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Synthesis, molecular docking and biological evaluation of metronidazole derivatives as potent *Helicobacter pylori* urease inhibitors

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ABSTRACT

Fourteen metronidazole derivatives (compounds **3a–f** and **4b–h**) have been synthesized by coupling of metronidazole and salicylic acid derivatives. All of them are reported for the first time. Their chemical structures are characterized by ^{1}H NMR, MS, and elemental analysis. The inhibitory activities against *Helicobacter pylori* urease have been investigated in vitro and many compounds have showed promising potential inhibitory activities of *H. pylori* urease. The effect of compounds **4b** (IC_{50} = 26 μM) and **4g** (IC_{50} = 12 μM) was comparable with that of acetohydroxamic acid, a well known *H. pylori* urease inhibitor used as a positive control. The experimental values of IC_{50} showed that inhibitor was potent urease inhibitor. A docking analysis using the AUTODOCK 4.0 program could explain the inhibitory activities of compound **4g** against *H. pylori* urease.

1. Introduction

Urease (urea amidohydrolase; E.C. 3.5.1.5) is an enzyme that catalyzes hydrolysis of urea to ammonia and carbamate, which is the final step of nitrogen metabolism in living organisms.[1] It is widely distributed in a variety of bacteria such as *Helicobacter pylori* and *Proteus mirabilis*. *H. pylori* is a Gram-negative microaerophilic bacterium that infects up to 50% of the world's human population.[2] Urease that comes from *H. pylori* is now accepted as a major cause of peptic ulcers. Many urease inhibitors have been investigated in the past decades, like phosphorodiamidates, hydroxamic acid derivatives, and imidazoles,[3] but part of them are prevented from using in vivo because of their toxicity or instability and part of them had side effects. Thus, seeking novel urease inhibitors with good bioavailability and low toxicity is significative.

Nitroimidazole derivative compounds exhibited broad variety of biological activities including antimicrobial,[4] antitubercular,[5] and urease inhibitors.[6] More attention has been focused on exploring novel biological properties of nitroimidazole derivative compounds. Metronidazole (1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole) (Scheme 1), one of the important nitroimidazole derivatives, has been widely used as an antimicrobial medicine. During recent years, the structure modification at the pendant hydroxy group of metronidazole has received much attention.[7]

Our previous research about the synthesis of new *H. pylori* urease inhibitors from nature products has been reported.[8,9] Many imidazoles have been designed as inhibitors against urease. Metronidazole in combination of other drugs has been used for the treatment of infections caused by *H. pylori*,[10] but it has not been reported as efficient inhibitor against *H. pylori* urease. Our interest in this area is to design *H. pylori* urease inhibitory active metronidazole derivatives at the pendant hydroxy group. Salicylic acid derivatives have wild biological activities. Some salicylic acid derivatives, such as anacardic acids and salicylhydroxamic acids (Scheme 2), were reported as urease inhibitors.[11,12] Herein several substituted salicylic acids were selected to join together with metronidazole and 14 new nitroimidazoles of two series were synthesized to test their *H. pylori* urease inhibitory activities. Fourteen nitroimidazoles were evaluated for their inhibitory activities against *H. pylori* urease. One nitroimidazole with the lowest IC_{50} value was chosen to dock with *H. pylori* urease using AUTODOCK 4.0.[13]

2. Results and discussion

2.1. Synthesis

Two series of compounds were designed and synthesized from metronidazole and salicylic acid derivatives for *H. pylori* urease inhibitors by the routes outlined in Scheme 3. The MET-OTs (2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl 4-methylbenzenesulfonate, compound **2**) was synthesized by the method in our previous paper.[14] In different reaction conditions, two series of products, including **3a–f** and **4a–h**, were first synthesized from MET-OTs and substituted salicylic acids. The research results showed that the ratio of MET-OTs and substituted salicylic acids and reaction temperature were the key factors of the synthesis of compounds

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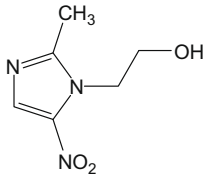
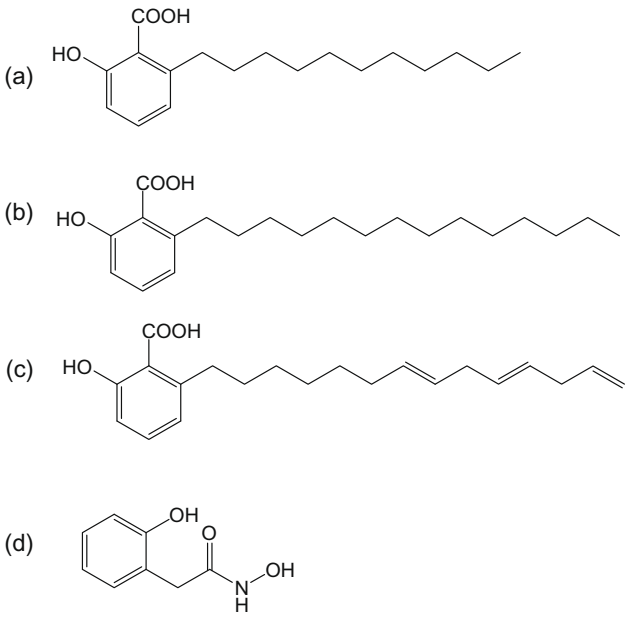


Scheme 1. Chemical structure of metronidazole.

3a–f or **4a–h.** When the ratio of the amount of substance of METOTs and substituted salicylic acid was 1:1.1, reaction temperature was 70–80 °C and the reaction time was 14–16 h, compounds **3a–f** were obtained. When the ratio of the amount of substance of METOTs and substituted salicylic acids is 2:1.1, reaction temperature was 100–110 °C and the reaction time was 20–22 h, compounds **4a–h** were obtained. All compounds were fully characterized by spectroscopic method and elemental analysis. All the nitroimidazoles **3a–f** and **4a–h** were synthesized for the first time.

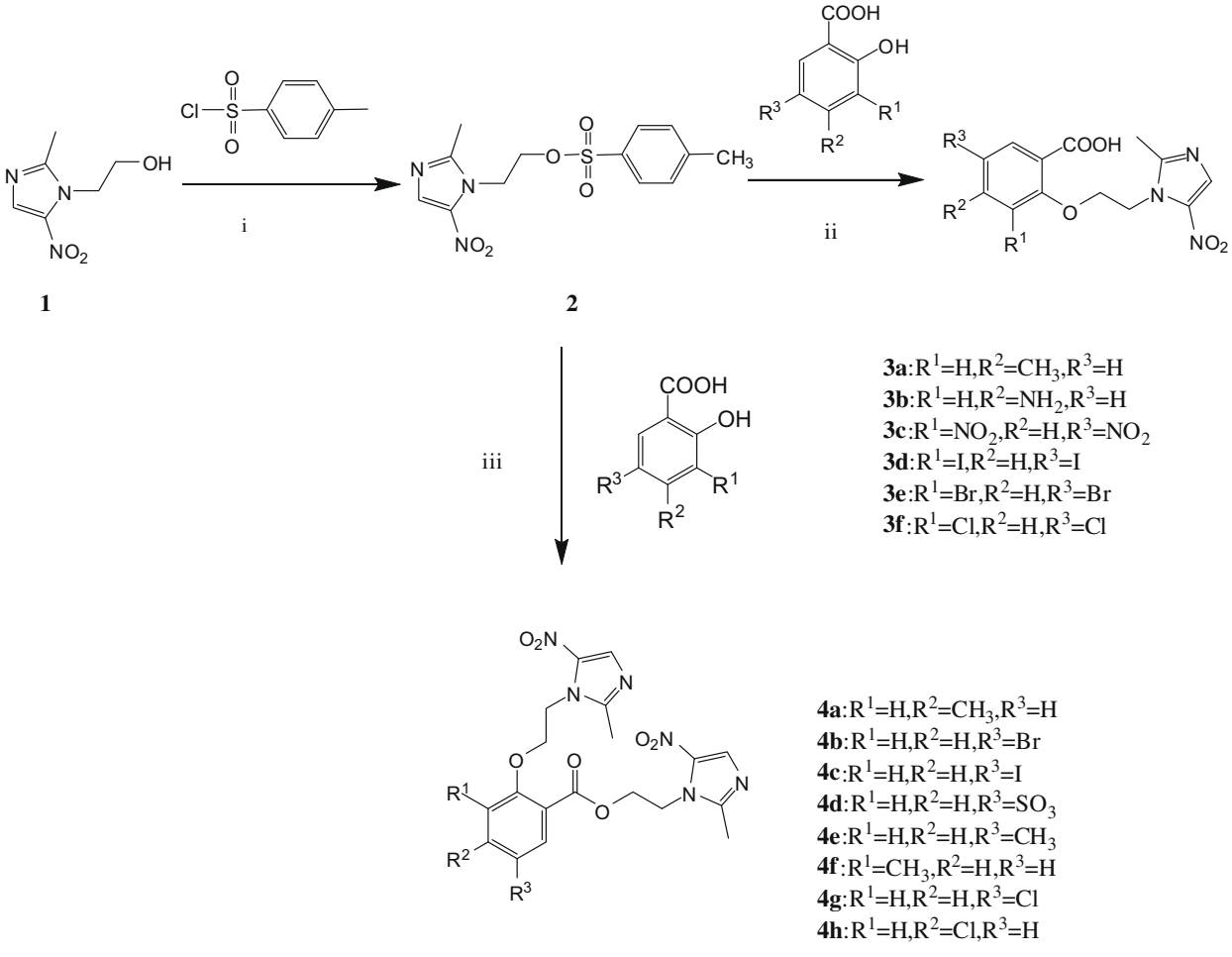
2.2. Biological activity

Fourteen nitroimidazoles **3a–f** and **4a–h** were evaluated for their inhibitory activities against *H. pylori* urease. Percent inhibition at a 1 mM concentration of compounds **3a–f** and **4a–h** was initially determined, and the results were reported in Table 1. Most of



Scheme 2. Chemical structures of some anti-urease salicylic acid derivatives: anacardic acids (a–c), salicylhydroxamic acid (d).

the nitroimidazoles showed good inhibitory activities, especially nitroimidazole **4b** (IC_{50} = 26 μM) and **4g** (IC_{50} = 12 μM) exhibited



Scheme 3. General method of the preparation of compounds **2**, **3a–f**, and **4a–h**. Reagents and conditions: (i) CH_2Cl_2, TEA, 0 °C, 5 h; (ii) DMF, K_2CO_3, 70–80 °C, 14–16 h; (iii) DMF, K_2CO_3, 100–110 °C, 22–24 h.

Table 1
Percent inhibition of compounds **1** (MET), **2** (MET-OTs), **3a–f** and **4a–h** against *H. pylori* urease at the concentration of 1 mM

Compounds	Percent inhibition (%)
3a	—[b]
3b	38.3
3c	41.5
3d	53.9
3e	56.1
3f	56.8
Metronidazole	—
MET-OTs	78.1
AHA[a]	91.2
4a	38.3
4b	93.9
4c	78.7
4d	61.2
4e	48.4
4f	42.1
4g	95.3
4h	82.2

[a] Used as a positive control.
[b] These compounds were found as inactive.

potent in vitro inhibitory activities, which were comparable to acetohydroxamic acid (AHA), a reversible urease inhibitor (Table 2).[3] In general, compounds **4a–h** with two imidazole groups exhibited better inhibitory activities than compounds **3a–f**. The comparison of the inhibitory activities of the synthetic nitroimidazoles and metronidazole indicated that the introduction of the substituted salicylic acids onto the 2-hydroxyethyl was responsible for the activities of the compounds.

In nitroimidazoles **3d–f**, compound **3e** exhibited better inhibitory activity than **3d** and the activity of compound **3f** was the highest. At the same time, in nitroimidazoles **4b**, **4c**, and **4g**, the order of inhibitory activities showed the potency of **4g** > **4b** > **4c**. It was possibly due to the difference between the electro affinities of chlorine, bromine, and iodine. This result indicated that the inhibitory activities increased with the increase of electron withdrawing ability of R^3. In addition, compound **4f** ($R^1 = CH_3$) and compound **4a** ($R^2 = CH_3$) showed weaker inhibitory activity than compound **4e** ($R^3 = CH_3$), which indicated that the position of the substitute group influenced the inhibitory activity. Compounds with halogen atoms in the benzene ring showed better inhibitory activity than those with methyl substitutes.

Generally, nitroimidazoles **4b** and **4g** based on metronidazole and salicylic acids exhibited good inhibitory activities against *H. pylori* urease (Table 2), which attracted our interest in further structure modification of them as lead compounds for new urease inhibitors or anti-*H. pylori* agents.

2.3. Molecular docking study

Bacterial ureases, including *H. pylori* urease, are large heteropolymeric metalloproteins with nickel (II) ions present in their active sites.[15,16] A significant amino acid sequence similarity has been observed between all ureases of a bacterial origin.[17] A superposition of the structures of uninhibited and inhibited *H. pylori* urease reveals a flap motion of the segment composed of residues α313–α346 forming a helix-turn-helix motif which opens the active site when the inhibitor is bound and closes it when not boun.[18] In the X-ray structures available for the *H. pylori* urease, the two nickels were coordinated by His136, His138, Kcx219, His248, His274, Asp362, and water molecules.[19–21] The residues of active site are showed together with nickel ions (NI3001 and NI3002) by AUTODOCK 4.0 in Figure 1.

Among the several known urease inhibitors, phosphorodiamidates were the most efficient. Phenylphosphorodiamidate (Scheme 4) inhibited the enzyme even when the molar concentration of the compound was comparable to that of the urease.[3] On the basis of the crystal structure of *H. pylori* urease (entry 1E9Z in the Protein Data Bank), the binding mode of phenylphosphorodiamidate in the enzyme active site was modeled (Fig. 2). In the binding model, amino hydrogen of His221 and amino formed hydrogen bond with the oxygen atoms of phenylphosphorodiamidate (length of the hydrogen bond: His221N–H···$O_{phenylphosphorodiamidate}$ = 2.167 Å; angle of the hydrogen bond: His221N–H···$O_{phenylphosphorodiamidate}$ = 171.6°). The benzene ring of phenylphosphorodiamidate may form hydrophobic interactions with Ala168, Ala169, and Ala365.

The binding model of compound **4g** and *H. pylori* urease was depicted in Figure 3 and the enzyme surface model was showed in Figure 4. All the amino acid residues which had interactions with *H. pylori* urease were showed. In the binding model, the nitro oxygen of compound **4g** formed hydrogen bond with the amino hydrogen of His221 similar to phenylphosphorodiamidate as shown in Figure 2 (length of the hydrogen bond: His221N–H···O_{4g} = 1.752 Å; angle of the hydrogen bond: His221N–H···O_{4g} = 160.5°). Moreover, hydrophobic interactions probably existed between compound **4g** and Ala168, Ala169, and Ala365. The novel interaction, in comparison to phenylphosphorodiamidate—urease complex, occurred in the model, the hydrogen bond formed by the oxygen atom and the NH of the inhibitor amide group with Met366 (length of the hydrogen bond: Met366N–H···O_{4g} = 1.975 Å; angle of the hydrogen bond: Met366N–H

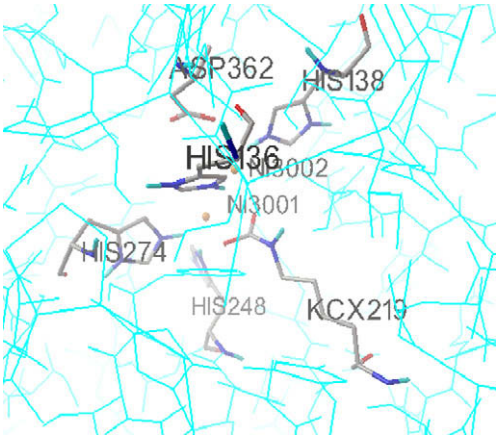
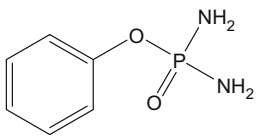


Figure 1. The active site of urease showed by AUTODOCK 4.0.

Table 2
Inhibition of *Helicobacter pylori* urease by compounds **4b** and **4g**

Compound	IC_{50} (μM)
4b	26 ± 3
4g	12 ± 1
Acetohydroxamic acid	17 ± 2

IC_{50} = 50% inhibitory concentration represents the mean ± S.D. from dose–response curves of at least three experiments.



Scheme 4. Chemical structure of phenylphosphorodiamidate.

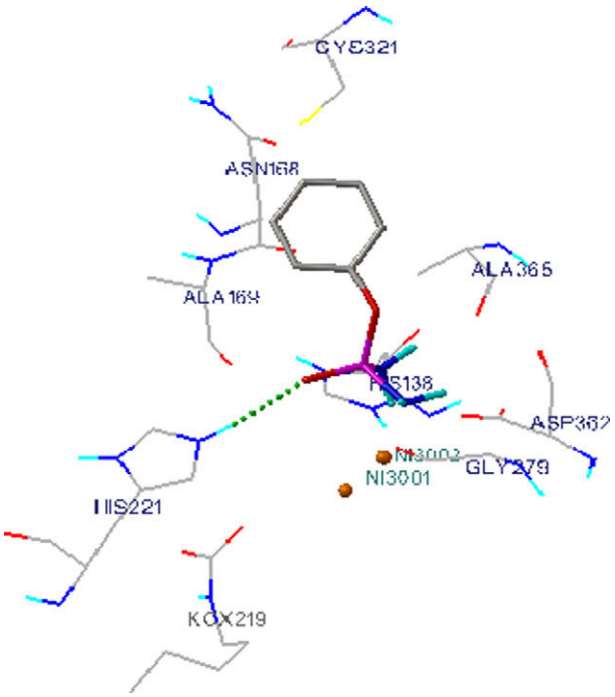


Figure 2. Computed structure of phenylphosphorodiamidate–*H. pylori* urease complex (colored by atom: carbons–gray; nitrogens–blue; oxygens–red; chlorine–green; phosphorus–purple). Hydrogen bonds are indicated as green dotted lines.

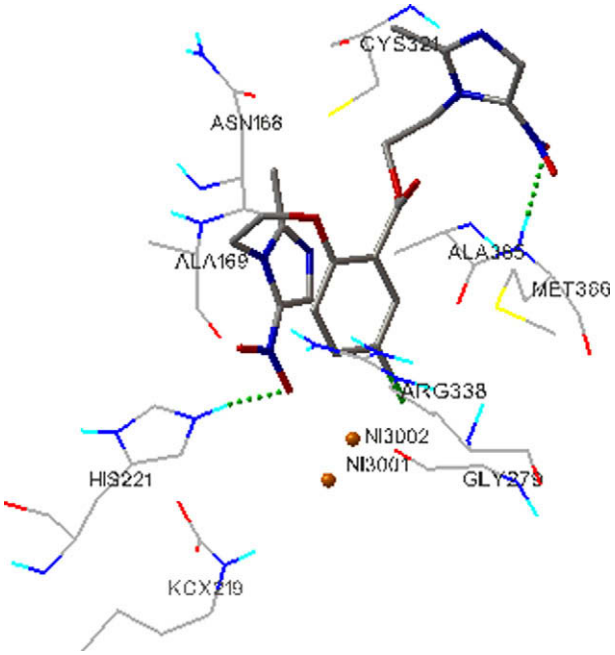


Figure 3. Compound **4g** (colored by atom: carbons–gray; nitrogens–blue; oxygens–red; chlorine–green) is bound into *H. pylori* urease (entry 1E9Z in the Protein Data Bank). The dotted lines show the hydrogen bond.

$\cdots O_{4g}$ = 140.8°). The result of molecular docking study could explain the inhibitory activities of compound **4g** against *H. pylori* urease.

3. Conclusions

Two series of new nitroimidazoles reacting by metronidazole and salicylic acid derivatives were synthesized and assayed for their inhibitory activities against *H. pylori* urease. Compound **4g**, 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethyl 5-chloro-2-(2-(5-nitro-1*H*-imidazol-1-yl) ethoxy) benzoate, showed the most potent inhibitory activity with IC_{50} of 12 μM against the urease. The introduction of hydrophobic and electron-withdrawing halogeno groups was conducive to the inhibitory activity. Docking simulation was performed to position compound **4g** into the *H. pylori* urease active site to determine the probable binding conformation and the result indicated that compound **4g** is a potent inhibitor of *H. pylori* urease.

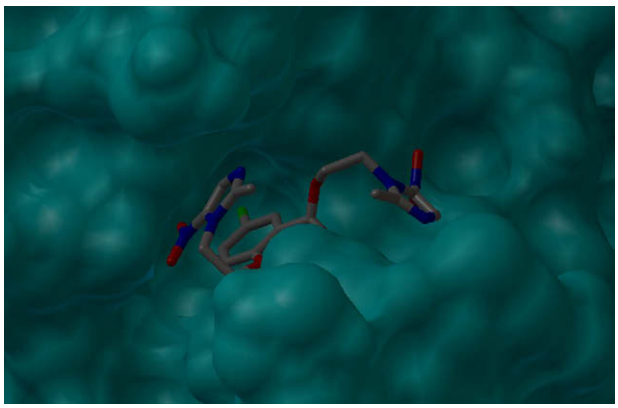


Figure 4. The enzyme surface model of structure of compound **4g**–*H. pylori* urease complex.

4. Experiment part

4.1. Materials

All the NMR spectra were recorded on a Bruker DPX 300 model spectrometer in DMSO-d_6. Chemical shifts (δ) for ^{1}H NMR spectra were reported in parts per million to residual solvent protons. Melting points were measured on a Boetius micro melting point apparatus. The ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. All chemicals and reagents used in current study were of analytical grade. All the substituted salicylic acids were purchased from Aladdin Company, Shanghai China, and metronidazole was purchased from Changzhou Dongsheng Company, Changzhou China. Protease inhibitors (Complete mini EDTA-free) were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and brucella broth was from Becton–Dickinson (Cockeysville, MD). Horse serum was from Hyclone (Utah, America).

4.2. Bacteria

H. pylori (ATCC 43504; American Type Culture Collection, Manassas, VA) was grown in brucella broth supplemented with 10% heat-inactivated horse serum for 24 h at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2), as previously described.[22]

4.3. Preparation of *H. pylori* urease

For urease inhibition assays, 50 mL broth cultures (2.0×10^8 CFU/mL) were centrifuged (5000*g*, 4 °C) to collect the bacteria, and after washing twice with phosphate-buffered saline (pH 7.4), the *H. pylori* precipitation was stored at −80 °C. *H. pylori* was returned to room temperature, and after addition of 3 mL of distilled water and protease inhibitors, sonication was performed for 60 s. Following centrifugation (15,000*g*, 4 °C), the supernatant

was desalted through SephadexG-25 column (PD-10 columns, Amersham–Pharmacia Biotech, Uppsala, Sweden). The resultant crude urease solution was added to an equal volume of glycerol and stored at 4 °C until use in the experiment.

4.4. Measurement of urease activity

The assay mixture, containing 25 μL (4 U) of *H. pylori* urease and 25 μL of the test compound, was preincubated for 3 h at room temperature in a 96-well assay plate. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn.[23]

4.5. Protocol of docking study

Molecular docking of compound **4g** into the three-dimensional X-ray structure of *H. pylori* urease (PDB code: 1E9Z) was carried out using the AUTODOCK software package (version 4.0) as implemented through the graphical user interface AUTODOCKTOOLS (ADT 1.4.6).[24]

The graphical user interface AUTODOCKTOOLS was employed to set-up the enzymes: all hydrogens were added, Gasteiger charges were calculated and nonpolar hydrogens were merged to carbon atoms. The Ni initial parameters are set as $r = 1.170$ Å, $q = +2.0$, and van der Waals well depth of 0.100 kcal/mol.[25] For macromolecules, generated pdbqt files were saved.

The 3D structures of ligand molecules were built, optimized (PM3) level, and saved in Mol2 format with the aid of the molecular modeling program SPARTAN (Wavefunction Inc.). These partial charges of Mol2 files were further modified by using the ADT package (version 1.4.6) so that the charges of the nonpolar hydrogens atoms assigned to the atom to which the hydrogen is attached. The resulting files were saved as pdbqt files.

AUTODOCK 4.0 was employed for all docking calculations. The AUTODOCKTOOLS program was used to generate the docking input files. In all docking a grid box size of $60 \times 60 \times 60$ points in *x*, *y*, and *z* directions was built, the maps were centered on N1 atom of the Kcx 219 in the catalytic site of the protein. A grid spacing of 0.375 Å (approximately one forth of the length of carbon–carbon covalent bond) and a distances-dependent function of the dielectric constant were used for the calculation of the energetic map. Ten runs were generated by using Lamarckian genetic algorithm searches. Default settings were used with an initial population of 50 randomly placed individuals, a maximum number of 2.5×10^6 energy evaluations, and a maximum number of 2.7×10^4 generations. A mutation rate of 0.02 and a crossover rate of 0.8 were chosen. Results differing by less than 0.5 Å in positional root–mean–square deviation (RMSD) were clustered together and the results of the most favorable free energy of binding were selected as the resultant complex structures.

4.6. Compounds

General procedure of preparation of compounds **2** (MET-OTs) and nitroimidazoles **3a–f** and **4a–h**: metronidazole (3.14 g, 20 mmol) and Et_3N (3.0 mL, 22 mmol) were dissolved in CH_2Cl_2 (20 mL), and 4-methyl-benzenesufonyl chloride (3.83 g, 20.1 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was stirred at 0 °C for 5 h followed by the addition of 30 mL ice water, and then the layer was separated and the aqueous layer was extracted with ethyl acetate (2×30 mL). The organic layer was combined and washed with saturated $NaHCO_3$, and dried with anhydrous Na_2SO_4 for 0.5 h. Removal of the solvent gave a slight-yellow crystal of compound **2** (MET-OTs). The compounds **3a–f** were prepared by following known procedure, MET-OTs (2 mmol) and K_2CO_3 (5 mmol) were dissolved in DMF (20 mL), and one kind of the substituted salicylic acid (2.2 mmol) in DMF (10 mL) was added. The reaction mixture was stirred at 70–80 °C for 14–16 h. After the reaction mixture was vacuum distillated, the target product was separated by column chromatography. When MET-OTs (4 mmol) and K_2CO_3 (5 mmol) were dissolved in DMF (20 mL), and one kind of the substituted salicylic acid (2.2 mmol) in DMF (10 mL) was added, then the reaction mixture was stirred at 110 °C for 22–24 h, compound **4a–h** were obtained.

4.6.1. 4-Methyl-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoic acid (3a)

White solid, mp 143–144 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.29 (s, 3H,); 2.46 (s, 3H); 4.65 (t, $J = 4.77$ Hz, 2H); 4.72 (t, $J = 4.77$ Hz, 2H); 6.77–6.79 (m, 2H); 7.49 (d, $J = 8.04$ Hz, 1H); 8.00 (s, 1H); 10.25 (s, 1H). ESI-MS $C_{14}H_{15}N_3O_5$ $[M+H]^+$ 306.1. Anal. Calcd for $C_{14}H_{15}N_3O_5$: C, 55.08; H, 4.95; N 13.76. Found: C, 55.30; H, 4.94; N, 13.83.

4.6.2. 4-Amino-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoic acid (3b)

Brown solid, mp 188–189 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.49 (s, 3H); 4.58 (t, $J = 4.95$ Hz, 2H); 4.70 (t, $J = 4.95$ Hz, 2H); 5.98 (s, 1H); 6.10 (s, $J = 8.76$ Hz, 1H); 6.20 (s, 2H); 728 (d, $J = 8.76$ Hz, 1H); 7.98 (s, 1H); 10.51 (s, 1H). ESI-MS $C_{13}H_{14}N_4O_5$ $[M+H]^+$ 307.2. Anal. Calcd for $C_{13}H_{14}N_4O_5$: C, 50.98; H, 4.61; N, 18.29. Found: C, 50.84; H, 4.69; N, 18.40.

4.6.3. 2-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)-3,5-dinitrobenzoic acid (3c)

Yellow solid, mp 155–156 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.50 (s, 3H); 4.51 (t, $J = 4.80$ Hz, 2H); 4.61 (t, $J = 4.80$ Hz, 2H); 8.02 (s, 1H); 8.30 (d, $J = 3.45$ Hz, 1H); 8.48 (d, $J = 3.45$ Hz, 1H). ESI-MS $C_{13}H_{11}N_5O_9$ $[M+H]^+$ 382.1. Anal. Calcd for $C_{13}H_{11}N_5O_9$: C, 40.95; H, 2.91; N, 18.37. Found: C. 40.98; H, 2.96; N, 18.30.

4.6.4. 3, 5-Diiodo-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl ethoxy) benzoic acid (3d)

Brown solid, mp 175–176 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.55 (s, 3H); 4.18 (t, $J = 4.89$ Hz, 2H); 4.76 (t, $J = 4.89$ Hz, 2H); 7.80 (s, 1H); 8.05 (s, 1H); 8.11 (s, 1H) ESI-MS $C_{13}H_{11}I_2N_3O_5$ $[M+H]^+$ 544.1. Anal. Calcd for $C_{13}H_{11}I_2N_3O_5$: C, 28.75; H, 2.04; I, 46.74; N 7.74. Found: C, 28.73; H, 2.05; I, 46.79; N, 7.72.

4.6.5. 3,5-Dibromo-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy) benzoic acid (3e)

White solid, mp 167–169 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.53 (s, 3H); 4.19 (t, $J = 4.86$ Hz, 2H); 4.76 (t, $J = 4.86$ Hz, 2H); 7.64 (s, 1H); 8.01 (s, 1H); 8.07 (s, 1H). ESI-MS $C_{13}H_{11}Br_2N_3O_5$ $[M+H]^+$ 449.9. Anal. Calcd for $C_{13}H_{11}Br_2N_3O_5$: C, 34.77; H, 2.47; Br, 35.59; N, 9.36. Found: C, 34.60; H, 2.47; Br, 35.71; N, 9.38.

4.6.6. 3,5-Dichloro-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoic acid (3f)

White solid, mp 153–155 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.51 (s, 3H); 4.16 (t, $J = 4.89$ Hz, 2H); 4.73 (t, $J = 4.89$ Hz, 2H); 7.56 (s, 1H); 7.94 (s, 1H); 8.01 (s, 1H). ESI-MS $C_{13}H_{11}Cl_2N_3O_5$ $[M+H]^+$ 360.1. Anal. Calcd for $C_{13}H_{11}Cl_2N_3O_5$: C, 43.35; H, 3.08; Cl, 19.69; N, 11.67. Found: C, 43.41; H, 3.10; Cl, 19.61; N, 11.64.

4.6.7. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl4-methyl-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoate (4a)

White solid, mp 159–160 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.28 (s, 3H); 2.38 (s, 3H); 2.47 (s, 3H); 4.36 (t, $J = 4.71$ Hz, 2H); 4.50 (t, $J = 4.77$ Hz, 2H); 4.65 (m, 4H); 6.82 (d, $J = 7.86$ Hz, 1H); 6.98 (s, 1H); 7.39 (d, $J = 7.86$ Hz, 1H); 8.01 (s, 1H); 8.03 (s, 1H). ESI-MS $C_{20}H_{22}N_6O_7$ $[M+H]^+$ 459.1. Anal. Calcd for $C_{20}H_{22}N_6O_7$: C, 52.40; H, 4.84; N, 18.33. Found: C, 52.42; H, 4.87; N, 18.30.

4.6.8. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl5-bromo-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoate (4b)

Brown solid, mp 160–161 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.26 (s, 3H); 2.39 (s, 3H); 4.39 (t, J = 4.74 Hz, 2H); 4.52 (t, J = 4.77 Hz, 2H); 4.65 (m, 4H); 7.13 (d, J = 8.97 Hz, 1H); 7.59 (s, 1H); 7.66 (d, J = 8.97 Hz, 1H); 8.00 (s, 1H); 8.03 (s, 1H). ESI-MS $C_{19}H_{19}BrN_6O_7$ $[M+H]^+$ 553.1. Anal. Calcd for $C_{19}H_{19}BrN_6O_7$: C, 43.61; H, 3.66; Br, 15.27; N, 16.06. Found: C, 43.55; H, 3.62; Br, 15.32; N, 16.12.

4.6.9. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl5-iodo-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoate (4c)

Brown solid, mp 166–168 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.42 (s, 3H); 4.37 (t, J = 4.74 Hz, 2H); 4.49 (t, J = 4.92 Hz, 2H); 4.65 (m, 4H); 6.98 (d, J = 8.76 Hz, 1H); 7.71 (d, J = 2.01 Hz, 1H); 7.78 (dd, J_1 = 8.76 Hz, J_2 = 2.01 Hz,1H); 7.98 (s, 1H); 8.01 (s, 1H). ESI-MS $C_{19}H_{19}IN_6O_7$ $[M+H]^+$ 571.1. Anal. Calcd for $C_{19}H_{19}IN_6O_7$: C, 40.01; H, 3.36; I, 22.25; N, 14.74. Found: C, 40.05; H, 3.38; I, 22.15; N, 14.76.

4.6.10. 4-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)-3-((2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)carbonyl) benzenesulfonic acid (4d)

Yellow solid, mp 140–141 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.73 (s, 3H); 2.89 (s, 3H); 4.29 (t, J = 4.774 Hz, 2H); 4.52 (t, J = 4.89 Hz, 2H); 4.70 (m, 4H); 7.39 (d, J = 8.82 Hz, 1H); 7.57 (s, 1H); 7.68 (d, J = 8.82 Hz, 1H); 7.99 (s, 1H); 8.03 (s, 1H). ESI-MS $C_{19}H_{20}N_6O_{10}S$ $[M+H]^+$ 525.2. Anal. Calcd for $C_{19}H_{20}N_6O_{10}S$: C, 43.51; H, 3.84; N, 16.02; S, 6.11. Found: C, 43.57; H, 3.85; N, 16.01; S, 6.08.

4.6.11. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl5-methyl-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoate (4e)

Yellow solid, mp 155–156 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.73 (s, 3H); 2.89 (s, 3H); 4.29 (t, J = 4.774 Hz, 2H); 4.52 (t, J = 4.89 Hz, 2H); 4.70 (m, 4H); 7.39 (d, J = 8.82 Hz, 1H); 7.57 (s, 1H); 7.68 (d, J = 8.82 Hz, 1H); 7.99 (s, 1H); 8.03 (s, 1H). ESI-MS $C_{20}H_{22}N_6O_7$ $[M+H]^+$ 459.2. Anal. Calcd for $C_{20}H_{22}N_6O_7$: C, 52.40; H, 4.84; N, 18.33. Found: C, 52.41; H, 4.84; N, 18.35.

4.6.12. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl3-methyl-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl ethoxy benzoate (4f)

White solid, mp 144–147 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 1.99 (s, 3H); 2.40 (s, 3H); 2.50 (s, 3H); 4.08 (t, J = 4.95 Hz, 2H); 4.51 (t, J = 4.95 Hz, 2H); 4.68 (m, 4H); 7.10 (d, J = 7.68 Hz, 1H); 7.40 (m, 2H); 8.03 (s, 1H); 8.07 (s, 1H). ESI-MS $C_{20}H_{22}N_6O_7$ $[M+H]^+$ 459.3. Anal. Calcd for $C_{20}H_{22}N_6O_7$: C, 52.40; H, 4.84; N, 18.33. Found: C, 52.46; H, 4.86; N 18.30.

4.6.13. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl5-chloro-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoate (4g)

White solid, mp 150–151 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.39 (s, 3H); 2.44 (s, 3H); 4.39 (t, J = 4.74 Hz, 2H); 4.52 (t, J = 4.92 Hz, 2H); 4.66 (m, 4H); 7.20 (d, J = 9.00 Hz, 1H); 7.47 (d, J = 2.55 Hz, 1H); 7.58 (dd, J_1 = 9.00 Hz, J_2 = 2.55 Hz,1H); 8.00 (s, 1H); 8.03 (s, 1H). ESI-MS $C_{19}H_{19}ClN_6O_7$ $[M+H]^+$ 479.3. Anal. Calcd for $C_{19}H_{19}ClN_6O_7$: C, 47.66; H, 4.00; Cl, 7.40; N, 17.55. Found: C, 47.59; H. 4.02; Cl, 7.43; N, 17.51.

4.6.14. 2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl4-chloro-2-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzoate (4h)

White solid, mp 101–102 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 2.36 (s, 3H); 2.45 (s, 3H); 4.44 (t, J = 2.94 Hz, 2H); 4.52 (t, J = 2.942 Hz, 2H); 4.66 (m, 4H); 7.10 (d, J = 5.10 Hz, 1H); 7.29 (s, 1H); 7.49 (d, J = 5.10 Hz,1H); 8.00 (s, 1H); 8.03 (s, 1H). ESI-MS: $C_{19}H_{19}ClN_6O_7$ $[M+H]^+$ 479.1. Anal. Calcd for $C_{19}H_{19}C_lN_6O_7$: C, 47.66; H, 4.00; Cl, 7.40; N, 17.55. Found: C, 47.70; H, 3.98; Cl, 7.38; N, 17.56.

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