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Bicyclic amidine inhibitors of nitric oxide synthase: discovery of perhydro-iminopyrindine and perhydro-iminoquinoline as potent, orally active inhibitors of inducible nitric oxide synthase

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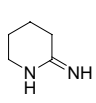
Abstract—Syntheses and nitric oxide synthase inhibitory activity of cyclic amidines containing 5,6- 6,6- and 7,6-fused systems are described. X-ray structure determination facilitated the assignment of the stereochemistry of the most active compounds perhydro-2-iminoisoquinoline (**8a**) and perhydro-2-iminopyrindine (**10a**). Both **8a** and **10a** are very potent inhibitors of iNOS, with excellent selectivity over eNOS and they are orally active in rats with long duration suitable for once or twice a day dosing.

The physiological roles of nitric oxide (NO), a reactive, free radical gas, have been extensively studied in many laboratories in recent years.[1] In mammalian systems NO is produced by a two-step oxidation of the terminal guanidine group of L-arginine by nitric oxide synthase (NOS).[2] Three distinct NOS isoforms have been identified in humans. The activities of the two constitutive enzymes, neuronal NOS (nNOS, or NOS-1) and endothelial NOS (eNOS or NOS-3) are regulated by Ca^{2+} calmodulin complex and they play important roles in neurotransmission and blood pressure regulation, respectively. The third isoform, inducible NOS (iNOS or NOS-2) is expressed in many cell types after stimulation by numerous inflammatory mediators such as cytokines and endotoxin. The NO produced by iNOS is thought to be critical in host defense mechanisms such as killing of intracellular pathogens.[3] The biological roles for individual NOS enzymes have been verified by producing transgenic mice lacking the gene for the respective NOS isoforms.

NO may also be involved in certain pathophysiological conditions. Since iNOS is induced by inflammatory stimuli which results in sustained production of NO by inflammatory cells, it has been suggested that iNOS could be responsible for the tissue damage observed in chronic inflammation.[4] NO derived from iNOS may also be involved in the organ damage and rapid drop of blood pressure seen in septic shock.[5] It is hoped that a selective iNOS inhibitor may be able to stop or reduce the NO mediated damage in such conditions and may be clinically useful in the treatment of septic shock and inflammatory diseases.



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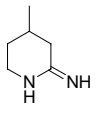

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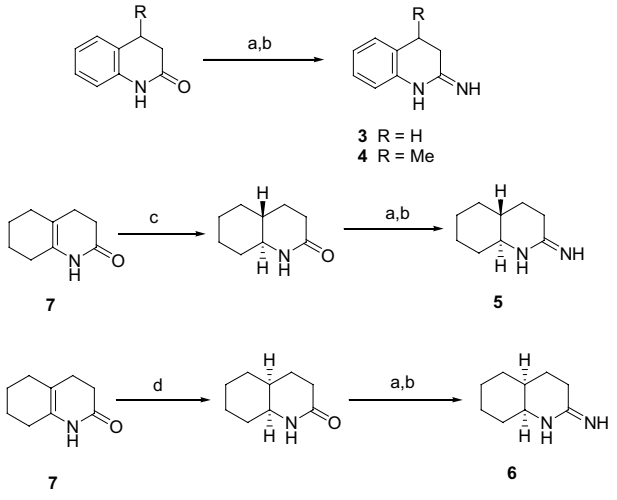
Many different types of NOS inhibitors have been described in the past few years. Some of them are analogs of the substrate L-arginine.[6] Potent non-amino acid inhibitors from isothiourea and amidine classes have also been reported.[7] We have been interested in potent and selective iNOS inhibitors with good pharmacokinetic properties that will allow their evaluation in animal models of inflammation.[8] Previously 2-aminopyridines as NOS inhibitors have been described from these laboratories wherein methyl substitution at C-4 and C-6 of the pyridine ring improved activity.[9] Selective nNOS inhibitors containing this scaffold were recently reported.[10]

The 2-aminopyridine leads were reduced to their saturated analogs 2-iminopiperidine (**1**) and 4-methyl-2-iminopiperidine (**2**) which are more active inhibitors.[11] Concurrently compounds **1** and **2** were also independently discovered as orally active NOS inhibitors.[7d,e] Bicyclic amidines were targeted to improve on the selectivity of **2**. It was hoped that by presenting the amidine pharmacophore in a more rigid framework subtle differences in the active sites of the three enzymes might be exploited. The synthesis and activity of such fused 2-iminopiperidines is reported herein.



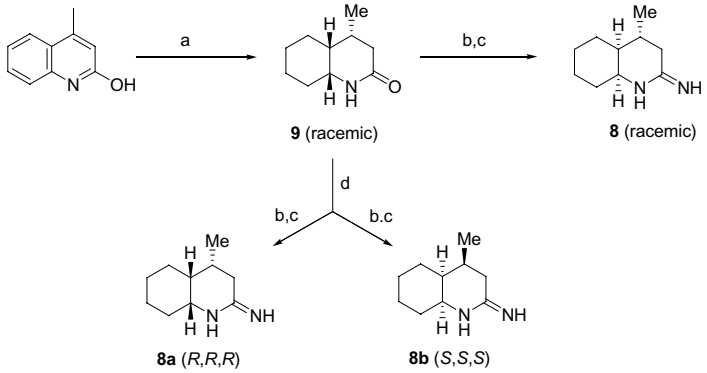
Scheme 1. Reagents and conditions: (a) Me_3OBF_4, CH_2Cl_2, rt; (b) NH_4Cl, EtOH, 80 °C; (c) HCOONa, HCOOH, 100 °C; (d) H_2, Pd/C, dioxane, ethanol.

The desired bicyclic amidines were prepared from the corresponding lactams via their imino ethers (Scheme 1). Thus, commercially available 3,4-dihydroquinolone was converted to its methyl imino ether by reaction with trimethyloxonium fluoroborate and the imino ether was subsequently treated with NH_4Cl in refluxing ethanol to afford the hydrochloride of amidine **3** as a crystalline solid. Similarly 4-methyl-3,4-dihydroquinolone was converted to **4**.

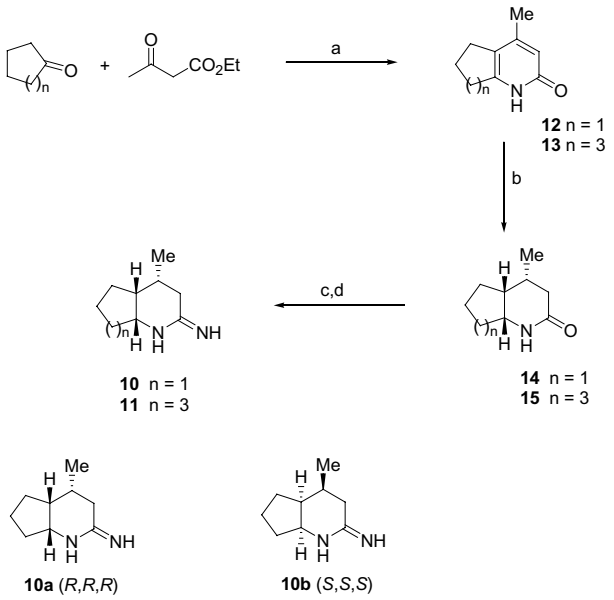
In order to prepare the saturated analogs **5** and **6**, hexahydro-2(1*H*)-quinolone (**7**) was first reduced by reaction with sodium formate in refluxing formic acid to afford a 9:1 mixture of *trans* and *cis* lactams, which were separated by chromatography.[12] The *trans* lactam was converted to amidine **5** via its imino ether. The *cis* lactam was more conveniently obtained by catalytic hydrogenation of **7** using 10% Pd/C[13] which was then carried through the two steps to give **6**.

The methyl substituted analog **8** was synthesized by hydrogenation of 2-hydroxy-4-methylquinoline (Scheme 2). In this case more forcing conditions (PtO_2 in acetic acid) were required to saturate the phenyl ring. The reduction was highly stereoselective and only the *cis*,*syn* isomer, **9**, was isolated. Reaction of **9** with Meerwein's reagent and amidine formation with NH_4Cl yielded **8**. Since **8** exhibited good activity and selectivity (see below) the individual enantiomers **8a** and **b** were prepared. It was found that the racemic lactam **9** could be separated into the two enantiomers (slower, **9a** and faster, **9b**) on a Chiralcel OD column using 10% isopropanol/hexane as eluent. Each isomer was then converted to the amidine (**8a**[14a] and **8b**) as described. The stereochemistry of **8a** (α_D −12.5 (*c* 0.2, EtOH)) derived from the slower lactam isomer (**9a**) was determined to be 4(*R*),4a(*R*),8a(*R*) by X-ray crystallography.[15]

The 5,6 fused analog **10**, and 7,6 fused analog **11**, were synthesized from cyclopentanone and cycloheptanone, respectively, as shown in Scheme 3. Condensation of cyclopentanone with ethyl acetoacetate and NH_4OAc by the method of Sakurai furnished **12**,[16] which was



Scheme 2. Reagents and conditions: (a) H_2, PtO_2, HOAc; (b) Me_3OBF_4, CH_2Cl_2, rt; (c) NH_4Cl, EtOH, 80 °C; (d) resolution on Chiralcel OD column, 10% isopropanol/hexane.



Scheme 3. (a) NH_4OAc, EtOH, 80 °C; (b) H_2, PtO_2, HOAc; (c) Me_3OBF_4, CH_2Cl_2, rt; (d) NH_4Cl, EtOH, 80 °C.

hydrogenated using PtO_2 in acetic acid. The reduction in this case was also highly stereoselective and only **14** (*cis*, *syn*) was isolated. This lactam was converted to the racemic amidine **10** via its imino ether. The individual enantiomers **10a** and **b** were obtained after resolution of **14** on Chiralcel OD column and the slower lactam, **14a**, gave **10a**[14b] (α_D −54.5 (*c* 0.2, EtOH)). The stereochemistry of **10a** as 4(*R*),4a(*R*),7a(*R*), was also confirmed by X-ray crystallography.[15] Analogous condensation of cycloheptanone with ethyl acetoacetate followed by reduction gave **15**, which was converted to racemic amidine **11** in two steps.

The inhibitory activities of these amidines against the three recombinant human NOS isoforms were measured by following the conversion of ^{3}H-arginine to ^{3}H-citrulline as previously described.[17] The observed NOS IC_{50} are listed in Table 1 and from these values selectivity for eNOS and nNOS defined as IC_{50}(eNOS)/IC_{50}(iNOS) and IC_{50}(nNOS)/IC_{50}(iNOS), respectively, were calculated and are shown in Table 1. Since the constitutive enzymes seems to be involved in important physiological processes our aim was to find a potent and selective iNOS inhibitor.

Although the benzo fused analogs **3** and **4** lost activity against all three enzymes; they had better eNOS selectivity than **1** and **2**. The selectivity was even better for the saturated cyclohexane fused amidines **5** and **6**. The *cis* isomer **6** (iNOS IC_{50} = 186 nM) was as good iNOS inhibitor as **1** but was 20-fold less active against eNOS. As we had observed for **1** and **2**, the activity of **6** was further improved by introduction of C-4 methyl (**8**, iNOS IC_{50} = 38 nM) and the selectivity was retained. Since **8** was a mixture of two enantiomers, it was resolved and the *R*,*R*,*R* isomer **8a** (iNOS IC_{50} = 22 nM,) was responsible for most of the inhibitory activity. Interestingly, the enantiomer **8b** was a selective nNOS inhibitor but it is not very potent. The 5,6-fused system, **10**, was somewhat more active than **8** but **11** with a fused cycloheptane ring lost activity against all three enzymes indicating a sterically restricted binding site. Separation of the enantiomers of **10** again provided the potent *R*,*R*,*R* isomer **10a** with iNOS IC_{50} = 9 nM. However, in this case the enantiomer, **10b** (iNOS IC_{50} = 133 nM) still had significant activity possibly due to its smaller size. Both **8a** and **10a** were 35-40x selective for iNOS over eNOS, indicating that the initial hypothesis about using a rigid bicyclic framework to discriminate between the active sites is probably valid in this case. The binding region for nNOS seems to be very similar to iNOS and it was very difficult to achieve selectivity in this series. This was also true for the monocyclic amidines. Nevertheless, the best compounds **8a** and **10a**, were 2-fold selective, while the leads **1** and **2** were more powerful inhibitors of nNOS than iNOS.

Table 1. NOS inhibitory activity and selectivity of bicyclic amidines

Compound	iNOS IC_{50} (nM)[a]	eNOS IC_{50} (nM)[a]	nNOS IC_{50} (nM)[a]	Selectivity[b] eNOS	Selectivity[c] nNOS
1	300	630	240	2.1	0.8
2	37	146	16	3.9	0.43
3	14,000	85,000	4900	6.1	0.35
4	974	15,300	240	15.7	0.25
5	528	5500	530	29.6	1
6	186	12,500	246	67	1.3
8	38	2000	190	52	5
8a	22	830	53	37	2.4
8b	>5000	>5000	610	ND[d]	ND[d]
10	15	470	31	31	2.1
10a	9	360	21	40	2.3
10b	133	870	36	6.5	0.27
11	860	4500	1300	5.2	1.5

[a] Inhibitory activity for human iNOS, eNOS, and nNOS was measured as described in Ref. 17 with a final L-arginine concentration of 1 μM. For each compound, the percent inhibition was determined in duplicate at 10 different concentrations and IC_{50} was calculated using SIGMAPLOT.
[b] Selectivity against eNOS is the ratio of eNOS IC_{50}/iNOS IC_{50}.
[c] Selectivity against nNOS is the ratio of nNOS IC_{50}/iNOS IC_{50}.
[d] ND = not determined.

Table 2. In vivo activity of NOS inhibitors in LPS treated rats

Compound	Plasma NO_x inhibition ED_{50}, mg/kg iv[a]
2	0.5
8a	0.19
10a	0.25

[a] The ability of test compounds to inhibit elevation of plasma nitrate/nitrite (NO_x) level following LPS challenge to rats. The drug was administered iv to the animals 2 h after LPS challenge and plasma was collected at 5 h after LPS. Total nitrate/nitrite levels in plasma were determined by first converting nitrate to nitrite by incubation with nitrate reductase followed by fluorometric measurement of nitrite as described in Ref. 18b. ED_{50} is the estimated dose causing 50% reduction in plasma nitrite relative to control animals.

Table 3. Duration of oral activity of **7a** and **8a** in rats

Time (h)[a]	%*I* with **8a**[b]	%*I* with **10a**[b]
0	78	99
−1	57	95
−2	69	80
−4	74	52
−16	42	39

[a] Time of dosing animals prior to LPS challenge.
[b] % Inhibition of plasma nitrite/nitrate following po administration of 10 mg/kg of the test compound to rats.

The ability of **8a** and **10a** to inhibit iNOS activity in vivo was measured in a rat endotoxin assay. It has been shown that the increase in plasma nitrate/nitrite (NO_x) levels following LPS administration to animals is mediated by iNOS and this model has been used to evaluate other iNOS inhibitors.[18] The results from an iv study are listed in Table 2. Both **8a** and **10a** are 2-fold more active than **2**, when they were dosed 2 h after LPS challenge. To assess the oral bioavailability and pharmacokinetics of these agents a time course of activity was performed. Thus 10 mg/kg po dose of the drug was administered to rats at various times before LPS challenge and the inhibition in plasma NO_x levels measured 5 h after LPS challenge is shown in Table 3. Both **8a** and **10a** were orally efficacious and at early times (0 and 1 h before LPS) almost complete inhibition was achieved with **10a**. The activity was sustained and when dosed 16 h before LPS, both **8a** and **10a** reduced plasma nitrite by 40% with a 10 mg/kg po dose. This result clearly demonstrated that high levels of **8a** and **10a** can be maintained in circulation for 16–20 h and therefore they are suitable for once or twice a day dosing for chronic studies in rats.

In summary, modification of the 2-iminopiperidine has lead to the identification of perhydro-iminoquinoline, **8a**, and perhydro-iminopyrindine, **10a**, as very potent iNOS inhibitors. The stereochemistry of the active enantiomer in each case was confirmed by X-ray crystallography. Both **8a** and **10a** are much poorer inhibitors of eNOS and are orally bioavailable in rats. Their potency against iNOS, selectivity, and pharmacokinetic properties make them useful tools to evaluate the role of iNOS in models of chronic inflammation.

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14. (a) Compound **8a**: ^{1}H NMR (CD_3OD): δ (ppm) 1.05 (d, J = 7 Hz, 3H), 1.1–2.2 (m, 10H), 2.30 (dd, J = 19 and 12 Hz, 1H), 2.63 (dd, J = 19 and 6 Hz, 1H), 3.72 (d, J = 3 Hz, 1H); ^{13}C NMR (CD_3OD): δ (ppm) 16.93, 18.55, 19.96, 24.58, 29.34, 29.59, 29.98, 37.71, 53.5, 167.63. (b) Compound **10a**: ^{1}H NMR (CD_3OD): δ (ppm) 1.07 (d, J = 7 Hz, 3H), 1.4–2.4 (m, 9H), 2.50 (dd, J = 18 and 5 Hz, 1H), 3.95 (t, J = 6 Hz, 1H); ^{13}C NMR (CD_3OD): δ (ppm) 18.05, 22.21, 22.49, 26.15, 28.17, 33.40, 41.38, 57.73, 166.71.
15. Crystallographic data (excluding structure factors) for **8a** and **10a** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 260319 for **8a** and CCDC 260320 for **10a**. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax:+44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].
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