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Selective Inhibition of iNOS by Benzyl- and Dibenzyl Derivatives of N-(3-Aminobenzyl)acetamidine

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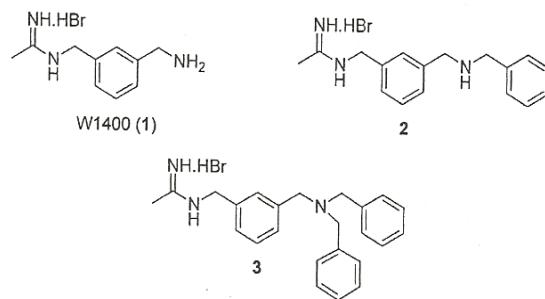
Nitric oxide (NO), one of the smallest known bioactive products of mammalian cells, can be produced by almost all cells.^[1] In mammals, NO is synthesized by a family of three NO synthases (NOS): neuronal nNOS, inducible iNOS and endothelial eNOS, that are products of three genes: *NOS1*, *NOS2*, and *NOS3*, respectively.^[2] The three isoforms generate NO by the conversion of L-arginine to L-citrulline; they differ in both structure and function but share about 50% sequence homology and are differentially regulated making the catalytic activity distinct for each isoform. nNOS and eNOS are constitutive and primarily expressed in neurons and endothelial cells, respectively. These isoforms are calcium/calmodulin dependent and generate low levels of NO for short periods of time in a pulsatile manner.^[3]

iNOS was first identified and characterized in cytokine-activated murine macrophages.^[4] This isoform is not dependent upon calcium/calmodulin for its enzymatic action and is expressed in a wide array of cells and tissues, for example, macrophages,^[5] hepatocytes,^[6] neutrophils, pulmonary epithelium, colonic epithelium, and vasculature.^[7] After induction, iNOS continuously produces NO until the enzyme is degraded. The overproduction of NO by iNOS may have detrimental consequences, and this activity seems to be involved in the pathophysiology of several human diseases, such as asthma, arthritis, multiple sclerosis, colitis, psoriasis, neurodegenerative diseases, tumor development, transplant rejection, and septic shock.^[8–10]

In recent years, considerable effort has been directed toward the selective inhibition of iNOS as a strategy for the prevention of excessive NO production, while maintaining the basal formation of NO from constitutive NOS that is required for normal physiological function. The first approach to NOS inhibitors included analogues of the natural substrate L-arginine that act competitively at the substrate binding site; however, these compounds are not selective enough over the eNOS and

nNOS isoforms, which limits their application *in vivo*. More recent studies have focused on the design and synthesis of non-amino acid analogues, such as amino heterocycles, amines, guanidines, isoquinolinamines, and isothioureas.^[11–14]

We previously reported the synthesis, biological evaluation, and docking studies of a series of N-substituted acetamidines,^[15] structurally related to the leading scaffold W1400^[16] (1), a potent and selective iNOS inhibitor. Starting from docking results previously obtained, and with the aim of extending the study of possible ligand–enzyme interactions, we have now evaluated the effect of the introduction of several substituents in *ortho*, *meta* or *para* positions of the leading scaffolds 2 and 3, differing from 1 by the amino substitution at the 3-aminomethyl group with one or two benzylic groups.



Acetamidines 2 and 3 were synthesized as previously described,^[15] and new compounds 7a–w were obtained according to the route shown in Scheme 1. Boc-protected *m*-xylylenediamine was treated with the appropriate *ortho*-, *meta*-, or *para*-substituted chloromethylbenzene to give *N*-benzyl derivatives 5a–u. Subsequent removal of the Boc group with trifluoroacetic acid (TFA) and treatment with sodium hydroxide gave free amines 6a–u. Finally, reaction of 6a–u with S-2-naphthylmethyl thioacetimidate hydrobromide or ethylacetimidate hydrochloride gave 7a–u as hydrobromide or hydrochloride salts. The syntheses of 5p and 5u also gave large amounts of *N,N*-dibenzyl analogues 5v–w, which were isolated by chromatographic separation; compounds 7v–w were obtained from 5v–w following the same synthetic scheme.

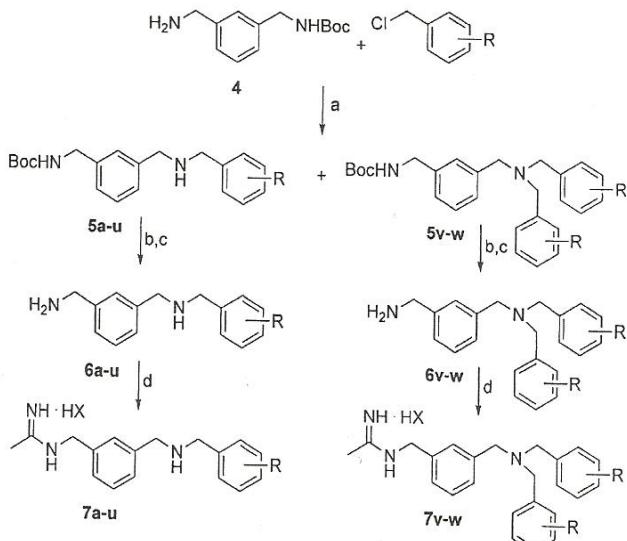
All new compounds are based on the common scaffolds 2 or 3, differing from them in the substitution at the benzyl or dibenzyl moiety with halogens or small groups, such as phenyl, methyl, methoxy, thiomethyl, and trifluoromethyl. In our study, we also considered the possible role of the position of the substituent (*ortho*, *meta* or *para*) on the aromatic ring. All new acetamidines were biologically evaluated for their ca-

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Scheme 1. Reagents and conditions: a) Et₃N, dry CH₃CN, N₂, 2–4 d, 43–75%; b) CF₃COOH, 0 °C, 1 h, 68–90%; c) NaOH, RT, 5 min, 100%; d) S-2-naphthylmethyltrioacetimidate hydrobromide or ethylacetimidate hydrochloride, abs EtOH, RT, 7–10 h, 64–97%.

pability to inhibit iNOS, eNOS, and nNOS, in an enzymatic assay,^[17] employing recombinant enzymes from different sources: murine macrophage iNOS, rat brain nNOS, and bovine eNOS. The inhibitory activity against NOS isoforms was measured by following the oxidation of oxyhemoglobin to methemoglobin by NO, and the IC₅₀ values were calculated from the data. The selectivities for iNOS over eNOS (e/i) and nNOS (n/i), and for nNOS over eNOS (e/n) were also evaluated. The known inhibitor 1 and the scaffolds 2 and 3 were also subjected to the same test system for comparison. The results of the enzymatic assays are reported in Table 1.

The parent compound 2, with an unsubstituted benzyl ring, showed an iNOS IC₅₀ value of 10 μM and selectivity over eNOS of fivefold. Considering mono-substitution of the primary amine group of 1, the introduction of a chloro (7a–c), trifluoromethyl (7g–i) or methyl (7j–k) group in the *ortho*, *meta* or *para* position of the benzyl ring did not improve the IC₅₀ value, which was greater than 30 μM. Introduction of a fluoro group in the *para* and *meta* positions (7d–e) gave compounds with iNOS IC₅₀ values of 15 μM, while the *ortho* derivative (7f) exhibi-

ed an iNOS IC₅₀ value of 40 μM. Furthermore, the presence of a thiomethyl group in the *para* position of the benzyl ring (7o) did not improve the potency (IC₅₀ = 18 μM). The introduction of a methoxy group in the *para* position led to a more potent iNOS inhibitor (7l; IC₅₀ = 5 μM) with improved selectivity when compared with the parent compound 2 (60-fold c.f. fivefold). However, when the methoxy group is in *meta* and *ortho* positions (7m–n), the IC₅₀ value is greater than 20 μM. Conversely, when the substituent is a phenyl ring in the *meta* or *ortho* position (7t–u), the iNOS IC₅₀ value is 5 and 1 μM, respectively, while the selectivity (e/i) is 100-fold and 50-fold. Compound 7s, with the phenyl ring in the *para* position, exhibited an IC₅₀ value of 30 μM against iNOS.

The most interesting substituent of the benzyl ring was certainly the nitro group. The most potent iNOS inhibitor of this series was compound 7r, containing a nitro group in the *ortho* position, with an IC₅₀ value of 0.1 μM against iNOS and 300-fold selectivity for iNOS over eNOS. Conversely, when the nitro group is in the *meta* (7q) or *para* (7p) position, the IC₅₀ values are 20 and 5 μM, respectively, with no significant selectivity for iNOS over other isoforms.

Parent scaffold 3, obtained by dibenzyl substitution of the primary amino group of 1, showed poor inhibition of iNOS, with an IC₅₀ value of 100 μM. However, *N,N*-dibenzyl derivatives

Table 1. IC₅₀ and selectivity values for the inhibition of NOS isoforms by compounds 1–3, and 7a–w.

Compd	R	IC ₅₀ ^[a] [μM]			Selectivity	
		iNOS	eNOS	nNOS	eNOS/iNOS	eNOS/nNOS
1	–	0.33 ± 0.06	1100 ± 104	7.3 ± 1.4	3333	150
2	H	10 ± 0.9	20 ± 2.2	300 ± 19.7	2	0.06
7a	4-Cl	100 ± 8.9	200 ± 13.2	150 ± 21.8	2	1.33
7b	3-Cl	100 ± 7.2	300 ± 27.5	300 ± 6.6	3	1
7c	2-Cl	30 ± 3.6	300 ± 16.2	200 ± 13.7	10	1.5
7d	4-F	15 ± 1.2	10 ± 1.1	20 ± 1.7	0.67	0.5
7e	3-F	15 ± 1.6	60 ± 4.3	50 ± 4.3	4	1.2
7f	2-F	40 ± 4.5	40 ± 3.4	30 ± 2.5	1	1.33
7g	4-CF ₃	40 ± 3.8	400 ± 24.5	100 ± 10.6	10	4
7h	3-CF ₃	100 ± 9.2	550 ± 50.7	200 ± 13.9	5.5	2.75
7i	2-CF ₃	100 ± 8.1	350 ± 13.2	100 ± 7.8	3.5	3.5
7j	4-CH ₃	30 ± 2.9	25 ± 2.3	30 ± 3.0	0.83	0.83
7k	3-CH ₃	50 ± 4.3	60 ± 3.2	100 ± 9.2	1.2	0.6
7l	4-OCH ₃	5 ± 0.3	300 ± 12.9	80 ± 7.8	60	3.75
7m	3-OCH ₃	20 ± 1.8	30 ± 3.2	100 ± 7.6	1.50	0.3
7n	2-OCH ₃	60 ± 5.6	100 ± 9.8	200 ± 12.6	1.67	0.5
7o	4-SCH ₃	18 ± 1.5	450 ± 33.2	200 ± 12.3	25	2.25
7p	4-NO ₂	5 ± 0.3	80 ± 7.2	100 ± 5.5	16	0.8
7q	3-NO ₂	20 ± 1.7	80 ± 8.7	200 ± 19.2	4	0.4
7r	2-NO ₂	0.1 ± 0.01	30 ± 2.5	200 ± 18.6	300	0.15
7s	4-Ph	30 ± 2.5	30 ± 3.1	80 ± 5.2	1	0.38
7t	3-Ph	5 ± 0.4	500 ± 37.7	200 ± 16.1	100	2.5
7u	2-Ph	1 ± 0.02	50 ± 2.1	300 ± 13.2	50	0.17
3	H	100 ± 9.2	400 ± 35.2	50 ± 4.3	4	8
7v	4-NO ₂	0.5 ± 0.04	100 ± 8.1	100 ± 11.3	200	1
7w	2-Ph	15 ± 1.3	400 ± 18.3	200 ± 15.6	27	2

[a] The NOS inhibition at 10 μM arginine. Values given are the mean ± SD of experiments performed in triplicate at 7–10 different concentrations.

7v-w showed improved iNOS inhibition compared with lead compound **3**. In particular, derivative **7v**, containing two *p*-nitro-benzyl rings, was a potent iNOS inhibitor ($IC_{50}=0.5\text{ }\mu\text{M}$), with high selectivity (200-fold) for iNOS over eNOS. All tested compounds were inactive against the nNOS isoform.

Docking simulations were performed to predict the binding mode of the two most potent inhibitors **7r** and **7v**, and the two most promising nitrobenzyl-containing derivatives **7p** and **7q**, in the active sites of iNOS and eNOS. Our aim was to shed light on the reasons for the potent inhibition of iNOS by **7r** and **7v**, and for their selectivity for iNOS over eNOS. Furthermore, we aimed to gain insight into the effects of the nitro group and the influence of its position on the benzyl ring (*ortho*, *meta*, or *para*) of the leading scaffold **2**. We also addressed the biphenyl derivative **7u** to investigate how this sterically demanding aryl group might be accommodated within the NOS binding cavity. The effectiveness of the Glide program in docking compound **1** to iNOS and eNOS isoforms was shown in previous work, with calculated poses in good agreement with the X-ray structures of the 1-iNOS (PDB code: 1QW5) and 1-eNOS (PDB code: 1F01) complexes, with a root mean square deviations (RMSD) of 0.341 \AA and 0.242 \AA , respectively.^[15] Comparison of the docked poses of **7p-r** and **7v** in iNOS with that of **1** (Figure 1), provides insight on the main effects of the presence of one or two nitro-benzyl groups on their interactions with this NOS isoform.

The predicted binding geometries of all these compounds, as that of **7u**, share common characteristics: hydrogen-bonding interactions between the amidine group and the carboxylate moiety of the Glu371 and Trp366 residues; orientation of the acetamidine-bound benzylic backbone atop of the heme pyrrole ring; and, for the mono-nitro-benzyl derivatives, interaction of the 3-aminomethyl group with one propionate arm of heme, also observed for the leading scaffold **1**.^[16] The

mono-nitro-benzyl derivatives **7p-r** are predicted to form a further hydrogen bond between one of the nitro group oxygen atoms and Asn348 (*ortho*- and *para*-nitro-benzyl derivatives **7r** and **7q**) or Asn115 (*meta*-nitro-benzyl **7p**) in the substrate access channel. The bis-nitro-benzyl derivative **7v** is predicted to form a hydrogen bond between the oxygen atom of one of the two *para*-nitro-benzyl groups and Arg382 in the catalytic site close to Glu371, while the remaining nitro-benzyl group assumes the same orientation as predicted for compounds **7p-r**, pointing in to the substrate access channel without, however, forming a second hydrogen bond with Asn115 or Asn348. The docked pose of **7u** does not show any further hydrogen bond but only an hydrophobic contact with Met114 in the substrate access channel, where the biphenyl moiety resides.

The docked structures of **7p-r** and **7v** in the eNOS isoform predict the same hydrogen-bonding interactions between the common amidine group and the carboxylate moiety of Glu363 and Trp358, and—for the mono-nitro-benzyl derivatives—between the 3-aminomethyl group and one propionate arm of the heme cofactor, observed for the lead compound **1**.^[18] However, none of these derivatives are predicted to form hydrogen bonds between a nitro group and a further amino acid; this difference could account for the weaker inhibitory potencies of these compounds against eNOS and thus their selectivity for iNOS over eNOS. In particular, the *ortho*- and *para*-nitro-benzyl derivatives **7r** and **7q** are not predicted to form any hydrogen bonds between the nitro group and Asn340 (corresponding to Asn348 in iNOS), presumably because the loop to which Asn340 belongs is shifted towards the heme cofactor in the eNOS isoform, contributing to the smaller size of its heme binding cavity.^[19] On the other hand, the *meta*-nitro-benzyl derivative **7p** is predicted to be incapable of forming any hydrogen bonds with eNOS because the polar Asn115 residue in iNOS is replaced with the hydrophobic Leu107 in eNOS. Interestingly, replacement of Asn115 (iNOS) by Leu107 (eNOS) and Leu337 (nNOS) has been proposed as the structural basis for the isoform selectivity (nNOS and eNOS over iNOS) of dipeptide inhibitors, *d*-Lys-*d*-ArgNO₂-NH₂ and *d*-Phe-*d*-ArgNO₂-NH₂.^[20]

Analysis of the calculated docking scores using the EModel score, which is based on the ChemScore function described by Eldridge et al.,^[21] provides support for the observed higher inhibitory potency of **7r** and **7v** against iNOS and their selectivity for iNOS over eNOS. The EModel score for the binding of **7r** and **7v** to iNOS (100–101) are significantly higher than those for **7q** and **7p** (90–94), and indeed, when the values calculated for the four compounds, together with those for **7u** and **1**, are plotted against the experimental pIC_{50} values, a reasonably good correlation is obtained with $R^2=0.84$ (figure S2 in the Supporting Information). Moreover, a comparative analysis of the EModel values for the NOS isoforms shows significantly higher docking scores for the iNOS isoform (90–101) than for the eNOS isoform (83–88), consistent with the observed selectivity for iNOS over eNOS.

In conclusion, a series of acetamidines structurally related to **1** was synthesized and evaluated for their biological activity as

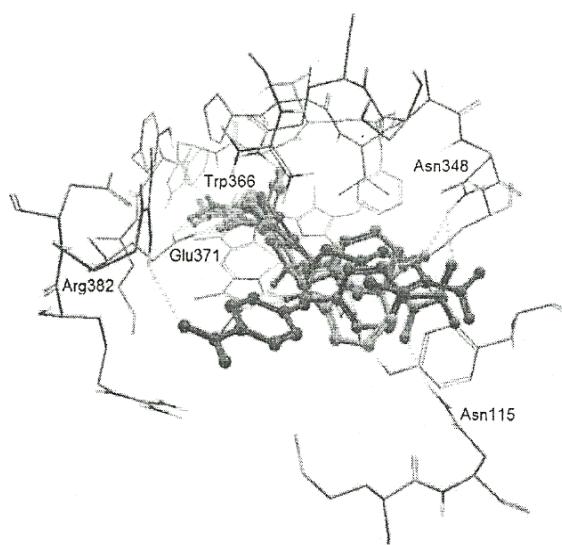


Figure 1. Superimposition of the docked conformation of derivatives **7p** (magenta), **7q** (orange), **7r** (yellow), and **7v** (green) in the active site of the iNOS isoform.

selective iNOS inhibitors. Compound **7r**, exhibiting an IC_{50} value of $0.1\ \mu\text{M}$, is a more potent inhibitor of iNOS than **1**, exhibiting good selectivity over eNOS. A docking study predicted the binding mode of the two most potent inhibitors **7r** and **7v** in the iNOS active site, suggesting that they form a hydrogen bond between their nitro group and an asparagine or arginine residue.

Experimental Section

General: All chemicals were commercially available and used without further purification. Flash chromatography was performed on silica gel 60 (Merck) and thin-layer chromatography (TLC) on F₂₅₄ silica gel 60 TLC plates. Melting points were determined on a Buchi apparatus and are uncorrected. Infrared spectra were recorded on a FT-IR 1600 Perkin–Elmer spectrometer. NMR spectra were run at 300 MHz on a Varian instrument; chemical shifts (δ) are reported in ppm. Mass spectra were obtained on a ThermoFinnigan LCQ Advantage spectrometer (ESI). Elemental analyses were carried out using an Eurovector Euro EA 3000 model analyzer. The purity of all compounds was $\geq 98\%$.

General procedure for the synthesis of 7a–u and 7v–w: A stirred solution of *tert*-butyl 3-(aminomethyl)benzylcarbamate (0.90 mmol) and Et₃N (0.60 mmol) in dry CH₃CN (10 mL) was treated dropwise with a solution of benzyl chloride derivative (0.60 mmol) in dry CH₃CN (20 mL) under N₂. The solution was stirred for 2–4 d then concentrated under reduced pressure. The residue was poured into water (20 mL) and extracted with CH₂Cl₂ (3 \times 20 mL). The organic layer was dried (Na₂SO₄), filtered and concentrated under reduced pressure. Purification by column chromatography on silica gel (CH₂Cl₂/MeOH, 95:5) gave compounds **5a–w**.

Compound **5a–w** (0.60 mmol) was dissolved in CH₂Cl₂ (20 mL) and treated with CF₃COOH (60 mmol) at 0 °C for 2 h. The mixture was concentrated, and the residue was re-dissolved in H₂O/EtOH (1:1, 1.5 mL) and then treated with NaOH (2 N). The solution was extracted with CH₂Cl₂ (2 \times 15 mL), and the organic layer was dried (Na₂SO₄), filtered and concentrated under reduced pressure to give pure product **6a–w**.

A solution of S-2-naphthylmethylthioacetimidate hydrobromide or ethylacetimidate hydrochloride (0.60 mmol) in EtOH (5 mL) was added to a solution of **6a–w** (0.60 mmol) in EtOH (2 mL) at RT. After stirring for 7–10 h, the mixture was concentrated. The crude residue was suspended in H₂O (15 mL) and washed with Et₂O (3 \times 15 mL) and EtOAc (2 \times 15 mL). The aqueous layer was lyophilized to give compound **7a–w**.

N-(3-[(2-Nitrobenzyl)amino]methyl)benzyl)ethanimidamide hydrobromide (7r): hygroscopic yellow solid (180 mg, 75%); ¹H NMR (300 MHz, D₂O): δ = 2.16 (s, 3 H, CH₃), 4.32 (s, 2 H, CH₂NH), 4.34 (s, 2 H, CH₂NH), 4.40 (s, 2 H, CH₂NH), 7.75 (m, 8 H, Ar); ¹³C NMR (300 MHz, D₂O): δ = 24.12 (CH₃), 40.85 (CH₂NH),

50.98 (CH₂NH), 55.62 (CH₂NH), 123.91, 125.53, 126.12, 127.73, 128.62, 128.93, 132.77, 137.54, 141.12, 146.54 (Car), 164.82 ppm (CNH); IR (KBr): $\tilde{\nu}$ = 1675, 1632 cm⁻¹; MS (ESI): *m/z* (%): 394.3 (100) [M + H⁺]; Anal. calcd for C₁₇H₂₁BrN₄O₂: C 64.41, H 6.08, N 18.78, O 10.73, found: C 64.21, H 6.09, N 18.83, O 10.76.

See the Supporting Information for experimental details of all other compounds, biological assays and docking studies.

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Keywords: acetamidines • inhibitors • inducible nitric oxide synthases (NOS) • isoform selectivity • nitric oxide

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