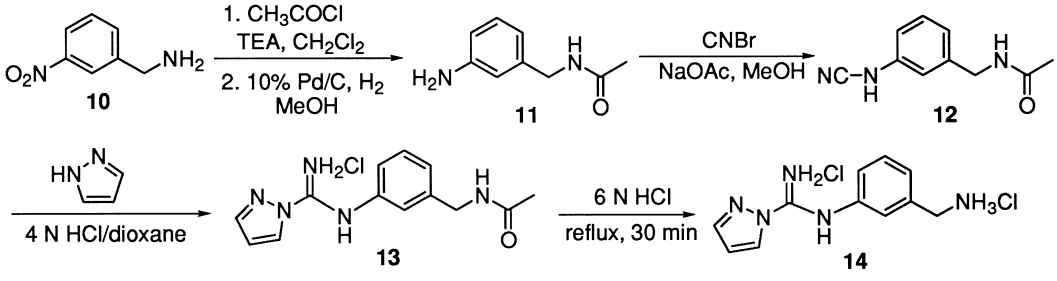
Results and Discussion

Compounds **2**, **4**, **5**, **9**, and **14** were evaluated as inhibitors of the three isoforms of NOS using the hemoglobin capture assay.[15] All of the NOS isoforms used were



6 7 8 9

Scheme 4.



10 11 12 13 14

Scheme 5.

recombinant enzymes overexpressed in *Escherichia coli* from difference sources; there is very high sequence identity for the isoforms from different sources. The murine macrophage iNOS was expressed and isolated according to the procedure of Hevel et al.[16] The rat neuronal NOS was expressed[17] and purified as described.[18] The bovine endothelial NOS was isolated as reported.[19] The 1*H*-pyrazole-1-carboxamidines (**2**) exhibit inhibitory activity against NOS. As summarized in Table 1, 1*H*-pyrazole-1-carboxamidine (**2a**) was found to be the most potent inhibitor of NOS among the series of compounds tested in this study. However, the isoform selectivity of **2a** was insignificant. Substitution of straight-chain alkyl groups (**2b**, **2c**, and **2d**) on the nitrogen resulted in decreased potency compared to **2a**. The decrease in potency was higher as the chain length of the alkyl group increased from propyl to pentyl, suggesting a steric hindrance to binding. There is a slight decrease in potency for the compounds with cyclo- or branched alkyl groups such as cyclopropyl (**2e**) (compared with **2b**) and cyclopropylmethyl (**2f**) and isobutyl (**2g**) (compared with the corresponding butyl analogue **2c**), again indicating a steric hindrance to binding.

Because of the reactivity of 1*H*-pyrazole-1-carboxamidines as guanylating agents, it was suspected that they may be inactivators as well as inhibitors. As a representative example, **2b** was shown to exhibit time-dependent inhibition of nNOS with a $K_i = 33\,\mu M$ and a $k_{inact} = 0.024\,min^{-1}$. The site of reactivity is under investigation.

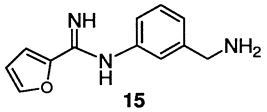
To investigate steric effects on the pyrazole ring, several methylated pyrazole analogues were made (**4a**–**c**). Appending methyl groups to the 3-, 4-, or 5-position of the pyrazole ring results in significant loss of inhibitory activity for all of the isoforms of NOS compared to the unsubstituted counterpart **2a**. The disubstituted analogue **4c** showed a dramatic decrease in potency (ca. 1000-fold) compared to its parent compound **2a**. This drop in potency for all isoforms is presumed to be steric in nature; eNOS is the most sensitive to methyl substitution on the pyrazole ring. Replacement of the pyrazole ring of **2b** with butylamine suffered about a 100-fold decrease in potency.

Table 1. Inhibition of NOS by 1*H*-pyrazole-1-carboxamidines

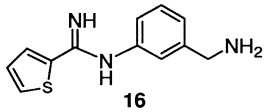
	K_i (μM)			Selectivity	
Compound	nNOS	iNOS	eNOS	nNOS/iNOS	nNOS/eNOS
2a	0.4	0.7	0.5	1.8	1.3
2b	3	5	9	1.7	3
2c	14	14	25	1	1.8
2d	60	50	700	0.8	14
2e	50	80	120	1.6	2.4
2f	10	22	80	2.2	8
2g	45	150	180	3.3	4
4a	8	9	100	1.1	13
4b	12	3	190	0.3	16
4c	350	750	1500	2.1	4.3
5	700	780	650	1.1	0.9
9	6	50	460	8.3	77
14	2	12	200	6	100
15[a]	0.0063	0.16	0.35	25	56
16[a]	0.0087	0.12	0.46	14	46

[a]Data taken from ref 16.

We found earlier that nitroarginine-containing dipeptides in which the other amino acid has an amino-containing side chain are quite potent and selective nNOS inhibitors.[13b] To mimic the potential binding of a side-chain amino group, **9** was synthesized, which was found to be 10 times more potent at inhibiting nNOS ($K_i = 6\,\mu M$) than its methyl isostere (i.e., **2d**, $K_i = 60\,\mu M$), again suggesting an important interaction of the side-chain amino group with nNOS. It also exhibited one of the highest selectivities (77-fold) of the compounds tested in this study. The conformationally-restricted analogue of **9**, namely **14**, also was synthesized. This compound was one of the more potent inhibitors of nNOS of the compounds studied here and was the most selective, 100-fold selective, for nNOS versus eNOS. A similar observation to our result was found in a report by the Glaxo Wellcome group in which the aryl amidine analogues **15** and **16** were selective for nNOS over iNOS and eNOS, although they were much more potent than **14** (Table 1).[20]



15



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Conclusion

1*H*-Pyrazole-1-carboxamidines exhibit only moderate potency and no selectivity for the NOS isozymes. Modification of the side chain by the addition of an amino group increases potency and selectivity for nNOS over iNOS and eNOS. These compounds show time-dependent inhibition, presumably the result of their guanylating reactivity.

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