Published in final edited form as:

Bioorg Med Chem. 2013 September 1; 21(17): 5323–5331. doi:10.1016/j.bmc.2013.06.014.

In search of potent and selective inhibitors of neuronal nitric oxide synthase with more simple structures

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Abstract

In certain neurodegenerative diseases damaging levels of nitric oxide (NO) are produced by neuronal nitric oxide synthase (nNOS). It, therefore, is important to develop inhibitors selective for nNOS that do not interfere with other NOS isoforms, especially endothelial NOS (eNOS), which is critical for proper functioning of the cardiovascular system. While we have been successful in developing potent and isoform-selective inhibitors, such as lead compounds **1** and **2**, the ease of synthesis and bioavailability have been problematic. Here we describe a new series of compounds including crystal structures of NOS-inhibitor complexes that integrate the advantages of easy synthesis and good biological properties compared to the lead compounds. These results provide the basis for additional structure–activity relationship (SAR) studies to guide further improvement of isozyme selective inhibitors.

Keywords

Nitric oxide; Neuronal nitric oxide synthase; Inhibition; Aminopyridines

1. Introduction

Nitric oxide (NO) is an important second-messenger molecule that plays many fundamental physiological roles. It is produced from L-arginine by the nitric oxide synthase (NOS) family of enzymes, which includes neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible

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Supplementary data Supplementary data associated (Detailed synthetic procedures and full characterization (¹H NMR, ¹³C NMR) of compounds **3–36**) with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2013.06.014>.

NOS (iNOS). However, overproduction of NO by the nNOS in the brain is closely associated with many neurodegenerative diseases, including chronic pathologies such as Parkinson's,¹ Alzheimer's,² Huntington's,³ headaches,⁴ and neuronal damage in stroke.⁵ Therefore, it has become a promising strategy to inhibit nNOS to block the excess generation of NO for the treatment of neurodegeneration.^{6–8} A large number of nNOS inhibitors have been reported⁹ but none of these inhibitors has entered clinical trials because of low potency or poor isoform selectivity.⁹ The isozymes of NOS share ~50% sequence homology and show a highly similar heme active site structure, which is why most inhibitors directed at the substrate binding site show limited isoform selectivity. While we have succeeded in developing highly selective nNOS inhibitors,¹⁰ combining ease of synthesis, bioavailability, and selectivity remains a challenging task.

We have previously developed two lead compounds (**1** and **2**) that exhibit excellent potency against nNOS. Compound **1** provides an excellent dual-selectivity of nNOS over the other two isoforms ($K_i = 7$ nM, $e/n = 2667$, $i/n = 806$),¹¹ but, unfortunately, the tedious synthesis limits its structure/activity optimization to improve bioavailability. The synthesis of **2** (three synthetic steps) is much easier than **1** (15 synthetic steps plus a chiral resolution), but its selectivity needs to be improved considerably.¹² On the basis of the current results, we attempted a new strategy to integrate both the advantages of easy synthesis and good activity for the next generation of inhibitors. The new compounds were designed with short synthetic routes, and their structures are ready for further optimization.

2. Chemistry

The synthesis of inhibitors **5** began with a condensation reaction to protect 4,6-dimethylpyridine, and the product, compound **3**, was deprotonated with *n*-BuLi then treated with a dibromide to obtain compound **4**. After removal of the protecting groups in the presence $\text{NH}_2\text{OH}\cdot\text{HCl}$, inhibitors **5a–c** were obtained, as described in Scheme 1.

Compound **6** was prepared according to the general method for amine protection (see details in Supplementary Data). Addition of a three-fold excess of 1,3-dihydroxybenzene over **6** provided mono-substituted **7**. Compound **9** was prepared according to a literature report¹³ as shown in Scheme 2. A standard ether synthesis linked fragments **7** and **9** together to produce **10** followed by deprotection of the amine to afford inhibitors **11**.

The synthesis of **14** and **15** followed a similar method as that for **11** but using NaH to deprotect 1,3-dihydroxybenzene without a large excess over 4-(bromomethyl)pyridine (Scheme 3). A second ether synthesis connected the 4-methyl aminopyridine head to generate **13** followed by deprotection of the amino group to yield **14**. Compound **15** followed the same pathway starting from 2,2'-biphenol.

In the synthesis of **18** and **19** (Scheme 4), a Mitsunobu reaction was used to add the *N*-Boc-piperidinemethyl fragment to dihydroxybenzene, and mono-substituted **16** was isolated in a 53% yield. An ether synthesis connected the 4-methylaminopyridine to generate **17**, followed by deprotection of the amino and Boc groups to yield **18**. Compound **19** followed the same pathway but started with 2,2'-biphenol.

The synthesis of inhibitor **24** started from 3-hydroxyaniline; the first step was protection of the amino group with Boc (Scheme 5). A standard ether synthesis linked the 4-methylpyridine head successfully in the presence of K_2CO_3 in acetone. The Boc group was removed in 1.5 M HCl methanol after reaction for four hours. The 3-fluorophenethyl aldehyde was prepared from 3-fluorophenethanol via Dess–Martin oxidation. Reductive

amination connected **22** and 3-fluorophenethyl aldehyde to provide **23**. After deprotection of the amino group, **24** was isolated in a 92% yield.

The synthesis of inhibitor **27** began with 3-hydroxybenzaldehyde, and the 4-methylpyridine head was added in the presence of **9** and K_2CO_3 in acetone (Scheme 6). Reductive amination connected **25** and 3-fluorophenethyl amine together to provide **26**. Compound **27** was obtained after deprotection of the amino group with $NH_2OH \cdot HCl$.

2,6-Di(hydroxymethyl)pyridine was used as the starting material for the synthesis of **32** (Scheme 7). It was oxidized to mono aldehyde **28** with Dess–Martin periodinane (DMP) followed by reductive amination with 3-fluorophenethyl amine. The resulting secondary amine (**29**) was Boc-protected and then treated with **9** in the presence of NaH in DMF to construct **31**. Inhibitor **32** was obtained after deprotection of dimethylpyrrole and Boc, respectively, in a yield of 71% for the two steps.

The synthesis of **36** is illustrated in Scheme 8. First, 5-hydroxy-3-pyridinecarboxylic acid methyl ester was linked to the 4-methyl-2-aminopyridine head, and then the methyl ester was reduced to aldehyde **34** with Dibal-H at $-78^\circ C$. Reductive amination successfully connected the second head, 3-fluorophenethyl amine. Compound **36** was afforded after removing the protecting group.

3. Results and discussion

All of the inhibitors were assayed against three different isoforms of NOS, rat nNOS, bovine eNOS, and murine macrophage iNOS, using L-arginine as a substrate. Although inhibitors **5a–c** have good potency with nNOS, selectivity over the other two isoforms remains at the same level as compound **2** (<100).¹¹ The crystal structures of these compounds in complex with nNOS or eNOS were determined to test the effects of the linker length between the two aminopyridine head groups. As shown in Figure 1, **5a** binds to nNOS and eNOS in a similar way with its first aminopyridine moiety H-bonded with active site glutamate residue Glu592 in nNOS and Glu363 in eNOS. The 5-carbon linker brings the second aminopyridine next to the heme propionate from pyrrole ring D (referred to as propionate D). The orientation of this second aminopyridine group in nNOS is clearly different from that seen in eNOS, even though the electron density for the second aminopyridine is less certain compared to that for the first aminopyridine. This aminopyridine bends over forming H-bonds with both Asn569 and heme propionate D in nNOS, while it forms only one H-bond with Asn340 in eNOS. The Met336 side chain in nNOS has two unusual rotamer conformations (Fig. 2A), which might prevent **5a** from adopting the more extended conformation seen in eNOS (Fig. 2B), where a smaller and more rigid Val106 residue is in the same location.

Since working with the double-headed aminopyridine inhibitors,¹³ we have noticed that inhibitors with a 7- or 8-atom spacer between the two aminopyridine groups are capable of having one aminopyridine form tight bifurcated H-bonds with the other aminopyridine bound to heme propionate D, in addition to the H-bonds between the first aminopyridine and the NOS active site glutamate (Fig. 3A). This is referred to as the double-headed binding mode. Consistent with the earlier observations, **5c**, with a 7-carbon linker, binds to both nNOS and eNOS in the double-headed binding mode, as shown in Figure 3. However, one binding conformation of **5c** cannot fully account for the electron density features in nNOS. The second conformation of **5c** also exists that makes a H-bond with Asn569. To match the alternate binding of **5c**, the Tyr706 side chain shows two alternate rotamers as well (Fig. 3A). Inhibitor **5b** has a 6-carbon linker, which is more flexible than **5a**, but the linker is not long enough to allow its second aminopyridine to H-bond with the heme propionate D

carboxylic acid. For the nNOS–**5b** complex, only the first aminopyridine that interacts with the active site Glu is ordered (data not shown).

Compound **11** has a potency of 60 nM against nNOS as well as selectivity of 298 and 140 over eNOS and iNOS, respectively, the highest among the inhibitors tested in this work. Unfortunately, we could only obtain partial information from the crystal structure of the nNOS–**11** complex because the aminopropyl tail is disordered. As shown in Figure 4, while the aminopyridine forms tight H-bonds with Glu592, the orientation of the phenyl ring is less certain. The ether oxygen can indirectly interact with the heme and H₄B through a bridging water molecule (Fig. 4A). It is reasonable to assume that the aminopropyl N atom does not have any favorable contact with the protein; otherwise, it would have been visible in the structure. No eNOS structure was attempted because of the poor binding of **11** to eNOS. The introduction of a rigid pyridine group in **14** did not work, but having a non-planar piperidine in inhibitor **18** led to better potency and selectivity. As seen in Figure 4B, although the density for the piperidine is not as good as that for other part of **18**, it does show the potential for the ring N atom to interact with both heme and H₄B, replacing a conserved water molecule that normally H-bonds to both the heme and H₄B (see Fig. 4A). The planar pyridine ring in **14** is not expected to establish similar interactions with the protein observed for piperidine in **18**. Inhibitors **11** and **18** share high structural similarity, thus exhibiting similar inhibitory activity. When the phenyl linker was replaced with a biphenyl linker, such as inhibitors **15** and **19**, the potency dropped dramatically. Their bulky and rigid structures might be the cause of the loss of activity, which is consistent with what we learned about the inhibitor binding pocket in the structures shown in Figure 4.

Compound **27**, which resulted by merging functional groups in lead compounds **1** and **2**, is the best inhibitor in this series, affording a potency of 40 nM with a dual selectivity of 267 (e/n) and 147 (i/n). A very similar inhibitor, **24**, with one carbon atom less than **27** in the right arm, exhibits a 54-fold decrease in potency. The structure of nNOS–**27**, shown in Figure 5, provides some clues as to why the benzylamine of **27** binds to the active site of nNOS much better than does the aniline fragment in **24**. Even though the density for the fluorophenyl tail of **27** is weaker than that for the other part of the inhibitor, the benzylamine N atom is likely involved in an H-bonding network with heme and H₄B through one bridging water molecule (Fig. 5A). The aniline N atom in **24** is co-planar with the ring and, therefore, is much less flexible. A rotation of the entire aniline ring would be required for the N atom to H-bond with the heme propionate. We have also tested inhibitors **32** and **36**, where the phenyl linker was replaced with a more hydrophilic pyridine ring. In addition, one more carbon was introduced between the aminopyridine and the middle ring in **32**.

However, those changes decrease the potency and selectivity relative to **27** (Table 1). For the nNOS–**32** structure only the aminopyridine ring that H-bonds to Glu592 is visible. The partial density indicates that the N atom of the middle pyridine might interact with the heme propionates, but the orientation of the ring is not well defined, which leads to total disordering of the tail (data not shown). Fortunately, structures of **36** with both nNOS and eNOS are available, as shown in Figure 5B and C, respectively. It is clear that the N atom in the middle pyridine ring of **36** does not interact with heme. The ring N atom is at the meta-position relative to the other substituents so that if the ring N atom had H-bonded with the heme by flipping 180° from what is shown in Figure 5B and C, the fluorophenyl tail would be forced to reposition, thereby losing important protein-inhibitor contacts. In the observed binding conformation, the amine N atom adjacent to the middle pyridine ring can join the H-bonding network, through two water molecules, with heme and H₄B. This two-water bridged H-bonding network in **36** is likely weaker than the one-water bridged network seen in **27** in terms of the binding energy gained by the inhibitor. The orientation of the fluorophenyl ring is different in nNOS vs. eNOS, which may be influenced by the nearby

amino acid variant, Met336 in nNOS vs. Val106 in eNOS; this may contribute to the observed isoform selectivity.

In conclusion, we designed and synthesized a series of inhibitors by using the functional groups from lead compounds **1** and **2**. The new series of inhibitors feature both the advantages of easy synthesis and improved isoform selectivity properties (up to 298-fold) compared to **2**. However, the high isoform selectivity of lead **1** has not yet been matched. Utilizing a planar aromatic ring as in **2**, whether polar or non-polar, as the middle bridging group increases rigidity, thereby restraining the possible positions for the remaining tail groups. Disadvantages of this approach seem to outweigh the advantages, as this greatly limits the variety of tails that can be used. The most critical, but challenging, task is to introduce more specific enzyme-inhibitor contacts into nNOS relative to the other isoforms in order to overcome the disordering of the tail group, which extends out of the active site. Certainly the structure–activity–relationship studies here will form a basis for further improvement. The main advantage of our current compounds is that the ether bond and heteroatom connecting the two heads simplify the chemistry required to probe a variety of tails for optimization of isoform selectivity.

4. Experimental section

4.1. General methods

All experiments were conducted under anhydrous conditions in an atmosphere of argon, using flame-dried apparatus and employing standard techniques in handling air-sensitive materials. All solvents were distilled and stored under an argon or nitrogen atmosphere before using. Non-synthesized reagents were purchased from Sigma–Aldrich Co., LLC. and used as received. Aqueous solutions of sodium bicarbonate, sodium chloride (brine), and ammonium chloride were saturated. Analytical thin layer chromatography was visualized by ultraviolet light, potassium permanganate or phosphomolybdic acid (PMA). Flash column chromatography was carried out under a positive pressure of air. ^1H NMR spectra were recorded on 500 MHz Varian or Bruker AVANCE spectrometers. Data are presented as follows: chemical shift (in ppm on the δ scale relative to $\delta = 0.00$ ppm for the protons in TMS), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (J/Hz). Coupling constants were taken directly from the spectra and are uncorrected. ^{13}C NMR spectra were recorded at 125 MHz, and all chemical shift values are reported in ppm on the δ scale with an internal reference of $\delta 77.0$ or 49.0 for CDCl_3 or CD_3OD , respectively. LC–MS (ESI) was conducted on Agilent LCQ mass spectrometer. High resolution mass spectra (HRMS) were measured with an Agilent 6210 LC-TOF (ESI) mass spectrometer. The enzyme assay was monitored on a BioTek Synergy 4 microplate reader.

4.2. NOS inhibition assays

IC_{50} values for inhibitors 5a–36 were measured for three different isoforms of NOS, rat nNOS, bovine eNOS, and murine macrophage iNOS using L-arginine as a substrate. The three enzyme isoforms were recombinant enzymes overexpressed in *Escherichia coli* and isolated as reported.¹⁵ The formation of nitric oxide was measured using a hemoglobin capture assay, as described previously.¹¹ All NOS isozymes were assayed at room temperature in a 100 mM Hepes buffer (pH 7.4) containing 10 μM L-arginine, 1.6 mM CaCl_2 , 11.6 $\mu\text{g/mL}$ calmodulin, 100 μM dithiothreitol (DTT), 100 μM NADPH, 6.5 μM H_4B , and 3.0 μM oxyhemoglobin (for iNOS assays, no CaCl_2 and calmodulin were added). The assay was initiated by the addition of enzyme, and the initial rates of the enzymatic reactions were determined on a UV–vis spectrometer by monitoring the formation of methemoglobin at 401 nm from 0 to 60 s after mixing. The corresponding K_i values of

inhibitors were calculated from the IC_{50} values using Eq. (1) with known K_m values (rat nNOS, 1.3 μ M; iNOS, 8.3 μ M; eNOS, 1.7 μ M).

$$K_i = IC_{50} / (1 + [S] / K_m) \quad (1)$$

4.3. Inhibitor complex crystal preparation

The nNOS or eNOS heme domain proteins used for crystallographic studies were produced by limited trypsin digest from the corresponding full length enzymes and further purified through a Superdex 200 gel filtration column (GE Healthcare) as described previously.¹⁶ The nNOS heme domain at 7–9 mg/mL containing 20 mM histidine or the eNOS heme domain at 12 mg/mL containing 2 mM imidazole were used for the sitting drop vapor diffusion crystallization setup under the conditions reported before.¹⁶ Fresh crystals (1–2 day old) were first passed stepwise through cryo-protectant solutions as described¹⁶ and then soaked with 10 mM inhibitor for 4–6 h at 4 °C before being flash cooled with liquid nitrogen.

4.4. X-ray diffraction data collection, processing, and structure refinement

The cryogenic (100 K) X-ray diffraction data were collected remotely at various beamlines at Stanford Synchrotron Radiation Lightsources (SSRL) or Advanced Light Source (ALS) through the data collection control software Blu-Ice¹⁷ and a crystal mounting robot. Raw data frames were indexed, integrated, and scaled using HKL2000.¹⁸ The binding of inhibitors was detected by the initial difference Fourier maps calculated with REFMAC.¹⁹ The inhibitor molecules were then modeled in COOT²⁰ and refined using REFMAC. Water molecules were added in REFMAC and checked by COOT. The TLS²¹ protocol was implemented in the final stage of refinements with each subunit as one TLS group. The omit $F_o - F_c$ density maps were calculated by repeating the last round of TLS refinement with inhibitor coordinate removed from the input PDB file to generate the map coefficients DELFT and SIGDELFT. The refined structures were validated in COOT before deposition in the RCSB protein data bank. The crystallographic data collection and structure refinement statistics are summarized in Table 2 with PDB accession codes included.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful for financial support from the National Institutes of Health (GM049725 to R.B.S. and GM057353 to T.L.P.). We thank Dr. Bettie Sue Siler Masters (NIH Grant GM52419, with whose laboratory P.M. and L.J.R. are affiliated). B.S.S.M. also acknowledges the Welch Foundation for a Robert A. Welch Distinguished Professorship in Chemistry (AQ0012). P.M. is supported by grants 0021620806 and 1M0520 from MSMT of the Czech Republic. We also thank the beamline staff at SSRL and ALS for their assistance during the remote X-ray diffraction data collections.

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Figure 1.
Structures of lead compounds **1** and **2**.

Figure 2.

Inhibitor **5a** bound to nNOS (A) or eNOS (B). The omit $F_o - F_c$ density for inhibitor is shown at a 2.5σ contour level. Relevant H-bonds are depicted as dashed-lines. In nNOS-**5a** structure a partially occupied (50%) zinc site is detected similar to what was observed in some other nNOS inhibitor complex structures¹⁴ Compound **5a** is small and flexible enough even to bind in a groove on the molecular surface of nNOS next to Glu705 (data not shown). Structure figures were made with PyMol (<http://www.pymol.org>).

Figure 3.

The active site of nNOS (A) or eNOS (B) in complex with inhibitor **5c**. The omit $F_o - F_c$ density for inhibitor is shown at a 2.5σ contour level. Relevant H-bonds are depicted as dashed-lines. Two alternate conformations are observed in nNOS for inhibitor **5c** and Tyr706.

Figure 4.

The active site of nNOS complexed with inhibitor **11** (A) and **18** (B). The omit $F_o - F_c$ density for inhibitor is shown at a 2.5σ contour level. Relevant H-bonds are depicted as dashed-lines.

Figure 5.
The active site of nNOS complexed with inhibitor **27** (A) and **36** (B), and that of eNOS complexed with **36** (C). The omit $F_o - F_c$ density for inhibitor is shown at a 2.5 σ contour level. Relevant H-bonds are depicted as dashed-lines.

Scheme 1.

Synthesis of **5a–c**. Reagents and conditions: (a) *p*-TsOH, toluene, reflux, 89%; (b) (i) *n*-BuLi, THF, -78 to 0 °C; (ii) dibromide, 51–55%; (c) $\text{NH}_2\text{OH}\cdot\text{HCl}$, EtOH/ H_2O (2/1), 100 °C, 20 h, 79–82%.

Scheme 2.

Synthesis of **11**. Reagents and conditions: (a) K_2CO_3 , acetone, reflux, 71%; (b) (i) $n\text{-BuLi}$, THF, -78 to 0 $^\circ\text{C}$; (ii) TMSCl , 0 $^\circ\text{C}$, 95%; (c) $\text{BrF}_2\text{CCF}_2\text{Br}$, CsF , DMF, 91%; (d) K_2CO_3 , acetone, reflux, 79%; (e) $\text{NH}_2\text{OH}\cdot\text{HCl}$, $\text{EtOH}/\text{H}_2\text{O}$ (2/1), 20 h, 100 $^\circ\text{C}$.

Scheme 3.

Synthesis of **14** and **15**. Reagents and conditions: (a) (i) NaH, DMF, 0 °C; (ii) 4-(bromomethyl)pyridine, 46%; (b) (i) NaH, DMF, 0 °C; (ii) compound **9**, 59%; (c) BrF₂CCF₂Br, CsF, DMF, 91%; (d) K₂CO₃, acetone, reflux, 79%; (e) NH₂OH-HCl, EtOH/H₂O (2/1), 100 °C, 20 h.

Scheme 4.

Synthesis of **18** and **19**. Reagents and conditions: (a) DIAD, Ph_3P , *N*-Boc-piperidinemethanol, THF, 53%; (b) K_2CO_3 , acetone, compound **9**, reflux, 73%; (c) (i) $\text{NH}_2\text{OH}\cdot\text{HCl}$, EtOH/ H_2O , 100 °C, 20 h; (ii) 2 M HCl in dioxane/ CH_3OH (1/1), 80%.

Scheme 5.

Synthesis of **24**. Reagents and conditions: (a) $(\text{Boc})_2\text{O}$, THF, reflux, 93%; (b) K_2CO_3 , acetone, compound **9**, reflux, 90%; (c) 1.5 M HCl in CH_3OH , 98%; (d) $\text{NaBH}(\text{OAc})_3$, 3-fluorophenethyl aldehyde, DCE, $3 \text{ \AA}$ MS, 67%; (e) $\text{NH}_2\text{OH}\cdot\text{HCl}$, $\text{EtOH}/\text{H}_2\text{O}$ (2/1), 100°C , 20 h, 92%.

Scheme 6.

Synthesis of **27**. Reagents and conditions: (a) K_2CO_3 , acetone, compound **9**, reflux, 72%; (b) $NaBH(OAc)_3$, 3-fluorophenethyl amine, DCE, 3 Å MS, 64%; (c) $NH_2OH-HCl$, EtOH/ H_2O (2/1), 100 °C, 20 h, 91%.

Scheme 7.

Synthesis of **32**. Reagents and conditions: (a) Dess–Martin periodinane (DMP), CH_2Cl_2 , 46%; (b) $\text{NaBH}(\text{OAc})_3$, 3-fluorophenethyl amine, DCE, 3 Å MS, 78%; (c) $(\text{Boc})_2\text{O}$, NaHCO_3 , CH_3OH , 76%; (d) NaH , compound **9**, DMF, 0 °C, 51%; (e) (i) $\text{NH}_2\text{OH}\cdot\text{HCl}$, $\text{EtOH}/\text{H}_2\text{O}$ (2/1), 100 °C, 20 h; (ii) 2 M HCl in dioxane/ CH_3OH (1/1), 71%.

Scheme 8.

Synthesis of **36**. Reagents and conditions: (a) NaH, compound **9**, DMF, 0 °C, 70%; (b) Dibal-H, -78 °C, 56%; (c) NaBH(OAc)₃, 3-fluorophenethyl amine, DCE, 3 Å MS, 90%; (d) NH₂OH-HCl, EtOH/H₂O (2/1), 100 °C, 20 h, 79%.

Table 1

 K_i^a values of inhibitors for rat nNOS, bovine eNOS and murine iNOS

Inhibitors	K_i^a (nM)			Selectivity	
	nNOS	eNOS	iNOS	e/n	i/n
5a	48	979	375	20	77
5b	33	2842	1798	86	54
5c	86	2154	2193	25	25
11	60	18018	8472	298	140
14	616	62839	27123	102	44
15	3847	ND ^b	ND ^b	ND ^b	ND ^b
18	117	27963	17124	239	146
19	4235	ND ^b	ND ^b	ND ^b	ND ^b
24	2156	ND ^b	ND ^b	ND ^b	ND ^b
27	40	10429	5886	261	147
32	270	5623	8427	21	31
36	140	10366	2862	74	21

^aThe IC₅₀ values were measured for three different isoforms of NOS including rat nNOS, bovine eNOS, and murine macrophage iNOS using L-arginine as a substrate. Nine data points (concentrations) were collected, and the IC₅₀ values were calculated using a nonlinear regression method. Each experiment was repeated three times, and the average numbers are presented in Table 1. The experimental standard deviations were less than 10%. The corresponding K_i values were calculated from the IC₅₀ values using the equation $K_i = \text{IC}_{50}/(1 + [S]/K_m)$ with known K_m values (rat nNOS, 1.3 μM ; iNOS, 8.3 μM ; eNOS, 1.7 μM).

^bNot determined.

Table 2

Crystallographic data collection and refinement statistics

Data set ^a	nNOS-5a	nNOS-5c	nNOS-11	nNOS-18
<i>Data collection</i>				
PDB code	4JSE	4JSF	4JSG	4JSH
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	51.7 110.2 163.9	51.8 110.5 164.3	52.2 111.2 164.2	51.7 111.6 164.3
Resolution (Å)	1.97 (2.00–1.97)	2.05 (2.09–2.05)	1.95 (1.98–1.95)	2.35 (2.39–2.35)
<i>R</i> _{merge}	0.060 (0.553)	0.072 (0.664)	0.066 (0.530)	0.076 (0.775)
<i>I</i> / σ <i>I</i>	25.7 (2.9)	22.7 (2.5)	27.9 (2.3)	18.9 (1.8)
No. unique reflections	66,633	59,677	71,051	40,870
Completeness (%)	99.3 (99.7)	99.1 (99.9)	99.6 (99.3)	99.0 (99.9)
Redundancy	4.0 (3.9)	4.0 (4.0)	4.0 (3.9)	3.7 (3.3)
<i>Refinement</i>				
Resolution (Å)	1.97	2.05	1.95	2.35
No. reflections used	63,104	56,506	67,268	38,227
<i>R</i> _{work} / <i>R</i> _{free} ^b	0.170/0.205	0.175/0.217	0.189/0.226	0.193/0.253
<i>No. atoms</i>				
Protein	6728	6711	6665	6832
Ligand/ion	219	197	172	177
Water	431	386	357	74
<i>R.m.s. deviations</i>				
Bond lengths (Å)	0.014	0.014	0.010	0.011
Bond angles (°)	1.42	1.42	1.36	1.52

Data set ^a	nNOS-27	nNOS-36	eNOS-5a	eNOS-5c	eNOS-36
<i>Data collection</i>					
PDB code	4JSI	4JSJ	4JSK	4JSL	4JSM
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	51.6, 110.8, 164.6	51.7, 111.3, 164.4	58.4, 106.6, 157.0	57.8, 106.6, 157.0	58.3, 106.4, 157.1
Resolution (Å)	2.09 (2.13–2.09)	1.92 (1.95–1.92)	2.28 (2.32–2.28)	2.04 (2.08–2.04)	2.25 (2.29–2.25)
<i>R</i> _{merge}	0.075 (0.656)	0.066 (0.620)	0.050 (0.585)	0.066 (0.669)	0.073 (0.674)
<i>I</i> / σ <i>I</i>	22.6 (2.3)	31.3 (2.8)	27.8 (2.3)	22.6 (2.0)	18.7 (1.8)
No. unique reflections	56,724	73,068	45,192	62,475	47,060
Completeness (%)	99.5 (99.9)	99.3 (100.0)	98.9 (100.0)	99.6 (99.7)	99.7 (100.0)
Redundancy	4.0 (4.1)	4.0 (4.0)	3.3 (3.3)	3.4 (3.4)	3.6 (3.6)
<i>Refinement</i>					
Resolution (Å)	2.09	1.92	2.28	2.04	2.25
No. reflections used	53,714	69,161	42,763	59,144	44,635
<i>R</i> _{work} / <i>R</i> _{free} ^b	0.193/0.241	0.193/0.225	0.205/0.258	0.167/0.208	0.186/0.244
<i>No. atoms</i>					
Protein	6668	6689	6427	6446	6455

Data set ^a	nNOS-27	nNOS-36	eNOS-5a	eNOS-5c	eNOS-36
Ligand/ion	183	183	197	205	201
Water	222	366	145	457	244
<i>R.m.s. deviations</i>					
Bond lengths (Å)	0.013	0.015	0.016	0.014	0.016
Bond angles (deg)	1.56	1.46	1.61	1.47	1.61

^aSee Table 1 for inhibitor chemical formulae.

^b R_{free} was calculated with the 5% of reflections set aside throughout the refinement. The set of reflections for the R_{free} calculation were kept the same for all data sets of each isoform according to those used in the data of the starting model.