Potent and Selective Double-Headed Thiophene-2-carboximidamide Inhibitors of Neuronal Nitric Oxide Synthase for the Treatment of Melanoma

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ABSTRACT: Selective inhibitors of neuronal nitric oxide synthase (nNOS) are regarded as valuable and powerful agents with therapeutic potential for the treatment of chronic neurodegenerative pathologies and human melanoma. Here, we describe a novel hybrid strategy that combines the pharmacokinetically promising thiophene-2-carboximidamide fragment and structural features of our previously reported potent and selective aminopyridine inhibitors. Two inhibitors, 13 and 14, show low nanomolar inhibitory potency (K_i = 5 nM for nNOS) and good isoform selectivities (nNOS over eNOS [440- and 540-fold, respectively] and over iNOS [260- and 340-fold, respectively]). The crystal structures of these nNOS—inhibitor complexes reveal a new hot spot that explains the selectivity of 14 and why converting the secondary to tertiary amine leads to enhanced selectivity. More importantly, these compounds are the first highly potent and selective nNOS inhibitory agents that exhibit excellent in vitro efficacy in melanoma cell lines.

INTRODUCTION

Nitric oxide synthases (NOSs) are responsible for the biological production of nitric oxide (NO), an important second messenger that regulates many biological processes such as neurotransmission, vasodilation, and immune response.¹ NOSs catalyze two sequential reactions that convert L-arginine to L-citrulline and the free radical NO in the presence of oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) with N6-hydroxy-L-arginine (NHA) as an intermediate.²⁻⁴

There are three mammalian NOS isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). All three isoforms are homodimers and share catalytic properties. However, according to various tissue distributions, NO generated from different NOS isoforms can play a wide range of physiological functions.⁵,⁶ Overproduction of NO by nNOS has been implicated in many chronic neurodegenerative pathologies. Our previous studies have shown that NO from nNOS plays an important role in increasing the invasion and proliferation of human melanoma cells, suggesting that targeting NO signaling may facilitate therapy and prevention.⁷ Although inhibition of nNOS activity is a promising strategy for the treatment of such diseases, inhibition of iNOS and eNOS, especially the latter, may cause undesired side effects.⁸,⁹ Therefore, to precisely control biological processes and improve the safety profile, selective inhibition of nNOS over the other two isoforms is important for any potential therapeutic agent. The close similarity of active site structures in all three isoforms¹⁰,¹¹ presents a critical barrier to the design of selective nNOS inhibitors.

In our continuous efforts to develop nNOS selective inhibitors, we discovered a series of highly potent and selective nNOS small molecule inhibitors with a 2-aminopyridinomethyl pyrrolidine scaffold (Figure 1, 1 and 2).¹²⁻¹⁶ Some showed excellent potency (K_i < 10 nM) and excellent selectivity for nNOS over eNOS (>2500-fold) and iNOS (>700-fold). Moreover, we have found that the 2-aminopyridine moiety not only can make hydrogen bonds (H-bonds) with the active site glutamate (Glu592 in nNOS or Glu363 in eNOS) but also can hydrogen bond with the heme propionate in a conformation flipped 180°, depending on the chirality of the

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pyrrolidine. To achieve the two possible enzyme–inhibitor H-bonding interactions with a single compound and to simplify the synthetic routes, we developed a series of symmetric double-headed aminopyridines (e.g., 3 in Figure 1). However, all of the aminopyridine-based inhibitors suffered from some limitations such as lack of oral bioavailability and/or poor membrane permeability. A fragment-based comparison of the in vitro enzyme assay versus the cellular assay clearly indicates that the aminopyridine moiety in these molecules may be an important factor that limits their membrane permeability.

In addition to the aminopyridine-based inhibitors, thiophene-2-carboximidamides (Figure 2, 4–6) represent another important class of nNOS inhibitors. Recently, some of these compounds were reported with promising pharmacological profiles. Despite their favorable pharmacokinetics, they have significant challenges to overcome with respect to their lower potency and isozyme selectivities in vitro compared with the aminopyridine-containing inhibitors. In addition, few crystal structures depicting the binding mode of these molecules to the NOS isoforms have been reported; therefore, more crystallographic studies of thiophene-2-carboximidamides would be highly desirable.

Both the 2-aminopyridine and thiophene-2-carboximidamide moieties exhibit similar H-bond interactions with the enzyme, acting as mimics of the guanidinium group of the natural substrate l-Arg. Taken together, the thiophene-2-carboximidamide fragment would serve as a bioisostere of the 2-aminopyridine fragment with the potential for improved pharmacological profiles. Here we adopted a hybrid strategy and designed a series of compounds that combines the thiophene-2-carboximidamide fragment and structural features of our potent and selective double-headed aminopyridine inhibitors (Figure 3). Furthermore, we conducted crystallographic studies to identify the inhibitor–enzyme interactions that promote nNOS-specific inhibition.

### CHEMISTRY

The synthesis of 7 began with 1-iodo-3-nitrobenzene (Scheme 1). Homocoupling of 1-iodo-3-nitrobenzene gave 18 in a moderate yield. The nitro groups of 18 were reduced to amino groups in a quantitative yield, and then coupling with methyl thiophene-2-carbimidothioate hydroiodide salt was used to generate final product 7. The coupling reaction of 3,3′-methyleneedianiline with methyl thiophene-2-carbimidothioate hydroiodide salt gave final product 8.

A synthetic route for target molecule 9 is shown in Scheme 2. Wittig reaction of 3-nitrobenzaldehyde with the corresponding phosphorus ylide of 1-(bromomethyl)-3-nitrobenzene allowed the isolation of the intermediate alkene in an 87% yield. Compound 23 was obtained by reduction of 21, followed by treatment with methyl thiophene-2-carbimidothioate hydroiodide salt. Catalytic hydrogenation of 23 reduced the double bond, giving final product 9 in a high yield.

Nucleophilic addition of 3-nitrophenol with 2-bromo-1-(3-nitrophenyl)ethanone yielded 24 (Scheme 3). The carbonyl group of 24 was reduced to a hydroxyl group. Reduction of 25 gave 26, which was allowed to react with methyl thiophene-2-carbimidothioate hydroiodide salt to give 10.

Inhibitors 11 and 12 were synthesized as illustrated in Scheme 4. Nucleophilic addition of 3-nitrophenol with the corresponding alkyl halides generated 27 and 28. Their nitro groups were reduced to give intermediates 29 and 30, which were subsequently coupled with methyl thiophene-2-carbimidothioate hydroiodide salt to give final products 11 and 12, respectively.

To synthesize inhibitors 13–17 (Scheme 5), reductive amination of 3-nitrobenzaldehyde with the corresponding amines generated 34–36. The amino groups of 34–36 were
protected with a Boc group. Reductive amination of 35 and 36 with acetaldehyde gave 40 and 41, respectively. Amino group reduction and coupling with methyl thiophene-2-carboximidamide hydroiodide salt were performed under conditions similar to those we adopted for the synthesis of 7, giving target molecules 15, 17, and intermediates 47–49. Deprotection of the Boc groups of 47–49 generated final products 13, 14, and 16.

**RESULTS AND DISCUSSION**

Compounds 7–12 were designed with double thiophene-2-carboximidamide heads having diverse linkers. These flexible linkers mainly vary in length and, therefore, can provide precursor structural features that fit the enzyme active site appropriately. Table 1 shows the results of inhibition assays using purified NOS enzymes with 7–12.

For this series, 7–10 display moderate inhibitory activity, while 11 and 12 are the best nNOS inhibitors. Compound 11 also shows outstanding nNOS selectivity over iNOS and good selectivity over eNOS. Compared to 11, addition of one methylene group leads to a compound (12), with half the potency and a sharp decrease in selectivity for nNOS over both eNOS and iNOS.

To gain more insight into the structural basis for relative potencies and selectivities, we determined the crystal structure of several inhibitors bound to nNOS. The crystal structures revealed that 7 and 9 bind to nNOS as designed with one thiophene-2-carboximidamide head interacting with Glu592, while the other head occupies a hydrophobic pocket surrounded by Met336, Leu337, and Tyr706 near the active site entrance (Figure 4). The two phenyl rings in 7 are in van der Waals contacts with both heme propionates (Figure 4A). In contrast, having a 2-carbon linker between the two phenyl rings in 9 pushes the second phenyl and thiophene-2-carboximidamide head farther away from the heme (Figure 4B). The Leu337 side chain must adopt an alternate rotamer to accommodate lengthy compound 9. The second carboximidamide in 9 does not make any direct contacts with the protein, while that in 7 makes only a weak H-bond with heme propionate D. Therefore, the submicromolar binding affinity is mainly attributed to the first phenyl ring and the thiophene-2-carboximidamide head that tightly anchors the inhibitor to the NOS active site. The structure of 10 bound to nNOS (data not shown) also supports this conclusion. While the first phenyl ring and thiophene-2-carboximidamide head is clearly shown in the electron density, the remainder of the compound is...
disordered. Nevertheless, 10 shares a similar affinity to nNOS as 7 and 9 (Table 1). Compounds with a longer (4-or 5-atom) linker between the two head groups, 11 and 12, exhibit much improved potency (Table 1). The 11-nNOS structure (Figure 5A) provides an explanation. Similar to the cases of 7 and 9, one phenyl ring and the thiophene-2-carboximidamide head of 11 recognizes the nNOS active site and H-bonds to Glu592 with the carboximidamide nitrogens. The O atom in the central linker distal to the active site forms a weak H-bond (3.4 Å) with Gln478. This brings the second phenyl ring next to Arg481. The Arg481 guanidinium plane has to rotate to make a better cation–aromatic stacking interaction with the phenyl ring of 11. In addition, the N atom in the second head joins a H-bonding network involving Arg596, Asp600, and Arg603 via a water molecule. Interestingly, this thiophene-2-carboximidamide head reaches to a pocket surrounded by Trp306 (in the other subunit of the dimer), Asp600, Ser602, and Arg603 (Figure 5B). This is the first time we have found an inhibitor reaching into this pocket. Sequence alignment reveals that Ser602 in nNOS is a His or a Gln at the corresponding positions in eNOS and iNOS, respectively. It is likely that interactions between the thiophene-2-carboximidamide and this unique hot spot affect the selectivity of 11 while the extensive aforementioned enzyme–inhibitor interactions account for the better potency. Linker length also is quite important because increasing the linker by one atom in going from 11 to 12 results in a 2-fold lower potency. The 12-nNOS structure (data not

Scheme 1. Synthesis of 7 and 8

\begin{center}
\begin{tikzpicture}
\node (1) at (0,0) {\includegraphics[width=0.8\textwidth]{Scheme1}};
\end{tikzpicture}
\end{center}

\textsuperscript{“Reagents and conditions: (a) Pd\textsubscript{2}(dba)\textsubscript{3}, tri(o-tolyl)phosphine, DIEA, 100 °C, 8 h, 61%; (b) Raney Ni, hydrazine hydrate, room temp, 30 min, quantitative; (c) EtOH, room temp, 24 h, 54–58%.”}

Scheme 2. Synthesis of 9

\begin{center}
\begin{tikzpicture}
\node (1) at (0,0) {\includegraphics[width=0.8\textwidth]{Scheme2}};
\end{tikzpicture}
\end{center}

\textsuperscript{“Reagents and conditions: (a) PPh\textsubscript{3}, toluene, 90 °C, 12 h, 97%; (b) LHMDS, THF, −78 °C to room temp, 4 h, 87%; (c) Raney Ni, hydrazine hydrate, room temp, 30 min, quantitative; (d) EtOH, room temp, 24 h, 46%; (e) H\textsubscript{2}, Pd/C, EtOH, room temp, 24 h, 91%.”}
shown) shows that while the first phenyl ring and thiophene-2-carboximidamide head clearly bind to the nNOS active site similarly to that of 11, the other head is too flexible to be modeled with certainty.

nNOS has two negative charges in the active site (Glu592 and Asp597) compared to one in eNOS (Glu363). As a result, nNOS provides better electrostatic stabilization for positively charged inhibitors. Inspired by our previously reported highly selective and potent pyrrolidine-containing nNOS inhibitors (1 and 2), we introduced a basic nitrogen atom mimicking the pyrrolidine N atom in the linker part of 11, leading to 13 and 14, to enhance the binding affinity and selectivity (Table 2). The results are impressive because both $K_i$ values of 13 and 14 for nNOS are 5 nM, which is 25-fold more potent than that of 11. Moreover, they display good selectivities of nNOS over iNOS and eNOS. The crystal structures of 13 and 14 bound to nNOS show that, as expected, both use the first phenyl ring and thiophene-2-carboximidamide head to anchor the inhibitor to the nNOS active site (Figure 6). The newly introduced amino group in the linker is situated between the two heme propionates. These electrostatic interactions are very likely the main source of binding affinity gained by the two compounds. However, 13 and 14 have a different phenyl ring in the second head. While 13 bears a meta-bisubstituted phenyl ring, the one in 14 is para-bissubstituted. The structural differences around the phenyl ring influence the way the second thiophene-2-carboximidamide headgroup interacts with the protein. The head in 13 reaches the same pocket next to Ser602 described for 11 but through a different "route". This is because the new inhibitor—heme electrostatic interactions direct the second phenyl ring of 13 to pack against Met336 (Figure 6A) rather than Arg481, as in the case of 11 (Figure 5A). The second thiophene-2-carboximidamide head in 14 is partially disordered because the para-bissubstituted phenyl forces the thiophene tail toward the position of Trp306 (in the other subunit). The tail has to make a sharp turn to avoid clashes (Figure 6B).
The basicity of the amine in the linker is important for potency, but this secondary amine is also a potential metabolic site that can be easily converted to a secondary hydroxylamine or serve as a substrate for flavin monooxygenases. Therefore, we introduced a small ethyl group at the basic N atom of 14 to block this potential metabolic site. Such a substitution (15) decreases the potency (leading to an almost 10-fold drop in nNOS inhibition) but affords an increase in selectivity of nNOS over eNOS (845-fold). To our surprise, the 15-nNOS structure reveals that 15 (Figure 6C) binds to nNOS in an orientation 180° flipped from that of 14 (Figure 6B). With 15, it is the para-bisubstituted phenyl ring and its adjacent thiophene-2-carboximidamide that binds to Glu592. This orientation still allows a H-bond between the ethylated tertiary amine and

**Scheme 5. Synthesis of 13−17**

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| Step | Reaction | Conditions |
|------|----------|------------|
| a    | Et<sub>3</sub>N, NaBH<sub>3</sub>CN, THF, room temp, 12 h, 69−72% | |
| b    | (Boc)<sub>2</sub>O, DMAP, MeOH, room temp, 12 h, 92−94% | |
| c    | NaBH<sub>3</sub>CN, THF, room temp, 12 h, 58−64% | |
| d    | Raney Ni, hydrazine hydrate, room temp, 30 min, quantitative | |
| e    | EtOH, room temp, 24 h, 48−53% | |
| f    | 3N HCl/MeOH, room temp, 24 h, quantitative | |
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**Reagents and conditions:** (a) Et<sub>3</sub>N, NaBH<sub>3</sub>CN, THF, room temp, 12 h, 69−72%; (b) (Boc)<sub>2</sub>O, DMAP, MeOH, room temp, 12 h, 92−94%; (c) NaBH<sub>3</sub>CN, THF, room temp, 12 h, 58−64%; (d) Raney Ni, hydrazine hydrate, room temp, 30 min, quantitative; (e) EtOH, room temp, 24 h, 48−53%; (f) 3N HCl/MeOH, room temp, 24 h, quantitative.
The compounds were evaluated for in vitro inhibition against three NOS isozymes: rat nNOS, bovine eNOS, and murine iNOS. Considering that three NOS isozymes are isolated from three different species, the activity or selectivity of these compounds may differ in human NOS isozymes.39

heme propionate D. Inhibitor 14 is close to both heme propionates while 15 is close to only one, which may be the structural basis for why 14 is a more potent inhibitor. Nevertheless, the second phenyl ring and thiophene-2-carboximidamide head in 15 are quite well-defined, making van der Waals contacts with Met336, Leu337, and Trp306 (in the other subunit).

To understand isoform selectivity, we also determined the structures of 14-nNOS and 15-eNOS. As shown in Figure 7A, 14 anchors its meta-bisubstituted phenyl ring and the connected thiophene-2-carboximidamide head to the eNOS active site next to Glu363, which is the same interaction seen in the nNOS structure (Figure 6C). That is, the para-bisubstituted phenyl ring brings the thiophene-2-carboximidamide next to Glu363 on one end and the tertiary amine next to heme propionate D on the other end, for H-bonds. This means that the 845-fold selectivity of 15 for nNOS over eNOS (Table 2) is associated with how the tail end of the inhibitor extending out of the active site interacts with the protein. Unfortunately, the position of this thiophene ring is less certain because of poorer density in this region in both the 15-eNOS and 15-nNOS structures. Even so, there are sufficient sequence differences in this area to provide a possible explanation for selectivity. The main difference is that Met336 in nNOS is Val106 in eNOS. The second phenyl ring of 15 in eNOS makes van der Waals contacts with Val106 and Leu107, while the same group in nNOS contacts Met336 and Leu337. As a result, there is the potential for greater nonpolar interactions in nNOS. Another factor is the contacts between the thiophene ring and a Trp side chain (Trp306 in nNOS or Trp76 in eNOS). These contacts are closer in nNOS than those in eNOS.

Changing the secondary amine in 14 to a tertiary amino in 15 improves selectivity. To explore whether such substitution is effective in other cases, especially in mono-thiophene-2-carboximidamide nNOS inhibitors, we designed and synthesized 16 and 17 (Table 3). One thiophene-2-carboximidamide head was replaced by a fluorobenzene fragment because fluorine has been widely used to alter the pharmacokinetic properties and overall drug-like properties of compounds.27 Compound 16 drops about half of the nNOS inhibitory activity compared to parent compound 14, and selectivity also decreases sharply, especially over eNOS. However, addition of an ethyl group at the basic nitrogen of fluorobenzene head of 16 further enhances the nNOS over eNOS selectivity although it decreases the potency (Table 3), which shows the same trend as 14 and 15. The reason for decreased potency may be the shorter length, thereby preventing additional van der Waals contacts with the protein that are afforded by the longer thiophene-2-carboximidamide head in 14. Detailed interactions of 16 and 17 are shown in Figure 8.

Indeed, the structure of 16-nNOS (Figure 8A) reveals that apart from the common features of NOS active site recognition through the thiophene-2-carboximidamide head seen for other analogues, the fluorobenzene head of 16 interacts only with Trp306 (other subunit). Compound 17 shows ~7-fold lower potency than 16, mainly because it maintains only one H-bond

Table 1. Inhibition of NOS Isozymes by Synthetic Inhibitors 7–12

| compd no. | Kᵢ (μM) nNOS | Kᵢ (μM) iNOS | Kᵢ (μM) eNOS | selectivity n/e | selectivity n/i |
|-----------|--------------|--------------|--------------|----------------|----------------|
| 7         | 0.787 ± 0.061| 66.4 ± 5.2   | 177.3 ± 10.9 | 84             | 225            |
| 8         | 0.774 ± 0.069| 79.0 ± 4.0   | 133.0 ± 12.4 | 102            | 171            |
| 9         | 0.736 ± 0.053| 103.8 ± 8.7  | 60.0 ± 5.8   | 141            | 89             |
| 10        | 0.819 ± 0.067| 101.1 ± 0.9  | 5.2 ± 0.4    | 12             | 6              |
| 11        | 0.110 ± 0.062| 66.8 ± 2.7   | 13.7 ± 0.7   | 513            | 105            |
| 12        | 0.237 ± 0.019| 12.6 ± 0.8   | 4.0 ± 0.3    | 53             | 17             |

Figures were generated using PyMol (www.pymol.org).
Figure 5. Crystallographic binding conformation of 11 with rat nNOS (A) and the new hot spot around the second thiophene head (B). The omit Fc − Fo electron density maps for inhibitors are shown at the 2.5 σ contour level. Relevant H-bonds are depicted with dashed lines.

Table 2. Inhibition of NOS Isozymes by Synthetic Inhibitors 13–15

| compd no. | K_i (µM) nNOS | K_i (µM) iNOS | K_i (µM) eNOS | selectivity | n/i | n/e |
|-----------|----------------|---------------|---------------|-------------|-----|-----|
| 13        | 0.005 ± 0.0005 | 1.3 ± 0.2     | 2.2 ± 0.1     | 260 ± 440   | 1.3 | 3.4 |
| 14        | 0.005 ± 0.0003 | 1.7 ± 0.1     | 2.7 ± 0.2     | 340 ± 540   | 1.3 | 3.4 |
| 15        | 0.049 ± 0.0002 | 14.2 ± 0.8    | 41.4 ± 2.2    | 290 ± 845   | 1.3 | 3.4 |

The compounds were evaluated for in vitro inhibition against three NOS isozymes: rat nNOS, bovine eNOS, and murine iNOS.

with heme propionate A via its tertiary amine (Figure 8B), while the secondary amine in 16 makes H-bonds/salt bridges with both heme propionates (Figure 8A). Moreover, the fluorobenzene in the 17-nNOS complex has higher flexibility with weaker electron density, making a new hydrophobic interaction with Tyr706. This interaction forces the phenyl group of Tyr706 to rotate about 70° from what is seen in the 16-nNOS structure. Although 17 is a weaker binder than 16, the former has better selectivity (Table 3). The crystal structure shows that 16 (Figure 8C) binds to eNOS in an almost identical manner as that found in the 16-nNOS structure (Figure 8A), and 17 also binds to eNOS (Figure 8D) similar to nNOS (Figure 8B). The only difference for 16 is the orientation of the F atom from fluorobenzene. In eNOS the F atom points toward Val106 and Leu107 for van der Waals contacts. To avoid clashes with the fluorobenzene of 16 (or 17), the Tyr477 side chain rotates, as we observed with 17-nNOS (Figure 8B). The subtle difference between 17-eNOS (Figure 8D) and 17-nNOS (Figure 8B) is also only in the position of fluorobenzene, which might be attributed to the amino acid variation in the vicinity. Met336 in nNOS is corresponds to Val106 in eNOS. Hydrophobic contacts (~3.5–3.9 Å) found between the fluorobenzene and Val106/Leu107 in 17-eNOS are closer than those contacts (~4.3–4.8 Å) to Met336/Leu337 seen with 17-nNOS. Although it is not clear that the differences near the tail end of the inhibitors is the explanation for isomform selectivity, it is clear that attaching an ethyl group to the secondary amine is a useful method to improve the nNOS/eNOS selectivity of these thiophene compounds.

Given the enzyme potency of 13 and 14, we next tested whether these inhibitors were effective in melanoma cell lines. Detailed in our previous study,7 nNOS is associated with the proliferation of melanoma and increased invasion, leading to subsequent development of metastases. nNOS inhibitors were able to efficiently inhibit melanoma proliferation and invasion with significant reduction of intracellular NO levels. Inhibitors 13 and 14 exhibited potent antimalanoma activity in vitro (Table 4). Their EC_{50} values in metastatic melanoma A375 cells are 1.3 and 3.4 µM (Table 4), which is better than that of the chemotherapeutic drug cisplatin (4.2 and 14.3 µM in A375 and Sk-Mel28 cells, respectively).7 Notably, the inhibition by 13 and 14 is more predominant in metastatic melanoma A375 cells compared to primary early stage Wm3211 cells, which supports our hypothesis that nNOS/NO signaling is more critical to melanoma progression than to the initiation phase. However, unexpectedly, these compounds also exhibited apparent inhibition of cell proliferation in primary normal cells, including melanocytes and fibroblast cells. Taken together, these results suggest that targeting nNOS provides a potential approach to block overactivated NO signaling in human melanoma.

**CONCLUSIONS**

In summary, we report the rational design of selective nNOS inhibitors using a hybrid strategy. Two compounds, 13 and 14, show excellent potency (5 nM) and good selectivities for nNOS over the other isozymes. The crystal structures of these inhibitors bound to nNOS or eNOS have identified a new hot spot near Ser602 in nNOS (Figure 5B). The equivalent residue in eNOS is His373, which may be a contributing structural factor for the selectivity observed for 14 (Figure 6). A small ethyl substituent attached to the amine of the inhibitor acts as an important component for improving isoform selectivity. Importantly, 13 and 14 achieve good cellular activity in two melanoma cell lines. Altogether, these inhibitors are promising candidates for further development of improved therapeutics, and their structural features provide new clues for the future design and development of new selective nNOS inhibitors.

**EXPERIMENTAL SECTION**

All reagents were purchased from Sigma-Aldrich, Alfa Aesar, or TCI and were used without further purification unless stated otherwise. Analytical thin layer chromatography was visualized by ultraviolet light. Flash column chromatography was carried out under a positive pressure of air. 1H NMR spectra were recorded on 500 or 400 MHz spectrometers. Data are presented as follows: chemical shift (in ppm on the δ scale, and the reference resonance peaks set at 0 ppm [TMS(CDCl_3)] and 3.31 ppm (CD_3OD), multiplicity (s = singlet, d
= doublet, t = triplet, q = quartet, p = quintet, m = multiplet), coupling constant ($J$/Hz), and integration. $^{13}$C NMR spectra were recorded at 125 or 100 MHz, and all chemical shift values are reported in ppm on the $\delta$ scale, with an internal reference of $\delta$ 49.0 for CD$_3$OD. High-resolution mass spectra were measured on liquid chromatography/time-of-flight mass spectrometry (LC-TOF). The purity of the tested compounds was determined by high-performance liquid chromatography (HPLC) analysis and was >95%.

**General Procedure A: Nitro Group Reduction.** To a solution of the reagent (1 mmol) in MeOH (10.0 mL) was added hydrazine hydrate (0.2 mL) and Raney nickel. The reaction mixture was stirred at room temperature for 30 min. Then the reaction mixture was filtered through Celite. The filtration was diluted with water, extracted with EtOAc, and washed with saturated NaHCO$_3$ and brine. The organic layer was concentrated in vacuo. The residue was purified by column chromatography to yield the product.

**General Procedure B: Reductive Amination.** To a solution of the aldehyde (1.0 mmol) and amine (1.0 mmol) in THF (20 mL) at 0 $^\circ$C was added NaBH$_3$CN (1.05 mmol) in three portions carefully. The reaction mixture was stirred at 0 $^\circ$C for 30 min and then stirred overnight at room temperature. The reaction mixture was quenched with MeOH in an ice bath, extracted with EtOAc, and washed with...
Table 3. Inhibition of NOS Isozymes by Synthetic Inhibitors 16 and 17a

| compd no. | Kᵢ (μM) nNOS | Kᵢ (μM) iNOS | Kᵢ (μM) eNOS | selectivity n/i | selectivity n/e |
|-----------|--------------|--------------|--------------|----------------|----------------|
| 16        | 0.011 ± 0.001 | 1.6 ± 0.07   | 0.9 ± 0.03   | 145            | 82             |
| 17        | 0.073 ± 0.005 | 12.1 ± 0.74  | 21.7 ± 1.57  | 166            | 297            |

a The compounds were evaluated for in vitro inhibition against three NOS isozymes: rat nNOS, bovine eNOS and murine iNOS.

saturated NaHCO₃ and brine. The organic layer was concentrated in vacuo. The residue was purified by column chromatography to yield product.

**General Procedure C: Boc-Protection.** To a solution of the amine (1 mmol) in MeOH (15 mL) was added di-tert-butyl dicarbonate (1.2 equiv) and DMAP (cat.). The reaction mixture was stirred at room temperature for 12 h. Then the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography to yield the product.

**General Procedure D: Coupling Reaction of Amine with Methyl Thiophene-2-carbimidothioate Hydroiodide Salt.** To a solution of the amine (1 mmol) in EtOH (15.0 mL) was added thiophene-2-carbimidothioate hydroiodide salt (1.5 equiv for mono-amine and 3.0 equiv for diamine). The reaction mixture was stirred at room temperature for 36 h. Then the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography to yield product.

**General Procedure E: Boc-Deprotection.** To a solution of the Boc-protected reagent (0.2 mmol) in MeOH (1.0 mL) was added 3N HCl (10.0 mL). The reaction mixture was stirred at room temperature for 24 h. Then the reaction mixture was concentrated in vacuo. The crude product was recrystallized with MeOH and cold diethyl ether to provide the product.

**N,N’-([1,1’-Biphenyl]-3,3’-diyl)bis(thiophene-2-carboximidamide) (7).** To a solution of 1-iodo-3-nitrobenzene (3.0 mmol) in dry DIEA (20 mL) was added Pd₂dba₃ (0.05 equiv) and tri(o-tolyl)phosphine (0.1 equiv). The reaction mixture was stirred under argon at 100 °C for 8 h and then diluted with H₂O, extracted with EtOAc, and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography to yield 18 (yield 61%).

7 was synthesized by general procedures A and D using 18 as the starting material (yield 54%). 1H NMR (500 MHz, CD₃OD): δ 7.68 (d, J = 3.5 Hz, 2H), 7.60 (d, J = 5.0 Hz, 2H), 7.46 (t, J = 7.5 Hz, 2H), 7.40 (d, J = 7.5 Hz, 2H), 7.26 (s, 2H), 7.15 (d, J = 5.0, 3.5 Hz, 2H), 7.00 (d, J = 7.5 Hz, 2H).

**13C NMR (125 MHz, CD₃OD):** δ 156.61, 140.59, 135.37, 134.96, 134.59, 130.65, 129.07, 128.62, 126.63, 125.25, 124.12. LC-TOF (M + H+) calcd for C₂₂H₁₉N₄S₂ 403.1046, found 403.1046.

**N,N’-(Methylenebis(3,1-phenylene))bis(thiophene-2-carboximidamide) (8).** Compound 8 was synthesized by general procedure D using 3,3’-methylenebiamine as the starting material (yield 58%).

1H NMR (500 MHz, CD₃OD): δ 8.10 − 8.00 (m, 4H), 7.53 (t, J = 7.5 Hz, 2H), 7.41 − 7.36 (m, 6H), 7.34 (d, J = 7.5 Hz, 2H), 4.18 (s, 2H).

**13C NMR (125 MHz, CD₃OD):** δ 159.39, 144.70, 135.82, 135.50, 135.15, 131.76, 130.92, 130.30, 130.02, 127.25, 124.71, 41.99. LC-TOF (M + H+) calcd for C₂₃H₂₁N₄S₂ 417.1202, found 417.1195.

**N,N’-(Ethane-1,2-diylbis(3,1-phenylene))bis(thiophene-2-carboximidamide) (9).** LHMDS (1.0 M in THF, 2.4 mmol) was added to a suspension of 20 (2 mmol) in anhydrous THF (15 mL) at −78 °C. The reaction mixture was stirred under argon for 30 min, then warmed to 0 °C over 30 min. To this reaction mixture was added 3-nitrobenzaldehyde (2.2 mmol) in anhydrous THF (3 mL) at −78 °C. The reaction mixture was warmed to room temperature and stirred for 4 h. The reaction mixture was then quenched with saturated NH₄Cl.

Figure 8. Crystallographic binding conformation of 16 (A) and 17 (B) with rat nNOS, as well as 16 (C) and 17 (D) with bovine eNOS. The omit Fₐ − Fₖ electron density maps for inhibitors are shown at the 2.5 σ contour level. Relevant H-bonds are depicted with dashed lines. Key distances are drawn with dashed lines and labeled in Å.
extracted with EtOAc, and washed with water and brine. The organic layer was concentrated in vacuo. The residue was purified by column chromatography to yield 21 (yield 87%). Intermediate 24 was synthesized by general procedures A and D using 25 as the starting material (yield 48%). A solution of 24 (0.5 mmol) in MeOH (20 mL) was treated with 10% Pd/C (1.5 mg). The reaction mixture was stirred at room temperature under a hydrogen atmosphere for 24 h. The catalyst was removed by filtration through Celite, and the resulting solution was concentrated in vacuo. The crude material was purified by column chromatography to yield 9 (yield 91%). 1H NMR (400 MHz, CD3OD): δ 8.17–8.02 (m, 4H), 7.56–7.51 (t, J = 7.5 Hz, 2H), 7.45–7.38 (m, 6H), 7.36–7.30 (d, J = 7.5 Hz, 2H), 3.10 (s, 4H). 13C NMR (100 MHz, CD3OD): δ 156.90, 143.97, 136.57, 133.15, 132.73, 130.64, 129.92, 128.32, 128.26, 125.00, 122.43, 36.92. LC-TOF (M + H+) calc for C18H13N3S: 341.1359, found 341.1359.

N-(1-Hydroxy-2-carboxydimidazolyl)-phenoxo)ethyl)phenyl)thiophene-2-carboximidamide (10). To a solution of 3-nitrophenol (2 mmol) in DMF (20 mL) was added K2CO3 (2.2 mmol) and 2-bromo-1-(3-nitrophenyl)ethanone (2.2 mmol). The suspension was stirred for 6 h at room temperature. The reaction mixture was then diluted with H2O (50 mL), extracted with EtOAc, and washed with water and brine. The organic layer was dried over Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography to yield 24 (yield 73%). To a solution of 24 (1 mmol) in THF (10 mL) was added NaBH4 (1.2 mmol) in three portions. The reaction mixture was stirred at room temperature for 8 h under N2 atmosphere. The reaction mixture was then quenched with H2O (20 mL), extracted with CH2Cl2, and washed with water and brine. The organic layer was dried over Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography to yield 25 (yield 80%). 10 was synthesized by general procedures A and D using 25 as the starting material (yield 41%). 1H NMR (500 MHz, CD3OD): δ 7.62 (dt, J = 7.5, 3.5 Hz, 2H), 7.58–7.51 (m, 2H), 7.37 (t, J = 7.5 Hz, 1H), 7.25 (t, J = 7.5 Hz, 1H), 7.20 (d, J = 7.5 Hz, 1H), 7.13–7.07 (m, 3H), 6.93 (d, J = 7.5, 2.5 Hz, 1H), 6.69 (dd, J = 7.5, 2.5 Hz, 1H), 6.60–6.54 (m, 2H), 5.05–5.00 (m, 1H), 4.15–4.00 (m, 2H). 13C NMR (125 MHz, CD3OD): δ 161.37, 144.06, 131.34, 130.62, 129.88, 128.51, 123.80, 123.20, 122.77, 121.79, 116.23, 110.97, 110.09, 74.18, 73.46. LC-TOF (M + H+) calc for C24H17N3OS 463.1257, found 463.1256.

N,N′-(Ethane-1,2-diyibis(oxi)bis(3,1-phenylene)bis-(thiophene-2-carboximidamide) (11). To a solution of 3-nitrophenol (2 mmol) in DMF (20 mL) was added K2CO3 (2.2 mmol) and 1,3-dibromopropane (1 mmol). The suspension was then stirred for 6 h at 60°C. The reaction mixture was then diluted with H2O (50 mL), extracted with EtOAc, and washed with water and brine. The organic layer was dried over Na2SO4 and concentrated in vacuo. The residue was purified by column chromatography to yield 27 (yield 80%). 11 was synthesized by general procedures A and D using 27 as the starting material (yield 59%). 1H NMR (500 MHz, CD3OD): δ 8.01 (t, J = 4.0 Hz, 4H), 7.47 (t, J = 8.0 Hz, 2H), 7.33 (t, J = 4.0 Hz, 2H), 7.03 (dd, J = 8.0, 2.0 Hz, 2H), 6.97 (t, J = 2.0 Hz, 2H), 6.94 (dd, J = 8.0, 2.0 Hz, 2H), 4.43 (s, 4H). 13C NMR (125 MHz, CD3OD): δ 161.52, 157.72, 133.75, 133.12, 132.15, 129.64, 129.49, 118.15, 114.83, 112.12, 68.12. LC-TOF (M + H+) calc for C36H26N6O6S2 643.1257, found 643.1257.

N,N′-(Propane-1,3-diylbis(oxi)bis(3,1-phenylene)bis-(thiophene-2-carboximidamide) (12). 12 was synthesized by the same procedures as those to prepare 11 using 1,4-dibromobutane as the starting material. 1H NMR (500 MHz, CD3OD): δ 8.09–7.99 (m, 4H), 7.49 (t, J = 8.0 Hz, 2H), 7.41–7.33 (m, 2H), 7.11–7.05 (dd, J = 8.0, 2.5 Hz, 2H), 7.03 (t, J = 2.5 Hz, 2H), 7.00 (dd, J = 8.0, 2.5 Hz, 2H), 4.28 (t, J = 6.0 Hz, 4H), 2.33 (p, J = 6.0 Hz, 2H). 13C NMR (125 MHz, CD3OD): δ 161.77, 158.79, 146.61, 134.93, 134.51, 132.32, 129.83, 118.43, 115.89, 112.62, 65.88, 50.21. LC-TOF (M + H+) calc for C19H11N3O2S2 344.1411, found 344.1141.

Table 4. EC50 Values (µM) of 13 and 14 with Human Melanoma Cells

| compd | primary melanoma WM3211 cell line | metastatic melanoma A375 cell line | primary fibroblast cells | primary keratinocytes | immortalized melanocytes |
|-------|-----------------------------------|-----------------------------------|--------------------------|-----------------------|-------------------------|
| 13    | 5.6                               | 3.4                               | 1.4                      | NDa                  | cannot calculateb      |
| 14    | 2.7                               | 1.3                               | NDa                      | 7.9                   | cannot calculateb      |

aN: Not done. bToxic even at lower concentration and no dose–activity curve occurred.
| Data Collection | nNOS-7 | nNOS-9 | nNOS-11 | nNOS-13 |
|----------------|--------|--------|---------|---------|
| PDB code       | 4KCH   | 4KCI   | 4KCJ    | 4KCK    |
| space group    | P2₁2₁₂₁ | P2₁2₁₂₁ | P2₁2₁₂₁ | P2₁2₁₂₁ |
| resolution (Å) | 2.15 (2.19–2.15) | 2.27 (2.31–2.27) | 2.05 (2.09–2.05) | 2.10 (2.14–2.10) |
| Rmerge         | 0.066 (0.559) | 0.055 (0.677) | 0.055 (0.799) | 0.069 (0.720) |
| I/σI           | 20.0 (1.3) | 29.7 (1.9) | 25.1 (2.1) | 20.7 (1.5) |
| no. unique reflns | 52573 | 44777 | 61070 | 52371 |
| completeness (%) | 99.4 (98.1) | 98.3 (97.8) | 99.6 (97.8) | 92.6 (100.0) |
| redundancy      | 3.6 (3.6) | 4.0 (4.2) | 3.6 (3.4) | 3.5 (3.7) |

Refinement

| resolution (Å) | 2.15 | 2.27 | 2.05 | 2.10 |
| no. reflns used | 49887 | 42147 | 57366 | 49419 |
| Rwork/Rfree   | 0.194/0.250 | 0.181/0.238 | 0.195/0.242 | 0.216/0.264 |
| no. atoms      | 6659 | 6659 | 6665 | 6681 |
| protein        | 185 | 189 | 193 | 179 |
| ligand/ion     | 167 | 122 | 259 | 379 |
| water          | 61898 | 58123 | 82346 | 81114 |
| completeness (%) | 98.0 (97.8) | 99.6 (100.0) | 99.3 (99.9) | 99.4 (100.0) |
| redundancy      | 3.3 (3.2) | 3.8 (3.8) | 4.0 (4.1) | 3.9 (3.8) |

RMS deviations

| bond lengths (Å) | 0.011 | 0.016 | 0.013 | 0.012 |
| bond angles (deg) | 2.02 | 2.27 | 2.11 | 2.10 |
| data set⁴ | eNOS-14 | eNOS-15 | eNOS-16 | eNOS-17 |

| Data Collection | eNOS-14 | eNOS-15 | eNOS-16 | eNOS-17 |
|----------------|--------|--------|---------|---------|
| PDB code       | 4KCL   | 4KCM   | 4KCN    | 4KCO    |
| space group    | P2₁2₁₂₁ | P2₁2₁₂₁ | P2₁2₁₂₁ | P2₁2₁₂₁ |
| resolution (Å) | 1.93 (1.96–1.93) | 2.08 (2.12–2.08) | 1.85 (1.88–1.85) | 1.86 (1.89–1.86) |
| Rmerge         | 0.057 (0.479) | 0.068 (0.762) | 0.050 (0.660) | 0.057 (0.680) |
| I/σI           | 26.3 (2.1) | 23.1 (1.7) | 29.2 (2.6) | 28.2 (2.1) |
| no. unique reflns | 71989 | 58123 | 8346 | 81114 |
| completeness (%) | 98.0 (97.8) | 99.6 (100.0) | 99.3 (99.9) | 99.4 (100.0) |
| redundancy      | 3.3 (3.2) | 3.8 (3.8) | 4.0 (4.1) | 3.9 (3.8) |

Refinement

| resolution (Å) | 2.10 | 2.07 | 1.85 | 1.86 |
| no. reflns used | 68,060 | 55,146 | 77,959 | 76,678 |
| Rwork/Rfree   | 0.182/0.213 | 0.191/0.236 | 0.185/0.217 | 0.187/0.220 |
| no. atoms      | 6666 | 6668 | 6677 | 6685 |
| protein        | 195 | 188 | 219 | 177 |
| ligand/ion     | 341 | 224 | 397 | 380 |
| water          | 61898 | 58123 | 82346 | 81114 |
| completeness (%) | 99.1 (99.9) | 99.6 (100.0) | 99.7 (100.0) | 100.0 |
| redundancy      | 4.0 (4.0) | 3.9 (3.9) | 4.0 (4.1) | 4.0 (4.0) |

RMS deviations

| bond lengths (Å) | 0.011 | 0.011 | 0.012 | 0.014 |
| bond angles (deg) | 1.45 | 2.05 | 1.52 | 1.62 |
| data set⁴ | eNOS-14 | eNOS-15 | eNOS-16 | eNOS-17 |

| Data Collection | eNOS-14 | eNOS-15 | eNOS-16 | eNOS-17 |
|----------------|--------|--------|---------|---------|
| PDB code       | 4KCP   | 4KCO   | 4KCR    | 4KCS    |
| space group    | P2₁2₁₂₁ | P2₁2₁₂₁ | P2₁2₁₂₁ | P2₁2₁₂₁ |
| resolution (Å) | 2.07 (2.07–2.07) | 2.03 (2.07–2.03) | 2.09 (2.13–2.09) | 2.05 (2.09–2.05) |
| Rmerge         | 0.060 (0.712) | 0.064 (0.494) | 0.059 (0.576) | 0.051 (0.528) |
| I/σI           | 24.2 (1.9) | 26.9 (2.6) | 27.3 (2.6) | 31.0 (2.9) |
| no. unique reflns | 59332 | 62254 | 57741 | 61246 |
| Completeness (%) | 99.1 (99.9) | 98.4 (99.7) | 99.7 (100.0) | 99.2 (100.0) |
| Redundancy      | 4.0 (4.0) | 3.9 (3.9) | 4.0 (4.1) | 4.0 (4.0) |

Refinement

| Resolution (Å) | 2.07 | 2.03 | 2.09 | 2.05 |
| No. reflns used | 56198 | 59095 | 54719 | 58050 |
| Rwork/Rfree   | 0.166/0.213 | 0.179/0.224 | 0.164/0.205 | 0.168/0.213 |
| No. atoms      | 6446 | 6429 | 6436 | 6446 |
and 2-(3-nitrophenyl)ethanamine as the starting materials. \(^1\)H NMR (400 MHz, CD\(_3\)OD): \(\delta\) 7.58–7.72 (m, 2H), 7.43 (t, \(J = 7.5\) Hz, 1H), 7.31–7.17 (m, 4H), 7.12 (dt, \(J = 7.5, 1.0\) Hz, 1H), 7.02 (dt, \(J = 7.5, 1.0\) Hz, 1H), 6.97 (dt, \(J = 9.0, 2.5\) Hz, 1H), 6.88 (td, \(J = 9.0, 2.5\) Hz, 1H), 3.96 (s, 2H), 2.98–2.92 (m, 6H), 1.18 (t, \(J = 7.0\) Hz, 3H). \(^{13}\)C NMR (100 MHz, CD\(_3\)OD): \(\delta\) 164.05, 161.62, 141.73 (d, \(J = 7.5\) Hz), 135.05, 130.92, 130.00, 129.91, 129.82, 129.80, 127.70, 126.86, 124.80, 124.44, 124.41, 123.27, 115.19 (d, \(J = 20\) Hz), 112.72 (d, \(J = 20\) Hz), 109.99, 56.82, 53.51, 47.10, 31.10, 9.67. LC-TOF (M + H\(^+\)) calcd for C\(_{22}\)H\(_{25}\)FN\(_3\)S 382.1748, found 382.1742.

The selectivity of an inhibitor was defined as the ratio of the respective \(K_i\) values.

**Enzyme Assays.** The three isozymes, rat nNOS, murine macrophage iNOS, and bovine eNOS, were recombinant enzymes, overexpressed (in *Escherichia coli*) and isolated as reported.\(^{28,30}\) **\(K_i\)** values for inhibitors 7–17 were measured for the three different isoforms of NOS using L-arginine as a substrate. The formation of nitric oxide was measured using the hemoglobin capture assay described previously.\(^{31}\) All NOS isoforms were assayed using the Synergy H1 hybrid multimode microplate reader (BioTek Instruments, Inc.) at 37 °C in a 100 mM Hepes buffer (pH 7.4) containing 10 \(\mu\)M L-arginine, 0.83 mM CaCl\(_2\), 320 units/mL calmodulin, 100 \(\mu\)M NADPH, 10 \(\mu\)M H\(_2\)O\(_2\), and 3.0 \(\mu\)M oxyhemoglobin (for NOS assays, no Ca\(^{2+}\) and calmodulin was added). The assay was initiated by the addition of enzyme, and the initial rates of the enzymatic reactions were determined by monitoring the formation of NO \(^{-}\) at 401 nm for 60 s. The apparent \(K_m\) values for L-arginine were 1.3 \(\mu\)M (nNOS), 8.2 \(\mu\)M (iNOS), and 1.7 \(\mu\)M (eNOS). The selectivity of an inhibitor was defined as the ratio of the respective \(K_i\) values.

**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.\(^{32,33}\) 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.\(^{32,33}\) Fresh crystals (1–2 day old) were first passed stepwise through cryoprotectant solutions as described\(^{32,33}\) and then soaked with 10 mM inhibitor for 4–6 h at 4 °C before being mounted on nylon loops and flash cooled by plunging into liquid nitrogen.

**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 Lightsource through the data collection control software Blu-Ice\(^{34}\) and a crystal mounting robot. Raw data frames were indexed, integrated, and scaled using HKL2000.\(^{35}\) The binding of inhibitors was detected by the initial difference Fourier maps calculated with REFMAC.\(^{36}\) The inhibitor molecules were then modeled in COOT\(^{37}\) and refined using REFMAC. Disordering in portions of inhibitor bound in the NOS active site was often observed, resulting in poor density quality. However, partial structural features usually could still be visible if the contour level of the sigmaA weighted 2\(|\text{F}_{o}\| - |\text{F}_{i}\|\) map dropped to 0.5 \(\sigma\), which afforded building of a reasonable model into the disordered regions. Water molecules were added in REFMAC and checked by COOT. The TLS\(^{38}\) protocol was implemented in the final stage of refinements with each subunit as one TLS group. The omit \(F_o - F_i\) density maps were calculated by repeating the last round of TLS refinement with inhibitor coordinate removed from the input PDB file to generate the coefficients DELFWT and SIGDELFWT. 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 5 with PDB accession codes included.

**ASSOCIATED CONTENT**

Supporting Information
Details of HPLC purity and copies of complete spectroscopic data of compounds 7–17. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

NOS, nitric oxide synthase; NO, nitric oxide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NHA, N\(^{\text{7+}}\)-hydroxy-L-arginine; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; H-bond, hydrogen bond

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