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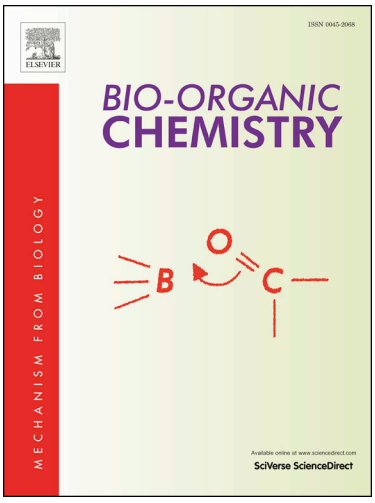

Discovery and optimization of novel N-benzyl-3,6-dimethylbenzo[d]-isoxazol-5-amine derivatives as potent and selective TRIM24 bromodomain inhibitors with potential anti-cancer activities

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Discovery and optimization of novel N-benzyl-3,6-dimethylbenzo[d]-isoxazol-5-amine derivatives as potent and selective TRIM24 bromodomain inhibitors with potential anti-cancer activities

Qingqing Hu,[a,b,c] Chao Wang,[a,b,c] Qiuping Xiang,[a,b,c] Rui Wang,[a] Cheng Zhang,[a] Maofeng Zhang,[d] Xiaoqian Xue,[e] Guolong Luo,[a] Xiaomin Liu,[a] Xishan Wu,[a] Yan Zhang,[a,b,c] Donghai Wu,[f] and Yong Xu[a,c,*]

[a]Guangdong Provincial Key Laboratory of Biocomputing, Joint School of Life Sciences, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences; Guangzhou Medical University, Guangzhou 510530, China.

[b]University of Chinese Academy of Sciences, No. 19 Yuquan Road, Beijing 100049, China.

[c]Guangzhou Regenerative Medicine and Health Guangdong Laboratory (GRMH-GDL), Guangzhou 510530, China

[d]Taizhou Polytechnic College, No. 8 Tianxing Road, Medical High-tech Development Zone, Taizhou 225300, Jiangsu Province, China

[e]School of Life Science, Huizhou University, Huizhou 516007, China

[f]Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences; Guangzhou 510530, China

[*]Corresponding Author: Yong Xu, PhD, E-mail: xu_yong@gibh.ac.cn

Abstract

Tripartite motif-containing protein 24 (TRIM24), recognized as an epigenetic reader for acetylated H3K23 (H3K23ac) *via* its bromodomain, has been closely involved in tumorigenesis or tumor progression of several cancers. Developing inhibitors of TRIM24 is significant for functional studies and drug discovery. Herein, we report the identification, optimization and evaluation of N-benzyl-3,6-dimethylbenzo[d]isoxazol-5-amines as TRIM24 bromodomain inhibitors starting from an in house library screening. Structure-based optimization leads to two potent and selective compounds **11d** and **11h** in an Alphascreen assay with IC_{50} values of 1.88 μM and 2.53 μM, respectively. The viability assay demonstrates the great potential of this series of compounds as inhibitors of proliferation of prostate cancer (PC) cells LNCaP, C4-2B. A colony formation assay further supports this inhibitory activity. Compounds **11d** and **11h** inhibit cell proliferation of other cancer types such as non-small cell lung cancer (NSCLC) cells A549 with IC_{50} values of 1.08 μM and 0.75 μM, respectively. These data suggests that compounds **11d** and **11h** are promising lead compounds for further research.

Keywords: TRIM24 bromodomain, inhibitor, prostate cancer, anti-cancer, structure-based drug design

1. Introduction

TRIM24 (Tripartite motif-containing protein 24) is a multifunctional protein involved in different biological processes [1] such as homologous or heterogeneous polymerization [2] and ubiquitylation [3], including the ubiquitylation of the tumor suppressor p53 as an E3 ubiquitin ligase due to its tripartite RBCC motif [4-6]. TRIM24 is also known as TIF1α (Transcription Intermediary Factor 1 alpha) because its nuclear receptor interacting LXXLL domain regulates multiple transcriptional activities [7, 8]. Another important domain of TRIM24 is the bromodomain which defines the whole BCP (bromodomain containing protein) superfamily which includes 61 members that are classified into eight groups [9]. TRIM24 belongs to the fifth subfamily and as a histone epigenetic reader, it can specifically recognize acetylated H3K23 (H3K23ac) *via* its bromodomain [10]. Previous studies have indicated that TRIM24 regulates tumorigenesis or tumor progression of various cancer types including prostate cancer [11], lung cancer [12], colorectal cancer [13, 14], breast cancer [10] and gliobastoma [15].

Prostate cancer (PC) is a common cancer in men worldwide. The clinically dominant treatment is androgen-deprivation approaches that inhibit the androgen synthesis or androgen receptor (AR) signaling pathways [16, 17]. Unfortunately, most patients eventually develop resistance to castration therapies and progress to castration-resistant prostate cancer (CRPC) [17]. CRPC is generally fatal and these patients are in significant need of more effective treatments such as targeted therapeutic drugs. In recent years, we have been focused on development of small molecule inhibitors of BCPs and nuclear receptors which are potential therapeutic targets for CRPC [18-22]. Pioneer work by Brown and others supported that the TRIM24 mediated CRPC growth through its bromodomain and LXXLL motif and suggested that the bromodomain was a potential drug target [11, 23-25]. The discovery of new potent and specific TRIM24 inhibitors will greatly enhance our understanding of the therapeutic potential of bromodomain inhibition for the treatment of CRPC.

LXXLL motif is a conserved short sequence [26] which interacts with the AF-2 domain of nuclear receptors [8, 27, 28]. From a general perspective of druggable targets, LXXLL motif is too small to provide sufficient protein surface to which drug molecules can bind, although it is large enough to bind with nuclear receptors [26]. Unlike the LXXLL motif, the bromodomain contains approximately 110 amino acids [9] which are folded into a conserved four helical bundle that forms an adequate protein surface for drug molecules' occupation [9, 10]. A separate evaluation of the druggability of TRIM24 bromodomain without involving the interaction between the bromodomain and the plant homeodomain (PHD) led to a low score, suggesting the difficulty for drugs to bind with this bromodomain [29]. Encouragingly, two groups initially disclosed a class of inhibitors targeting the TRIM24 bromodomain [30, 31], which casted a new light on the druggability of the bromodomain of TRIM24. To date, the best inhibitor, IACS-9571, has nanomolar activity in a protein-level assay [30], but the activity data of TRIM24 bromodomain inhibitors tested on various cancer cells is still limited and deficient [31, 32].

In view of the significance of TRIM24 in cancer development and the limited structure types, it is worthwhile to discover novel molecules as tool molecules or lead compounds for drug discovery. Herein, we reported identification of a series of novel TRIM24 bromodomain inhibitors through a sequential process including in-house library screening, structure-based optimization and molecular docking studies. Two potent and selective leads also inhibited the growth of PC cells and other cancer cells including non-small cell lung cancer cells, suggesting the clinical anti-cancer potential of these compounds.

2. Results and discussion

2.1. Discovery of hit compounds through in-house library screening

In this work, we performed an Alphascreen assay [19, 21] to screen our in-house compound library against a recombinant human TRIM24 bromodomain produced in-house. As shown in Figure 1A, we identified three compounds with inhibition rates

above 90% by conducting the screening at a concentration of 20 μM. These compounds (**1**, **2** and **3**) have dose-dependent inhibitory activities against the TRIM24 bromodomain with an IC_{50} value of 11.18 μM, 14.22 μM, and 13.76 μM, respectively (Figure 1B, Table 1). These compounds exhibit fair binding capability and their inhibition rates were comparable with that of IACS-9571, making them perfect hits in the traditional medicinal chemistry sense. We identified these three compounds as hit compounds for subsequent structure optimization.

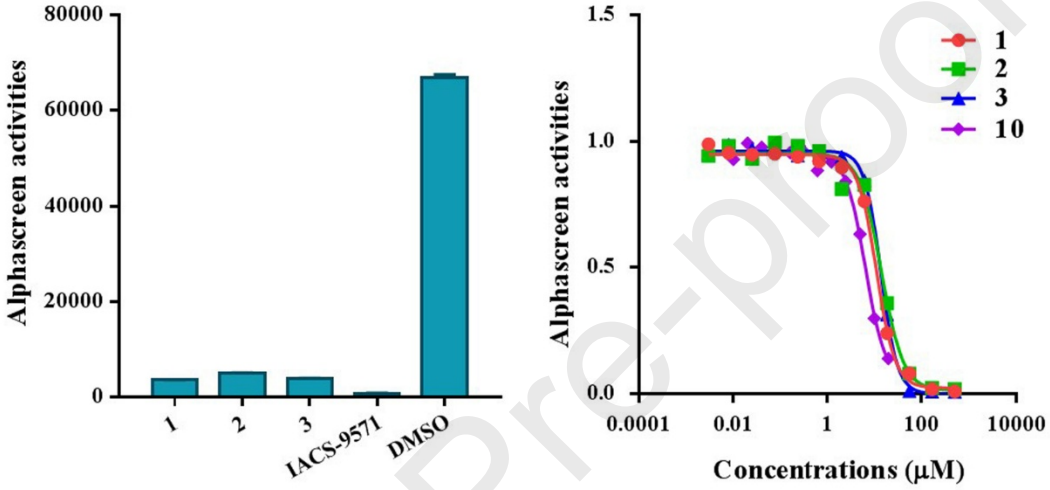
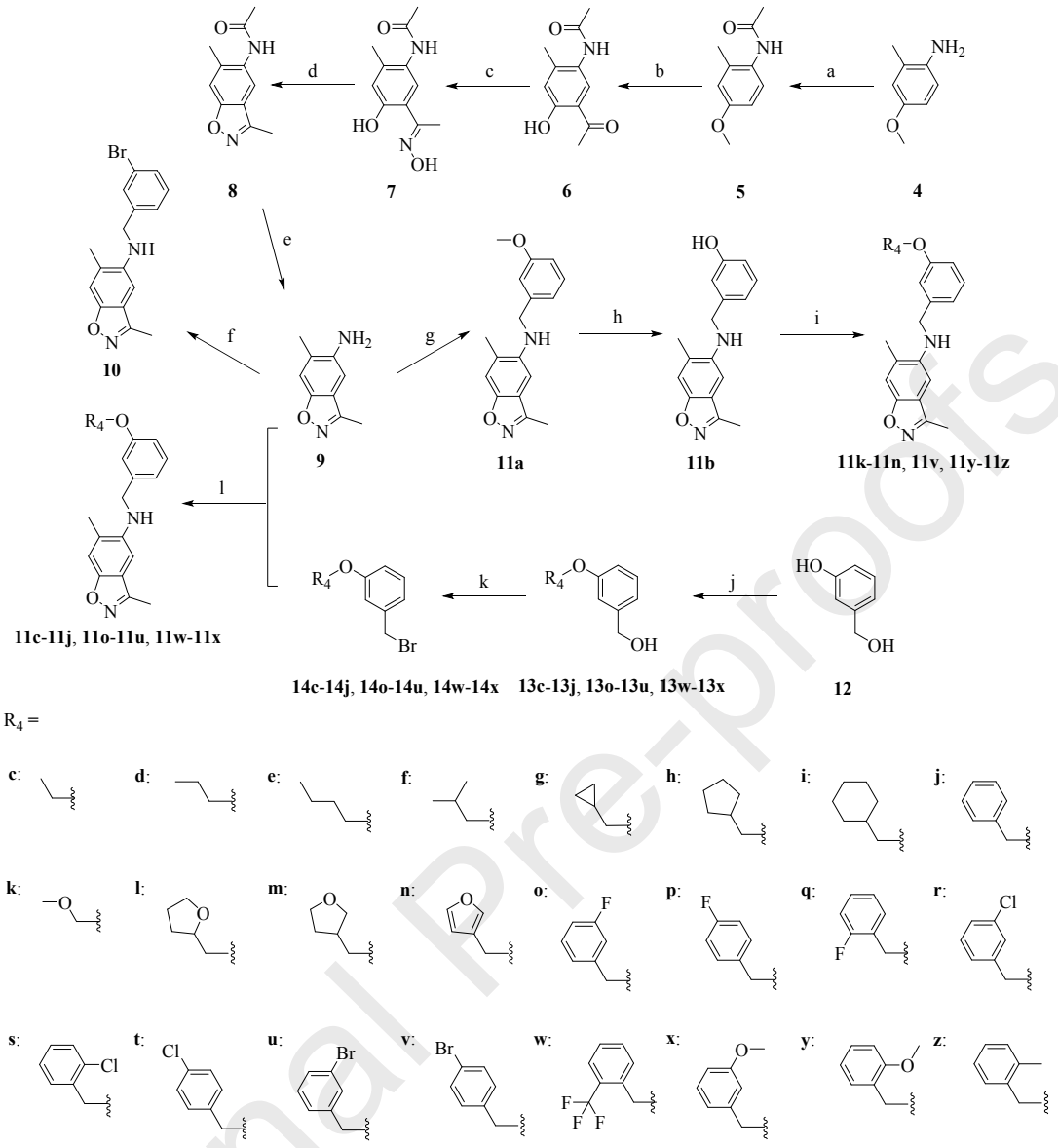


Figure 1. The discovery of hit compounds. (A) The inhibitory rate of three hit compounds at a concentration of 20 μM in an in-house library screening. (B) The dose-dependent inhibitory activities of three hit compounds. All experiments were carried out in an Alphascreen assay.

2.2. Synthesis

Based on three hit compounds mentioned above, a series of derivatives were designed and synthesized. Two important intermediates **8** (for synthesizing compounds **11a'**, **11b'**, **11d'** and **11x'**) and **9** (for synthesizing compounds **10**, **11a-11z**) were prepared as depicted in scheme 1 [20].

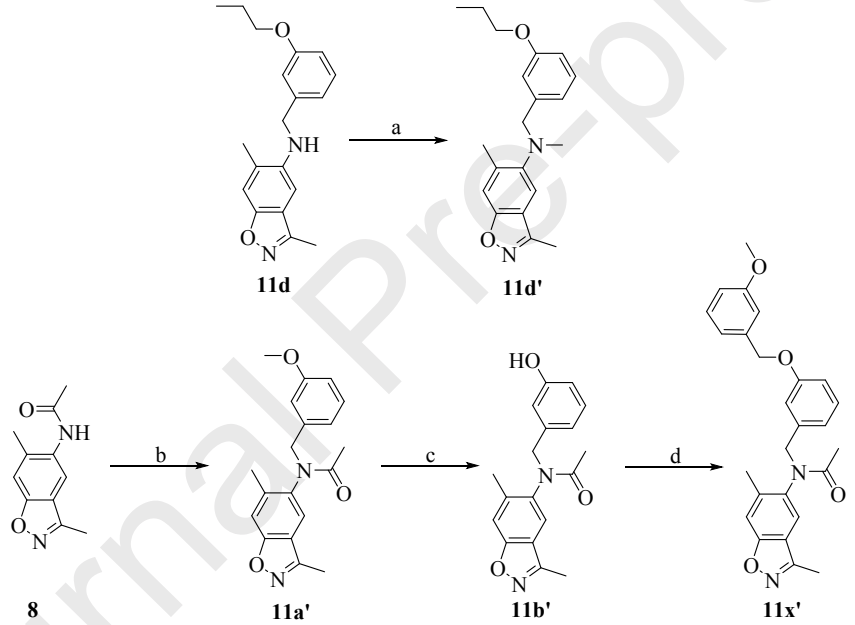


Scheme 1. Synthesis of compounds **10**, **11a-11z**.

Reagents and conditions: (a) Acetyl chloride, Et_3N, DCM, 0 °C, 15 min, 78%; (b) Acetyl chloride, $AlCl_3$, DCM, 0-44 °C, 2 h, 68%; (c) H_2NOH-HCl, AcONa, EtOH/H_2O (3:1), 80 °C, reflux, 70 min, 100%; (d) DMF-DMA, 1,4-dioxane, 100 °C, 10 min, 78%; (e) HCl/H_2O, 100 °C, reflux, 2.5 h, 100%; (f) 1-bromo-3-(bromomethyl)benzene, Cs_2CO_3, DMF, 60 °C, overnight, 40-65%; (g) 1-(bromomethyl)- 3-methoxybenzene, Cs_2CO_3, DMF, 60 °C, overnight, 40-65%; (h) BBr_3, DCM, 0 °C; RT, 2.5 h, 57%; (i) R_4Br, K_2CO_3, 80 °C, 40 min-overnight, 2-47% (for **11k-11l**, **11v**, **11z**) or KOH, RT, DMF, 1h, 28-43% (for **11n** and **11y**); (tetrahydrofuran-3-yl)methyl 4-methylbenzenesulfonate (for **11m**), K_2CO_3, NMP, 95 °C, 4 h, 97%; (j) R_4Br or R_4I, K_2CO_3, DMF, 80 °C, 0.5-1.0 h, 49-100%; (k) PBr_3, DCM, RT, 0.5-2.0 h, 47-98%; (l) Cs_2CO_3, DMF, 60 °C, overnight, 13-57%.

To obtain the final products **10** and **11a-11z**, shown in Scheme 1, we adopted two slightly different strategies, mostly through a chain of substitution reactions. The raw material R_4Br or R_4I can be directly purchased or synthesized in-house from the corresponding alcohols that also are commercially available.

After obtaining compound **11d** by the steps shown in Scheme 1, its simple methylation led to the synthesis of the compound **11d'** (Scheme 2). The synthetic procedure of **11x'** used a strategy similar to the step i shown in Scheme 1 and additionally, we obtained two intermediates **11a'** and **11b'** (Scheme 2).



Scheme 2. Synthesis of compounds **11a'**, **11b'**, **11d'**, **11x'**.

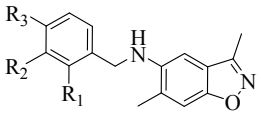
Reagents and conditions: (a) MeI, NaH, 0 °C, 15 min; rt, 10.5 h, 63%; (b) 1-(bromo- methyl)-3-methoxybenzene, Cs_2CO_3, DMF, 60 °C, 10.5 h, 91%; (c) BBr_3, DCM, 0 °C, rt, 0.5-2.5 h, 92%; (d) 1-(bromomethyl)-3-methoxybenzene, K_2CO_3, 80 °C, 3 h, 61%.

2.3. Structure-activity relationship (SAR) studies

We identified three hit compounds (**1**, **2** and **3**) with equivalent inhibitory activities against the TRIM24 bromodomain. Based on their similar inhibitory activities and structures, it was difficult for us to initiate a structure optimization process (Table 1). When we attempted to replace the carboxyl group of compound **3** with a halogen atom,

the newly obtained compound **10** (IC_{50} = 6.46 μM) exhibited an approximately 2-fold increase in inhibitory activity versus compounds **1-3** (Table 1). Comparing the structure and activities of compounds **1**, **2**, and **10** might be helpful to determine the optimal position of substituents, thus we chose compound **10** as the initial compound and R_2 of compound **10** as the next optimization direction.

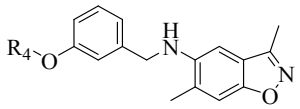
Table 1. The discovery of hit compounds

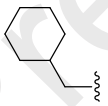
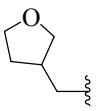
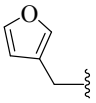
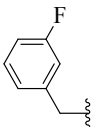
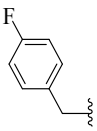
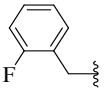


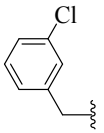
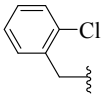
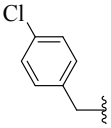
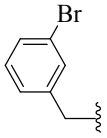
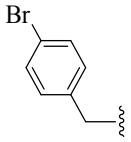
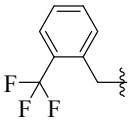
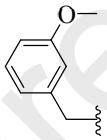
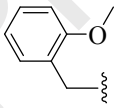
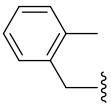
No.	R_1	R_2	R_3	Alphascreen IC_{50} (μM)
IACS-9571				0.24
1	Cl	H	H	11.18
2	H	H	Cl	14.22
3	H	COOMe	H	13.76
10	H	Br	H	6.46

Based on the docking results, we supposed that the bromine atom of compound **10** was directed towards the hydrophobic "upper pocket" (Figure 2A, 3A, see the next section). We synthesized compounds **11a-11j** to explore the optimal space available to the R_4 group (Table 2). The inhibitory activity was improved from **11a** to **11d** and then decreased from **11d** to **11e**, suggesting that three-carbon atom length was most suitable for this "upper pocket" (Table 2) [30]. To further explore the optimal size of the R_4 group, we synthesized **11f-11j** in which the R_4 group has a relatively fixed length of 3 or 4 carbon atoms (Table 2). An R_4 group larger than n-propyl (**11d**) was found to be disadvantageous for improving the activity. Introduction of a hydrophilic oxygen atom into the R_4 group (**11k-11n**, Table 2) failed to improve the activity, which suggested hydrophilic R_4 group was also disadvantageous for improving the activity. Based on compounds **11a-11n**, we could verify the hydrophobic requirement of the R_4 group for better occupation of the hydrophobic "upper pocket".

Table 2. Structure−activity relationships of compounds **11a-11z**



No.	R_4	Alphascreen IC_{50} (μM)
IACS-9571		0.24
11a	CH_3	7.30
11b	H	7.37
11c		3.84
11d		1.88
11e		4.19
11f		3.34
11g		3.47
11h		2.53
11i		12.22
11j		2.86
11k		9.01
11l		4.3
11m		2.54
11n		2.72
11o		70.76
11p		78.36
11q		65.05

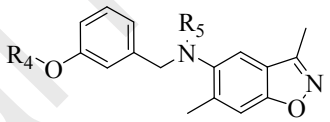
11r		76.09
11s		76.41
11t		24.15
11u		87.31
11v		54.49
11w		64.42
11x		8.42
11y		2.57
11z		2.33

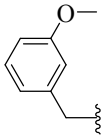
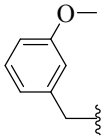
It was found that the activity was promoted more than 4-fold when the cyclohexyl methyl group (**11i**) was replaced by the benzyl group (**11j**). This suggested a potential opportunity to improve the activity by optimizing the structure of **11j**. Since benzene ring of the R_4 group in compound **11j** could be easily substituted, we introduced electron-donating or electron-accepting groups in the *o*-, *m*- and *p*- positions of the phenyl ring to explore the optimization space (Table 2). The activities of compounds **11o-11w** were drastically decreased comparing with the reference compound (**11j**), which implied the disadvantage of electron-attracting substituents. While an electron-donating methyl or methoxyl group was added in the *o*-position of the phenyl ring, the inhibitory activities of **11y** and **11z** were only slightly improved over that of the

reference compound (**11j**), revealing the weak and insignificant advantage of electron-donating substituents (Tables 2). Eventhough optimization of the phenyl ring of the starting compound (**11j**) failed to distinctly improve activity, the unimpressive success of the hydrophobic ortho-methyl substituted **11z** further supported the hydrophobicity requirement of the R_4 group. Taking these findings together, we concluded that introducing hydrophobic groups with appropriate length and shape into the R_4 group is an efficient strategy to improve activity. The *n*-propyl group (**11d**) was identified as the best substituent [30]. A cyclopentyl methyl group (**11h**) and an *o*-tolylmethyl group (**11z**) were suboptimal substituents.

Based on the common N-benzyl-3,6-dimethylbenzo-[d]isoxazol-5-amine skeleton, we synthesized four methyl or acetyl derivatives **11a'**, **11b'**, **11d'** and **11x'** to further investigate the importance of the hydrogen atom in the backbone structure. However, replacement of the hydrogen atom led to significant loss of activity, indicating the essential role of the hydrogen atom of these derivatives (Table 3).

Table 3. Exploration of Substitutability in R_5



No.	R_4	R_5	Alphascreen IC_{50} (μM)
11a		H	7.30
11a'			32.79
11b	H	H	7.37
11b'	H		34.19
11d		H	1.88
11d'			34.97
11x		H	8.42
11x'			39.61

2.4. Binding mode analysis

To further reveal the molecular mechanism of inhibitory activity of these compounds, we analyzed the binding mode from several perspectives. As shown in Figure 2A, the "upper pocket" is defined by hydrophobic residues Ala989, Met920 and a hydrophobic LAF/V shelf (Leu922, Ala923, Phe924/Val986) as well as hydrophilic residues Glu919 and Glu985 with both carboxyl groups extending into the solvent area, making the "upper pocket" highly hydrophobic. As previously reported by Anderson et al., there was one conserved water molecule in the "upper pocket" (see Figure 2B water molecules depicted in red) [30]. After aligning all crystal structures of the TRIM24 bromodomain revealed by Anderson et al., it was found that there actually was a second water molecule relatively conserved in the "upper pocket" (see Figure 2B water molecules depicted in magenta) for two reasons: Firstly, almost every available crystal structures of TRIM24-ligand retain two water molecules in relatively fixed positions in the "upper pocket", and secondly, in most aligned PDBs, two water molecules and residue Glu919 could form a hydrogen-bond network (Figure 2A, 2B).

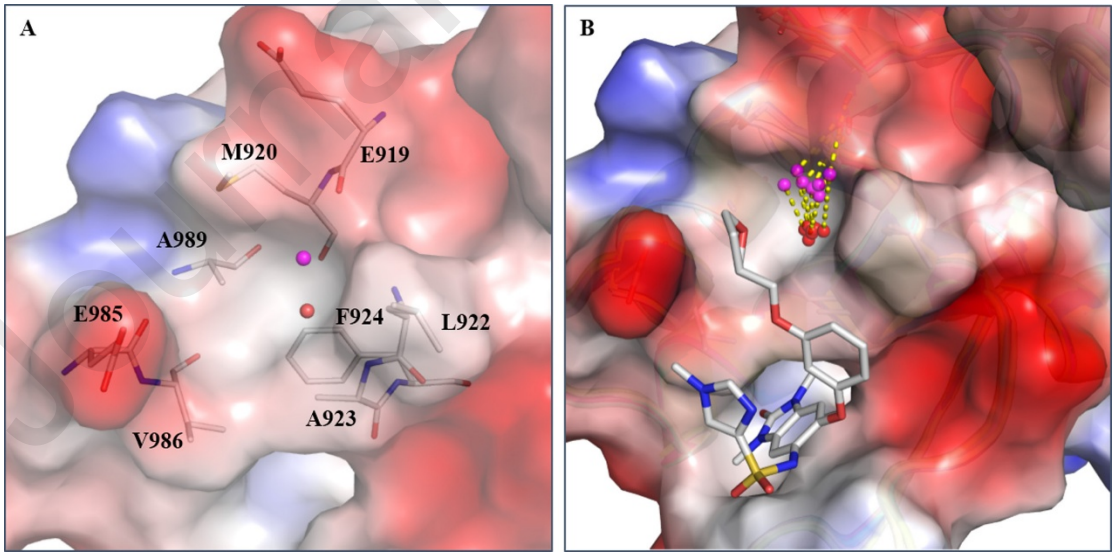


Figure 2. Structure of the "upper pocket". (A) Constitutive residues of the "upper pocket". PDB 4YBT was used as an example. (B) Conserved water molecules in the "upper pocket" based on alignment of released PDBs (4YAB, 4YAD, 4YAT, 4YAX, 4YBM, 4YBS, 4YBT, 4YC9; all PDBs were aligned with 4YBT) [30]. The reported conserved water molecules are depicted as red spheres and the second conserved water molecules are depicted as magenta spheres. All residues are shown as lines. The main

chain of 4YBT was generated as an electrostatic potential surface. The ligand of PDB 4YBT was shown as sticks. All aligned PDBs were shown as transparent cartoons under surface for clarity.

We investigated the binding mode after docking compound **10** into the TRIM24 bromodomain (PDB: 4YBT, with two conserved water molecules in the "upper pocket") with the Glide Standard Precision module [33]. The bromine atom of compound **10** extended into the "upper pocket" (Figure 3A). Due to hydrophobicity of the "upper pocket", introduction of hydrophobic ether groups which could occupy this hydrophobic pocket should benefit activity. Among a series of ether derivatives **11a-11j**, the activity increased from **11a** to **11d** but subsequently declined from **11d** to **11e** as the R_4 group became longer, suggesting that a three-carbon-atom length is the optimal choice of the R_4 group to occupy the "upper pocket" (Figure 3B). Keeping the length fixed and enlarging the shape of the R_4 group to increase the hydrophobic interaction from **11e** to **11j** led to a "rise-fall-rise" trend in activity, revealing that larger R_4 group led to improvement of activity because the activity of **11h** increased versus that of **11f** or **11g**. But the clash in the "upper pocket" caused by increasing the volume of the R_4 group was also important. As shown in Figures 3B, 3C and 3D, **11d** docked its R_4 group precisely into the "upper pocket" without any clash while the phenyl ring of **11j** clashed with the first conserved water in the pocket and **11i** entirely failed to enter into the pocket because of its relatively massive cyclohexyl group. Taken together, **11d** displayed the best balance between scale and hydrophobicity of the R_4 group, and showed optimal inhibitory activity against the TRIM24 bromodomain. Compounds **11h** and **11z** were the suboptimal compounds also achieving that balance.

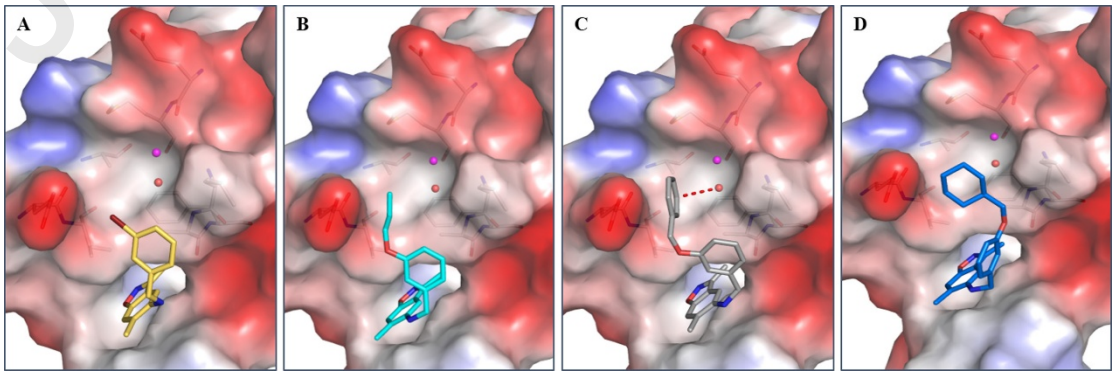
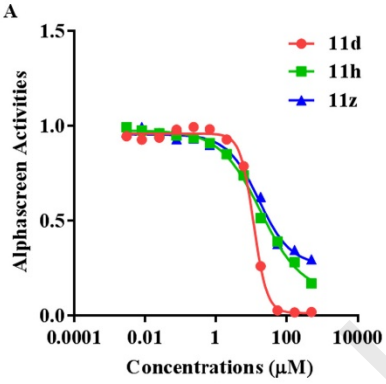


Figure 3. Predicted binding modes of **11d**, **11h**, **11j**, and **11i** in the "upper pocket". 4YBT was prepared for docking each compound: (A) **11d**, (B) **11h**, (C) **11j**, (D) **11i**. All compounds are shown as sticks and all residues as lines. Water molecules in the "upper pocket" are shown as spheres. An electrostatic potential surface was shown as well. The red dashed line represents a serious conflict.

2.5. Evaluation of bromodomain selectivity

To further investigate the selectivity profile, we used an Alphascreen assay to test three optimal compounds **11d**, **11h** and **11z** against representative BCPs produced in house. These compounds showed excellent selectivity for TRIM24 bromodomain over BRD4 (1) and BAZ2B, and moderate selectivity over CREBBP and BRD9 (Figure 4A, 4B) and compound **11h** exhibited the best selectivity profile.

A



B

Compounds	TRIM24 (IC_{50}/ μM)	BRD4(1) (IC_{50}/ μM)	CREBBP (IC_{50}/ μM)	BRD9 (IC_{50}/ μM)	BAZ2B (IC_{50}/ μM)
IACS-9571	0.24	815.6	24.89	184.4	44.18
11d	1.88	18.5 (18%)	3.85	15.02	153.3 (23%)
11h	2.53	51.95 (30%)	32.06	14.94	447 (34%)
11z	2.33	4.91 (11%)	37.81	11.85	16.5 (26%)

Figure 4. The activities and selectivity profiles of three optimal compounds. (A) Dose-dependent inhibitory activities of three optimal compounds. (B) Selectivity profiles of three optimal compounds against four representative BCPs. All experiments were carried out in an Alphascreen assay. Compounds with lower than 50% inhibition rate at concentration of 100 μM were attached corresponding values in brackets.

2.6. Cell-based evaluation of inhibitory effects

The bromodomain of TRIM24 has been reported to be highly correlated with progression of prostate cancers [11]. To generally evaluate the inhibitory effects of our compounds on prostate cancer cell proliferation, compounds whose IC_{50} values in the Alphascreen assay were lower than 10 μM were selected to conduct a luminescent assay in prostate cancer cells LNCaP and C4-2B respectively, with the CellTiter-Glo reagent serving as a detection reagent. As shown in Table S1, most compounds exhibited higher inhibitory potency than enzalutamide (an androgen receptor antagonist approved for

CRPC [34-36]) on proliferation of LNCaP and C4-2B cells, demonstrating the overall inhibition capability of this series of compounds. Three optimal compounds **11d**, **11h** and **11z** showed low to high micromolar activities on the proliferation of LNCaP and C4-2B cells. And compound **11h** was the optimal compound for both cells (Table S1, Figure 5A).

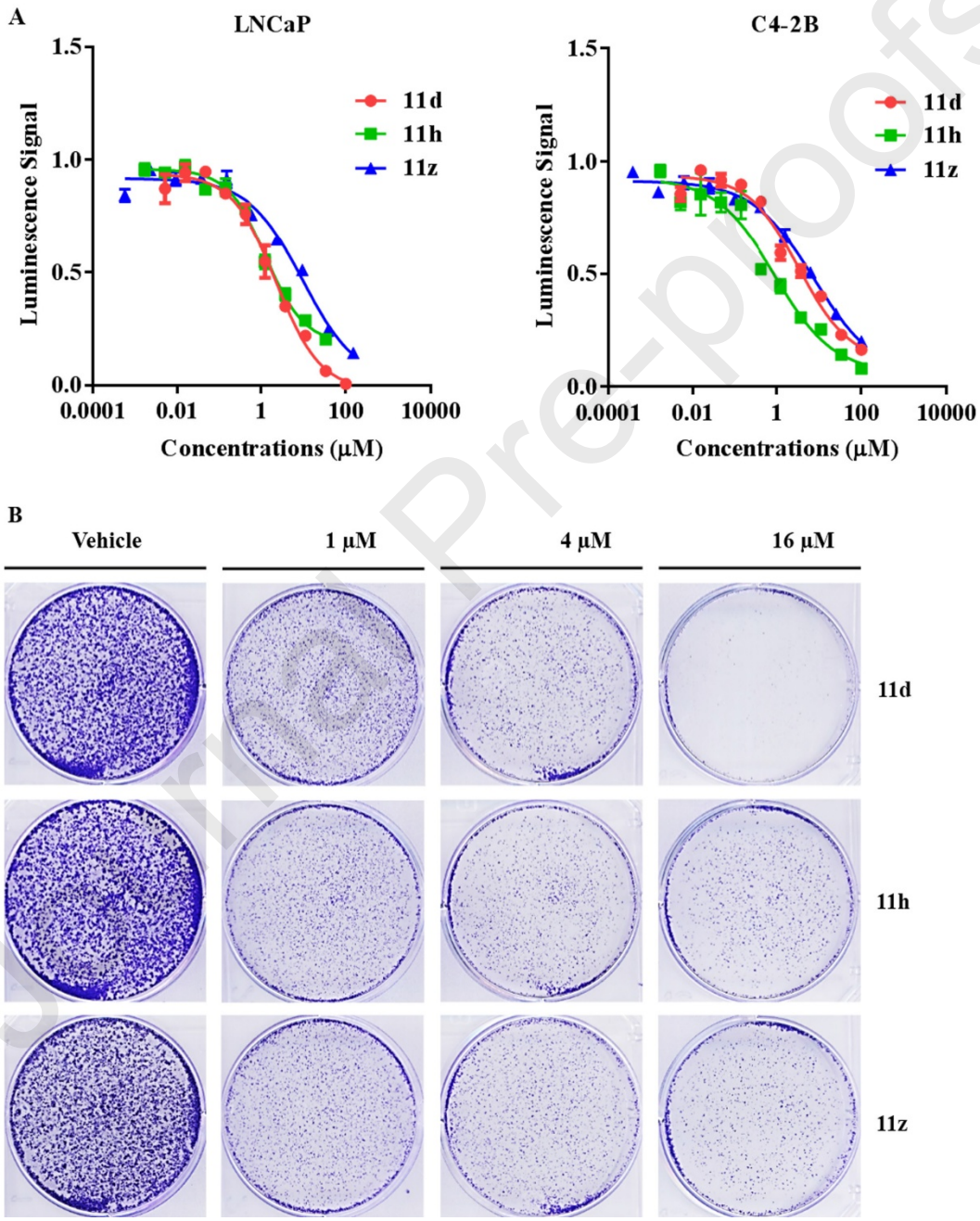


Figure 5. The dose-dependent inhibitory activities of three optimal compounds in PC cell lines. (A) The inhibitory activities on the proliferation of LNCaP cells and C4-2B cells. (B) The inhibitory activities on the colony formation of C4-2B cells.

To further investigate the long-term cell growth inhibitory effects, we tested the inhibitory capacity of compounds **11d**, **11h** and **11z** on PC cell colony formation. Consistent with the effects of the viability assay, treatment of C4-2B cells with any of these compounds reduced the colony formation in a dose-dependent manner (Figures 5A, 5B). Compounds **11d** showed a clearer dose-dependent effect in inhibiting the colony formation (Figure 5B). Based on effects in the above two assays, compounds **11d** and **11h** were selected for the subsequent cell-based assays.

Previous studies have reported that TRIM24 is involved in the development of various other cancer types such as lung cancer [12], colorectal cancer [13, 14] and breast cancer [10]. Accordingly, we tentatively tested the effects of compounds **11d** and **11h** in NSCLC cells A549, colorectal cancer cells HT-29, breast cancer cells MCF-7 and MDA-MB-231 (Figure 6). Both compounds exhibited inhibitory effects on the growth of each kind of cancer cells. Moreover, compounds **11d** and **11h** both showed encouraging low micromolar inhibitory activities in A549 cells with IC_{50} values of 1.08 μM and 0.75 μM, respectively (Figure 6).

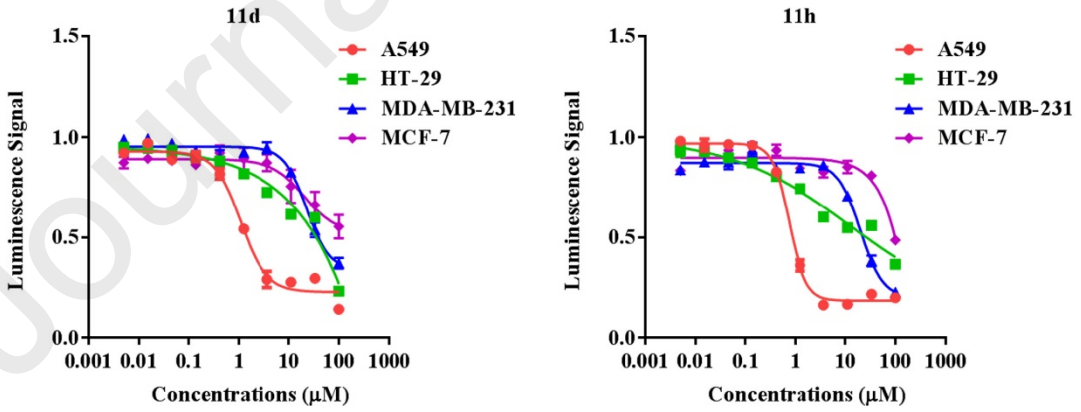


Figure 6. Dose-dependent inhibitory activities of compounds **11d** and **11h** in diverse cell lines as shown above.

Overall, our compounds had preference for prostate cancer cells LNCaP and C4-2B and NSCLC cells A549. The general inhibitory effects on these types of cancer cells

highlighted the therapeutic potential of TRIM24 bromodomain inhibitors to treat cancers clinically in the future.

3. Conclusions

In this study, we have reported a novel class of N-benzyl-3,6-dimethylbenzo-[d]-isoxazol-5-amine derivatives as TRIM24 bromodomain inhibitors. Through a sequential process including in-house library screening, structure-based medicinal chemistry optimization and biological evaluation, we obtained two molecules with low micromolar activities in both protein and cell viability assays. The results from the colony formation assay further supported the inhibitory effects shown in the viability assay. Taken together, we reported a novel series of TRIM24 bromodomain inhibitors and revealed for the first time the inhibitory effects of our inhibitors on proliferation of previously reported cancer cells, particularly PC cells and NSCLC cells. We obtained two compounds **11d** and **11h** which can be used as probe molecules for studying the biological function of TRIM24 bromodomain in cell-based assays. These compounds can also be used as lead compounds for further optimization to develop novel cancer targeted drug candidates especially for intractable CRPC.

4. Methods

4.1. Plasmid construction, protein expression and purification

All BCPs were expressed as His6-tagged fusion proteins using the pET24a expression vector (Novagen). cDNA encoding bromodomain of human TRIM24 (residues Gly896-Glu1014), BRD4 (1) (residues Asn44-Glu168), CREBBP (residues Arg1081-Gly1197), BRD9 (residues Leu14-Gln134) and BAZ2B (residues Ser1858-Ser1972) were synthesized by Genscript. BL21 (DE3) cells transformed with these expression plasmids were grown in LB broth at 25 °C to an OD_{600} of ~1.0 and then induced with 0.1 mM isopropyl- β-D-1-thiogalactopyranoside (IPTG) at 16 °C for 16 h. Cells were harvested by centrifugation (6000g for 15 min at 4 °C, JLA 81000 rotor, on a Beckman Coulter Avanti J-20 XP centrifuge) and were frozen at -20 °C for storage as pellets. Cells were resuspended in extract buffer (50 mM HEPES, pH 7.5 at 25 °C, 500 mM

NaCl, 5 mM imidazole, 5% glycerol and 0.5 mM TCEP (Tris (2-carboxyethyl)phosphine hydrochloride) and were lysed by high pressure homogenization using an JN3000 PLUS high pressure homogenizer (JNBIO, Guangzhou, China) at 4 °C. The lysate was collected on ice and centrifuged at 12,000 rpm for 40 min at 4 °C. The supernatant was loaded onto a 5 mL $NiSO_4$-loaded HisTrap HP column (Ni-NTA, GE Healthcare, NJ). The column was washed with 20 mL of extract buffer (50 mM HEPES, pH 7.5 at 25 °C, 500 mM NaCl, 50 mM imidazole). The protein was eluted with a 50-500 mM imidazole gradient in elute buffer with (50 mM HEPES, pH 7.5 at 25 °C, 500 mM NaCl, 500 mM imidazole). The protein was collected during elution, and concentrated and further purified by a gel filtration column (HiLoad, Superdex 75, 16/60, GE Healthcare). The sample purity of each fraction was examined by SDS-PAGE. Purified proteins were concentrated and stored in the gel filtration buffer (10 mM HEPES pH 7.5 at 25 °C, 150 mM NaCl, 0.5 mM TCEP) at -80 °C used for the Alphascreen assay.

4.2. Alphascreen assay

Interactions between bromodomain-containing proteins (BCPs) and ligands were assessed by luminescence-based Alphascreen technology (PerkinElmer) as previously described [19-21] using a histidine detection kit from Perkin Elmer (Norwalk, CT). All of the reactions contained bromodomain-containing proteins bound to nickel acceptor beads (5 μg/mL) and a biotinylated acetylated histone H4 or H3 peptide bound to streptavidin donor beads (5 μg/mL) in the presence of the indicated amounts of control compound IACS-9571 (MCE, hydrochloride form) or candidate compounds. The sequences of biotinylated acetylated histone H4 or H3 peptide were listed as follows: bH4Kac4: SGRGK(ac)GGK(ac)GLGK(ac)GGAK(ac)RHRK-biotin, bH3K14ac: YQTARKSTGGK(ac)APRKQLATKAK-biotin, bH3K23ac: GKAPRKQLATK(ac)AARKSAPATYKK-biotin (both produced by Genscript). The experiments were conducted with protein/peptide ratio as follows for sensitive signal: BRD4 (1): bH4Kac4 = 50 nM: 50 nM, BRD9: bH4Kac4 = 150 nM: 50 nM, CREBBP: bH4Kac4 = 150 nM: 50 nM, BAZ2B: bH3K14ac = 150 nM: 100 nM, TRIM24:

bH3K23ac = 500 nM: 100 nM.

All reagents were diluted in the buffer (50 mM MOPS, pH 7.4, 50 mM NaF, 50 μM CHAPS, and 0.1 mg/mL bovine serum albumin) and allowed to equilibrate at RT prior to their addition to low-volume 384-well plates (ProxiPlate-384 Plus, PerkinElmer, USA). Plates were sealed in foil to protect them from light, incubated at RT for 1.5 h and read on an EnSpire plate reader (PerkinElmer, USA). When excited by a laser beam of 680 nm, the donor beam emits singlet oxygen that activates thioxene derivatives in the acceptor beads, which releases photons of 520–620 nm as the binding signal. All experiments were carried out in triplicate. The IC_{50} values were calculated using GraphPad Prism 7 software.

4.3. Molecular docking

The molecular docking was carried out using the Glide module in Maestro software v11.7. The crystal structure of hTRIM24 (PDB: 4YBT) was used as the receptor for docking. The protein structure was prepared with the Protein Preparation Wizard in Maestro. A receptor grid was generated with the Receptor Grid Generation program leaving all settings on the default parameters. All ligands were prepared with the LigPrep for generating all ionization states and stereoisomers. A standard precision (SP) module was used to perform the molecular docking.

4.4. Cell viability and cell colony formation assays

For the viability assay, LNCaP, C4-2B, A549, HT-29 cells were cultured in RPMI-1640 supplemented with 10% FBS; MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS. Cells were seeded in white 384-well microplates at their optimum density for growth (500 or 1000 cells per well) in a total volume of 20 μL of media. After cultivating cells in an incubator for 12 h, the control compound enzalutamide (Selleck) and compounds were added in a total volume of 10 μL of media (triple diluted) to each well with final concentration between 5 nM and 100 μM. Then the plate was incubated for 72-120 h. Each plate and its contents was equilibrated at RT

for 20-30 min then 25 μL of Cell-Titer GLO reagents (Promega) was added to each well. The contents were mixed on an orbital shaker for 20 min and incubated at RT for 10 min. Luminescence was measured on an EnSpire plate reader (PerkinElmer, USA), according to the manufacturer's instructions. All experiments were carried out in triplicate. The estimated IC_{50} values were calculated using GraphPad Prism 7 software.

C4-2B cells were seeded in a well of 6-well plates with 4500 cells per well and cultured with vehicle or indicated concentrations of candidate compounds for 14 days with the 3 mL medium. When the cell clone grew visible, the medium was removed and the plates were washed once with 2 mL PBS. The cell colonies were stained with 2.5% crystal violet in MeOH for 2 h. The numbers of colonies were counted after being washed 3 times with twice-distilled water.

4.5. Chemistry

All solvents and reagents were purchased from commercially available sources and unless otherwise specified, were used without purification. Column chromatography was performed using 300-400 mesh silica gel. All reactions were monitored by TLC, using silica gel plates with fluorescence F254 and UV light visualization. All products were characterized by their NMR and MS spectra. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker Avance 400 or 500 instruments at 400 MHz or 500 MHz, respectively. Chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane (TMS) as the internal standard control and coupling constants (J values) are reported in Hertz (Hz). Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m). ESI-MS were recorded on an Agilent 1200 HPLC-MSD mass spectrometer. Compound purities were determined by reverse-phase high-performance liquid chromatography (HPLC) with solvent A (H_2O) and solvent B (MeOH) as eluents. The purity tests used a Dionex Summit HPLC column

(Inertsil ODS-SP, 5.0 μm, 4.6 mm × 250 mm (GL Sciences Inc.)) with a UVD170U detector, and a manual injector, a P680 pump with a detection wavelength of 254 nm and a flow rate of 1.0 mL/min. The purity of all the final compounds was determined by HPLC to be >95%.

4.5.1. General procedure for synthesis of skeleton structures shown in Scheme 1

4.5.1.1 General procedure for synthesis of skeleton structures **8** and **9**

As shown in Scheme 1, commercially available **4** was N-acetylated with acetyl chloride to give **5**, which was converted to **6** *via* a Friedel-Crafts reaction. The intermediate **6** was used to prepare the oxime precursor **7**, which was cyclized by high temperature intramolecular dehydration to afford the key skeleton structure **8**. Further deacylation of compound **8** can yield another important skeleton structure **9**.

4.5.1.1.1. N-(4-Methoxy-2-methylphenyl)acetamide (**5**).

4-Methoxy-2-methylaniline (**4**) (11.152 g, 81.29 mmol) and Et_3N (13.161 g, 130.06 mmol) were dissolved in DCM (70 mL) in an ice bath. Acetyl chloride (8.934 g, 113.81 mmol) was added to the cold mixture sequentially and slowly. The mixture was stirred at 0 °C for 15 min. Then ice water was used to quench the reaction and the mixture was extracted with DCM. The organic layer was washed with saturated brine, dried over Na_2SO_4 and concentrated under reduced pressure. Adding petroleum ether to the concentrated solution crystallized N-(4-methoxy-2-methylphenyl) acetamide (**5**) as light purple crystals (11.398 g, 78% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.16 (s, 1H), 7.18 (d, J = 8.6 Hz, 1H), 6.77 (d, J = 2.6 Hz, 1H), 6.70 (dd, J = 8.6, 2.8 Hz, 1H), 3.71 (s, 3H), 2.14 (s, 3H), 2.00 (s, 3H). MS (ESI) m/z $[M + H]^+$ calcd. 180.09; found 180.0.

4.5.1.1.2. N-(5-Acetyl-4-hydroxy-2-methylphenyl)acetamide (**6**).

N-(4-Methoxy-2-methylphenyl)acetamide (**5**) (11.398 g, 63.6 mmol) was dissolved in DCM. Acetyl chloride (14.977 g, 190.8 mmol) and aluminum chloride (33.922 g, 254.4

mmol) were added in succession to the reaction in an ice bath with continuous stirring. Then the mixture was heated at 45 °C and refluxed for 2 h. The mixture was cooled to RT and the reaction was quenched with ice water. The mixture was extracted with DCM and the organic layer was washed with water, then with saturated brine, and dried over Na_2SO_4. Decolorizing the organic solution by suction filtration with silica gel powder to give light yellow filtrate. The residue was washed with DCM twice and the filtrate was concentrated to produce the product which was collected as white crystals by suction filtration again to yield N-(5-acetyl- 4-hydroxy-2-methylphenyl) acetamide (**6**) (9.03 g, 68% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.80 (s, 1H), 9.31 (s, 1H), 7.77 (s, 1H), 6.83 (s, 1H), 2.57 (s, 3H), 2.18 (s, 3H), 2.03 (s, 3H). MS (ESI) m/z $[M + H]^+$ calcd. 208.09; found 208.0.

4.5.1.1.3. N-(4-Hydroxy-5-(1-(hydroxyiminoethyl)-2-methylphenyl)acetamide (**7**).

N-(5-acetyl-4-hydroxy-2-methylphenyl)acetamide (**6**) (9.03 g, 43.6 mmol) was dissolved in an EtOH-H_2O mixture (3:1, 100 mL). Hydroxylamine hydrochloride (4.84g, 69.7 mmol) and NaOAc (5.72 g, 69.7 mmol) were added successively and the mixture was refluxed at 80 °C for 70 min. Then the mixture was cooled to RT and the EtOH was removed under reduced pressure to produce crystals. Water (~ 60 mL) was added to dissolve ionic impurities and then the crystals were filtered and the filter cake was washed with water. The cake was dried to afford N-(4-hydroxy- 5-(1-(hydroxyimo)ethyl)-2-methylphenyl)acetamide (**7**) as a white powder containing solid NaCl (12.3 g, 100% yield,). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.48 (s, 1H), 11.37 (s, 1H), 9.21 (s, 1H), 7.35 (s, 1H), 6.72 (s, 1H), 2.19 (s, 3H), 2.12 (s, 3H), 2.01 (s, 3H). MS (ESI) m/z $[M - H]^-$ calcd. 221.09; found 221.0.

4.5.1.1.4. N-(3,6-Dimethylbenzo[d]isoxazol-5-yl)acetamide (**8**).

To a solution of N-(4-hydroxy-5-(1-(hydroxyimino)ethyl)-2-methylphenyl)acetamide (**7**) (12.3 g, 55.35 mmol, theoretically 43.6 mmol) dissolved in 1,4-dioxane (46 mL) was added DMF-DMA (33.8 mL) under vigorous stirring. The mixture was stirred at 100 °C for 37 min. Then the reaction mixture was cooled to RT. The product was

extracted between the water layer and EtOAc layer. The organic layer was washed with once with water and saturated brine, then dried over Na_2SO_4. The organic solution was concentrated under reduced pressure and the concentrated solution was filtered to give N-(3,6-dimethylbenzo-[d]isoxazol-5-yl)acetamide (**8**) as a brown solid (6.955 g, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.99 (s, 1H), 7.35 (s, 1H), 7.13 (s, 1H), 2.52 (s, 3H), 2.39 (s, 3H), 2.24 (s, 3H). MS (ESI) m/z $[M + H]^+$ calcd. 205.09; found 205.0.

4.5.1.1.5. 3,6-Dimethylbenzo[d]isoxazol-5-amine (**9**).

To the compound **8** (6.955 g, 34.05 mmol) was added 3N solution of hydrochloric acid (204 mL). The mixture was stirred and refluxed at 100 °C for 2.5 h. Then the reaction was terminated and the mixture was cooled to RT. Unreacted compound **8** was removed by extraction from the water layer with EtOAc. In an ice bath, the pH of the aqueous solution was adjusted to pH 7~8 with sodium hydroxide solution to precipitate the product. The solid was collected by filtration, washed with water and dried to give 3,6-dimethylbenzo-[d]isoxazol-5-amine (**9**) as a brown powder (6.54 g, 100% yield, containing NaCl solid). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.29 (s, 1H), 6.83 (s, 1H), 4.89 (s, 2H), 2.41 (s, 3H), 2.21 (s, 3H). MS (ESI) m/z $[M + H]^+$ calcd. 163.08; found 163.0.

4.5.1.2. General procedure for synthesis of compounds **11c-11j**, **11o-11u**, **11w-11x**

4.5.1.2.1. General procedure for synthesis of intermediates **14c-14j**, **14o-14u**, **14w-14x**

As shown in Scheme 1, commercially available **12** was etherified on the phenolic hydroxyl group to give compounds **13c-13j**, **13o-13u**, **13w-13x** in 49-100% yield with corresponding R_4Br or R_4I purchased from reagent companies or synthesized in-house from corresponding commercially available alcohols with the same reaction reagents and conditions as were used in step k. The synthesized R_4Br was used for next reactions without further purification and characterization. Compounds **13c-13j**, **13o-13u**, **13w-13x** were then transformed to the corresponding bromides **14c-14j**, **14o-14u**, **14w-14x** (47-98% yield) with PBr_3. The following monosubstitution of **9** with **14c-14j**, **14o-14u**, **14w-14x** finally yielded the target products **11c-11j**, **11o-11u**, **11w-11x** (13-57% yield).

For steps j and k, we used synthetic procedures of intermediates **13c** and **14c** as examples.

4.5.1.2.1.1. (3-Ethoxyphenyl)methanol (**13c**).

To a solution of compound **12** (200 mg, 1.61 mmol, 1.0 eq) dissolved in DMF were added K_2CO_3 (668 mg, 4.83 mmol, 3.0 eq), bromoethane (193 mg, 1.77 mmol, 1.1 eq) (for most other intermediates, R_4Br or R_4I could be 1.1 eq or 1.5 eq). The mixture was stirred at 60 °C overnight. For most other intermediates, the stirring could be 80 °C for 0.5-1.0 h. Then the mixture was cooled, poured into water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$ and concentrated under reduced pressure to give the crude (3-ethoxyphenyl)methanol (**13c**) as a colorless or light yellow liquid (210 mg, 86% yield). For most other intermediates, the crude product was further purified by silica gel chromatography with petroleum ether/EtOAc (1/5~1/2) to obtain products which were monitored by TLC. The intermediates **13c-13j**, **13o-13u**, **13w-13x** were not characterized but were used directly in the next steps.

4.5.1.2.1.2. 1-(Bromomethyl)-3-ethoxybenzene (**14c**).

To a solution of compound 13c (210 mg, 1.38 mmol, 1.0 eq) dissolved in DCM was added PBr_3 (374 mg, 1.38 mmol, 1.0 eq). The mixture was stirred at RT for 2 h. For most other intermediates, the stirring time could be 0.5~2.0 h. Then water was added to quench the reaction and the DCM was removed under reduced pressure. The water solution was extracted with EtOAc, washed with saturated brine, dried over Na_2SO_4, and concentrated to give the crude product 1-(bromomethyl)-3-ethoxybenzene (**14c**) as a light yellow oil (150 mg, 51% yield). All intermediates **14c-14j**, **14o-14u**, **14w-14x** were not characterized but used directly in the next steps.

4.5.1.2.2. General procedure for synthesis of compounds **11c-11j**, **11o-11u**, **11w-11x**

To a solution of compound **9** (1.0 eq) dissolved in DMF (3 mL) were added Cs_2CO_3 (1.5 eq) and 1.0 eq of intermediates **14c-14j**, **14o-14u**, **14w-14x**. The mixture was stirred at 60 °C overnight. Then the mixture was cooled, poured into water and extracted

with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/ EtOAc (1/10~1/5) to give the target products**11c-11j**, **11o-11u**, **11w-11x**.

4.5.1.2.2.1. N-(3-Ethoxybenzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11c**).

Compound **11c** was prepared from **9** and 1-(bromomethyl)-3-ethoxybenzene (**14c**) according to the general procedure and isolated as a solid, in 30% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 – 7.27 (m, 2H), 7.01 – 6.96 (m, 2H), 6.84 (dd, J = 8.2, 2.1 Hz, 1H), 6.63 (s, 1H), 4.36 (s, 2H), 4.04 (q, J = 7. 0 Hz, 2H), 3.84 (s, 1H), 2.49 (s, 3H), 2.31 (s, 3H), 1.42 (t, J = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.61, 155.92, 154.02, 143.51, 141.64, 129.31, 128.61, 120.22, 119.25, 113.27, 112.39, 110.02, 97.89, 62.77, 46.89, 18.83, 14.63, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 319.14; found 319.3. HPLC analysis: MeOH − H_2O (85:15), 10.26 min, 95.21% purity.

4.5.1.2.2.2. 3,6-Dimethyl-N-(3-propoxybenzyl)benzo[d]isoxazol-5-amine (**11d**).

Compound **11d** was prepared from **9** and 1-(bromomethyl)-3-propoxybenzene (**14d**) according to the general procedure and isolated as a liquid in 28% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 – 7.27 (m, 2H), 7.01 – 6.95 (m, 2H), 6.85 (d, J = 8.0 Hz, 1H), 6.63 (s, 1H), 4.36 (s, 2H), 3.93 (t, J = 6.5 Hz, 2H), 3.84 (s, 1H), 2.49 (s, 3H), 2.31 (s, 3H), 1.80 (dt, J = 13.9, 7.0 Hz, 2H), 1.04 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 159.22, 156.37, 154.46, 143.98, 142.10, 129.76, 129.06, 120.67, 119.71, 113.81, 112.94, 110.47, 98.37, 69.19, 47.37, 22.46, 19.27, 10.84, 10.04. MS (ESI) m/z $[M + Na]^+$ calcd. 333.39; found.333.4. HPLC analysis: MeOH − H_2O (85:15), 10.95 min, 99.03% purity.

4.5.1.2.2.3. N-(3-Butoxybenzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11e**).

Compound **11e** was prepared from **9** and 1-(bromomethyl)-3-butoxybenzene (**14e**) according to the general procedure. **11e** was isolated as a liquid in 57% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.21 (t, J = 8.1 Hz, 1H), 7.03 – 6.94 (m, 2H), 6.83

– 6.74 (m, 1H), 6.60 (s, 1H), 5.61 (t, *J* = 6.0 Hz, 1H), 4.35 (d, *J* = 5.9 Hz, 2H), 3.93 (t, *J* = 6.5 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H), 1.71 – 1.60 (m, 2H), 1.40 (dq, *J* = 14.7, 7.4 Hz, 2H), 0.90 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.79, 155.93, 154.02, 143.53, 141.65, 129.31, 128.61, 120.23, 119.25, 113.33, 112.49, 110.02, 97.91, 66.92, 46.91, 30.74, 18.84, 18.74, 13.67, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 347.17; found 347.4. HPLC analysis: MeOH − H_2O (85:15), 11.22 min, 97.93% purity.

4.5.1.2.2.4. N-(3-Isobutoxybenzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11f**). Compound **11f** was prepared from **9** and 1-(bromomethyl)-3-isobutoxybenzene (**14f**) according to the general procedure and isolated as a liquid in 32% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.21 (t, J = 7.8 Hz, 1H), 7.01 – 6.95 (m, 2H), 6.77 (dd, J = 8.1, 1.6 Hz, 1H), 6.60 (s, 1H), 5.61 (t, J = 6.0 Hz, 1H), 4.35 (d, J = 5.9 Hz, 2H), 3.70 (d, J = 6.5 Hz, 2H), 2.36 (s, 3H), 2.32 (s, 3H), 2.05 – 1.90 (m, 1H), 0.96 (s, 3H), 0.94 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.88, 155.92, 154.02, 143.54, 141.67, 129.32, 128.62, 120.23, 119.29, 113.44, 112.57, 110.03, 97.93, 73.60, 46.93, 27.67, 19.06 (2 × C), 18.84, 9.59. MS (ESI) m/z $[M + Na]^+$ calcd. 347.17; found 347.4. HPLC analysis: MeOH − H_2O (85:15), 11.06 min, 96.14% purity.

4.5.1.2.2.5. N-(3-(Cyclopropylmethoxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11g**).

Compound **11g** was prepared from **9** and 1-(bromomethyl)-3-(cyclopropylmethoxy)benzene (**14g**) according to the general procedure. Product **11g** was isolated as a liquid in 24% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.27 (m, 2H), 7.01 – 6.97 (m, 2H), 6.86 – 6.83 (m, 1H), 6.62 (s, 1H), 4.36 (s, 2H), 3.85 (s, 1H), 3.81 (d, J = 6.9 Hz, 2H), 2.49 (s, 3H), 2.31 (s, 3H), 1.29 – 1.25 (m, 1H), 0.67 – 0.61 (m, 2H), 0.35 (q, J = 4.8 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.73, 155.92, 154.02, 143.52, 141.62, 129.29, 128.62, 120.22, 119.26, 113.41, 112.48, 110.02, 97.90, 71.81, 46.90, 18.84, 10.15, 9.60, 3.06 (2 × C). MS (ESI) m/z $[M + Na]^+$ calcd. 345.16; found 345.9. HPLC analysis: MeOH − H_2O (85:15), 11.18 min, 96.02% purity.

4.5.1.2.2.6. N-(3-(Cyclopentylmethoxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11h**).

Compound **11h** was prepared from **9** and 1-(bromomethyl)-3-(cyclopentylmethoxy)benzene (**14h**) according to the general procedure. The product **11h** was isolated as a liquid in 35% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.27 (m, 2H), 7.01 – 6.96 (m, 2H), 6.88 – 6.83 (m, 1H), 6.63 (s, 1H), 4.35 (s, 2H), 3.87 – 3.80 (m, 3H), 2.49 (s, 3H), 2.41 – 2.33 (m, 1H), 2.31 (s, 3H), 1.88 – 1.79 (m, 2H), 1.70 – 1.58 (m, 4H), 1.41 – 1.32 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.91, 155.92, 154.02, 143.53, 141.64, 129.31, 128.62, 120.23, 119.28, 113.44, 112.55, 110.03, 97.93, 71.46, 46.92, 38.54, 29.01 (2 × C), 24.93 (2 × C), 18.85, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 373.19; found 373.4. HPLC analysis: MeOH − H_2O (85:15), 11.70 min, 97.54% purity.

4.5.1.2.2.7. N-(3-(Cyclohexylmethoxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11i**).

Compound **11i** was prepared from **9** and 1-(bromomethyl)-3-(cyclohexylmethoxy)benzene (**14i**) according to the general procedure. The product **11i** was isolated as a liquid in 53% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.20 (t, J = 8.1 Hz, 1H), 6.99 – 6.95 (m, 2H), 6.76 (d, J = 7.4 Hz, 1H), 6.60 (s, 1H), 5.61 (t, J = 5.9 Hz, 1H), 4.35 (d, J = 5.9 Hz, 2H), 3.73 (d, J = 6.4 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H), 1.80 – 1.73 (m, 2H), 1.72 – 1.65 (m, 3H), 1.66 – 1.60 (m, 1H), 1.25 – 1.13 (m, 3H), 1.04 – 0.94 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.92, 155.93, 154.02, 143.53, 141.65, 129.31, 128.62, 120.23, 119.25, 113.32, 112.58, 110.03, 97.93, 72.57, 46.90, 37.04, 29.28 (2 × C), 26.04, 25.26 (2 × C), 18.85, 9.61. MS (ESI) m/z $[M + Na]^+$ calcd. 387.20; found 387.5. HPLC analysis: MeOH − H_2O (85:15), 12.52 min, 95.38% purity.

4.5.1.2.2.8. N-(3-(Benzyloxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11j**).

Compound **11j** was prepared from **9** and 1-(benzyloxy)-3-(bromomethyl)benzene (**14j**) according to the general procedure and isolated as a liquid in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.43 – 7.39 (m, 2H), 7.38 – 7.28 (m, 4H), 7.23 (t, J = 7.9 Hz, 1H),

7.07 (s, 1H), 7.00 (d, J = 7.5 Hz, 1H), 6.87 (dd, J = 8.1, 1.8 Hz, 1H), 6.59 (s, 1H), 5.60 (t, J = 5.9 Hz, 1H), 5.07 (s, 2H), 4.36 (d, J = 5.8 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.50, 155.95, 154.06, 143.51, 141.77, 137.12, 129.37, 128.64, 128.39 (2 × C), 127.78, 127.66 (2 × C), 120.24, 119.62, 113.69, 112.85, 110.04, 97.89, 69.09, 46.89, 18.85, 9.63. MS (ESI) m/z $[M + Na]^+$ calcd. 381.16; found 381.4. HPLC analysis: MeOH − H_2O (85:15), 11.69 min, 95.02% purity.

4.5.1.2.2.9. N-(3-((3-Fluorobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11o**).

Compound **11o** was prepared from **9** and 1-(bromomethyl)-3- ((3-fluorobenzyl)oxy)benzene (**14o**) according to general procedure and isolated as a liquid in 33% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.43 – 7.35 (m, 2H), 7.27 – 7.21 (m, 3H), 7.15 – 7.09 (m, 1H), 7.07 (s, 1H), 7.01 (d, J = 7.5 Hz, 1H), 6.87 (dd, J = 8.0, 1.8 Hz, 1H), 6.57 (s, 1H), 5.61 (t, J = 5.9 Hz, 1H), 5.10 (s, 2H), 4.36 (d, J = 5.8 Hz, 2H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.14, 161.20, 158.26, 155.95, 154.04, 143.48, 141.83, 140.15, 140.09, 130.45, 130.38, 129.40, 128.63, 123.42, 123.40, 120.22, 119.78, 114.57, 114.40, 114.20, 114.03, 113.68, 112.88, 110.04, 97.88, 68.21, 46.87, 18.83, 9.61. MS (ESI) m/z $[M + Na]^+$ calcd. 399.15; found 399.3. HPLC analysis: MeOH − H_2O (95:5), 11.98 min, 95.89% purity.

4.5.1.2.2.10. N-(3-((4-Fluorobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11p**).

Compound **11p** was prepared from **9** and 1-(bromomethyl)-3-((4-fluorobenzyl)oxy)benzene (**14p**) according to the general procedure. The product was isolated as a white solid in 15% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 – 7.41 (m, 2H), 7.37 (s, 1H), 7.26 – 7.21 (m, 1H), 7.20 – 7.13 (m, 2H), 7.06 (s, 1H), 7.01 (d, J = 7.2 Hz, 1H), 6.87 (d, J = 8.1 Hz, 1H), 6.58 (s, 1H), 5.67 – 5.56 (m, 1H), 5.05 (s, 2H), 4.36 (d, J = 4.9 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 162.66, 160.73, 158.37, 155.93, 154.01, 143.48, 141.76, 133.33, 133.31, 129.90, 129.83, 129.36, 128.62, 120.22, 119.66, 115.26, 115.09, 113.65, 112.84, 110.02, 97.86,

68.33, 46.86, 18.82, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 399.15; found 399.3. HPLC analysis: MeOH − H_2O (95:5), 11.98 min, 95.44% purity.

4.5.1.2.2.11. N-(3-((2-Fluorobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11q**).

Compound **11q** was prepared from **9** and 1-((3-(bromomethyl)- phenoxy)methyl)-2-fluorobenzene (**14q**) according to the general procedure. The product was isolated as a liquid in 47% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.54 – 7.48 (m, 1H), 7.42 – 7.35 (m, 2H), 7.28 – 7.16 (m, 3H), 7.08 (s, 1H), 7.02 (d, J = 7.5 Hz, 1H), 6.89 (dd, J = 8.0, 1.8 Hz, 1H), 6.59 (s, 1H), 5.61 (t, J = 5.8 Hz, 1H), 5.11 (s, 2H), 4.37 (d, J = 5.7 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 161.33, 159.37, 158.32, 155.95, 154.05, 143.49, 141.86, 130.62, 130.59, 130.35, 130.28, 129.43, 128.64, 124.48, 124.45, 120.23, 119.84, 115.44, 115.27, 113.56, 112.80, 110.04, 97.89, 63.36, 63.34, 46.85, 18.84, 9.61. MS (ESI) m/z $[M + Na]^+$ calcd. 399.15; found 399.3. HPLC analysis: MeOH − H_2O (95:5), 10.78 min, 98.60% purity.

4.5.1.2.2.12. N-(3-((3-Chlorobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11r**).

Compound **11r** was prepared from **9** and 1-(bromomethyl)-3-((3-chloro-benzyl)oxy)benzene (**14r**) according to the general procedure. The product was isolated as a liquid in 13% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.47 (s, 1H), 7.40 – 7.33 (m, 4H), 7.24 (t, J = 7.9 Hz, 1H), 7.07 (s, 1H), 7.01 (d, J = 7.6 Hz, 1H), 6.87 (dd, J = 8.0, 2.1 Hz, 1H), 6.57 (s, 1H), 5.60 (t, J = 5.9 Hz, 1H), 5.09 (s, 2H), 4.36 (d, J = 5.8 Hz, 2H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.21, 155.93, 153.99, 143.45, 141.81, 139.74, 133.06, 130.27, 129.38, 128.60, 127.62, 127.13, 126.01, 120.20, 119.79, 113.68, 112.86, 110.01, 97.88, 68.14, 46.88, 18.79, 9.58. MS (ESI) m/z $[M + Na]^+$ calcd. 415.12; found 414.9. HPLC analysis: MeOH − H_2O (95:5), 10.19 min, 95.47% purity.

4.5.1.2.2.13. N-(3-((2-Chlorobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-

amine (**11s**).

Compound **11s** was prepared from **9** and 1-((3-(bromomethyl)phenoxy)- methyl)-2-chlorobenzene (**14s**) according to the general procedure. The product was isolated as a liquid in 32% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.57 – 7.51 (m, 1H), 7.50 – 7.44 (m, 1H), 7.40 – 7.31 (m, 3H), 7.26 (t, J = 7.8 Hz, 1H), 7.07 (s, 1H), 7.03 (d, J = 7.5 Hz, 1H), 6.89 (dd, J = 8.1, 1.6 Hz, 1H), 6.57 (s, 1H), 5.63 (t, J = 5.8 Hz, 1H), 5.12 (s, 2H), 4.37 (d, J = 5.8 Hz, 2H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.30, 155.94, 154.03, 143.47, 141.90, 134.34, 132.48, 129.97, 129.76, 129.45, 129.34, 128.62, 127.28, 120.23, 119.89, 113.51, 112.89, 110.03, 97.90, 66.69, 46.83, 18.84, 9.61. MS (ESI) m/z $[M + Na]^+$ calcd. 415.12; found 415.0. HPLC analysis: MeOH − H_2O (95:5), 10.65 min, 95.77% purity.

4.5.1.2.2.14. N-(3-((4-Chlorobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11t**).

Compound **11t** was prepared from **9** and 1-(bromomethyl)-3-((4-chloro-benzyl)oxy)benzene (**14t**) according to the general procedure. The product was isolated as a brown solid in 23% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.47 – 7.35 (m, 5H), 7.24 (t, J = 7.8 Hz, 1H), 7.09 – 6.97 (m, 2H), 6.91 – 6.82 (m, 1H), 6.58 (s, 1H), 5.59 (t, J = 5.1 Hz, 1H), 5.07 (s, 2H), 4.36 (d, J = 5.2 Hz, 2H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.28, 155.93, 154.01, 143.47, 141.79, 136.17, 132.32, 129.40 (2 × C), 129.37, 128.61, 128.37 (2 × C), 120.21, 119.73, 113.65, 112.86, 110.02, 97.86, 68.20, 46.85, 18.82, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 415.12; found 415.2. HPLC analysis: MeOH − H_2O (95:5), 10.17 min, 95.71% purity.

4.5.1.2.2.15. N-(3-((3-Bromobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11u**). Compound **11u** was prepared from **9** and 1-bromo-3-((3-(bromomethyl)-phenoxy)methyl)benzene (**14u**) according to general procedure. The product was isolated as a liquid in 45% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.61 (s, 1H), 7.49 (d, J = 7.9 Hz, 1H), 7.41 (d, J = 7.6 Hz, 1H), 7.37 (s, 1H), 7.31 (t, J = 7.8 Hz, 1H), 7.24 (t, J = 7.8 Hz, 1H), 7.07 (s, 1H), 7.01 (d, J = 7.5 Hz, 1H), 6.87 (dd, J = 8.0, 1.7 Hz, 1H),

6.58 (s, 1H), 5.60 (t, J = 5.8 Hz, 1H), 5.09 (s, 2H), 4.36 (d, J = 5.8 Hz, 2H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.23, 155.95, 154.04, 143.48, 141.84, 140.01, 130.59, 130.57, 130.07, 129.42, 128.64, 126.46, 121.68, 120.23, 119.81, 113.70, 112.84, 110.05, 97.88, 68.08, 46.88, 18.85, 9.63. MS (ESI) m/z [M + Na]$^+$ calcd. 459.07; found 459.3. HPLC analysis: MeOH − H_2O (95:5), 10.65 min, 96.50% purity.

4.5.1.2.2.16. 3,6-Dimethyl-N-(3-((2-(trifluoromethyl)benzyl)oxy)benzyl)benzo[d]isoxazol-5-amine (**11w**).

Compound **11w** was prepared from **9** and 1-((3-(bromomethyl)- phenoxy)methyl)-2-(trifluoromethyl)benzene (**14w**) according to the general procedure. The product was isolated as a liquid in 34% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.75 – 7.62 (m, 3H), 7.51 – 7.48 (m, 1H), 7.55 (s, 1H), 7.26 (t, *J* = 8.0 Hz, 1H), 7.09 – 7.00 (m, 2H), 6.87 (dd, J = 8.0, 1.7 Hz, 1H), 6.54 (s, 1H), 5.61 (t, *J* = 5.9 Hz, 1H), 5.20 (s, 2H), 4.37 (d, *J* = 5.9 Hz, 2H), 2.34 (s, 3H), 2.30 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.14, 155.92, 154.01, 143.43, 141.95, 135.03, 132.69, 130.10, 129.50, 128.57, 128.55, 126.76, 126.52, 126.09, 126.05, 126.00, 125.96, 125.38, 123.21, 120.21, 119.94, 113.34, 112.91, 110.00, 97.85, 65.92, 46.78, 18.79, 9.56. MS (ESI) m/z [M + Na]$^+$ calcd. 449.15; found 449.3. HPLC analysis: MeOH − H_2O (95:5), 10.43 min, 95.53% purity.

4.5.1.2.2.17. N-(3-((3-Methoxybenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11x**).

Compound **11x** was prepared from **9** and 1-(bromomethyl)-3-((3-methoxy-benzyl)oxy)benzene (**14x**) according to the general procedure. The product was isolated as a liquid in 36% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.29 – 7.20 (m, 2H), 7.06 (s, 1H), 7.03 – 6.94 (m, 3H), 6.91 – 6.82 (m, 2H), 6.58 (s, 1H), 5.64 – 5.54 (m, 1H), 5.05 (s, 2H), 4.36 (d, *J* = 5.0 Hz, 2H), 3.73 (s, 3H), 2.35 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 159.31, 158.46, 155.95, 154.05, 143.50, 141.77, 138.73, 129.51, 129.37, 128.64, 120.23, 119.65, 119.64, 113.72, 113.15, 113.08, 112.84, 110.04, 97.89, 68.93, 55.01, 46.89, 18.84, 9.61. MS (ESI) m/z [M + Na]$^+$ calcd.

411.17; found 411.3. HPLC analysis: MeOH − H_2O (95:5), 10.25 min, 96.51% purity.

4.5.1.2.3. General procedure for synthesis of compounds **10**, **11k-11n**, **11v**, **11y-11z**

As shown in Scheme 1, the synthesized skeleton (**9**) was monosubstituted on the aniline group with 1-bromo-3-(bromomethyl)benzene or 1-(bromomethyl)-3-methoxybenzene to give compounds **10** or **11a** in 40% or 65% yield. Demethylation of compound **11a** with BBr_3 gave an intermediate (**11b**) for subsequent substitution reactions affording compounds **11k-11n**, **11v**, **11y-11z** in 2-97% yield with a bromide reagent R_4Br which was commercially available or synthesized from corresponding commercially available alcohols in-house as described in the step k.

4.5.1.2.3.1. N-(3-Bromobenzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**10**) or N-(3-methoxy- benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11a**).

To a solution of **9** (1.86 g, 11.46 mmol, 1.0 eq) dissolved in DMF (24 mL) were added Cs_2CO_3 (5.6 g, 17.19 mmol, 1.5 eq) and or 1-(bromomethyl)-3-methoxybenzene (2.3 g, 11.46 mmol, 1.0 eq). The mixture was stirred at 60 °C overnight and then cooled, poured into water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/EtOAc (1/10~1/5) to give N-(3-methoxy- benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11a**) as a light yellow solid (2.1 g, 65% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.23 (t, J = 7.7 Hz, 1H), 6.99 (s, 2H), 6.79 (d, J = 7.7 Hz, 1H), 6.61 (s, 1H), 5.58 (s, 1H), 4.36 (s, 2H), 3.72 (s, 3H), 2.36 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 159.36, 155.92, 154.03, 143.52, 141.69, 129.32, 128.62, 120.22, 119.37, 112.88, 111.91, 110.02, 97.91, 54.91, 46.91, 18.83, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 305.13; found 305.6. HPLC analysis: MeOH − H_2O (85:15), 10.11 min, 98.45% purity.

Displacement of 1-(bromomethyl)-3-methoxybenzene with 1-bromo-3-(bromo-methyl)benzene led to N-(3-bromobenzyl)-3,6-dimethyl-benzo[d]isoxazol-5-amine (**10**) as a solid (40% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57 (s, 1H), 7.44 (d, J = 8.1 Hz, 1H), 7.34 (d, J = 7.6 Hz, 1H), 7.31 (s, 1H), 7.25 – 7.22 (m, 1H), 6.57 (s, 1H), 4.39 (d, J

= 4.4 Hz, 2H), 3.87 (s, 1H), 2.48 (s, 3H), 2.33 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 155.98, 154.04, 143.22, 143.12, 130.45, 129.79, 129.50, 128.65, 126.24, 121.73, 120.23, 110.13, 97.92, 46.24, 18.84, 9.60. MS (ESI) m/z $[M + Na]^+$ calcd. 353.03; found 353.6. HPLC analysis: MeOH − H_2O (85:15), 9.23 min, 99.55% purity.

4.5.1.2.3.2. 3-(((3,6-Dimethylbenzo[d]isoxazol-5-yl)amino)methyl)phenol (**11b**).
To a solution of compound **11a** (1.121 g, 3.97 mmol) dissolved in DCM (25 mL) in ice-bath was added BBr_3 (2.98 g, 11.9 mmol). The mixture was stirred at RT for 2.5 h. Then ice-water was added to quench the reaction and the mixture was extracted with DCM, washed with water, dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/EtOAc (1/4~1/2) to give 3-(((3,6-dimethylbenzo[d]isoxazol-5-yl)amino)methyl)phenol (**11b**) as a yellow solid (0.606 g, 57% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.28 (s, 1H), 7.37 (s, 1H), 7.15 – 7.07 (m, 1H), 6.86 – 6.76 (m, 2H), 6.64 – 6.53 (m, 2H), 5.54 (s, 1H), 4.31 (s, 2H), 2.35 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 157.39, 155.87, 153.97, 143.59, 141.43, 129.19, 128.50, 120.21, 117.67, 113.73, 113.58, 109.97, 97.78, 46.93, 18.78, 9.57. MS (ESI) m/z $[M + Na]^+$ calcd. 291.11; found 291.5. HPLC analysis: MeOH − H_2O (85:15), 9.55 min, 98.98% purity.

4.5.1.2.3.3. General procedure for synthesis of compounds **11k-11n**, **11v**, **11y-11z**
11k-11l, **11v**, **11z**. To a solution of compound **11b** (1.0 eq) dissolved in DMF (3 mL) were added K_2CO_3 (1.5 eq) and 1.0 eq of R_4Br. The mixture was stirred at 60 °C for 1-8 h and then cooled, poured into water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/EtOAc (1/5~1/1) to give the target products **11k-11l**, **11v**, **11z** in 2-47% yield.

11n, **11y**. To a solution of compound **11b** (1.0 eq) dissolved in DMF (3 mL) were added KOH (1.0 eq) and 1.0 eq of RBr. The mixture was stirred at RT for 1 h. Then the mixture was cooled, poured into water and extracted with EtOAc. The organic layer was washed

with saturated brine, dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/EtOAc (1/5~1/1) to give the target products **11n** and **11y** (28-43% yield).

11m. To a solution of compound **11b** (120 mg, 0.45 mmol) dissolved in NMP (5 mL) were added K_2CO_3 (513 mg, 3.71 mmol) and (tetrahydrofuran-3-yl)methyl 4-methylbenzenesulfonate (crude product 427 mg, 1.67 mmol). (Tetrahydrofuran-3-yl)methyl 4-methylbenzenesulfonate was synthesized through a simple and regular sulfonylation on hydroxyl group of (tetrahydrofuran-3-yl)methanol (1.0 eq) with 4-methylbenzenesulfonyl chloride (2.0 eq) using pyridine as base and solvent at RT stirring for 5.5 h. The mixture was stirred at 95 °C for 4 h. Then the mixture was cooled, poured into water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$, concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/ EtOAc (1/1) to give the target product **11m** (154 mg, 97% yield).

4.5.1.2.3.3.1. N-(3-(Methoxymethoxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11k**).

Compound **11k** was prepared from **11b** and bromo(methoxy)methane according to the general procedure. The product was isolated as a liquid in 2% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.27 (m, 2H), 7.13 – 7.04 (m, 2H), 6.99 (dd, J = 8.2, 2.2 Hz, 1H), 6.63 (s, 1H), 5.18 (s, 2H), 4.37 (s, 2H), 3.48 (s, 3H), 2.49 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 156.91, 155.92, 153.98, 143.47, 141.75, 129.29, 128.60, 120.49, 120.20, 115.11, 114.27, 109.98, 97.95, 93.81, 55.48, 46.89, 18.76, 9.52. MS (ESI) m/z $[M + Na]^+$ calcd. 335.37; found 335.3. HPLC analysis: MeOH − H_2O (85:15), 12.22 min, 96.78% purity.

4.5.1.2.3.3.2. 3,6-Dimethyl-N-(3-((tetrahydrofuran-2-yl)methoxy)benzyl)benzo[d]isoxazol-5-amine (**11l**).

Compound **11l** was prepared from **11b** and 2-(bromomethyl)tetra- hydrofuran

according to the general procedure and isolated as a liquid in 20% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.22 (t, J = 8.0 Hz, 1H), 7.01 – 6.96 (m, 2H), 6.78 (dd, J = 8.0, 1.8 Hz, 1H), 6.59 (s, 1H), 5.62 (t, J = 6.0 Hz, 1H), 4.35 (d, J = 5.9 Hz, 2H), 4.15 – 4.08 (m, 1H), 3.94 – 3.84 (m, 2H), 3.79 – 3.72 (m, 1H), 3.68 – 3.61 (m, 1H), 2.35 (s, 3H), 2.32 (s, 3H), 2.03 – 1.91 (m, 1H), 1.91 – 1.74 (m, 2H), 1.68 – 1.58 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.49, 157.41, 154.63, 143.24, 140.66, 129.89, 127.97, 121.02, 120.20, 114.24, 113.48, 110.87, 98.58, 77.21, 70.61, 68.72, 49.08, 28.40, 25.84, 18.98, 10.30. MS (ESI) m/z $[M + Na]^+$ calcd. 375.17; found 375.5. HPLC analysis: MeOH − H_2O (95:5), 11.56 min, 95.90% purity.

4.5.1.2.3.3.3. 3,6-Dimethyl-N-(3-((tetrahydrofuran-3-yl)methoxy)benzyl)benzo[d]isoxazol-5-amine (**11m**).

Compound **11m** was prepared from **11b** and (tetrahydrofuran-3-yl)- methyl 4-methylbenzenesulfonate synthesized in-house according to the general procedure. The product was isolated as a liquid in 97% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.37 (s, 1H), 7.22 (t, *J* = 7.9 Hz, 1H), 7.10 – 6.96 (m, 2H), 6.79 (d, J = 8.1 Hz, 1H), 6.60 (s, 1H), 5.58 (t, *J* = 5.8 Hz, 1H), 4.35 (d, *J* = 5.7 Hz, 2H), 3.94 – 3.82 (m, 2H), 3.80 – 3.70 (m, 2H), 3.66 – 3.60 (m, 1H), 3.53 – 3.48 (m, 1H), 2.67 – 2.57 (m, 1H), 2.36 (s, 3H), 2.32 (s, 3H), 2.03 – 1.94 (m, 1H), 1.69 – 1.58 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.66, 155.92, 154.01, 143.51, 141.71, 129.33, 128.61, 120.22, 119.52, 113.48, 112.54, 110.02, 97.91, 69.85, 69.31, 66.85, 46.91, 38.22, 28.53, 18.82, 9.58. MS (ESI) m/z $[M + Na]^+$ calcd. 375.17; found 375.5. HPLC analysis: MeOH − H_2O (95:5), 11.47 min, 99.29% purity.

4.5.1.2.3.3.4. N-(3-(Furan-3-ylmethoxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11n**).

Compound **11n** was prepared from **11b** and 3-(bromomethyl)furan synthesized in-house according to the general procedure. The product was isolated as a liquid in 43% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73 (s, 1H), 7.62 (s, 1H), 7.37 (s, 1H), 7.23 (t, J = 7.8 Hz, 1H), 7.05 (s, 1H), 7.00 (d, J = 7.5 Hz, 1H), 6.86 (dd, J = 8.0, 1.9 Hz, 1H),

6.59 (s, 1H), 6.53 (s, 1H), 5.58 (t, J = 5.7 Hz, 1H), 4.92 (s, 2H), 4.36 (d, J = 5.7 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.30, 155.92, 154.00, 143.61, 143.47, 141.68, 141.39, 129.30, 128.60, 121.19, 120.21, 119.57, 113.60, 112.79, 110.63, 110.00, 97.89, 60.92, 46.88, 18.79, 9.57. MS (ESI) m/z [M + Na]$^+$ calcd. 371.14; found 371.4. HPLC analysis: MeOH − H_2O (85:15), 11.43 min, 99.97% purity.

4.5.1.2.3.3.5. N-(3-((4-Bromobenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11v**).

Compound **11v** was prepared from **11b** and 1-bromo-4-(bromomethyl)benzene according to the general procedure. The product was isolated as a solid in 47% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.59 – 7.50 (m, 2H), 7.40 – 7.32 (s, 3H), 7.27 – 7.20 (m, 1H), 7.05 (s, 1H), 7.00 (d, J = 6.9 Hz, 1H), 6.86 (d, J = 7.9 Hz, 1H), 6.58 (s, 1H), 5.62 – 5.52 (m, 1H), 5.05 (s, 2H), 4.36 (s, 2H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.26, 155.93, 154.01, 143.47, 141.80, 136.60, 131.29 (2 × C), 129.71 (2 × C), 129.37, 128.62, 120.85, 120.21, 119.73, 113.65, 112.86, 110.02, 97.86, 68.23, 46.85, 18.83, 9.61. MS (ESI) m/z [M - H]$^-$ calcd. 435.07; found 435.4. HPLC analysis: MeOH − H_2O (95:5), 10.53 min, 96.65% purity.

4.5.1.2.3.3.6. N-(3-((2-Methoxybenzyl)oxy)benzyl)-3,6-dimethylbenzo[d]isoxazol-5-amine (**11y**).

Compound **11y** was prepared from **11b** and 1-(bromomethyl)-2-methoxybenzene according to the general procedure. The product was isolated as a liquid in 28% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.38 – 7.33 (m, 2H), 7.33 – 7.28 (m, 1H), 7.23 (t, J = 7.9 Hz, 1H), 7.06 – 6.98 (m, 3H), 6.92 (t, J = 7.4 Hz, 1H), 6.85 (dd, J = 8.1, 2.1 Hz, 1H), 6.59 (s, 1H), 5.58 (t, J = 5.9 Hz, 1H), 5.02 (s, 2H), 4.36 (d, J = 5.9 Hz, 2H), 3.79 (s, 3H), 2.35 (s, 3H), 2.31 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.65, 156.85, 155.93, 154.02, 143.49, 141.74, 129.34, 129.28, 128.98, 128.59, 124.67, 120.22, 119.52, 113.48, 112.77, 110.85, 110.01, 97.92, 64.40, 55.38, 46.90, 18.79, 9.58. MS (ESI) m/z [M + Na]$^+$ calcd. 411.17; found 411.3. HPLC analysis: MeOH − H_2O (95:5),

10.27 min, 96.73% purity.

4.5.1.2.3.3.7. 3,6-Dimethyl-N-(3-((2-methylbenzyl)oxy)benzyl)benzo[d]isoxazol-5-amine (**11z**).

Compound **11z** was prepared from **11b** and 1-(bromomethyl)-2-methylbenzene according to the general procedure. The product was isolated as a liquid in 29% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.38 – 7.34 (m, 2H), 7.27 – 7.13 (m, 4H), 7.08 (s, 1H), 7.01 (d, J = 7.6 Hz, 1H), 6.89 (dd, J = 8.0, 2.1 Hz, 1H), 6.60 (s, 1H), 5.59 (t, J = 5.9 Hz, 1H), 5.05 (s, 2H), 4.37 (d, J = 5.9 Hz, 2H), 2.35 (s, 3H), 2.32 (s, 3H), 2.29 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 158.60, 155.94, 154.02, 143.50, 141.76, 136.46, 134.97, 130.08, 129.36, 128.62, 128.42, 127.97, 125.71, 120.23, 119.61, 113.68, 112.84, 110.01, 97.92, 67.78, 46.90, 18.82, 18.41, 9.59. MS (ESI) m/z $[M + Na]^+$ calcd. 395.17; found 395.3. HPLC analysis: MeOH − H_2O (95:5), 10.35 min, 96.32% purity.

4.5.2. Synthesis of compounds shown in Scheme 2

4.5.2.1. N,3,6-trimethyl-N-(3-propoxybenzyl)benzo[d]isoxazol-5-amine (**11d'**).

To a solution of compound **11d** (70 mg, 0.23 mmol) dissolved in DMF (2 mL) was added NaH (60% in oil) (27 mg, 0.68 mmol) in ice bath and the mixture was stirred for 15 min. Iodomethane (152 mg, 1.07 mmol) was subsequently added. Then the mixture was warmed to RT stirring for another 10.5 h. Water was added to quench the reaction and the mixture was extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/EtOAc (1/10~1/5) to give the target product N,3,6-trimethyl-N-(3-propoxybenzyl)benzo[d]isoxazol-5-amine (**11d'**) as a liquid (46 mg, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (s, 1H), 7.25 – 7.20 (m, 2H), 6.99 – 6.92 (m, 2H), 6.83 – 6.78 (m, 1H), 4.01 (s, 2H), 3.91 (t, J = 6.6 Hz, 2H), 2.63 (s, 3H), 2.56 (s, 3H), 2.54 (s, 3H), 1.81 (dt, J = 14.1, 7.1 Hz, 2H), 1.03 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 159.05, 158.68, 154.78, 148.75, 140.21, 137.46, 129.29, 120.30, 114.19, 112.96, 112.28, 110.63, 68.73, 60.26, 41.88, 22.00, 18.91, 10.38, 9.62 (s). MS (ESI) m/z $[M + Na]^+$ calcd. 347.17; found

347.4. HPLC analysis: MeOH − H_2O (85:15), 18.75 min, 98.76% purity.

4.5.2.2. N-(3,6-dimethylbenzo[d]isoxazol-5-yl)-N-(3-methoxybenzyl)acetamide (**11a'**).

To a solution of compound **8** (619 mg, 3.03 mmol) dissolved in DMF (6 mL) was added NaH (60% in oil) (121 mg, 3.03 mmol) in ice bath and the mixture was stirred for 15 min. 1-(bromomethyl)-3-methoxybenzene (753 mg, 3.75 mmol) was subsequently added and the mixture was warmed to RT with stirring for another 40 min. Water was added to quench the reaction and the mixture was extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/ EtOAc (1/3~1/1) to give the target product N-(3,6-dimethylbenzo[d]isoxazol- 5-yl)-N-(3-methoxybenzyl)acetamide (**11a'**) as a liquid (895 mg, 91% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.66 (s, 1H), 7.45 (s, 1H), 7.19 (t, J = 7.8 Hz, 1H), 6.83 (dd, J = 8.2, 2.1 Hz, 1H), 6.78 – 6.73 (m, 2H), 5.05 (d, J = 14.3 Hz, 1H), 4.42 (d, J = 14.3 Hz, 1H), 3.67 (s, 3H), 2.43 (s, 3H), 2.16 (s, 3H), 1.70 (s, 3H). MS (ESI) m/z $[M + Na]^+$ calcd. 347.14; found 347.4. HPLC analysis: MeOH − H_2O (75:25), 14.22 min, 99.60% purity.

4.5.2.3. N-(3,6-Dimethylbenzo[d]isoxazol-5-yl)-N-(3-hydroxybenzyl)acetamide (**11b'**).

To a solution of compound **11a** (825 mg, 2.54 mmol) dissolved in DCM (10 mL) was added BBr_3 (1.911 g, 7.63 mmol) in ice bath. The mixture was then stirred at RT for 30 min. Then ice-water was added to quench the reaction and the mixture was extracted with DCM, washed with adequate water, dried over $NaSO_4$, concentrated under reduced pressure to give N-(3,6-dimethylbenzo[d]isoxazol-5-yl)-N-(3-hydroxybenzyl)-acetamide (**11b'**) as a white solid (720 mg, 92%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.28 (s, 1H), 7.66 (s, 1H), 7.42 (s, 1H), 7.05 (t, J = 7.7 Hz, 1H), 6.66 – 6.61 (m, 2H), 6.56 (d, J = 7.6 Hz, 1H), 4.96 (d, J = 14.2 Hz, 1H), 4.39 (d, J = 14.2 Hz, 1H), 2.44 (s, 3H), 2.15 (s, 3H), 1.69 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.50, 161.27,

157.25, 155.21, 139.28, 138.52, 137.59, 129.22, 122.17, 120.81, 119.46, 115.69, 114.31, 111.07, 51.35, 22.07, 18.06, 9.51. MS (ESI) m/z $[M + Na]^+$ calcd. 333.12; found 333.4. HPLC analysis: MeOH − H_2O (65:35), 12.95 min, 96.36% purity.

4.5.2.4. N-(3,6-Dimethylbenzo[d]isoxazol-5-yl)-N-(3-((3-methoxybenzyl)oxy)benzyl)acetamide (**11x'**).

To a solution of compound **11x'** (172 mg, 0.55 mmol) dissolved in DMF (4 mL) were added K_2CO_3 (126 mg, 0.91 mmol) and 1-(bromomethyl)-3-methoxybenzene (181 mg, 0.9 mmol). The mixture was stirred at 80 °C for 3 h and then cooled, poured into water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over $NaSO_4$, concentrated under reduced pressure. The residue was purified by silica gel chromatography with petroleum ether/EtOAc (1/3~1/1) to give the target product N-(3,6-dimethylbenzo[d]isoxazol-5-yl)-N-(3-((3-methoxybenzyl)-oxy)-benzyl)acetamide (**11x'**) as a yellow liquid (146 mg, 61% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.65 (s, 1H), 7.38 (s, 1H), 7.27 (t, J = 8.1 Hz, 1H), 7.18 (t, J = 7.9 Hz, 1H), 6.97 – 6.93 (m, 2H), 6.92 – 6.85 (m, 2H), 6.83 (s, 1H), 6.74 (d, J = 7.5 Hz, 1H), 5.07 – 5.00 (m, 3H), 4.41 (d, J = 14.3 Hz, 1H), 3.74 (s, 3H), 2.42 (s, 3H), 2.13 (s, 3H), 1.68 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.65, 161.28, 159.31, 158.18, 155.15, 139.20, 138.78, 138.64, 137.49, 129.49, 129.33, 122.16, 121.26, 120.76, 119.50, 115.30, 113.79, 113.18, 112.95, 111.08, 68.95, 55.00, 51.24, 22.01, 18.01, 9.45. MS (ESI) m/z $[M + Na]^+$ calcd. 453.18; found 453.4. HPLC analysis: MeOH − H_2O (75:25), 22.40 min, 99.05% purity.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/xxxxxx.

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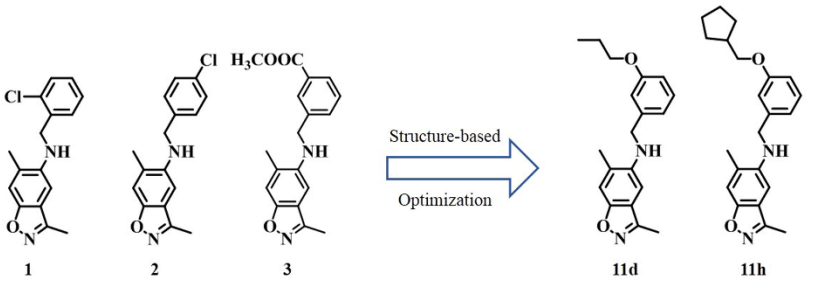

1 **2** **3** **11d** **11h**

TRIM24 bromodomain IC_{50}:

Hit **1** (11.18 μM)

Hit **2** (14.22 μM)

Hit **3** (13.76 μM)

TRIM24 bromodomain IC_{50}:

11d (1.88 μM), **11h** (2.53 μM)

LNCaP IC_{50}: **11d** (2.23 μM), **11h** (1.26 μM)

C4-2B IC_{50}: **11d** (3.37 μM), **11h** (0.96 μM)

A549 IC_{50}: **11d** (1.08 μM), **11h** (0.75 μM)

Highlights

- TRIM24 bromodomain is an attractive therapeutic target for the treatment of cancers.
- Thirty one novel N-benzyl-3,6-dimethylbenzo[d]isoxazol-5-amine derivatives were designed and synthesized.
- The structure-activity relationship study revealed high hydrophobic requirements of the "upper pocket".
- Two potent and selective compounds **11d** and **11h** exhibited low micromolar inhibitory activities against TRIM24 bromodomain.
- Compounds **11d** and **11h** exhibited low micromolar inhibitory activities on the proliferation of several cancer cells.