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Subnanomolar Inhibitor of Cytochrome *bc*₁ Complex Designed via Optimizing Interaction with Conformationally Flexible Residues

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Abstract

Cytochrome *bc*₁ complex (EC 1.10.2.2, *bc*₁), an essential component of the cellular respiratory chain and the photosynthetic apparatus in photosynthetic bacteria, has been identified as a promising target for new drugs and agricultural fungicides. X-ray diffraction structures of the free *bc*₁ complex and its complexes with various inhibitors revealed that the phenyl group of Phe274 in the binding pocket exhibited significant conformational flexibility upon different inhibitors binding to optimize respective π - π interactions, whereas the side chains of other hydrophobic residues showed conformational stability. Therefore, in the present study, a strategy of optimizing the π - π interaction with conformationally flexible residues was proposed to design and discover new *bc*₁ inhibitors with a higher potency. Eight new compounds were designed and synthesized, among which compound **5c** with a K_i value of 570 pM was identified as the most promising drug or fungicide candidate, significantly more potent than the commercially available *bc*₁ inhibitors including azoxystrobin (AZ), kresoxim-methyl (KM), and pyraclostrobin (PY). To our knowledge, this is the first *bc*₁ inhibitor discovered from structure-based design with a potency of subnanomolar K_i value. For all of the compounds synthesized and assayed, the calculated binding free energies correlated reasonably well with the binding free energies derived from the experimental K_i values with a correlation coefficient of $r^2 = 0.89$. The further inhibitory kinetics studies revealed that compound **5c** is a non-competitive inhibitor with respect to substrate cytochrome *c*, but is a competitive inhibitor with respect to substrate ubiquinol. Due to its subnanomolar K_i potency and slow dissociation rate constant ($k_{-0} = 0.00358 \text{ s}^{-1}$), compound **5c** could be used as a specific probe for further elucidation of the mechanism of *bc*₁ function and as a new lead compound for future drug discovery.

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Supporting Information Available. Complete citations of references 8 and 20. Figure 1s for Sequence alignment of the Q0 pocket residue. Figure 2s for Inhibitory kinetics of porcine *bc*₁ complex by compound **5c**, Figure 3s for effect of lauryl maltoside on k_{obs} , Figure 4s for the enzyme kinetic of AZ. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Understanding of the drug-receptor interactions is the molecular basis of designing new compounds with the higher potency.¹ In fact, the interaction between drug and receptor is the interaction between the bioactive conformation of a drug molecule and the residues in the binding pocket of its receptor. A drug molecule will display the highest potency when its bioactive conformation matched very well with the binding pocket. Generally speaking, some residues in the binding pocket of a receptor protein might be conformationally flexible, while the others are rigid. Therefore, it should be an effective way to improve the potency of a drug by optimizing the interactions with the conformationally flexible residues in the binding pocket.

Cytochrome *bc*₁ complex (EC 1.10.2.2, *bc*₁) is well known as an essential component of the cellular respiratory chain and the photosynthetic apparatus in photosynthetic bacteria. Its function has been known to catalyze the electron transfer from quinol to a soluble cytochrome *c* (cyt *c*) and couple this electron transfer to the translocation of protons across the membrane.^{2–5} The *bc*₁ complex has been found in the plasma membrane of bacteria and in the inner mitochondrial membrane of eukaryotes. Due to its crucial role in life cycle, the *bc*₁ complex has been identified as a promising target for new drugs and agricultural fungicides.^{6,7} For example, malaria, especially chloroquine- and multidrug-resistant *P. falciparum*-malaria, remains a major threat to over 40% of the world's population and is associated with about hundred million clinical cases every year. Atovaquone, as shown in Scheme 1, has been used in treating multidrug-resistant malaria and for prophylaxis in areas with chloroquine resistance for many years.^{8–12} The action mechanism of Atovaquone has been known to inhibit the *bc*₁ complex by binding to the ubiquinol oxidation pocket of the *bc*₁ complex, where it interacts with the Rieske iron-sulfur protein.^{11,12} In addition, a number of Q₀-specific inhibitors of the *bc*₁ complex, always named Q₀I fungicides, have been introduced into the agricultural fungicide market.^{13,14} The first Q₀I fungicides entered the market in 1996 include azoxystrobin (AZ) and kresoxim-methyl (KM) depicted in Scheme 1, two synthetic analogues of the natural antifungal compound strobilurin A. With a distributor sale value of over US\$900 million apiece in 2008, AZ currently represents one of the most important fungicides in the world.

According to the structural information determined by the crystallographic studies, Xia *et al.*⁵ suggested that the existing *bc*₁ inhibitors could be grouped into three classes: class P, class N, and class PN. Class P inhibitors binding at the Q₀ site include famoxadone, stigmatellin, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), methoxyacrylate-stilbene (MOAS), myxothiazol, AZ, KM, pyraclostrobin (PY), and many others. Class N inhibitors targeting the Q_i site include antimycin A and diuron. Class PN inhibitors with the capability of binding to both the Q₀ and Q_i sites contain 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (NQNO) and possibly funiculosin. Among these inhibitors, MOA-type inhibitors, such as strobilurin, oudemansin and AZ, are very important due to their great commercial success and potential role in elucidating the molecular mechanism of *bc*₁ function. However, these commercial inhibitors as agricultural fungicides or drugs are suffering from the rapid development of resistance associated with site mutations of *bc*₁ complex. Therefore, discovery of new *bc*₁ inhibitors with a higher potency is of great interest.^{15–17}

X-ray diffraction structures of the free *bc*₁ complex and its complexes with various MOA-type inhibitors revealed that the conformation of MOA moiety in the binding pocket is well conserved,^{3,5} especially for the methoxy and carbonyl oxygen atoms, which makes it possible to identify common structural features that are important for binding. In addition, the Q₀ pocket is hydrophobic and exceptionally rich in highly conserved residues (Supporting Information, Figure 1s). Most importantly, the Ar-Ar stacking interactions with the hydrophobic pocket formed by the side chains of Phe274, Phe128, Ile146, Pro270, Ala277, Leu294, Met124, and

Ile298 (numbered according to the cyt *b* subunit of the bovine *bc*₁) have an important contribution to the binding affinity of inhibitors.^{3, 5} It is noteworthy that the side chains of all these hydrophobic residues showed the conformational stability during the simulation, except that the phenyl group of Phe274 exhibited the significant conformational flexibility in different complexes. The conformational flexibility of Phe274 side chain allows to optimize the corresponding Ar-Ar interactions. However, due to the existence of the linear cyano group in AZ, the phenyl ring of the cyanophenoxy group is almost vertical to the pyrimidyl ring. As a result, the pyrimidyl ring does not parallel well with the phenyl ring of Phe274 to reach an ideal Ar-Ar interaction. On the contrary, myxothiazol has higher potency than AZ because its thiazoles ring parallels well with the phenyl group of Phe274. These facts suggest that improving the Ar-Ar interactions between the side-chain of inhibitors and the phenyl of Phe274 may be an effective way to design and discover new, more potent inhibitors of *bc*₁ complex.

On the basis of the above premises, the development of new, more potent *bc*₁ inhibitors by improving the Ar-Ar interactions of AZ is the main strategy of this work. Without altering the main structural features of MOA-type inhibitors, we first designed the molecular framework **I** (Scheme 2). As an important kind of five-membered aromatic scaffold with broad-spectrum biological activities, pyrazole ring has appeared in many of commercial drugs.^{18–20} Hence, we replaced the pyrimidine ring of AZ with a methylpyrazole ring as shown in Scheme 2. Meanwhile, an *ortho*-hydroxy group was designed to replace the cyano group with the expectation that an intramolecular hydrogen bond between the hydroxy group and the *N*(1) atom of pyrazole ring could be formed, which enables it possible for the pyrazole ring to perfectly parallel with the phenyl group of Phe274 to improve the Ar-Ar interactions. In addition, in order to adjust the hydrophobic property of the designed compounds, some functional groups, such as Cl and CH₃, were also introduced into the R-position of the phenyl moiety.

The strategy we proposed for the selection of compounds to be synthesized makes use of computational simulation protocols. These allow us to analyze the key interactions at the atomic level between the selected compounds and the *bc*₁ complex. As a result, one compound with a *K*_i value of 570 pM against porcine *bc*₁ complex was discovered. To our knowledge, it is so far the first *bc*₁ inhibitor discovered through structure-based design with subnanomolar *K*_i potency. The synthesis, *in vitro* enzymatic assay, and subsequent enzymatic kinetic study are reported in this work. Its excellent potency and interesting characteristics of enzymatic kinetic behavior indicate that this compound could be used as a specific probe for further elucidating the mechanism of *bc*₁ function and as a new lead compound for future drug discovery.

Materials and Methods

1. Computational methods

Homology Modeling of the Porcine Cytochrome *b* Subunit (cyt *b*)—In order to study the binding of a set of representative inhibitors with the porcine mitochondrial cytochrome *bc*₁ complex, a homology model of the cyt *b* was built by using the complete crystal structure of bovine heart cytochrome *bc*₁ in complex with AZ (PDB entry of 1SQB) as the template and the Homology module of InsightII program (version 2000, Accelrys, Inc., San Diego, CA). As one of the available cytochrome *bc*₁ crystal structures containing inhibitors, the AZ-bound *bc*₁ complex was selected as the template because of the structural similarity among the compounds used in this study. The sequence alignment between the porcine cyt *b* and the same subunit of the template was generated by ClusterW with the Blosom scoring function. The best alignment was selected according to both the alignment score and the reciprocal positions of the conserved residues, especially those in or close to the inhibitor-binding sites of the template. The coordinates of the conserved regions, including the two heme groups at *b*_L and *b*_H sites were transformed directly from the template structure, whereas the

non-equivalent residues were mutated from the template to the corresponding ones in the porcine cyt *b*. The side chains of these non-conserved residues were relaxed by using the Homology module of InsightII program, in order to remove any possible steric overlap (or hinderance) with the neighboring conserved residues. The structures of other subunits of the porcine cyt *bc*₁ complex were not modeled due to the shortage of their amino acid sequences of some of the subunits. For the purpose of viewing the structural integrity, the structures of other subunits of the porcine cyt *bc*₁ complex were directly copied from the template providing their high homology to the bovine heart cyt *bc*₁ complex.

After initial coordinates were generated, all the ionizable residues of cyt *b* were set to the standard protonation states. A careful check of the structural model allowed the proton to be assigned to the N_{D1} atoms for all the His residues under the physiological condition (*pH* = ~7.4), including His83, His97, His182, and His196 coordinated with the central irons of the two heme groups. As the His residues are coordinated optimally with the two heme groups as seen from the crystal structure, these residues and heme groups were always fixed in the following energy minimization steps. The initial 3D model of the porcine cyt *b* was energy-minimized by using the Sander module of the Amber8 program²¹ suite with a non-bonded cutoff of 12 Å and a conjugate gradient minimization method. The heme groups were treated at its oxidized state, and the input parameters of these heme groups were directly adopted from Izrailev *et al.*²² in their steered molecular dynamics simulations. The energy minimization was performed in the gas phase first for 5000 steps with the backbone atoms fixed, while the side chain atoms were relaxed, and then for another 1000 steps with the side chain atoms constrained in order to relax the backbone. After two rounds of these partial energy-minimization runs, the convergence criterion of 0.001 kcal mol⁻¹ Å⁻¹ was quickly achieved in a full energy minimization.

Molecular Docking—As observed from the crystal structure, AZ is bound in the Q_o site which is formed by loop *ef*, helices cd1 and cd2, and part of the helices B, C, E, and F. Considering the structural similarity of AZ with other inhibitors, this Q_o site was also selected as a binding site for other inhibitors. Based on the 3D model of the porcine cyt *b*, the AutoDock 4.0 program²³ was applied to dock these inhibitors into the Q_o binding site. The Gasteiger charges were used for these inhibitors. To select the best set of docking parameters and to test the reliability of the docking results, the AZ was first docked into the Q_o binding site. In the docking process, conformational search was performed for AZ molecule using the Solis and Wets local search method, and the Lamarkian genetic algorithm (LGA) was applied for the conformational search of binding complex of AZ with porcine cyt *b*. Among a series of docking parameters, the grid size was set to be 40 × 40 × 40, and the used grid space was the default value of 0.375 Å. The interaction energy resulted from probing of the porcine cyt *b* with AZ molecule was assessed by the standard AutoDock scoring function. Among a set of 50 candidates of the docked complex structures, the best one was selected first according to the interaction energy, and was then compared with the conformation of AZ extracted from the crystal structure. By tuning the docking parameters, the final AZ-cyt *b* complex was obtained based on the smallest root-mean-square deviation (rmsd) of the docked conformation of AZ from its conformation in the crystal structure. The same set of docking parameters were adopted in the molecular docking of other inhibitors, including KM, PY, 5a~d, and 6a~d, into the Q_o binding site of cyt *b*.

All the complex structures derived from molecular docking were used as starting structures for further energy minimizations using the Sander module of the Amber8 program before the final binding structures were achieved. The atomic charges used for these inhibitors were the restrained electrostatic potential (RESP) charges determined by using the standard RESP procedure implemented in the Antechamber module of the Amber8 program following the electronic structure and electrostatic potential calculations at the HF/6-31G* level. The energy

minimization process was similar to that used for modeling the porcine cyt *b*, *i.e.* first fixing the backbone atoms of the protein, in order to relax the side chains and the docked inhibitor molecule. Then the energy minimization was performed on the whole complex until the convergence criterion of 0.001 kcal mol⁻¹ Å⁻¹ was reached.

Binding Energy Calculations—Due to the fact that only the structure of cyt *b* of the porcine *bc*₁ complex was modeled, and due to the expected geometric symmetry of the dimer structure of the porcine *bc*₁ complex, the binding free energy for each inhibitor with the porcine *bc*₁ complex was represented by the binding free energy of each inhibitor with the cyt *b*. Such an approximation should be reasonable, as all the other subunits are far away from the Q_o binding site and have no direct interactions with the inhibitors binding at the Q_o site. Their effects on the inhibitor binding belong to the long-range interactions and, therefore, such long-range interactions can be treated as the background without significant influence on the calculated binding free energy (ΔG_{bind}). Based on the modeled complex structure of porcine cyt *b* bound with inhibitors, the binding free energy for each of the minimized complexes was estimated by using the molecular mechanics-Possion-Boltzmann surface area (MM-PBSA) method.²⁴

In the MM-PBSA calculations, the free energy of inhibitor binding, ΔG_{bind} , was calculated from the difference between the free energies of the complex (G_{complex}), the free cyt *b* (G_{cytb}), and free inhibitor ($G_{\text{inhibitor}}$) as Eq.(1):

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{cytb}} + G_{\text{inhibitor}}) \quad (1)$$

The ΔG_{bind} was evaluated as a sum of the changes in the MM gas-phase binding energy (ΔE_{bind}), solvation free energy (ΔG_{solv}), and entropy contribution ($-T\Delta S$).

$$\Delta G_{\text{bind}} = \Delta E_{\text{bind}} - T\Delta S \quad (2)$$

$$\Delta E_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} \quad (3)$$

$$\Delta G_{\text{solv}} = \Delta G_{\text{PB}} + \Delta G_{\text{np}} \quad (4)$$

$$\Delta G_{\text{np}} = \gamma \text{SASA} \quad (5)$$

Electrostatic solvation free energy was calculated by the finite-difference solution to the Possion-Boltzmann (PB) equation (ΔG_{PB}) as implemented in the Delphi program.^{25, 26} The dielectric constants used were 1 for the solute and 78.5 for the solvent. The SASA was calculated by the default surface area calculation program in the MM-PBSA module of Amber8 program with the default $\gamma = 0.0072$ kcal Å⁻². The entropy contribution, $-T\Delta S$, to the binding free energy was calculated at $T = 300$ K by using an in-house program which has been used and proven to be reliable in our other studies.^{27–33}

2. Syntheses of the title compounds

Unless noted otherwise, reagents and starting materials 1a~c were purchased from commercial suppliers and used without further purification whereas all solvents were redistilled before use. ¹H NMR spectra were recorded on a Mercury-Plus 400 spectrometer in CDCl₃ with TMS

as the internal reference. MS spectra were determined using a Trace MS 2000 organic mass spectrometry. Elementary analyses were performed on a Vario EL III elemental analysis instrument. Melting points were taken on a Buchi B-545 melting point apparatus and uncorrected. Intermediates **2a-c**, **3a-c**, and **4a-b** were prepared according to the reported methods.^{34–36}

Preparation of 4-hydroxycoumarins (2a-c)—In a 250 mL two-neck round-bottom flask, 36 mmol o-hydroxyacetophenone **1** was added to 6.3 g of NaH (180 mol, 70%, dispersion in mineral oil) in anhydrous toluene (100 mL). After the evolution of hydrogen had ceased, 6 mL of diethyl carbonate in anhydrous toluene (20 mL) was dropwise added during 30 min, and the mixture was refluxed with stirring for 3 h. After cooling to room temperature, the precipitate was filtered off and washed with toluene 20 mL. The combined toluene solutions were concentrated on a rotary evaporator, and the residua was poured into 100 mL of water, and acidified with 2N hydrochloric acid. The resulting precipitate was filtered off and recrystallized from ethanol to give white solid. Yields for **2a**, **2b**, and **2c** are 85%, 72%, and 75%, respectively.

Preparation of 3-(2-hydroxy-5-substitutedphenyl)-1-methyl-1H-pyrazol-5(4H)-one (3a-c)—In a 100 mL three-neck round-bottom flask, 6 mmol of 6-substituted 4-hydroxycoumarins **2**, 5.6 g (12 mmol) of methylhydrazine and 40 mL of ethanol were added gradually. The reaction was refluxed under N₂ flow until the material was completely transformed by TLC. The resulting mixture was concentrated on a rotary evaporator to give the crude products. The white crystal **3** could be obtained by recrystallization of the crude products from ethanol. Yields for **3a**, **3b**, and **3c** are 81%, 92%, and 90%, respectively.

General Procedure for the Synthesis of Target Compounds (5a-d)—5.0 mmol of 3-(2-hydroxy-5-substitutedphenyl)-1-methyl-1H-pyrazol-5(4H)-one **3** and 0.82 g (6.0 mmol) of anhydrous K₂CO₃ in dry acetone (20 mL) was stirred and refluxed for 0.5 h, followed by the addition of 5.0 mmol of intermediate **4**. The reaction was continued for 5–8 h under reflux. The resulting mixture was cooled to room temperature, filtered off by suction and the solvent was evaporated to give the crude product followed by chromatography purification on silica using a mixture of petroleum ether and acetone (12:1) as an eluent to give the target compounds **5a-d** in yields of 67%–84%.

Data for 5a: Yield, 74%; M.p. 129–131 °C; ¹H NMR (400 MHz, CDCl₃) δ: 3.69 (s, 3H, NCH₃), 3.73 (s, 3H, COOCH₃), 3.85 (s, 3H, =CH-OCH₃), 5.07 (s, 2H, CH₂), 5.82 (s, 1H, HetH), 6.87 (t, *J* = 8.0 Hz, 1H, ArH), 6.99 (d, *J* = 8.0 Hz, 1H, ArH), 7.15–7.23 (m, 3H, ArH), 7.38–7.43 (m, 2H, ArH), 7.53 (dd, *J* = 3.6 Hz, *J* = 4.8 Hz, 1H, ArH), 7.63 (s, 1H, =CH-OCH₃). EI MS: *m/z* (%) 395 ([M+1]⁺, 17), 394 (M⁺, 100), 362 (19), 330 (14), 205 (60), 204 (42), 188 (13), 145 (83), 131 (23), 117 (38), 115 (25), 102 (51). Anal. Calcd for C₂₂H₂₂N₂O₅: C, 66.99; H, 5.62; N, 7.10. Found: C, 67.19; H, 5.38; N, 7.21.

Data for 5b: Yield, 84%; M.p. 143–145 °C; ¹H NMR (400 MHz, CDCl₃) δ: 2.29 (s, 3H, Ar-CH₃), 3.68 (s, 3H, NCH₃), 3.70 (s, 3H, COOCH₃), 3.84 (s, 3H, =CH-OCH₃), 5.06 (s, 2H, CH₂), 5.81 (s, 1H, HetH), 6.88 (d, *J* = 8.0 Hz, 1H, ArH), 6.98 (d, *J* = 8.4 Hz, 1H, ArH), 7.20–7.24 (m, 2H, ArH), 7.37–7.41 (m, 2H, ArH), 7.52 (dd, *J* = 3.2 Hz, *J* = 4.8 Hz, 1H, ArH), 7.62 (s, 1H, =CH-OCH₃). EI-MS: *m/z* (%) 409 ([M+1]⁺, 6), 408 (M⁺, 44), 205 (23), 204 (24), 175 (10), 146 (11), 145 (100), 131 (28), 103 (34). Anal. Calcd for C₂₃H₂₄N₂O₅: C, 67.63; H, 5.92; N, 6.86. Found: C, 67.36; H, 5.83; N, 6.90.

Data for 5c: Yield, 81%; M.p. 189–191 °C; ¹H NMR (600 MHz, CDCl₃) δ: 3.68 (s, 3H, NCH₃), 3.73 (s, 3H, COOCH₃), 3.88 (s, 3H, =CH-OCH₃), 5.06 (s, 2H, CH₂), 5.73 (s, 1H, HetH), 6.88 (d, *J* = 8.8 Hz, 1H, ArH), 7.08 (dd, *J* = 1.4 Hz, *J* = 9.0 Hz, 1H, ArH), 7.21 (dd, *J*

= 3.6 Hz, $J = 5.2$ Hz, 1H, ArH), 7.33 (d, $J = 2.4$ Hz, 1H, ArH), 7.37 (dd, $J = 3.2$ Hz, $J = 5.6$ Hz, 2H, ArH), 7.50 (dd, $J = 3.6$ Hz, $J = 5.2$ Hz, 1H, ArH), 7.64 (s, 1H, =CH-OCH₃). EI-MS: m/z (%) 428 (M⁺, 2), 204 (36), 189 (14), 160 (32), 144 (100), 128 (29), 115 (26), 101 (29), 91 (34). Anal. Calcd for C₂₂H₂₁ClN₂O₅: C, 61.61; H, 4.94; N, 6.53. Found: C, 61.35; H, 5.15; N, 6.59.

Data for 5d: Yield, 67%; M.p. 150–152 °C; ¹H NMR (600 MHz, CDCl₃) δ: 3.66 (s, 3H, NCH₃), 3.86 (s, 3H, COOCH₃), 4.08 (s, 3H, =N-OCH₃), 5.07 (s, 2H, CH₂), 5.82 (s, 1H, HetH), 6.86 (t, $J = 4.4$ Hz, 1H, ArH), 6.98 (d, $J = 7.8$ Hz, 1H, ArH), 7.15–7.25 (m, 2H, ArH), 7.40–7.54 (m, 4H, ArH). EI MS: m/z (%) 395 (M⁺, 42), 206 (10), 205 (20), 189 (28), 160 (41), 146 (31), 131 (96), 118 (37), 116 (100), 115 (19), 89 (27). Anal. Calcd for C₂₁H₂₁N₃O₅: C, 63.79; H, 5.35; N, 10.63. Found: C, 63.52; H, 5.58; N, 10.44.

General Procedure for the Synthesis of Target Compounds (6)—A reaction system with 1.0 mmol of compounds 5a–b and 0.16 g (1.2 mmol) of anhydrous K₂CO₃ or 0.12 g (1.2 mmol) of triethylamine in dry N,N-dimethylformamide (DMF) (6 mL) was stirred at room temperature for 0.5 h, followed by the addition of 1.1 mmol of halogenated compounds or substituted benzoyl chloride. The reaction was continued for 2–24 h. The resulting mixture was poured into 20 mL of water, extracted with ethyl acetate (10 mL×3), washed with saturated sodium chloride (10 mL×3), dried with anhydrous magnesium sulfate, filtered off with suction and the solvent was evaporated to give the crude, purified by chromatography on silica using a mixture of petroleum ether and acetone (20:1) as an eluent to give the target compounds 6a–d in yields of 46–82%.

Data for 6a: Yield, 78%; M.p. 117–119 °C; ¹H NMR (400 MHz, CDCl₃) δ: 3.68 (s, 3H, NCH₃), 3.70 (s, 3H, COOCH₃), 3.73 (s, 3H, =CH-OCH₃), 3.86 (s, 3H, Ar-OCH₃), 5.06 (s, 2H, CH₂), 5.81 (s, 1H, HetH), 6.85 (t, $J = 8.0$ Hz, 1H, ArH), 6.98 (d, $J = 7.2$ Hz, 1H, ArH), 7.07 (dd, $J = 3.6$ Hz, $J = 5.6$ Hz, 1H, ArH), 7.16–7.24 (m, 2H, ArH), 7.38–7.42 (m, 2H, ArH), 7.54 (dd, $J = 3.6$ Hz, $J = 5.6$ Hz, 1H, ArH), 7.64 (s, 1H, =CH-OCH₃). EI MS: m/z (%) 410 ([M + 1]⁺, 3), 408 (M⁺, 2), 394 (100), 378 (10), 376 (31), 375 (83), 361 (17), 318 (13), 161 (16), 145 (26), 118 (21), 115 (14), 102 (29). Anal. Calcd for C₂₃H₂₄N₂O₅: C, 67.63; H, 5.92; N, 6.86. Found: C, 67.83; H, 6.01; N, 6.76.

Data for 6b: Yield, 46%; M.p. 152–153 °C; ¹H NMR (400 MHz, CDCl₃) δ: 2.32 (s, 3H, Ar-CH₃), 3.70 (s, 3H, NCH₃), 3.77 (s, 3H, COOCH₃), 3.84 (s, 3H, =CH-OCH₃), 3.87 (s, 3H, Ar-OCH₃), 5.06 (s, 2H, CH₂), 6.08 (s, 1H, HetH), 6.85 (d, $J = 8.4$ Hz, 1H, ArH), 7.09 (t, $J = 7.2$ Hz, 1H, ArH), 7.21 (d, $J = 7.8$ Hz, 1H, ArH), 7.36–7.40 (m, 2H, ArH), 7.53 (t, $J = 7.2$ Hz, 1H, ArH), 7.60 (s, 1H, =CH-OCH₃), 7.71 (s, 1H, ArH). EI MS: m/z (%) 423 ([M + 1]⁺, 8), 422 (M⁺, 9), 217 (13), 206 (18), 205 (91), 173 (14), 146 (13), 145 (100), 131 (19), 130 (20), 116 (24), 103 (12). Anal. Calcd for C₂₄H₂₆N₂O₅: C, 68.23; H, 6.20; N, 6.63. Found: C, 68.43; H, 5.99; N, 6.65.

Data for 6c: Yield, 82%; M.p. 83–85 °C; ¹H NMR (400 MHz, CDCl₃) δ: 3.65 (s, 3H, NCH₃), 3.67 (s, 3H, COOCH₃), 3.70 (s, 3H, =CH-OCH₃), 4.91 (s, 2H, Ph-OCH₂), 5.15 (s, 2H, Het-OCH₂), 6.13 (s, 1H, HetH), 6.99–7.04 (m, 2H, ArH), 7.18 (dd, $J = 4.0$ Hz, $J = 5.2$ Hz, 1H, ArH), 7.31–7.39 (m, 6H, ArH), 7.43–7.48 (m, ArH), 7.51 (s, 1H, =CH-OCH₃), 7.94 (d, $J = 7.6$ Hz, 1H, ArH). EI MS: m/z (%) 484 (M⁺, 9), 280 (13), 204 (100), 177 (21), 174 (14), 173 (23), 144 (98), 131 (32), 117 (16), 114 (54), 103 (37), 101 (35). Anal. Calcd for C₂₉H₂₈FN₂O₅: C, 71.88; H, 5.82; N, 5.78. Found: C, 71.72; H, 5.62; N, 5.62.

Data for 6d: Yield, 81%; M.p. 76–77 °C; ¹H NMR (400 MHz, CDCl₃) δ: 3.61 (s, 3H, NCH₃), 3.75 (s, 3H, COOCH₃), 3.87 (s, 3H, =CH-OCH₃), 5.39 (s, 2H, CH₂), 5.76 (s, 1H,

HetH), 7.11–7.19 (m, 2H, ArH), 7.28–7.33 (m, 3H, ArH), 7.34–7.40 (m, 3H, ArH), 7.61 (d, $J = 7.2$ Hz, 1H, ArH), 7.69 (s, 1H, =CH–OCH₃), 7.87 (d, $J = 7.8$ Hz, 1H, ArH), 7.99 (d, $J = 7.8$ Hz, 1H, ArH), 8.23 (t, $J = 7.2$ Hz, 1H, ArH). EI MS: m/z (%) 517 ([M+1]⁺, 6), 516 (M⁺, 8), 484 (20), 456 (24), 147 (14), 145 (43), 144 (23), 131 (17), 123 (100), 115 (22), 103 (23), 102 (14). Anal. Calcd for C₂₉H₂₅N₂O₆: C, 67.43; H, 4.88; N, 5.42. Found: C, 67.15; H, 4.69; N, 5.12.

X-ray Diffraction—A colorless block of **5b** (0.20 mm × 0.20 mm × 0.10 mm) was mounted on a quartz fiber. Cell dimensions and intensities were measured at 299 K on a Bruker SMART CCD area detector diffractometer with graphite mono-chromated Mo K α radiation ($\lambda = 0.71073$ Å); $\theta_{\max} = 22.13$; 6204 measured reflections; 3658 independent reflections ($R_{\text{int}} = 0.0229$) of which 2701 had $|F_o| > 2|F_c|$. Data were corrected for Lorentz and polarization effects and for absorption ($T_{\min} = 0.9819$; $T_{\max} = 0.9909$). The structure was solved by direct methods using SHELXS-97;³⁷ all other calculations were performed with Bruker SAINT System and Bruker SMART programs.³⁸ Full-matrix least-squares refinement based on F^2 using the weight of $1/[\sigma^2(F_o^2) + (0.0934P)^2 + 0.1767P]$ gave final values of $R = 0.0570$, $wR = 0.1576$, and GOF (F) = 1.055 for 289 variables and 1634 contributing reflections. Maximum shift/error = 0.000 (3), max/min residual electron density = 0.209/−0.169 e Å^{−3}. Hydrogen atoms were observed and refined with a fixed value of their isotropic displacement parameter.

3. Inhibitory Kinetics of Porcine *bc*₁ Complex

The preparation of succinate-cytochrome *c* reductase (SCR, mixture of respiratory complex II and *bc*₁ complex) from porcine heart was essentially as reported.³⁹ The activity of SCR in catalyzing the oxidation of succinate by cytochrome *c* was determined in 1.8 mL of reaction mixture containing 100 mM PBS (pH 7.4), 0.3 mM EDTA, 20 mM succinate, 60 μM oxidized cytochrome *c* and an appropriate amount of SCR at 23°C. The enzymatic assay for succinate-ubiquinone reductase (complex II)-catalyzed oxidation of succinate by 2,6-dichloroindophenol (DCIP) was carried out in essentially the same reaction mixture, except the replacement of the oxidized cytochrome *c* with 53 μM DCIP. The reaction was initiated by the addition of enzyme, and monitored continuously by following the absorbance change at certain wavelength on a lambda 45 Perkin-Elmer spectrophotometer equipped with a magnetic stirrer. The extinction coefficients used were 18.5 mM^{−1}cm^{−1} for $A_{\text{red-ox}}^{550}$ for cytochrome *c* reduction and 21 mM^{−1}cm^{−1} for $A_{\text{red-ox}}^{600}$ for DCIP reduction. For the inhibitory kinetic studies, the reaction was carried out in the presence of varying concentration of the inhibitor.

The Ubiquinol-cytochrome *c* reductase (*bc*₁ complex) activity in catalyzing the oxidation of decylubiquinol (DBH₂) by cytochrome *c* was assayed in 100 mM PBS (pH 6.5), 2 mM EDTA, 750 μM lauryl maltoside (*n*-dodecyl-β-*D*-maltoside), 20–115 μM DBH₂, 100 μM oxidized cytochrome *c*, and an appropriate amount of SCR at 23°C. The preparation of DBH₂ from decylubiquinone (DB) was carried out according to the procedure described in previous publications,^{40,41} and the concentration of DBH₂ was determined by measuring the absorbance difference between 288 nm and 320 nm using an extinction coefficient of 4.14 mM^{−1}cm^{−1} for the calculation.^{42,43} The nonionic detergent lauryl maltoside was used to decrease the interfering non-enzymatic activity,^{42–45} though it was expected to affect the K_m value of DBH₂ to *bc*₁ complex.⁴⁵ For each reaction, the non-enzymatic rate for cytochrome *c* reduction was followed for at least 100 seconds before enzyme was added to initiate the reaction.

The reaction mechanism could be considered as follows (Scheme 3): where *E*, *S*, and *I* represent enzyme, substrate, and inhibitor, respectively. According to the substrate reaction kinetic theory, the concentration of product formed at time *t* is calculated by Eq.(6).⁴⁶

$$[P] = v_s t + \frac{v_0 - v_s}{k_{obs}} (1 - e^{-k_{obs}t}) \quad (6)$$

where v_0 and v_s are the initial and steady-state velocities of the reaction in the presence of inhibitor, and k_{obs} is the observed first-order rate constant:

$$\begin{aligned} k_{obs} &= A[I]_0 + B, \\ A &= \frac{k_{+0}K_m + k_{+0}[S]}{K_m + [S]}, \\ B &= \frac{k_{-0}K_m^* + k_{-0}[S]}{K_m^* + [S]} \end{aligned}$$

We have K_m and K_m^* as the Michaelis-Menten constants:

$$\begin{aligned} K_m &= \frac{k_{-1} + k_2}{k_1} \\ K_m^* &= \frac{k_{-1}^*}{k_1^*} \end{aligned}$$

The slow-tight binding inhibitors can also be classified as competitive, noncompetitive, and uncompetitive on the basis of similar considerations, as in the case of the classical inhibitors. Experimentally, the type of inhibition can be ascertained by studying the effect of $[S]$ on the apparent rate constants A and B

$$\text{Competitive: } A = \frac{k_{+0}K_m}{K_m + [S]}, \quad B = k_{-0} \quad (7)$$

$$\text{Noncompetitive: } A = k_{+0}, \quad B = k_{-0} \quad (8)$$

$$\text{Uncompetitive: } A = \frac{k_{+0}'[S]}{K_m + [S]}, \quad B = \frac{k_{-0}'[S]}{K_m^* + [S]} \quad (9)$$

Results and Discussion

Homology Modeling and Computational Prediction

Because the K_i values of all compounds were tested based on the porcine bc_1 complex in this work, we firstly used the crystal structure of bovine heart cytochrome bc_1 in complex with AZ (PDB entry of 1SQB at resolution of 2.69 Å) as a template to establish the three-dimensional (3D) structural model of porcine bc_1 complex. The sequence alignment between the porcine cyt b and the same subunit of the template indicate that only 38 out of 379 residues were found to be different from each other, showing very high homology of the porcine cyt b with that of the template. The 3D structural models for porcine bc_1 in complex with inhibitors were simulated by integrating homology modeling, molecular docking, and energy minimizations as described in the experimental section. As shown in Figure 1A, the porcine bc_1 complex is a dimer and has the C_2 symmetry. Each monomer contains one cyt b subunit in which the Q_o site is situated. This Q_o site is located near the inter-membrane space, and partially interacts with the b_L heme group. The E - β -methoxy methyl acrylate group and the phenyl group of AZ

are orientated at the deep bottom of the binding pocket. The *E*- β -methoxy methyl acrylate group interacts with residues Phe128, Tyr131, Val132, Gly142, and Ala143 from helix C and helix cd1, and residues Glu271 and Tyr 273 from the *ef* loop. This head group of the inhibitor blocks the way to the *b_L* heme group with the shortest distances around 6 Å, while the distances of carbonyl oxygen atom of this head group of the inhibitor to the heme iron is about 10.5 Å. The carbonyl oxygen atom of this head group also forms a hydrogen bond with the backbone –NH group of residue Glu271 from the *ef* loop, further stabilizing the orientation of the head group of the inhibitor inside the Q_o binding pocket. The phenyl group of the inhibitor packs residue Gly142, Val145, Ile146, Pro270, Ile268, and Tyr278 through extensive hydrophobic interactions. Compared with the conformation of AZ in the crystal structure (blue colored in Figure 1B), the rmsd for its docked conformation (red colored in Figure 1B) is only 0.77 Å. In addition, the calculated binding free energies for KM, AZ, and PY are in good agreement with the corresponding experimental data (Table 1, see below), indicating that our computational protocol is reliable.

Then, the above computational protocol was used to predict the potency of the designed compounds. The calculated binding free energies ($\Delta G_{\text{cal.}}$), summarized in Table 1, indicate that compounds **5a**~**d** should have a significantly higher inhibitory potency than AZ and KM. In addition, compounds **5a**, **5b**, and **5d** should have a similar inhibitory potency compared to PY, whereas compound **5c** should have an inhibitory potency higher than PY. For an ideal computational protocol, it can be used to predict not only compounds with a higher activity, but also compounds with a lower activity. Therefore, in order to validate the predictability of the computational protocol, some hydroxy-protected compounds **6a**~**d** were also predicted to have a lower potency due to the lack of the intramolecular hydrogen bond.

Table 1 also summarizes the K_i values against the porcine *bc₁* complex for the inhibition by the synthesized compounds and also by the commercial compounds that were used as the control. Interestingly, the calculated binding free energies correlated reasonably well with the binding free energies derived from the corresponding experimental K_i values with a correlation coefficient of $r^2 = 0.89$ (Figure 2), further confirming the reliability of our computational models. As shown in Table 1, compound **5c** distinguishes itself as the most promising candidate with the highest potency, with about 522-fold, 280-fold, and 5.8-fold improved binding affinity compared to AZ, KM, and PY, respectively.

Structural Basis of the Higher Binding Affinity of Compounds **5a**~**d**

As shown in Figure 3A and 3B, similar to that of AZ (Figure 3B), the methoxy methyl acrylate group of compounds **5a**, **5b**, **5c**, and **5d** form a hydrogen bond with the backbone amide group of Glu271 (Figure 3A and 3B). However, compared to that of AZ (Figure 3C), the π - π interactions between the Phe274 and the pyrazole ring of compound **5** are improved greatly because the pyrazole ring and the 2-hydroxyphenyl are totally coplanar due to the intramolecular hydrogen bond between the hydroxyl group and the N(1) atom of pyrazole moiety (Figure 3D). In other words, compared with the conformation in the AZ-complexed *bc₁*, the phenyl side-chain of Phe274 undergoes a rotation of $\sim 4^\circ$ for in order to enhance the π - π interactions with compound **5c**. Figure 3E shows the results of the superimposition of compounds **5a**, **5b**, **5c**, and **5d** based on the binding pocket. As a result, with about 522-fold, 280-fold, and 5.8-fold improvement of the binding affinity compared respectively with AZ, KM, and PY, compound **5c** is clearly the most potent inhibitor. On the contrary, the hydroxy-protected derivatives, such as **6a**, **6b**, **6c**, and **6d**, all have a lower binding affinity due to the lack of such an intramolecular hydrogen bond.

Synthesis of the Designed Compounds

The synthetic route for the designed compounds **5** and **6** is shown in Scheme 4. 4-hydroxy-2H-chromen-2-one (also named 4-hydroxycoumarin) **2** was prepared easily from 2'-hydroxy acetophenone **1a-c** as starting material in good overall yield (85% for **2a**, 72% for **2b**, and 75% for **2c**). Then, compound **2** reacted with methylhydrazine to afford the key intermediate **3a-c**, 3-(2-hydroxyphenyl)-1-methyl-1H-pyrazol-5(4H)-ones. It should be noted that compound **3** underwent tautomerization under basic condition to produce the enol isomer. Fortunately, maybe due to the existence of intramolecular hydrogen bond, the resulted enol-hydroxy was apparently more active than the phenol-hydroxy group and reacted with (*E*)-methyl 2-(2-bromomethyl-phenyl)-3-methoxyacrylate **4** under the basic condition to produce the target compound **5** in yields of 67%~84%. In addition, in order to examine the importance of the intramolecular hydrogen bond, we also synthesized some hydroxy-protected compounds **6a-d** by the reactions of compounds **5a** and **5b** with various alkylation or acetylation reagents. The structures of compounds **2-6** were characterized by ¹H NMR, EI-MS spectrum, and elemental analyses. In addition, the crystal structure (CCDC 709734) of **5b** was determined by X-ray diffraction analyses. As shown in Figure 4, the β-methoxyacrylate group adopts *E*-configuration. Most interestingly, an intramolecular hydrogen bond between the hydroxyl group and the N(1) atom was formed as expected. As a result, the pyrazole ring and the 2-hydroxyphenyl planes are coplanar to form a bigger aromatic system.

Enzymatic Kinetics

In order to understand the action mechanism, we further studied the inhibitory kinetics of succinate-cytochrome *c* reductase (SCR, mixture of respiratory complex II and *bc*₁ complex) with compounds **5a-d**. Complex II first passes electrons from succinate to ubiquinone, and then cytochrome *bc*₁ complex passes electrons from reduced ubiquinone to cytochrome *c*. We measured the succinate-ubiquinone reductase (complex II) activity of SCR using succinate and DCIP as substrates and the succinate-cytochrome *c* reductase (both complex II and *bc*₁ complex) activity of SCR using succinate and cytochrome *c* as substrates. All of the compounds exhibited no effect on the activity of complex II (data not shown), whereas they inhibited the activity of succinate-cytochrome *c* reductase considerably (Figure 5). Thus, the inhibition of these compounds on the activity of SCR may be attributed to their effects on the function of *bc*₁ complex, and the assay for the SCR-catalyzed oxidation of succinate by cytochrome *c* was used to elucidate the inhibitory kinetics of the *bc*₁ complex. Like many slow-binding inhibitors, the progress curves in the presence of compounds **5a-d** did not display a simple linear product-versus-time relationship as seen for classical reversible inhibitors like AZ and KM. Rather, the product formation *versus* time shows a curvilinear function. For example, Figure 5A shows the time courses of the *bc*₁ complex-catalyzed reaction in the presence of different concentrations of compound **5c**. The progress curves start off linear (the initial-rate phase) but fall off with increasing time. When time (*t*) approaches the “infinity”, the concentration of product (*[P]*) approaches an asymptote with a positive slope, which decreases with increasing concentration of compound **5c**. The initial-rate phase reflects the slow onset of inhibition by these compounds since it can be simply eliminated by preincubating the inhibitor with the enzyme (data not shown). Under the conditions employed in the present study, the time-dependent behavior of enzyme-catalyzed reaction can be well described according to the substrate reaction kinetic theory.⁴⁶ The best-fit curves in Figure 5A were obtained by nonlinear regression analysis from which the kinetic parameters, the initial (*v*₀) and steady-state (*v*_s) velocities of the reaction in the presence of inhibitor and the observed first-order rate constant (*k*_{obs}) can be determined. The inset of Figure 5A shows the plot of *k*_{obs} against the concentration of compound **5c** (*[5c]*). Noticeably, *k*_{obs} is proportional to the inhibitor concentration. From the slope and intercept of this plot, the apparent rate constants *A* = 0.00631 ± 0.00036 s⁻¹nM⁻¹ and *B* = 0.00358 ± 0.00110 s⁻¹ were then determined.

Subsequently, we monitored the time courses of the bc_1 complex-catalyzed reaction in the presence of different concentrations of cytochrome c and at a fixed concentration of compound **5c** (Figure 5B). Using procedures described above, the values of k_{obs} can be obtained by fitting the progress curves according to the substrate reaction kinetic theory and were plotted against the concentration of cytochrome c in the inset of Figure 5B. The data clearly revealed that k_{obs} was independent of the concentration of substrate cytochrome c under the experimental conditions used, indicating that compound **5c** is a non-competitive inhibitor with respect to cytochrome c . Thus, the values of A and B determined from Figure 5A are equal to the true association and dissociation rate constants k_{+0} and k_{-0} , respectively, and the inhibition constant for compound **5c** can be calculated as $K_i = k_{-0} / k_{+0} = 0.57 \pm 0.21$ nM.

To study the effect of substrate ubiquinol on the inhibition kinetics of compound **5c**, we directly measured the ubiquinol-cytochrome c reductase (bc_1 complex) activity of SCR using decylubiquinol (DBH_2) and cytochrome c as substrates in the absence and presence of compound **5c**. This reaction exhibited the Michaelis-Menten kinetics towards substrate DBH_2 , and the kinetic parameters were determined ($K_m = 58 \pm 4$ μM and $k_{\text{cat}} = 238 \pm 8$ s^{-1}). In the presence of compound **5c**, with succinate used as substrate, the progress curves exhibited a similar curvilinear pattern due to the slow onset of inhibition (Figure 6). However, when the experiments were performed at a fixed concentration of compound **5c**, the k_{obs} value decreased with increasing concentration of DBH_2 (Supporting Information, Figure 2s), which is different from that observed in Figure 5B. To further elucidate the inhibitory mechanism, we carried out six sets of inhibitory experiments in the presence of varying concentrations of DBH_2 and compound **5c** (Figure 6, panels A and B). Similarly, from these progress curves of product formation at fixed concentrations of DBH_2 , the values of v_0 , v_s , and k_{obs} can be determined as described previously. Plot of k_{obs} against the inhibitor concentration gives a straight line (insets of Figure 6A–B), from which the values of A (slope) and B (intercept) at indicated DBH_2 concentrations were obtained. Panels C and D of Figure 6 show the dependence of the A and B values on the DBH_2 concentration. Clearly, A decreased with increasing the concentration of DBH_2 , while B was not affected, suggesting that compound **5c** is a competitive inhibitor with respect to substrate ubiquinol. Thus, with the K_m value of 58 μM , the microscopic inhibition rate constants $k_{+0} = 0.00185 \pm 0.00003$ $\text{s}^{-1}\text{nM}^{-1}$ and $k_{-0} = 0.00480 \pm 0.00018$ s^{-1} can be obtained by fitting Eq.(2) to the experimental data, and the inhibition constant $K_i = k_{-0}/k_{+0} = 2.6 \pm 0.1$ nM can be derived. The K_i value determined is about 5-fold higher than that measured from the succinate-cytochrome c reductase activity inhibition, which is mainly due to the presence of lauryl maltoside. This nonionic detergent was reported to affect the K_m value of DBH_2 to bc_1 complex,⁴⁵ and it possibly has an impact on the microscopic inhibition rate constants and the K_i value. Indeed, we found that the k_{obs} value decreased with increasing the concentration of lauryl maltoside at fixed DBH_2 and compound **5c** concentrations (Supporting Information, Figure 3s).

Conclusion

In summary, we have successfully designed and synthesized a series of pyrazole derivatives as inhibitors of cytochrome bc_1 complex. By introducing an intramolecular hydrogen bond to form a big aromatic system, the π - π interactions with the phenyl of Phe274, an important residue with conformational flexibility located in the binding pocket, were significantly improved. As a result, a new bc_1 inhibitor with subnanomolar K_i potency was discovered. Compound **5c** with a K_i value of 570 pM was found to display about 522-fold, 280-fold, and 5.8-fold improved binding affinity compared to the commercial inhibitors AZ, KM, and PY, respectively. The further inhibitory kinetics studies showed that compound **5c** is a non-competitive inhibitor with respect to substrate cytochrome c , but a competitive inhibitor with respect to substrate ubiquinol. Due to its subnanomolar K_i potency and slow dissociation rate ($k_{-0} = 0.00358$ s^{-1}), compound **5c** could be used as a specific probe for further elucidating the mechanism of

bc_1 function and as a new lead compound for future drug discovery. In addition, the present work provides a successful case study concerning how to improve binding affinity of a ligand with a receptor by optimizing the interactions of the ligand with conformationally flexible residues of the receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
(A) The modeled 3D-structure of porcine bc_1 complex with bound inhibitor. (B)
Superimposition of the conformations of AZ in the crystal (blue) and modeled (red) structures.

Figure 2.
Correlation between the calculated and experimental binding free energies of compounds **5**, **6**, KM, AZ, and PY.

Figure 3.

Comparison of the binding modes of compound **5c** and AZ. (A), the simulated binding model of *bc*₁ in complex with **5c**; (B), the simulated binding model of *bc*₁ in complex with AZ; (C), the π - π stacking interaction between the side chain of F274 and the pyrimidyl ring of AZ (the interaction energy between F274 and AZ is -9.48 kcal/mol); (D), the π - π stacking interaction between the side chain of F274 and the enlarged aromatic system of **5c** (the interaction energy between F274 and **5c** is -10.28 kcal/mol); (E) Superimposition of the binding-conformation of compounds **5a** (green), **5b** (white), **5c** (magenta) and **5d** (blue).

Figure 4.
Crystal structure of compound **5b** (the dotted line shows the intramolecular H-bond)

Figure 5.

(A) Inhibitory kinetics of bc_1 complex by compound **5c**. Each reaction mixture contains 100 mM PBS (pH 7.4), 0.3 mM EDTA, 20 mM succinate, 60 μM cytochrome *c*, 0.1 nM SCR, and certain amount of compound **5c** (1, 0 nM; 2, 1 nM; 3, 1.5 nM; 4, 2 nM; and 5, 5 nM).

Experimental data were shown as colored dots and theoretical values as black solid lines. Inset: plot of k_{obs} against concentration of compound **5c**. (B) Effect of cytochrome *c* concentration on the inhibition of bc_1 complex by compound **5c**. The assays were carried out in the presence of 5 nM compound **5c** and various concentrations of cytochrome *c* (1, 4 μM ; 2, 12 μM ; and 3, 48 μM). Each reaction was initiated by adding 0.1 nM SCR. Inset: plot of k_{obs} against concentration of cytochrome *c*.

Figure 6.

(A–B) Inhibitory kinetics of porcine bc_1 complex by compound **5c**. Each reaction mixture contains 100 mM PBS (pH 6.5), 2 mM EDTA, 750 μM lauryl maltoside, 100 μM oxidized cytochrome c , 0.05 nM SCR, certain amount of DBH_2 (A, 20 μM ; B, 115 μM), and compound **5c** (1, 7.5 nM; 2, 15 nM; 3, 25 nM). Experimental data were shown as colored dots and theoretical values as black solid lines. Insets: plots of k_{obs} against concentration of compound **5c**. (C) Plot of the apparent rate constant A against concentration of DBH_2 . Inset: plot of $1/A$ against concentration of DBH_2 . (D) Plot of the apparent rate constant B against concentration of DBH_2 .

Scheme 1. Chemical structures of some representative *bc₁* inhibitors

Scheme 2.

Proposed structures of new *bc*₁ inhibitors with an intramolecular hydrogen bond.

Scheme 3.

Scheme 4.

Table 1

K_i values of compounds **5**, **6**, KM, AZ, and PY against porcine bc₁ complex

No.	R	R'	X	K_i (nM)	ΔG (kcal/mol)	Redise Interaction Energies (kcal/mol)			
						$\Delta G_{cal.}^a$	$\Delta G_{exp.}^b$	F274	P270
5a	H	H	CH	1.20	-11.11	-12.19	-10.55	-10.55	-4.97
5b	CH ₃	H	CH	3.10	-11.40	-11.62	-10.24	-10.24	-4.91
5c	Cl	H	CH	0.57	-12.50	-12.63	-10.28	-10.28	-4.86
5d	H	H	N	3.80	-10.17	-11.50	-10.49	-10.49	-4.88
6a	H	CH ₃	CH	29.10	-9.08	-10.29	-10.30	-10.30	-4.95
6b	CH ₃	CH ₃	CH	19.90	-9.59	-10.52	-11.46	-11.46	-5.06
6c	H	C ₆ H ₅ CH ₂	CH	12.70	-9.95	-10.79	-8.17	-8.17	-4.70
6d	H	<i>p</i> -FC ₆ H ₄ CO	CH	97.70	-9.09	-9.56	-8.57	-8.57	-4.95
AZ	/	/	/	297.60	-8.57	-8.92	-9.48	-9.48	-4.53
KM	/	/	/	159.60	-8.90	-9.28	-9.13	-9.13	-4.54

No.	R	R'	X	K_i (nM)	ΔG (kcal/mol)	Redissue Interaction Energies (kcal/mol)			
						$\Delta G_{\text{cal.}}^a$	$\Delta G_{\text{exp.}}^b$	F274	P270
PY	/	/	/	3.30	-10.95	-11.59	-7.92	-6.23	

^a $\Delta G_{\text{cal.}}$ was calculated according to our modified MM-PBSA method.

^b $\Delta G_{\text{exp.}} = -RT \ln K_i$