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Structure-based design, synthesis, and biological evaluation of lipophilic-tailed monocationic inhibitors of neuronal nitric oxide synthase

Fengtian Xue [a,b,†,‡], Jinwen Huang [a,b,†], Haitao Ji [a,b], Jianguo Fang [a,b], Huiying Li [c,d], Pavel Martásek [e,f], Linda J. Roman [e], Thomas L. Poulos [c,d,*], Richard B. Silverman [a,b,*]

[a] Department of Chemistry, Center for Molecular Innovation and Drug Discovery, and Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208-3113, USA
[b] Department of Biochemistry, Molecular Biology, and Cell Biology, Center for Molecular Innovation and Drug Discovery, and Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208-3113, USA
[c] Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697-3900, USA
[d] Department of Pharmaceutical Chemistry, and Chemistry, University of California, Irvine, CA 92697-3900, USA
[e] Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX, USA
[f] Department of Pediatrics and Center for Applied Genomics, 1st School of Medicine, Charles University, Prague, Czech Republic

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ABSTRACT

Selective inhibitors of neuronal nitric oxide synthase (nNOS) have the potential to develop into new neurodegenerative therapeutics. Recently, we described the discovery of novel nNOS inhibitors (**1a** and **1b**) based on a *cis*-pyrrolidine pharmacophore. These compounds and related ones were found to have poor blood–brain barrier permeability, presumably because of the basic nitrogens in the molecule. Here, a series of monocationic compounds was designed on the basis of docking experiments using the crystal structures of **1a**,**b** bound to nNOS. These compounds were synthesized and evaluated for their ability to inhibit neuronal nitric oxide synthase. Despite the excellent overlap of these compounds with **1a**,**b** bound to nNOS, they exhibited low potency. This is because they bound in the nNOS active site in the normal orientation rather than the expected flipped orientation used in the computer modeling. The biphenyl or phenoxyphenyl tail is disordered and does not form good protein–ligand interactions. These studies demonstrate the importance of the size and rigidity of the side chain tail and the second basic amino group for nNOS binding efficiency and the importance of the hydrophobic tail for conformational orientation in the active site of nNOS.

1. Introduction

Many lines of biological evidence have shown that overproduction of nitric oxide (NO) in the central nervous system (CNS) is implicated in various types of neurodegenerative diseases, including Parkinson's,[1–3] Alzheimer's,[4] and Huntington's disease.[5] Neuronal nitric oxide synthase (nNOS), the enzyme responsible for the generation of neuronal NO, is, therefore, a promising target for the development of new neurodegenerative therapeutics.[6–9] Over the past two decades, significant research has been devoted to developing nNOS selective inhibitors.[10,11] Recently, we described the discovery of the novel nNOS inhibitors **1a**,**b** (Fig. 1)[12] based on a *cis*-pyrrolidine pharmacophore.[13,14] Inhibitor **1b** has excellent potency (K_i = 15 nM), as well as high isozyme selectivity for nNOS (2100-fold over endothelial NOS; 630-fold over inducible NOS), which makes this compound a promising lead for neurodegenerative therapeutics.[12]

Inhibition of an enzyme that is implicated in the CNS, such as nNOS, however, requires the designed molecules to cross the blood–brain barrier (BBB).[15] Generally, there are three criteria for a small molecule to cross the BBB: (1) molecular weight <450 Da;[16] (2) reasonably good lipid solubility;[16] and (3) no affinity for one of BBB active efflux transporters (e.g., *p*-glycoprotein).[17–21] Recent animal tests demonstrated that **1a**,**b** have low BBB permeability, which strongly impedes further application of this compound as a neurodegenerative therapeutic.[12] Among the mechanisms that could limit **1b** from crossing the BBB, the dicationic character of this molecule under physiological conditions, as a result of the two high pK_a secondary amino groups, is a crucial factor prohibiting passive diffusion.[16] To improve the BBB permeability, prodrug approaches have been carried out to partially decrease

Abbreviations: nNOS, neuronal nitric oxide synthase; CNS, central nervous system; BBB, blood–brain barrier; TEA, triethylamine; TBAF, tetrabutylammonium fluoride; TBS, *t*-butyldimethylsilyl.

* Corresponding authors. Tel.: +1 847 491 5653; fax: +1 847 491 7713 (R.B.S.).
E-mail addresses: poulos@uci.edu (T.L. Poulos), r-silverman@northwestern.edu, agman@chem.northwestern.edu (R.B. Silverman).
† These authors contributed equally to the research.
‡ Present address: Department of Chemistry, University of Louisiana at Lafayette, PO Box 44370, Lafayette, LA 70504, USA.

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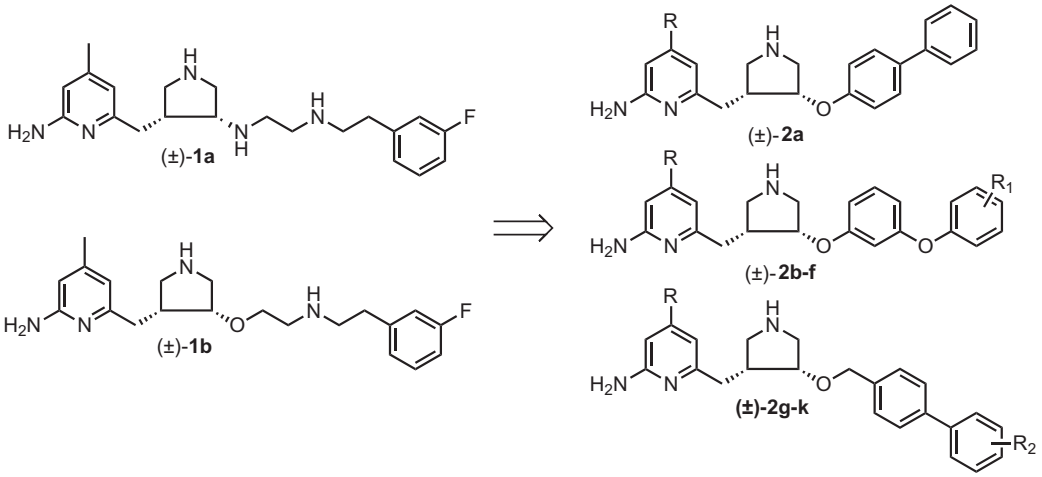


Figure 1. Design of monocationic inhibitors **2** based on **1b**.

the cationic character of **1b**.[22] Herein, we describe the structure-based design and synthesis of a new series of nNOS inhibitors (**2**, Fig. 1) with the aminoalkyl tail of **1b** replaced by a lipophilic biphenyl fragment or a phenoxyphenyl fragment. We reasoned that these compounds would be effective, given that they were designed based on a recent unexpected finding on how the (3*R*,4*R*) enantiomer of **1a** binds to nNOS.[23] It was anticipated that the (3*R*,4*R*)-**1a** would bind such that the aminopyridine ring stacks over the heme and H-bonds with the active site Glu592. Instead, we found that it binds in a flipped orientation with the aminopyridine extending out of the active site, where it H-bonds with a heme propionate. We also found that the (3*R*,4*R*)-**1b** binds the same as its counterpart of **1a** (to be published).[23,24] To make room for the aminopyridine, Tyr706 must swing out of the way, where it forms a π-stacking interaction with the aminopyridine (Fig. 2).[23,24] Because of this new binding orientation, compounds with fewer basic amino groups could overlay nicely on the flipped binding mode of **1b**. There are two potential advantages to this new design. First, one of the two high pK_a amino groups is removed from the lead compound (**1b**), resulting in a monocationic compound, which should have a better chance to cross the BBB by passive diffusion.[25] Second, using a phenoxyphenyl or biphenyl fragment, new inhibitors **2** possess a more rigid structure compared to **1b**, which can potentially improve the potency of inhibitors and be less susceptible to metabolic degradation. The 4-methyl group on the aminopyridine ring of **1b** was removed in compounds **2a**–**f** because the methyl group of the eutomer of **1b** is stabilized at the hydrophobic pocket of rat nNOS lined with Met336, Leu337, and Trp306 (chain B).[23,24] In human NOS, the residue that corresponds to rat nNOS Leu337 is His342, and it is the only residue in the active site that differs between rat nNOS and human nNOS.[26] It was hypothesized that demethylated compound **2a**–**f** might fit better in the active site of human nNOS, which does not have this hydrophobic pocket.

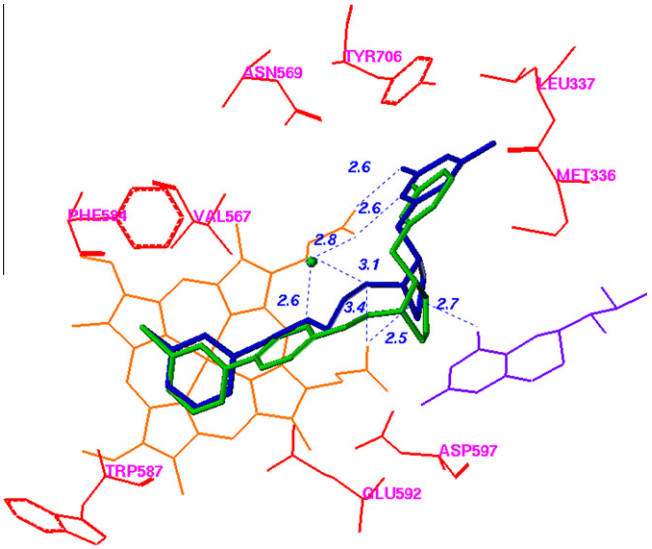


Figure 2. Docking conformation of **2h** (green) in the active site of nNOS. The potent and selective inhibitor N^1-[(3*R*,4*R*)-4′-((6″-amino-4″-methylpyridin-2″-yl)methyl)-pyrrolidin-3′-yl]-N^2-(3′-fluorophenethyl)ethane-1,2-diamine (blue), the (3*R*,4*R*) enantiomer of **1a** in Figure 1 in the crystal structure of rat nNOS (PDB code: 3JWT)[23] is overlaid for comparison.

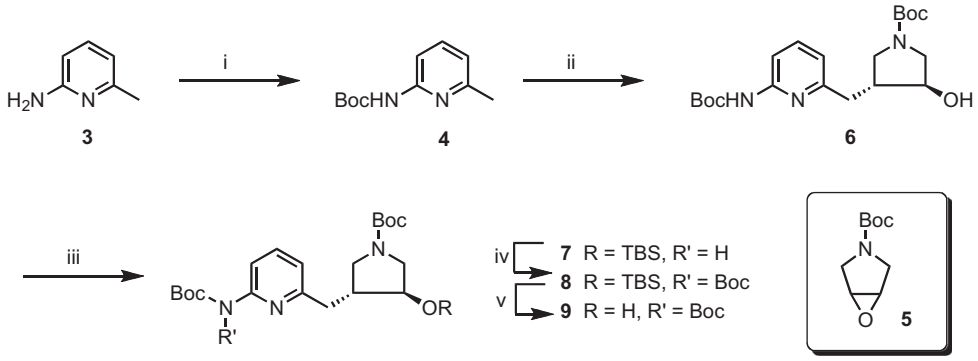
2. Chemistry

The synthesis of key intermediate **9** began with 2-amino-6-methylpyridine (**3**) (Scheme 1). Protection of the amino group in **3** with $(Boc)_2O$ in the presence of triethylamine (TEA) gave **4** in an excellent yield. Boc-protected aminopyridine **4** was treated with 2 equiv of *n*-BuLi, and the resulting dianion was allowed to react with epoxide **5**[13] to give secondary alcohol **6** in good yields. Next, **6** was converted to a bis-Boc-protected alcohol using a three-step procedure.[27] First, the hydroxyl group was protected as the *t*-butyldimethylsilyl (TBS)-ether (**7**) using TBSCl in the presence of imidazole. Then, **7** was treated with $(Boc)_2O$ in the presence of *N*,*N*-dimethylpyridine (DMAP) to provide bis-Boc-protected silyl ether **8**. Finally, the TBS-protecting group was removed using tetrabutylammonium fluoride (TBAF) to generate **9** in high yields.

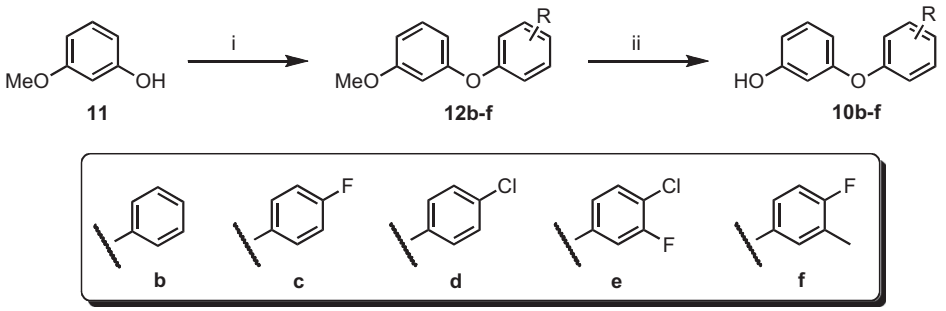
The substituted phenoxyphenols (**10b–f**) were synthesized using a two-step procedure (Scheme 2). First, Ullmann couplings of *m*-methoxyphenol (**11**) with iodobenzene derivatives using Cs_2CO_3 in the presence of a catalytic amount of CuBr provided **12b**–**f**.[28] Then, the methyl ethers were cleaved using BBr_3 to give **10b**–**f** in high yields.

With both **9** and **10a**–**f** in hand, the syntheses of inhibitors **2a**–**f** were completed (Scheme 3). After screening a series of reaction conditions, the Mitsunobu reaction using **9** and **10a**–**f** as starting materials went smoothly at room temperature to generate **12a**–**f** in modest to good yields. Then, the three Boc-protecting groups of **12a**–**f** were removed in TFA to generate inhibitors **2a**–**f**.

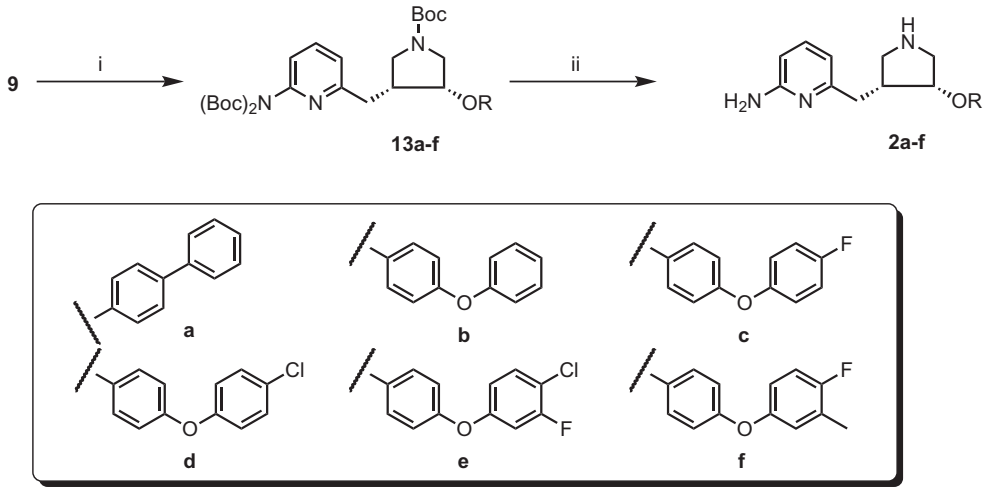
The synthesis of single enantiomer **14a** and **14b** is shown in Scheme 4. The free NH group on the pyridine ring of **15** was protected with a SEM-protecting group using SEM-Cl using NaH as a



Scheme 1. Synthesis of **9**. Reagents and conditions: (i) $(Boc)_2O$, TEA, rt, 12 h, 97%; (ii) (1) *n*-BuLi, −78 °C to 0 °C; (2) **5**, −78 °C to rt, 16 h, 70%; (iii) TBSCl, imidazole, rt, 16 h, 78%; (iv) $(Boc)_2O$, DMAP, rt, 30 h, 92%; (v) TBAF, rt, 30 min, 97%.



Scheme 2. Synthesis of **10b–f**. Reagents and conditions: (i) iodobenzene, Cs_2CO_3, CuBr, 60 °C, 48 h, 87–95%; (ii) BBr_3, −78 °C to rt, 85–92%.



Scheme 3. Syntheses of inhibitors **2a–f**. Reagents and conditions: (i) phenol, PPh_3, DEAD, 0 °C to rt, 72 h, 35–51%; (ii) TFA/CH_2Cl_2 (1:2), rt, 4 h, 91–95%.

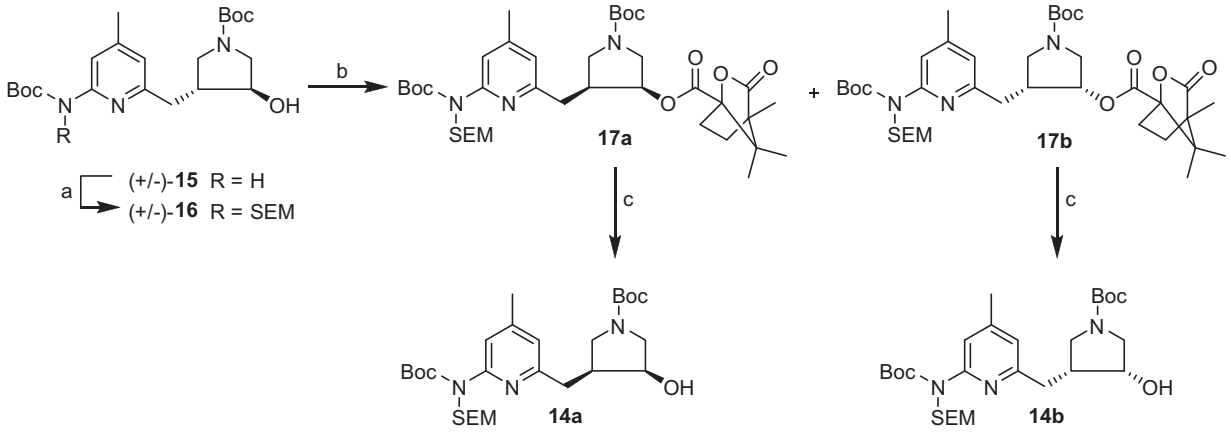
base to yield **16** in good yields. The two enantiomers were resolved through camphanic ester derivatives using a Mitsunobu reaction to generate two separable diastereomers **17a** and **17b** in reasonable yields. Finally, the ester linkage was hydrolyzed using Na_2CO_3 to provide chiral precursor **14a** and **14b** in excellent yields.

The bi-aryl fragment **19a–e** was synthesized using a two-step procedure (Scheme 5). Suzuki coupling of 4-(bromomethyl)phenol with a series of boronic acid generated to **18a–e**. Then, the hydroxyl group of **18a–e** was converted to bromide (**19a–e**) by refluxing in HBr in high yields.

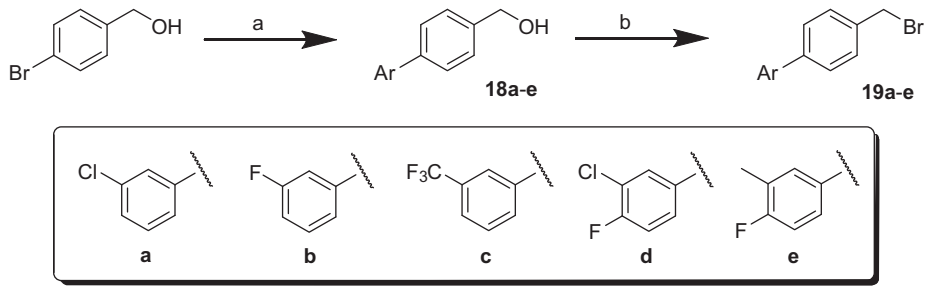
The syntheses of inhibitors **2g–k** were finished as shown in Scheme 6. Compound **14b** was treated with NaH, and the resulting anion was reacted with **19a–e** to provide **13g–k** in excellent yields. Then, global deprotection of SEM-protecting group and Boc-protecting groups generated inhibitors **2g–k** in high yields.

3. Results and discussion

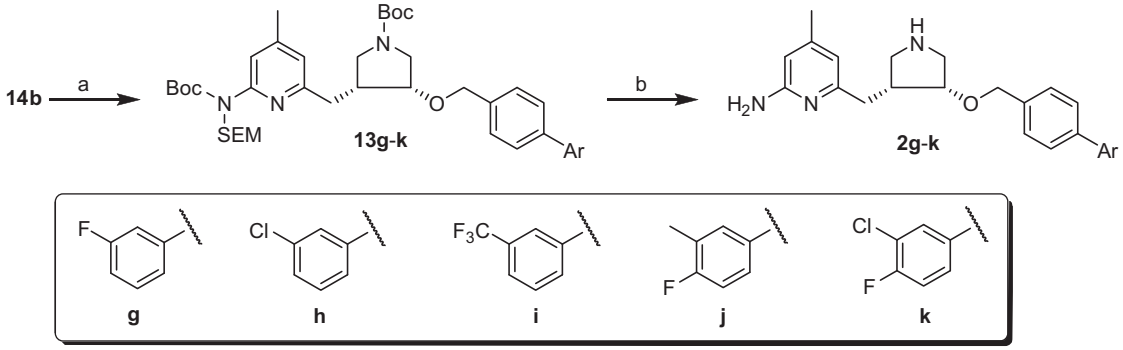
Inhibitors **2a–k** were evaluated for in vitro inhibition activity against three isozymes of NOS: rat nNOS, bovine eNOS, and murine iNOS using known methods.[22] The results are summarized in Table 1. Inhibitor **2a**, with a biphenyl group in place of the aminoalkyl tail of **1b**, had a K_i value of 10 μM for nNOS, while the phenoxyphenyl analog **2b** had slightly better inhibitory activity (K_i = 3.0 μM for nNOS). Inhibitors **2c** and **2d**, with halogen-substituents at the



Scheme 4. Synthesis of **14a** and **14b**. Reagents and conditions: (a) NaH, SEM-Cl, rt, 16 h, 68%; (b) (*S*)-(−) camphanic acid, PPh_3, DEAD, rt, 16 h, 88%; (d) Na_2CO_3, rt, 4 h, 95%.



Scheme 5. Synthesis of **19a–e**. Reagents and conditions: (a) $ArB(OH)_2$, $Pd(Ph_3P)_4$, 2 N Na_2CO_3, DME, 80 °C, 48 h; (b) HBr, reflux, 3 h.



Scheme 6. Synthesis of **2g–k**. Reagents and conditions: (a) NaH, 4-arylbenzyl bromide, rt, 6 h; (b) 4 N HCl in MeOH, rt, 16 h, 90%.

Table 1
K_i[a] values of inhibitors for rat nNOS, bovine eNOS, and murine iNOS

Compound	nNOS (μM)	eNOS (μM)	iNOS (μM)	Selectivity[b]	
				n/e	n/i
2a	10.0	>200	>200	>20	>20
2b	3.0	>200	>200	>67	>67
2c	7.8	18	221	2.3	28
2d	8.4	16	79.5	1.9	10
2e	2.2	8.3	81.8	1.8	18
2f	4.3	15	79.1	1.7	9
2g	0.75	13.3	39.5	18	53
2h	1.2	26.0	55.6	22	46
2i	0.82	13.6	37.3	17	45
2j	0.62	8.0	22.1	13	36
2k	0.76	13.3	50.0	18	66

[a] The K_i values were calculated based on the directly measured IC_{50} values, which represent at least duplicate measurements with standard deviations of ±10%.
[b] The ratio of K_i (eNOS or iNOS) to K_i (nNOS).

para position of the terminal phenyl ring, are weaker inhibitors than the non-substituted analog (**2b**). However, the additional substituent at the *meta* position on the ring, inhibitors **2e** and **2f**, showed slightly enhanced potency relative to **2a** against nNOS. We believe that the additional fluorine or methyl substituent fits into a small hydrophobic pocket at Met336, providing additional binding energy. Inhibitors **2g–k**, with the installation of a 4-methyl group on the aminopyridine ring, indicated some improved inhibitory activity. All new inhibitors are significantly less potent (300–600-fold) than lead compound **1b**, with K_i values in the low micromolar range, although the shape and size of the active site of nNOS can fit this series of compounds according to the docking calculation. These results were initially disappointing, given that this series overlaid very well with the crystal structure of the (3*R*,4*R*)-**1b** bound to nNOS in the flipped mode (Fig. 2). However, the crystal structure of a representative phenoxyphenyl analogue (**2b**) bound to nNOS showed that although the racemic mixture of **2b** was used in crystal preparation,

only the (3*R*,4*R*) enantiomer was observed in the structure, and it did not bind in the flipped orientation (Fig. 3). Instead, the aminopyridine was observed to bind in the normal orientation in which the aminopyridine interacts with Glu592, and Tyr706 remains in its native position. This is the first case in the pyrrolidine series of compounds in which the (3*R*,4*R*) stereochemistry does not bind in the flipped orientation. It appears that the configuration around the (3,4) positions of the pyrrolidine, therefore, is not the sole determinant controlling the binding orientation of these inhibitors; the size and rigidity of the tail also are very important. When the tail is bulky and rigid, as that in **2b**, the inhibitor cannot adopt the flipped mode because its tail no longer fits into the active site over the heme distal surface. Instead, the aminopyridine ring interacts with Glu592, and the tail end of the inhibitor extends out of the active site. The absence of electron density for the phenoxyphenyl tail indicates poor interactions with protein groups, which may account, in part, for the relatively weak affinity seen for these compounds. It is interesting that the (3*S*,4*S*)-**1b**[24] was bound in the same orientation as was observed for (3*R*,4*R*)-**2b** in this structure. The pyrrolidine nitrogen of (3*S*,4*S*)-**2b** would have H-bonded directly to Glu592, which is not observed here (Fig. 3). It is likely that the bulky phenoxyphenyl tail in (3*S*,4*S*)-**2b** would have an even poorer fit than what was seen for the tail in (3*R*,4*R*)-**2b**. The bulkiness of this hydrophobic tail, apparently, does not permit the molecule from assuming the flipped binding mode.

4. Conclusion

A new series of nNOS inhibitors (**2a**–**k**) has been designed and synthesized based on docking experiments with the crystal structure of the lead compound, (3*R*,4*R*)-**1b,** which bound to nNOS in a flipped binding mode. Because of the bulky tail of **2a–k**, they actually prefer the normal binding mode with a disordered tail, and, therefore, these compounds are only weak inhibitors of nNOS. The secondary amino group in the lipophilic tail also might be critical for tight binding of **1** to nNOS in the flipped mode, as compounds without this amino group, to date, bind poorly to nNOS.

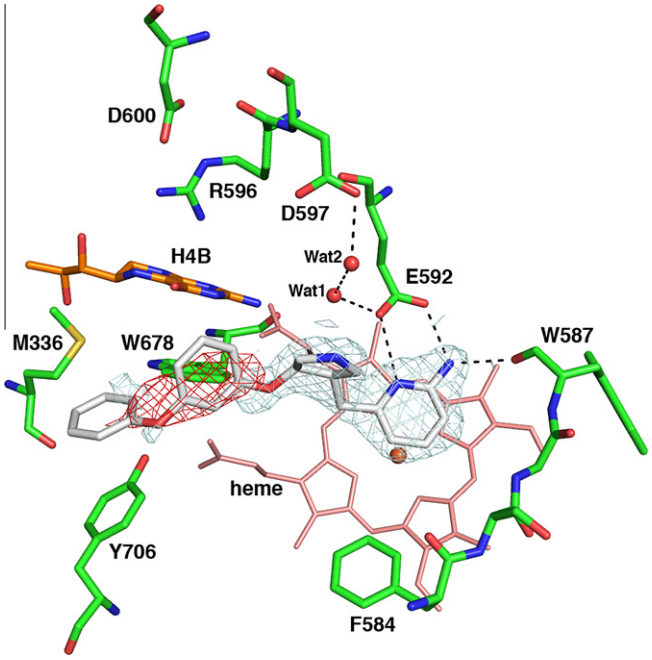


Figure 3. The active site crystal structure of nNOS with **2b** bound (PDB code: 3N2R) showing some relevant hydrogen bonds as dashed lines. The 2Fo–Fc (gray) and Fo–Fc (red) electron density map of **2b** are contoured at 1.0 σ and −3.0 σ, respectively. Although crystals were soaked in a racemic mix of **2b**, the electron density is consistent with only the (3*R*,4*R*) enantiomer. The pyrrolidine ring nitrogen of the (3*S*,4*S*) enantiomer would have directly hydrogen bonded to Glu592, replacing the position of Wat1. The electron density for the aminopyridine group is very clear and partially so for the pyrrolidine. However, there is no electron density for the phenoxyphenyl tail, indicating disordering and, therefore, the absence of any tight protein–phenoxyphenyl interactions.

5. Experimental procedures

5.1. General methods

All syntheses 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 use. All reagents were 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. Flash column chromatography was carried out under a positive pressure of nitrogen. ^{1}H NMR spectra were recorded on 500 MHz spectrometers. Data are presented as follows: chemical shift (in ppm on the δ scale relative to δ = 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 δ 77.0 or 49.0 for $CDCl_3$ or CD_3OD, respectively. High-resolution mass spectra were measured on liquid chromatography/time-of-flight mass spectrometry (LC–TOF).

5.2. General procedure A (Ullmann reaction)

To a mixture of Cs_2CO_3 (1.37 g, 4.2 mmol), CuBr (29 mg, 0.2 mmol), and ethyl 2-oxocyclohexanecarboxylate[28] (64 μL, 0.4 mmol) was added DMSO (1.0 mL). The mixture was allowed to stir at room temperature for 30 min, followed by addition of a mixture of 3-methoxyphenol (2.0 mmol) and iodobenzene (2.0 mmol) as a solution in DMSO (1.0 mL) through a cannula. The flask was sealed and heated at 60 °C for 24 h and then cooled to room temperature. The reaction was filtered through Celite, and the resulting cake was washed with EtOAc (4 × 25 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, and concentrated. The crude product was purified by flash chromatography (EtOAc/hexanes, 1:9) to yield **12b–f** (87–95%) as pale yellow oils.

5.3. General procedure B (demethylation)

To a solution of a methyl ether (**12b–f**, 1.0 mmol) in dichloromethane at −78 °C was added BBr_3 (1 M solution, 1.2 mL, 1.2 mmol) dropwise. The mixture was warmed to room temperature over a period of 2 h and kept stirring overnight. The solvent was removed by rotary evaporation, and the resulting material was purified by flash chromatography (EtOAc/hexanes, 1:9) to yield **10b–f** (85–92%) as colorless oils.

5.4. General procedure C (Mitsunobu reaction)

A mixture of **9** (100 mg, 0.2 mmol) and PPh_3 (100 mg, 0.3 mmol) was dissolved in THF (5 mL). To the resulting solution was added a phenol (**10a–f**, 0.3 mmol) as a solution in THF (1.0 mL), followed by DEAD (60 μL, 0.3 mmol) dropwise. The mixture was allowed to stir at room temperature for 40 h and then concentrated. The crude product was purified by flash chromatography to yield **13a–f** (35–51%) as white solids.

5.5. General procedure D (Boc-deprotection)

To a solution of **13a**–**f** (50 μmol) in dichloromethane (1.0 mL) was added TFA (1.0 mL). The reaction mixture was allowed to sit at room temperature for 4 h and then concentrated. The crude product was purified by flash chromatography (5–10% methanol in dichloromethane) to yield **2a**–**f** (91–95%) as white solids.

5.6. General procedure E (Suzuki coupling)

To a solution of 3-fluorophenylboronic acid (462 mg, 3.3 mmol) and 4-bromophenylmethanol (561 mg, 3 mmol) in DME (10 mL) was added $Pd(Ph_3P)_4$ (173 mg, 0.15 mmol) and 2 N Na_2CO_3 (1.5 mL). The mixture was stirred at 80 °C for 48 h. After cooling to room temperature, the reaction mixture was filtered through Celite. The filtrate was concentrate, and the resulting residue was purified by flash column chromatography (EtOAc/hexane, 1:4) to generate **18a**–**e** as white solids (80–84%).

5.7. General procedure F

To 2.0 mmol of **18a**–**e** was added HBr solution (48%, 5 mL). The reaction was heated under reflux for 3 h, and then cooled to room temperature. The mixture was partitioned between EtOAc (100 mL) and H_2O (30 mL). The organic layer was washed with H_2O (2 × 30 mL), dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography on silica gel to provide the product **19a**–**e** (84–90%).

5.8. General procedure G

To a solution of **14b** (0.1 mmol) in DMF (2 mL) was added NaH (60% in mineral oil, 10 mg, 0.25 mmol) at 0 °C. After 10 min, **19a**–**e** (0.105 mmol) was added as a solution DMF (1 mL). The reaction was stirred at room temperature for 16 h, and concentrated in vacuo. The resulting residue was partitioned between EtOAc (100 mL) and H_2O (10 mL). The organic layer was washed with H_2O (2 × 10 mL), dried over Na_2SO_4, and concentrated. The crude product was purified by flash column chromatography on silica gel (EtOAc/hexanes, 5:2) to generate **13g**–**k** as colorless oils (60–66%).

5.9. General procedure H

A solution of **13g**–**k** (0.1 mmol) in 3 N HCl in methanol (3 mL) was stirred at room temperature for 24 h. The mixture was concentrated, and the resulting crude material was purified by Sephdex LH-20 to give final inhibitors **2g**–**k** (95–99%) as hydrochloride salts.

5.9.1. *tert*-Butyl 6-methylpyridin-2-ylcarbamate (4)

To a solution of 2-amino-6-methylpyridine (4.32 g, 40 mmol) in *t*-butanol (40 mL) was added $(Boc)_2O$ (10.9 g, 50 mmol) and triethylamine (7.0 mL, 50 mmol). The solution was heated at 50 °C for 24 h. The solvent was removed by rotary evaporation, and the crude product was purified using flash column chromatography (EtOAc/hexanes, 1:18–1:9) to generate **4** (7.5 g, 36 mmol, 97%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42 (s, 9H), 2.40 (s, 3H), 7.46–7.50 (dd, J = 7.5, 8.5 Hz, 1H), 6.72–6.74 (d, J = 8.0 Hz, 1H), 7.71–7.73 (d, J = 8.5 Hz, 1H), 8.79 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.0, 28.4, 80.7, 109.6, 118.0, 138.6, 152.0, 153.0, 156.9; LCQ-MS ($M+H^+$) calcd for $C_{11}H_{17}N_2O_2$ 209, found 209.

5.9.2. *tert*-Butyl 3-((6-(*tert*-butoxycarbonylamino)pyridin-2-yl)-methyl)-4-hydroxypyrrolidine-1-carboxylate (6)

To a solution of **4** (1.0 g, 4.81 mmol) in THF (30 mL) at −78 °C was added *n*-BuLi (1.6 M in hexanes, 6.3 mL, 10.0 mmol) dropwise. The resulting dark red solution was stirred at the same temperature for an additional 1 h, and then the reaction flask was transferred to an ice-bath for another 30 min. The reaction was cooled to −78 °C, to which a solution of epoxide **5** (890 mg, 4.81 mmol) in THF (10 mL) was added dropwise over a period of 30 min along the side of the reaction flask. After addition, the Dry Ice-acetone bath was removed, and the reaction was kept stirring for an additional 2.5 h. The reaction was quenched with H_2O (1.0 mL) and concentrated by rotary evaporation. The crude product was purified using flash column chromatography (EtOAc/hexanes, 2:3–1:1) to provide **6** (1.32 g, 3.37 mmol, 70%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.45 (s, 9H), 1.49 (s, 9H), 2.30–2.50 (m, 1H), 2.60–2.80 (m, 2H), 3.00–3.30 (m, 2H), 3.50–3.80 (m, 2H), 4.00–4.20 (m, 1H), 5.04 (br s, 1H), 6.80–6.90 (m, 1H), 7.50–7.80 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.3, 28.6, 39.2, 44.8, 45.2, 49.2, 49.7, 52.3, 52.7, 60.5, 64.5, 74.5, 75.2, 79.5, 81.1, 110.3, 118.2, 139.2, 151.6, 152.4, 154.6, 157.9; LCQ-MS ($M+H^+$) calcd for $C_{20}H_{32}N_3O_5$ 394, found 394.

5.9.3. *tert*-Butyl 3-((6-(*tert*-butoxycarbonylamino)pyridin-2-yl)-*tert*-butyldimethylsilyloxy)pyrrolidine-1-carboxylate (7)

To a solution of **6** (850 mg, 2.16 mmol) in DMF (5.0 mL) were added TBSCl (408 mg, 2.7 mmol) and imidazole (367 mg, 5.4 mmol). The reaction mixture was stirred at 40 °C for 24 h and then partitioned between EtOAc (150 mL) and aqueous NH_4Cl (100 mL). The organic layer was washed with brine (100 mL), dried over Na_2SO_4, and concentrated. The crude product was purified using flash column chromatography (EtOAc/hexanes, 1:9–1:6) to provide **7** (855 mg, 1.68 mmol, 78%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.03 (s, 6H), 0.81 (s, 9H), 1.40 (s, 9H), 1.47 (s, 9H), 2.40–2.50 (m, 2H), 2.75–2.85 (m, 1H), 2.98–3.14 (m, 2H), 3.38–3.62 (m, 2H), 3.90–3.98 (m, 1H), 6.73 (d, J = 7.5 Hz, 1H), 7.38–7.54 (m, 2H), 7.71 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ −4.6, 18.2, 25.9, 28.4, 28.7, 39.3, 46.1, 46.7, 48.8, 49.1, 52.7, 53.0, 74.5, 75.3, 79.4, 81.0, 109.9, 117.9, 138.7, 151.6, 152.5, 154.9, 158.3; LCQ-MS ($M+H^+$) calcd for $C_{26}H_{46}N_3O_5Si$ 508, found 508.

5.9.4. *tert*-Butyl 3-((6-(bis(*tert*-butoxycarbonyl)amino)pyridin-2-yl)-methyl)-4-(*tert*-butyldimethylsilyloxy)pyrrolidine-1-carboxylate (8)

To a solution of **7** (507 mg, 1.0 mmol) in THF (10 mL) was added $(Boc)_2O$ (327 mg, 1.5 mmol) and DMAP (30 mg, 0.25 mmol). The solution was stirred at room temperature for 16 h. The solvent was removed by rotary evaporation, and the crude product was purified using flash column chromatography (EtOAc/hexanes, 1:9–1:6) to generate **8** (558 mg, 0.92 mmol, 92%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ −0.01 (s, 6H), 0.84 (s, 9H), 1.40–1.45 (m, 27H), 2.45–2.55 (m, 2H), 2.83–3.14 (m, 3H), 3.40–3.64 (m, 2H), 3.98–4.04 (m, 1H), 6.98–7.02 (m, 1H), 7.06–7.14 (m, 1H), 7.55–7.65 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ −4.5, 18.2, 25.9, 28.1, 28.7, 39.1, 46.1, 46.7, 48.5, 48.9, 52.6, 53.2, 74.5, 75.1, 79.4, 83.0, 118.9, 121.5, 138.3, 151.5, 152.0, 154.9, 159.2; LCQ-MS ($M+H^+$) calcd for $C_{31}H_{54}N_3O_7Si$ 608, found 608.

5.9.5. *tert*-Butyl 3-((6-(bis(*tert*-butoxycarbonyl)amino)pyridin-2-yl)-methyl)-4-hydroxypyrrolidine-1-carboxylate (9)

To a solution of **8** (607 mg, 1.0 mmol) in THF (10 mL) was added TBAF (1 M in THF, 1.25 mL, 1.25 mmol). The solution was stirred at room temperature for 16 h. The solvent was removed by rotary evaporation, and the resulting crude product was purified using flash column chromatography (EtOAc/hexanes, 1:1) to generate **9** (476 mg, 0.97 mmol, 97%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42–1.47 (m, 27H), 2.43–2.50 (m, 1H), 2.85–2.95 (m, 2H), 3.03–3.12 (m, 1H), 3.15–3.24 (m, 1H), 3.61–3.80 (m, 2H), 4.14–4.18 (m, 1H), 7.06–7.14 (m, 2H), 7.70 (dd, J = 7.5, 8.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.1, 28.7, 39.5, 39.7, 44.9, 45.7, 49.7, 50.0, 52.5, 53.0, 74.6, 75.3, 79.5, 83.7, 119.8, 120.0,

122.2, 122.4, 139.1, 151.5, 151.8, 154.7, 159.4; LCQ-MS ($M+H^+$) calcd for $C_{25}H_{40}N_3O_7$ 494, found 494.

5.9.6. 1-(4-Fluorophenoxy)-3-methoxybenzene (12c)

Compound **12c** was synthesized using general procedure A (95%): ^1H NMR (500 MHz, $CDCl_3$) δ 3.76 (s, 3H), 6.52–6.54 (d, J = 8.5 Hz, 2H), 6.62–6.64 (d, J = 10.0 Hz, 1H), 6.90–7.10 (m, 4H), 7.18–7.22 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.5, 104.5, 108.9, 110.5, 116.4, 116.6, 120.9, 121.0, 130.4; LC–MS ($M+H^+$) calcd for $C_{13}H_{12}FO_2$ 219, found 219.

5.9.7. 1-(4-Chlorophenoxy)-3-methoxybenzene (12d)

Compound **12d** was synthesized using general procedure A (87%): ^1H NMR (500 MHz, $CDCl_3$) δ 3.76 (s, 3H), 6.56–6.67 (m, 2H), 6.66–6.68 (d, J = 10.0 Hz, 1H), 6.94–6.96 (d, J = 11.0 Hz, 2H), 7.15–7.35 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.6, 105.2, 109.5, 111.2, 120.5, 129.9, 130.5; LC–MS ($M+H^+$) calcd for $C_{13}H_{12}ClO_2$ 235, found 235.

5.9.8. 1-Chloro-2-fluoro-4-(3-methoxyphenoxy)benzene (12e)

Compound **12e** was synthesized using general procedure A (88%): ^1H NMR (500 MHz, $CDCl_3$) δ 3.76 (s, 3H), 6.56–6.67 (m, 2H), 6.66–6.68 (d, J = 10.0 Hz, 1H), 6.94–6.96 (d, J = 11.0 Hz, 2H), 7.15–7.35 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.6, 105.2, 109.5, 111.2, 120.5, 129.9, 130.5; LC–MS ($M+H^+$) calcd for $C_{13}H_{11}ClFO_2$ 253, found 253.

5.9.9. 1-Fluoro-4-(3-methoxyphenoxy)-2-methylbenzene (12f)

Compound **12f** was synthesized using general procedure A (87%): ^1H NMR (500 MHz, $CDCl_3$) δ 3.76 (s, 3H), 6.56–6.67 (m, 2H), 6.66–6.68 (d, J = 10.0 Hz, 1H), 6.94–6.96 (d, J = 11.0 Hz, 2H), 7.15–7.35 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 55.6, 105.2, 109.5, 111.2, 120.5, 129.9, 130.5; LC–MS ($M+H^+$) calcd for $C_{14}H_{14}FO_2$ 233, found 233.

5.9.10. 3-(4-Fluorophenoxy)phenol (10c)

Compound **10c** was synthesized using general procedure B (85%): ^1H NMR (500 MHz, $CDCl_3$) δ 5.51 (br s, 1H), 6.44 (s, 1H), 6.49–6.55 (m, 2H), 6.90–7.10 (m, 4H), 7.12–7.16 (dd, J = 10.0, 10.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 105.6, 110.3, 110.6, 116.4, 116.7, 121.15, 121.24, 130.7; LC–MS ($M+H^+$) calcd for $C_{12}H_{10}FO_2$ 205, found 205.

5.9.11. 3-(4-Chlorophenoxy)phenol (10d)

Compound **10d** was synthesized using general procedure B (85%): ^1H NMR (500 MHz, $CDCl_3$) δ 5.79 (br s, 1H), 6.47 (s, 1H), 6.52–6.58 (dd, J = 11.0, 14.5, 2H), 6.91–6.94 (d, J = 11.0, 2H), 7.13–7.17 (dd, J = 10.0, 11.0 Hz, 1H), 7.23–7.25 (d, J = 11.0, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 106.4, 110.9, 111.2, 120.7, 128.8, 130.0, 130.8, 155.6, 157.1, 158.5; LC–MS ($M+H^+$) calcd for $C_{12}H_{10}ClO_2$ 221, found 221.

5.9.12. 3-(4-Chloro-3-fluorophenoxy)phenol (10e)

Compound **10e** was synthesized using general procedure B (92%): ^1H NMR (500 MHz, $CDCl_3$) δ 5.79 (br s, 1H), 6.47 (s, 1H), 6.52–6.58 (dd, J = 11.0, 14.5, 2H), 6.91–6.94 (d, J = 11.0, 2H), 7.13–7.17 (dd, J = 10.0, 11.0 Hz, 1H), 7.23–7.25 (d, J = 11.0, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 107.1, 107.6, 107.8, 110.7, 110.8, 115.27, 115.29, 131.0, 131.1, 157.2, 157.4; LC–MS ($M+H^+$) calcd for $C_{12}H_9ClFO_2$ 239, found 239.

5.9.13. 3-(4-Fluoro-3-methylphenoxy)phenol (10f)

Compound **10f** was synthesized using general procedure B (86%): ^1H NMR (500 MHz, $CDCl_3$) δ 5.79 (br s, 1H), 6.47 (s, 1H), 6.52–6.58 (dd, J = 11.0, 14.5, 2H), 6.91–6.94 (d, J = 11.0, 2H), 7.13–7.17 (dd, J = 10.0, 11.0 Hz, 1H), 7.23–7.25 (d, J = 11.0, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.9, 150.0, 105.6, 110.2, 110.5, 116.0, 116.2, 118.46, 118.52, 122.67, 122.71, 126.5, 126.7, 130.7, 152.09, 152.11, 157.0, 158.9, 159.5; LC–MS ($M+H^+$) calcd for $C_{13}H_{12}FO_2$ 219, found 219.

5.9.14. (3*R*,4*R*/3*S*,4*S*)-*tert*-Butyl 3-(biphenyl-4-yloxy)-4-((6-(bis(*tert*-butoxycarbonyl)amino)pyridin-2-yl)methyl)pyrrolidine-1-carboxylate (13a)

Compound **13a** was synthesized using general procedure C (40%): ^1H NMR (500 MHz, $CDCl_3$) δ 1.40–1.60 (m, 27H), 2.90–3.10 (m, 2H), 3.15–3.25 (m, 1H), 3.35–3.45 (m, 1H), 3.50–3.65 (m, 2H), 3.66–3.80 (m, 1H), 4.68–4.71 (m, 1H), 6.90–6.92 (d, J = 8.0 Hz, 2H), 7.02–7.06 (m, 1H), 7.07–7.12 (m, 1H), 7.30–7.35 (m, 1H), 7.40–7.65 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.9, 28.2, 28.7, 28.8, 30.0, 34.9, 42.8, 76.7, 79.7, 79.8, 83.2, 100.0, 116.1, 116.2, 119.3, 120.0, 126.9, 127.0, 128.5, 128.9, 129.0, 134.6, 138.6, 140.9, 151.5, 152.1, 154.6, 154.8, 157.0, 159.3; LCQ-MS ($M+H^+$) calcd for $C_{37}H_{48}N_3O_7$ 646, found 646.

5.9.15. (3*R*,4*R*/3*S*,4*S*)-*tert*-Butyl 3-((6-(bis(tert-butoxycarbonyl)-amino)pyridin-2-yl)methyl)-4-(3-phenoxyphenoxy)pyrrolidine-1-carboxylate (13b)

Compound **13b** was synthesized using general procedure C (35%): ^1H NMR (500 MHz, $CDCl_3$) δ 1.40–1.60 (m, 27H), 2.80–3.00 (m, 2H), 3.10–3.20 (dd, J = 7.5, 16.5 Hz, 1H), 3.25–3.35 (m, 1H), 3.45–3.50 (m, 1H), 3.55–3.60 (m, 1H), 3.61–3.70 (m, 1H), 4.57–4.60 (d, J = 10.5 Hz, 1H), 7.00–7.60 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.6, 25.1, 28.6, 32.9, 113.0, 113.2, 113.4, 113.6, 122.4, 129.9, 130.0, 130.1, 143.77, 143.83, 162.2, 164.1; LC–MS ($M+H^+$) calcd for $C_{37}H_{48}N_3O_8$ 662, found 662.

5.9.16. (3*R*,4*R*/3*S*,4*S*)-*tert*-Butyl 3-((6-(bis(*tert*-butoxycarbonyl)-amino)pyridin-2-yl)methyl)-4-(3-(4-fluorophenoxy)phenoxy)-pyrrolidine-1-carboxylate (13c)

Compound **13c** was synthesized using general procedure C (35%): ^1H NMR (500 MHz, $CDCl_3$) δ 1.40–1.60 (m, 27H), 2.80–3.00 (m, 2H), 3.10–3.20 (dd, J = 7.5, 16.5 Hz, 1H), 3.25–3.35 (m, 1H), 3.45–3.50 (m, 1H), 3.55–3.60 (m, 1H), 3.61–3.70 (m, 1H), 4.57–4.60 (d, J = 10.5 Hz, 1H), 7.00–7.60 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.6, 25.1, 28.6, 32.9, 113.0, 113.2, 113.4, 113.6, 122.4, 129.9, 130.0, 130.1, 143.77, 143.83, 162.2, 164.1; LC–MS ($M+H^+$) calcd for $C_{37}H_{48}N_3O_8$ 662, found 662.

5.9.17. (3*R*,4*R*/3*S*,4*S*)-*tert*-Butyl 3-((6-(bis(*tert*-butoxycarbonyl)-amino)pyridin-2-yl)methyl)-4-(3-(4-chlorophenoxy)phenoxy)-pyrrolidine-1-carboxylate (13d)

Compound **13d** was synthesized using general procedure C (46%): ^1H NMR (500 MHz, $CDCl_3$) δ 1.40–1.60 (m, 27H), 2.80–3.00 (m, 2H), 3.10–3.20 (dd, J = 7.5, 16.5 Hz, 1H), 3.25–3.35 (m, 1H), 3.45–3.50 (m, 1H), 3.55–3.60 (m, 1H), 3.61–3.70 (m, 1H), 4.57–4.60 (d, J = 10.5 Hz, 1H), 7.00–7.60 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.6, 25.1, 28.6, 32.9, 113.0, 113.2, 113.4, 113.6, 122.4, 129.9, 130.0, 130.1, 143.77, 143.83, 162.2, 164.1; LC–MS ($M+H^+$) calcd for $C_{37}H_{48}N_3O_8$ 662, found 662.

5.9.18. (3*R*,4*R*/3*S*,4*S*)-*tert*-Butyl 3-((6-(bis(*tert*-butoxycarbonyl)-amino)pyridin-2-yl)methyl)-4-(3-(4-chloro-3-fluorophenoxy)-phenoxy)pyrrolidine-1-carboxylate (13e)

Compound **13e** was synthesized using general procedure C (48%): ^1H NMR (500 MHz, $CDCl_3$) δ 1.40–1.60 (m, 27H), 2.80–3.00 (m, 2H), 3.10–3.20 (dd, J = 7.5, 16.5 Hz, 1H), 3.25–3.35 (m, 1H), 3.45–3.50 (m, 1H), 3.55–3.60 (m, 1H), 3.61–3.70 (m, 1H), 4.57–4.60 (d, J = 10.5 Hz, 1H), 7.00–7.60 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.6, 25.1, 28.6, 32.9, 113.0, 113.2, 113.4, 113.6, 122.4, 129.9, 130.0, 130.1, 143.77, 143.83, 162.2, 164.1; LC–MS ($M+H^+$) calcd for $C_{37}H_{48}N_3O_8$ 662, found 662.

5.9.19. (3*R*,4*R*/3*S*,4*S*)-*tert*-Butyl 3-((6-(bis(*tert*-butoxycarbonyl)-amino)pyridin-2-yl)methyl)-4-(3-(4-fluoro-3-methylphenoxy)-phenoxy)pyrrolidine-1-carboxylate (13f)

Compound **13f** was synthesized using general procedure C (51%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.40–1.60 (m, 27H), 2.80–3.00 (m, 2H), 3.10–3.20 (dd, J = 7.5, 16.5 Hz, 1H), 3.25–3.35 (m, 1H), 3.45–3.50 (m, 1H), 3.55–3.60 (m, 1H), 3.61–3.70 (m, 1H), 4.57–4.60 (d, J = 10.5 Hz, 1H), 7.00–7.60 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.6, 25.1, 28.6, 32.9, 113.0, 113.2, 113.4, 113.6, 122.4, 129.9, 130.0, 130.1, 143.77, 143.83, 162.2, 164.1; LC–MS ($M+H^+$) calcd for $C_{37}H_{48}N_3O_8$ 662, found 662.

5.9.20. 6-(((3*R*,4*R*/3*S*,4*S*)-4-(Biphenyl-4-yloxy)pyrrolidin-3-yl)-methyl)pyridin-2-amine (2a)

Compound **2a** was synthesized using general procedure D (95%): ^{1}H NMR (500 MHz, CD_3OD) δ 3.00–3.20 (m, 2H), 3.21–3.30 (dd, J = 9.0, 14.0 Hz, 1H), 3.61–3.72 (m, 3H), 5.01 (s, 1H), 6.69–6.71 (d, J = 7.5 Hz, 1H), 6.81–6.83 (d, J = 9.0 Hz, 1H), 7.05–7.07 (d, J = 7.5 Hz, 2H), 7.29–7.32 (dd, J = 7.0, 7.5 Hz, 1H), 7.40–7.43 (dd, J = 7.5, 8.0 Hz, 2H), 7.55–7.59 (m, 3H), 7.73–7.77 (dd, J = 7.5, 9.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 21.3, 41.5, 47.7, 50.4, 75.6, 99.9, 116.2, 119.3, 122.0, 127.0, 129.0, 134.6, 138.6, 140.9, 151.5, 152.1, 154.6, 154.8, 157.0, 158.3; LC–MS ($M+H^+$) calcd for $C_{22}H_{24}N_3O$ 346.1919, found 346.1911.

5.9.21. 6-(((3*R*,4*R*/3*S*,4*S*)-4-(3-Phenoxyphenoxy)pyrrolidin-3-yl)-methyl)pyridin-2-amine (2b)

Compound **2b** was synthesized using general procedure D (91%): ^{1}H NMR (500 MHz, D_2O) δ 2.70–3.00 (m, 3H), 3.14–3.19 (dd, J = 11.0, 11.0 Hz, 1H), 3.25–3.40 (m, 2H), 3.45–3.55 (dd, J = 7.5, 11.5 Hz, 1H), 6.29 (s, 1H), 6.41–6.50 (m, 2H), 6.51–6.55 (dd, J = 1.5, 8.0 Hz, 1H), 6.56–6.60 (d, J = 8.5 Hz, 1H), 6.79–6.81 (d, J = 8.0 Hz, 2H), 6.98–7.01 (dd, J = 7.0, 7.0 Hz, 1H), 7.06–7.10 (dd, J = 8.0, 8.0 Hz, 1H), 7.17–7.21 (d, J = 8.0 Hz, 2H), 7.45–7.55 (dd, J = 7.5, 8.5 Hz, 1H); ^{13}C NMR (125 MHz, D_2O) δ 21.3, 41.5, 47.7, 50.4, 75.6, 106.1, 110.9, 111.5, 112.4, 119.4, 124.4, 130.3, 131.1, 144.7, 146.7, 154.2, 156.2, 157.5, 158.4; LC–MS ($M+H^+$) calcd for $C_{22}H_{24}N_3O_2$ 362.18685, found 362.18597.

5.9.22. 6-(((3*R*,4*R*/3*S*,4*S*)-4-(3-(4-Fluorophenoxy)phenoxy)-pyrrolidin-3-yl)methyl)pyridin-2-amine (2c)

Compound **2c** was synthesized using general procedure D (95%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.99–3.08 (m, 2H), 3.09–3.16 (dd, J = 8.5, 14.5 Hz, 1H), 3.30–3.35 (m, 1H), 3.50–3.52 (m, 2H), 3.60–3.72 (dd, J = 8.0, 11.5 Hz, 1H), 4.94–4.95 (t, J = 2.0 Hz, 1H), 6.56 (s, 1H), 6.64–6.66 (d, J = 7.0 Hz, 1H), 6.67–6.70 (m, 1H), 6.80–6.82 (d, J = 8.5 Hz, 1H), 6.98–7.00 (m, 2H), 7.07–7.11 (m, 2H), 7.23–7.27 (dd, J = 8.0, 8.5 Hz, 1H), 7.72–7.74 (dd, J = 7.0, 8.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 28.5, 30.5, 43.4, 51.5, 77.2, 107.2, 111.0, 112.7, 112.8, 112.9, 117.4, 117.5, 122.15, 122.22, 132.0, 145.5, 148.7, 153.91, 153.93, 156.8, 159.0, 159.6, 160.8, 161.5, 162.4, 162.6; LC–MS ($M+H^+$) calcd for $C_{22}H_{23}FN_3O_2$ 380.1774, found 380.1777.

5.9.23. 6-(((3*R*,4*R*/3*S*,4*S*)-4-(3-(4-Chlorophenoxy)phenoxy)-pyrrolidin-3-yl)methyl)pyridin-2-amine (2d)

Compound **2d** was synthesized using general procedure D (95%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.99–3.16 (m, 3H), 3.30–3.35 (m, 1H), 3.50–3.52 (m, 2H), 3.60–3.72 (m, 1H), 4.96 (s, 1H), 6.60–6.95 (m, 6H), 6.96–6.97 (d, J = 2.5 Hz, 2H), 6.97–6.98 (d, J = 2.5 Hz, 2H), 7.27–7.29 (m, 1H), 7.73–7.76 (dd, J = 7.5, 9.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 27.7, 30.5, 43.4, 51.5, 77.2, 107.8, 111.6, 112.7, 112.9, 113.0, 116.5, 118.8, 121.6, 130.9, 132.1, 145.5, 145.6, 147.9, 148.7, 156.8, 156.9, 157.0, 159.0, 159.9, 162.3, 162.6; LC–MS ($M+H^+$) calcd for $C_{22}H_{23}ClN_3O_2$ 396.1479, found 396.1465.

5.9.24. 6-(((3*R*,4*R*/3*S*,4*S*)-4-(3-(4-Chloro-3-fluorophenoxy)-phenoxy)pyrrolidin-3-yl)methyl)pyridin-2-amine (2e)

Compound **2e** was synthesized using general procedure D (92%): ^{1}H NMR (500 MHz, CD_3OD) δ 3.00–3.20 (m, 3H), 3.30–3.35 (m, 1H), 3.50–3.75 (m, 3H), 5.02 (s, 1H), 6.64–6.75 (m, 3H), 6.80–6.90 (m, 3H), 6.88–6.91 (m, 2H), 7.34–7.37 (dd, J = 8.0, 8.5 Hz, 1H), 7.75–7.79 (dd, J = 7.0, 8.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 28.5, 30.5, 43.4, 51.5, 77.2, 108.3, 108.4, 108.5, 112.4, 112.7, 112.8, 113.0, 114.2, 116.0, 116.1, 116.4, 116.7, 119.0, 132.3, 132.4, 145.5, 145.6, 147.9, 148.7, 156.8, 156.9, 158.5, 158.6, 158.7, 158.9, 159.1, 160.8, 162.6, 162.9; LC–MS ($M+H^+$) calcd for $C_{22}H_{22}ClFN_3O_2$ 414.1385, found 414.1397.

5.9.25. 6-(((3*R*,4*R*/3*S*,4*S*)-4-(3-(4-Fluoro-3-methylphenoxy)-phenoxy)pyrrolidin-3-yl)methyl)pyridin-2-amine (2f)

Compound **2f** was synthesized using general procedure D (95%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.24 (s, 3H), 2.95–3.18 (m, 3H), 3.30–3.35 (m, 1H), 3.50–3.75 (m, 3H), 4.95–4.96 (t, J = 2.0 Hz, 1H), 6.56–6.58 (m, 1H), 6.67–6.70 (m, 1H), 6.80–6.85 (m, 3H), 6.86–6.90 (m, 3H), 7.01–7.07 (m, 1H), 7.72–7.74 (dd, J = 7.0, 8.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.6, 27.7, 30.5, 43.4, 51.5, 77.1, 107.1, 110.8, 111.8, 112.7, 112.8, 113.0, 116.6, 116.8, 117.0, 118.9, 119.4, 119.5, 123.5, 127.5, 127.6, 131.9, 145.5, 147.9, 148.7, 153.5, 156.8, 156.9, 158.1, 158.9, 161.0, 162.4, 162.6, 162.9; LC–MS ($M+H^+$) calcd for $C_{23}H_{25}FN_3O_2$ 394.1931, found 394.1920.

5.9.26. (±)-*tert*-Butyl-{{6-{tert-butoxycarbonyl{[2-(trimethylsilyl)ethoxy]methyl}amino}-4-methylpyridine-2-yl}methyl}-4-hydroxypyrrolidine-1-carboxylate (16)

To an ice-cooled solution of **15**[12] (1.328 g, 3.26 mmol) in DMF (10 mL) was added sodium hydride (0.157 g, 60% in mineral oil, 3.92 mmol). After 15 min, SEM-Cl (0.59 g, 0.64 mL, 3.59 mmol) was added dropwise. The mixture was stirred at room temperature for 16 h, and then quenched with. H_2O (5 mL). The solvent was removed by rotary evaporation, and the resulting material was partitioned between EtOAc (30 mL) and H_2O (20 mL). The organic layer was extracted with EtOAc (2 × 30 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, EtOAc/hexanes, 2:3) to yield a white foamy solid (1.25 g, 68%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.0 (s, 9H), 0.93 (t, J = 8.0 Hz, 2H), 1.47 (s, 9H), 1.52 (s, 9H), 2.35 (s, 3H), 2.38–2.58 (m, 1H), 2.78–2.98 (m, 2H), 3.01–3.30 (m, 2H), 3.63 (t, J = 8.0 Hz, 2H), 3.64–3.90 (m, 2H), 4.10–4.25 (m, 1H), 4.85 + 5.12 (brs, OH), 5.29 (s, 2H), 6.82 and 6.84 (s, 1H), 7.17 (s, 1H); LC–TOF ($M+H^+$) calcd for $C_{27}H_{47}N_3O_6Si$ 537.3234, found 537.3244.

5.9.27. (3*S*,4*S*)-*tert*-Butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)-methyl)-4-((4*S*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]-heptane-1-carbonyloxy)pyrrolidine-1-carboxylate (17a) and (3*R*,4*R*)-*tert*-butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)methyl)-4-((4*S*)-4,7,7-trimethyl-3-oxo-2-oxabicyclo[2.2.1]heptane-1-carbonyloxy)pyrrolidine-1-carboxylate (17b)

To an ice-cooled solution of **16** (0.935 g, 1.74 mmol), (1*S*)-(−)-camphanic acid (0.4 g, 2.02 mmol) and Ph_3P (0.75 g, 2.86 mmol) in THF (10 mL) was added DEAD (0.9 mL, 3.0 mmol) dropwise. The mixture was stirred at room temperature for 6 h, and then concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, EtOAc/hexanes, 1:5) to obtain the two diastereomers in 88% overall yield.

Compound 17a (Foamy solid, 44%; 0.55 g): ^{1}H NMR (400 MHz, $CDCl_3$) δ 0 (s, 9H), 0.92 (t, J = 8.0 Hz, 2H), 0.97 + 0.99 (s, 3H), 1.07 + 1.09 (s, 3H), 1.16 (s, 3H), 1.25–1.4 (m, 1H), 1.46 (s, 9H),

1.54 (s, 9H), 1.65–1.80 (m, 1H), 1.90–2.10 (m, 2H), 2.326 + 2.336 (s, 3H), 2.40–2.55 (m, 1H), 2.75–3.05 (m, 2H), 3.18–3.30 (m, 1H), 3.50–3.70 (m, 3H), 4.10–4.44 (m, 2H), 5.36 (s, 2H), 6.79 (s, 1H), 7.18 (s, 1H); LC–MS ($M+H^+$) calcd for $C_{37}H_{59}N_3O_9Si$ 718, found 718.

Compound 17b (Foamy solid, 0.55 g, 44%): ^1H NMR (400 MHz, $CDCl_3$) δ 0 (s, 9H), 0.92 (t, J = 8.8 Hz, 2H), 0.99 + 1.01 (s, 3H), 1.08 (s, 3H), 1.34 + 1.60 (s, 3H), 1.25–1.40 (m, 1H), 1.70–1.80 (m, 1H), 1.90–2.04 (m, 1H), 2.06–2.19 (m, 1H), 2.33 (s, 3H), 2.39–2.54 (m, 1H), 2.70–3.04 (m, 2H), 3.25 (t, J = 10.4 Hz, 1H), 3.50–3.70 (m, 3H), 4.22–4.42 (m, 3H), 5.36 (s, 1H), 6.74 (s, 1H), 7.17 (s, 1H); LC–MS ($M+H^+$) calcd for $C_{37}H_{59}N_3O_9Si$ 718, found 718.

5.9.28. (3*R*,4*R*)-*tert*-Butyl-3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl) amino)-4-methylpyridin-2-yl)-methyl)-4-hydroxypyrrolidine-1-carboxylate (14b)

To a solution of **17b** (397 mg, 0.55 mmol) in methanol (5 mL) and water (0.5 mL) was added Na_2CO_3 (0. 11 g, 1 mmol). The mixture was stirred at room temperature overnight, and concentrated. The resulting residue was partitioned between EtOAc (20 mL) and H_2O (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by flash column chromatography (silica gel, EtOAc/hexanes, 2:3) to yield compound **17b** as a foamy solid (0.291 g, 98%): ^1H NMR (400 MHz, $CDCl_3$) δ 0 (s, 9H), 0.95 (t, J = 8.0 Hz, 2H), 1.46 (s, 9H), 2.35 (s, 3H), 2.80 (t, J = 12.0 Hz, 1H), 2.96 (t, 1H), 3.22 (t, 1H), 3.40–3.70 (m, 4H), 4.05–4.25 (m, 2H), 4.53 + 4.63 (br s, OH), 5.25 (s, 2H), 6.85 (s, 1H), 7.21 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): 17.7, 20.8, 28.0, 28.3, 34.8, 44.3, 45.0, 48.8, 49.2, 53.3, 53.6, 61.6, 65.6, 69.8, 70.6, 76.6, 78.8, 81.4, 118.8, 120.8, 120.9, 149.5, 149.6, 153.1, 153.7, 154.2, 157.8; ESI-MS: calcd for $C_{27}H_{47}N_3O_6Si$ 537.3234, found 537.32437.

5.9.29. (3′-Chlorobiphenyl-4-yl)methanol (18a)

Compound **18a** was synthesized using general procedure E (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.75 (s, 2H), 7.30–7.34 (m, 1H), 7.37 (t, J = 8.0 Hz, 1H), 7.42–7.48 (m, 3H), 7.54–7.58 (m, 3H).

5.9.30. (3′-Fluorobiphenyl-4-yl)methanol (18b)

Compound **18b** was synthesized using general procedure E (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.75 (s, 2H), 7.00–7.08 (m, 1H), 7.22–7.32 (m, overlapped by $CDCl_3$, 1H), 7.34–7.42 (m, 2H), 7.45 (d, J = 8.0 Hz, 2H), 7.57 (d, J = 8.0 Hz, 2H).

5.9.31. (3′-(Trifluoromethyl)biphenyl-4-yl)methanol (18c)

Compound **18c** was synthesized using general procedure E (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.75 (s, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 7.2 Hz, 1H), 7.59 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 7.2 Hz, 1H), 7.83 (s, 1H).

5.9.32. (3′-Chloro-4′-fluorobiphenyl-4-yl)methanol (18d)

Compound **18d** was synthesized using general procedure E (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.75 (s, 2H), 7.20 (t, J = 8.8 Hz, 1H), 7.40–7.47 (m, 3H), 7.49–7.55 (m, 2H), 7.61 (dd, J = 7.8, 2.4 Hz, 1H).

5.9.33. (4′-Fluoro-3′-methylbiphenyl-4-yl)methanol (18e)

Compound **18e** was synthesized using general procedure E (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 2.33 (s, 3H), 4.73 (s, 2H), 7.06 (t, J = 9.2 Hz, 1H), 7.25–7.54 (m, 6H).

5.9.34. 4′-(Bromomethyl)-3-chlorobiphenyl (19a)

Compound **19a** was synthesized using general procedure F (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.54 (s, 2H), 7.29–7.33 (m,1H), 7.35 (t, J = 8.0 Hz, 1H), 7.41–7.43 (m, 1H), 7.45 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H), 7.53–7.55 (m, 1H).

5.9.35. 4′-(Bromomethyl)-3-fluorobiphenyl (19b)

Compound **19b** was synthesized using general procedure F (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.55 (s, 2H), 7.0–7.10 (m, 1H), 7.22–7.3 (m, 1H, overlapped by CDCl3), 7.35–7.44 (m, 2H), 7.50 (d, J = 8.0 Hz, 2H), 7.55 (d, J = 8.0 Hz, 2H).

5.9.36. 4′-(Bromomethyl)-3-(trifluoromethyl)biphenyl (19c)

Compound **19c** was synthesized using general procedure F (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.55 (s, 2H), 7.50 (d, J = 7.6 Hz, 2H), 7.57 (d, J = 7.6 Hz, 2H), 7.62 (d, J = 8.0 Hz, 1H), 7.75 (d, J = 8.0 Hz, 1H), 7.82 (s, 1H).

5.9.37. 4′-(Bromomethyl)-3-chloro-4-fluorobiphenyl (19d)

Compound **19d** was synthesized using general procedure F (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 4.52 (s, 2H), 7.18 (t, J = 8.8 Hz, 1H), 7.38–7.47 (m, 5H), 7.57 (dd, J = 8.8, 2.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): 33.0, 116.8 (d, J_{C-F} = 84 Hz), 121.2, 126.6 (d, J_{C-F} = 29.2 Hz), 127.3, 129.1, 129.6, 137.3, 137.5 (d, J_{C-F} = 12 Hz), 138.9, 156.4, 158.9; ^{19}F NMR (376 MHz, $CDCl_3$): −119.5.

5.9.38. 4′-(Bromomethyl)-4-fluoro-3-methylbiphenyl (19e)

Compound **19e** was synthesized using general procedure F (87%): ^1H NMR (400 MHz, $CDCl_3$) δ 2.32 (s, 3H), 4.53 (s, 2H), 7.05 (t, J = 8.8 Hz, 1H), 7.30–7.40 (m, 2H), 7.44 (d, J = 8.8 Hz, 2H), 7.49 (d, J = 8.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): 314.5, 32.2, 115.2 (d, J_{C-F} = 90.8 Hz), 125.0 (d, J_{C-F} = 69.2 Hz), 125.8 (d, J_{C-F} = 29.2 Hz), 127.2, 129.4, 130.0 (d, J_{C-F} = 18.4 Hz), 136.0 (d, J_{C-F} = 14.4 Hz), 136.5, 140.3, 159.8, 162.3; ^{19}F NMR (376 MHz, $CDCl_3$): −117.8.

5.9.39. (3*S*,4*S*)-*tert*-Butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)-methyl)-4-((3′-chlorobiphenyl-4-yl)methoxy)pyrrolidine-1-carboxylate (13g)

Compound **13g** was synthesized using general procedure G (87%): ^1H NMR (400 MHz, D_2O) δ 0 (s, 9H), 0.94 (t, J = 8.0 Hz, 2H), 1.49 (s, 9H), 1.53 (s, 9H), 2.31 + 2.32 (s, 3H), 2.66–2.82 (m, 1H), 2.83–2.94 (m, 1H), 3.08 (dd, J = 14.0, 6.8 Hz, 1H), 3.22–3.33 (m, 1H), 3.35–3.42 (m, 1H), 3.48–3.60 (m, 1H), 3.63 (t, J = 8.0 Hz, 2H), 3.78 (d, J = 12.0 Hz, 1H), 3.97–4.03 (m, 1H), 4.38–4.48 (m, 1H), 4.68 (t, J = 11.6 Hz, 1H), 5.37 (s, 2H), 6.79 (s, 1H), 7.16 (d, J = 9.6 Hz, 1H), 7.33–7.46 (m, 4H), 7.50 (d, J = 7.2 Hz, 1H), 7.55–7.63 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ −1.48, 18.0, 21.0, 28.2, 28.5, 28.9, 34.9, 42.6, 43.3, 49.0, 49.3, 50.2, 50.8, 65.7, 70.6 + 70.7, 76.5, 78.0, 79.0, 79.16 + 79.21, 81.3, 119.2, 121.3, 125.2, 127.0, 127.1, 127.2, 127.9, 128.2, 130.0, 134.6, 137.8, 138.0, 139.1, 142.7, 148.8, 153.4, 154.1, 154.5, 154.8, 158.4; ESI-MS: m/z = 738 $[M+H]^+$; HR-ESI-MS: 737.3627 calcd for $C_{40}H_{56}ClN_3O_6Si$, found: 737.3634.

5.9.40. (3*S*,4*S*)-*tert*-Butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)-methyl)-4-((3′-fluorobiphenyl-4-yl)methoxy)pyrrolidine-1-carboxylate (13h)

Compound **13h** was synthesized using general procedure G (87%): ^1H NMR (400 MHz, D_2O) δ 0 (s, 9H), 0.92 (t, J = 8.0 Hz, 2H), 1.49 (s, 9H), 1.54 (s, 9H), 2.31 + 2.33 (s, 3H), 2.68–2.83 (m, 1H), 2.84–2.93 (m, 1H), 3.04–3.13 (m, 1H), 3.22–3.34 (m, 1H), 3.35–3.43 (m,1H), 3.48–3.60 (m,1H), 3.63 (t, J = 8.0 Hz, 2H), 3.78 (d, J = 12.0 Hz, 1H), 3.96–4.04 (m, 1H), 4.38–4.49 (m, 1H), 3.68 (t, J = 11.2 Hz, 1H), 5.37 (s, 2H), 6.80 (s, 1H), 7.04–7.12 (m, 1H), 7.16 (d, J = 8.8 Hz, 1H), 7.32–7.35 (m, 1H), 7.38–7.50 (m, 4H), 7.55–7.62 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ −1.46, 18.0, 21.0, 28.2, 28.5, 29.7, 34.9 + 35.0, 42.6, 43.3, 49.0, 49.3, 50.2, 50.8, 64.8, 65.7, 70.6 + 70.7, 76.5, 78.0, 78.9, 79.17 + 79.22, 81.23 + 81.28, 113.8, 113.9, 114.0, 114.1, 119.2, 121.3, 122.6 + 122.7, 127.0, 127.2, 127.4, 127.9, 128.2, 130.16 + 130.24, 137.9, 138.0, 139.2,

143.0, 148.8, 153.4, 154.2, 154.5, 154.8, 158.4, 161.9, 164.4; ESI-MS: m/z = 700 $[M+H]^+$; HR-ESI-MS: 699.4119 calcd for $C_{41}H_{57}F_3N_3O_6Si$, found: 699.4113.

5.9.41. (3*S*,4*S*)-*tert*-Butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)-methyl)-4-((3′-(trifluoromethyl)biphenyl-4-yl)methoxy)-pyrrolidine-1-carboxylate (13i)

Compound **13i** was synthesized using general procedure G (87%): ^{1}H NMR (400 MHz, D_2O) δ 0 (s, 9H), 0.92 (t, J = 8.0 Hz, 2H), 1.49 (s, 9H), 1.54 (s, 9H), 2.31 + 2.33 (s, 3H), 2.65–2.83 (m, 1H), 2.84–2.93 (m, 1H), 3.02–3.13 (m, 1H), 3.22–3.34 (m, 1H), 3.35–3.43 (m, 1H), 3.47–3.60 (m, 1H), 3.63 (t, J = 8.0 Hz, 2H), 3.79 (d, J = 12.8 Hz, 1H), 4.00 (s, 1H), 4.40–4.50 (m, 1H), 4.69 (t, J = 10.8 Hz, 1H), 5.37 (s, 2H), 6.80 (s, 1H), 7.16 (d, J = 8.8 Hz, 1H), 7.46 (d, J = 7.2 Hz, 2H), 7.55–7.67 (m, 4H), 7.80 (d, J = 7.2 Hz, 1H), 7.86 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ −1.47, 18.0, 21.0, 28.2, 28.5, 29.7, 34.85 + 34.95, 42.6, 43.3, 49.0, 49.3, 50.2, 50.8, 65.7, 70.6 + 70.7, 76.5, 78.1, 79.0, 79.16 + 79.23, 81.23 + 81.27, 119.2, 121.3, 123.8 + 123.9, 127.1, 128.0, 128.3, 129.2, 130.3, 138.1, 138.2, 139.0, 141.6, 148.8, 153.4, 154.2, 154.8, 158.4; ^{19}F NMR (100 MHz, $CDCl_3$): −63.0; ESI-MS: m/z = 772 $[M+H]^+$; HR-ESI-MS: 771.3891 calcd for $C_{41}H_{57}F_3N_3O_6Si$, found: 771.3898.

5.9.42. (3*S*,4*S*)-*tert*-Butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)-methyl)-4-((4′-fluoro-3′-methylbiphenyl-4-yl)methoxy)-pyrrolidine-1-carboxylate (13j)

Compound **13j** was synthesized using general procedure G (87%): ^{1}H NMR (400 MHz, D_2O) δ 0 (s, 9H), 0.92 (t, J = 8.0 Hz, 2H), 1.49 (s, 9H), 1.54 (s, 9H), (2.31 + 2.33) (s, 3H), 2.37 (s, 3H), 2.67–2.83 (m, 1H), 2.84–2.94 (m,1H), 3.08 (dd, J = 14.0, 6.8 Hz, 1H), 3.22–3.34 (m, 1H), 3.35–3.42 (m, 1H), 3.48–3.59 (m, 1H), 3.63 (t, J = 8.0 Hz, 2H), 3.78 (d, J = 12.0 Hz, 1H), 3.96–4.04 (m, 1H), 4.38–4.46 (m, 1H), 4.67 (t, J = 11.2, 10.8 Hz, 1H), 5.37 (s, 2H), 6.79 (s, 1H), 7.10 (t, J = 8.8 Hz, 1H), 7.16 (d, t, J = 9.2 Hz, 1H), 7.36–7.45 (m, 4H), 7.51–7.58 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ −1.46, 14.669 + 14.696, 18.0, 20.983 + 20.965, 28.2, 28.5, 29.7, 34.9 + 35.0, 42.6, 43.3, 49.0, 49.3, 50.2, 50.8, 65.7, 70.7 + 70.8, 76.5, 77.9, 78.9, 79.1, 79.2, 81.22 + 81.26, 115.1, 115.3, 119.2, 121.3, 125.80 + 125.88, 126.9, 127.9, 128.1, 130.09 + 130.14, 136.6, 137.0, 137.1, 139.8, 148.8, 153.4, 154.2, 154.5, 154.8, 158.4, 159.8, 162.2; ESI-MS: m/z = 736 $[M+H]^+$; HR-ESI-MS: 735.4079 calcd for $C_{24}H_{26}ClN_3OS$, found: 735.4082.

5.9.43. (3*S*,4*S*)-*tert*-Butyl 3-((6-(*tert*-butoxycarbonyl((2-(trimethylsilyl)ethoxy)methyl)amino)-4-methylpyridin-2-yl)-methyl)-4-((3′-chloro-4′-fluorobiphenyl-4-yl)methoxy)-pyrrolidine-1-carboxylate (13k)

Compound **13k** was synthesized using general procedure G (87%): ^{1}H NMR (400 MHz, D_2O) δ 0 (s, 9H), 0.92 (t, J = 8.0 Hz, 2H), 1.49 (s, 9H), 1.54 (s, 9H), 2.31 + 2.33 (s, 3H), 2.65–2.82 (m,1H), 2.83–2.94 (m, 1H), 3.08 (dd, J = 14.0, 7.2 Hz, 1H), 3.21–3.34 (m, 1H), 3.34–3.43 (m, 1H), 3.48–3.60 (m, 1H), 3.63 (t, J = 8.0 Hz, 2H), 3.78 (d, J = 12.0 Hz, 1H), 3.96–4.05 (m, 1H), 4.38–4.49 (m, 1H), 4.67 (t, J = 12.0, 11.6, 9.2 Hz, 1H), 5.38 (s, 2H), 6.79 (s, 1H), 7.16 (d, J = 9.2 Hz, 1H), 7.24 (t, J = 8.8 Hz, 1H), 7.40–7.49 (m, 3H), 7.50–7.56 (m, 2H), 7.64 (dd, J = 6.8, 2.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ −1.47, 17.9, 21.0, 28.2, 28.5, 29.6, 34.9, 42.6, 43.3, 48.9, 49.3, 50.2, 50.8, 65.7, 70.5 + 70.6, 76.5, 78.0, 79.0, 79.1 + 79.2, 81.3, 116.7, 116.9, 119.2, 121.2, 126.6, 126.7, 126.9, 128.0, 128.2, 129.1, 137.8, 138.0, 138.1, 138.3, 148.8, 153.4, 154.1, 154.8, 156.3, 158.4, 158.8; ^{19}F NMR (100 MHz, $CDCl_3$): −120.4; ESI-MS: m/z = 756 $[M+H]^+$; HR-ESI-MS: 755.3533 calcd for $C_{24}H_{26}ClN_3OS$, found: 755.3540.

5.9.44. 6-(((3*S*,4*S*)-4-((3′-Chlorobiphenyl-4-yl)methoxy)pyrrolidin-3-yl)methyl)-4-methylpyridin-2-amine hydrochloride salt (2g)

Compound **2g** was synthesized using general procedure H (100%): ^{1}H NMR (400 MHz, D_2O) δ 1.80 (s, 3H), 2.50–2.72 (m, 3H), 3.04–3.10 (m, 1H), 2.32–2.34 (m, 1H), 3.48–3.59 (m, 1H), 3.71 (d, J = 12.8 Hz, 1H), 4.01 (s, 1H), 4.15 (d, J = 12 Hz, 1H), 4.62 (d, J = 12 Hz, 1H), 5.86 (s, 1H), 6.24 (s, 1H), 7.09 (d, J = 7.2 Hz, 1H), 7.17–7.35 (m, 7H); ^{13}C NMR (100 MHz, D_2O) δ 21.1, 28.4, 40.7, 47.4, 49.8, 70.1, 75.5, 110.0, 113.3, 124.9, 126.2, 126.4, 127.4, 130.5, 134.2, 136.8, 138.2, 141.4, 145.4, 153.4, 157.1; ESI-MS: m/z = 408 $[M+H]^+$; HR-ESI-MS: 407.1764 calcd for $C_{24}H_{26}ClN_3OS$, found: 407.1773.

5.9.45. 6-(((3*S*,4*S*)-4-((3′-Fluorobiphenyl-4-yl)methoxy)pyrrolidin-3-yl)methyl)-4-methylpyridin-2-amine hydrochloride salt (2h)

Compound **2h** was synthesized using general procedure H (100%): ^{1}H NMR (400 MHz, D_2O) δ 1.86 (s, 3H), 2.55–2.80 (m, 3H), 3.04–3.15 (m, 1H), 3.30–3.40 (m, 1H), 3.50–3.61 (m, 1H), 3.68–3.75 (m, 1H), 4.06 (s, 1H), 4.17 (d, J = 12 Hz, 1H), 4.62 (d, J = 12 Hz, 1H), 6.97 (s, 1H), 6.20 (s, 1H), 6.95 (s, 1H), 7.11–7.44 (m, 7H); ^{13}C NMR (100 MHz, D_2O) δ 21.0, 28.1, 40.0, 47.5, 49.9, 70.0, 75.0, 109.8, 113.0, 114.2, 122.3, 126.4, 129.6, 130.8, 136.6, 138.4, 141.8, 145.5, 153.4, 157.2, 161.8, 164.3; ^{19}F NMR (100 MHz, D_2O): −113.7; ESI-MS: m/z = 392 $[M+H]^+$; HR-ESI-MS: 391.2071 calcd for $C_{24}H_{26}FN_3OS$, found: 391.2072.

5.9.46. 4-Methyl-6-(((3*S*,4*S*)-4-((3′-(trifluoromethyl)biphenyl-4-yl)methoxy)pyrrolidin-3-yl)methyl)pyridin-2-amine hydrochloride salt (2i)

Compound **2i** was synthesized using general procedure H (100%): ^{1}H NMR (400 MHz, D_2O) δ 1.67 (s, 3H), 2.42–2.68 (m, 3H), 2.94–3.08 (m, 1H), 3.24–3.32 (m, 1H), 3.42–3.54 (m, 1H), 3.69 (d, J = 12.4 Hz, 1H), 3.91 (s, 1H), 4.07 (d, J = 11.2 Hz, 1H), 4.57 (d, J = 11.2 Hz, 1H), 5.70 (s, 1H), 6.23 (s, 1H), 7.08–7.28 (m, 6H), 7.37–7.50 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 20.8, 28.7, 41.4, 47.3, 49.5, 70.1, 76.2, 110.1, 113.5, 122.6, 122.8, 123.7, 126.4, 129.1, 129.5, 129.9, 130.3, 130.6, 137.3, 137.9, 140.4, 145.6, 153.6, 156.8; ^{19}F NMR (100 MHz, D_2O): −63.2; ESI-MS: m/z = 442 $[M+H]^+$; HR-ESI-MS: 441.2028 calcd for $C_{25}H_{26}F_3N_3O$, found: 441.2032.

5.9.47. 6-(((3*S*,4*S*)-4-((4′-Fluoro-3′-methylbiphenyl-4-yl)methoxy)-pyrrolidin-3-yl)methyl)-4-methylpyridin-2-amine hydrochloride salt (2j)

Compound **2j** was synthesized using general procedure H (100%): ^{1}H NMR (400 MHz, D_2O) δ 1.84 (s, 3H), 2.06 (s, 3H), 2.50–2.78 (m, 3H), 3.05–3.12 (m, 1H), 3.32–3.34 (m, 1H), 3.51–3.60 (m, 1H), 3.72 (d, J = 12.8 Hz, 1H), 4.04 (s, 1H), 4.17 (d, J = 12.0 Hz, 1H), 4.63 (d, J = 12.0 Hz, 1H), 5.92 (s, 1H), 6.25 (s, 1H), 6.86 (t, J = 8.8 Hz, 1H), 7.14–7.29 (m, 6H); ^{13}C NMR (100 MHz, D_2O) δ 13.9, 21.1, 28.4, 40.6, 47.4, 49.8, 70.1, 75.3, 110.0, 113.3, 115.1, 115.3, 125.0, 125.2, (125.34 + 125.42), 126.2, 129.4, 135.5, 135.9, 138.9, 145.5, 153.4, 157.2, 159.6, 162.1; ESI-MS: m/z = 406 $[M+H]^+$; HR-ESI-MS: 405.2216 calcd for $C_{25}H_{28}FN_3O$, found: 405.2285.

5.9.48. 6-(((3*S*,4*S*)-4-((3′-Chloro-4′-fluorobiphenyl-4-yl)methoxy)-pyrrolidin-3-yl)methyl)-4-methylpyridin-2-amine hydrochloride salt (2k)

Compound **2k** was synthesized using general procedure H (100%): ^{1}H NMR (400 MHz, D_2O) δ 1.86 (s, 3H), 2.55–2.74 (m, 3H), 3.06–3.12 (m, 1H), 3.32 (d, J = 12.8 Hz, 1H), 3.50–3.57 (m, 1H), 4.05 (s, 1H), 4.17 (d, J = 12.0 Hz, 1H), 4.62 (d, J = 12.0 Hz, 1H), 5.95 (s, 1H), 6.24 (s, 1H), 7.06 (t, J = 8.8 Hz, 1H), 7.20–7.40

(m, 6H); ^{13}C NMR (100 MHz, D_2O) δ 21.0, 28.6, 40.7, 47.4, 49.8, 70.1, 75.5, 109.8, 113.3, 116.8, 117.0, (120.5 + 120.7), 126.3, 126.5, 128.2, 129.4, 136.6, 137.0, 137.5, 146.1, 153.7, 156.1, 156.8, 158.5; ESI-MS: m/z = 426 $[M+H]^+$; HR-ESI-MS: 425.1670 calcd for $C_{24}H_{25}ClFN_3O$, found: 425.1674.

5.10. Enzyme assays

IC_{50} values for inhibitors **2a**–**d** were measured for the three different isoforms of NOS, including rat nNOS, bovine eNOS, and murine macrophage iNOS using L-arginine as the substrate. The three isozymes were recombinant enzymes, which were overexpressed (in *E. coli*) and isolated as reported.[23] The formation of nitric oxide was measured using a hemoglobin capture assay described previously.[29] 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 μg/mL calmodulin, 100 μM DTT, 100 μM NADPH, 6.5 μM H_4B, 3.0 μM oxyhemoglobin (for iNOS 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-hemoglobin complex at 401 nm from 0 to 60 s after. 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 μM; iNOS, 8.3 μM; eNOS, 1.7 μM).

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

5.11. Molecular docking

Docking simulations were carried out as described by Ji et al. with AutoDock 3.0.5.[30] Polar hydrogen atoms were added to the nNOS crystal structure (PDB code: 3JWT),[24] and Kollman united atom charges[31] were assigned. Hydrogens were also added to the heme and H_4B, and charges were calculated by the Gasteiger–Marsili method.[32] The charge of the Fe atom bound to heme was assigned +3. The nonpolar hydrogen atoms of heme and H_4B were removed manually, and their charges were united with the bonded carbon atoms. Atomic solvation parameters and fragmental volumes were assigned using the AddSol utility. The 3D structures of the ligands were built and partial atomic charges were also calculated using the Gasteiger–Marsili method. The rotatable bonds in the ligands were defined using AutoTors, which also unites the nonpolar hydrogens and partial atomic charges to the bonded carbon atoms. The grid maps were calculated using AutoGrid. The dimensions of the grid box was 31 × 28 × 31 Å, and the grid spacing was set to 0.375 Å. Docking was performed using the Lamarckian genetic algorithm (LGA), and the pseudo-Solis and Wets method were applied for the local search.

5.12. Crystal preparation, X-ray diffraction data collection, and structure refinement

The nNOS heme domain protein used for crystallographic studies was produced by limited trypsin digest from the corresponding full length enzyme and further purified through a Superdex 200 gel filtration column (GE Healthcare) as described previously.[33,34] The nNOS heme domain at 7–9 mg/mL containing 20 mM histidine was used for the sitting drop vapor diffusion crystallization setup under the conditions reported before.[33] Fresh crystals (1–2 day old) were first passed stepwise through cryo-protectant solutions described [33] and then soaked with 10 mM inhibitor for 4–6 h at 4 °C before being flash cooled by plunging into liquid nitrogen.

The cryogenic (100 K) X-ray diffraction data were collected remotely at the Stanford Synchrotron Radiation Lightsource through the data collection control software Blu-Ice[35] and the crystal mounting robot. Raw data frames were indexed, integrated, and scaled using HKL2000.[36]

Table 2
Crystallographic data collection and refinement statistics

	nNOS-**2b**
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	52.28, 111.56, 164.85
Resolution (Å)	1.90 (1.93–1.90)
R_{sym}	0.075 (0.572)
$I/\sigma I$	24.2 (2.0)
No. unique reflections	75,896
Completeness (%)	99.3 (99.7)
Redundancy	4.0 (4.0)
Refinement	
Resolution (Å)	1.90
No. reflections used	71,875
R_{work}/R_{free} [a]	0.182/0.215
Mean *B* value	44.53
No. atoms	
Protein	6,671
Ligand/ion	186
Water	380
Rms deviations	
Bond lengths (Å)	0.014
Bond angles (°)	1.350

[a] The 5% of reflections were set aside for the free *R* calculation throughout the refinement according to the free *R* flags used in the refinement of the starting model (1OM4).

The binding of inhibitors was detected by the initial difference Fourier maps calculated with REFMAC.[37] The inhibitor molecules were then modeled in O[38] or COOT[39] and refined using REFMAC. Water molecules were added in REFMAC and checked by COOT. The TLS[40] protocol was implemented in the final stage of refinements with each subunit as one TLS group. The refined structures were validated in COOT before deposition to RCSB protein data bank with PDB accession code 1OM4. The crystallographic data collection and structure refinement statistics are summarized in Table 2.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.074. These data include MOL files and InChiKeys of the most important compounds described in this article.

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