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Synthetic receptor and its association with CTD peptides

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ABSTRACT

In this study, a receptor for phosphorylated peptide was developed. The receptor contains Zn^{2+}–dipicolylamine (Zn–Dpa) moiety at one end and guanidiniocarbonyl pyrrole at the other. The association between the receptor and CTD (carboxy-terminal domain of RNA polymerase II) peptides with different phosphorylated patterns was investigated by isothermal titration calorimetry and molecular docking, and the receptor showed much higher affinity towards bis-phosphorylated CTD peptide with affinity constant $K=1.09\times10^5$.

1. Introduction

Phosphorylation is one of the most important post-translational modifications of protein. It regulates many important biological processes, such as the growth and differentiation of living cell. Developing receptors, which can tightly associate with phosphorylated peptides is not only necessary for the regulation of protein phosphorylation but also for the disruption of protein–protein interaction, additionally, when conjugated with some signal groups, the receptor can also find applications in sensors and imaging.[1]

Pioneering studies by Hamachi and co-workers provide fundamental insight into recognition of phosphorylated peptides using artificial receptors,[2,3] after that, Andreas grauer and co-workers reported the binding of carboxylate side-chain-containing phosphorylated peptide using synthetic receptors,[4] and all these receptors employ zinc(II) complexes as the phosphate binding moieties. Schmuck and co-workers developed receptors based on guanidiniocarbonyl pyrrole, because of the electro-withdrawing nature of the carbonyl and the hydrogen-bond-donating role of pyrrole, guanidiniocarbonyl pyrrole shows much higher affinity towards carboxylate and phosphate groups in comparison with simple guanidinium ion.[5] Besides, there are also some important fundamental researches in the field of artificial receptors for peptides.[6–17]

In eukaryotes, transcription of mRNA is controlled by polymerase II. RNA polymerase II contains a carboxy-terminal domain (CTD) composed of up to 52 heptapeptide repeats (Tyr-Ser-Pro-Thr-Ser-Pro-Ser), and this sequence is thought to be phosphorylated at several positions in vivo, predominantly at serines 2 and 5.[18] CTD regulates the activities of the polymerase by phosphorylation of different patterns, and many important biological events are considered to be related to this process, also, some antitumour drugs have been proved to take effect by regulating the phosphorylation of CTD.[19] Hence, developing receptors, which can effectively associate with CTD is of great biological importance.

In this study, we synthesized a receptor bearing Zn^{2+}–dipicolylamine at one end and guanidiniocarbonyl pyrrole at the other end, and investigated its association with CTD peptides with different phosphorylated patterns.

2. Results and discussion

Molecular structures of the receptor and the controls were shown in Fig. 1a, and the detailed synthesis was provided in experimental section. Receptors **1**, **2** and **3** were controls. Receptor **1** was Zn^{2+}–dipicolylamine (Zn–Dpa), which can selectively bound phosphate group with moderate affinity in aqueous solution. Receptor **2** was guanidiniocarbonyl pyrrole (GCP), which can bind oxygen-containing anions via electrostatic force and hydrogen bond. Receptor **3**, which does not have guanidine group, was precursor of receptor **4**. Receptor **4** was the anticipant receptor with Zn–Dpa at one end and GCP at the other end, a benzene was employed as the linker, which is considered to provide the receptor with rigidity as well as hydrophobic property.

The association between the receptors and CTD peptides with different phosphorylated patterns was investigated by isothermal

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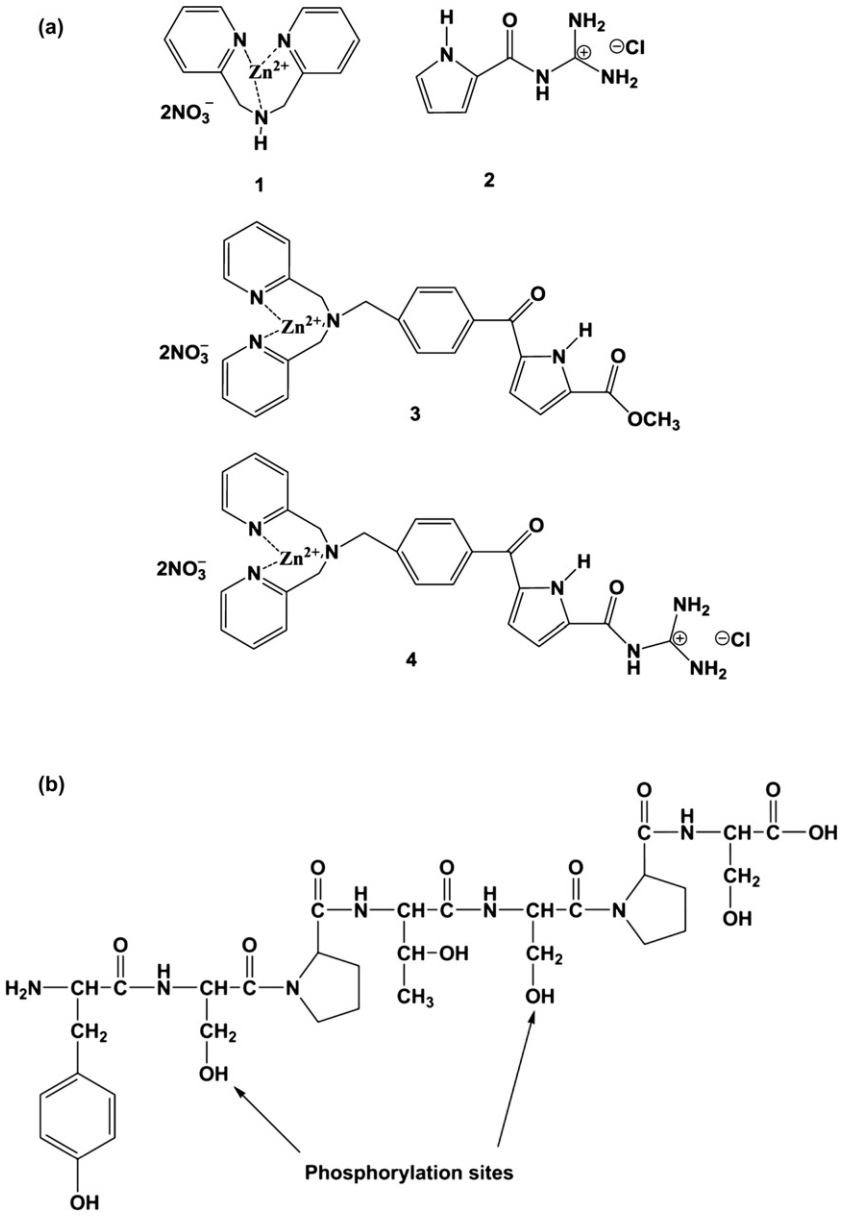

Fig. 1. (a) Molecular structures of the synthetic receptors, (b) showed the phosphoralated patterns of CTD.

titration calorimetry (ITC), and the results were summarized in Table 1 '—' Indicate that the reaction heat is too small to be accurately fitted in Origin. For receptor **1** and **2**, 20 mM receptor was loaded in the syringe and 1 mM peptide was loaded in the cell. For receptor **3** and **4**, limited by their solubility, 1–2 mM peptide was loaded in the syringe and 80–100 μM receptor was loaded in the cell. YS, $Y_{\mathbf{2p}}S$, $Y_{\mathbf{5p}}S$ and $Y_{\mathbf{2p5p}}S$ denote non-phosphorylated CTD peptide, CTD peptide phosphorylated at 2 site, 5 site, and at both 2,5 site, respectively. Ac–$Y_{\mathbf{2p5p}}S$–NH_2 denotes $Y_{\mathbf{2p5p}}S$ with the terminal groups are protected.

Receptor **1** is Zn–Dpa, which has been extensively used as the coordination moiety of receptors/sensors for the recognition of biologically relevant phosphate compounds. From Table 1, we can see that receptor **1** shows moderate affinity towards the phosphorylated CTD peptides, and the association constant is about 10^3, while the association between receptor **1** and non-phosphorylated CTD peptide is too weak to be detected. The association between receptor **1** and bis-phosphorylated CTD peptide $Y_{\mathbf{2p5p}}S$ was well fitted using 'two set of sites' mode, and thus have two association constants, and K2 is much higher than K1, this is because $Y_{\mathbf{2p5p}}S$ has two phosphate groups and more negative charge, the association of the first Zn–Dpa decreased the net negative charge of the peptide, and thus reduced the electrostatic attraction. The associations between Zn–Dpa and the phosphorylated CTD peptides were all endothermic, which are considered to be entropy-driven.

Receptor **2** is a simple GCP, which can bind oxygen-containing anions through electrostatic and hydrogen-bonding interaction.[5] In comparison with Zn–Dpa, the associations between GCP and the peptides were much weaker, with all the association constants below 100. Thus, GCP have no selectivity towards the peptides. The association is exothermic.

Receptor **3** is composed of Zn–Dpa, pyrrole and benzene groups, thus, it could probably associate with the peptides through multiple interactions (coordination, hydrophobic and hydrogen-bond interactions). In comparison with simple Zn–Dpa, receptor

Table 1
Stoichiometry (n), binding constant (K, M^{-1}), enthalpy (ΔH, cal mol^{-1}) and entropy (ΔS, cal mol^{-1} K^{-1}) for the association between the receptors and the CTD peptides at 25 °C

Receptor	Peptide	n[a]	K	ΔH	ΔS
1	$Y_{\mathbf{2p}}S$	1.0	392±46.1	4.62 E3±0.76 E3	27.2
1	$Y_{\mathbf{5p}}S$	1.0	435±42.5	4.74 E3±0.80 E3	28.1
1	$Y_{\mathbf{2p5p}}S$	1.0	4.93 E3±1.29E3	2.89 E3±0.58 E3	26.4
		1.0	448±211	1.39 E3±1.46 E3	16.8
1	YS		—	—	—
2	$Y_{\mathbf{2p}}S$	1.0	20.8±1.03	−7.55 E3±0.51 E3	−24.4
2	$Y_{\mathbf{5p}}S$	1.0	23.4±1.05	−9.46 E3±0.43 E3	−25.7
2	$Y_{\mathbf{2p5p}}S$	1.0	25.5±0.31	−2.9 E4±0.56 E3	−23.1
2	YS	1.0	20.5±0.83	−9.56 E3±0.36 E3	−26.1
3	$Y_{\mathbf{2p}}S$	1.0	1.48 E3±0.73 E3	1.8 E3±0.13 E3	24.9
3	$Y_{\mathbf{5p}}S$	1.0	5.57 E3±0.59 E3	1.7 E3±0.15 E3	22.6
3	$Y_{\mathbf{2p5p}}S$	1.0	2.04 E4±0.27 E4	4.1 E3±0.36 E3	22.5
3	YS		—	—	—
4	$Y_{\mathbf{2p}}S$	0.45± 0.12	2.13 E4±0.88 E3	447.8±88.6	21.3
		4.4±1.6	917±74.9	917±74.9	14.7
4	$Y_{\mathbf{5p}}S$	1.02±0.42	2.98 E4±0.47 E3	2.68 E3±1.11 E3	25.9
		1.65±1.10	3.13 E3±0.15 E3	1.39 E3±70.7	19.4
4	$Y_{\mathbf{2p5p}}S$	0.09±0.06	1.09 E5±0.81 E4	2.20 E3±0.42 E3	29.6
		0.19±0.05	5.3 E3±0.12 E3	9.2 E3±0.44 E3	27.8
4	YS		—	—	—
4	Ac−$Y_{\mathbf{2p5p}}S$−NH_2	0.14±0.06	4.74 E4±0.14 E3	1.15 E4±56.85	24.0
		0.34+0.11	2.33 E3±0.78 E3	1.14 E4±0.33 E3	60.2

[a] For receptor **1**, **2** and **3**, the stoichiometry was fixed to 1.0, and the data plots were well fitted to the corresponding model. For receptor **4**, the data plots were fitted without fixing stoichiometry.

3 shows increased affinity towards all the phosphorylated CTD peptides, this could be reasonably ascribed to the additional force provided by benzene and pyrrole, but its association with non-phosphorylated peptide YS is still weak, and can not be measured. Additionally, receptor **3** shows higher affinity towards $Y_{\mathbf{5p}}S$ (K_a=5.57 E3) in comparison with $Y_{\mathbf{2p}}S$ (K_a=1.48 E3), and this could probably because that the phosphate of $Y_{\mathbf{5p}}S$ is closer to *C*-terminal carboxylate thus has more local net negative charge than phosphate of $Y_{\mathbf{2p}}S$.

Receptor **4** is the anticipant receptor, which contains Zn−Dpa, benzene and GCP, and is expected to associate phosphorylated CTD peptide with higher affinity. All the associations between receptor **4** and the phosphorylated CTD peptides were endothermic (typical ITC titration curve of receptor **4** to $Y_{\mathbf{2p5p}}S$ is shown in Fig. 2), indicating the entropy-driven nature of the association. In comparison with receptor **1**, **2** and **3**, receptor **4** shows much higher affinity towards bis-phosphorylated CTD peptide $Y_{\mathbf{2p5p}}S$ with association constant of about 10^5. This indicates that GCP and Zn−Dpa are synergistically involved in the association between receptor **4** and $Y_{\mathbf{2p5p}}S$.

The association between receptor **4** and $Y_{\mathbf{2p5p}}S$ were further investigated by molecular docking. The results in Fig. 3 show that: Zn−Dpa coordination group will associate with phosphate on 5-Ser, and this is independent of the initial position of Zn−Dpa, indicating the higher affinity of Zn−Dpa towards 5-Ser. This is consistent with the higher affinity (measured by ITC) of receptor **3** towards $Y_{\mathbf{5p}}S$ in comparison with $Y_{\mathbf{2p}}S$. Additionally, it can be observed in Fig. 3 that GCP on receptor **4** is far from either the terminal carboxylate or phosphate on 2-Ser, indicating no direct association occurs between GCP and these two groups, furthermore, no hydrogen bond was found between GCP and the peptide in Autodock, thus, GCP probably only provides electrostatic force for the association.

To further understand the interaction between receptor **4** and $Y_{\mathbf{2p5p}}S$, a terminal groups-protected bis-phosphorylated CTD peptide, i.e., Ac−$Y_{\mathbf{2p5p}}S$−NH_2, was introduced. ITC data showed that the association between receptor **4** and Ac−$Y_{\mathbf{2p5p}}S$−NH_2 (K=4.74×10^4) is much weaker in comparison with $Y_{\mathbf{2p5p}}S$ (K=1.09×10^5), indicating the terminal carboxylate group is also actively involved in the association. Therefore, receptor **4** tend to associate with *C*-terminal bis-phosphorylated CTD peptide, i.e., $Y_{\mathbf{2p5p}}S$, in comparison with other CTD peptides. The schematic

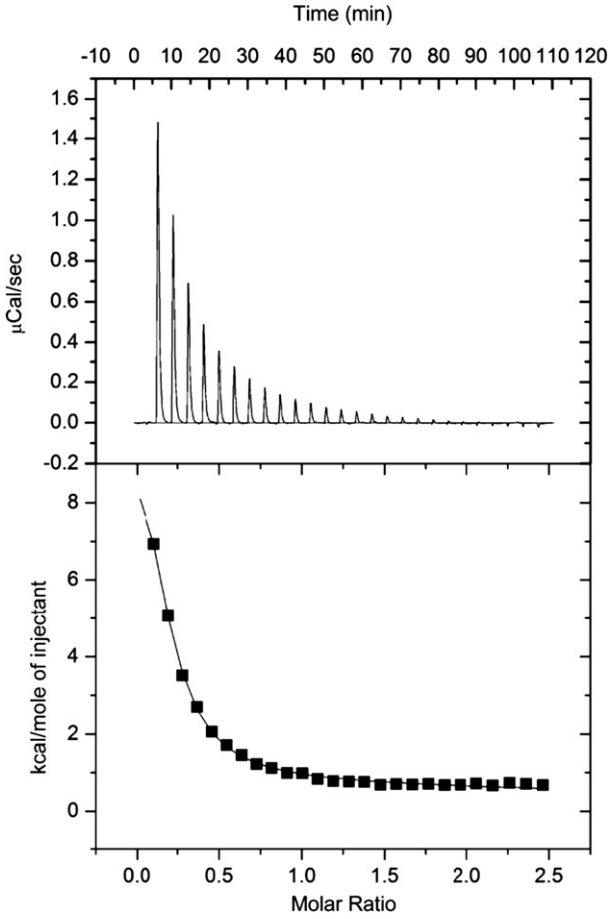

Fig. 2. ITC data for titration of 80 μM receptor **4** with 1 mM $Y_{\mathbf{2p5p}}S$ at 25 °C.

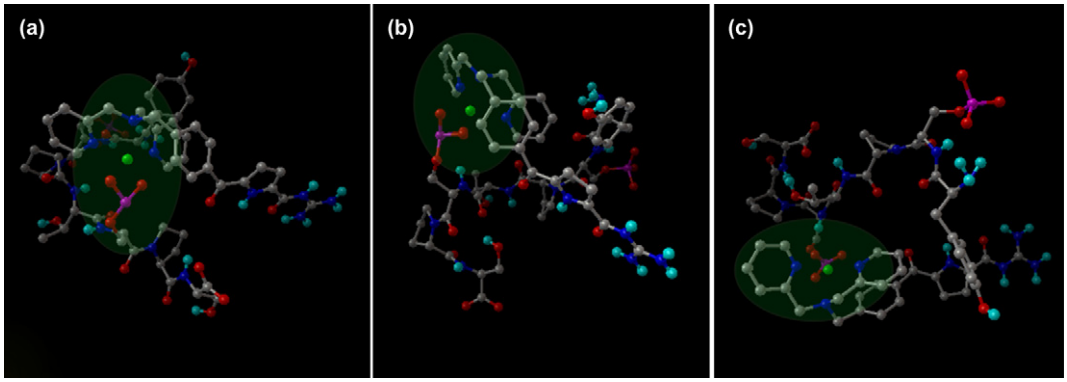

Fig. 3. Molecular docking between receptor **4** and $Y_{\mathbf{2p5p}}S$ with different initial fitting modes: (a) phosphate on 2-Ser was fitted to Zn–Dpa before blind docking, (b) phosphate on 5-Ser was fitted to Zn–Dpa before blind docking, (c) no fitting was performed before blind docking. The final association between phosphate on $Y_{\mathbf{2p5p}}S$ and Zn–Dpa on receptor **4** are highlighted, and all the three figures show that the Zn–Dpa will associate with phosphate on 5-Ser independent of the initial fitting mode.

illustration of the association between receptor **4** and $Y_{\mathbf{2p5p}}S$ is shown in Fig. 4.

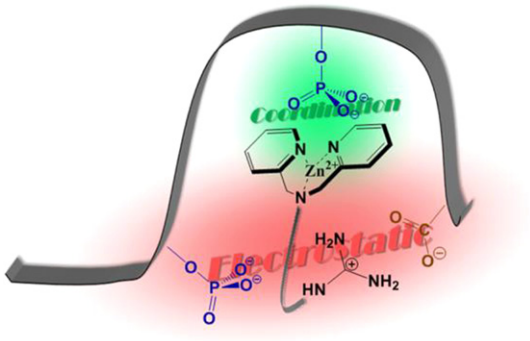

Fig. 4. Schematic illustration of the association between receptor **4** and $Y_{\mathbf{2p5p}}S$.

3. Conclusions

We have synthesized receptor based on guanidiniocarbonyl pyrrole and Zn^{2+}–dipicolylamine groups, and investigated its association with CTD peptides with different phosphorylated patterns. The receptor showed much higher affinity towards *C*-terminal bis-phosphorylated CTD peptide $Y_{\mathbf{2p5p}}S$ in comparison with other forms of CTD peptides as revealed by isothermal titration calorimetry. In combination with molecular docking, the association between the receptor and peptide was well elucidated, and the results indicate that the coordination occurs between Zn–Dpa and phosphate on 5-Ser, while the *C*-terminal carboxylate are synergistically involved in the association via electrostatic interaction.

4. Experimental section

4.1. Materials

Peptides with purity >98% were purchased from GL Biochem (Shanghai) Ltd. All other reagents were commercially available analytical grade and used as received.

4.2. Instrumentation

NMR characterization was performed on VARIAN UNITY-plus 400, molecular mass were measured on Thermo Finnigan LCQ-Advantage MS. Isothermal titration calorimetry measurement was conducted using VP-ITC.

4.3. Synthesis of the receptors

The schematic illustration of the synthesis of the receptors was shown in Fig. 5.

Preparation of receptor **1**: Receptor **1** can be easily obtained by dissolving certain amount of dipicolylamine by $Zn(NO_3)_2$ methanol solution.

Preparation of receptor **2**: Receptor **2** was prepared as reported by Schmuck's group.[5]

Preparation of receptor **3**: 4-(Bromomethyl)benzoyl chloride (10.2 g), 4.0 g methyl 1*H*-pyrrole-2-carboxylate and 8.72 g $ZnCl_2$ were dissolved in 100 mL $CHCl_3$, and the reaction was kept at 50 °C for 72 h under N_2 atmosphere. Then the mixture was filtrated, and the solvent was removed under reduced pressure, and a red solid was obtained. This raw product was further purified by flash chromatography (silica, $CH_2Cl_2/CH_3COOCH_2CH_3$=30:1), the first fraction was collected, and compound **3** was obtained as a white solid (yield: 3.24 g, 31.4%). ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.93 (s, 3H), 4.53 (s, 1.3H), 4.65 (s, 0.7H), 6.83 (d, 1H), 6.93 (d, 1H), 7.52 (d, 2H), 7.90 (t, 2H), 10.04 (br, 1H).

To a solution of 3.22 g compound **3**, 2.19 g 2, 2′-dipicolylamine and 0.67 g K_2CO_3 in 50 mL dry DMF, was added dropwise 1.66 g KI in 10 mL DMF, and the reaction was kept at 40 °C for 10 h under N_2 atmosphere. After removal of the solvent, a brown oil-like product was obtained. The product was dissolved in 100 mL $CHCl_3$ and filtrated, and then was washed with 50 mL 1 N HCl, 50 mL 2 N NaOH and 100 mL H_2O, the organic phase was then dried with $MgSO_4$. After removal of the solvent, a brown solid was obtained, and was further purified by flash chromatography (silica, $CHCl_3$/CH_3OH=30:1), the second fraction was collected and compound **4** was obtained as a red solid (yield: 2.25 g, 51%). ^{1}H NMR (400 MHz, $CDCl_3$): δ=3.75 (s, 6H), 3.82 (s, 3H), 6.78 (d, 2H), 6.88 (d, 2H), 7.26 (t, 2H), 7.69 (m, 4H), 7.80 (m, 4H), 8.50 (d, 2H). EI-MS: *m*/*e* calcd for $C_{26}H_{25}N_4O_3$ [M] 277.7, found 277.0.

Compound **4** (0.30 g) was dissolved in 24 mL CH_3CN/THF (v/v=5:1), then 0.20 g $Zn(NO_3)_2$ in 11.96 mL CH_3OH was added, after the solution was further stirred for 0.5 h at room temperature, the white precipitation was filtrated and washed with CH_3CN. Receptor **3** (compound **5**) was obtained, ^{1}H NMR (400 MHz, DMSO): δ=3.70 (s, 2H), 3.97 (s, 6H), 4.10 (s, 1H), 4.14 (s, 1H), 4.32 (s, 1H), 4.36 (s, 1H), 6.64 (d, 2H), 6.74 (d, 2H), 7.22 (d, 2H), 7.50 (m, 4H), 7.65 (m, 2H), 7.99 (m, 2H), 8.08 (d, 2H), 8.95 (d, 2H), 10.08 (s, 2H) ppm, MALDI-TOF: *m*/*e* calcd for $C_{26}H_{26}N_5O_6$ Zn $[M]^+$ 567.91, found 568.2.

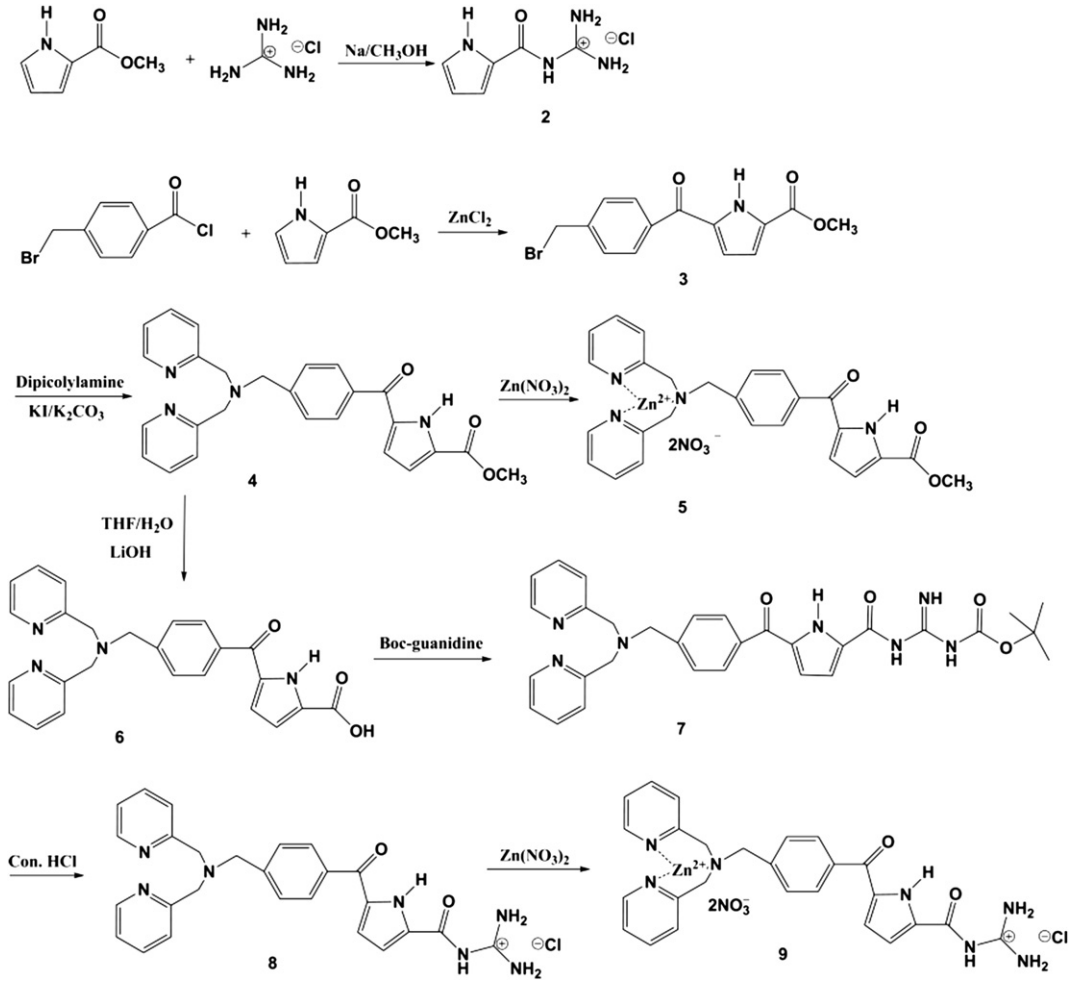

Fig. 5. Synthesis of the receptors.

Preparation of receptor **4**: Compound **4** (1.32 g) and 0.63 g $LiOH·H_2O$ were dissolved in 100 mL THF/H_2O (v/v=4:1), the solution was then stirred at room temperature for two days. After the concentrated to 10 mL, the solution was acidified to pH=3, and yellow precipitation was formed and filtrated, after washed with water, the solid was dried in vacuum and 1.02 g compound **6** was obtained, yield 79.8%. ^{1}H NMR (400 MHz, DMSO): δ=3.77 (s, 6H), 6.77 (s, 2H), 6.82 (s, 2H), 7.26 (t, 2H), 7.61 (m, 4H), 7.80 (m, 4H), 8.50 (d, 2H), 11.68 (br s, 0.1H), 12.35 (br s, 1H) ppm, ESI-MS: *m*/*e* calcd for $C_{25}H_{23}N_4O_3$ $[M+H]^+$ 427.17565, found 427.1764.

Compound **6** (0.93 g), 1.14 g PyBOP and 2 mL NMM were dissolved in 30 mL dry DMF, after stirred at room temperature for 1 h, 0.35 g *N*-Boc-Guanidine was added, the reaction was kept at 40 °C for 24 h, then the solution was concentrated to 20 mL and poured into 200 mL H_2O under vigorous stirring, and a yellow precipitation was formed. After vacuum dried, 0.9 g compound **7** was obtained, yield 79.3%. ^{1}H NMR (400 MHz, DMSO): δ=1.48 (s, 9H), 3.77 (s, 6H), 6.77 (s, 2H), 6.82 (s, 2H), 7.26 (t, 2H), 7.61 (m, 4H), 7.80 (m, 4H), 8.50 (d, 2H), 11.68 (br s, 0.1H), 12.35 (br s, 1H) ppm, ESI-MS: *m*/*e* calcd for $C_{25}H_{23}N_4O_3$ $[M+H]^+$ 568.267, found 568.2677.

Compound **7** (0.60 g) was dissolved in 10 mL concentrated HCl, and the solution was kept stirring for 1 h at room temperature. After vacuum dried, 0.49 g compound **8** was obtained as a yellow solid, yield 92.5%. ^{1}H NMR (400 MHz, DMSO): δ=3.91 (s, 6H), 4.37 (s, 4H), 6.76 (s, 0.34H), 6.77 (m, 0.52H), 7.56 (d, 2H), 7.68 (m, 2H), 7.90 (t, 2H), 8.13 (d, 2H), 8.48 (m, 2H), 8.77 (br s, 3H), 8.85 (m, 1H), 12.38 (br s, 0.49H) ppm, ESI-MS: *m*/*e* calcd for $C_{26}H_{26}N_7O_2$ $[M+H]^+$ 468.2142, found 468.2130.

Compound **8** (0.23 g) was dissolved in 20 mL CH_3OH, then 0.15 g $Zn(NO_3)_2$ in 10 mL CH_3OH was added, after stirred for 0.5 h at room temperature, a red precipitation was formed, after washed with CH_3CN, receptor **4** (compound **9**) was obtained, yield 93.0%. MALDI-TOF: *m*/*e* calcd for $C_{26}H_{25}N_8O_5Zn$ $[M+H_3O]^+$ 612.9, found 612.3.

4.4. ITC measurement

All the peptides and the receptors were dissolved in 50 mM HEPES (pH=7.2). The titration contains an initial injection of 2 μL followed by 26 injections of 10 μL receptors. Heat of dilution was substracted from titration data before curve-fitting in Origin.

4.5. Computational methodologies

Molecules of the receptors and the peptides were generated on Silicon Graphic Indio workstation using Sybyl 7.3 software package, and the conformations of the molecules were optimized and selected according to the previously reported method.[20] The receptors were then fitted to the peptides, typically, for one mode of the fitting between receptor **4** and $Y_{\mathbf{2p5p}}S$: oxygen atom on the phosphate of 2-Ser was fitted to Zn^{2+} of Zn–Dpa.

Subsequently, the fitted molecules were subjected to blind docking in Autock 4.0. The running parameters were according to our previous work.[20]

Acknowledgements

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