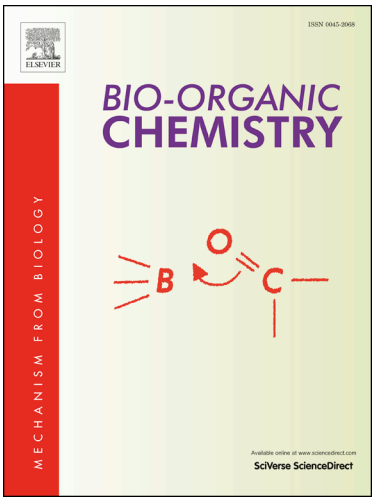

Identification of Novel and Selective Non-peptide Inhibitors Targeting the Polo-Box Domain of Polo-Like Kinase 1

Yanhong Chen, Zhiyan Li, Yu Liu, Tongyuan Lin, Huiyong Sun, Dasong Yang, Cheng Jiang

PII: S0045-2068(18)30765-X
DOI: https://doi.org/10.1016/j.bioorg.2018.08.030
Reference: YBIOO 2487

To appear in: *Bioorganic Chemistry*

Received Date: 24 July 2018
Revised Date: 15 August 2018
Accepted Date: 21 August 2018

Please cite this article as: Y. Chen, Z. Li, Y. Liu, T. Lin, H. Sun, D. Yang, C. Jiang, Identification of Novel and Selective Non-peptide Inhibitors Targeting the Polo-Box Domain of Polo-Like Kinase 1, *Bioorganic Chemistry* (2018), doi: https://doi.org/10.1016/j.bioorg.2018.08.030

Identification of Novel and Selective Non-peptide Inhibitors Targeting the Polo-Box Domain of Polo-Like Kinase 1

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Abstract:

A series of non-peptide inhibitors targeting the polo-box domain (PBD) of polo-like kinase 1 (Plk1) was designed based on the potent and selective minimal tripeptide Plk1 PBD inhibitor. Seven compounds were designed, synthesized and evaluated for fluorescence polarization (FP) assay. The most promising compound **10** bound to Plk1 PBD with IC_{50} of 3.37 μM and had no binding to Plk2 PBD or Plk3 PBD at 100 μM. Molecular docking study was performed and possible binding mode was proposed. MM/GBSA binding free energy calculation were in agreement with the observed experimental results. These novel non-peptide selective Plk1 PBD inhibitors provided new lead compounds for further optimization.

Key words: Polo like kinase 1; Polo-box domain; Inhibitors; Non-peptide; Molecular docking

1. Introduction

Polo-like kinase 1 (Plk1) is a serine/threonine protein kinase which acts as a key regulator in multiple stages of mitotic progression [1]. Plk1 modulates the transition through the G2/M checkpoint by influencing the activation of the CDC25C phosphatase and cyclin B1 [2]. The overexpression of Plk1 is required for the viability of broad spectrum of cancer cells and interference with Plk1 function induces cell apoptosis in most cancer cells [3]. Therefore Plk1 has been considered as an attractive anti-cancer drug target for anticancer drug development.

Five Plks in mammalian cells have been identified (Plk1-5) so far. Among them, Plk1-4 consists of an N-terminal catalytic domain and a C-terminal domain having 1 or 2 highly conserved sequences, termed polo-box domains (PBDs) [4]. A large body of evidence suggests that PBD directs the N-terminal catalytic domain for specific subcellular localization through interacting with phosphoserine/phosphothreonine (pS/pT)-containing motifs. Moreover, the subcellular targeting binding site in PBD forms a compact and druggable interface [5].

Plk1, Plk2 and Plk3 are three closely related members while Plk4 is a distantly related kinase with apparently different expression patterns and physiological functions [3,4]. In contrast to the role of Plk1 in cell proliferation and tumorigenesis, the two most closely related kinases, Plk2 and Plk3, seem to have a role in checkpoint-mediated cell-cycle arrest to ensure genetic stability and prevent oncogenic transformation [3-5]. Thus, the development of Plk1-specific inhibitors could be important for anti-Plk1 cancer therapy.

For a long period, several potent orthosteric Plk1 inhibitors targeting the ATP-binding catalytic domain have been reported [3]. However, the ATP-binding sites of the catalytic domains of protein kinases are closely related, these efforts suffered from a lack of selectivity. Rigosertib (ON01910) was reported as a Plk1 allosteric inhibitor, which showed potent Plk1 inhibitory activity and antitumor activity [6-8]. However, it has an inhibitory effect on multiple target (Plk3, Chk1, Chk2, BCR-ABL, Fyn, Src and Ras) [9], among which Plk3 was considered as a negative regulator responsible for the inhibition of cell cycle progression and tumor cell development at multiple stages of mitosis. Based on the structure of PBIP1 which was isolated as a PBD-interacting protein crucial for centromeric localization of Plk1, a minimal peptide (PLHSpT, **1**) was identified as an inhibitor with high affinity toward the Plk1 PBD and high selectivity against Plk2 PBD and Plk3 PBD [10].

After the identification of minimal peptide (**1**) with high affinity toward the Plk1 PBD, several phosphate peptides and peptidomimetics were reported (**Fig. 1**, **2**-**4**) [11-19]. Then a series of (2*S*,3*R*)-2-amino-3-methyl-4-phosphonobutanoic acid (Pmab) incorporated peptidomimetic was reported (**Fig. 1**, **5**) to produce a phosphatase-stable peptidomimetic with complete retention of inhibitory potency [20]. To date, most of the modification made to peptides and peptidomimetics acting as Plk1 PBD inhibitors focusing on the 1-3 *N*-terminal residues of PLHSpT. Very few modification has been made to the C-terminal SpT part which was considered essential for high-affinity binding [10]. In this study, we make the modification on the C-terminal SpT residue based on the highly potent Plk1 tripeptide (**Fig. 1**, **4**) reported [17] and provide new

non-peptide skeleton for further modification toward the discovery and development of highly potent and selective Plk1 PBD inhibitors.

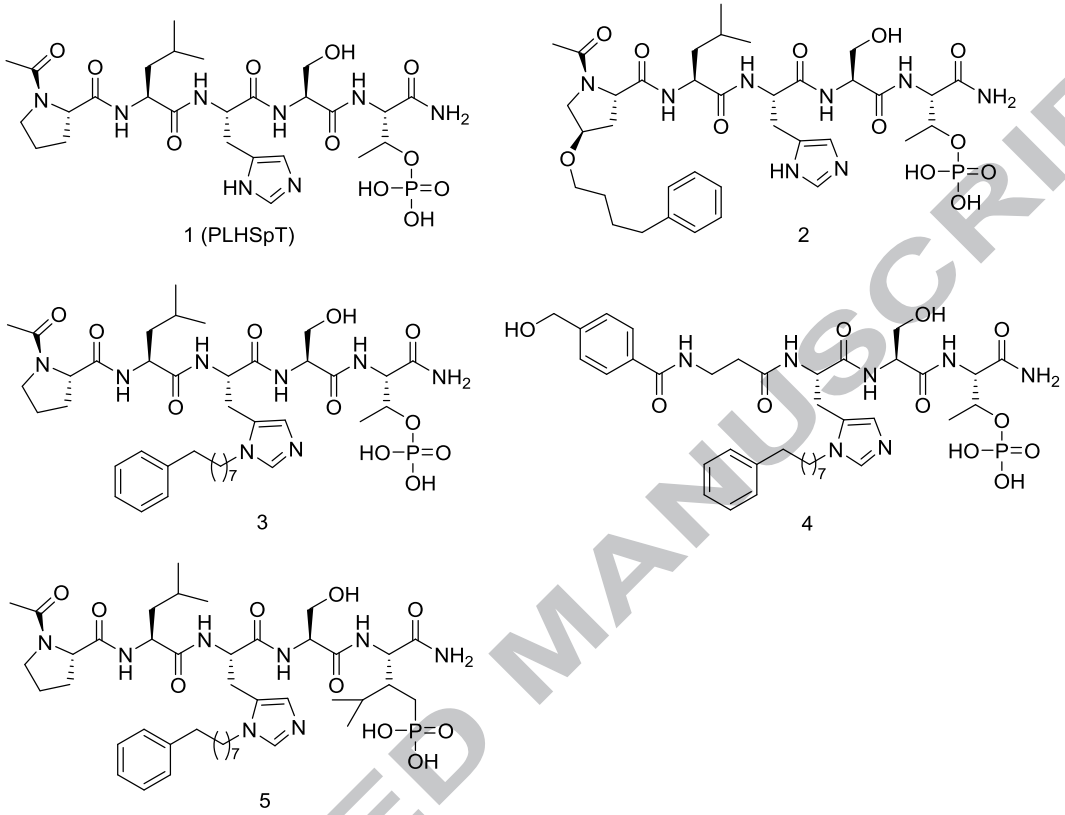


Fig. 1. Examples of the reported phosphate peptides and peptidomimetics as Plk1 PBD inhibitors.

2. Results and discussion

2.1. Design, synthesis and evaluation

According to the binding mode of **4** with Plk1 PBD (PDB ID: 4WHH), the *N*-terminal fragment of peptidomimetic **4** and $C_6H_5(CH_2)_8$-group on the imidazole ring were directed toward the Tyr-rich hydrophobic channel, whereas the negatively charged pThr phosphoryl group mediated strong electrostatic interactions with the H538 and K540 residues [17]. The residues of Ser and Thr in **4** acted as a linker and limited the direction of the phosphate acid substitution. In our research, the imidazole ring of the His in **4** was substituted by a triazole ring for easier synthesis (blue in **Fig.2**). The

linker formed by Ser and Thr was substituted by a rigid linker (red in **Fig.2**) which may also limit the direction of the terminal phosphate acid. It was reported that Pmab incorporated peptidomimetic could produce a phosphatase-stable analogue of phosphothreonine (pThr) [20]. Thus 3-phosphonopropanoic acid was attached to the aromatic ring as compound **5**. Then compound **6**, **7** and **8** were designed as shown in **Fig.2**.

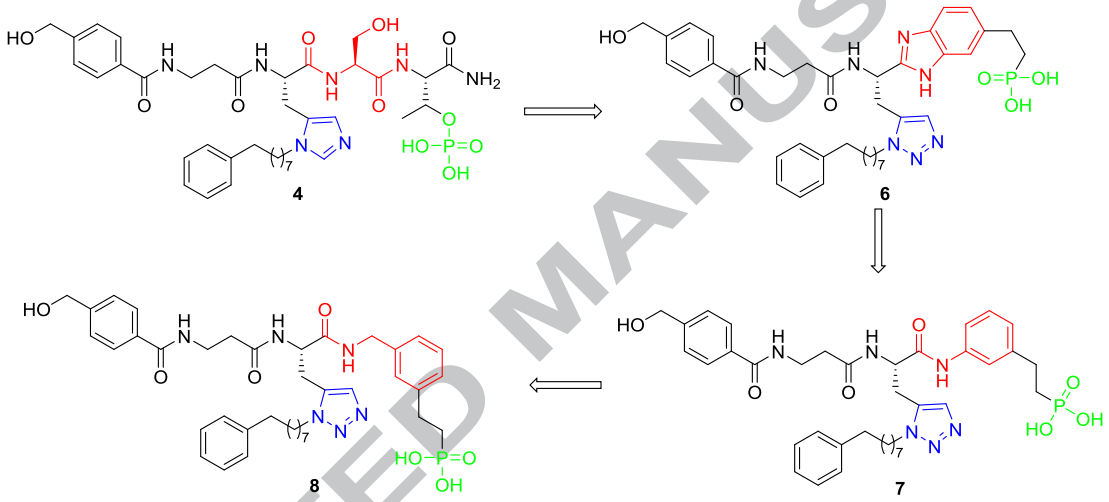
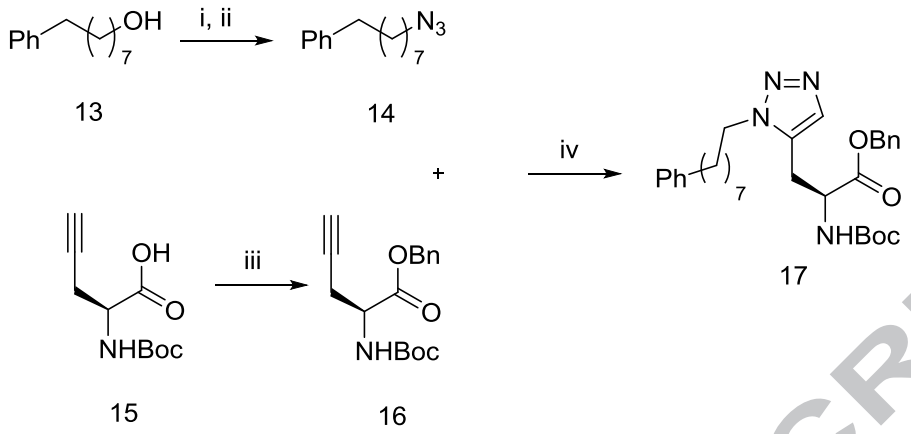
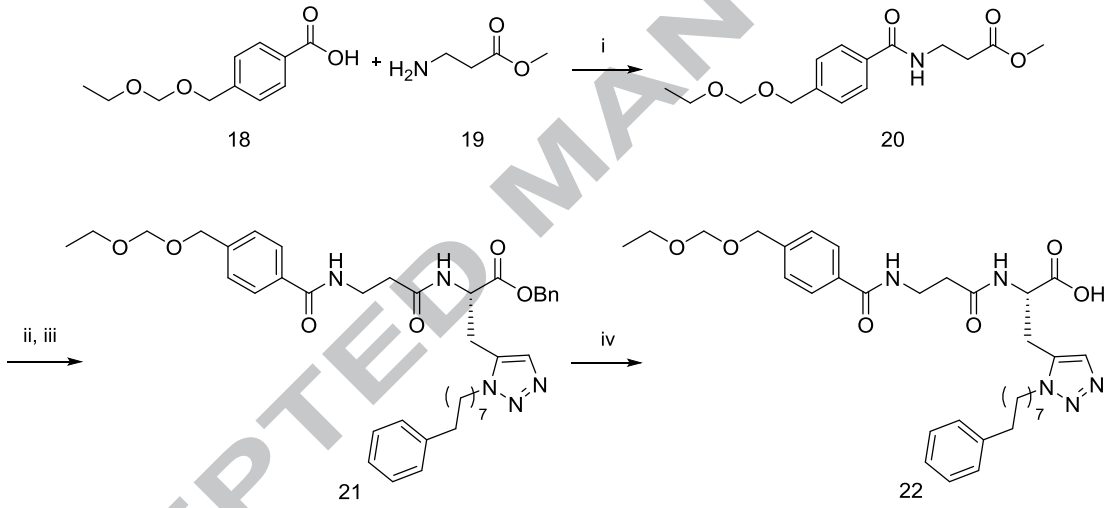


Fig. 2. Design strategy of compound **6**, **7** and **8** with new skeleton based on **4**.

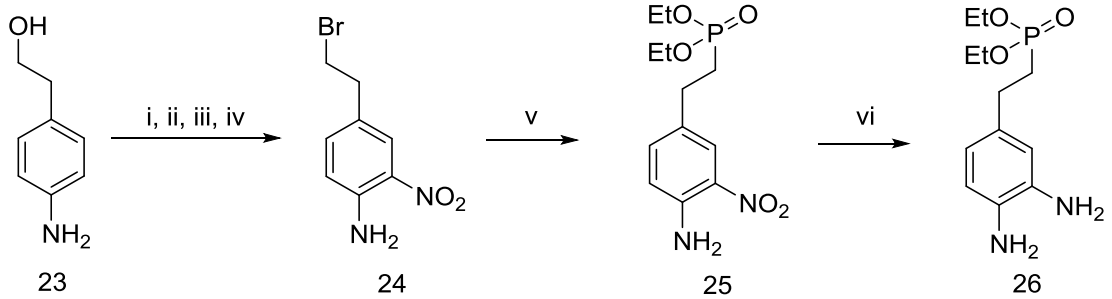
The key intermediate **22** was synthesized using the method as shown in **Scheme 1** and **Scheme 2**. Compound **6** was synthesized according to the route shown in **Scheme 3** and **Scheme 4**. The intermediate of **7** and **8** was prepared according to the method as shown in **Scheme 5**. And the target compound **7** and **8** were readily obtained using the route in **Scheme 8**.



Scheme 1. Reagents and conditions: (i) MsCl, Et_3N, DCM, rt, 2 h; (ii) NaN_3, DMF, 80℃, 5 h; (iii) K_2CO_3, BnBr, DMF, rt, 1 h; (iv) Cp*$RuCl(PPh_3)_2$, DCE, reflux, 12 h.

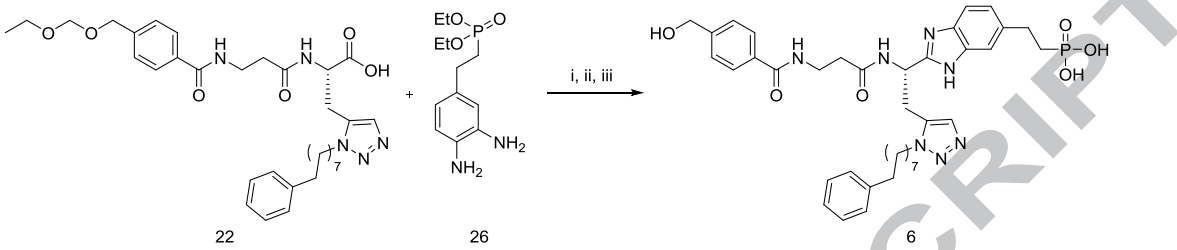


Scheme 2. Reagents and conditions: (i) EDCI, HOBt, Et_3N, DCM, rt, 5 h; (ii) 3N NaOH, 1,4-dioxane, rt, 2 h; (iii) **17**, EDCI, HOBt, Et_3N, DCM, rt, 5 h; (iv) H_2, 10% Pd/C, MeOH, rt, 40 min.

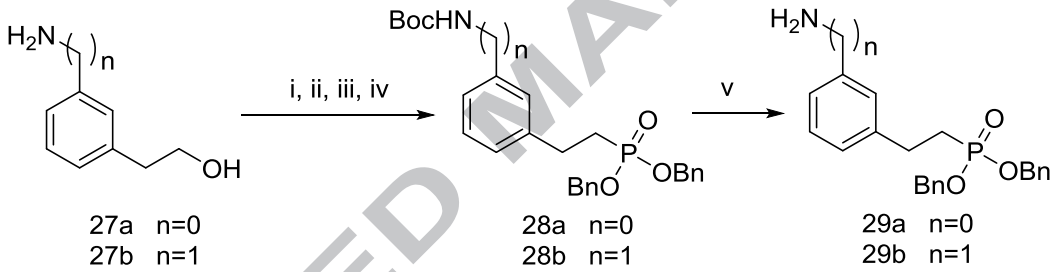


Scheme 3. Reagents and conditions: (i) Ac_2O, Et_3N, toluene, 80 ℃, 1 h; (ii) HNO_3, Ac_2O, 0℃, 1

h; (iii) 10N NaOH, H_2O, MeOH, 15 min; (iv) HBr, reflux, 10 h; (v) Triethyl phosphite, reflux, 5 h; (vi) H_2, 10 % Pd/C, MeOH, r.t., 4 h.



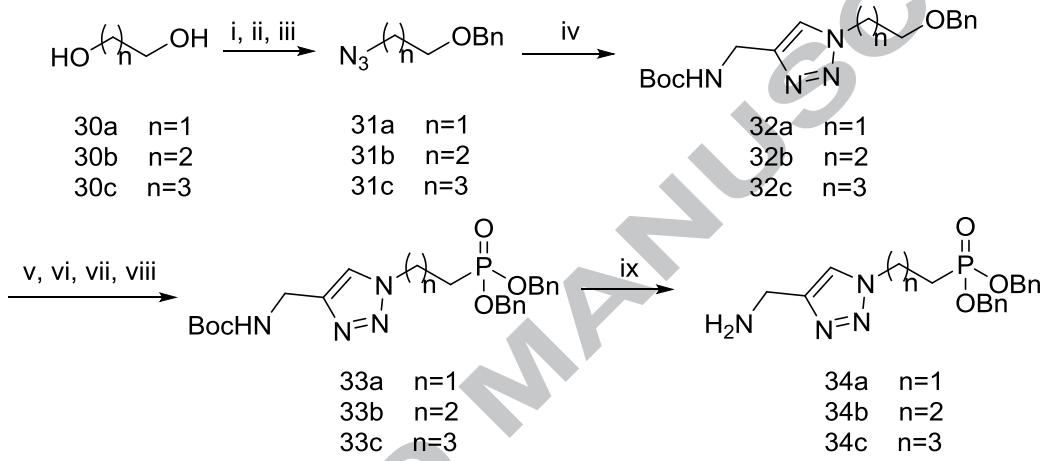
Scheme 4. Reagents and conditions: (i) HATU, HOBt, DIPEA, DCM, rt, 3 h; (ii) Ac_2O, 65℃, 6 h; (iii) TMSBr, DCM, rt, 10 h.



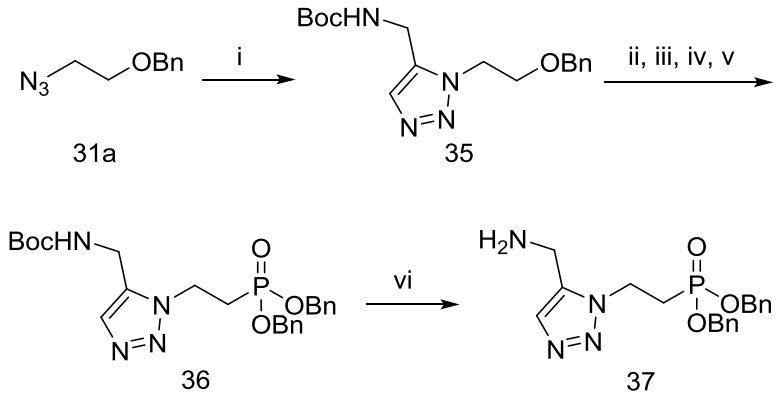
Scheme 5. Reagents and conditions: (i) Boc_2O, NaOH, $NaHCO_3$, rt, 5 h; (ii) MsCl, Et_3N, rt, 2 h; (iii) NaBr, TBAB, DMF, 36 h; (iv) Dibenzyl phosphite, TBAI, Cs_2CO_3, DMF, rt, 10 h; (v) TFA, DCM, rt, 0.5 h.

The binding affinity of **6**, **7** and **8** to Plk1 PBD was evaluated using our optimized fluorescein polarization (FP) binding assay [21]. As shown in **Table 1**, none of the three compound (**6**, **7** and **8**) showed binding affinity to Plk1 PBD. The binding of the pT residue is essential for high-affinity binding, a slight change may cause complete loss of binding. Further modification was focused on how to adjust the direction of the 3-phosphonopropanoic acid side chain. Triazole group was introduced to mimic the aromatic ring (red in **Fig.2**) linked with 3-phosphonopropanoic acid.

Appropriate azide was reacted with alkyne in the presence of copper sulfate and sodium ascorbate (VcNa) to give the protected triazolyl contained intermediate (**Scheme 6**). The alternative 1,5-regioisomer was achieved using the ruthenium catalyst Cp*RuCl(PPh_3)$_2$ (**Scheme 7**) [22,23]. Then compound **9** and **10** were synthesized using the similar method as **7** and **8** as shown in **Scheme 8**.

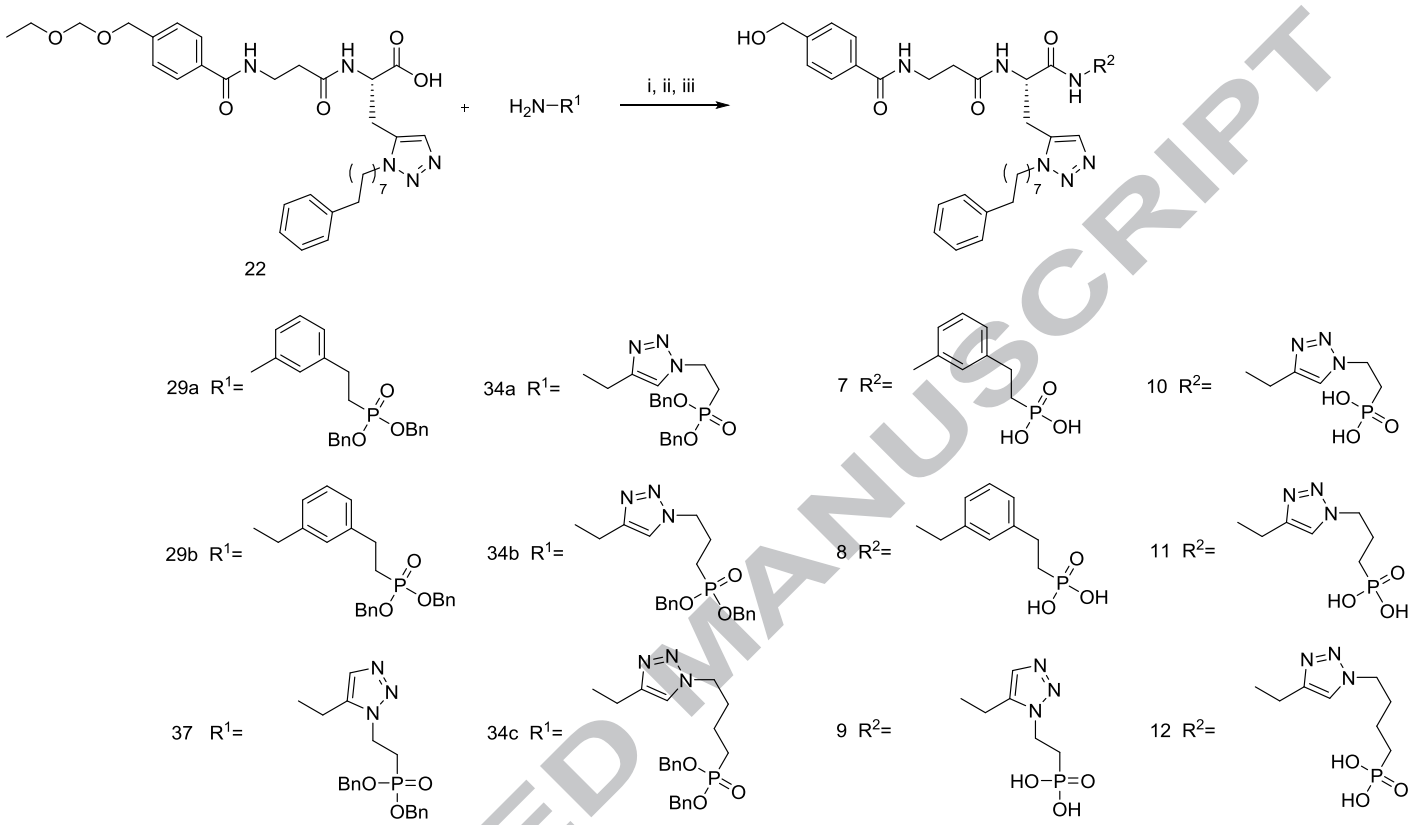


Scheme 6. Reagents and conditions: (i) NaH, BnBr, 0℃, 2 h; (ii) MsCl, Et_3N, rt, 2 h; (iii) $TMSN_3$, K_2CO_3, DMF, 100 ℃, 5 h; (iv) *N*-Boc-propargylamine, $CuSO_4$, sodium ascorbate, t-BuOH:H_2O= 1:1, 3 h; (v) H_2, Pd/C, MeOH, rt, 5 h; (vi) MsCl, Et_3N, rt, 2 h; (vii) NaBr, TBAB, DMF, 36 h; (viii) Dibenzyl phosphite, TBAI, Cs_2CO_3, DMF, rt, 10 h; (ix) TFA, DCM, rt, 0.5 hr.



Scheme 7. Reagents and conditions: (i) *N*-Boc-propargylamine, Cp*RuCl(PPh_3)$_2$, DCE, reflux, 5 h; (ii) H_2, Pd/C, MeOH, rt, 5 h; (iii) MsCl, Et_3N, rt, 2 h; (iv) NaBr, TBAB, DMF, 36 h; (v)

Dibenzyl phosphite, TBAI, Cs_2CO_3, DMF, rt, 10 h; (vi) TFA, DCM, rt, 0.5 h.

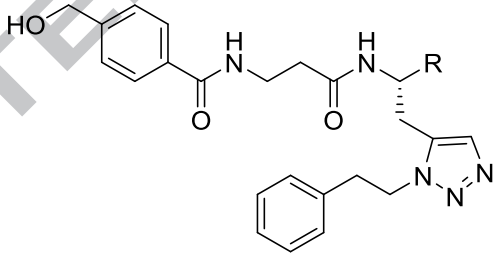


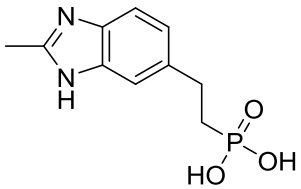
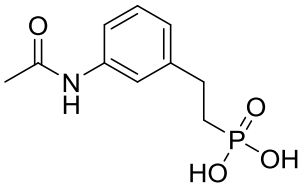
Scheme 8. Reagents and conditions: (i) HATU, HOBt, DIPEA, DCM, rt, 3 h; (ii) H_2, 10% Pd/C, MeOH, rt, 40 min; (iii) CF_3COOH, rt, 1 h.

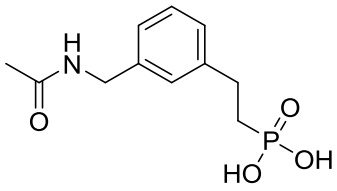
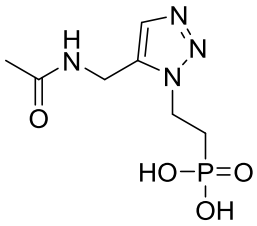
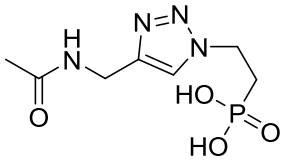
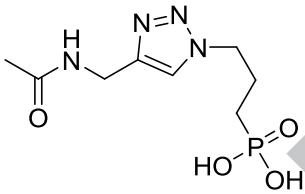
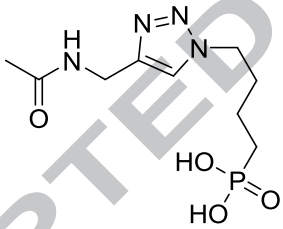
The binding affinity of **9** and **10** to Plk1 PBD was evaluated using FP assay. As shown in **Table 1**, the 1,5-regioisomer **9** showed no binding affinity to Plk1 PBD, while the 1,4-regioisomer **10** showed moderate Plk1 PBD binding affinity (IC_{50} = 3.37 μM). Thus the 1,4-regioisomer **10** was selected for further exploration. Compounds with different length of the linker between the triazolyl and the phosphonic acid was made to find the optimal length. Compound **11** and **12** was

synthesized using the same method as **10**. The binding affinity of **11** and **12** was evaluated using FP assay. As shown in **Table 1**, **11** showed similar Plk1 PBD binding affinity (IC_{50} = 5.45 μM) as **10**, while the binding affinity of **12** was slightly decreased (IC_{50} = 11.12 μM), suggesting that the 1,4-regioisomer triazolyl attached with ethyl or propyl could be an optimal length of linker for the phosphonic acid. The binding affinity of **10**, **11** and **12** with Plk2 PBD and Plk3 PBD was also evaluated using FP assay. As shown in **Table 1**, none of the tested compounds showed binding affinity to Plk2 PBD and Plk3 PBD. The new identified non-peptide inhibitors showed excellent selectivity to Plk1 PBD against Plk2 PBD and Plk3 PBD.

Table 1. Structures of designed compounds and their binding affinity to Plk1 PBD, Plk2 PBD and Plk3 PBD



Compound	R	Plk1 PBD IC_{50} (μM)[a]	Plk2 PBD IC_{50} (μM)[a]	Plk3 PBD IC_{50} (μM)[a]
6		>100	ND	ND
7		>100	ND	ND

8		>100	ND	ND
9		>100	ND	ND
10		3.37	>100	>100
11		5.45	>100	>100
12		11.12	>100	>100
PLHSpT(**1**)	-	0.62	>100	>100

[a] IC_{50} Values represent the mean of at least two independent determinations.

N.D.: Not Determined

2.2. Structural and energetic analyses of the potential inhibitors

Routine methods of Molecular docking (Autodock 4.2) [24] and MM/GBSA binding free energy calculation were used for the analyses [25-30]. As shown in **Table 2**, compound **10** exhibits the strongest predicted binding affinity among the four investigated compounds, which is consistent with the experimental data. Compound **9**

fails in the docking simulation because of the neighborhood substitution of the phosphate-containing tail, which may markedly affect the binding orientation of the compound. Nevertheless, all the other compounds show a similar binding pose in Plk1 with the phosphate-containing tail toward K540 to match the electrostatic environment (**Fig. 3 A~C**), which is consistent with the binding mode of the co-crystallized ligands in 4WHH (**Fig.3D**) and 3RQ7 (**Fig.3F**).

Table 2. Predicted binding affinity of four investigated compounds.

compound	docking score (kcal/mol)	MM/GBSA (kcal/mol)	IC_{50} (μM)
10	-9.69	-52.47	3.37
6	-8.97	-46.17	>100
8	-9.30	-39.62	>100
9	N.A.	N.A.	>100

To investigate the binding affinity difference between compounds **10** and **6**/**8**, we decomposed the total binding free energy into residue-inhibitor pairs [31-33]. **Fig.3E** and **Fig.3G** shows the energetic difference spectrums of compounds **10**&**6** and **10**&**8**, respectively, where a positive value means attenuated interaction of the corresponding residue to compound **6**/**8** compared with compound **10**. Compared with compounds **6** and **8**, compound **10** shows a 180º-rotated phenylmethanol-containing head (**Fig.3A**), which forms strong interaction with R516 and F535 (**Fig.3E** and **Fig.3G**). One may concern that the docking pose of the phenylmethanol-containing head in compound **10** cannot overlap well with the co-crystallized ligand in 4WHH (**Fig.3D**), which contains a similar group as compound **10**. This is because the modified backbone of compound **10** hinders the phenylmethanol-containing head staying in the original

place (red box in **Fig.3D**). Nevertheless, the new binding position of the phenylmethanol-containing head is similar with another co-crystallized ligand in 3RQ7 (red box in **Fig.3F**), indicating that the predicted binding mode of compound **10** is reasonable for analysis. Besides the weakened interaction with R516 and F535 of compounds **6** and **8**, the benzoimidazole-containing tail of compound **6** may be too rigid to adopt a suitable binding conformation in Plk1, which also leads to the attenuated interaction with N533 and K540 (**Fig.3E**) compared with compound **10**; and the meta substitution of the phosphate-containing tail to benzene in compound **8** may modify the orientation of the backbone of the compound and thus leads to the decreased interaction with Y481 and F482 (**Fig.3C**).

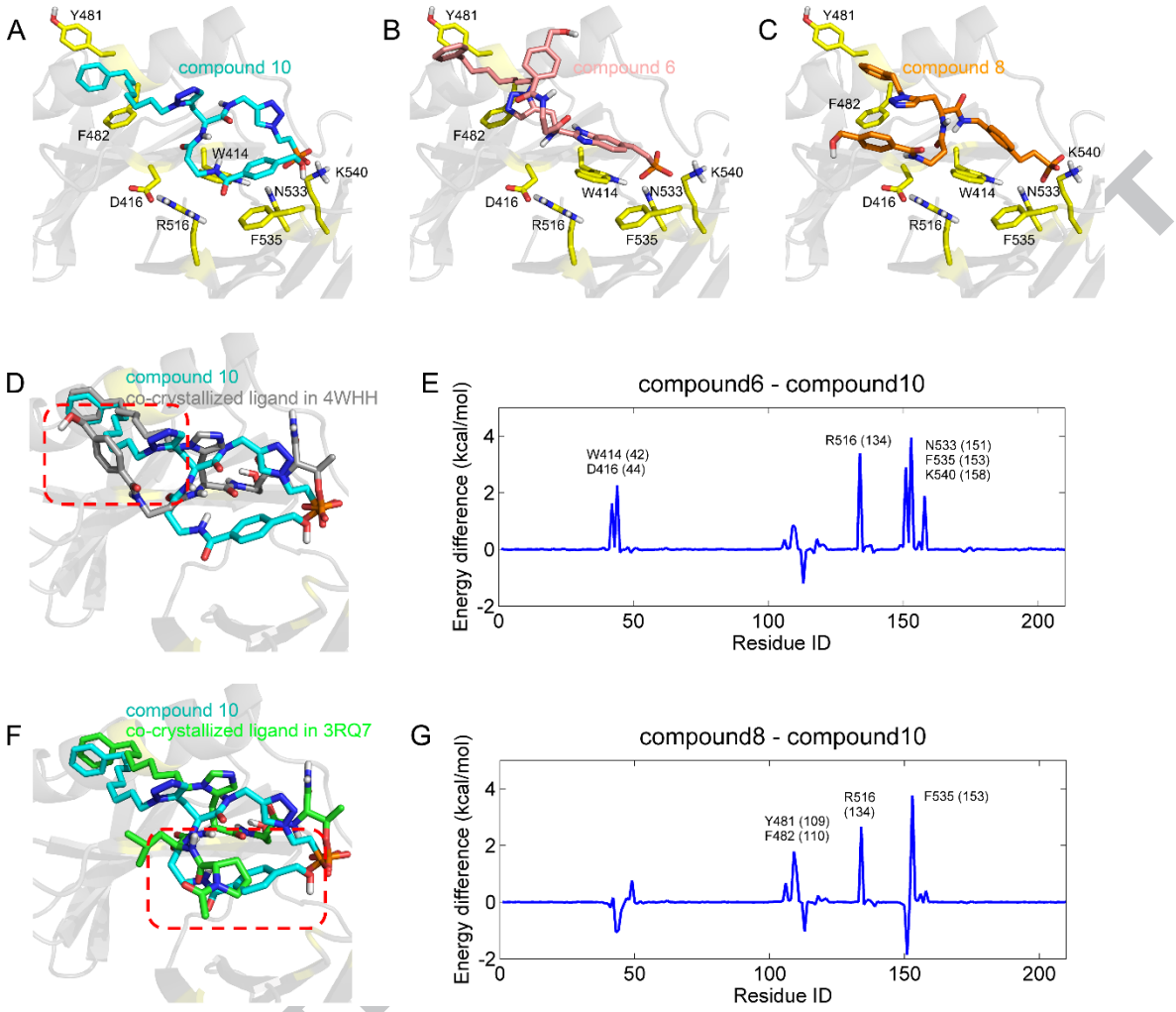


Fig.3. Binding modes and energetic analyses of compounds **10** (**A**), **6** (**B**) and **8** (**C**). Binding energy difference based on residue-inhibitor pairs for compounds **6**&**10** and **8**&**10** were shown in panel **E** and **G**, respectively (calculated by $\Delta\Delta G=\Delta G_{\mathbf{6/8}}-\Delta G_{\mathbf{10}}$), where a positive value means attenuated binding affinity of the corresponding residue to compound **6**/**8** compared with compound **10**. Here, vital residues contributing to the energetic difference were colored in yellow stick model in panels **A**~**C**. To validate the docking pose, compound **10** was overlapped with the co-crystallized ligands in 4WHH (gray in panel **D**) and 3RQ7 (green in panel **F**).

3. Conclusion

In summary, we have designed and synthesized a new series of non-peptide inhibitors

targeting the polo-box domain of Polo-like kinase 1 based on the reported tripeptide Plk1 PBD inhibitor. All of the synthesized compounds were evaluated for their *in vitro* Plk1 PBD inhibitory activity. Among them, compounds **10**, **11** and **12** containing the 1,4-regioisomer triazolyl group were significantly active with IC_{50} values ranging from 3.37 to 11.12 μM. Molecule docking studies were performed and suggested possible binding orientation, MM/GBSA binding free energy calculation were in agreement with the observed experimental results.

Comparing to the reported phosphate peptides and peptidomimetics, our compounds showed moderate activity to Plk1 PBD. However, the non-peptide skeleton may have advantage over other peptide based inhibitors. The newly identified compounds represent a novel non-peptide Plk1 PBD inhibitors with high selectivity to Plk2 PBD and Plk3 PBD. These preliminary research findings provided novel lead compounds for further optimization towards the discovery and development of novel non-peptide Plk1 PBD inhibitors as potential anticancer agents.

4. Experimental section

4.1 Chemistry.

All reagents were purchased from commercial sources. Organic solutions were concentrated in a rotary evaporator (Büchi Rotavapor) below 50 °C under reduced pressure. Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates(GF-2.5) and visualized under UV light. The ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AV-300 instrument using deuterated solvents with

tetramethylsilane (TMS) as internal standard. ESI-MS and high-resolution mass spectra (HRMS) were recorded on a Water Q-Tof micro mass spectrometer.

*4.1.1. (8-azidooctyl)benzene (**14**)*

8-Phenyl-1-octanol (**13**) (0.50 g, 2.42 mmol) was dissolved in dichloromethane (10 ml), triethylamine (0.49 mg, 4.85 mmol) was added. Methanesulfonyl chloride (0.42 g, 3.63 mmol) was added dropwise at 0-5 ℃. The mixture was stirred at room temperature for 2 hrs. Then dichloromethane (20 ml) was added and the mixture was washed with water, dried, evaporated and used without further purification. The residue was then dissolved in DMF (10ml), NaN_3 (0.47 g, 7.27 mmol) was added and the mixture was stirred at 80℃ for 5 hrs. Water (100ml) was added and the mixture was extracted by ethyl acetate (50 ml*3). The organic phase was collected, dried, evaporated and the purified by column chromatography (PE). **14** was obtained as a colorless oil (0.45 g, yield 80.3% for two steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.60-6.89 (m, 5H), 3.31 (t, J = 6.9 Hz, 2H), 2.70 (t, J = 6.9 Hz, 2H), 1.80-1.56 (m, 4H), 1.41 (s, 8H) ppm.

*4.1.2. Benzyl (S)-2-(Boc-amino)pent-4-ynoate (**16**)*

(*S*)-*N*-Boc-Propargyl glycine (**15**) (0.80 g, 3.75 mmol) was dissolved in DMF (5 ml), K_2CO_3 (1.04 g, 7.50 mmol) was added. Then benzyl bromide (0.40 ml, 3.38 mmol) was added and stirred at room temperature for 1 hr. Water (50ml) was added and the mixture was extracted by ethyl acetate (30 ml*3), the organic phase was collected,

dried, evaporated and the purified by column chromatography (PE:EA=10:1). **16** was obtained as a colorless oil (0.89 g, yield 78.2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35 (s, 5H), 5.38 (d, J = 7.2 Hz, 1H), 5.29-5.07 (m, 2H), 4.58-4.40 (m, 1H), 2.82-2.59 (m, 2H), 2.01 (s, 1H), 1.44 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{17}H_{21}NO_4$ $[M+H]^+$ 304.1471, found 304.1815.

*4.1.3. (S)-2-(N-Boc)-3-(1-(8-phenylhexyl)-1H-1,2,3-triazol-5-yl)alanine benzyl ester (**17**)*

14 (2.52 g, 10.88 mmol), **16** (3.30 g, 10.88 mmol) and catalytic amount of Cp*RuCl$(PPh_3)_2$ was dissolved in dichloromethane (50 ml). The mixture was heated to reflux for 12 hrs and then purified by column chromatography (PE:EA=2:1). **17** was obtained as a colorless oil (3.00 g, yield 63.5%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.42-7.08 (m, 11H), 5.67-5.51 (m, 1H), 5.24-4.99 (m, 2H), 4.58 (d, J = 5.3 Hz, 1H), 4.13 (dd, J = 13.0, 6.5 Hz, 2H), 3.28-3.04 (m, 2H), 2.57 (t, J = 7.6 Hz, 2H), 1.88-1.66 (m, 2H), 1.66-1.50 (m, 2H), 1.40 (s, 9H), 1.34-1.15 (m, 8H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{31}H_{42}N_4O_4$ $[M+H]^+$ 535.3206, found 535.3320.

*4.1.4. Methyl 3-(4-((ethoxymethoxy)methyl)benzamido)propanoate (**20**)*

4-((Ethoxymethoxy)methyl)benzoic acid (**18**) (0.67 g, 3.19 mmol), HOBt (0.43 g, 3.19 mmol), EDCI(0.60 g, 3.19 mmol) was mixed with dichloromethane (20 ml). DIPEA(1.29 g, 12.75 mmol) dissolved in dichloromethane (10 ml) was added in drop, the mixture was stirred for 0.5 hr and then methyl 3-amino propanoate(0.33 g, 3.19

mmol) was added. The mixture was stirred at room temperature for 5 hrs and then washed using 1 N HCl (10ml*2) followed by water (10ml*2). The organic phase was dried, concentrated and purified by column chromatography (PE:EA=2:1). **20** was obtained as a colorless oil 0.80 g, yield 85.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.76 (d, *J* = 7.3 Hz, 2H), 7.41 (d, *J* = 7.4 Hz, 2H), 6.97 (s, 1H), 4.76 (s, 2H), 4.64 (s, 2H), 3.83-3.52 (m, 7H), 2.66 (t, *J* = 5.4 Hz, 2H), 1.23 (t, *J* = 7.0 Hz, 3H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{15}H_{21}NO_5$ $[M+H]^+$ 296.1420, found 296.1505.

*4.1.5. Benzyl (S)-2-(3-(4-((ethoxymethoxy)methyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1 H-1,2,3-triazol-4-yl)propanoate (**21**)*

20 (0.38 g, 1.35 mmol) was dissolved in 1,4-dioxane (10ml), 3N NaOH a.q. (10ml) was added. The mixture was stirred at room temperature for 2 hrs. The solution was extracted by ethyl acetate (20 ml*3). The organic phase was collected, dried, evaporated to get the corresponding acid. The acid, HOBt (0.18 g, 1.35 mmol), EDCI(0.26 g, 1.35 mmol) was mixed with dichloromethane (10 ml). DIPEA(0.58 g, 4.49 mmol) dissolved in dichloromethane (5 ml) was added in drop, the mixture(**A**) was stirred for 0.5 hr. At the same time, (*S*)-2-(*N*-Boc)-3-(1-(8-phenylhexyl)-1*H*-1,2,3-triazol-5-yl)alanine benzyl ester (**17**) (0.33 g, 3.19 mmol) was dissolved in dichloromethane (10 ml), trifluoroacetic acid (1 ml) was added. The mixture was stirred at room temperature for 0.5 hr and evaporated to dry. Then saturated $NaHCO_3$ solution (10 ml) was added and extracted using

dichloromethane (10 ml*3). The organic phase was collected, dried and contracted and then mixture(**A**) was added in. After stirred at room temperature for 5 hrs, the solution was diluted using dichloromethane (20 ml), washed by 1 N HCl (10 ml*2), saturated $NaHCO_3$ solution (10 ml*2) and water (10 ml*2). The organic layer was purified by column chromatography (PE:EA=2:1). **21** was obtained as a colorless oil (0.78 g, yield 87.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.82-7.55 (m, 3H), 7.40-7.06 (m, 14H), 5.24-5.03 (m, 2H), 4.85 (dd, J = 13.3, 6.4 Hz, 1H), 4.78 (s, 2H), 4.61 (s, 2H), 4.18-3.99 (m, 2H), 3.72-3.53 (m, 4H), 3.26-3.03 (m, 2H), 2.55 (dd, J = 15.5, 7.5 Hz, 4H), 1.80-1.65 (m, 2H), 1.65-1.46 (m, 2H), 1.33-1.09 (m, 11H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{40}H_{51}N_5O_6$ $[M+H]^+$ 698.3839, found 698.3995.

4.1.6.

*(S)-2-(3-(4-((ethoxymethoxy)methyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1 H-1,2,3-triazol-4-yl)propanoic acid (**22**)*

21 was dissolved in methanol, 10%Pd-C (catalytic amount) was added in. The mixture was hydrogenated at normal pressure for 40 min. The insoluble was filtered off and the solvent was removed under vacuum and the residue was used directly for the next step without further purification.

*4.1.7. Diethyl (4-amino-3-nitrophenethyl)phosphonate (**25**)*

2-(4-Aminophenyl)ethan-1-ol (**23**) (3.00 g, 21.87 mmol) was dissolved in toluene (50 ml). Acetic anhydride (5.6 g, 54.67 mmol), triethylamine (11.06 g, 109.34 mmol) was

added. The mixture was heated to 80℃ and stirred for 1 hr. The solution was then washed using water (50ml*3), dried and evaporated to dry. Then the residue was dissolved in dichloromethane (50 ml), nitric acid (1.60 ml, 35.43 mmol) was added at 0 ℃, the mixture was stirred at 0 ℃ for 1 hr and then poured into crashed ice (50 g). The mixture was extracted using dichloromethane (50 ml*3). The organic layer was collected, dried and evaporated to get yellow oil. The yellow oil was then added to 10 N NaOH (30ml), and stirred at room temperature for 15 min. The mixture was extracted by ethyl acetate (30 ml*3). The organic phase was collected, dried, evaporated to dry. The residue was mixed with 45% HBr (40 ml), and heated to reflux for 10 hrs. After cooled to room temperature, the mixture was extracted by ethyl acetate (30 ml*3), washed by water (10 ml*2) and then saturated $NaHCO_3$ solution (10 ml*2). The organic layer was dried to get yellow oil and used for next step without further purification. The yellow oil was dissolved in triethyl phosphite (8.14 g, 48.96 mmol), and the mixture was stirred at 120℃ for 5 hrs. The target compound (**25**) was purified by column chromatography (EA) as a yellow oil (4.00g, yield 81.1%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.91 (d, J = 1.0 Hz, 1H), 7.33-7.05 (m, 1H), 6.80 (d, J = 8.6 Hz, 1H), 6.23 (s, 2H), 4.28-3.84 (m, 4H), 2.97-2.66 (m, 2H), 2.26-1.85 (m, 2H), 1.31 (t, J = 7.1 Hz, 6H) ppm.

*4.1.8. Diethyl (3,4-diaminophenethyl)phosphonate (**26**)*

25 (2.00g) was dissolved in methanol (30 ml), 10%Pd-C (0.05 g) was added in. The mixture was hydrogenated at normal pressure for 4 hrs. The insoluble was filtered off

and the solvent was removed to gain **26** as a brown oil (1.80 g, yield 91.6%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.67-6.55 (m, 1H), 6.53-6.44 (m, 2H), 4.19-3.94 (m, 4H), 3.52 (s, 4H), 2.83-2.64 (m, 2H), 2.10-1.82 (m, 2H), 1.30 (t, J = 7.1 Hz, 6H) ppm.

*4.1.9. Dibenzyl (3-(N-Boc-amino)phenethyl)phosphonate (**28a**)*

2-(3-Aminophenyl)ethan-1-ol (**27a**) (5.00 g, 36.46 mmol) was dissolved in 1,4-dioxane (250 ml), Boc_2O(7.96 g, 36.46 mmol) was added. The mixture was stirred at room temperature for 10 hrs and then extracted by ethyl acetate (250 ml*3), washed by water (250 ml*2) and then saturated $NaHCO_3$ solution (250 ml*2). The organic layer was dried and concentrated to get pale yellow oil. The pale yellow oil was then dissolved in dichloromethane (200 ml), triethylamine (6.94 g, 68.61 mmol) was added and the mixture stirred with ice bath for 10 min. Methanesulfonyl chloride (5 ml, 51.45 mmol) was added in drop and then stirred at room temperature for 2 hrs. The mixture was washed by water (100 ml*2). The organic layer was dried and evaporated to get yellow oil and used for next step without further purification. The yellow oil was dissolved in DMF (40 ml), NaBr (14.12 g, 137.21 mmol) and catalytic amount of TBAB was added. The mixture was stirred at room temperature for 36 hrs. Water (400 ml) was added and the mixture was extracted by ethyl acetate (250 ml*3). The organic layer was collected and washed by water (250 ml*2), dried and the solvent removed to get white solid. The white solid (1.42 g, 4.66 mmol), TBAI(5.13 g, 13.99 mmol), Cs_2CO_3(4.56 g, 13.99 mmol) and dibenzyl phosphite (1.47 g, 5.60 mmol) were mixed with dry DMF (5 ml). The mixture was stirred at room temperature for 10 hrs and the

water (50 ml) added, extracted by ethyl acetate (30 ml*3). The organic layer was collected and washed by water (50 ml*2), dried and purified by column chromatography (PE:EA=2:1) as a white powder (**28a**) (4.97 g, yield 43.0% for four steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.44-7.17 (m, 13H), 7.00 (d, J = 8.3 Hz, 2H), 5.20-4.89 (m, 4H), 2.82 (dd, J = 16.9, 10.1 Hz, 2H), 2.11-1.87 (m, 2H), 1.52 (s, 9H) ppm.

*4.1.10. Dibenzyl (3-(N-Boc-aminomethyl)phenethyl)phosphonate (**28b**)*

28b was prepared under similar conditions as described for **28a** (34.0 % yield for four steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.51-6.88 (m, 14H), 5.02-4.74 (m, 5H), 4.18 (d, J = 5.7 Hz, 2H), 2.88-2.64 (m, 2H), 2.05-1.80 (m, 2H), 1.39 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{28}H_{34}NO_5P$ $[M+H]^+$ 496.2175, found 496.2522.

*4.1.11. Dibenzyl (3-aminophenethyl)phosphonate (**29a**)*

28a was dissolved in dichloromethane (10 ml), trifluoroacetic acid (1 ml) was added. The mixture was stirred at room temperature for 0.5 hr and evaporated to dry. Then saturated $NaHCO_3$ solution (10 ml) was added and extracted using dichloromethane (10 ml*3). The organic phase was collected, dried and evaporated to get **29a** as a yellow oil and used directly for next step without further purification.

*4.1.12. Dibenzyl (3-aminomethylphenethyl)phosphonate (**29b**)*

29b was prepared under similar conditions as described for **29a** and used directly for

next step without further purification.

*4.1.13. ((2-Azidoethoxy)methyl)benzene (**31a**)*

Ethylene glycol (**30a**) (7.36 g, 116.93 mmol) was dissolved in DMF (100 ml), NaH (2.34 g, 58.47 mmol) was added in part with ice bath. After 0.5 hr, benzyl bromide (3.47 ml, 29.23 mmol) was added slowly and the mixture was stirred at room temperature for 2 hrs. Water (500 ml) was added and the mixture was extracted by ethyl acetate (300 ml*3). The organic layer was collected, dried and evaporated. The residue (3.00 g, 19.71 mmol) and dissolved in dichloromethane (50 ml), triethylamine (3.99 g, 39.42 mmol) was added and the mixture was stirred with ice bath for 5 min. Methanesulfonyl chloride (3.39 g, 29.57 mmol) was added in drop and then stirred at room temperature for 1 hrs. The mixture was washed by water (30 ml*2). The organic layer was dried and evaporated to get yellow oil. The yellow oil was dissolved in DMF (20 ml), azidotrimethylsilane (4.55 g, 39.42 mmol) and K_2CO_3(7.27 g, 15.49 mmol) were added. The mixture was stirred at 100℃ for 5 hrs. Water (100 ml) was added and the mixture was extracted by ethyl acetate (80 ml*3). The organic layer was collected and washed by water (100 ml*2) and dried. **31a** was purified by column chromatography (PE:EA=20:1) as a colorless oil (2.60g, yield 76.4% for three steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.09-7.96 (m, 2H), 7.56-7.46 (m, 1H), 7.44-7.35 (m, 2H), 4.60 (s, 2H), 4.36 (t, J = 6.2 Hz, 2H), 3.41 (t, J = 6.7 Hz, 2H) ppm.

*4.1.14. ((3-Azidopropoxy)methyl)benzene (**31b**)*

31b was prepared under similar conditions as described for **31a** (88.1% yield for three steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.13-7.90 (m, 2H), 7.65-7.34 (m, 3H), 4.42 (t, J = 6.1 Hz, 2H), 3.48 (t, J = 6.7 Hz, 2H), 2.17-1.90 (m, 2H) ppm.

*4.1.15. ((4-azidobutoxy)methyl)benzene (**31c**)*

31c was prepared under similar conditions as described for **31a** (80.1% yield for three steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.45-7.23 (m, 5H), 4.56 (s, 2H), 3.63-3.49 (m, 2H), 3.42-3.23 (m, 2H), 1.87-1.67 (m, 4H) ppm.

*4.1.16. 2-(4-(N-Boc-aminomethyl)-1H- 1,2,3-triazol-1-yl)ethyl benzyl ether (**32a**)*

***N*-Boc-2-Propynylamine** (0.45 g, 2.88 mmol), **31a** (0.59 g, 2.88 mmol) was dissolved in *tert*-butanol (5 ml). Catalytic amount of $CuSO_4$ was dissolved in water (5 ml), Vc-Na was added to change the color from blue to yellow. Then the solution was added to the *tert*-butanol solution and stirred for 2 hrs. **32a** was purified by column chromatography (PE:EA=1:1) as a pale yellow oil (0.82g, yield 79.8 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.63 (s, 1H), 7.36-7.19 (m, 5H), 5.29 (s, 1H), 4.58-4.45 (m, 4H), 4.38 (m, 2H), 3.80 (t, J = 5.0 Hz, 2H), 1.42 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{17}H_{24}N_4O_3$ $[M+H]^+$ 333.1848, found 333.1938.

*4.1.17. 3-(4-(N-Boc-aminomethyl)-1H- 1,2,3-triazol-1-yl)propyl benzyl ether (**32b**)*

32b was prepared under similar conditions as described for **32a** (yield 86.9%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.95-7.84 (m, 2H), 7.59-7.31 (m, 4H), 5.47 (s, 1H),

4.54-4.38 (m, 2H), 4.33-4.21 (m, 4H), 2.37-2.23 (m, 2H), 1.34 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{18}H_{24}N_4O_4$ $[M+H]^+$ 361.1798, found 361.1892.

*4.1.18. 4-(4-(N-Boc-aminomethyl)-1H- 1,2,3-triazol-1-yl)butyl benzyl ether (**32c**)*

32c was prepared under similar conditions as described for **32a** (yield 80.3%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.50 (s, 1H), 7.33 (m, 5H), 5.27 (s, 1H), 4.49 (s, 2H), 4.43-4.29 (m, 4H), 3.49 (t, J = 6.0 Hz, 2H), 2.11-1.87 (m, 2H), 1.77-1.57 (m, 2H), 1.44 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{19}H_{28}N_4O_3$ $[M+H]^+$ 361.2161, found 361.2263.

*4.1.19. Dibenzyl (2-(4-(N-Boc-aminomethyl)-1H-1,2,3-triazol-1-yl)ethyl)phosphonate (**33a**)*

33a was prepared under similar conditions as described for **28a** (yield 79.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.53-7.11 (m, 11H), 5.21-4.80 (m, 5H), 4.54-4.17 (m, 4H), 2.50-2.24 (m, 2H), 1.41 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{25}H_{33}N_4O_5P$ $[M+H]^+$ 487.2032, found 487.2118.

*4.1.20. Dibenzyl (3-(4-(N-Boc-aminomethyl)-1H-1,2,3-triazol-1-yl)propyl) phosphonate (**33b**)*

33b was prepared under similar conditions as described for **28a** (yield 50.4%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.72-7.20 (m, 11H), 5.28 (s, 1H), 5.09-4.91 (m, 4H), 4.43-4.24 (m, 4H), 2.21-2.02 (m, 2H), 1.79-1.62 (m, 2H), 1.45 (s, 9H) ppm; HRMS

(ESI-TOF) *m/z* calc'd for $C_{25}H_{33}N_4O_5P$ $[M+H]^+$ 501.2189, found 501.2275.

*4.1.21. Dibenzyl (4-(4-(N-Boc-aminomethyl)-1H-1,2,3-triazol-1-yl)butyl)phosphonate (**33c**)*

33c was prepared under similar conditions as described for **28a** (yield 74.5%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.72-7.20 (m, 11H), 5.42 (s, 1H), 5.08-4.77 (m, 4H), 4.40-4.06 (m, 4H), 2.48-2.20 (m, 1H), 1.80-1.47 (m, 2H), 1.39 (s, 9H), 0.93 (d, J = 5.8 Hz, 3H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{26}H_{35}N_4O_5P$ $[M+H]^+$ 515.2345, found 515.2448.

*4.1.22. Dibenzyl (2-(4-aminomethyl-1H-1,2,3-triazol-1-yl)ethyl)phosphonate (**34a**)*

34a was prepared under similar conditions as described for **29a** and used directly for next step without further purification.

*4.1.23. Dibenzyl (3-(4-aminomethyl-1H-1,2,3-triazol-1-yl)propyl)phosphonate (**34b**)*

34b was prepared under similar conditions as described for **29a** and used directly for next step without further purification.

*4.1.24. Dibenzyl (4-(4-aminomethyl-1H-1,2,3-triazol-1-yl)butyl)phosphonate (**34c**)*

34c was prepared under similar conditions as described for **29a** and used directly for next step without further purification.

*4.1.25. 2-(5-(N-Boc-aminomethyl)-1H- 1,2,3-triazol-1-yl)ethyl benzyl ether (**35**)*

***N*-Boc-2-Propynylamine** (0.45 g, 2.88 mmol), **31a** (0.59 g, 2.88 mmol) and catalytic amount of Cp*RuCl(PPh_3)$_2$ was dissolved in DCE (5 ml). The mixture was heated to reflux and stirred for 10 hrs and **35** was purified by column chromatography (PE:EA=1:1) as a pale yellow oil (0.57g, yield 55.2 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.49 (d, J = 12.0 Hz, 1H), 7.27-7.00 (m, 5H), 5.25 (s, 1H), 4.50-4.17 (m, 6H), 3.82-3.65 (m, 2H), 1.28 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{17}H_{24}N_4O_3$ $[M+H]^+$ 333.1848, found 333.1936.

*4.1.26. Dibenzyl (2-(5-(N-Boc-aminomethyl)-1H-1,2,3-triazol-1-yl)ethyl)phosphonate (**36**)*

36 was prepared under similar conditions as described for **28a** (yield 64.5%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.30-7.15 (m, 11H), 4.95-4.75 (m, 5H), 4.43-4.24 (m, 2H), 4.20-4.11 (m, 2H), 2.34-2.18 (m, 2H), 1.41 (s, 9H) ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{25}H_{33}N_4O_5P$ $[M+H]^+$ 487.2032, found 487.2105.

*4.1.27. Dibenzyl (2-(5-aminomethyl-1H-1,2,3-triazol-1-yl)ethyl)phosphonate (**37**)*

37 was prepared under similar conditions as described for **29a** and used directly for next step without further purification.

4.1.28.

(S)-(2-(2-(1-(3-(4-(hydroxymethyl)benzamido)propanamido)-2-(1-(8-phenyloctyl)-1H-

*1,2,3-triazol-5-yl)ethyl)-1H-benzo[d]imidazol-6-yl)ethyl)phosphonic acid (**6**)*

22 (0.26 g, 0.37 mmol), HATU(0.12 g, 0.37 mmol), DIPEA(0.14 g,1.10 mmol) were mixed with CH_2Cl_2 (10 ml). After stirred with ice bath for 0.5 h, **26** (0.10 g, 0.37 mmol) was added in and the mixture was stirred at room temperature for 3 hrs. The solution was washed by water (10 ml*2), dried and purified by column chromatography (PE:EA=20:1). The colorless oil gained was then dissolved in acetic anhydride (5 ml) and the mixture was stirred at 65 ℃ for 6 hrs. Then saturated $NaHCO_3$ solution (20 ml) was added and the mixture was extracted by CH_2Cl_2 (20 ml*3), the organic layer was collected and the solvent was removed under vacuum and the residue was then dissolved in CH_2Cl_2 (1 ml). Bromotrimethylsilane (2 ml) was added to the solution and the mixture was stirred at room temperature for 10 hrs. Methanol (10 ml) was added and stirred at room temperature for more 0.5 hr. **6** was purified by the preparative RP-HPLC (XBridge^{TM}, Prep C18, 50 × 250 mm, 10 μm) using an appropriate water/acetonitrile gradient in the presence of 0.05% TFA (10.0mg, yield 3.7% for three steps). ^{1}H NMR (300 MHz, MeOD) δ: 7.80-7.03 (m, 13H), 5.53-5.37 (m, 1H)，4.62 (s, 2H), 4.25 (t, J = 6.9 Hz, 2H), 3.72-3.46 (m, 4H), 3.03 (s, 2H), 2.63-2.50 (m, 4H), 2.07-1.90 (m, 2H), 1.80-1.68 (m, 2H), 1.58-1.46 (m, 2H), 1.27-1.07 (m, 8H) ppm; ^{13}C NMR (75 MHz, MeOD) δ: 173.3, 167.2, 147.5, 146.0, 142.1, 141.2, 138.5, 136.0, 134.7, 133.3, 131.0, 129.8, 128.6, 128.1, 127.7, 1255, 124.3, 115.0, 114.2, 64.7, 51.5, 49.9, 37.3, 36.2, 35.8, 35.3, 34.6, 31.2, 29.8, 29.2, 28.7, 27.4, 27.1, 20.8 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{38}H_{48}N_7O_6P$ $[M+H]^+$ 730.3407, found 730.3463.

4.1.29.

*(S)-(3-(2-(3-(4-(hydroxymethyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1H-1,2,3-triazol-5-yl)propanamido)phenethyl)phosphonic acid (**7**)*

22 (0.26 g, 0.37 mmol), HATU(0.12 g, 0.37 mmol), DIPEA(0.14 g,1.10 mmol) were mixed with CH_2Cl_2 (10 ml). After stirred with ice bath for 0.5 h, **29a** (0.10 g, 0.37 mmol) was added in and the mixture was stirred at room temperature for 3 hrs. The solution was washed by water (10 ml*2), dried and purified by column chromatography (PE:EA=20:1). The colorless oil gained was then dissolved in methanol (30 ml), 10%Pd-C (0.05 g) was added in. The mixture was hydrogenated at normal pressure for 40 min. The insoluble was filtered off and TFA (10 ml) was added and the mixture was stirred at room temperature for more 2 hrs. **7** was purified by the preparative RP-HPLC (XBridge^{TM} , Prep C18, 50 × 250 mm, 10 μm) using an appropriate water/acetonitrile gradient in the presence of 0.05% TFA (22.2 mg, yield 12.6% for three steps). ^{1}H NMR (300 MHz, MeOD) δ: 7.76 (d, J = 8.3 Hz, 2H), 7.63 (s, 1H), 7.48-7.34 (m, 4H), 7.27-6.96 (m, 7H), 4.64 (s, 2H), 4.35 (t, J = 7.2 Hz, 2H), 3.65 (d, J = 3.7 Hz, 2H), 3.22-2.79 (m, 5H), 2.57 (t, J = 7.5 Hz, 4H), 2.07-1.91 (m, 2H), 1.91-1.77 (m, 2H), 1.65-1.48 (m, 2H), 1.30 (s, 8H) ppm; ^{13}C NMR (75 MHz, MeOD) δ: 173.4, 172.8, 167.7, 147.5, 146.5, 142.1, 139.0, 138.4, 133.8, 132.1, 129.5, 129.0, 128.6, 128.1, 127.7, 125.4, 123.3, 120.0, 118.8, 64.7, 56.9, 49.2, 37.3, 35.8, 31.6, 30.7, 29.4, 28.7, 27.3, 26.8, 20.8 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{38}H_{49}N_6O_7P$ $[M+H]^+$ 733.3400, found 733.3499.

4.1.30.

*(S)-(3-((2-(3-(4-(hydroxymethyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1H-1,2,3-triazol-5-yl)propanamido)methyl)phenethyl)phosphonic acid (**8**)*

8 was prepared under similar conditions as described for **7** (20.7 % yield for three steps). ^{1}H NMR (300 MHz, MeOD) δ: 7.71-6.62 (m, 14H), 4.59 (d, J = 7.6 Hz, 1H), 4.53 (s, 2H), 4.32-4.02 (m, 4H), 3.51 (s, 2H), 3.25-3.04 (m, 1H), 3.03-2.88 (m, 1H), 2.81-2.64 (m, 2H), 2.56-2.24 (m, 1H), 1.96-1.75 (m, 2H), 1.78-1.55 (m, 1H), 1.53-1.34 (m, 2H), 1.18 (s, 8H) ppm; ^{13}C NMR (75 MHz, MeOD) δ: 173.4, 172.5, 167.5, 147.7, 146.5, 142.6, 139.2, 137.5, 133.2, 131.9, 129.3, 128.8, 128.3, 127.9, 127.7, 125.9, 125.2, 124.1, 64.7, 57.1, 49.3, 43.9, 37.5, 35.9, 35.6, 31.2, 29.6, 29.3, 28.7, 27.3, 26.6, 21.1 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{39}H_{51}N_6O_7P$ $[M+H]^+$ 747.3557, found 747.3675.

4.1.31.

*(S)-(2-(5-((2-(3-(4-(hydroxymethyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1H-1,2,3-triazol-5-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)ethyl)phosphonic acid (**9**)*

9 was prepared under similar conditions as described for **7** (8.3 % yield for three steps). ^{1}H NMR (500 MHz, MeOD) δ: 7.91-6.99 (m, 11H), 4.77-4.57 (m, 5H), 4.50-4.28 (m, 4H), 3.66 (dq, J = 13.8, 6.8 Hz, 2H), 3.21 (m, 2H), 2.59 (m, 4H), 2.41-2.26 (m, 2H), 1.91-1.81 (m, 2H), 1.70-1.55 (m, 2H), 1.35 (s, 8H) ppm; ^{13}C NMR

(75 MHz, MeOD) δ: 173.3, 172.3, 167.5, 147.6, 146.5, 142.7, 133.1, 131.5, 129.5, 128.5, 128.0, 127.7, 125.6, 65.0, 57.5, 49.9, 41.6, 37.3, 36.9, 35.8, 33.5, 31.2, 29.6, 29.2, 28.7, 27.3, 27.1 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{35}H_{48}N_9O_7P$ $[M+H]^+$ 738.3414, found 738.3524.

4.1.32.

*(S)-(2-(5-((2-(3-(4-(hydroxymethyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1H-1,2,3-triazol-4-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)ethyl)phosphonic acid (**10**)*

10 was prepared under similar conditions as described for **7** (14.0 % yield for three steps). ^{1}H NMR (300 MHz, MeOD) δ: 7.79-7.69 (m, 3H), 7.46 (t, J = 18.6 Hz, 3H), 7.28-7.08 (m, 5H), 4.74-4.63 (m, 2H), 4.47-4.27 (m, 5H), 3.67-3.57 (m, 2H), 3.31-3.22 (m, 4H), 3.14-3.02 (m, 1H), 2.63-2.49 (m, 3H), 2.16 (s, 2H), 1.82 (s, 2H), 1.71-1.53 (m, 4H) ppm; ^{13}C NMR (75 MHz, MeOD) δ: 174.2, 171.9, 168.1, 147.8, 146.5, 142.5, 134.1, 131.2, 130.7, 129.5, 128.7, 128.1, 127.4, 125.9, 122.9, 64.7, 57.3, 50.6, 43.1, 39.8, 37.3, 36.2, 33.2, 31.2, 29.8, 29.4, 28.7, 27.3, 27.0 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{35}H_{48}N_9O_7P$ $[M+H]^+$ 738.3414, found 738.3506.

4.1.33.

*(S)-(3-(5-((2-(3-(4-(hydroxymethyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1H-1,2,3-triazol-4-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)propyl)phosphonic acid (**11**)*

11 was prepared under similar conditions as described for **7** (14.3 % yield for three steps). ^{1}H NMR (300 MHz, MeOD) δ: 7.79-7.69 (m, 3H), 7.46 (t, J = 18.6 Hz, 3H), 7.28-7.08 (m, 5H), 4.74-4.63 (m, 2H), 4.47-4.27 (m, 5H), 3.67-3.57 (m, 2H), 3.31-3.22 (m, 4H), 3.14-3.02 (m, 1H), 2.63-2.49 (m, 3H), 2.16 (s, 2H), 1.82 (s, 2H), 1.71-1.53 (m, 4H) ppm; ^{13}C NMR (75 MHz, MeOD): δ 173.8, 172.3, 167.4, 148.1, 147.0, 142.6, 133.8, 131.4, 130.1, 129.5, 128.5, 128.1, 127.7, 125.9, 122.9, 64.7, 57.3, 53.5, 50.2, 43.1, 37.3, 35.8, 33.8, 31.6, 30.2, 29.1, 28.7, 27.5, 12.1 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{36}H_{46}N_9O_7P$ $[M+H]^+$ 752.3571, found 752.3621.

4.1.34.

(S)-(4-(5-((2-(3-(4-(hydroxymethyl)benzamido)propanamido)-3-(1-(8-phenyloctyl)-1H-1,2,3-triazol-4-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)butyl)phosphonic acid ***(12)***

12 was prepared under similar conditions as described for **7** (12.3 % yield for three steps). ^{1}H NMR (300 MHz, MeOD) δ: 7.81-7.05 (m, 11H), 4.78-4.57 (m, 3H), 4.49-4.24 (m, 5H), 3.61 (d, J = 3.6 Hz, 2H), 3.28-3.02 (m, 3H), 2.65-2.45 (m, 4H), 1.90-1.51 (m, 7H), 1.41-1.20 (m, 8H), 1.03 (d, J = 6.5 Hz, 3H) ppm; ^{13}C NMR (75 MHz, MeOD): δ 174.1, 172.5, 167.9, 148.5, 146.8, 142.5, 133.6, 131.2, 129.8, 129.0, 128.3, 128.0, 127.7, 125.0, 123.2, 65.1, 57.7, 52.1, 49.9, 43.0, 37.3, 36.0, 35.2, 31.2, 29.7, 29.6, 29.0, 28.4, 27.9, 12.0 ppm; HRMS (ESI-TOF) *m/z* calc'd for $C_{37}H_{52}N_9O_7P$ $[M+H]^+$ 766.3727, found 766.3850.

4.2 Fluorescence polarization (FP) assays

The binding experiments were performed on a SpectraMax MultiMode Microplate Reader (Molecular Devices) using the excitation at 485 nm and emission filters at 535 nm, respectively. In the fluorescein polarization assays, FP was determined by measuring intensities parallel (Intparallel, F∥) and the perpendicular fluorescence intensity (Intperpendicular, F⊥). The percentage inhibition of the phosphopeptides at each concentration was defined as = 1- (P_{obs} - P_{min}) / (P_{max} - P_{min}). Where, P_{max} was the polarization of the wells containing Plk1 PBD and the probe, P_{min} was referred to the polarization of the free probe, and the P_{obs} was the polarization for the wells containing the inhibitors at a range of concentrations under the assay conditions [19]. Briefly, FITC-GPMQSpTPLNG-OH was used as the Fluorescein-labeled peptides (fluorescent probe, purity>95%) as previously reported, which was dissolved in dimethyl sulfoxide (DMSO) and the finally optimized concentration was set at 20 nM [15]. Phosphopeptides used for competition binding assays were dissolved in assay buffer. The buffer makes up 10 mM NaCl, 50 mM Tris (pH 8.0), 1 mM EDTA-2Na . We further performed the sensitivity by synthesizing two new fluorescent probes, FITC-GPMQTSpTPKNG-OH for Plk2 PBD and FITC-GPLATSpTPKNG-OH for Plk3 PBD, respectively.

Binding-affinities experiments were performed in 384-well, black, round microtiter bottom plates (Corning 3575, Thermo Scientific), which were filled with 20 μL of 1.8 nM FITC-GPMQSpTPLNG-OH, 20 μL of 4 350nM Plk1 PBD, 20 μL of tested phosphopeptides at varying concentrations in assay buffer. A well containing no Plk1

PBD was served as a blank control whereas the negative control including Plk1 PBD, probe complex and assay buffer (equivalent to 0% inhibition). The 384-well black plate was incubated at room temperature for 30 min with gentle shaking prior to FP values measurements. However, the Plk2 PBD or Plk3 PBD was introduced into the selectivity assays to replace Plk1 PBD. All experiments were performed in triplicate. Competition binding data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA) and the inhibition constants (IC_{50}) were calculated by nonlinear curve fitting.

Acknowledgments

This work has been financially supported by National Natural Science Foundation of China (No. 81573278) and Excellent Science and Technology Innovation Team Projects of Jiangsu Province Universities in 2017.

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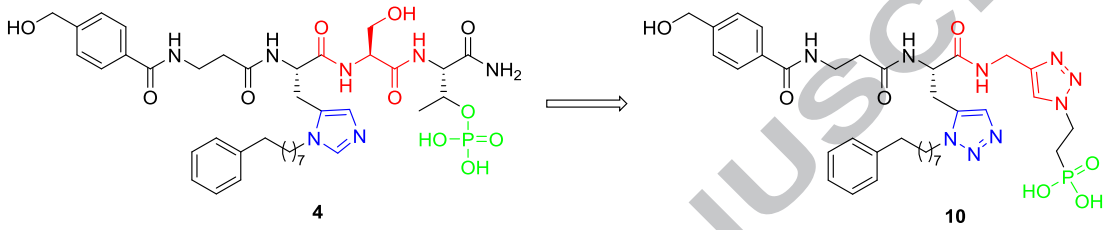
Highlights

1. A series of non-peptide inhibitors targeting the Plk1 PBD was identified.
2. Optimized compound showed moderate Plk1 PBD binding affinity and high selectivity.
3. Molecular docking study was performed and possible binding mode was proposed.

Graphic abstract

Identification of Novel and Selective Non-peptide Inhibitors Targeting the Polo-Box Domain of Polo-Like Kinase 1



tripeptide **4** Plk1 PBD IC_{50} = 0.04 μM

non-peptide **10** Plk1 PBD IC_{50} = 3.37 μM

Plk2 PBD IC_{50} > 100 μM

Plk3 PBD IC_{50} > 100 μM