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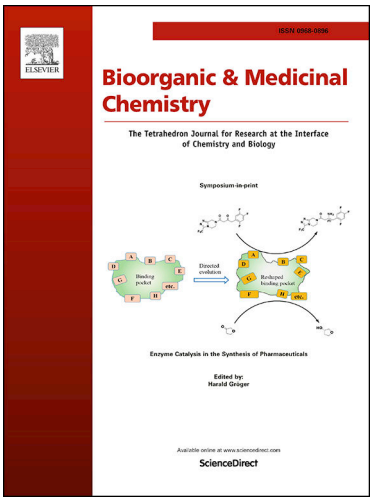

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Design, Synthesis, and Biological Evaluation of Novel Bcr-Abl^{T315I} Inhibitors Incorporating Amino Acids as Flexible Linker

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Abstract

Despite the success of imatinib in CML therapy through Bcr-Abl inhibition, acquired drug resistance occurs over time in patients. In particular, the resistance caused by T315I mutation remains a challenge in clinic. Herein, we embarked on a structural optimization campaign aiming at discovery of novel Bcr-Abl inhibitors toward T315I mutant based on previously reported dibenzoylpiperazin derivatives. We proposed that incorporation of flexible linker could achieve potent inhibition of Bcr-Abl^{T315I} by avoiding steric clash with bulky sidechain of Ile315. A library of 28 compounds with amino acids as linker has been developed and evaluated. Among them, compound **AA2** displayed the most potent activity against Bcr-Abl^{WT} and Bcr-Abl^{T315I}, as well as toward Bcr-Abl driven K562 and K562R cells. Further investigations indicated that **AA2** could induce apoptosis of K562 cells and down regulate phosphorylation of Bcr-Abl. In summary, the compounds with amino acid as novel flexible linker exhibited certain antitumor activities, providing valuable hints for the discovery of novel Bcr-Abl inhibitors to overcome T315I mutant resistance, and **AA2** could be considered as a candidate for further optimization.

Keywords: Bcr-Abl, T315I mutant, Flexible linker, Amino acids, Biological activity, Modeling study

1. Introduction

Protein kinases are crucial in the regulation of critical cellular processes, and deregulation of protein kinases function has been implicated in cancer and other disorders, such as immunological and metabolic disease [1,2]. Bcr-Abl, resulting from a reciprocal chromosome translocation between chromosomes 9 and 22, known as Philadelphia (Ph) chromosome, plays a vital role in the pathogenesis and progression of chronic myeloid leukemia (CML) [3]. It is found in >90% of patients with CML. Meanwhile, it is also presented in 25% of adult patients with acute lymphocytic leukemia (ALL) [4]. This fusion protein, characterized by constitutively active tyrosine kinase activity, promotes growth and replication through downstream

pathways such as JAK/STAT, RAS/RAF/ MEK/ERK, PI3K/AKT, and BAD/BCL-XI [5]. Thus, it represents potential therapeutic target for the treatment of CML. Imatinib, the first Bcr-Abl inhibitor, could induce apoptosis of Bcr-Abl positive CML cells, and it has been used as a first-line treatment for CML [6].

Despite the great success in clinical use of imatinib, many patients eventually develop acquired resistance. Several possible mechanisms of imatinib resistance are suggested, such as amplification of the fusion gene, mutation in Bcr-Abl, activation of compensatory pathways, binding of α1 acid glycoprotein, and so on [7]. Among these mechanisms, point mutation in Bcr-Abl especially in the kinase domain that interferes with imatinib binding is most important, which accounts for 60-80% of the case of imatinib resistance [8]. To date, over 100 mutations have been reported in resistance to imatinib which lead to >50 different amino acid substitutions, primarily centered around or within the P-loop, ATP binding site and activation loop [9]. The degree of resistance ranges from a few fold for some of the A-loop mutants, up to complete resistance for the T315I mutation.

To overcome imatinib-resistance, four second generation Bcr-Abl tyrosine kinase inhibitors were developed, including dasatinib (Sprycel), nilotinib (Tasigna), bosutinib (Bosulif), and bafetinib [10-13]. These inhibitors retain efficacy against the majority of Bcr-Abl mutant forms. However, none is capable of inhibiting T315I mutation, which accounts for 15-20% in all clinical required resistance [14]. Ponatinib (Iclusig), a third generation Bcr-Abl inhibitor, was then developed for the treatment of resistant or intolerant CML and Ph+ ALL patients against imatinib, especially those harboring T315I mutation [15]. It is presently the only clinically available Bcr-Abl inhibitor effective against T315I. However, due to its serious side effects seen in ~12% of the patient population, ponatinib has been permitted to resume sales to a limited patient population with an expanded black box warning [16]. Thus, there is still an urgent need to develop novel kinase inhibitors to overcome Bcr-Abl^{T315I} drug resistance.

T315 is referred as the “gatekeeper” residue, which controls the type II inhibitors’ access to the back cavity generated in inactive conformation [17]. However, the substitution of T315 with a bulkier and more hydrophobic isoleucine results in the loss of an important hydrogen bond with inhibitors and creates a steric hindrance that could disrupt the inhibitor binding [18]. In our previous research, pharmacophores for type II Bcr-Abl inhibitors are summarized in three parts: the hinge binding moiety (HBM), linker, and the selective site binding moiety (SBM) [19]. Under this analysis, flexible linker strategy is proposed based on the hypotheses that improving linker flexibility might avoid the steric clash with Ile315 to make inhibitor access to the back

cavity. Meanwhile, to compensate the decreased affinity caused by T315I mutation, hydrogen bond network strategy is applied to HBM modification to improve the affinity with hinge region which is critical for inhibitor binding. **(Figure 1)**

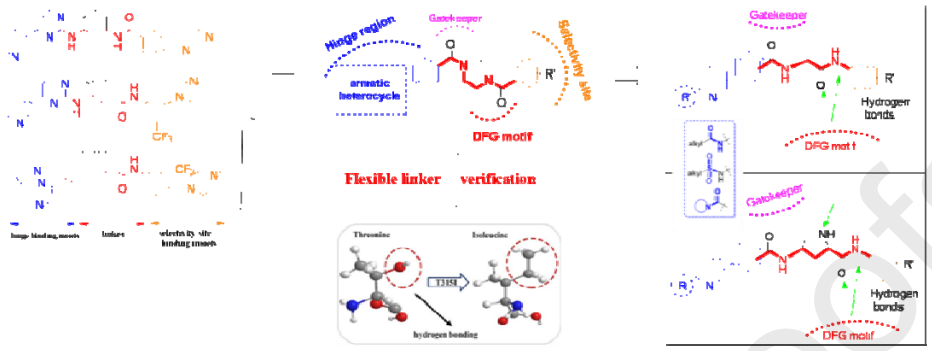


Figure 1. The design of Bcr-Abl inhibitors with amino acids as flexible linker.

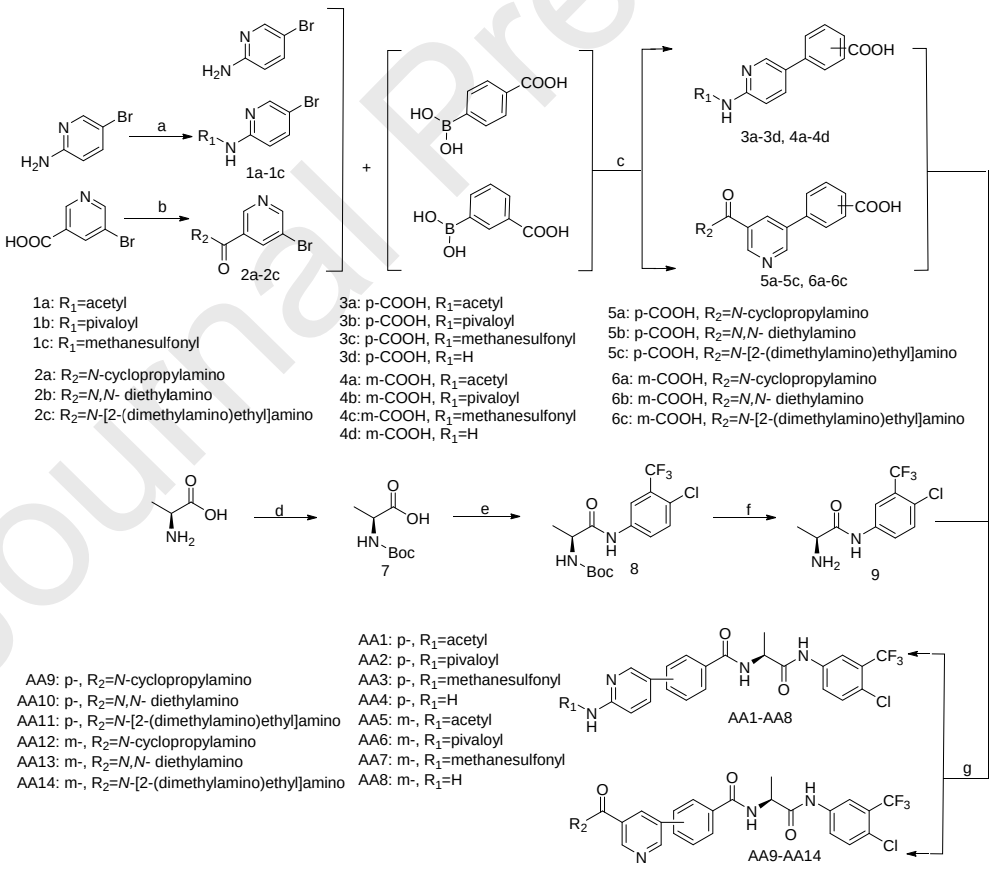
The feasibility of flexible linker strategy for Bcr-Abl inhibitors design has been verified in the modification with diethylamine or piperazine as linker [20]. As a continuous to our work, the further exploration is to expand the diversity of flexible linker, developing Bcr-Abl inhibitors with new scaffold. Studies have indicated that the introduction of amino acid could enhance the lipid solubility and relieve the toxicity for antitumor drugs, as well as improving the selectivity to tumor cells [21, 22]. Hence, based on bioelectronics isometric strategy, amino acids are expected to be used as flexible linker. Meanwhile, phenylpyridine with various amide side chain, generating hydrogen bonds with hinge region, is incorporated as HBM. As for selectivity binding site moiety, 4-chloro-3-trifluoromethylaniline is used according to our previous biological results. **(Figure 1)**

Herein, a series of compounds were designed and synthesized with two amino acids, alanine and hydroxyproline, as flexible linker. The biological activities were evaluated through enzyme inhibition experiment and MTT assay. Furthermore, the compound with the highest activity was studied to reveal the anticancer mechanisms by western blot. In addition, molecular docking and molecular dynamics simulations were performed using the most potent compounds to study the dynamic behavior of this complex involved in the inhibition process and investigate the possible binding interactions between synthesized compounds and Bcr-Abl active site.

2. Chemistry

The general synthetic procedures for two classes of title compounds were outlined in **Scheme 1** and **2**. All of the title compounds were prepared using Pd-catalyzed Suzuki coupling and amide condensation as key reactions. The preparation of compounds **AA1-AA14** started with the construction of key

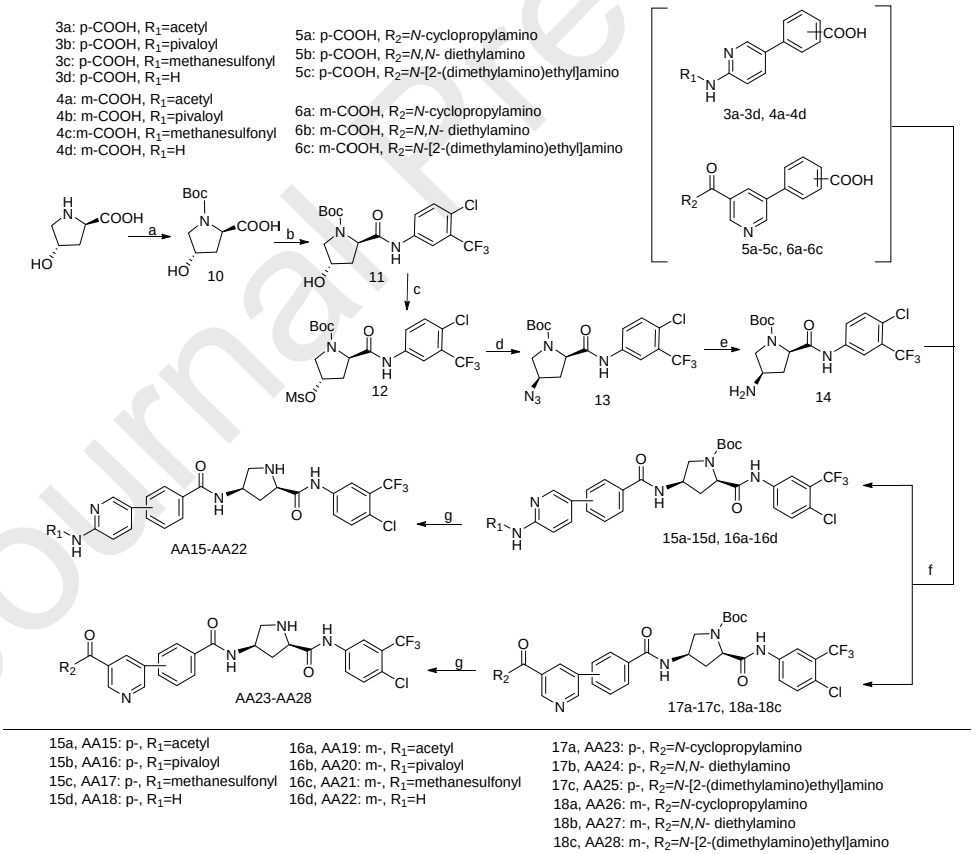
intermediates **3a-3d**, **4a-4d**, **5a-5c** and **6a-6c** from commercially available 5-bromopyridin-2-amine and 5-bromonicotinic acid, respectively. 5-bromopyridin-2-amine was firstly acylated using various acyl chloride [23], followed by coupling with 4(3)-carboxyphenyl-boronic acid, to generate intermediates **3a-3d** and **4a-4d** [24, 25]. For other intermediates **5a-5c** and **6a-6c**, 5-bromonicotinic acid was activated by thionyl chloride to generate active benzoyl chloride, then reacted with various aliphatic amines [26], followed by coupling with 4(3)-carboxyphenyl-boronic acid to obtain **5a-5c** and **6a-6c**. *L*-alanine was used as starting material to generate another key intermediate **9**. At first, the amine group of *L*-alanine was protected by $(Boc)_2O$ [27]. Next, Boc-Ala-OH was reacted with 5-amino-2-chlorobenzotrifluoride using a mixed anhydride method with isobutyl chloroformate as condensation reagent [28], and then deprotected by trifluoroacetic acid to yield compound **9** [29]. Consequently, treatment of **9** with various biphenyl carboxylic acid **3a-3d**, **4a-4d**, **5a-5c** and **6a-6c** in the presence of *N*-methylmorpholine and isobutyl chloroformate afforded title compounds **AA1-AA14** [30]. **(Scheme 1)**



Scheme 1. Synthesis route of the title compounds **AA1-AA14**.

Reagents and conditions: a. acyl chloride, Et_3N, CH_2Cl_2, 0ºC→r.t.; b. $SOCl_2$, reflux, amine, CH_2Cl_2, 0ºC→r.t.; c. $Pd(PPh_3)_4$, Cs_2CO_3, CH_3CN/H_2O (V:V=3:2), 100ºC; d. $(Boc)_2O$, $NaOH/H_2O$, THF, 25ºC; e. ClCOO-*i*Bu, Et_3N, CH_2Cl_2, -20ºC, 5-amino-2-chlorobenzotrifluoride; f. CH_2Cl_2, TFA; g. ClCOO-*i*Bu, **9**, NMM, THF, 0ºC→r.t.

Title compounds **AA15-AA28**, with proline as linker, were readily prepared using the synthetic route described in **Scheme 2**. Commercially available *L-(4S)*-hydroxyproline was firstly NH_2-protected by $(Boc)_2O$ [27], and reacted with 4-chloro-3-(trifluoromethyl)aniline using ethyl chloroformate as condensation reagent [31]. Then, the hydroxyl group of compound **11** was mesylated, followed by reaction with sodium azide to convert into azide group. Meanwhile, the chirality of C4 was reversed from *S* to *R*. Next, the azide group was hydrated by Pd/C under H_2, to generate the key intermediate **14** [32]. Consequently, using the same condensation method as described for the preparation of title compounds in **Scheme 1**, compounds **3a-3d**, **4a-4d**, **5a-5c** and **6a-6c** were reacted with compound **14** to get corresponding compounds **15a-15d**, **16a-16d**, **17a-17c** and **18a-18c**, which were deprotected by trifluoroacetic acid to obtain target compounds **AA15-AA28**.



Scheme 2. Synthesis route of the title compounds **AA15-AA28**.

Reagents and conditions: a. $(Boc)_2O$, $NaOH/H_2O$, THF, 25ºC; b. ClCOOEt, Et_3N, CH_2Cl_2, 0ºC, 5-amino-2-chlorobenzotrifluoride; c. methylsulfonyl chloride, Et_3N, CH_2Cl_2, 0ºC→r.t.; d. NaN_3,

DMF, 65-70ºC; e. 10% Pd/C, MeOH; f. ClCOO-*i*Bu, **14**, NMM, THF, 0ºC→r.t.; g. CH_2Cl_2, TFA.

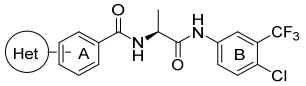
All of the title compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, LC-MS, HRMS and melting point. Detailed synthetic procedures are described in Experimental Section, and the NMR, MS and MP data are listed in **Supplementary Material**. In addition, to standard the ^{13}C NMR resonance signals of CF_3 group and nearby C atom in phenyl ring, the ^{13}C-NMR data of 4-chloro-3-trifluoromethyl-aniline, used as starting material in synthesis of **AA** series, is provided. Furthermore, HSQC-NMR date and ^{19}F-NMR data of two representative compounds (**AA2** and **AA19**) are depicted in **Supplementary Material**.

3. Results and discussion

3.1 In Vitro kinases inhibitory activity and SAR

Enzymatic inhibition evaluation of the title compounds against Bcr-Abl^{WT} and Bcr-Abl^{T315I} was performed using ADP-Glo kinase assay kit (Promega, Wisconsin, USA) [33, 34]. The results were depicted in **Table 1** and **Table 2**, with imatinib as positive control. The majority of the title compounds exhibited moderate to good inhibitory activity. As shown in **Table 1**, with alanine as linker, HBM was diversified, including various pyridinyl amide and nicotinamide at *m*- or *p*- position of **A** ring. The biological results indicated that *p*- substitution of heterocycle on **A** ring was more favorable than *m*- substitution for Bcr-Abl^{WT} inhibition, especially for Bcr-Abl^{T315I} inhibition. With pyridine on *p*- position of phenyl ring as hinge binding group, compound **AA4** displayed good activity toward Bcr-Abl^{WT} and Bcr-Abl^{T315I} kinases with IC_{50} of 0.77 μM and 7.51 μM, respectively. Furthermore, the pivaloylation of pyridine yielded the most potent compound, **AA2**, with IC_{50} of 0.041 μM and 0.53 μM against Bcr-Abl^{WT} and Bcr-Abl^{T315I}, respectively, comparable to that of imatinib. When the hinge binding group turned out to be nicotinamide, the inhibitory potency against Bcr-Abl^{T315I} was enhanced, while the Bcr-Abl^{WT} inhibition activity was retained. However, compounds **AA10** and **AA13** displayed poor activity against T315I mutant, which may be attributed to the bulky and basic sidechain, *N*,*N*-diethylamino.

Table 1 Structures and kinase inhibitory activities of title compounds **AA1**-**AA14**.

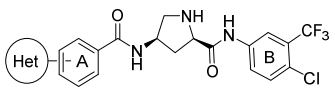


Compd	Het	Py	R_1/R_2	Bcr-Abl^{WT} IC_{50} (μM)	Bcr-Abl^{T315I} IC_{50} (μM)
AA1		p-	acetyl	0.20	22.45
AA2		p-	pivaloyl	0.041	0.53

AA3		p-	methanesulfonyl	3.77	9.95
AA4		p-	H	0.77	7.51
AA5		m-	acetyl	0.60	38.64
AA6		m-	pivaloyl	4.48	243.91
AA7		m-	methanesulfonyl	3.34	129.31
AA8		m-	H	4.40	86.84
AA9		p-	*N*-cyclopropylamino	9.38	4.99
AA10		p-	*N*,*N*- diethylamino	0.011	59.87
AA11		p-	*N*-[2-(dimethylamino)ethyl]amino	6.62	2.28
AA12		m-	*N*-cyclopropylamino	1.46	7.83
AA13		m-	*N*,*N*-diethylamino	0.29	86.01
AA14		m-	*N*-[2-(dimethylamino)ethyl]amino	0.64	2.41
Imatinib				0.054	0.28

For compounds with proline as linker, the results are depicted in **Table 2**. Compared with the series above, the majority of these compounds exhibited decreased Bcr-Abl^{WT} and Bcr-Abl^{T315I} inhibitory activities. Compound **AA17** showed the most potent inhibition against Bcr-Abl with IC_{50} of 0.15 μM. As for Bcr-Abl^{T315I} inhibition, most compounds exhibited poor inhibitory potency with IC_{50} up to dozens micromolar. The results indicated that alanine, branched chain amino acid, might be more favorable than proline as flexible linker for Bcr-Abl inhibitors. Besides, in compounds (**AA15-AA22**) with pyridinyl amide as hinge binding group, the same phenomenon was observed that compounds bearing pyridine on *p*- position of **A** ring had better potency than compounds with pyridine on *m*- position. It may be concluded that the heterocycle in *p*-position of linker was more beneficial for improving Bcr-Abl inhibition than *m*-position of each other, when heterocyclic biphenyl group was adopted as HBM. Interestingly, it was also found that compounds **AA24** and **AA27**, bearing *N*,*N*- diethylamino as a sidechain, had poor potency against T315I mutant. We suspected that the bulky diethylamino group resulted in large spatial conformation, which was not preferred for binding to the ATP pocket of Bcr-Abl^{T315I}. Overall, compound **AA2** was the most potent against Bcr-Abl^{WT} and Bcr-Abl^{T315I}, which can be used for further modification.

Table 2 Structures and kinase inhibitory activities of title compounds **AA15-AA28**.



Compd	Het	Py	R_1/R_2	Bcr-Abl^{WT} IC_{50} (μM)	Bcr-Abl^{T315I} IC_{50} (μM)
AA15		p-	acetyl	34.47	12.99
AA16		p-	pivaloyl	2.64	50.26

AA17		p-	methanesulfonyl	0.15	36.92
AA18		p-	H	9.18	14.10
AA19		m-	acetyl	0.49	24.59
AA20		m-	pivaloyl	4.63	147.17
AA21		m-	methanesulfonyl	6.48	16.16
AA22		m-	H	11.96	103.11
AA23		p-	*N*-cyclopropylamino	61.21	39.03
AA24		p-	*N*,*N*- diethylamino	7.40	50.47
AA25		p-	*N*-[2-(dimethylamino)ethyl]amino	4.23	22.10
AA26		m-	*N*-cyclopropylamino	16.00	26.61
AA27		m-	*N*,*N*-diethylamino	3.75	42.67
AA28		m-	*N*-[2-(dimethylamino)ethyl]amino	2.41	21.75
Imatinib				0.054	0.28

3.2 Anti-proliferative activity against CML cell lines

Based on the inhibition data from kinases assay, all of title compounds were screened for their antiproliferative potency against Bcr-Abl positive K562 cells, using MTT method with imatinib as positive control [35]. The results were presented in **Table 3**. Meanwhile, for further understanding of these compounds' antiproliferative activity, the determined ClogP values were also provided. As shown in **Table 3**, the majority of these compounds displayed good antiproliferative activities with IC_{50} value at micromolar level. Compared with imatinib, most compounds had an increased ClogP value, which indicated that the amino acids motif has indeed improved the lipophilicity for these compounds. In particular, compounds **AA7**, **AA11**, and **AA25** accounted for the most significant effect with IC_{50} values of 4.66 μM, 4.65 μM and 4.56 μM, respectively, comparable to imatinib with IC_{50} of 4.26 μM. In addition, the antiproliferative activities of compounds (**AA1-AA14**), with alanine as linker, were generally better than that of compounds (**AA15-AA28**) with proline as linker. These results were consistent with their enzymatic inhibition against Bcr-Abl kinase. Moreover, the potent activities of Ala-based inhibitors might also be attributed to their general higher ClogP values compared with Pro-based series. Since increased ClogP values meant enhanced lipid solubility, compounds with Ala-linker could easier penetrate the cell membrane and enter into cytoplasm to interact with Bcr-Abl kinase. Consequently, they exhibited good activity in cell assays, in accordance with the results shown in **Table 3**. More importantly, compound **AA2**, the most potent compound in kinase assay, also had a good antiproliferative activity with IC_{50} of 7.45 μM, confirming its rationality as a lead compound for next optimization.

Table 3 Antiproliferative activities of title compounds against K562 cell lines and ClogP values.

Compd	K562 IC_{50}(μM)	ClogP	Compd	K562 IC_{50}(μM)	ClogP
AA1	106.77	4.23	AA15	22.80	3.39
AA2	7.45	5.45	AA16	11.55	4.61
AA3	7.24	3.64	AA17	44.99	2.84
AA4	5.95	4.15	AA18	9.13	3.95
AA5	42.81	4.34	AA19	8.71	4.13
AA6	18.40	5.57	AA20	28.03	4.72
AA7	4.66	3.75	AA21	24.62	2.95
AA8	8.09	4.26	AA22	20.04	4.06
AA9	396.00	3.79	AA23	65.30	3.63
AA10	20.21	3.80	AA24	55.48	3.59
AA11	4.65	3.46	AA25	4.56	3.33
AA12	15.12	3.90	AA26	27.29	3.74
AA13	29.93	3.91	AA27	24.89	3.70
AA14	7.92	3.57	AA28	19.76	3.44
Imatinib	4.26	3.28	Imatinib	4.26	3.28

*3.3 In virto activity of **AA2** against K562R cells and its cytotoxicity*

According to the Bcr-Abl inhibitory potency and anti-proliferative activity, compound **AA2** was further evaluated for its activity toward K562R cells expressing T315I mutation [36], with ponatinib and imatinib as positive control. The result was displayed in **Figure 2A**, and imatinib was less potent against K562R cells, exhibiting about 45% inhibition rate at the concentration of 100 μM. For compound **AA2**, it was found to moderately inhibit the growth of K562R cells with IC_{50} value of 9.10 μM, while ponatinib had an IC_{50} value of 0.093 μM toward K562R cells. In addition, we identified the cytotoxicity of compound **AA2** on two normal cells, including Hek293 (human embryonic kidney 293 cells) and EA.hy 926 (human vascular endothelial cells), with imatinib as control. As shown in **Figure 2B** and **2C**, compound **AA2** exhibited less toxicity toward these two cell lines when its concentration increased up to 100 μM. In brief, compound **AA2** displayed selectivity growth inhibitory activity against Bcr-Abl positive K562 and K562R cells compared to normal human cell lines.

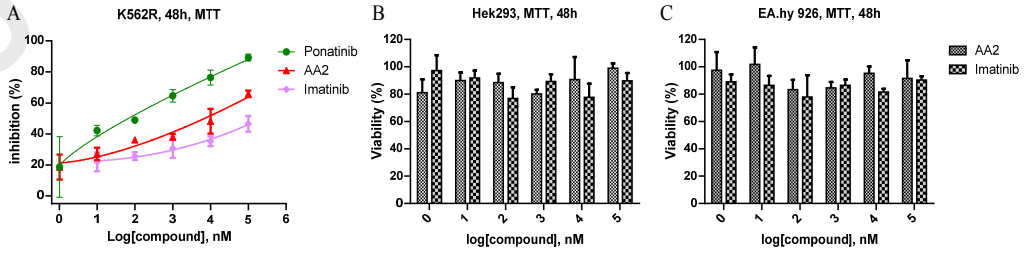


Figure 2. Anti-proliferative activity of compound **AA2** against K562R cells (**A**) expressing T315I mutation, and cytotoxicity of compound **AA2** toward two normal human cells, including Hek293 cells (**B**) and EA.hy926 cells (**C**).

*3.4 Effects of compound **AA2** on apoptosis in K562 cell lines*

Compound **AA2** was also selected to do the flow cytometry analysis to investigate its effect on cell proliferation. K562 cells was used, and the apoptosis assay was performed by Annexin V/PI staining [37]. A substantial and dose-dependent apoptotic activity in K562 cells at various concentrations for 48 h was observed (**Figure 3A)**. The apoptotic K562 cells (15.58%) of negative control group were depicted in **Figure 3B**. After treatment of compound **AA2** at the concentration of 1, 10, 20 μM, the percentage of cell apoptosis was 24.5%, 58.99%, and 62.42%, respectively. These data indicated that compound **AA2** could induce K562 cells apoptosis in a dose-dependent manner. Next, western blot assay of compound **AA2** was performed to explore its inhibitory mechanism. K562 cells were treated with compound **AA2** at 1, 5, 10, 15 μM for 48 h, and the phosphorylation status of Bcr-Abl in CML cells was determined. As expected, we observed that compound **AA2** dose-dependently decreased tyrosine phosphorylation of Bcr-Abl in K562 cells compared with the control group (**Figure 3C**). Meanwhile, as illustrated in **Figure 3D**, at the concentration of 10 μM, compound **AA2** begin to remarkably suppress the phosphorylation of Bcr-Abl. This result suggested that compound **AA2** may inhibit the growth of K562 cells and further induce K562 cells apoptosis through down regulation of phosphorylation level of Bcr-Abl.

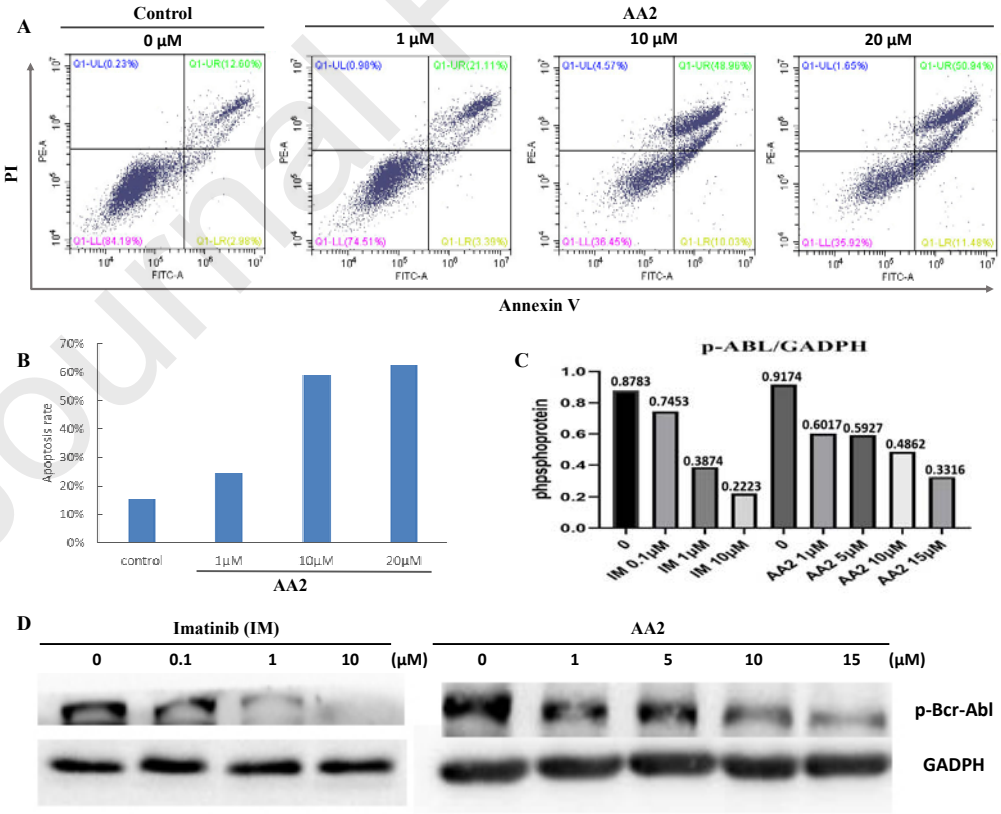


Figure 3. Apoptosis analysis of K562 cells after treatment with **AA2** (**A**. Flow cytometry analysis, **B**. Apoptotic ratio), and effect of **AA2** on the phosphorylated level of Bcr-Abl in K562 cells (**C**. p-Bcr-Abl/GADPH ratio, **D**. western blotting results).

3.5 Assessment of the simulation stability via RMSD analysis

For further structural optimization and investigation of the potential binding mode, the most potent compound **AA2** were applied to do the molecular modeling study with Bcr-Abl^{WT} and Bcr-Abl^{T315I} kinases, respectively [15, 38]. In addition, compound **AA19**, with proline as linker, was also employed in the modeling studies. Molecular docking was conducted using Sybyl-X to generate the initial conformations of protein-ligand complex, and then the constructed systems were subjected to 20 ns MD simulation to check for their structural stability and movements during the course of simulation [39]. The RMSD values of the backbone atoms of protein, and the heavy atoms of ligands were used to monitor the dynamic stabilities of the studied systems.

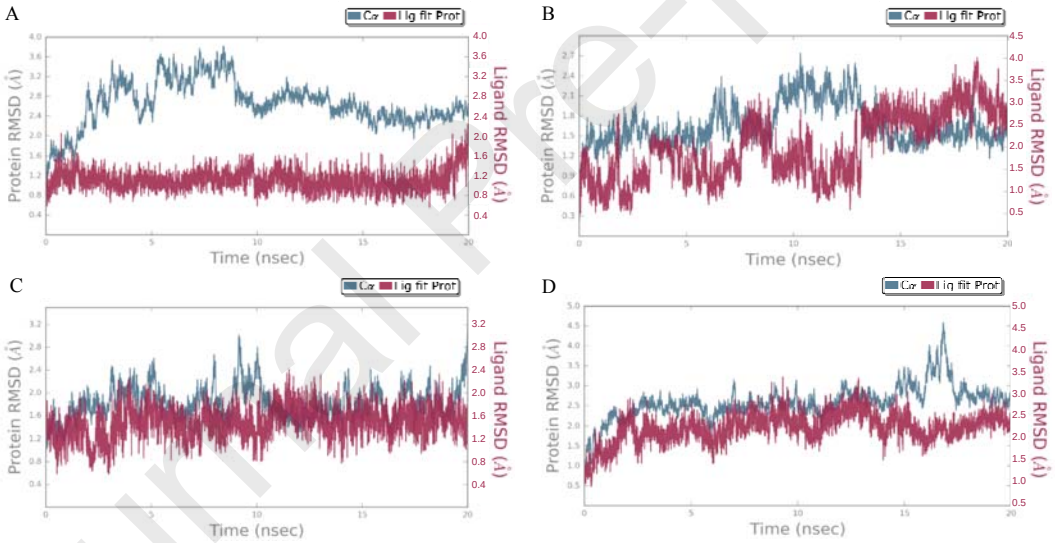


Figure 4. Protein-Ligand RMSD plot for Abl kinases and its inhibitors as a function of time in MD simulations (**A**. Bcr-Abl^{WT} and **AA2**, **B**. Bcr-Abl^{WT} and **AA19**, **C**. Bcr-Abl^{T315I} and **AA2**, **D**. Bcr-Abl^{T315I} and **AA19**). The protein backbone is presented in blue, and the ligand is presented in red.

As shown in **Figure 4A**, in **AA2**-1IEP (Bcr-Abl^{WT}) complex, protein RMSD started from 1.2 Å and later stabilized at 2.4 Å, whereas, ligand RMSD stabilized around 1.0 Å. As for **AA2**-3IK3 (Bcr-Abl^{T315I}) complex (**Figure 4B**), the protein RMSD fluctuated at around 2.2 Å while the ligand RMSD at about 0.9 Å when the system reached to its equilibrium state. The dynamic study of **AA19** with Bcr-Abl kinases were depicted in **Figure 4C** and **4D**. The system of **AA9** with 1IEP was stable

during the MD process, with the average values of protein RMSD and ligand RMSD at 2.0 Å and 1.4 Å, respectively. In terms of **AA19**-3IK3 complex, the average value for protein RMSD was 2.5 Å with a maximum of 4.0 Å at 18 ns, and the ligand RMSD was stable around 2.5 Å. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins. Overall spectrum of RMSD for above studied complexes did not show any considerable structural shifts during MD process, which explains the stability of the kinases structure and strength of ligand attachment inside the active site pocket.

3.6 The interaction analysis between target compounds and Bcr-Abl kinases

The interaction mode of compounds **AA2** and **AA19** with Bcr-Abl kinases after MD processed were applied and presented by PyMOL softwore. The interaction analysis of **AA2** with Bcr-Abl kinases were displayed in **Figure 5**. Favorable binding interactions of **AA2** with Bcr-Abl^{WT} included three hydrogen bonds (**Figure 5A**): *N*-(pyridin-2-yl)pivalamide in **AA2** formed one hydrogen bond with Met318 in the hinge region of Bcr-Abl with distance of 2.4Å. In addition, the terminal NH of linker part generated one hydrogen bond with conserved Glu286 for the bond length of 2.1Å, while CO formed a hydrogen bond with Asp381 of DFG-motif with distance of 2.1Å.

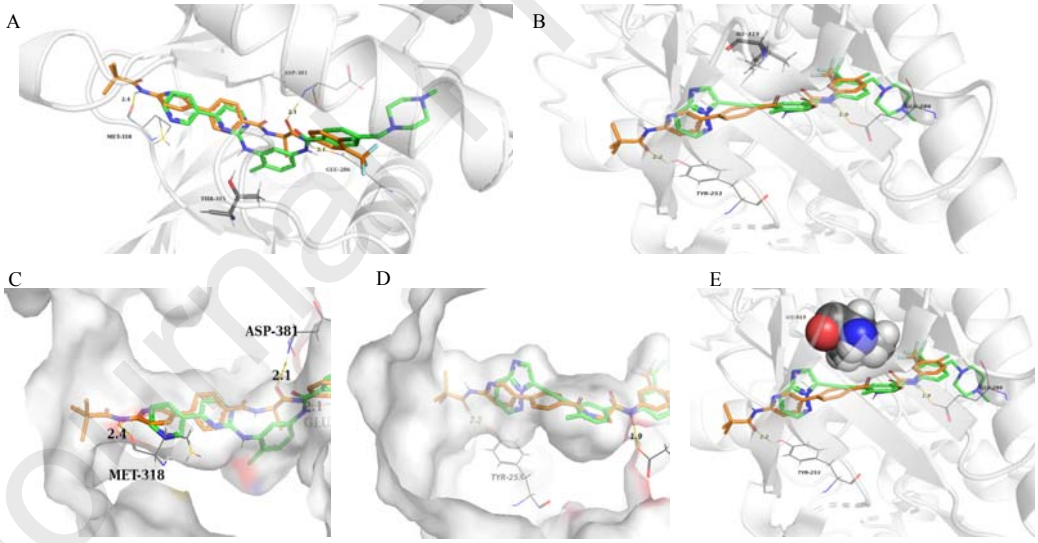


Figure 5. Docking results of compound **AA2** (Orange). **A** and **C**, Bcr-Abl^{WT} docking with imatinib (Green) as control. **B** and **D**, Bcr-Abl^{T315I} docking with ponatinib (Green) as control. **A** and **B** are represented as Ribbon model, while **C** and **D** are shown as surface for the binding site. **E**, Bcr-Abl^{T315I} docking with Ile315 presented as CPK models.

As for Bcr-Abl^{T315I}, the binding mode was depicted in **Figure 5B**. The CO of pivalamide generated one hydrogen bond with Tyr253 for the bond length of 2.2Å, while the terminal NH of linker part also formed one hydrogen bond with conserved Glu286 with distance of 1.9Å. Compared with Bcr-Abl^{WT} docking, the hydrogen bond

with DFG motif has been diminished. Meanwhile, as shown in **5D**, the channel, where HBM occupied, came to be smaller when it went to the Gatekeeper residue part, compared with that of Bcr-Abl^{WT} (**Figure 5C**). This narrowed channel made the phenyl ring in HBM a little crash with its around residues when it was binding, resulting in a certain loss of interaction between HBM and adenine pocket. Consequently, the decreased affinity might lower the activity of **AA2** against Bcr-Abl^{T315} observed in enzymatic assays.

The above molecular docking results indicated that alanine could generate hydrogen bond with conserved Glu286 or DFG-motif, which was important for Bcr-Abl binding, and avoid steric clash with Ile315 in Bcr-Abl^{T315I} seen in **Figure 5E**, thus providing novel scaffolds for developing anti-leukemia agents. Besides, as we expected, *N*-(pyridin-2-yl)pivalamide generated hydrogen bonds with hinge region, beneficial for improving affinity with Bcr-Abl.

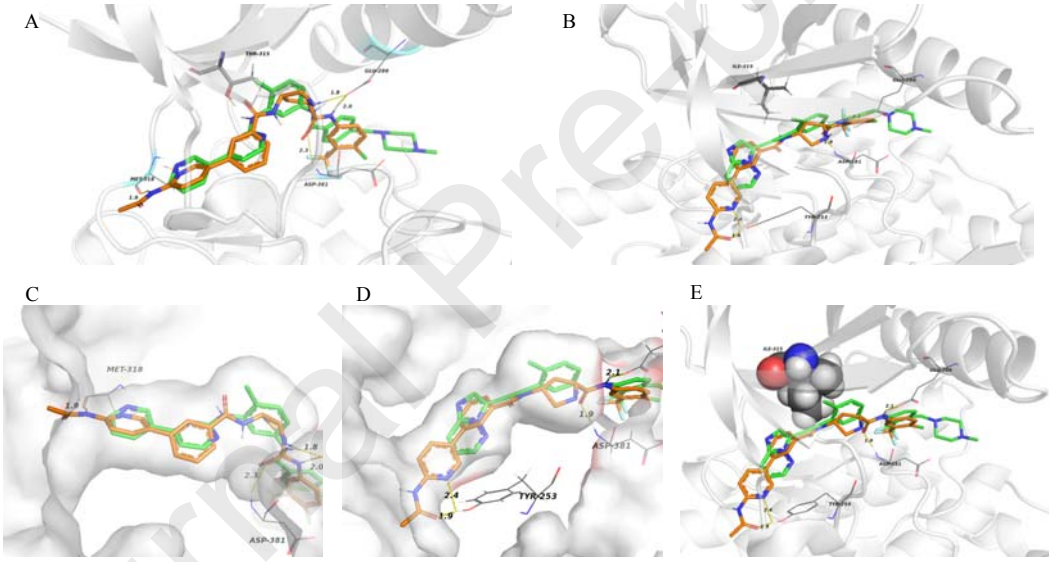


Figure 6. Docking results of compound **AA19** (Orange). **A** and **C**, Bcr-Abl^{WT} docking with imatinib (Green) as control. **B** and **D**, Bcr-Abl^{T315I} docking with ponatinib (Green) as control. **A** and **B** are represented as Ribbon model, while **C** and **D** are shown as surface for the binding site. **E**, Bcr-Abl^{T315I} docking with Ile315 presented as CPK models.

For compound **AA19**, the docking results with Bcr-Abl kinases (WT and T315I) were depicted in **Figure 6**. Similar as Ala-linker, Pro-linker could avoid Ile315 bulky steric clash (**Figure 6E**), attributing to its flexibility. Compound **AA19** fitted the ATP pocket of Bcr-Abl^{WT} well, and formed five hydrogen bonds with kinase protein (**Figure 6A**). As for Bcr-Abl^{T315I}, although there were four hydrogen bonds generated **(Figure 6B)**, the HBM of **AA19** pointed almost out of the pocket and was exposed to the solvent compared with that of ponatinib, which may result in the deletion of

hydrophobic interaction with hinge region (**Figure 6D**). The decreased affinity with Bcr-Abl^{T315I} might explain the decreased activity of compound **AA19** toward T315I mutant.

In addition, we also calculated the CScore of these two compounds, **AA2** and **AA19**, which was used as criterion to assess the inhibitors' binding affinity in Sybyl-X. The CScore of **AA2** was 11.8549, while **AA19** had a CScore value of 9.1113. It was indicated that compound **AA2**, with Ala-linker, had better affinity with Bcr-Abl, than Pro-based compound **AA19**. We suspected that Pro-linker, containing a five-membered ring, had a certain degree of rigidity, making Pro-based compounds requiring more energy to fit in the ATP pocket. This may explain why most Ala-based compounds had better enzymatic activity than compounds with Pro-linker.

4. Conclusion

In this study, a series of twenty-eight compounds was developed to overcome drug resistance caused by T315I mutation in Bcr-Abl. Amino acids were firstly introduced as flexible linker to avoid steric clash with the isoleucine gatekeeper residue in Bcr-Abl^{T315I}, with heterocyclic structure applied as HBM to generate hydrogen bonds with hinge region. The biological results indicated that compound **AA2** displayed the most potent inhibition of Bcr-Abl^{WT} and Bcr-Abl^{T315I}, comparable to that of imatinib. Meanwhile, it displayed potent antiproliferative activity against Bcr-Abl positive K562 cells. In addition, compound **AA19**, with proline as linker, also exhibited promising activity against Bcr-Abl^{WT} and Bcr-Abl^{T315I} as well as toward K562 cells. Meanwhile, compound **AA2** exhibited the potent antiproliferative activity toward K562R cells with T315I mutant. These compounds, especially compound **AA2**, might be considered as novel lead compounds for further development of Bcr-Abl inhibitors as anti-leukemia agents. Furthermore, cytometry analysis suggested that the representative compound **AA2** could induce the apoptosis of K562 cells, and western blot revealed that **AA2** exerted the growth inhibition of K562 cells through down regulation of Bcr-Abl phosphorylation. Moreover, molecular docking studies identified the rationality of amino acids as linker for Bcr-Abl inhibitor design, and phenyipyridine with amide sidechain was beneficial for potency of these inhibitors. Together with these prior studies, our study strongly supports the notion that introducing flexible linker can be a general strategy for developing inhibitors of protein kinases containing a bulky gatekeeper residue. Specially, our study raises the interesting possibility that applying amino acids as linker lead to promising inhibitors for Bcr-Abl^{T315I}. This study is currently ongoing in our laboratory.

5. Experimental section

5.1. Chemistry: General procedure

Chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. All anhydrous solvents were dried according to the standard methods and parts of them were freshly distilled prior to use. Melting points were measured on an X-4 micromelting apparatus. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker Advance 400 at 400 and 100 HZ, respectively. Chemical shifts (δ) in DMSO-d_6 were referenced to tetra-methyl silane (TMS 0.0 ppm). The solvent peak and H_2O peak were set to 2.50 ppm and 3.33 ppm. Mass spectra were partly measured using liquid chromatography mass spectrometry (LC-MS) with electrospray ionization (ESI), while others were measured using gas chromatography mass spectrometry (GC-MS) with electron impact ionization (EI). All reactions progress was monitored by TLC (GF_{254}), and visualized with UV (254 nm) except Boc-protection of amino acids using ninhydrin reaction. All compounds were purified by chromatography with silica gel (300-400 mesh).

*5.1.1 N-(5-bromopyridin-2-yl)acetamide (**1a**)*

5-bromopyridin-2-amine (5.19 g, 30 mmol) and Et_3N (20 mL) were dissolved in anhydrous CH_2Cl_2 (100 mL), cooled to 0□, followed by dropwise addition of acetyl chloride (2.54 mL, 36 mmol). The reaction solution was warmed to room temperature and stirred overnight. Then the solution was diluted with CH_2Cl_2, and washed with water (30 mL×3), saturated $NaHCO_3$ aq (30 mL×3), and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered, and purified by column chromatography (PE/ EtOAc=5:1) to give a white solid (5.65 g, 88%). Mp 78-81□. EI-MS (m/z) 214$[M]^+$.

*5.1.2 N-(5-bromopyridin-2-yl)pivalamide (**1b**)*

The method described above was used with 4.43 mL pivaloyl chloride. Elution with petroleum ether gave a white solid (7.37 g, 96%). Mp 42-44□. EI-MS (m/z) 256$[M]^+$.

*5.1.3 N-(5-bromopyridin-2-yl)methanesulfonamide (**1c**)*

Same method as compound **1a** was used with 2.78 mL methanesulfonyl chloride. Elution with PE/EtOAc (5/1, v/v) yield a white solid (5.52 g, 86%). Mp 159-160□. EI-MS (m/z) 250$[M]^+$.

*5.1.4 5-bromo-N-cyclopropylnicotinamide (**2a**)*

Sulfoxide chloride (36 mL) was added dropwise into 5-bromonicotinoc acid (5.0 g, 24.7 mmol) under N_2 at room temperature. The resulting mixture was refluxed for 2 h

till the solution was clear, and then the volatiles were removed under vacuum. The generated acid chloride was dissolved in anhydrous CH_2Cl_2 (30 ml), and the solution was added slowly at 0□ to a solution of cyclopropylamine (3.77 mL, 54.4 mmol) in CH_2Cl_2 (30 mL). Stirring was continued overnight. K_2CO_3 aq (2 M, 20 mL) was added and the aqueous layer was extracted with CH_2Cl_2 (10 mL×3). The combined organic layer was washed with brine (30 mL), dried over Na_2SO_4, filtered, concentrated *in vacuo*, and purified by chromatography column (PE/EtOAc=1:1) to obtain a white solid (5.27 g, 89%). Mp 140-142□. EI-MS (m/z) 240$[M]^+$.

*5.1.5 5-bromo-N,N-diethylnicotinamide (**2b**)*

Same method as described above was used with 5.60 mL diethylamine. Elution with PE/EtOAc (1/1, v/v) gave an oil (5.50 g, 87%). EI-MS (m/z) 256$[M]^+$.

*5.1.6 5-bromo-N-(2-(dimethylamino)ethyl)nicotinamide (**2c**)*

Same method as compound **2a** was used with 4.80 mL *N,N*-dimethyl-1,2-ethanediamine. Elution with ethyl acetate gave a colorless oil (5.35 g, 80%). EI-MS (m/z) 271$[M]^+$.

*5.1.7 4-(6-(acetylamino)pyridin-3-yl)benzoic acid (**3a**)*

N-(5-bromopyridin-2-yl)acetamide (**1a**) (4.30 g, 20 mmol), 4-boronobenzoic acid (3.66 g, 22 mmol), Cs_2CO_3 (13.0 g, 40 mmol), and Pd $(PPh_3)_4$ (1.2 g, 1 mmol) were suspended in a mixture of CH_3CN/H_2O (200 mL, v/v=3:2) under N_2. The mixture was heated in oil bath at 90□ for 48 h. Then the resulting solution was filtered immediately after the reaction completed. The filtrate was adjusted to pH4 using HCl aq (6M). The precipitate was filtered out and dried overnight to get the product as white solid (3.89 g, 76%). Mp 156-158□. EI-MS (m/z) 256$[M]^+$.

Compounds **3b-3d**, **4a-4d**, **5a-5c**, and **6a-6c** were prepared using the same method as described above.

*5.1.8 4-(6-pivalamidopyridin-3-yl)benzoic acid (**3b**).*

N-(5-bromopyridin-2-yl)pivalamide (**1b**) (5.14 g) was reacted with 4-boronobenzoic acid, giving a solid (4.77 g, 80%). Mp 276-277□. EI-MS (m/z) 298$[M]^+$.

*5.1.9 4-(6-(methylsulfonamido)pyridin-3-yl)benzoic acid (**3c**)*

N-(5-bromopyridin-2-yl)methanesulfonamide (**1c**) (5.02 g) was reacted with 4-boronobenzoic acid, giving a solid (4.32 g, 74%). Mp 294-295□. EI-MS (m/z)

292$[M]^+$.

*5.1.10 4-(6-aminopyridin-3-yl)benzoic acid (**3d**)*

5-bromopyridin-2-amine (3.46 g) was reacted with 4-boronobenzoic acid, giving a solid (3.12 g, 73%). Mp >300□. EI-MS (m/z) 216.0765$[M+H]^+$, 214.0622$[M-H]^-$.

*5.1.11 3-(6-acetamidopyridin-3-yl)benzoic acid (**4a**)*

N-(5-bromopyridin-2-yl)acetamide (**1a**) (4.30 g) was reacted with 3-boronobenzoic acid, giving a solid (3.32 g, 65%). Mp 238-240□. EI-MS (m/z) 255.30$[M-H]^+$.

*5.1.12 3-(6-pivalamidopyridin-3-yl)benzoic acid (**4b**)*

N-(5-bromopyridin-2-yl)pivalamide (**1b**) (5.14 g) was reacted with 3-boronobenzoic acid, giving a solid (4.29 g, 72%). Mp 222-223□. EI-MS (m/z) 297.25$[M-H]^+$.

*5.1.13 3-(6-(methylsulfonamido)pyridin-3-yl)benzoic acid (**4c**)*

N-(5-bromopyridin-2-yl)methanesulfonamide (**1c**) (5.02 g) was reacted with 3-boronobenzoic acid, giving a solid (3.67 g, 63%). Mp 264-265□. EI-MS (m/z) 291.20$[M-H]^+$.

*5.1.14 4-(6-aminopyridin-3-yl)benzoic acid (**4d**)*

5-bromopyridin-2-amine (3.46 g) was reacted with 3-boronobenzoic acid, giving a solid (2.60g, 61%). Mp 240-241□. EI-MS (m/z) 215.10$[M]^+$.

*5.1.15 4-(5-(cyclopropylcarbamoyl)pyridin-3-yl)benzoic acid (**5a**)*

5-bromo-*N*-cyclopropylnicotinamide (**2a**) (4.82 g) was reacted with 4-boronobenzoic acid, giving a solid (4.79 g, 85%). Mp 258-260□. EI-MS (m/z) 282$[M]^+$.

*5.1.16 4-(5-(diethylcarbamoyl)pyridin-3-yl)benzoic acid (**5b**)*

5-bromo-*N*,*N*-diethylnicotinamide (**2b**) (5.12 g) was reacted with 4-boronobenzoic acid, giving a solid (4.53 g, 76%). Mp 235-236□. EI-MS (m/z) 297$[M-H]^-$.

*5.1.17 4-(5-((2-(dimethylamino)ethyl)carbamoyl)pyridin-3-yl)benzoic acid (**5c**)*

5-bromo-*N*-(2-(dimethylamino)ethyl)nicotinamide (**2c**) (5.44 g) was reacted with 4-borono-benzoic acid, giving a solid (4.38 g, 70%). Mp 160-162□. EI-MS (m/z)

313.95$[M+H]^+$.

*5.1.18 3-(5-(cyclopropylcarbamoyl)pyridin-3-yl)benzoic acid (**6a**)*

5-bromo-*N*-cyclopropylnicotinamide (**2a**) (4.82 g) was reacted with 3-boronobenzoic acid, giving a solid (4.39 g, 78%). Mp 263-264□. EI-MS (m/z) 281.10$[M-H]^+$.

*5.1.19 3-(5-(diethylcarbamoyl)pyridin-3-yl)benzoic acid (**6b**)*

5-bromo-*N*,*N*-diethylnicotinamide (**2b**) (5.12 g) was reacted with 3-boronobenzoic acid, giving a solid (3.93 g, 66%). Mp 176-178□. EI-MS (m/z) 297.25$[M-H]^+$.

*5.1.20 3-(5-((2-(dimethylamino)ethyl)carbamoyl)pyridin-3-yl)benzoic acid (**6c**)*

5-bromo-*N*-(2-(dimethylamino)ethyl)nicotinamide (**2c**) (5.44 g) was reacted with 3-borono-benzoic acid, giving a solid (3.63 g, 58%). Mp 167-168□. EI-MS (m/z) 299.15$[M-CH_3]^-$.

*5.1.21 (tert-butoxycarbonyl)-L-alanine (**7**)*

L-alanine (7.14 g, 80 mmol) was dissolved in NaOH aq (40 M, 80 mL), followed by adding 80 mL THF, and cooled to 0□. $(Boc)_2O$ (19.2 g, 88 mmol) was added dropwise into the above solution, and stirring was continued at room temperature untill *L*-alanine was consumed completely by TLC (ninhydrin reaction). Then THF was removed under vacuum, and the concentrated solution was adjusted to pH 2-3 under water-ice bath using citric acid, extracted with EtOAc (60 mL×3). The combined organic phase was washed with brine (60 mL), dried over Na_2SO_4. The crude product was recrystallized using PE/EtOAc, yielding Boc-*L*-alanine (13.6 g, 90%). Mp 81-83□.

*5.1.22 tert-butyl-(R)-(1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl)carbamate (**8**)*

(*tert*-butoxycarbonyl)-*L*-alanine (**7**) (1.9 g, 10 mmol) was dissolved in 50 mL anhydrous CH_2Cl_2, cooled to -20□, and stirred for 5-10 min, followed by adding triethylamine (2.77 mL, 20 mmol). Then isobutyl chloroformate (1.95 mL, 15 mmol) in CH_2Cl_2 (3 mL) was added slowly, and stirred for 10-20 min to generate activated anhydride. Next, 4-chloro-3-(trifluoro-methyl)aniline (1.95 g, 10 mmol) in CH_2Cl_2 (10 mL) was added dropwise to the above solution, and stirring was continued for 2 h. After that, 5% $NaHCO_3$ aq (50 M, 100 mL) was added, and the reaction solution was warmed to room temperature, extracted with CH_2Cl_2 (30 mL×3). The combined

organic layer was washed with 5% $NaHCO_3$ aq, 5% HCl aq, brine, and dried over Na_2SO_4. The crude product was purified by column chromatography (PE/EtOAc=20:1) to obtain compound **8** (3.23 g, 88%). EI-MS (m/z): 367 $[M+H]^+$.

*5.1.23 (R)-2-amino-N-(4-chloro-3-(trifluoromethyl)phenyl)propanamide (**9**)*

Under ice bath, compound **8** (3.23 g, 8.8 mmol) was dissolved in 100 mL anhydrous CH_2Cl_2, and trifluoroacetic acid (6.5 mL, 88 mmol) in CH_2Cl_2 (10 mL) was added slowly to the above solution. The reaction solution was stirred under room temperature overnight. Then water (150 mL) was added, stirred for 20 min, and the organic layer was discarded. The aqueous phase was adjusted to pH 8 using saturated $NaHCO_3$ aq, and extracted with EtOAc (60 mL×3). The combined EtOAc was washed with brine, dried over Na_2SO_4, filtered, concentrated, and the residue was obtained, which was used next without purification. EI-MS (m/z) 267.05 $[M+H]^+$, 265.00$[M-H]^-$.

*5.1.24 (R)-4-(6-acetamidopyridin-3-yl)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl) amino)-1-oxopropan-2-yl)benzamide (**AA1**)*

In a 100 mL flask, compound **3a** (2.5 mmol) was dissolved in anhydrous THF (60 mL), followed by adding 4-methylmorpholine (0.85 mL, 7.5 mmol). At 0□, isobutyl chloroformate (0.55 mL, 3.75 mmol) in anhydrous THF (5 mL) was dropped slowly into the above suspension. Then the reaction mixture was reacted under 0□ for 1 h. Next, the THF (10 mL) solution of compound **9** (0.8 g, 3 mmol) and 4-methylmorpholine (0.85 mL) was added dropwise into the above solution, and stirring was continued under room temperature overnight. THF was removed in vacuo, and the residue was dissolved with EtOAc (60 mL). The organic layer was washed with water (15 mL), brine (15 mL), dried over Na_2SO_4. The crude product was purified by column chromatography (PE/EtOAc=1:1) to get a white solid (0.34 g, 27%). Mp 224-225□. EI-MS (m/z): 505 $[M+H]^+$, 503 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{24}H_{21}ClF_3N_4O_3$: 505.12543, found 505.12735. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.67 (s, 1H), 10.55 (s, 1H), 8.81 (d, J = 6.6 Hz, 1H), 8.74 (s, 1H), 8.25 (d, J = 2.4 Hz, 1H), 8.19 (s, 2H), 8.04 (d, J = 8.4 Hz, 2H), 7.90 (dd, J = 8.8, 2.3 Hz, 1H), 7.86 (d, J = 8.4 Hz, 2H), 7.69 (d, J = 8.8 Hz, 1H), 4.63 – 4.56 (m, 1H), 2.13 (s, 3H), 1.47 (d, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.71, 169.87, 166.43, 152.33, 146.47, 140.07, 139.04, 136.79, 133.08, 132.60, 130.33, 128.85 (2C), 127.63/127.32/ 127.02/126.71 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF3), 126.42 (2C), 127.26/124.55/121.84/119.13 (q, J = 273.7 Hz, $\underline{C}F_3$), 124.44, 124.32, 118.33/118.28 ($\underline{C}H_{phenyl\text{-}CF3}$), 113.61, 50.64, 24.41, 17.89.

Compound **AA2-AA14** was prepared using the same method as described above.

*5.1.25 (R)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl)-4-(6-pivalamido-pyridin-3-yl)benzamide (**AA2**)*

Compound **3b** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (3/1, v/v) to give a white solid (0.29 g, 21%). Mp 179-181□. EI-MS (m/z): 547 $[M+H]^+$, 545 $M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{27}H_{27}ClF_3N_4O_3$: 547.117238, found 547.17132. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 9.99 (s, 1H), 8.82 (d, *J* = 6.6 Hz, 1H), 8.76 (s, 1H), 8.25 (d, *J* = 2.2 Hz, 1H), 8.19 (s, 2H), 8.05 (d, *J* = 8.3 Hz, 2H), 7.90 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.87 (d, *J* = 8.3 Hz, 2H), 7.68 (d, *J* = 8.7 Hz, 1H), 4.64 – 4.57 (m, 1H), 1.48 (d, *J* = 7.1 Hz, 3H), 1.27 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.78, 172.72, 166.42, 152.59, 146.17, 140.04, 139.04, 136.68, 133.12, 132.59, 130.45, 128.86 (2C), 127.64/127.33/127.03/126.72 (q, *J* = 30.3Hz, $\underline{C}_{phenyl}$-CF_3), 126.45 (2C), 127.26/124.55/121.84/119.13 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.42, 124.32, 118.33/118.27, 114.38, 50.64, 39.91, 27.34 (3C), 17.90.

*5.1.26 (R)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl)-4-(6-(methyl-sulfonamido)pyridin-3-yl)benzamide (**AA3**)*

Compound **3c** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/1, v/v) to give a white solid (0.63 g, 47%). Mp 155-157□. EI-MS (m/z): 541 $[M+H]^+$, 539 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{23}H_{21}ClF_3N_4O_4S$: 541.09241, found 541.09284. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 10.54 (s, 1H), 8.80 (d, *J* = 6.7 Hz, 1H), 8.68 (s, 1H), 8.24 (d, *J* = 2.3 Hz, 1H), 8.16 (dd, *J* = 8.6, 2.4 Hz, 1H), 8.04 (d, *J* = 8.4 Hz, 2H), 7.90 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 1H), 4.66 – 4.54 (m, 1H), 1.47 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.70, 166.45, 152.55, 145.67, 139.91, 139.03, 137.57, 133.15, 132.59, 129.08, 128.86 (2C), 127.63/127.32/127.02/126.71 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 126.41 (2C), 127.27/124.55/121.84/119.10 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.43, 124.32, 118.34/118.28 ($\underline{C}H_{phenyl\text{-}CF3}$), 112.73, 50.64, 42.23, 17.89.

*5.1.27 (R)-4-(6-aminopyridin-3-yl)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl) amino)-1-oxopropan-2-yl)benzamide (**AA4**)*

Compound **3d** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/2, v/v) to give a white solid (0.52 g, 45%). Mp

137-138□. EI-MS (m/z): 464 $[M+H]^+$, 462 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{22}H_{19}ClF_3N_4O_2$: 464.12269, found 464.11249. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.54 (s, 1H), 8.76 (d, *J* = 6.6 Hz, 1H), 8.68 (s, 2H), 8.24 (d, *J* = 2.5 Hz, 1H), 8.00 (d, *J* = 8.3 Hz, 2H), 7.90 (dd, *J* = 8.8, 2.5 Hz, 1H), 7.77 (d, *J* = 8.5 Hz, 2H), 7.68 (d, *J* = 8.8 Hz, 1H), 6.91 (s, 2H), 4.62 – 4.55 (m, 1H), 1.47 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.75, 166.50, 163.64, 156.66 (3C), 139.03, 138.67, 132.55, 132.34, 128.84 (2C), 127.63/127.32/127.02/126.71 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 125.12 (2C), 127.26/124.54/121.83/ 119.12 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.42, 124.31, 121.45, 118.33/118.28 ($\underline{C}H_{phenyl\text{-}CF3}$), 50.63, 17.88.

*5.1.28 (R)-3-(6-acetamidopyridin-3-yl)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl) amino)-1-oxopropan-2-yl)benzamide (**AA5**)*

Compound **4a** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/1, v/v) to give a white solid (0.30 g, 24%). Mp 128-131□. EI-MS (m/z): 505 $[M+H]^+$, 503 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{24}H_{21}ClF_3N_4O_3$: 505.12543, found 505.12689. ^{1}H NMR (400 MHz, DMSO-d_6) 1H NMR (400 MHz, DMSO) δ 10.66 (s, 1H), 10.57 (s, 1H), 8.89 (d, *J* = 6.6 Hz, 1H), 8.74 (s, 1H), 8.29 – 8.23 (m, 2H), 8.23 – 8.16 (m, 2H), 7.92 – 7.89 (m, 3H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.62 – 7.58 (m, 1H), 4.65 – 4.58 (m, 1H), 2.13 (s, 3H), 1.48 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.68, 169.84, 166.63, 152.16, 146.41, 139.01, 137.30, 136.77, 134.95, 132.58, 130.81, 129.73, 129.59, 127.46, 127.64/ 127.33/127.02/126.71 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 125.69, 124.45, 124.34, 127.25/124.55/121.83/119.11 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 118.35/118.28 ($\underline{C}H_{phenyl\text{-}\ CF3}$), 113.62, 50.68, 24.40, 17.92.

*5.1.29 (R)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl)-3-(6-pivalamido-pyridin-3-yl)benzamide (**AA6**)*

Compound **4b** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (3/1, v/v) to give a white solid (0.45 g, 33%). Mp 115-116□. EI-MS (m/z): 547 $[M+H]^+$, 545 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{27}H_{27}ClF_3N_4O_3$: 547.17238, found 547.17277. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 9.97 (s, 1H), 8.90 (d, *J* = 6.6 Hz, 1H), 8.75 (s, 1H), 8.25 (d, *J* = 2.5 Hz, 2H), 8.20 (d, *J* = 1.3 Hz, 2H), 7.92 (d, *J* = 7.6 Hz, 2H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.64 – 7.59 (m, 2H), 4.65 – 4.58 (m, 1H), 1.49 (d, *J* = 7.2 Hz, 3H), 1.27 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.75, 172.68, 166.65, 152.40, 146.10, 139.02, 137.25, 136.65, 134.98, 132.00, 130.90, 129.59, 129.27, 127.48, 127.64/127.33/127.03/126.72 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 125.71, 124.45, 124.36, 127.26/124.55/121.83/ 119.12 (q,

J = 273.7 Hz, $\underline{C}F_3$), 118.34/118.25 ($\underline{C}H_{phenyl\text{-}CF3}$), 114.41, 50.69, 39.89, 27.35 (3C), 17.92.

*5.1.30 (R)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl)-3-(6-(methyl-sulfonamido)pyridin-3-yl)benzamide (**AA7**)*

Compound **4c** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/1, v/v) to give a white solid (0.33 g, 24%). Mp 246-247□. EI-MS (m/z): 541 $[M+H]^+$, 539 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{23}H_{21}ClF_3N_4O_4S$: 541.09241, found 541.09142. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H), 10.57 (s, 1H), 8.87 (d, *J* = 6.7 Hz, 1H), 8.69 (s, 1H), 8.28 – 8.22 (m, 2H), 8.17 – 8.14 (m, 1H), 7.92 – 7.87 (m, 3H), 7.69 (d, *J* = 8.8 Hz, 1H), 7.62 – 7.58 (m, 1H), 7.11 (d, *J* = 8.6 Hz, 1H), 4.67 – 4.55 (m, 1H), 3.34 (s, 3H), 1.48 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.67, 166.57, 152.39, 139.01, 137.59, 137.11, 134.93, 132.61 (2C), 129.69, 129.62 (2C), 127.46, 127.62/127.32/ 127.01/126.70 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 125.73, 127.27/124.55/ 121.84/119.13 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.46, 124.34, 118.33/118.29 ($\underline{C}H_{phenyl\text{-}CF3}$), 112.75, 50.66, 42.28, 17.93.

*5.1.31 (R)-3-(6-aminopyridin-3-yl)-N-(1-((4-chloro-3-(trifluoromethyl)phenyl) amino)-1-oxopropan-2-yl)benzamide (**AA8**)*

Compound **4d** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/2, v/v) to give a white solid (0.43 g, 37%). Mp 215-216□. EI-MS (m/z): 464 $[M+H]^+$, 462 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{22}H_{19}ClF_3N_4O_2$: 464.12269, found 464.11103. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 8.84 (d, *J* = 6.2 Hz, 1H), 8.70 (s, 2H), 8.25 (s, 1H), 8.19 (s, 1H), 7.91 –7.81 (m, 3H), 7.67 (d, *J* = 8.7 Hz, 1H), 7.56 – 7.52 (m, 1H), 6.88 (s, 2H), 4.68 – 4.57 (m, 1H), 1.49 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 172.69, 166.65, 163.57, 156.61 (3C), 139.01, 135.76, 134.86, 132.57, 129.53, 128.57, 127.63/127.33/127.02/127.62 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 126.78, 124.51, 124.45, 124.36, 127.26/124.54/121.83/119.11 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 121.93, 118.35/118.30 ($\underline{C}H_{phenyl\text{-}CF3}$), 50.67, 17.93.

*5.1.32 (R)-5-(4-((1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl) carbamoyl)phenyl)-N-cyclopropylnicotinamide (**AA9**)*

Compound **5a** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/1→1/2, v/v) to give a white solid (0.59 g, 44%). Mp 207-209□. EI-MS (m/z): 531 $[M+H]^+$, 529 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$

$C_{26}H_{23}ClF_3N_4O_3$: 531.14108, found 531.14209. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 9.10 (d, *J* = 2.1 Hz, 1H), 8.99 (d, *J* = 1.9 Hz, 1H), 8.87 (d, *J* = 6.6 Hz, 1H), 8.77 (d, *J* = 3.9 Hz, 1H), 8.49 – 8.48 (m, 1H), 8.25 (d, *J* = 2.4 Hz, 1H), 8.10 (d, *J* = 8.4 Hz, 2H), 7.95 (d, *J* = 8.4 Hz, 2H), 7.90 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.69 (d, *J* = 8.8 Hz, 1H), 4.68 – 4.53 (m, 1H), 2.93 – 2.88 (m, 1H), 1.48 (d, *J* = 7.2 Hz, 3H), 0.79 – 0.72 (m, 2H), 0.66 – 0.58 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.67, 166.36, 166.28, 150.30, 148.35, 139.63, 139.03, 134.55, 133.95, 133.16, 132.58, 130.37, 128.92 (2C), 127.30 (2C), 127.64/127.33/ 127.03/126.72 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 127.26/124.55/121.84/ 119.12 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.43, 124.35, 118.34/118.28 ($\underline{C}H_{phenyl\text{-}CF3}$), 50.68, 23.54, 17.89, 6.23 (2C).

*5.1.33 (R)-5-(4-((1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl) carbamoyl)phenyl)-N,N-diethylnicotinamide (**AA10**)*

Compound **5b** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/1, v/v) to give a white solid (0.38 g, 28%). Mp 104-106□. EI-MS (m/z): 547 $[M+H]^+$, 545 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{27}H_{27}ClF_3N_4O_3$: 547.17238, found 547.17580. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 9.05 (d, *J* = 2.0 Hz, 1H), 8.87 (d, *J* = 6.6 Hz, 1H), 8.60 (d, *J* = 1.7 Hz, 1H), 8.25 (d, *J* = 2.1 Hz, 1H), 8.18 (s, 1H), 8.07 (d, *J* = 8.3 Hz, 2H), 7.95 (d, *J* = 8.3 Hz, 2H), 7.90 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.69 (d, *J* = 8.7 Hz, 1H), 4.65 – 4.54 (m, 1H), 3.50 (d, *J* = 6.4 Hz, 2H), 3.25 (d, *J* = 6.6 Hz, 2H), 1.47 (d, *J* = 7.1 Hz, 3H), 1.19 (d, *J* = 6.9 Hz, 3H), 1.09 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.67, 167.87, 166.36, 148.59, 146.48, 139.49, 139.03, 134.74, 133.95, 133.71, 132.58, 132.36, 128.91 (2C), 127.31 (2C), 127.63/127.32/127.02/126.72 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 127.26/124.55/121.84/119.13 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.44, 124.32, 118.34/118.29 ($\underline{C}H_{phenyl\text{-}CF3}$), 50.67, 43.49, 39.56, 17.87, 14.52, 13.28.

*5.1.34 (R)-5-(4-((1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl) carbamoyl)phenyl)-N-(2-(dimethylamino)ethyl)nicotinamide (**AA11**)*

Compound **5c** was reacted with compound **9**, and the crude product was purification with EtOAc/MeOH (7/1, v/v) to give a white solid (0.26 g, 18%). Mp 126-129□. EI-MS (m/z): 562 $[M+H]^+$, 560 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{27}H_{28}ClF_3N_5O_3$: 562.18328, found 562.18362. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.11 (s, 1H), 9.02 (s, 1H), 8.90 (s, 1H), 8.81 (s, 1H), 8.53 (s, 1H), 8.26 (s, 1H), 8.10 (d, *J* = 6.4 Hz, 2H), 7.96 (d, *J* = 7.0 Hz, 2H), 7.91 (d, *J* = 7.3 Hz, 1H), 7.69 (d, *J* = 8.4 Hz, 1H), 4.61 (s, 1H), 3.32 – 3.26 (m, 2H), 2.48 (s, 2H), 2.22 (s, 6H), 1.49 (d, *J* = 5.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.71, 166.38, 165.01,

150.32, 148.39, 139.62, 139.04, 134.59, 133.97, 133.23, 132.60, 130.46, 128.93 (2C), 127.32 (2C), 127.62/127.32/127.01/126.71 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 127.25/124.56/121.86/119.14 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.48, 124.34, 118.34/118.30 ($\underline{C}H_{phenyl\text{-}CF3}$), 58.54, 50.71, 45.67 (2C), 37.91, 17.89.

*5.1.35 (R)-5-(3-((1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl) carbamoyl)phenyl)-N-cyclopropylnicotinamide (**AA12**)*

Compound **6a** was reacted with compound **9**, and the crude product was purified with PE/EtOAc (1/1→1/2, v/v) to give a white solid (0.79 g, 60%). Mp 191-192□. EI-MS (m/z): 531 $[M+H]^+$, 529 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{26}H_{23}ClF_3N_4O_3$: 531.14108, found 531.14293. ^{1}H NMR (400MHz, DMSO-d_6) δ 10.58 (s, 1H), 9.12 (d, *J* = 2.1 Hz, 1H), 8.99 (d, *J* = 1.9 Hz, 1H), 8.94 (d, *J* = 6.7 Hz, 1H), 8.78 (d, *J* = 4.0 Hz, 1H), 8.50 – 8.49 (m, 1H), 8.34 (s, 1H), 8.25 (d, *J* = 2.4 Hz, 1H), 7.99 (d, *J* = 7.8 Hz, 2H), 7.90 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.70 – 7.64 (m, 2H), 4.70 – 4.56 (m, 1H), 2.94 – 2.85 (m, 1H), 1.49 (d, *J* = 7.2 Hz, 3H), 0.78 – 0.73 (m, 2H), 0.64 – 0.62 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.65, 166.50, 166.35, 150.38, 148.08, 139.01, 136.92, 135.08, 135.03, 133.29, 132.61, 130.50, 130.43, 129.72, 128.30, 127.63/127.33/127.02/126.72 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF3), 126.49, 127.27/124.55/121.83/ 119.12 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.46, 124.36, 118.34/118.29 ($\underline{C}H_{phenyl\text{-}CF3}$), 50.71, 23.55, 17.93, 6.22 (2C).

*5.1.36 (R)-5-(3-((1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl) carbamoyl)phenyl)-N,N-diethylnicotinamide (**AA13**)*

Compound **6b** was reacted with compound **9**, and the crude product was purification with PE/EtOAc (1/1, v/v) to give a white solid (1.05 g, 77%). Mp 114-116□. EI-MS (m/z): 547 $[M+H]^+$, 545 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{27}H_{27}ClF_3N_4O_3$: 547.17238, found 547.17466. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 9.08 (d, *J* = 2.2 Hz, 1H), 8.94 (d, *J* = 6.7 Hz, 1H), 8.61 (d, *J* = 1.9 Hz, 1H), 8.32 (s, 1H), 8.25 (d, *J* = 2.4 Hz, 1H), 8.20 – 8.19 (m, 1H), 8.01 – 7.96 (m, 2H), 7.90 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.68 (d, *J* = 8.8 Hz, 1H), 7.66 – 7.62 (m, 1H), 4.67 – 4.60 (m, 1H), 3.50 (d, *J* = 6.8 Hz, 2H), 3.25 (d, *J* = 6.6 Hz, 2H), 1.49 (d, *J* = 7.2 Hz, 3H), 1.19 (d, *J* = 7.1 Hz, 3H), 1.10 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.63, 167.93, 166.48, 148.67, 146.24, 139.01, 136.71, 135.18, 135.06, 133.66, 132.57, 132.37, 130.45, 129.74, 128.38, 127.64/ 127.33/127.03/ 126.72 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 126.43, 127.26/ 124.54/121.83/119.11 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.45, 124.37, 118.35/118.30 ($\underline{C}H_{phenyl\text{-}CF3}$), 50.67, 43.50, 39.60, 17.94, 14.52, 13.27.

*5.1.37 (R)-5-(3-((1-((4-chloro-3-(trifluoromethyl)phenyl)amino)-1-oxopropan-2-yl) carbamoyl)phenyl)-N-(2-(dimethylamino)ethyl)nicotinamide (**AA14**)*

Compound **6c** was reacted with compound **9**, and the crude product was purification with EtOAc/MeOH (7/1, v/v) to give a white solid (0.43 g, 30%). Mp 114-117□. EI-MS (m/z): 562 $[M+H]^+$, 560 $[M-H]^-$. HRMS (ESI): calcd for $[M+H]^+$ $C_{27}H_{28}ClF_3N_5O_3$: 562.18328, found 562.18576. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.13 (d, *J* = 1.9 Hz, 1H), 9.02 (d, *J* = 1.7 Hz, 1H), 8.97 (d, *J* = 6.6 Hz, 1H), 8.85 – 8.82 (m, 1H), 8.56 (s, 1H), 8.37 (s, 1H), 8.26 (d, *J* = 2.3 Hz, 1H), 8.00 (d, *J* = 7.6 Hz, 2H), 7.91 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.71 – 7.63 (m, 2H), 4.68 – 4.57 (m, 1H), 3.45 – 3.41 (m, 2H), 2.49 (s, 2H), 2.24 (s, 6H), 1.50 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.68, 166.49, 165.08, 150.37, 148.15, 139.04, 136.91, 135.09, 135.03, 133.33, 132.55, 130.45 (2C), 129.70, 128.31, 127.63/127.32/127.02/126.71 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 126.52, 127.26/124.54/121.83/119.12 (q, *J* =273.7 Hz, $\underline{C}F_3$), 124.45, 124.33, 118.35/118.29 ($\underline{C}H_{phenyl\text{-}CF3}$), 58.42, 50.79, 45.48 (2C), 37.74, 17.93.

*5.1.38 (2R,4S)-1-(tert-butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid (**10**)*

The prepare procedure was same as compound **7**.

*5.1.39 tert-butyl(2R,4S)-2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)-4-hydroxypyrrolidine-1-carboxylate (**11**)*

Compound **10** (3.48 g, 15.31 mmol) was dissolved in anhydrous CH_2Cl_2 (60 mL), and triethylamine (1.5 mL, 15.31 mmol) was added. Under 0□, ethyl chloroformate (2 mL, 15.31 mmol) in CH_2Cl_2 (5 mL) was dropped into the solution. Stirring was continued for 30 min to generate activated anhydride. Then 4-chloro-3-(trifluoromethyl)aniline (2.7 g, 13.92 mmol) in CH_2Cl_2 (15 mL) was added dropwise into the above reaction solution. The reaction was performed overnight under room temperature. After that, the resulting solution was diluted with CH_2Cl_2 (30 mL), and washed with saturated $NaHCO_3$ (30 mL), water (30 mL), and brine (30 mL). The organic layer was dried over Na_2SO_4, filtered, concentrated, and purified by column chromatography (PE/EtOAc=3:1) to provide product (4.26 g, 75%). EI-MS (m/z): 407 $[M-H]^-$.

*5.1.40 tert-butyl(2R,4S)-2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)-4-((methylsulfonyl)oxy)pyrrolidine-1-carboxylate (**12**)*

In a 100 mL flask, compound **11** (3.79 g, 9.27 mmol) was dissolved in anhydrous

CH_2Cl_2, and cooled to 0□, followed by adding triethylamine (1.55 mL, 11.12 mmol). Stirring was continued for 15 min, and methylsulfonyl chloride (0.86 mL, 11.12 mmol) was dropped slowly into the solution. Then the reaction was performed overnight under room temperature. Water was added to quench the reaction, and the organic layer was washed with brine, dried over Na_2SO_4. Solvents was removed in vacuo to get product (3.87 g, 86%). EI-MS (m/z): 485 $[M-H]^-$.

*5.1.41 tert-butyl(2R,4R)-4-azido-2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl) pyrrolidine-1-carboxylate (**13**)*

Compound **12** (3.78g, 7.76 mmol) was dissolved in anhydrous DMF (10 mL), and sodium azide (0.99g, 15.52 mmol) was added. Then the reaction was continued at 65-70□ under N_2 protection for 16 h. Next, the reaction solution was poured into 100 mL ice-water, and extracted with EtOAc (80 mL×3). The combined organic phase was washed with brine (50 mL), dried over Na_2SO_4. The crude product was purified by column chromatography (PE/EtOAc=5:1) to give a white solid (2.69 g, 80%). EI-MS (m/z): 434 $[M+H]^+$, 432 $[M-H]^-$.

*5.1.42 tert-butyl(2R,4R)-4-amino-2-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl) pyrrolidine-1-carboxylate (**14**)*

Compound **13** (2.77 g) was dissolved in anhydrous methanol (25 mL), and 5% Pd/C (0.54g) was added. The solution was purged with H_2 for 3 times, and the reaction was continued under H_2 overnight. Then the reaction was filtered, and washed with methanol. The combined methanol was concentrated under vacuum, and the residue was obtained for next step. EI-MS (m/z): 408 $[M+H]^+$.

*5.1.43 Compounds **15a-15d, 16a-16d, 17a-17c,** and **18a-18c***

These compounds were prepared using the same method as compound **AA1**. All the compounds were purified by column chromatography. The elution solvents for **17c** and **18c** was EtOAc/MeOH (7/1, v/v), while others were eluted with PE/EtOAc (1/1 or 1/2, v/v).

*5.1.44 (2R,4R)-4-(4-(6-acetamidopyridin-3-yl)benzamido)-N-(4-chloro-3-(trifluoromethyl)phenyl)pyrrolidine-2-carboxamide (**AA15**)*

Compound **15a** was used as starting material, and then deprotection of Boc group was performed using the method same as compound **9**. The overall yield of last two steps was 14%. Mp 249-251□. EI-MS (m/z): 546 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{26}H_{24}ClF_3N_5O_3$: 546.15198, found 546.17135. ^1H NMR (400 MHz,

DMSO-d_6) δ 10.65 (s, 1H), 10.42 (s, 1H), 8.67 (d, *J* = 2.4 Hz, 1H), 8.45 (d, *J* = 6.6 Hz, 1H), 8.21 – 8.10 (m, 3H), 7.95 (d, *J* = 8.1 Hz, 1H), 7.89 (d, *J* = 8.4 Hz, 2H), 7.73 (d, *J* = 8.5 Hz, 2H), 7.58 – 7.54 (m, 1H), 7.41 (d, *J* = 7.7 Hz, 1H), 4.39 – 4.34 (m, 1H), 3.87 – 3.83 (m, 1H), 3.23 – 3.18 (m, 1H), 2.90 – 2.86 (m, 1H), 2.48 – 2.44 (m, 1H), 2.12 (s, 3H), 2.02 – 1.95 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.37, 169.87, 166.20, 152.28, 146.36, 139.81, 139.79, 136.71, 133.66, 130.37, 130.36, 130.32/130.01/129.69/129.38 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 128.49 (2C), 126.32 (2C), 128.67/125.96/123.25/120.54 (q, *J* = 273.3 Hz, $\underline{C}F_3$), 123.47, 120.16/120.11 ($CH_{phenyl\text{-}CF3}$), 116.02/115.98 ($\underline{C}H_{phenyl\text{-}CF3}$), 113.61, 60.05, 51.97, 51.67, 36.77, 24.40.

*5.1.45 (2R,4R)-N-(4-chloro-3-(trifluoromethyl)phenyl)-4-(4-(6-pivalamidopyridin-3-yl)benzamido)pyrrolidine-2-carboxamide (**AA16**)*

Compound **15b** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 57%. Mp 178-180□. EI-MS (m/z): 588 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{29}H_{30}ClF_3N_5O_3$: 588.19893, found 588.20236. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.72 (s, 1H), 9.98 (s, 1H), 8.70 (d, *J* = 1.2 Hz, 1H), 8.57 (d, *J* = 6.5 Hz, 1H), 8.19 – 8.15 (m, 3H), 7.92 (d, *J* = 8.4 Hz, 3H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.60 – 7.56 (m, 1H), 7.44 (d, *J* = 7.8 Hz, 1H), 4.49 – 4.44 (m, 1H), 4.11 – 4.07 (m, 1H), 3.32 – 3.29 (m, 1H), 3.08 – 3.04 (m, 1H), 2.67 – 2.55 (m, 1H), 2.10 – 2.05 (m, 1H), 1.26 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.77, 171.99, 166.31, 152.53, 146.08, 139.89, 139.73, 136.60, 133.47, 130.46, 130.42, 130.37/130.06/129.74/129.43 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 128.56 (2C), 126.39 (2C), 128.61/125.90/123.19/120.48 (q, *J*= 273.3 Hz, $\underline{C}F_3$), 123.51, 120.37/120.34 ($\underline{C}H_{phenyl\text{-}CF3}$), 116.07/116.03 ($\underline{C}H_{phenyl\text{-}CF3}$), 114.39, 59.73, 51.11, 50.76, 36.25, 27.32 (3C), 21.59.

*5.1.46 (2R,4R)-N-(4-chloro-3-(trifluoromethyl)phenyl)-4-(4-(6-(methylsulfonamido) pyridin-3-yl)benzamido)pyrrolidine-2-carboxamide (**AA17**)*

Compound **15c** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 29%. Mp 130-133□. EI-MS (m/z): 582$[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{25}H_{24}ClF_3N_5O_4S$: 582.11896, found 582.13965. ^{1}H NMR (400MHz, DMSO-d_6) δ 10.43 (s, 1H), 8.61 (s, 1H), 8.45 (d, J = 6.6 Hz, 1H), 8.21 (s, 1H), 8.09 (d, *J* = 8.6 Hz, 1H), 7.95 (d, *J* = 8.1 Hz, 1H), 7.89 (d, *J* = 8.3 Hz, 2H), 7.70 (d, *J* = 8.2 Hz, 2H), 7.58 – 7.54 (m, 1H), 7.42 (d, *J* = 7.7 Hz, 1H), 7.08 (d, *J* = 8.6 Hz, 1H), 4.39 – 4.34 (m, 1H), 3.88 – 3.84 (m, 1H), 3.33 (s, 3H), 3.23 – 3.18 (m, 1H), 2.90 – 2.86 (m, 1H), 2.48 – 2.44 (m, 1H), 2.04 – 1.94 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ

174.31, 166.23, 152.59, 145.60, 139.81, 139.65, 137.48, 133.71, 130.34, 130.32/130.02/129.70/129.39 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.05, 128.49 (2C), 126.30 (2C), 128.67/125.96/123.25/120.54 (q, J = 273.3 Hz, $\underline{C}F_3$), 123.47, 120.16/120.12 ($\underline{C}H_{phenyl\text{-}CF3}$), 116.03/115.98 ($\underline{C}H_{phenyl\text{-}CF3}$), 112.76, 60.04, 51.95, 51.65, 42.20, 36.78.

*5.1.47 (2R,4R)-4-(4-(6-aminopyridin-3-yl)benzamido)-N-(4-chloro-3-(trifluoromethyl)phenyl)pyrrolidine-2-carboxamide (**AA18**)*

Compound **15d** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 35%. Mp 170-172□. EI-MS (m/z): 504 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{24}H_{22}ClF_3N_5O_2$: 504.14141, found 504.14154. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 9.02 (s, 1H), 8.62 (s, 2H), 8.20 (s, 1H), 7.95 – 7.91 (m, 2H), 7.85 (d, J = 8.5 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H), 7.58 – 7.54 (m, 1H), 7.42 (d, J = 7.8 Hz, 1H), 6.90 (s, 2H), 4.40 – 4.35 (m, 1H), 3.90 – 3.87 (m, 1H), 3.24 – 3.20 (m, 1H), 2.94 – 2.85 (m, 1H), 2.48 – 2.41 (m, 1H), 2.04 – 1.94 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.63, 166.26, 156.57 (2C), 139.79, 138.39, 132.87, 130.38, 130.33/130.02/129.70/129.39 (q, J = 30.3 Hz), 128.57, 128.47 (2C), 126.37, 125.04 (2C), 128.65/125.94/123.24/120.53 (q, J = 273.3 Hz, $\underline{C}F_3$), 123.48, 121.45, 120.20/120.17 ($\underline{C}H_{phenyl\text{-}CF3}$), 116.03/115.99 ($\underline{C}H_{phenyl\text{-}CF3}$), 59.97, 51.78, 51.43, 36.65.

*5.1.48 (2R,4R)-4-(3-(6-acetamidopyridin-3-yl)benzamido)-N-(4-chloro-3-(trifluoromethyl)phenyl)pyrrolidine-2-carboxamide (**AA19**)*

Compound **16a** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 37%. Mp 149-151□. EI-MS (m/z): 546 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{26}H_{24}ClF_3N_5O_3$: 546.15198, found 546.15245. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.63 (s, 1H), 10.43 (s, 1H), 8.66 (s, 1H), 8.50 (d, J = 5.9 Hz, 1H), 8.17 (d, J = 9.1 Hz, 2H), 8.08 (d, J = 8.5 Hz, 2H), 7.92 (d, J = 7.9 Hz, 1H), 7.84 – 7.79 (m, 2H), 7.50 (d, J = 3.9 Hz, 2H), 7.37 (d, J = 7.5 Hz, 1H), 4.40 – 4.36 (m, 1H), 3.91 – 3.82 (m, 1H), 3.25 – 3.20 (m, 1H), 2.91 – 2.87 (m, 1H), 2.50 – 2.45 (m, 1H), 2.13 (s, 3H), 2.08 – 1.96 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.33, 169.83, 166.40, 152.09, 146.33, 139.76, 137.32, 136.66, 135.52, 130.80, 130.24, 130.30/129.99/129.68/129.37 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.46, 129.41, 127.11, 128.60/125.90/123.19/ 120.49 (q, J = 273.7 Hz, $\underline{C}F_3$), 125.30, 123.42, 120.12/120.09 ($\underline{C}H_{phenyl\text{-}CF3}$), 115.98/115.94 ($\underline{C}H_{phenyl\text{-}CF3}$), 113.58, 59.99, 51.93, 51.72, 36.74, 24.39.

*5.1.49 (2R,4R)-N-(4-chloro-3-(trifluoromethyl)phenyl)-4-(3-(6-pivalamidopyridin-3-yl)benzamido)pyrrolidine-2-carboxamide (**AA20**)*

Compound **16b** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 25%. Mp 155-157□. EI-MS (m/z): 588 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{29}H_{30}ClF_3N_5O_3$: 588.19893, found 588.21917. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 9.94 (s, 1H), 8.68 (s, 1H), 8.52 (d, J = 5.9 Hz, 1H), 8.15 (d, J = 8.8 Hz, 2H), 8.10 (d, J = 7.0 Hz, 2H), 7.91 (d, J = 8.3 Hz, 1H), 7.85 (d, J = 8.2 Hz, 1H), 7.80 (d, J = 7.1 Hz, 1H), 7.51 (s, 2H), 7.38 (s, 1H), 4.41– 4.39 (m, 1H), 3.98 – 3.84 (m, 1H), 3.26 – 3.22 (m, 2H), 3.00 – 2.86 (m, 1H), 2.10 – 1.99 (m, 1H), 1.27 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.73, 172.69, 166.45, 152.32, 146.04, 139.75, 137.28, 136.56, 135.54, 130.91, 130.28, 130.31/130.00/129.68/129.37 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.51, 129.42, 127.11, 128.60/125.89/123.19/120.48 (q, J = 273.7 Hz, $\underline{C}F_3$), 125.37, 123.44, 120.16/120.12 ($\underline{C}H_{phenyl\text{-}CF3}$), 115.99/115.95 ($\underline{C}H_{phenyl\text{-}CF3}$), 114.36, 59.95, 51.80, 51.60, 36.64, 27.35 (3C), 21.65.

*5.1.50 (2R,4R)-N-(4-chloro-3-(trifluoromethyl)phenyl)-4-(3-(6-(methylsulfonamido) pyridin-3-yl)benzamido)pyrrolidine-2-carboxamide (**AA21**)*

Compound **16c** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 38%. Mp 140-142□. EI-MS (m/z): 582 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{25}H_{24}ClF_3N_5O_4S$: 582.11896, found 582.13934. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.43 (s, 1H), 8.60 (s, 1H), 8.48 (d, J = 5.9 Hz, 1H), 8.18 (s, 1H), 8.06 (s, 2H), 7.91 (d, J = 7.7 Hz, 1H), 7.80 (d, J = 7.1 Hz, 2H), 7.50 (d, J = 7.3 Hz, 2H), 7.38 (d, J = 6.9 Hz, 1H), 7.06 (d, J = 7.8 Hz, 1H), 4.44 – 4.31 (m, 1H), 3.91 – 3.81 (m, 1H), 3.34 (s, 3H), 3.27 – 3.13 (m, 2H), 2.94 – 2.85 (m, 1H), 2.05 – 1.96 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.36, 166.34, 152.35, 145.58, 139.75, 137.47, 137.13, 135.49, 130.28, 130.27/129.97/129.67/129.37 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.50, 129.44 (2C), 127.11, 128.63/125.91/123.20/120.49 (q, J = 273.7 Hz, $\underline{C}F_3$), 125.30, 123.44, 120.14/120.10 ($\underline{C}H_{phenyl\text{-}CF3}$), 115.96/115.92 ($\underline{C}H_{phenyl\text{-}CF3}$), 112.72, 59.98, 51.95, 51.71, 42.24, 36.76.

*5.1.51 (2R,4R)-4-(3-(6-aminopyridin-3-yl)benzamido)-N-(4-chloro-3-(trifluoromethyl)phenyl)pyrrolidine-2-carboxamide (**AA22**)*

Compound **16d** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two

steps was 50%. Mp 165-167□. EI-MS (m/z): 504 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{24}H_{22}ClF_3N_5O_2$: 504.14141, found 504.19911. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 8.61 (s, 2H), 8.45 (s, 1H), 8.17 (s, 1H), 8.01 (s, 1H), 7.91 (d, *J* = 8.1 Hz, 1H), 7.77 – 7.70 (m, 2H), 7.53 – 7.49 (m, 1H), 7.46 – 7.43 (m, 1H), 7.38 (d, *J* = 7.3 Hz, 1H), 6.85 (s, 2H), 4.41 – 4.35 (m, 1H), 3.95 – 3.83 (m, 1H), 3.27 – 3.21 (m, 1H), 2.93 – 2.87 (m, 1H), 2.49 – 2.41 (m, 1H), 2.05 – 1.95 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.74, 166.44, 163.52, 156.53 (3C), 139.74, 135.78, 135.40, 130.27, 130.31/130.00/129.69/129.38 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.38, 128.35, 126.43, 128.60/125.89/123.19/120.48 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 124.14, 123.43, 121.94, 120.14/120.11 ($\underline{C}H_{phenyl\text{-}CF3}$), 115.98/115.94 ($\underline{C}H_{phenyl\text{-}CF3}$), 59.93, 51.83, 51.60, 36.68.

*5.1.52 5-(4-(((3R,5R)-5-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)pyrrolidin-3-yl)carbamoyl)phenyl)-N-cyclopropylnicotinamide (**AA23**)*

Compound **17a** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 13%. The overall yield of last two steps was 13%. Mp 162-165□. EI-MS (m/z): 572 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{28}H_{26}ClF_3N_5O_3$: 572.16763, found 572.18422. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.42 (s, 1H), 9.04 (d, *J* = 2.1 Hz, 1H), 8.97 (d, *J* = 1.9 Hz, 1H), 8.75 (d, *J* = 3.8 Hz, 1H), 8.51 (d, *J* = 6.6 Hz, 1H), 8.43 – 8.42 (m, 1H), 8.21 (s, 1H), 7.95 (d, *J* = 8.4 Hz, 3H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.58 – 7.54 (m, 1H), 7.41 (d, *J* = 7.8 Hz, 1H), 4.40 – 4.35 (m, 1H), 3.88 – 3.84 (m, 1H), 3.22 – 3.19 (m, 1H), 2.91 – 2.86 (m, 2H), 2.49 – 2.41 (m, 1H), 2.04 – 1.95 (m, 1H), 0.77 – 0.71 (m, 2H), 0.64 – 0.58 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.33, 166.28, 166.11, 150.21, 148.28, 139.82, 139.36, 134.58, 134.51, 133.16, 130.38, 130.35, 130.32/130.01/129.70/129.39 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 128.55 (2C), 127.20 (2C), 128.65/ 125.95/123.24/120.54 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 123.47, 120.15/120.12 ($\underline{C}H_{phenyl\text{-}CF3}$), 116.02/115.98 ($\underline{C}H_{phenyl\text{-}CF3}$), 60.08, 51.98, 51.72, 36.72, 23.53, 6.22 (2C).

5.1.53 5-(4-(((3R,5R)-5-((4-chloro-3-(trifluoromethyl)phenyl)*carbamoyl)pyrrolidin-3-yl)carbamoyl)phenyl)-N,N-diethylnicotinamide (**AA24**)*

Compound **17b** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 34%. Mp 145-146□. EI-MS (m/z): 588 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{29}H_{30}ClF_3N_5O_3$: 588.19893, found 588.21751. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.42 (s, 1H), 8.99 (d, *J* = 2.2 Hz, 1H), 8.59 (d, *J* = 1.9 Hz, 1H), 8.50 (d,

J = 6.6 Hz, 1H), 8.21 (s, 1H), 8.10 – 8.09 (m, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.81 (d, *J* = 8.4 Hz, 2H), 7.58 – 7.54 (m, 1H), 7.40 (d, *J* = 7.7 Hz, 1H), 4.39 – 4.35 (m, 1H), 3.87 – 3.83 (m, 1H), 3.51 – 3.47 (m, 2H), 3.24 – 3.22 (m, 2H), 3.22 – 3.17 (m, 1H), 2.90 – 2.86 (m, 1H), 2.49 – 2.46 (m, 1H), 2.05 – 1.94 (m, 1H), 1.19 (t, *J* = 6.4 Hz, 3H), 1.08 (t, *J* = 6.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.47, 167.84, 166.09, 148.50, 146.47, 139.83, 139.20, 134.74, 134.53, 133.68, 132.25, 130.33, 130.31/130.00/129.69/129.37 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 128.54 (2C), 127.18 (2C), 128.66/125.95/123.25/120.54 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 123.46, 120.10/120.07 ($\underline{C}H_{phenyl\text{-}CF3}$), 116.02/115.98 ($\underline{C}H_{phenyl\text{-}CF3}$), 60.07, 52.01, 51.75, 43.48, 39.56, 36.81, 14.50, 13.26.

*5.1.54 5-(4-(((3R,5R)-5-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl) pyrrolidin-3-yl)carbamoyl)phenyl)-N-(2-(dimethylamino)ethyl)nicotinamide (**AA25**)*

Compound **17c** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 14%. Mp 216-218□. EI-MS (m/z): 639 $[M+HCl]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{29}H_{31}ClF_3N_6O_3$: 603.20983, found 603.23241. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.74 (s, 1H), 9.08 (s, 1H), 9.04 (s, 1H), 8.73 (s, 1H), 8.60 (s, 1H), 8.17 (s, 1H), 8.13 (s, 1H), 8.01 (d, *J* = 8.3 Hz, 2H), 7.93 (d, *J* = 8.3 Hz, 2H), 7.90 – 7.86 (m, 1H), 7.59 – 7.55 (m, 1H), 7.41 (d, *J* = 7.6 Hz, 1H), 4.56 – 4.52 (m, 1H), 4.49 – 4.44 (m, 1H), 3.92 – 3.80 (m, 1H), 3.48 – 3.43 (m, 3H), 2.76 (s, 2H), 2.70 – 2.61 (m, 1H), 2.43 (s, 6H), 2.18 – 2.08 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.84, 166.09, 165.16, 150.32, 148.49, 140.15, 139.55, 134.53, 134.18, 133.35, 130.46, 130.27, 130.31/130.01/129.70/129.38 (q, *J* = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 128.58 (2C), 127.36 (2C), 128.62/125.91/123.20/120.50 (q, *J* = 273.7 Hz, $\underline{C}F_3$), 123.32, 120.23, 115.99/115.84 ($\underline{C}H_{phenyl\text{-}CF3}$), 61.29, 57.76, 49.04, 48.31, 44.67 (2C), 36.89, 35.57.

*5.1.55 5-(3-(((3R,5R)-5-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl) pyrrolidin-3-yl)carbamoyl)phenyl)-N-cyclopropylnicotinamide (**AA26**)*

Compound **18a** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 61%. Mp 180-182□. EI-MS (m/z): 572 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{28}H_{26}ClF_3N_5O_3$: 572.16763, found 572.18624. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.48 (s, 1H), 9.03 (d, *J* = 1.9 Hz, 1H), 8.98 (d, *J* = 1.8 Hz, 1H), 8.81 (s, 1H), 8.56 (s, 1H), 8.46 (s, 1H), 8.20 (s, 1H), 7.93 (s, 1H), 7.91 – 7.86 (m, 2H), 7.73 (d, *J* = 8.1 Hz, 1H), 7.59 – 7.53 (m, 1H), 7.47 – 7.43 (m, 1H), 7.30 (d, *J* = 7.7 Hz, 1H), 4.42 – 4.39 (m, 1H), 4.33 – 4.29 (m, 1H), 3.83 – 3.79 (m, 1H), 3.12 – 3.08 (m, 1H),

2.92 – 2.87 (m, 1H), 2.49 – 2.41 (m, 1H), 2.29 – 2.23 (m, 1H), 0.79 – 0.71 (m, 2H), 0.65 – 0.57 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.22, 166.31, 162.53, 150.24, 148.11, 140.60, 136.99, 135.66, 134.98, 133.20, 130.27 (2C), 130.16, 130.26/129.95/129.63/ 129.32 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.55, 127.76, 126.07, 128.61/125.90/123.19/120.49 (q, J = 273.7 Hz, $\underline{C}F_3$), 123.06, 119.40, 115.49/115.45 ($\underline{C}H_{phenyl\text{-}CF3}$), 60.12, 52.74, 49.58, 33.91, 23.55, 6.18 (2C).

*5.1.56 5-(3-(((3R,5R)-5-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)pyrrolidin-3-yl)carbamoyl)phenyl)-N,N-diethylnicotinamide (**AA27**)*

Compound **18b** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 34%. Mp 97-99□. EI-MS (m/z): 588 $[M+H]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{29}H_{30}ClF_3N_5O_3$: 588.19893, found 588.22202. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.39 (s, 1H), 8.99 (d, J = 2.2 Hz, 1H), 8.59 (d, J = 1.9 Hz, 1H), 8.52 (d, J = 6.7 Hz, 1H), 8.17 (d, J = 7.2 Hz, 2H), 8.12 – 8.11 (m, 1H), 7.94 – 7.88 (m, 2H), 7.86 (d, J = 7.8 Hz, 1H), 7.56 – 7.52 (m, 1H), 7.50 – 7.46 (m, 1H), 7.36 (d, J = 7.7 Hz, 1H), 4.40 – 4.35 (m, 1H), 3.87 – 3.83 (m, 1H), 3.53 – 3.44 (m, 2H), 3.24 – 3.20 (m, 3H), 2.89 – 2.85 (m, 1H), 2.49 – 2.45 (m, 1H), 2.03 – 1.96 (m, 1H), 1.19 (s, 3H), 1.08 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.48, 167.91, 166.22, 148.62, 146.21, 139.77, 136.75, 135.60, 135.15, 133.61, 132.33, 130.24 (2C), 130.28/129.97/129.65/129.34 (q, J = 30.3 Hz, $\underline{C}_{phenyl}$-CF_3), 129.59, 128.01, 125.99, 128.61/125.91/123.20/120.49 (q, J = 273.7 Hz, $\underline{C}F_3$), 123.42, 120.09, 115.94/115.90 ($CH_{phenyl\text{-}CF3}$), 60.03, 52.00, 51.83, 43.48, 39.60, 36.74, 14.50, 13.28.

*5.1.57 5-(3-(((3R,5R)-5-((4-chloro-3-(trifluoromethyl)phenyl)carbamoyl)pyrrolidin-3-yl)carbamoyl)phenyl)-N-(2-(dimethylamino)ethyl)nicotinamide(**AA28**)*

Compound **18c** was used as starting material, and then deprotection of Boc group was performed using the same method as compound **9**. The overall yield of last two steps was 16%. Mp 207-209□. EI-MS (m/z): 639 $[M+HCl]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{29}H_{31}ClF_3N_6O_3$: 603.23054, found 603.20983. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.73 (s, 1H), 9.10 (s, 1H), 9.05 (s, 1H), 8.91 (s, 1H), 8.80 (s, 1H), 8.40 (s, 1H), 8.13 (s, 1H), 8.11 (s, 1H), 8.03 (d, J = 7.7 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.87 (s, 1H), 7.62 – 7.58 (m, 1H), 7.49 (d, J = 7.4 Hz, 1H), 7.36 (s, 1H), 4.57 – 4.52 (m, 1H), 4.50 – 4.40 (m, 1H), 3.90 – 3.83 (m, 1H), 3.58 – 3.45 (m, 3H), 3.05 (s, 2H), 2.75 – 2.65 (m, 1H), 2.62 (s, 6H), 2.28 – 2.13 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ171.99, 166.21, 165.36, 150.29, 148.42, 140.10, 136.81, 135.18, 134.73, 133.51, 130.33, 130.29, 129.93, 129.73, 129.93/129.62/ 129.31/129.00 (q, J = 30.3 Hz,

$\underline{C}_{phenyl}$-CF_3), 128.11, 126.20, 128.57/125.87/123.16/120.45 (q, J = 273.7 Hz, $\underline{C}F_3$), 123.38, 120.11, 115.99/115.90 ($\underline{C}H_{phenyl\text{-}CF3}$), 61.27, 56.95, 49.21, 48.44, 43.71 (2C), 35.92, 35.63.

5.2. In vitro enzymatic assays

The *in vitro* Bcr-Abl inhibition assays were carried out by ADP-Glo™ kinase assay (Promega, Madison, WI) according to the manufacturer's instructions, with Imatinib as the positive control. General procedures were as follows: Kinases (4 ng/μl) were incubated with substrates (0.2 μg /μl), compounds (1.2×10^{-4}–12 μM) and ATP (25 μM) in a final buffer of Tris 40 mM, $MgCl_2$ 10 mM, BSA 0.1 mg/mL, and DTT 1 mM in a 384-well plate with a total volume of 5 μL. The assay plate was incubated at 30 °C for 1 h. Once the kinase reaction was complete, 5 μL of ADP-Glo reagent was added to terminate the reaction and deplete the remaining ATP. The mixture was incubated at room temperature for 40 min. At the end, 10 μL of kinase detection reagent was added and incubated for 30 min, to convert ADP to ATP and allow the newly synthesized ATP to be measured using luciferase/luciferin reaction. The luminescence generated was read by a VICTORX multiple plate reader. The signal was correlated with the amount of ATP present in the reaction and was inversely correlated with the kinase activity.

5.3 Cell growth inhibition assays

The MTT assays were performed to evaluate the antiproliferative activity and identify the cytotoxicity of the title compounds *in vitro*. Imatinib and ponatinib were used as the positive control. The K562R cells expressing T315I mutation was kindly provided by Professor Libo Yao (Fourth Military Medical University, Xi'an, China). The Hek293 cells was provided by Dr. Tao Zhang (Xi'an Jiaotong University, Xi'an, China). The EA.hy926 cells was provided by Dr. Wen Lu (Xi'an Jiaotong University, Xi'an, China). The cancer cell lines (K562 and K562R cells) were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). The two normal human cells (Hek293 and EA.hy926 cells) were cultured in DMEM medium with 10% FBS.

The cells in exponential phase of growth were transferred to the 96-well plate, and it is about 2.5×10^3 cells in each well. The plate was incubated in 5% CO_2 at 37□ for 12 h. The test compounds at serial diluted concentrations were added to the cells solution and incubated for 48 h. Then MTT was added to each well at a final concentration of 0.5 mg/mL and incubated for 4 h at 37□. Supernatant was discarded, and 150 μL DMSO was added to each well. After shaking 15 min, absorbance values

at 490 nm were determined by a microplate reader (Bio-Rad, Hercules, USA). The IC_{50} values were calculated according to inhibition ratios.

5.4 Cell apoptosis assay

First, K562 cells (5×10^5 cells/mL) were seeded into 6-well plates, and treated with compounds at different concentrations for 48 h. The cells were then harvested and washed with cold PBS three times. After centrifugation and removal of the supernatant, cells were resuspended in 400 μL binding buffer (1×). After that, 5 μL annexin V-FITC was added and incubated at room temperature for 15 min, followed by adding 10 μL PI and incubated at room temperature for 15 min in the dark. Finally, the stained cells were analyzed by flow cytometer (BD Accuri C6).

5.5 Western blot assay

K562 cells were treated with vehicle (DMSO) or drug at desired concentration for 48 h. The cells were lysed in SDS sample buffer, collected, and normalized using BCA protein assay kit before being diluted in SDS loading buffer. Then the samples containing equal amounts of protein were separated by SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes and blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20. Membranes were incubated with antibodies p-Bcr-Abl and GADPH at 4□ overnight. The membranes were washed with PBS three times, and incubated with the appropriate anti-HRP secondary antibodies for 2 h at room temperature. Finally, immunoreactive proteins were visualized using the enhanced chemiluminescence system from Pierce Chemical.

5.6 Molecular docking

In order to investigate the interaction between the title compounds and Abl kinases (Bcr-Abl and Bcr-Abl^{T315I}), molecular docking was performed using Surflex-Dock Module of Sybyl-X 2.0. Crystal structures of Bcr-Abl^{WT} (PDB ID: 1IEP) and Bcr-Abl^{T315I} (PDB ID: 3IK3) were imported, and protein structure preparation was carried out including removing water molecules, adding missing atom and hydrogen, and minimized with Pullman charges under Tripos Force Field. Then the corresponding ligand in the prepared protein structure was extracted and used to define the binding cavity. The residues in a radius 5.0Å around the ligand were selected as active site and generated as docking promotal. The inhibitor molecules were drawn with Sketch and minimized by Powell's method for 1000 iterations under Tripos Force Field with Gasteiger-Huckel charges. In the end, the prepared molecules were docking into the active sites of protein promotal using ligand-based mode with

other docking parameters as default.

5.7 Molecular Dynamics

We carried out Molecular Dynamic (MD) simulation studies using Dynamics module (Desmond v53011) of Schrodinger software. At the beginning, the complex was processed by Protein Prepare Module according to the default parameters. The bonding information is corrected, hydrogen atoms are added, and water molecules are removed in order to get minimized protein structure. Next, in the System Builder plate, the processed protein was imported to be solvated with the water model of TIP3P and the force field of OPLS3. In order to ensure that the complex is completely wrapped in the simulated solvent environment, we set some parameters of Boundary conditions, including Box shape of Orthorhombic, Box size calculation method of Buffer, and Minimize Volume. The Na^+ was added to neutralize the negative charge of protein. Then, we used the Molecular Dynamics to simulate the dynamic simulation of complex with default parameters. Finally we use the function of Simulation Interactions Diagram The Root to analysis the output of Molecular Dynamics. Mean Square Deviation (RMSD) was used to measure the average change in displacement of a selection of atoms for a particular frame concerning a reference frame. This was calculated for all structures in the trajectory.

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Design, Synthesis, and Biological Evaluation of Novel Bcr-Abl^{T315I} Inhibitors Incorporating Amino Acids as Flexible Linker

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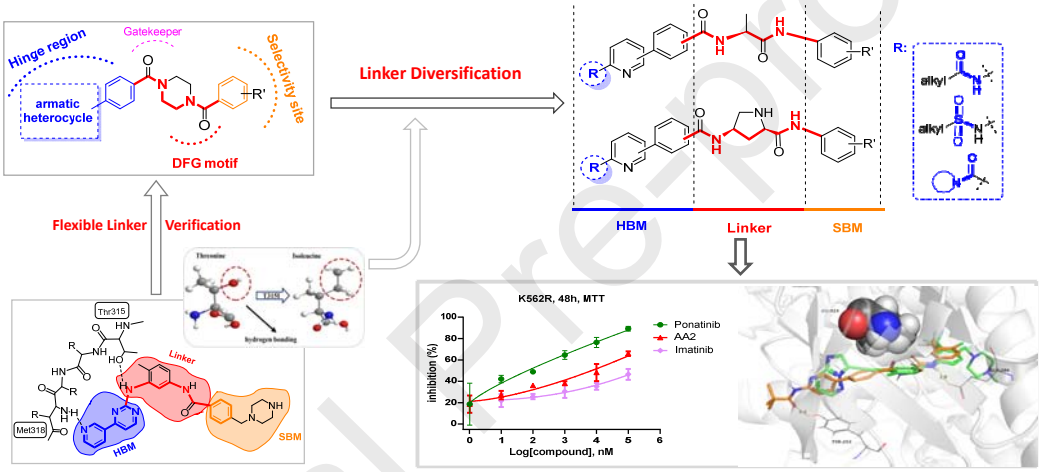

A series of novel Bcr-Abl^{T315I} inhibitors with amino acids as flexible linker were developed and performed biological evaluation *in vitro*.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: