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Oxadiazole derivatives containing 1,4-benzodioxan as potential immunosuppressive agents against RAW264.7 cells

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ABSTRACT

A series of oxadiazole derivatives containing 1,4-benzodioxan (**4a**–**4s**) have been first synthesized for their potential immunosuppressive activity. Among the compounds, compound **4i** showed the most potent biological activity against RAW264.7 cells (inhibition = 37.66 ± 2.34% for NO overproduction and IC_{50} = 0.05 μM for iNOS). Docking simulation was performed to position compound **4i** into the iNOS structure active site to determine the probable binding model. RT-PCR experiment results demonstrated that some of these compounds possessed good immunosuppressive activity against iNOS, especially for compound **4i**. Therefore, compound **4i** with potent inhibitory activity may be a potential agent.

1. Introduction

Immunosuppressant is an important class of clinical drugs for an array of medical processes, including transplant rejection and treatment of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and psoriasis.[1,2] Nitric oxide (NO), an endogenous free radical is an important signaling molecule involved in a wide range of physiological functions, as well as pathophysiological states. NO is generated from L-arginine by a family of nitric oxide synthases (NOSs) including major of isozymes, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).[3] Both eNOS and nNOS are constitutively expressed and produce NO at a low level. However, iNOS is often expressed at high level and is essentially unregulated once expressed. If activated by many immunological stimuli such as lipopolysaccharide (LPS), interferon (IFN-*c*) and a variety of proinflammatory cytokines, iNOS produces a high level of NO to exert defense against pathogens.[4] It is well known that NO plays a major role in anti-inflammatory and immune reactions, however, an extremely high level of NO induced by iNOS causes inflammatory diseases such as rheumatoid arthritis. Therefore, as drug development targets, inhibitors of NO overproduction and over expression of iNOS might be beneficial for treatment of inflammatory disorders caused by excessive production of NO.

Compounds containing a 1,4-benzodioxan template have received significant attention in chemical, medicinal and pharmaceutical research as this structural scaffold is found in a variety of drugs. For example (Fig. 1), the mesylate salt of doxazosin (A) is an effective drug for treatment of hypertension.[5] The 6-position substituted 1,4-benzodioxan (B) is known as a nonsteroidal anti-inflammatory drug (NSAID).[6] WB 4104 (C) is recognized as a selective α-adrenoceptor antagonist.[7–11]

Besides, 1,3,4-oxadiazoles are an important class of heterocyclic compounds. The widespread use of them as a scaffold in medicinal chemistry establishes this moiety as a member of the privileged structures class.[12] They possess a variety of biological activities.[13–17] In particular, a few differently substituted 1,3,4-oxadiazoles have been found to exhibit immunosuppressive activities.[18–20] Further, 1,3,4-oxadiazole heterocycles are very good bioisosteres of amides and esters, which can contribute substantially in increasing pharmacological activity by participating in hydrogen bonding interactions with the receptors.[21,22]

Recently, it was reported that a number of other compounds (D, E and F) containing the 1,4-benzodioxan template showed potent anti-inflammatory activity (Fig. 1).[23–25] However, to our knowledge, few reports have been dedicated to the synthesis and iNOS inhibitory activity of oxadiazole derivatives containing 1,4-benzodioxan. Herein, in continuation to extend our research on compounds with iNOS inhibitory activity,[26] we reported in the present work the synthesis and structure-activity relationships of a series of oxadiazole derivatives containing 1,4-benzodioxan as potential immunosuppressive agents. Biological evaluation indicated that some of the synthesized compounds were potent inhibitors of iNOS.

2. Results and discussion

2.1. Chemistry

Nineteen oxadiazole derivatives containing 1,4-benzodioxan (**4a**–**4s**) were synthesized to screen for the immunosuppressive

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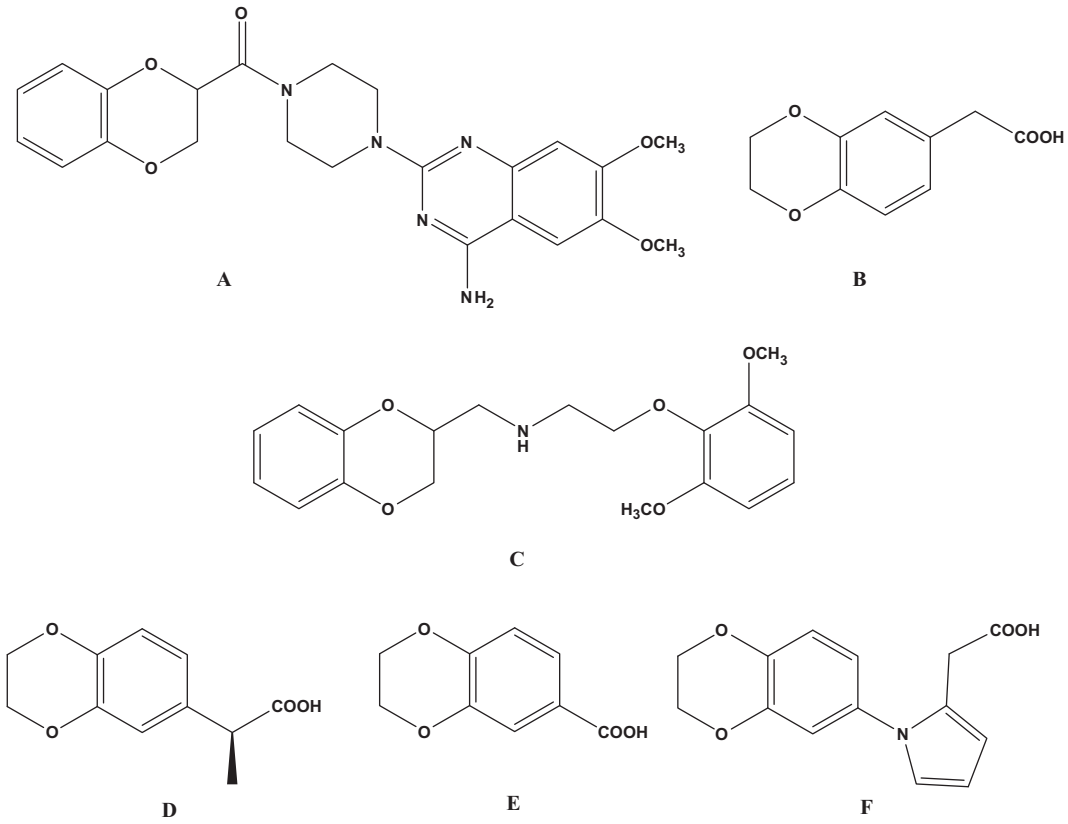


Figure 1. The structure of compounds A–F.

activity. They were prepared in a four step process (Scheme 1) and all were reported for the first time. Firstly, the 2,3-dihydrobenzo[*b*][1,4] dioxine-6-carboxylic acid on treatment with methanol containing concentrated H_2SO_4 was refluxed overnight. This step can yield the corresponding ester. Secondly, the ester was treated with hydrazine hydrate in ethanol overnight, refluxing. Thirdly, the coupling reaction between the obtained compound **3a** and the different substituted phenyl acetic acid or benzoic acid was performed by using carbodiimide hydrochloride and *N*-hydroxybenzotriazole in anhydrous CH_2Cl_2 ,then refluxed in phosphoryl chloride afforded the target compounds. Or the coupling reaction between the obtained compound **3a** and the different substituted phenyl acetic acid or benzoic acid was directly refluxed in phosphoryl chloride afforded the corresponding target compounds 2-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-substituted-1,3,4-oxadiazole. Then compounds **4a**–**4s** were obtained by subsequent purification with recrystallisation. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were full accordance with their depicted structures.

2.2. Biological activity

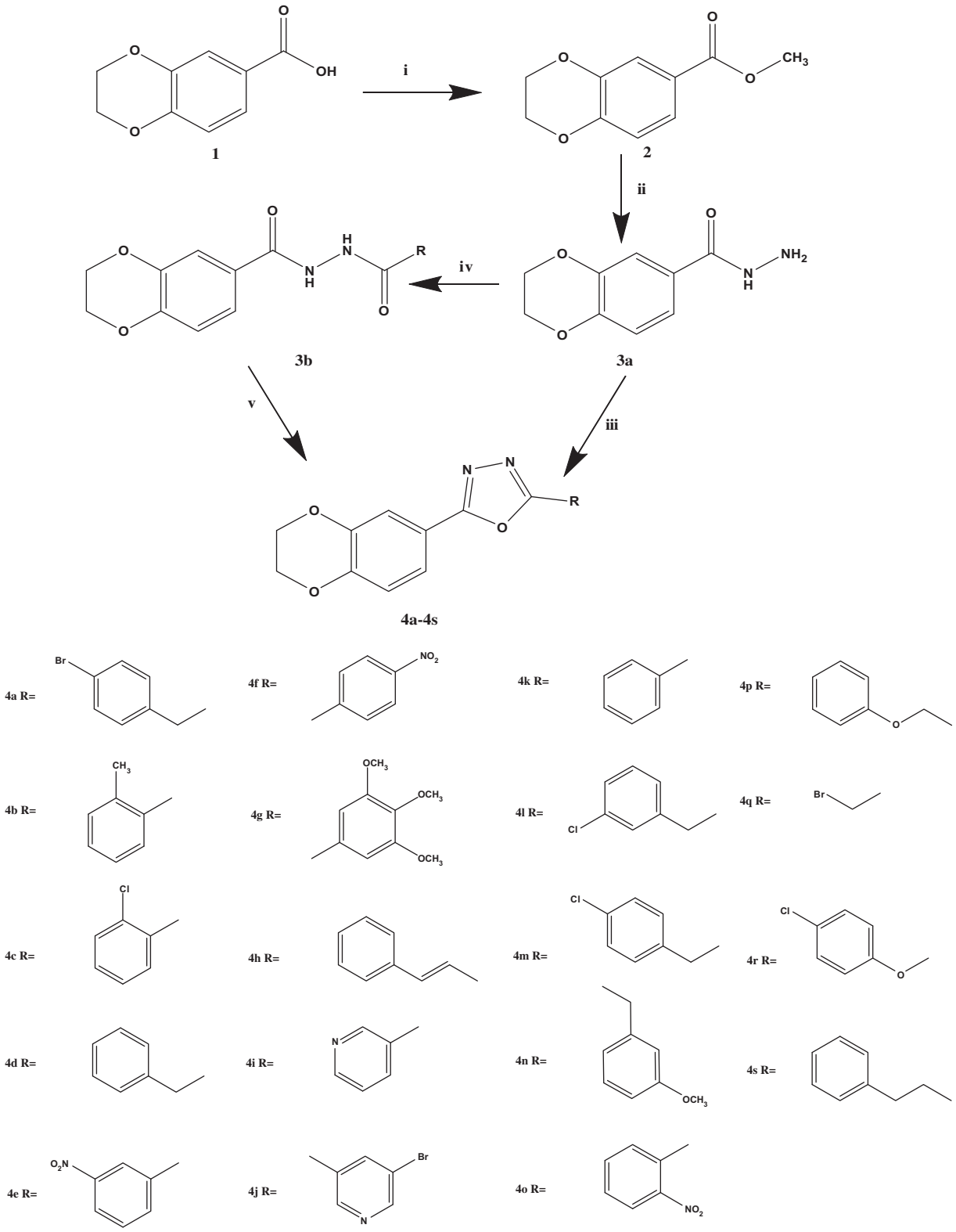
2.2.1. Acute toxicity test

The inhibitory activity of the compounds is sometimes a result of their toxic effect and consequently might cause an erroneous conclusion,[27–29] prior to the following bioactivity analyses, all the synthesized oxadiazole derivatives containing 1,4-benzodioxan were evaluated using LPS-activated murine macrophages RAW264.7 for acute toxicity test.[30] The pharmacological results were summarized in Table 1. The data showed that most of the oxadiazole derivatives containing 1,4-benzodioxan moiety having a quite low toxicity.

2.2.2. Inhibitory activity on NO production

To evaluate the inhibitory effects on the excessive NO production, all the compounds were assayed using LPS-activated murine macrophage-like RAW264.7 cells culture systems. The results were summarized in Table 2. Among them, compound **4i** (inhibition = 37.66%) exhibited significant immunosuppressive activity.

Structure–activity relationships in these oxadiazole derivatives containing 1,4-benzodioxan demonstrated that compounds with different acids substituents led to different immunosuppressive activity, and the order was benzoic acid (**4k**) > phenylacetic acid (**4d**). However, when introduced a withdrawing group, the situation was changed. The compounds with Cl substituted in the phenylacetic acid group (**4m**, **4l**) exhibited excellent immunosuppressive activity compared with that in the benzoic acid group (**4c**), and substituent Cl at the *para* (**4l**) position showed a little stronger activity than that at the *meta* (**4m**) position in the benzene ring. Meanwhile, a significant loss of activity was observed when the NO_2 substituent was introduced at the benzoic acid group (**4e**, **4f**, **4o**), and the order of the activities is that substituent at the *ortho* (**4o**) position > substituent at the *para* (**4f**) position > substituent at the *meta* (**4e**) position. However, compound **4a** (*p*-Br in the phenylacetic acid group) displayed poor activity. On the contrary, when introduced a donor group in the benzoic acid (**4b**, **4g**) or phenylacetic acid group (**4n**), a remarkable increase in the immunosuppressive activity was observed. Compound **4i** with substituent of pyridine group showed the most potent activity in these compounds, interestingly, introduction of Br moiety, afforded **4j** with a remarkable decrease in the immunosuppressive activity. Besides, compound **4h** with definic bond substituted on benzene ring showed better immunosuppressive activity than compound **4s** with the same chain length, however, a significant loss of activity was observed when the carbon atom of the carbon chain was replaced by oxygen atom (**4p**).



Scheme 1. General synthesis of compounds **4a**–**4s**. Reagents and conditions: (i) methanol, concentrated sulfuric acid; 90 °C; (ii) $NH_2NH_2{\cdot}H_2O$, ethanol; 90 °C; (iii) aliphatic or aromatic carboxylic acids, $POCl_3$; 110 °C; iv) EDC·HCl, HOBt, dichloromethane, rt; (v) $POCl_3$; 100 °C.

2.2.3. iNOS inhibitory assay

The iNOS inhibitory potency of the oxadiazole derivatives containing 1,4-benzodioxan were examined and the results were summarized in Table 3. Most of the tested compounds displayed potent iNOS inhibitory. Among them, compound **4i** showed the most potent inhibitory with IC_{50} of 0.05 μM. The results of iNOS inhibitory activity of the tested compounds were corresponding to the structure relationships (SAR) of their inhibitory effects on the excessive NO production. This demonstrated that the potent inhibitory effects on the excessive NO production activities of the synthetic

Table 1
The acute toxicity test, most of the compounds exhibited nontoxic

Compound	OD_{540}
4a	0.99
4b	1.03
4c	1.45
4d	0.45
4e	0.73
4f	0.57
4g	1.02
4h	1.15
4i	1.31
4j	1.22
4k	1.50
4l	0.20
4m	0.63
4n	0.76
4o	0.30
4p	0.36
4q	0.94
4r	0.89
4s	0.10
LPS	1.17
Control	0.83

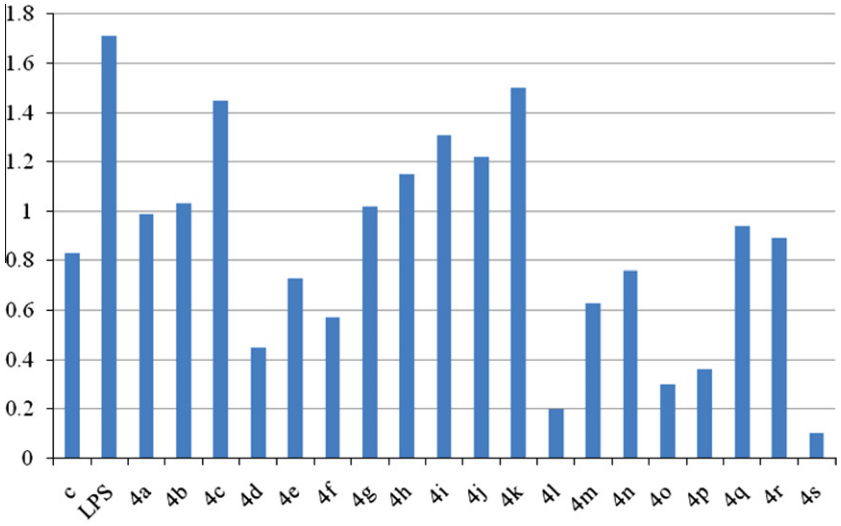


Table 2
The effect of compounds on LPS-induced NO production in RAW264.7 cells

Compound	Inhibition (%)
4a	−8.53 ± 5.52
4b	21.58 ± 3.71
4c	−57.48 ± 8.32
4d	−3.63 ± 2.17
4e	−33.82 ± 6.25
4f	1.34 ± 1.12
4g	20.03 ± 2.14
4h	25.54 ± 1.98
4i	37.66 ± 2.34
4j	−42.39 ± 7.92
4k	5.08 ± 0.35
4l	23.70 ± 2.84
4m	28.11 ± 1.94
4n	21.66 ± 4.32
4o	3.65 ± 3.12
4p	6.98 ± 2.15
4q	6.57 ± 2.41
4r	10.49 ± 1.95
4s	3.71 ± 3.03
LPS	−30.97 ± 4.13
Control	0.00 ± 5.76

compounds were probably correlated to their iNOS inhibitory activities.

2.2.4. RT-PCR experiment

The high level of NO is mainly generated by iNOS, so iNOS expression is one of the key steps during the process of LPS-activated NO production. In an effort to study the preliminary mechanism of the compounds with potent inhibitory activity, the RT-PCR experi-

Table 3
iNOS inhibitory activity of synthetic compounds

Compound	IC_{50} (μM)
4b	0.10 ± 3.03
4g	0.12 ± 2.12
4h	0.09 ± 0.78
4i	0.05 ± 1.14
4k	2.35 ± 2.32
4q	2.02 ± 2.01
4r	2.16 ± 0.12
LPS	>100
Control	0.00

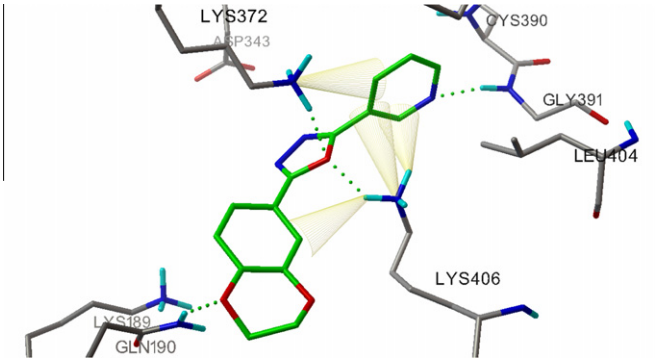


Figure 2. Molecular docking modeling of compound **4i** with inducible NOS: compound **4i** was nicely bound to the iNOS with its nitrogen atom of pyridine group project towards the amino hydrogen of GLY 391, with the hydroxyl group forming a more optimal H-bond (H–N···H: 1.95 Å, 167.83°) interaction, and the oxygen atom of benzene group of **4i** also forms hydrogen bond (H–O···H: 2.08 Å, 156.72°) with hydrogen atom of GLN 190. Besides, the oxygen atom of oxadiazole group project towards the amino hydrogens of GLY 406 and GLY 372, forming two H-bond interactions, H–O···H: 1.81 Å, 137.81° and H-O...H: 2.09 Å, 144.55°, respectively. Meanwhile, π-cation interactions were shown as yellow column.

ment was performed to assay the effect of four selected compounds on mRNA expression of iNOS.[31] The RT-PCR results were summarized in Figure 4. Among them, compound **4i** strongly inhibited the expression of iNOS mRNA. However, the other compounds expressed little inhibitory activity, suggesting that they might inhibit NO production through other mechanisms.

2.3. Binding model of compounds 4i into iNOS structure

In an effort to elucidate the possible mechanism by which the title compounds can induce immunosuppressive activity against RAW264.7 cells and guide further SAR studies, molecular docking of the potent inhibitor **4i** into binding site of iNOS were performed on the binding model based on the iNOS complex structure (1M9T.pdb). The binding models of compound **4i** and iNOS were depicted in Figure 2 and Figure 3. In the binding model, compound **4i** was nicely bound to the iNOS with its nitrogen atom of pyridine group project towards the amino hydrogen of GLY 391, with the hydroxyl group forming a more optimal H-bond (H–N···H: 1.95 Å, 167.83°) interaction, and the oxygen atom of benzene group of **4i** also forms hydrogen bond (H–O···H: 2.08 Å, 156.72°) with hydrogen atom of GLN 190. Besides, the oxygen atom of oxadiazole

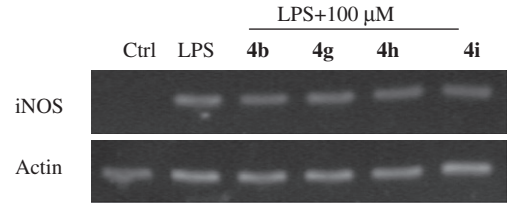


Figure 4. The RT-PCR experiment was performed to assay the effect of selected compounds on mRNA expression of iNOS.

group project towards the amino hydrogens of GLY 406 and GLY 372, forming two H-bond interactions, H–O···H: 1.81 Å, 137.81° and H–O···H: 2.09 Å, 144.55°, respectively. Meanwhile, π-cation interaction was shown as yellow column. This molecular docking result, along with the biological assay data, suggesting that compound **4i** was a potential inhibitor of iNOS.

3. Conclusions

A series of new oxadiazole derivatives containing 1,4-benzodioxan moiety were synthesized. Preliminary results showed that most of the compounds displayed enhanced inhibitory activities and low toxicity. Compound **4i** demonstrated the most potent inhibitory activity that inhibited the excessive NO production of RAW264.7 cells with inhibition of 37.66 ± 2.34% and inhibited the activity of iNOS with IC_{50} of 0.05 μM.

In order to gain more understanding of the structure–activity relationships observed at the iNOS, molecular docking of the most potent inhibitor **4i** into the binding site of iNOS was performed on the binding model based on the iNOS complex structure. Analysis of the compound **4i**'s binding conformation demonstrated that compound **4i** was stabilized by hydrogen bonding interaction with GLN190, GLY 372, GLY 391 and GLY 406, as well as four π-cation interactions. RT-PCR results showed the compound **4i** was a potential agent, as well.

4. Experimental section

4.1. Methods of synthesis

All chemicals used were purchased from Aldrich (USA). The eluates were monitored using TLC. Melting points (uncorrected) were determined on a XT4MP apparatus (Taike Corp., Beijing, China). ESI mass spectra were obtained on a Mariner System 5304 mass

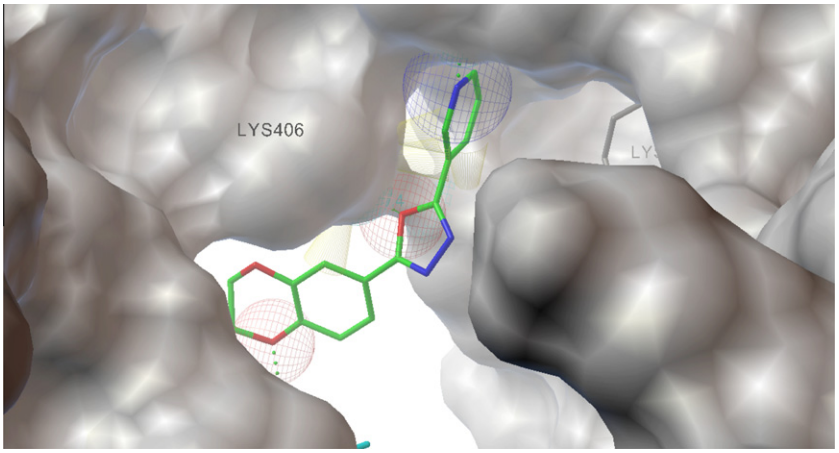


Figure 3. 3D model of the interaction between compound **4i** and the inducible NOS binding site. The inducible NOS was represented by molecular surface. Compound **4i** was depicted by sticks and balls.

spectrometer, and ^{1}H NMR spectra were recorded on a Bruker DPX300 spectrometer at 25 °C with TMS and solvent signals allotted as internal standards, Chemical shifts are reported in ppm (δ). Elemental analyses were performed on a CHN–O–Rapid instrument and were within 0.4% of the theoretical values.

4.1.1. Synthesis of 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylate (2)

The 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylic acid (9.0 g, 50 mmol) in methanol (50 mL) containing concentrated H_2SO_4 (5 mL) was refluxed overnight. Water (100 mL) was added, the organic phases were washed with saturated NaCl (100 mL) and dried over Na_2SO_4, and the solvents were evaporated.

4.1.2. Synthesis of 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carbohydrazide (3a)

To compound **2** (0.01 mol) dissolved in dry ethanol (50 mL) 99% hydrazine hydrate (1 mL) was added and the mixture was refluxed for 8–10 h. The reaction mixture was cooled and the solid obtained was filtered, washed with small quantity of ethanol to give **3a**.

4.1.3. Synthesis of N-substituted-2,3-dihydrobenzo[b][1,4] dioxine-6-carbohydrazide (3b)

A stirred solution of compound **3a** (0.1 mol) in CH_2Cl_2 (50 mL) was treated with the appropriate substituted phenyl acetic acid or benzoic acid, EDC·HCl (0.15 mol), HOBt (0.05 mol) and refluxed overnight. Then purification with recrystallisation afforded the corresponding compound as white powder.

4.1.4. Synthesis of 2-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-substituted-1,3,4-oxadiazole. (4a–4s)

General method. An equimolar compound **3a** (0.001 mol) and substituted carboxylic acid in phosphoryl chloride was refluxed for 10–15 h. Or compound **3b** in phosphoryl chloride was refluxed for 5–7 h. Then reaction mixture was cooled, poured into ice-cold water and neutralized with 20% $NaHCO_3$ solution. The resultant solid was filtered, washed with water and recrystallized from ethanol to give the title compounds.

4.1.4.1. 2-(4-Bromophenyl)-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-1,3,4-oxadiazole (4a). White powder. Mp: 140–141 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.30 (s, 2H); 4.32–4.33 (m, 4H); 6.92–6.95 (m, 1H); 7.21–7.24 (d, J = 5.4 Hz, 2H); 7.46–7.50 (m, 4H). MS (ESI): 373.01 ($C_{17}H_{14}BrN_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{13}BrN_2O_3$: C, 54.71; H, 3.51; N, 7.51. Found: C, 54.43; H, 3.90; N, 7.26.

4.1.4.2. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-o-tolyl-1,3,4-oxadiazole (4b). White powder. Mp: 116–117 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 2.76 (s, 3H); 4.34 (s, 4H); 6.98–7.01 (m, 1H); 7.34–7.42 (m, 3H); 7.63–7.65 (m, 2H); 8.01–8.03 (d, J = 7.5 Hz, 1H). MS (ESI): 295.10 ($C_{17}H_{15}N_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.14; H, 4.99; N, 9.21.

4.1.4.3. 2-(2-Chlorophenyl)-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-1,3,4-oxadiazole (4c). White powder. Mp: 121–123 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 4.35–4.35 (m, 4H); 7.09–7.12 (d, J = 8.4 Hz, 1H); 7.59–7.60 (m, 1H); 7.60–7.61 (m, 2H); 7.62–7.68 (m, 1H); 7.72–7.75 (d, J = 7.8 Hz, 1H); 8.11–8.14 (m, 1H). MS (ESI): 315.05 ($C_{16}H_{12}ClN_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{11}ClN_2O_3$: C, 61.06; H, 3.52; N, 8.90. Found: C, 61.35; H, 3.83; N, 8.68.

4.1.4.4. 2-Benzyl-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-1,3,4-oxadiazole (4d). White powder. Mp: 82–83 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.25 (s, 2H); 4.27–4.32 (m, 4H); 6.91–6.94 (m, 1H); 7.28–7.33 (m, 1H); 7.34–7.36 (m, 4H); 7.48–7.51 (m, 2H). MS (ESI): 295.10 ($C_{17}H_{15}N_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{14}N_2O_3$: C, 69.38; H, 4.79; N, 9.52. Found: C, 69.64; H, 4.99; N, 9.36.

4.1.4.5. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(3-nitrophenyl)-1,3,4-oxadiazole (4e). White powder. Mp: 236–237 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.33–4.36 (m, 4H); 7.00–7.03 (m, 1H); 7.65–7.68 (m, 2H); 7.72–7.75 (m, 1H); 8.39–8.41 (d, J = 5.7 Hz, 1H); 8.47–8.50 (d, J = 7.9 Hz, 1H); 8.92 (s, 1H). MS (ESI): 326.07 ($C_{16}H_{12}N_3O_5$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{11}N_3O_5$: C, 59.08; H, 3.41; N, 12.92. Found: C, 59.33; H, 3.69; N, 12.76.

4.1.4.6. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(4-nitropheneyl)-1,3,4-oxadiazole (4f). White powder. Mp: 273–275 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.48–3.50 (m, 4H); 4.32–4.35 (m, 3H); 6.91–7.04 (m, 1H); 7.60–7.67 (m, 1H); 8.30–8.43 (m, 2H). MS (ESI): 326.07 ($C_{16}H_{12}N_3O_5$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{11}N_3O_5$: C, 59.08; H, 3.41; N, 12.92. Found: C, 59.36; H, 3.11; N, 12.65.

4.1.4.7. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(3,4,5-trimethoxyphenyl)-1,3,4-oxadiazole (4g). White powder. Mp: 205–206 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.93–3.97 (m, 9H); 4.21–4.34 (m, 4H); 6.97–7.02 (m, 1H); 7.26–7.35 (m, 2H); 7.64–7.65 (m, 2H). MS (ESI): 371.12 ($C_{19}H_{19}N_2O_6$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_6$: C, 61.62; H, 4.90; N, 7.56. Found: C, 61.93; H, 4.68; N, 7.78.

4.1.4.8. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-styryl-1,3,4-oxadiazole (4h). Yellow powder. Mp: 131–132 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.33(s, 4H); 6.98–7.00 (d, J = 8.9 Hz, 1H); 7.06–7.11 (d, J = 16.8 Hz, 1H); 7.38–7.43 (m, 3H); 7.57–7.64 (m, 5H). MS (ESI): 307.10 ($C_{18}H_{15}N_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{14}N_2O_3$: C, 70.58; H, 4.61; N, 9.15. Found: C, 70.77; H, 4.87; N, 8.89.

4.1.4.9. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(pyridin-3-yl)-1,3,4-oxadiazole (4i). White powder. Mp: 170–171 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.32–4.36 (m, 4H); 7.00–7.03 (m, 1H); 7.46–7.50 (m, 1H); 7.63–7.66 (m, 2H); 8.39–8.43 (m, 1H); 8.78 (s, 1H); 9.34 (s, 1H). MS (ESI): 282.08 ($C_{15}H_{12}N_3O_3$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{11}N_3O_3$: C, 64.05; H, 3.94; N, 14.94. Found: C, 64.40; H, 3.82; N, 14.69.

4.1.4.10. 2-(5-Bromopyridin-3-yl)-5-(2,3-dihydrobenzo[*b*][1,4] dioxin-6-yl)-1,3,4-oxadiazole(4j). White powder. Mp: 222–223 °C. ^{1}H NMR (300 MHz, DMSO-d_6): 4.34 (s, 4H); 7.07–7.10 (d, J = 9.0 Hz, 1H); 7.65–7.67 (m, 2H); 8.74 (s, 1H); 8.93–8.94 (d, J = 2.0 Hz, 1H); 9.25–9.26 (d, J = 1.5 Hz, 1H); MS (ESI): 359.99 ($C_{15}H_{11}BrN_3O_3$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{10}BrN_3O_3$: C, 50.02; H, 2.80; N, 11.67. Found: C, 50.34; H, 2.49; N, 11.97.

4.1.4.11. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-phenyl-1,3,4-oxadiazole (4k). Yellow powder. Mp: 162–161 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.33–4.34 (m, 4H); 6.98–7.01 (m, 1H); 7.52–7.54 (m, 3H); 7.63–7.66 (m, 2H); 8.10–8.13 (m, 2H). MS (ESI): 281.08 ($C_{16}H_{13}N_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 9.99. Found: C, 68.79; H, 4.66, N, 10.21.

4.1.4.12. 3-(3-Chlorobenzyl)-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl-4H-pyrazole (4l). White powder. Mp: 75–76 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.22 (s, 2H); 4.27–4.33 (m, 4H); 6.92–6.95 (m, 1H); 7.21–7.23 (m, 1H); 7.27–7.29 (m, 2H); 7.35 (s, 1H); 7.48–7.51 (m, 2H). MS (ESI): 329.06 ($C_{17}H_{14}ClN_2O_3$, $[M+H]^+$). Anal. Calcd

for $C_{17}H_{13}ClN_2O_3$: C, 62.11; H, 3.99; N, 8.52. Found: C, 62.37; H, 4.21; N, 8.77.

4.1.4.13. 2-(4-Chlorobenzyl)-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-1,3,4-oxadiazole (4m). Yellow powder. Mp: 109–110 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.22 (s, 2H); 4.27–4.32 (m, 4H); 6.92–6.95 (m, 1H); 7.01–7.06 (m, 2H); 7.29–7.34 (m, 2H); 7.47–7.50 (m, 2H). MS (ESI): 329.06 ($C_{17}H_{14}ClN_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{13}ClN_2O_3$: C, 62.11; H, 3.99; N, 8.52. Found: C, 62.33; H, 4.24; N, 8.33.

4.1.4.14. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(3-methoxybenzyl)-1,3,4-oxadiazole (4n). White powder. Mp: 82–83 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.80 (s, 3H); 4.22 (s, 2H); 4.27–4.31 (m, 4H); 6.82–6.84 (m, 1H); 6.85 (s, 1H); 6.91–6.94 (m, 2H); 7.23–7.28 (m, 1H); 7.48–7.51 (m, 2H). MS (ESI): 325.11 ($C_{18}H_{17}N_2O_4$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.97; N, 8.64. Found: C, 66.91; H, 4.74; N, 8.79.

4.1.4.15. 2-((4-Chlorophenoxy)methyl)-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)-1,3,4-oxadiazole (4o). White powder. Mp: 145–146 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.31–4.35 (m, 4H); 5.30 (s, 2H); 6.97–7.02 (m, 3H); 7.30–7.31 (m, 2H); 7.56–7.59 (m, 2H). MS (ESI): 345.06 ($C_{17}H_{14}ClN_2O_4$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{13}ClN_2O_4$: C, 59.23; H, 3.80; N, 8.13. Found: C, 59.46; H, 3.62; N, 8.42.

4.1.4.16. 2-(2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-phenethyl-1,3,4-oxadiazole (4p). White powder. Mp: 79–80 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.18–3.25 (m, 4H); 4.31–4.33 (m, 4H); 6.94–6.96 (d, J = 8.4 Hz, 1H); 7.23 (s, 1H); 7.31–7.34 (m, 3H); 7.48–7.49 (m, 1H); 7.52 (s, 2H). MS (ESI): 309.12 ($C_{18}H_{17}N_2O_3$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{16}N_2O_3$: C, 70.12; H, 5.23; N, 9.09. Found: C, 70.36; H, 5.03; N, 9.35.

4.1.4.17. 2-(2,3- Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(2-nitrophenyl)-1,3,4-oxadiazole (4q). Yellow powder. Mp: 122–124 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.29–4.35 (m, 4H); 6.96–6.99 (m, 1H); 7.54–7.57 (m, 2H); 7.70–7.81 (m, 2H); 8.00–8.09 (m, 2H). MS (ESI): 326.07 ($C_{16}H_{12}N_3O_5$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{11}N_3O_5$: C, 59.08; H, 3.41; N, 12.9. Found: C, 59.33; H, 3.74; N, 12.69.

4.1.4.18. 2-(2,3- Dihydrobenzo[*b*][1,4]dioxin-6-yl)-5-(phenoxymethyl)-1,3,4-oxadiazole (4i). White powder. Mp: 186–187 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.27–4.32 (m, 4H); 4.68 (s, 2H); 6.91–6.93 (d, J = 8.2 Hz, 1H); 6.96–6.99 (m, 2H); 7.02–7.07 (m, 1H); 7.31–7.37 (m, 3H); 7.39–7.40 (m, 1H). MS (ESI): 311.10 ($C_{17}H_{15}N_2O_4$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.55; N, 9.03. Found: C, 65.99; H, 4.31; N, 8.83.

4.1.4.19. 3-(Bromometyl)-5-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl-4H-pyrazole (4s). White powder. Mp: 118–120 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.33–4.34 (m, 4H); 4.59 (s, 1H); 4.77 (s, 1H); 6.98–7.01 (m, 1H); 7.57–7.64 (m, 2H). MS (ESI): 295.00 ($C_{12}H_{12}BrN_2O_2$, $[M+H]^+$). Anal. Calcd for $C_{12}H_{11}BrN_2O_2$: C, 48.84; H, 3.76; N, 9.49. Found: C, 48.65; H, 3.98; N, 9.80.

4.2. Acute toxicity test

RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. In all cell cultures, each compound was prepared in dimethyl sulphoxide (DMSO) followed by dilution with culture medium to desired concentrations, and DMSO final concentration was 0.1%. DMSO at 0.1% was added into control (RAW264.7 cells were treated with LPS only) and blank (RAW264.7 cells only, without any treatments) groups and showed no effects on cells.

Cells were plated at a density of 5×10^4 cells in a 96-well plate, and compounds were added to each plate at the indicated concentrations. After a 24 h incubation period, 20 μL MTT reagent (5 mg/mL) was added, and the cells were incubated for 4 h. The supernatants were aspirated, and the formazan crystals in each well were dissolved in 200 μL of dimethyl sulfoxide for 30 min at 37 °C. The absorbance value was monitored by microplate reader at 540 nm.

4.3. Inhibitory activity on NO production

The activity of the prepared compounds **4a–4s** against RAW264.7 cells was evaluated as described in the literature[32] with some modifications. Target cells were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were incubated in a 96-well plate at a density of 5×10^5 cells per well and were left untreated or treated with compounds at various concentrations (0.5–100 μM) for 24 h. For the proliferation assay, 20 mL MTT (Sigma, 4 mg/mL in PBS) was added per well 4 h before the end of incubation. After removing the supernatant, 200 mL DMSO was added to dissolve the formazan crystals. The absorbance at 540 nm (OD_{540}) was read on an ELISA reader (Tecan, Austria).

NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent according to manufacturer's instructions. Briefly, 100 μL culture supernatant was transferred into a new 96-well plate and mixed with 100 μL of Griess reagent. After 10 min, the absorbance value at 540 nm was collected by microplate reader. NO inhibitory activity was calculated using the following formula:

$$\text{NO inhibitory activity} = [\text{Control}(OD_{540nm}) - \text{Compounds}(OD_{540nm})/(\text{Control}(OD_{540nm})] \times 100\%$$

4.4. INOS inhibitory assay

Mouse RAW 264.7 cells were grown in RPMI 1640 medium supplemented by 10% fetal bovine serum under 5% CO_2 atmosphere at 37 °C. Then, LPS and interferon-γ were added to this medium, the final concentrations were 0.2 μg/mL and 100 unit/mL, respectively. Tris and dithiothreitol was added to this cell culture after the grown cells were collected from this medium, with the final concentration of 50 mM and 100 μM, respectively. And then the culture was centrifuged at 10,000*g* for 10 min. After that Dowex HCRW2 added to the obtained supernatant and stirred for 30 min at 4 °C. The obtained supernatant was used as crude enzyme. 20 μL of Tris (pH 7.5) including 1 mM of NADPH, 10 μL of compounds, 20 μL of 10 μCi/mL L-[H^3]-arginine (0.5 μM) and 80 μL of Tris (pH 7.5) were added into the 70 mL obtained crude enzyme. After incubated for 30 min at 37 °C, 200 μL of 0.1 M (pH 5.0) including 2 mM EDTA and 2 mM EGTA were added. To the resulting was added Dowex 50 W-8X and stirred for 5 min. iNOS activity was measured by monitoring the conversion level of L-citrulline from L-arginine. Therefore, L-[H^3]-citrulline in the supernatant was monitored by scintillation counting.

4.5. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression

To examine iNOS mRNA expression level in RAW264.7 cells, total RNA was extracted using the Trizol reagent, and cDNA was synthesized from 1 g RNA utilizing M-MLV reverse transcriptase according to manufacturer's instruction (TOYOBO, JAPAN). The PCR reaction was performed by the following reaction conditions: 94 °C for 5 min and 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with a final elongation step of 72 °C for 5 min. The PCR products were run on a 1% agarose gel and were visualized by ethidium bromide staining. The produced bands in the gel were then photographed. The primer sequences used in this study were as follows: iNOS: forward, 5'-CAACATCAGGTCGGCCATCACT-3'; reverse, 5'-ACCAGAG GCAGCACATCAAAGC-3; β-actin: forward, 5'-TGCTGTCCCTGTATGCCTCT-3'; reverse, 5'-TTTGATGTCACGCACG-ATTT-3'.

4.6. Experimental protocol of docking study

The automated docking studies were carried out using Auto Dock version 4.0. First, AutoGrid component of the program precalculates a three-dimensional grid of interaction energies based on the macromolecular target using the AMBER force field. Then automated docking studies were carried out to evaluate the binding free energy of the inhibitors within the macromolecules.

The three-dimensional structures of the aforementioned compounds were constructed using Chem. 3D ultra 11.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2009)], then they were energetically minimized by using MOPAC with 100 iterations and minimum RMS gradient of 0.10. The Gasteiger-Hückel charges of ligands were assigned. The crystal structures of telomerase (PDB code: 1M9T) complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound waters and ligands were eliminated from the protein and the polar hydrogens and the Kollman-united charges were added to the proteins.

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