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Design and Synthesis of Orally Bioavailable Inhibitors of Inducible Nitric Oxide Synthase. Part 1: Synthesis and Biological Evaluation of Dihydropyridin-2-imines

Yasufumi Kawanaka,[a,*] Kaoru Kobayashi,[b] Shinya Kusuda,[b] Tadashi Tatsumi,[b] Masanori Murota,[b] Toshihiko Nishiyama,[b] Katsuya Hisaichi,[b] Atsuko Fujii,[b] Keisuke Hirai,[b] Masao Naka,[b] Masaharu Komeno,[b] Hisao Nakai[b] and Masaaki Toda[b]

[a]*Fukui Research Institute, Ono Pharmaceutical Co., Ltd., Technoport, Yamagishi, Mikuni, Sakai, Fukui 913-8538, Japan*
[b]*Minase Research Institute, Ono Pharmaceutical Co., Ltd., Shimamoto, Mishima, Osaka 618-8585, Japan*

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Abstract—Dihydropyridin-2-imines were synthesized and biologically evaluated both in vitro and in vivo using a nitric oxide inhibition assay. Compounds **1**, **4**, **5** and **7**–**11** exhibited potent activity in the inducible nitric oxide (iNOS) inhibition assay. Of these **5**, **6**, **9** and **10** showed 5- to 11-fold increases in isoform selectivity. Compounds **1**, **5**, **9** and **10** showed potent inhibitory activity in the NOx accumulation assay in mice. Compounds **1** and **5** also showed good bioavailability (BA) when given orally.

Introduction

Nitric oxide (NO) is a mediator of normal and pathophysiological processes.[1–3] NO is synthesized by two major classes of nitric oxide synthase (NOS): constitutive and inducible. The constitutive enzymes require Ca^{2+}/calmodulin for their activity and are further divided into the neuronal and endothelial isoforms. The endothelial isoform (eNOS) is found predominantly in the vascular endothelium and it produces low concentrations of NO, which reduce the blood pressure and inhibit platelet aggregation. NO produced by the neuronal enzyme (nNOS) appears to function as a neurotransmitter which regulates neuronal transmission. The inducible isoform is found in activated macrophages as well as many other cells and it produces NO, which plays a role in the host defense system. The overexpression of NO which destroys functional tissue during acute and chronic inflammation is caused by this inducible NOS (iNOS). Identification of potent and selective inhibitors for the above-mentioned iNOS has been the subject of intense interest because of their therapeutic potential in the treatment of diseases mediated by the overexpression of NO.[4,5] The natural substrate for NOS, arginine, has been the obvious basis for the molecular design of NOS inhibitors.

Structural analogues of L-arginine such as N^G-methyl-L-arginine (L-NMA),[6,7] N^G-nitro-L-arginine,[8] *N*-iminoethyl-L-ornithine (L-NIO),[9] and L-thiocitrulline[10] have been shown to inhibit the various forms of NOS. Much pharmacology and biology data have been determined utilizing some of the early relatively nonspecific inhibitors of arginine analogues as described above. A number of non-amino acid inhibitors of iNOS such as aminoguanidine,[11] isothioureas,[12] 2-amino-5,6-dihydro-6-methyl-*4H*-1,3-thiazine,[13] 2-iminopiperidine[14] and 2-aminopyridine[15] have also been described. As illustrated in Chart 1, our molecular design started with a structural hybridization of 2-amino-4-methylpyridine **13**[15] and (4*R*)-4-methylpiperidin-2-imine **14**.[14] This modification led us to the discovery of 4-methyl-5,6-dihydropyridin-2-imine **1**, which is structurally new and possesses no asymmetric center. A series of 5,6-dihydropyridin-2-imines were synthesized and biologically evaluated with the expectation of identifying a new selective inhibitor of human iNOS (hiNOS). We now report a series of 5,6-dihydropyridin-2-imines which are structurally new inhibitors of iNOS.

*Corresponding author. Tel.: +81-776-82-6161; fax: +81-776-82-8420; e-mail: kawanaka@ono.co.jp

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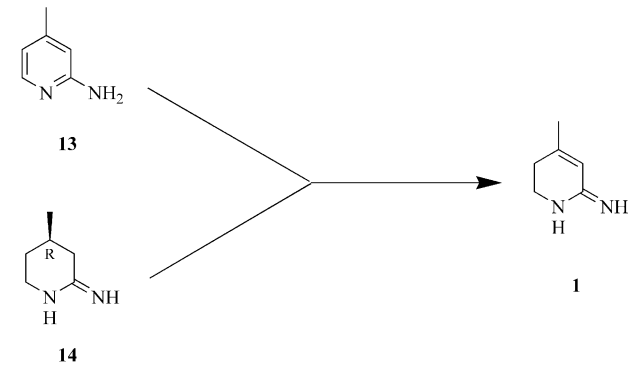
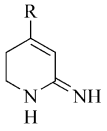
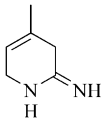
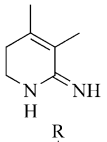
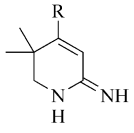
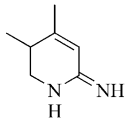
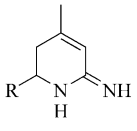
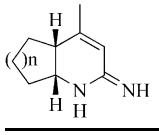


Chart 1. Molecular design of 5,6-dihydropyridin-2-imine.

Table 1. Inhibitory activities of **1**–**12** against hiNOS and heNOS and their isoform selectivity[e]

Structure	Compd (synthetic method)	hiNOS IC_{50} (μM)[a]	heNOS IC_{50} (μM)[a]	Selectivity heNOS/hiNOS[b]
	**1** R = Me (A) **2** R = Et[c]	0.20 1.2	0.23 1.5	1.2 1.3
	**3**[c]	0.70	0.44	0.6
	**4** (B)	0.31	0.32	1.0
	**5** R = Me (B) **6** R = H (C)	0.42 2.8	2.1 31.0	5.0 11.1
	**7** (B)[d]	0.51	0.52	1.0
	**8** R = Me (A)[d] **9** R = *n*-Pr (A)[d] **10** R = Allyl (A)[d]	0.18 0.06 0.05	0.24 0.58 0.36	1.3 9.7 7.2
	**11** *n* = 1 (A)[d] **12** *n* = 2 (A)[d]	0.17 1.4	0.51 7.4	3.0 5.3

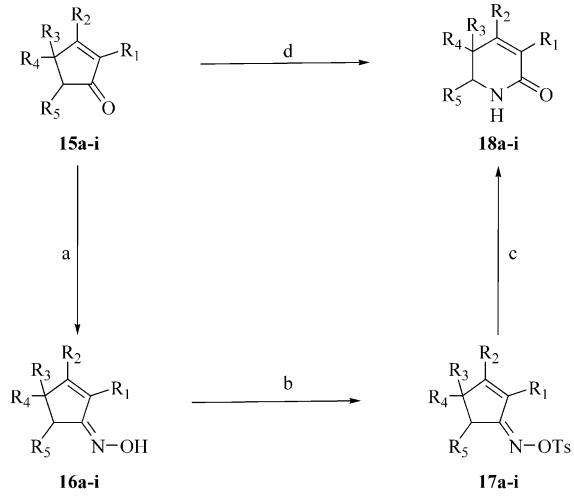
[a]Preparation of partially purified enzyme and determination of K_i values. Human eNOS was overexpressed in Sf-21 cells, by infecting the cells with baculovirus carrying heNOS cDNA. The hiNOS was overexpressed in A549 by stimulation with LPS (10 μg/mL) plus cytokines (10 ng/mL TNF-α, 5 ng/mL IL-1β and 100 ng/mL interferon-γ). Human eNOS and iNOS were partially purified by chromatography on 2′, 5′-ADP-Sepharose gels. NOS activity was determined by the method for the conversion of [^{14}C]-L-arginine to L-citrulline with a minor modification. The conversion rates for various concentrations of the test compounds and L-arginine were measured. Dixon and Lineweaver–Bulk plots were constructed to determine the K_i values and the mode of inhibition.
[b]Selectivity was evaluated as the rate of the IC_{50} values for heNOS and hiNOS.
[c]Compounds **2** and **3** were prepared according to a modified Birch reduction of 2-amino-4-methylpyridine and 2-amino-4-ethylpyridine, respectively.[16]
[d]Synthesized as a mixture of racemates.
[e]All compounds were prepared as their hydrochlorides.

Chemistry

Synthesis of the compounds described in Table 1 is outlined in Schemes 1–3. As described in Scheme 1, the common intermediates, substituted 5,6-dihydropyridin-2-ones **18a**–**i**, were prepared by the conventional Beckmann rearrangement reaction of oximes prepared from the corresponding cyclopentenones **15a**–**i** via **16a**–**i** and **17a**–**i** (Method A: 22–48% yield) or directly from **15a**–**i** (Method B: 44–60% yield), respectively. The common intermediates **18a**–**i** were used to prepare **1** and **8**–**12** as well as **4**–**5** and **7** according to Methods A and B, respectively. As described in Scheme 2, another intermediate **21** used to prepare **6** was obtained by the oxidation of **20** with a 70% yield, which was prepared by the Beckmann rearrangement of an oxime prepared from the cyclopentanone **19** with a 52% yield (Method C). As described in Scheme 3, **18a**–**i** and **21** were converted to the test compounds **1** and **4**–**12** (8–70% yield) via their corresponding ethyl imidates **22a**–**i** and **23**, respectively.

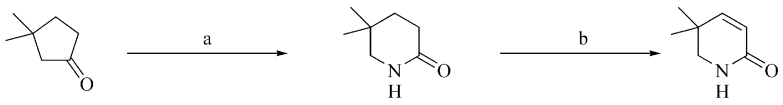
Results and Discussion

As described in Table 1, the compounds were evaluated for their ability to inhibit the two isoforms of human NOS. As expected, 4-methyl-5,6-dihydropyridin-2-imine **1** demonstrated potent inhibitory activity against both the human iNOS (hiNOS) and human eNOS (heNOS) isoforms without any selectivity.



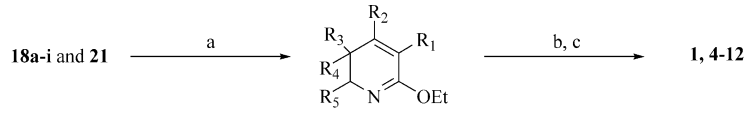
Scheme 1. Synthesis of 5,6-dihydropyridin-2-ones. Method A: (a) NH_2OH·HCl, NaOAc, MeOH, reflux; (b) *p*-TsCl, pyridine, 0 °C; (c) concd HCl, MeOH, refux (**18a**, **18e**–**i**: 22–48% in three steps); Method B: (d) NaN_3, TFA, reflux (**18b**–**d**: 44–60%).

Compd	R_1	R_2	R_3	R_4	R_5
a	H	Me	H	H	H
b	Me	Me	H	H	H
c	H	Me	Me	Me	H
d	H	Me	(H,Me)		H
e	H	Me	H	H	Me
f	H	Me	H	H	*n*-Pr
g	H	Me	H	H	Allyl
h	H	Me	H	–$(CH_2)_3$–	
i	H	Me	H	–$(CH_2)_4$–	



19 20 21

Scheme 2. Synthesis of 5,5-dimethyl-5,6-dihydropyridin-2-one (**21**). Method C: (a) NH_2OSO_3H, HCO_2H, reflux (52%); (b) DDQ, BSTFA, dioxane, reflux (70%).



22a-i
23 R_1=R_2=H, R_3=R_4=Me, R_5=H

Scheme 3. Synthesis of 5,6-dihydropyridine-2-imines (**1**, **4**–**12**) Reagents: (a) $Et_3O^+BF_4^-$,CH_2Cl_2; (b) NH_3, EtOH; (c) 4 M HCl dioxane (8–70% in three steps)

Compd	R_1	R_2	R_3	R_4	R_5
a	H	Me	H	H	H
b	Me	Me	H	H	H
c	H	Me	Me	Me	H
d	H	Me	(H,Me)		H
e	H	Me	H	H	Me
f	H	Me	H	H	*n*-Pr
g	H	Me	H	H	Allyl
h	H	Me	H	$-(CH_2)_3-$	
i	H	Me	H	$-(CH_2)_4-$	

Replacement of the 4-methyl group with a 4-ethyl group afforded **2**[16] with a nearly 6-fold reduction in the inhibitory activity and still no isoform selectivity. Shifting the double bond of **1** from position 3,4- to 4,5- provided **3**[16] with reduced inhibitory activity against both of the isoforms as well as a reduced selectivity. Based on this information, further chemical modification was carried out by fixing the double bond in position 3,4- as illustrated in compounds **4**–**12**. Introduction of another methyl group into position 3 of **1** afforded **4** with little change in the inhibitory activity or isoform selectivity. Introduction of a *gem*-dimethyl group into position 5 of **1** provided **5** with a 2-fold reduction in the inhibitory activity against hiNOS and 10-fold reduction against heNOS. As a result, the isoform selectivity was 5 times more than that of **1**. Removal of the 4-methyl group from **5** afforded **6** with a 14-fold reduction in the potency of the hiNOS inhibition and a 135-fold reduction in the heNOS inhibition. As a result, the isoform selectivity (heNOS/hiNOS) of **6** increased to nearly 10 times greater than that of **1**. Introduction of another methyl group into position 5 of **1** afforded **7** with a nearly 2-fold reduction in potency against both the hiNOS and heNOS isoforms but with the isoform selectivity retained. Introduction of another methyl group into position 6 of **1** afforded **8** which retained the inhibitory activity for both isoforms as well as the selectivity. Replacement of the 6-methyl group of **8** with a 6-propyl group afforded **9** with a 3-fold increase in potent inhibitory activity against hiNOS and a 2.4-fold reduction against heNOS resulting in an isoform selectivity which was 7.5-fold higher than **8**. A similar result was obtained in the corresponding allyl derivative **10**.

Finally, two of the *cis*-fused bicyclic analogues were prepared and biologically evaluated as inhibitors of NOS. The *cis*-fused 5,6-bicyclic analogue **11** demonstrated nearly the same potency of hiNOS inhibition as compared with **1** but with a 2.2-fold decrease in the potency of heNOS inhibition, while the isoform selectivity of **11** was higher than that of **1**. This was also true for the *cis*-fused 6,6-bicyclic analogue **12**, although its IC_{50} values for the isoforms were much less potent than those of **11**.

Among the tested compounds described in Table 1, the compound **1**, which is a structurally representative compound of this series with potent iNOS inhibitory activity was selected together with **5**, **9** and **10** for further biological and pharmacodynamic evaluations in animal models because of their in vitro potency and isoform selectivity, as well as their mouse acute toxicity (MTD: maximum tolerated dose) and structural features. Compounds **9** and **10** exhibited greater potency and more isoform selectivity than the above-mentioned compounds **1** and **5**. As shown in Table 2, compounds **9** and **10** demonstrated higher acute toxicity than **1** and **5**, as evaluated by the ratio between their maximum tolerated doses (MTDs) and the ID_{50} values of NOx[17] accumulation (MTD/NOx).

In order to assess compounds **1**, **5**, **9** and **10** for their ability to inhibit iNOS in vivo, mice were given a single compound subcutaneously (sc) 3 h after lipopolysaccharide (LPS) injection. The plasma NOx accumulation from 3 to 6 h after the LPS injection was then determined. As described in Table 2, these test compounds **1**, **5**, **9** and **10** inhibited NOx accumulation in plasma and their ID_{50} values were 0.16, 0.28, 0.36 and 0.16 mg/kg, sc, respectively. To assess the acute toxicity, the MTDs were determined. As shown in Table 2, the MTDs of **1** and **5**, when a single intravenous (iv) dose

Table 2. Pharmacological evaluation of **1**, **5**, **9** and **10**

Compd	Mouse iNOS	NOx	MTD	MTD/NOx
	IC_{50} (μM)[a]	ID_{50} (mg/kg, sc)[b]	mg/kg, iv[c]	
1	0.022	0.16	20	130
5	0.10	0.28	20	71
9	0.10	0.36	5	14
10	0.025	0.16	5	31
L-NMMA	3.5	26	3000	120

[a]Enzyme assay with recombinant mouse iNOS. Recombinant mouse iNOS was purchased from Cayman Chemical (Cat. No. 60862) and the inhibitory activities of the test compounds were evaluated by measuring the conversion rate from [^{14}C]-L-arginine to [^{14}C]-L–citrulline, and then the IC_{50} values were determined.

[b]The ID_{50} value was determined from a log–logit transformation of the dose–response curves (**1**, **5**, **9** and **10**; 0.1, 0.3, 1 mg/kg, sc L-NMMA; 10, 30 and 100 mg/kg, sc). The ID_{50} value was defined as the dose of test compound that produced a 50% inhibition in the NOx accumulation induced by LPS treatment alone. The MTD was defined as the maximum dose at which no death was observed within 24 h after an intravenous injection administration. The doses used were 5, 10, 20, 30, 40, and 50 mg/kg for **1**, **5**, **9** and **10** and 1000, 2000, 3000, 4000, and 5000 mg/kg for L-NMMA.

[c]Inhibition of NOx accumulation and the maximum tolerated dose (MTD) in mice. The test compounds or saline were administered subcutaneously 3 h after the LPS (10 mg/kg, iv) injection into 7 week old Balb/c mice (Charles River Japan, Inc.). Blood was collected by venipuncture from the abdominal aorta under light anesthesia at 6 h after LPS treatment. Plasma was obtained by centrifugation and the concentration of accumulated NOx over 3 h was determined by the method described below. To evaluate the acute toxicity, the MTD (iv maximum dose where no death was observed within 24 h after the administration) of the test compound was determined.

was given to normal mice, were 20 and 20 mg/kg, respectively. The MTD values of all four compounds were lower than that of L-NMMA (3000 mg/kg), although the ratio of MTD/ID_{50} for NOx accumulation in mice was 130 for **1**, 71 for **5**, 14 for **9** and 31 for **10**, respectively. The oral bioavailability of **1** and **5** in rats was excellent (**1**: 88% and **5**: 84%).

In summary, we have explored the structure–activity relationship (SAR) for a series of substituted 5,6-dihydropyridin-2-imines and obtained significant increases in the isoform selectivity and potency of iNOS inhibition. 4-Methyl and 4-methyl-5,5-dimethyl substituents provided compounds **1** and **5** which were potent and/or selective inhibitors of the iNOS isoform. Compounds **9** and **10** exhibited greater potent inhibitory activity and isoform selectivity than those of **1** and **5** but their acute toxicity in mice was also higher. Full details will soon be reported in *Bioorganic Medicinal Chemistry*.

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