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Research paper

Discovery of 3-phenyl- and 3-*N*-piperidinyl-isothiazolo[4,3-*b*] pyridines as highly potent inhibitors of cyclin G-associated kinase

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ABSTRACT

Structural modifications at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold afforded a new series of cyclin G-associated kinase (GAK) inhibitors. It was shown that the insertion of a carboxamide residue at position 3 of a phenyl or piperidinyl moiety generated potent GAK inhibitors with IC_{50} values in a low nanomolar range. This potent GAK binding affinity was rationalized by molecular modelling demonstrating that the carboxamide moiety engages in an extra hydrogen bond with GAK. Moreover, this new series of compounds was also endowed with antiviral activity against dengue virus, highlighting the potential utility of GAK as a target for the development of antiviral drugs.

1. Introduction

The Numb-associated kinase (NAK) family includes four serine/threonine kinases: adaptor protein 2-associated kinase 1 (AAK1), cyclin G-associated kinase (GAK), BMP-2-inducible kinase (BIKE/BMP2K) and myristoylated and palmitoylated serine/threonine kinase 1, also known as serine/threonine kinase 16 (MPSK1 or STK16) that play important roles in a variety of cellular functions [1]. Both AAK1 and GAK are regulators of the host adaptor protein complexes 1 and 2 (AP1 and AP2, respectively), which are involved in the formation of vesicles for the transport in the secretory pathway [2–4]. GAK-mediated phosphorylation of the Thr156 residue of the μ-subunit of AP2 is important in the regulation of clathrin-mediated endocytosis [2,5,6]. Phosphorylation of Thr144 of AP1 by GAK plays an important role in *trans*-Golgi network to lysosome trafficking [7]. Overall, GAK-mediated phosphorylation of the APs enhances their coupling to cargo proteins and assists in the coordination and recruitment of clathrin to the membrane to form clathrin-coated vesicles (CCV) [8]. GAK is also involved in the uncoating of CCV in the cytoplasm. Due to the importance of these events for the cell cycle, GAK has been studied as drug target for the treatment of various disorders such as Parkinson's disease [9], prostate cancer [10] and osteosarcoma [11]. In addition, intracellular GAK-mediated events, are often hijacked by viruses for their own replication [12], and hence, GAK inhibition is a promising strategy for the development of broad-spectrum antiviral agents. Several studies have shown that GAK is implicated in early and late stages of the life cycle of different viruses including hepatitis C virus (HCV), Ebola virus (EBOV) and dengue virus (DENV) among others [13].

Dengue virus is an enveloped, single-stranded RNA virus that belongs to the Flaviviridae family and is transmitted by female mosquitoes, mainly of the species *Aedes aegypti*. DENV is the causative agent of dengue, an acute febrile illness that can develop into a serious illness called severe dengue in 5–20% of symptomatic patients [14,15]. Dengue is now endemic in more than 130 countries, including European ones. Local transmission was reported for the first time in France and Croatia in 2010, and several cases are observed annually in many European countries. The number of dengue cases reported to the World Health Organization (WHO) have increased by over 8-fold in the last two decades, reaching 4.2

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million cases in 2019 [16]. Unfortunately, the development of an effective treatment or vaccine to protect against DENV infection remains unsuccessful [17].

Most of the drug discovery programs searching for novel treatment options for DENV infection, focus on the inhibition of viral enzymes, with the major drawback of the rapid emergence of viral resistance [18]. In contrast, a host-targeted approach is attractive, as it gives the opportunity to develop broad-spectrum antiviral drugs with a high barrier to drug resistance [13]. We have previously demonstrated that erlotinib (Fig. 1), an approved anticancer drug, shows *in vitro* and *in vivo* antiviral activity against DENV and other unrelated viruses including HCV, EBOV, Zika virus (ZIKV), and Chikungunya (CHIKV) [19]. Erlotinib is endowed with strong GAK affinity (dissociation constant or K_D value of 3.1 nM) [20]. However, this compound showed cross activity with epidermal growth factor receptor (EGFR, $K_D = 0.70$ nM) [21] and a number of other kinases [22,23]. Therefore, it is not clear whether the antiviral efficacy of erlotinib is due to GAK inhibition or if there are any other factors involved. Despite the fact that erlotinib has been selected as reference compound for GAK inhibition in multiple studies [19,20], the lack of kinase selectivity has limited its use as a chemical tool to study the importance of GAK in viral replication and has stimulated medicinal chemists to search for potent, more selective and drug-like GAK inhibitors. In the past years, the chemistry of 4-anilinoquin(az)olines has been studied in an effort to develop chemical probes for GAK [24]. In 2019, compound SGC-GAK-1 (Fig. 1), a 4,6-disubstituted quinoline, was described as a potent and selective GAK inhibitor with a K_D of 1.9 nM [25] and a IC_{50} (half-maximal inhibitory concentration) of 48 nM [26] using a live cell NanoBRET target engagement assay [27]. Recent studies have also shown that SGC-GAK-1 displays a potent antiviral activity against DENV in low micromolar range ($EC_{50} = 0.080$ μM) [28].

We have previously discovered a series of isothiazolo[4,3-*b*]pyridines as potent and selective GAK inhibitors with antiviral activity against various viruses [19,29–31]. The main prior focus has been on structural modifications at positions 3, 5 and 6 of the isothiazolo[4,3-*b*]pyridine scaffold [29–32]. Compound **1** (Fig. 1), bearing a 4-amino-3-methoxyphenyl residue at position 6 and a morpholino ring at position 3, displayed potent GAK affinity ($K_D = 9.0$ nM) [29] and low micromolar antiviral activity against HCV ($EC_{50} = 2.8$ μM) and DENV ($EC_{50} = 1.8$ μM) [19]. The introduction of a variety of nucleophiles at position 3 revealed that close mimics of morpholine were optimal for GAK affinity and antiviral activity. This was exemplified by the discovery of compound **2** (Fig. 1), an isothiazolo[4,3-*b*]pyridine with a *cis*-2,6-dimethylmorpholine residue at position 3. Although this compound showed a decreased GAK affinity ($K_D = 89$ nM, $IC_{50} = 39$ nM), it displayed an improved antiviral activity against DENV ($EC_{50} = 0.82$ μM) [30]. Unfortunately, commercially available morpholine analogues are expensive and their number is very limited. In addition, the synthesis of these morpholine analogues is cumbersome, posing an additional hurdle in the exploration of the structure-activity relationship (SAR). Piperidine is closely related to morpholine with the advantage that a large number of piperidine analogues is commercially available. Moreover, various 3-*N*-piperidinyl-isothiazolo[4,3-*b*]pyridines were synthesized before (i.e. *N*-piperidine, *N*-(4-amino)piperidine, *N*-(4-cyano)piperidine, *N*-(4,4-difluoro)piperidine, *N*-(3-carboxamide)piperidine, etc) and found to display potent GAK affinity [30].

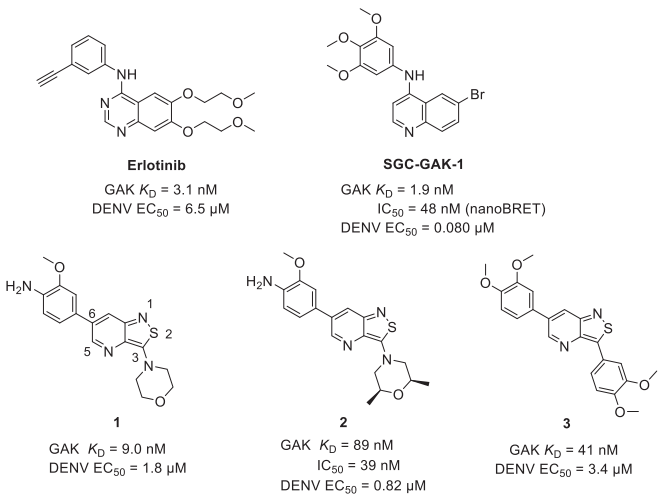


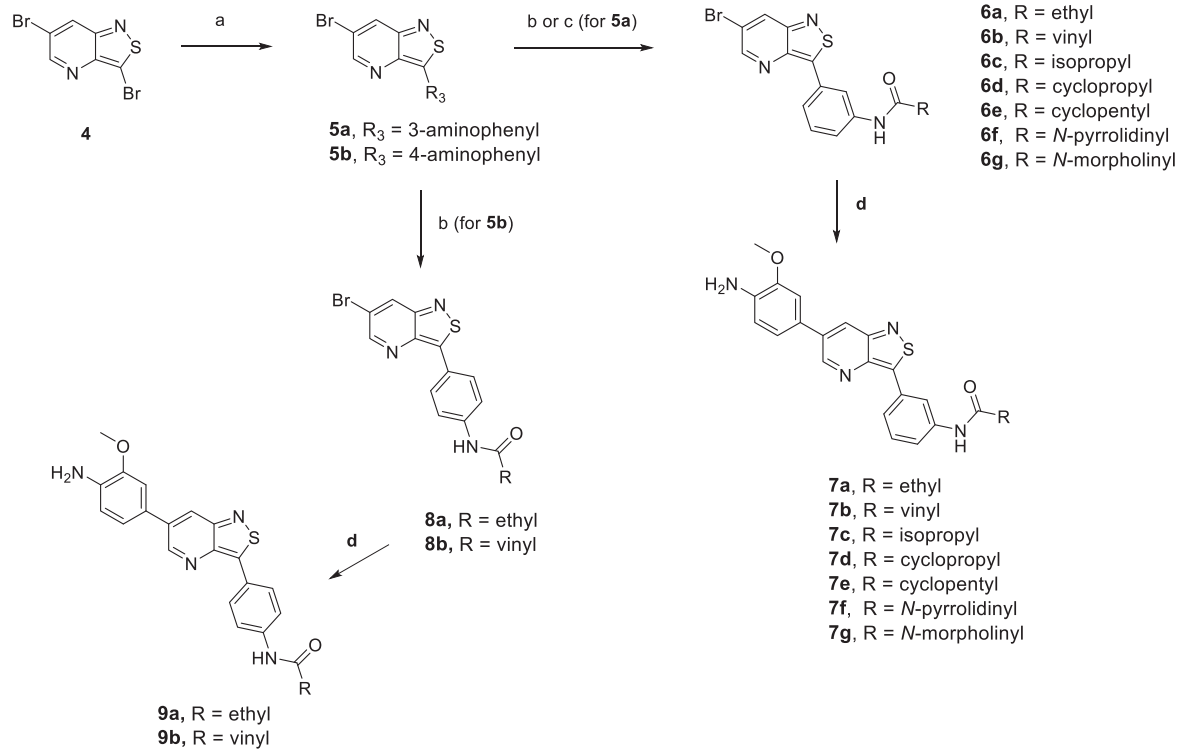
Fig. 1. Previously reported GAK inhibitors.

In order to expand the structural variety at position 3 of the isothiazolo[4,3-*b*]pyridine skeleton, we previously applied various palladium-catalyzed cross-couplings such as Suzuki and Sonogashira reactions. This effort led to the discovery of 3,6-bis(3,4-dimethoxyphenyl)isothiazolo[4,3-*b*]pyridine **3**, that was endowed with strong affinity for GAK ($K_D = 41$ nM) [31]. Molecular docking showed that the 3,4-dimethoxyphenyl residue at position 3 forms an additional hydrogen bond with Lys69 of the GAK enzyme. In the present study, the aim was to further exploit potential hydrogen bond interactions with GAK and hence to increase GAK affinity. Therefore, various carboxamides were introduced on the phenyl ring at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold. The same carboxamide substituents were also appended to a piperidinyl ring yielding a novel series of 3-*N*-piperidinyl isothiazolo[4,3-*b*]pyridines.

2. Chemistry

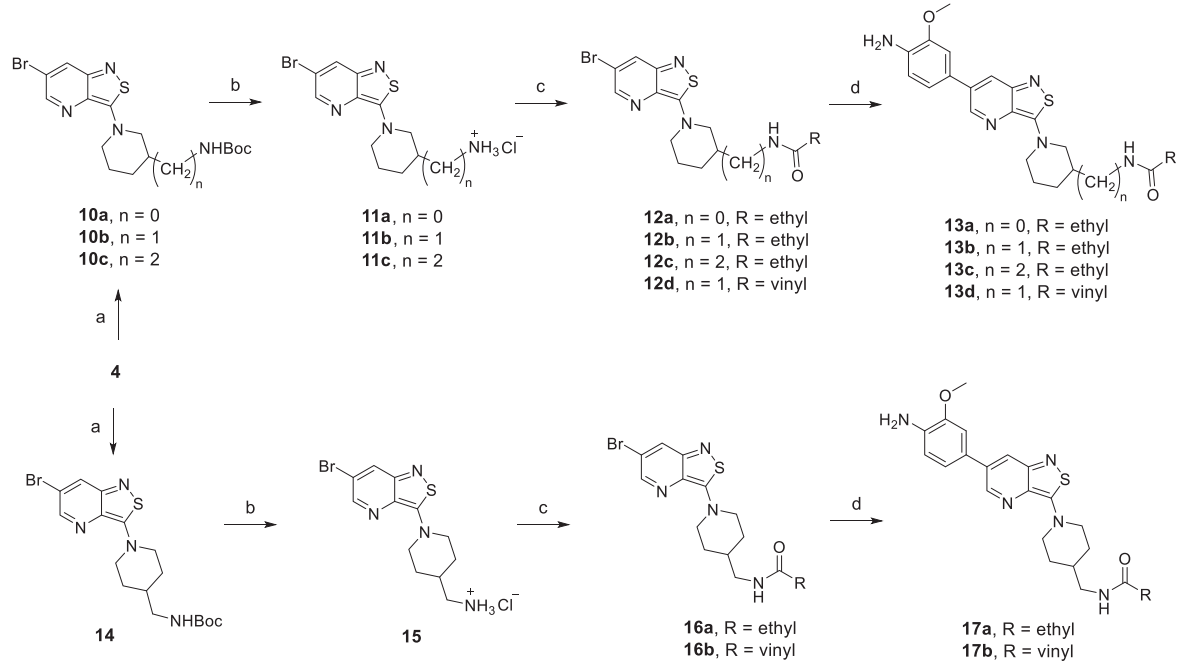
The synthesis of the target isothiazolo[4,3-*b*]pyridines started from the known key intermediate 3,6-dibromoisothiazolo[4,3-*b*]pyridine **4** [29]. Firstly, a 3- and 4-aminophenyl residue was introduced at position 3 of the 3,6-dibromoisothiazolo[4,3-*b*]pyridine following the standard Suzuki cross-coupling reaction conditions [33,34]: the appropriate boronic acid, $Pd(PPh_3)_4$ as catalyst and potassium carbonate as base in a mixture of dioxane/water (Scheme 1). A continuous flow of argon was conveniently passed through the mixture (for 5–10 min) before the addition of the catalyst. This prevents the inactivation of the palladium catalyst and allowed the isolation of the desired compounds **5a** and **5b** in excellent yields (88% and 86%, respectively). Subsequently, the amino group was coupled with various acid chlorides following two different conditions (pathways b and c, Scheme 1) [35]. Compounds **6a-e** and **8a-b** were obtained using mild reaction conditions (pathway b: the appropriate acid chloride, dry triethylamine as base and dry dichloromethane at room temperature) furnishing the target compounds in good yields (82–87%). On the other hand, the synthesis of compounds **6f** and **6g** (pathway c) required the addition of DMAP as catalyst and harsher reaction conditions were needed. These reactions were performed in dry *N*,*N*-dimethylformamide (DMF) for compound **6f** and dry 1,4-dioxane for **6g** at 100 °C, yielding the desired compounds in lower yields (71–73%). Finally, 4-amino-3-methoxyphenylboronic acid pinacol ester was introduced at position 6 of the isothiazolo[4,3-*b*]pyridine scaffold using the palladium-catalyzed cross-coupling conditions described before, yielding the final compounds **7a-g** and **9a** in good yields (71–83%). DMF was used as a solvent for the synthesis of compound **9b** and the reaction mixture was stirred at 95 °C overnight.

To carry out the insertion of modified piperidines at position 3 of



Scheme 1. *Reagents and conditions*: a) $ArB(OH)_2$, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 85 °C; b) acid chloride, Et_3N, dry DCM, 0 °C to rt; c) acid chloride, Et_3N, DMAP, dry dioxane or dry DMF, 100 °C; d) 4-amino-3-methoxyphenylboronic acid pinacol ester, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 85 °C or DMF at 95 °C for compound **9b**.

the isothiazolo[4,3-*b*]pyridine scaffold, four Boc-protected amino-piperidines (as racemic mixtures) with a different chain length between the piperidine ring and the exocyclic amino group were selected: 3-(Boc-amino)piperidine, 3- and 4-(Boc-aminomethyl) piperidine and 3-(Boc-aminoethyl)piperidine (Scheme 2). Nucleophilic addition of these piperidines to 3,6-dibromoisothiazolo[4,3-*b*]pyridine **4** was carried out using triethylamine as base in absolute ethanol [29], yielding the desired compounds **10a-c** and **14** in excellent yields (87–91%). Acidic cleavage (4 M HCl in dioxane) of the *tert*-butoxycarbonyl (Boc)



Scheme 2. *Reagents and conditions*: a) appropriate piperidine, Et_3N, EtOH, reflux, overnight; b) HCl/dioxane 4 M, 0 °C to rt, 3–4 h; c) propionyl or acryloyl chloride, dry Et_3N, dry DCM, 0 °C to rt; d) 4-amino-3-methoxyphenylboronic acid pinacol ester, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 85 °C.

moiety [36] afforded compounds **11a-c** and **15** that were used as such for the next step. Reaction with propionyl and acryloyl chloride, followed by a Suzuki coupling reaction using the standard conditions (4-amino-3-methoxyphenylboronic acid pinacol ester, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 85 °C) furnished the final compounds **13a-d** and **17a-b**, in yields ranging from 75 to 87%.

Compound **11b**, with a (±)-3-(aminomethyl)piperidinyl residue at position 3 of the isothiazolo[4,3-*b*]pyridine, was chosen as synthon for coupling with various acid chlorides, a sulfonyl chloride and an isocyanate (Scheme 3). These reactions were carried out using dry triethylamine as base in dry dichloromethane at room temperature or at 35 °C, yielding the corresponding compounds **18a-h** in good yields (63−88%). In the last step, a Suzuki cross-coupling reaction allowed the insertion of 4-amino-3-methoxyphenyl moiety at position 6 of isothiazolo[4,3-*b*]pyridine, giving rise to compounds **19a-h** in yields ranging from 72 to 83%.

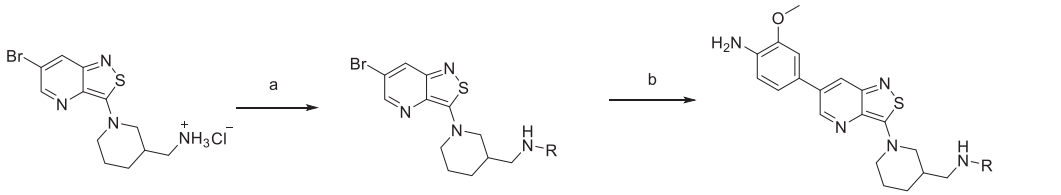
Following a similar scheme as for the synthesis of racemate **19b** (Scheme 3), the (*S*)- and (*R*)-enantiomers (compounds **23a** and **23b**, respectively) were prepared separately (Scheme 4), starting from the commercially available, optically pure building blocks (*R*)-3-(Boc-aminomethyl)piperidine and (*S*)-3-(Boc-aminomethyl)piperidine, respectively. Their regioselective introduction at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold yielded compounds **20a** (89%) and **20b** (87%). Acidic removal of the Boc group, followed by amide formation by reaction with cyclopropanecarbonyl chloride and Suzuki cross-coupling reaction using 4-amino-3-methoxyphenylboronic acid pinacol ester, afforded the (*S*)-enantiomer (**23a**) and the (*R*)-enantiomer (**23b**) in moderate yields (75% and 78%, respectively).

3. GAK binding affinity

Compounds **7a-g**, **9a-b**, **13a-d**, **17a-b**, **19a-h** and **23a-b** were evaluated for GAK binding affinity (Tables 1 and 2) *via* the commercially available LanthaScreen® Europium kinase binding assay [37]. In this assay, an Alexa Fluor 647-labeled conjugate or tracer competes with the GAK ligand for the ATP binding site. Detection is dependent on the time-resolved fluorescence resonance energy transfer (TR-FRET) signal resulting from the antibody and tracer binding. When the ATP site is occupied by the tracer, there is a high TR-FRET signal. When the tracer is displaced by a GAK inhibitor, a reduction in TR-FRET signal is observed. The IC_{50} value for the binding affinity of compound **1**, as determined *via* this LanthaScreen assay, was 51 nM, which correlates with the K_D value of 9.0 nM, as previously measured via the DiscoverX screening assay [29]. Staurosporine, a microbial alkaloid with potent binding affinity for various human protein kinases including GAK, has been used in research as a universal kinase inhibitor [38,39] and was also included as positive control for the LanthaScreen assay, displaying an IC_{50} value of 11 nM.

The goal of this optimization campaign was to improve GAK affinity of our previously reported isothiazolo[4,3-*b*]pyridines. In the present study, the 4-amino-3-methoxyphenyl moiety at position 6 of the isothiazolo[4,3-*b*]pyridine scaffold was fixed, as it was previously shown that electron-donating substituents (such as methoxy or amino groups) on the phenyl ring were optimal for GAK affinity and antiviral activity [29]. Previous molecular modelling demonstrated that an appropriate substitution pattern at position 3 of the scaffold can engage in additional interactions with Lys69 of GAK [31]. Given the synthetic feasibility, we opted for the insertion of phenyl and piperidine residues at position 3 of the isothiazolo [4,3-*b*]pyridine scaffold, and inserted carboxamide groups at different positions in order to exploit hydrogen bond interactions. The synthesis started with a 3-phenyl-isothiazolo[4,3-*b*]pyridine carrying a propionamide residue. To probe the optimal position, the 3- and 4-propionamide derivatives (compounds **7a** and **9a**, respectively) were initially prepared (Table 1). Compound **9a** was devoid of GAK affinity (IC_{50} > 1.1 μM), whereas its 3-substituted phenyl congener **7a** shows very potent GAK affinity (IC_{50} = 7.7 nM). This potent GAK affinity prompted us to prepare a small series of 3-*N*-acyl (compounds **7c-e**) and 3-*N*-carbamoyl (compounds **7f-g**) derivatives. All these compounds behaved as highly potent GAK binders (IC_{50} < 20 nM).

A second series of isothiazolo[4,3-*b*]pyridines carried a piperidinyl moiety at position 3 (Table 2). In a first round of synthesis, a set of 3-modified piperidines (as racemic mixture), with different chain lengths between the piperidine ring and the propionamide group, was prepared. Compound **13b**, having a methylene group as a linker moiety, was the most potent GAK ligand within this series, displaying an IC_{50} value of 74 nM. A direct attachment of the propionamide moiety to the piperidine ring (compound **13a**) or elongation of the linker to an ethylene group (compound **13c**) led to a decreased activity (IC_{50} values of 0.90 and 0.19 μM, respectively). In order to define the optimal position of the propionamidomethyl residue at the piperidine ring, compound **17a** was prepared, showed a drop in GAK affinity (IC_{50} = 1.8 μM) when compared to its 3-substituted congener **13b**, suggesting that the position of the amido group is important for GAK affinity. To expand the SAR study, the 3-(aminomethyl)piperidine analogue was selected as building block for the synthesis of a number of 3-*N*-acyl- (compounds **19a-**

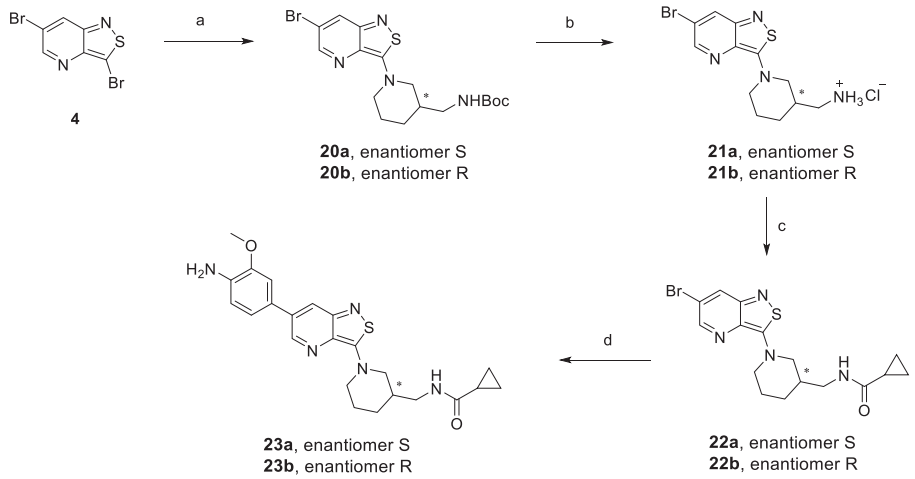


11b

18a, R = 3-methylbutanoyl
18b, R = cyclopropanecarbonyl
18c, R = cyclopentanecarbonyl
18d, R = benzoyl
18e, R = *N*-pyrrolidinecarbonyl
18f, R = *N*-morpholinecarbonyl
18g, R = cyclopropanesulfonyl
18h, R = cyclopropanylaminecarbonyl

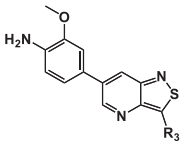
19a, R = 3-methylbutanoyl
19b, R = cyclopropanecarbonyl
19c, R = cyclopentanecarbonyl
19d, R = benzoyl
19e, R = *N*-pyrrolidinecarbonyl
19f, R = *N*-morpholinecarbonyl
19g, R = cyclopropanesulfonyl
19h, R = cyclopropanylaminecarbonyl

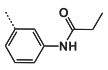
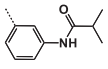
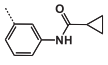
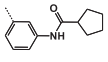
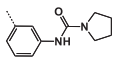
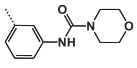
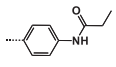
Scheme 3. *Reagents and conditions.* a) acid chloride, sulfonyl chloride or isocyanate, dry Et_3N, dry DCM, 0 °C to rt or 35 °C; b) 4-amino-3-methoxyphenylboronic acid pinacol ester, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 85 °C.



Scheme 4. *Reagents and conditions.* a) (*R*) or (*S*)-3-(Boc-aminomethyl)piperidine, Et_3N, EtOH, reflux, overnight; b) HCl/dioxane 4 M, 0 °C to rt, 3–4 h; c) cyclopropanecarbonyl chloride, dry Et_3N, dry DCM, 0 °C to rt; d) 4-amino-3-methoxyphenylboronic acid pinacol ester, $Pd(PPh_3)_4$, K_2CO_3, dioxane/water, 85 °C.

Table 1
GAK binding affinity of 3-aryl isothiazolo[4,3-*b*]pyridines.



Comp.	R_3	GAK IC_{50} (μM)[a]
Staurosporine	–	0.011 ± 0.0012
1		0.051 ± 0.0031
7a		0.0077 ± 0.0023
7c		0.0038 ± 0.0012
7d		0.0088 ± 0.00061
7e		0.017 ± 0.0013
7f		0.0022 ± 0.0016
7g		0.0032 ± 0.0024
9a		>1.1

[a] IC_{50} = half-maximal inhibitory concentration. Values are the mean ± S.D. of two independent experiments.

d) and 3-*N*-carbamoyl (compounds **19e-f**), that displayed IC_{50} values in the range of 10–90 nM. The most potent derivative in this series was the 3-*N*-cyclopropylcarbonyl derivative **19b** that was endowed with an IC_{50} value of 14 nM. The corresponding sulfonamide (compound **19g**) and urea (compound **19h**) derivatives showed both a 5-fold drop in GAK affinity, when compared to the amido congener **19b**. The potent GAK affinity of racemate **19b** encouraged us to evaluate the enantiopure compounds **23a-b** for GAK affinity, with the (*R*)-enantiomer **23b** (IC_{50} = 13 nM) being 28-fold more potent than the (*S*)-enantiomer **23a** (IC_{50} = 0.36 μM).

4. Molecular modelling

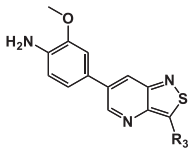
In order to characterize the binding mode of this new series of isothiazolo[4,3-*b*]pyridines, the most potent GAK inhibitors were docked into the ATP binding site, using the X-ray structure of GAK (PDB entry 4Y8D) [29]. Compound **7a** binds in a similar manner as the co-crystallized compound **1**, but is anchored by an extra hydrogen bond between the amide carbonyl group and the protonated side chain of Lys69 (Fig. 2A). The improved GAK affinity of compound **7f**, relative to compound **7a**, can be explained by the presence of a larger pyrrolidine ring, leading to a better contact with the GAK enzyme (supporting information; Figs. S1 and S2).

The (*R*)- and (*S*)-enantiomers of racemic mixture **13b** were docked separately into the ATP binding pocket of GAK. Only the (*R*)-isomer was able to form extra hydrogen bond interactions with the side chain of Lys69 (Fig. 2B), because of the elongated conformation of the (*R*)-enantiomer relative to the (*S*)-enantiomer. To rationalise the difference in binding affinity between the enantiomers **23a** and **23b**, both compounds were docked into the ATP binding pocket of GAK (Fig. 3). The (*R*)-enantiomer **23b** steers the amide closer to the side chains of Lys69 and Asp191 establishing extra hydrogen bonds. These interactions lead to an improvement of GAK affinity of compound **23b**, when compared to the (*S*)-enantiomer **23a**. This is in complete agreement with the molecular modelling performed with the enantiomers of compound **13b**.

5. Attempts towards the discovery of irreversible GAK inhibitors

Molecular modelling of compounds **7a** and **13b** can bring the propionamide group in close proximity to the Cys190 side chain of GAK (Fig. 2B). The rotation of this propionamide residue was more

Table 2
GAK binding affinity of 3-*N*-piperidinyl isothiazolo[4,3-*b*]pyridines.



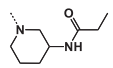
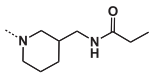
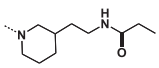
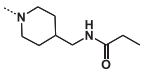
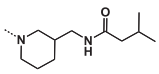
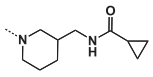
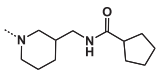
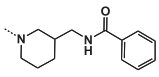
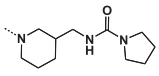
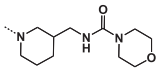
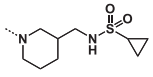
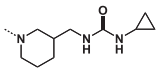
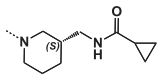
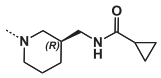
Comp.	R_3	GAK IC_{50} (μM)[a]
Staurosporine	–	0.011 ± 0.0012
1		0.051 ± 0.0031
13a		0.90 ± 0.33
13b		0.074 ± 0.019
13c		0.19 ± 0.029
17a		1.8 ± 0.23
19a		0.069 ± 0.0089
19b		0.014 ± 0.0043
19c		0.039 ± 0.018
19d		0.035 ± 0.0063
19e		0.037 ± 0.0015
19f		0.085 ± 0.00033
19g		0.078 ± 0.012
19h		0.062 ± 0.015

Table 2 (*continued*)

Comp.	R_3	GAK IC_{50} (μM)[a]
23a		0.36 ± 0.068
23b		0.013 ± 0.00052

[a] IC_{50} = half-maximal inhibitory concentration. Values are the mean ± S.D. of two independent experiments.

convenient for compound **13b**, since the presence of an extra methylene residue between the amide group and the piperidine moiety allows for more conformational flexibility.

With the aim to develop irreversible GAK inhibitors, compounds **7b**, **9b**, **13d** and **17b** were prepared. These compounds contain an *α,β*-unsaturated carbonyl moiety that can act as an electrophilic warhead to irreversibly bind the sulfur atom of Cys190 in the ATP binding pocket of GAK. Biological evaluation for GAK affinity of these compounds demonstrated that especially an acrylamide at *meta* position of the phenyl ring gives rise to very potent GAK affinity (compound **7b**), with an IC_{50} of 11 nM, whereas the corresponding *para* congener, compound **9b**, is 50-fold less active (Table 3). Within the piperidine series, the 3-substituted analogue (compound **13d**) is a potent GAK ligand (IC_{50} = 78 nM), whereas the presence of an acrylamide motif at position 4 of the piperidine ring (compound **17b**) gives rise to a much less active compound (IC_{50} = 1.7 μM).

In order to determine whether the two most potent Michael acceptors (compounds **7b** and **13d**) irreversibly bind to GAK, mass spectrometry experiments were performed, in which a well-known irreversible inhibitor of GAK ((5*Z*)-7-oxozeaenol) was included as positive control [37]. Incubation of (5*Z*)-7-oxozeaenol with recombinant GAK protein led to a shift of 362.5 Da in the observed molecular weight of the protein (Figs. S3–B), confirming its covalent binding to GAK. In contrast, incubation of compounds **7b** and **13d** with GAK did not reveal any differences in the molecular weight (Figures S3-C and S3-D), indicating no covalent binding to GAK.

6. Anti-DENV activity of 3-modified isothiazolo[4,3-*b*] pyridines

The most potent GAK inhibitors (IC_{50} < 40 nM) were evaluated for antiviral activity in human hepatoma (Huh7) cells infected with DENV2 (Table 4). The effect of each compound on DENV2 infection was measured at 48 h following infection with a reporter virus *via* luciferase assays, and the EC_{50} (half-maximal effective concentration) values were calculated. In parallel, the CC_{50} (half-maximal cytotoxic concentration) values were measured in the same wells *via* AlamarBlue assays. Erlotinib and compound **2** were both included as reference compounds [30]. Compound **2**, rather than compound 1, was selected as reference for antiviral studies due to its improved activity/cytotoxicity profile [29,30]. Despite the fact that some of the analogues were endowed with very potent GAK affinity, that did not translate into an improved antiviral efficacy when compared to reference compound **2** (EC_{50} = 0.96 μM). All 3-modified isothiazolo[4,3-*b*]pyridines showed comparable antiviral activity against DENV2, with EC_{50} values in the range of 1.8–7.5 μM. Several derivatives with an amidophenyl moiety at position 3 of the isothiazolo[4,3-*b*]pyridine scaffold (compounds **7a**, **7c-e**) were

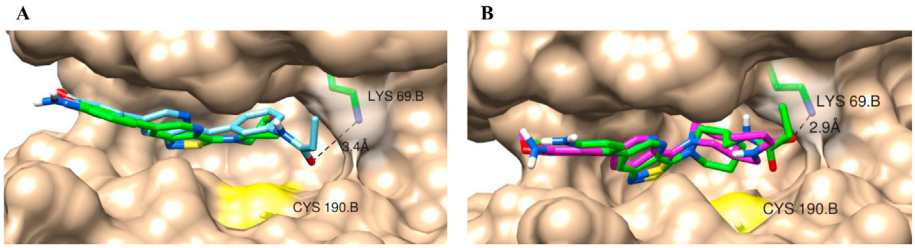


Fig. 2. A) Docking of **7a** (light blue carbons) and the reference compound **1** (green carbons) into the ATP binding site of GAK; B) Docking of **13b** as both (*R*)-enantiomer (green carbons) and (*S*)-enantiomer (magenta carbons) into the ATP binding site of GAK.

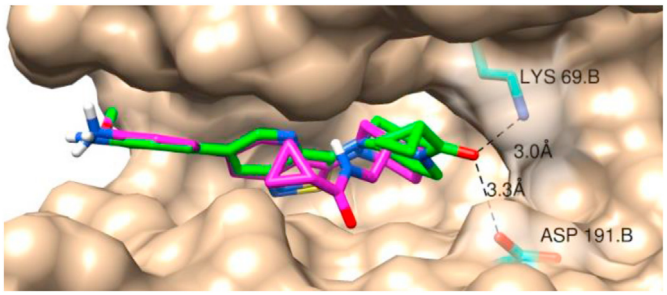
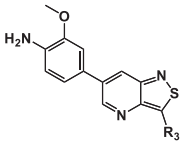
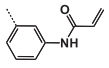
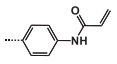
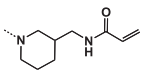
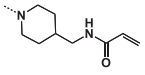


Fig. 3. Docking of compounds **23a** ((*S*)-enantiomer, magenta carbons) and **23b** ((*R*)-enantiomer, green carbons) into the ATP binding site of GAK.

Table 3
GAK binding affinity of isothiazolo[4,3-*b*]pyridines with an acrylamide residue.

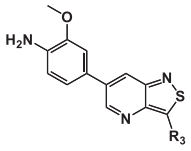


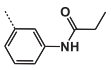
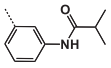
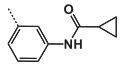
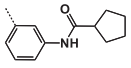
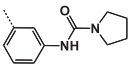
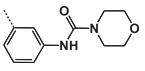
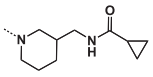
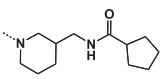
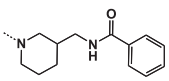
Comp.	R_3	GAK IC_{50} (μM)[a]
Staurosporine	–	0.011 ± 0.0012
1		0.051 ± 0.0031
7b		0.012 ± 0.00070
9b		0.50 ± 0.21
13d		0.078 ± 0.0069
17b		1.7 ± 0.41

[a] IC_{50} = half-maximal inhibitory concentration. Values are the mean ± S.D. of two independent experiments.

endowed with CC_{50} values close to their antiviral activity. In contrast, compounds **7f** and **7g** had more favourable selectivity indices, with reduced cytotoxicity (CC_{50} > 20 μM). All 3-*N*-

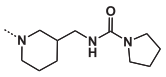
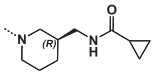
Table 4
Antiviral activity against DENV2 of 3-modified isothiazolo[4,3-*b*]pyridines.



Comp	R_3	DENV2 EC_{50} (μM)[a]	Huh7 cells CC_{50} (μM)[b]
Erlotinib	–	12 ± 7.3	>20
2		0.96 ± 0.12	>20
7a		1.9 ± 0.92	9.9 ± 4.87
7c		3.8 ± 2.1	14 ± 7.5
7d		3.6 ± 0.92	15 ± 7.3
7e		1.8 ± 1.2	13 ± 11
7f		6.0 ± 3.0	>20
7g		7.9 ± 3.6	>20
19b		4.0 ± 1.1	>20
19c		2.6 ± 1.3	>20
19d		3.9 ± 2.5	>20

(*continued on next page*)

Table 4 (*continued*)

Comp	R_3	DENV2 EC_{50} (μM)[a]	Huh7 cells CC_{50} (μM)[b]
19e		4.1 ± 1.1	>20
23b		7.5 ± 4.1	>20

[a] EC_{50} = half-maximal effective concentration.
[b] CC_{50} = half-maximal cytotoxic concentration. Values are the mean ± S.D. of two or three independent experiments.

piperidinyl-isothiazolo[4,3-*b*]pyridines (**19b**, **19c-e** and **23b**) were less cytotoxic for Huh7 cells, with CC_{50} values greater than 20 μM.

7. Kinase selectivity profile

In order to determine whether these novel isothiazolo[4,3-*b*]pyridines are selective GAK inhibitors, the 3-*N*-piperidinyl analogue **23b** was selected for a kinase selectivity screening due to its potent GAK affinity (IC_{50} = 13 nM) and low cytotoxicity (CC_{50} > 20 μM). In addition, the reduced number of aromatic rings of 3-*N*-piperidinyl-isothiazolo[4,3-*b*]pyridines is advantageous to avoid future development issues like poor aqueous solubility, high lipophilicity or high serum albumin binding [40]. Compound **23b** was screened against a panel of 97 kinases distributed across the kinome using the DiscoverX *scan*EDGE℠ Kinase Assay Panel platform [21]. Although screening a compound against a full kinase panel is ideal, it is costly and a time-consuming exercise. However, it has been shown before that a mini-kinome panel provides sufficient information to estimate kinase selectivity [41–43]. When the tested compound binds the kinase active site, it prevents the kinase binding to the immobilized ligand and hence reduces the amount of kinase captured on the solid support [21]. The results are reported as the percentage of kinase remaining bound to the ligand relative to a control (%Ctrl), with the lower numbers indicating stronger binding affinity of the compound to the kinase. Compound **23b** was tested at a single concentration of 10 μM and its selectivity profile is illustrated as a kinome tree (Fig. 4). This compound has a selectivity score for kinases with a percentage control lower than 10 (S(10)) of 0.078 and only targets seven non-mutant kinases with greater than 90% inhibition (% Ctrl <10): CDK7, FLT3, KIT, MKNK2, PDGFRA, PDGFRB and TRKA. We reported before that structurally related isothiazolo[4,3-*b*]pyridines, when assayed against 468 kinases in the *scan*MAX℠ Kinase Assay Panel, also showed potent FLT3, KIT and PDGFRB binding affinity [29,30]. KIT suppression reduced DENV infection but also reduced cellular viability [19], thus the role of KIT in DENV replication is not clear. These data suggest that KIT can be an extra target (beyond GAK) of these isothiazolo[4,3-*b*]pyridines that may contribute to the observed antiviral effect. The observed cytotoxicity in the Huh7 cell line might also be due to targeting of these kinases.

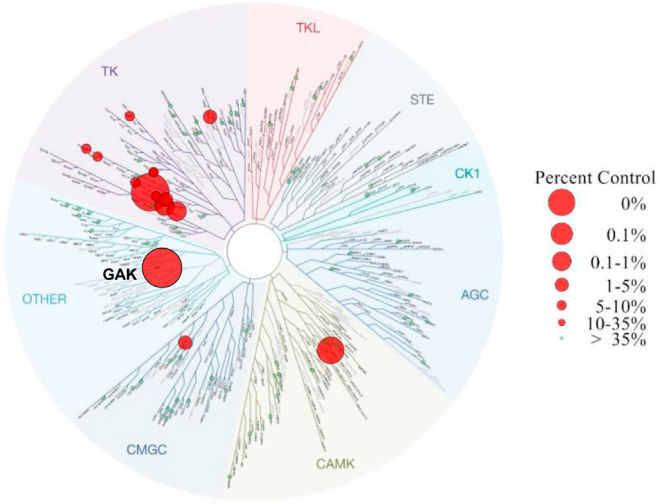


Fig. 4. Kinome tree of compound **23b**. Red circles indicate kinases inhibited.

8. Conclusion

In this paper, the synthesis of a novel series of isothiazolo[4,3-*b*]pyridines carrying a phenyl and a *N*-piperidinyl moiety at position 3 of the heterocyclic scaffold is described. All compounds were evaluated for their binding affinity towards GAK. The most potent GAK inhibitors within this series, display IC_{50} values in the low nanomolar range. A systematic SAR study showed that the insertion of a carboxamide group at position 3 of the phenyl or piperidinyl ring was crucial to increase the binding affinity for GAK. Molecular modelling revealed that this carboxamide residue engaged in a hydrogen bond interaction with Lys69 in the ATP binding pocket of GAK. The most potent GAK inhibitors from this series were also evaluated for anti-DENV activity, showing antiviral activities in the low micromolar range, very similar to the reference compound **2**. It has been shown before that isothiazolo[4,3-*b*]pyridines show only low micromolar GAK cellular potency when investigated in a NanoBRET target engagement assay [25]. In addition, previous research demonstrated that the isothiazolo[4,3-*b*]pyridine derivatives are usually endowed with good permeability [29]. Therefore, the competition with the intracellular ATP concentration in cells might be an issue for this type of compounds and might be responsible for the moderate antiviral activity.

In summary, we have developed a new family of potent and selective GAK inhibitors with promising antiviral activity. The synthetic accessibility, high potency and low cytotoxicity of these analogues, make them good leads for further studies to understand the role of GAK in viral replication.

9. Experimental section

9.1. Chemistry

9.1.1. Materials and methods

Analytical grade solvents were used for all reactions. Argon was used to carry out reactions under an inert atmosphere. Melting points were recorded with a Stuart SMP20 melting point apparatus. Optical rotations were recorded with a polarimeter Model 341 using a Na gas lamp with a wavelength of 589 nm and a standard temperature of 20 °C. The concentrations are expressed in g/100 mL and the specific rotations ($[\alpha]_D$) are reported in deg·mL·dm^{-1} g^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz instrument (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz), 500 MHz instrument (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) or a 600 MHz instrument (^{1}H NMR, 600 MHz; ^{13}C NMR, 150 MHz), using tetramethylsilane as internal standard for ^{1}H NMR spectra and DMSO-d_6 (39.5 ppm) or $CDCl_3$ (77.2 ppm) for ^{13}C NMR spectra. Abbreviations used are s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. Coupling constants are expressed in Hz. High resolution mass spectra were acquired on a quadrupole orthogonal acceleration time-of-flight mass

spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 mL/min and spectra were obtained in positive or negative ionization mode with a resolution of 15000 (FWHM) using leucine enkephalin as lock mass. Precoated aluminum sheets (Fluka silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography was performed on silica gel 0.060–0.200 mm, 60 (Acros Organics). Purity of final compounds was verified to be >95% by HPLC analysis. HPLC conditions to assess purity were as follows: Shimadzu HPLC equipped with a LC-20AT pump, DGU-20A5 degasser, and a SPD-20A UV-VIS detector; Symmetry C18 column (5 μm, 4.6 mm × 150 mm); gradient elution of H_2O/CH_3CN from 95/5 or 70/30 to 5/95 over 25 min; flow rate 1 mL/min; wavelength, UV 254 nm.

9.1.2. Suzuki reaction at 3 position of isothiazolo[4,3-b]pyridines (**5a-b**)

General Procedure: A solution of 3,6-dibromoisothiazolo[4,3-*b*] pyridine (1 equiv) in a mixture of 1,4-dioxane/water (ratio 9:1) was degassed with argon and, subsequently, the corresponding boronic acid (1.2 equiv), $Pd(PPh_3)_4$ (0.02 equiv) and K_2CO_3 (2 equiv) were added. The mixture was degassed a second time, filled with argon and stirred at 85 °C for 3–6 h. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was purified by silica gel, yielding the corresponding compounds 5a-b. The following compounds were made according to this procedure.

*9.1.2.1. 3-(3-Aminophenyl)-6-bromoisothiazolo[4,3-b]pyridine (**5a**).* This compound was obtained using 3-aminophenylboronic acid. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an orange solid in 88% yield (91.5 mg, 0.30 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.88 (bs, 2H, NH_2), 6.77–6.85 (m, 1H, arom H), 7.31 (t, J = 7.8 Hz, 1H, arom H), 7.41–7.46 (m, 1H, arom H), 7.50–7.55 (m, 1H, arom H), 8.29 (d, J = 2.1 Hz, 1H, arom H), 8.77 (d, J = 2.1 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{12}H_8BrN_3S$ 305.9696, found 305.9695.

*9.1.2.2. 3-(4-Aminophenyl)-6-bromoisothiazolo[4,3-b]pyridine (**5b**).* This compound was obtained using 4-aminophenylboronic acid hydrochloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as an orange solid in 86% yield (91.4 mg, 0.29 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 5.94 (bs, 2H, NH_2), 6.71 (d, J = 8.7 Hz, 2H, arom H), 8.00 (d, J = 8.6 Hz, 2H, arom H), 8.50 (d, J = 2.1 Hz, 1H, arom H), 8.79 (d, J = 2.1 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{12}H_8BrN_3S$ 305.9696, found 305.9684.

9.1.3. Synthesis of 3-(amidophenyl)-6-bromoisothiazolo[4,3-b] pyridines (**6a-g** and **8a-b**)

9.1.3.1. General procedure A. To a solution of the precursor 3-(3-aminophenyl)-6-bromoisothiazolo[4,3-*b*]pyridine (5a) or 3-(4-aminophenyl)-6-bromoisothiazolo[4,3-*b*]pyridine (5b) (1 equiv) in dry dichloromethane (15 mL), dry triethylamine (2 equiv) was added. The reaction mixture was cooled at 0 °C and then the appropriated acid chloride (1.2 equiv) was added. The resulting mixture was stirred at room temperature overnight or at reflux for compounds 8a and 8b. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was purified by silica gel, yielding the corresponding compounds. The following compounds were made according to the general procedure A.

*9.1.3.1.1. 3-(3-Propylamidophenyl)-6-bromoisothiazolo[4,3-b]pyridine (**6a**).* This compound was obtained using the precursor 5a and propionyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a light yellow solid in 83% yield (98 mg, 0.27 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 1.11 (t, J = 7.5 Hz, 3H, CH_3), 2.38 (q, J = 7.5 Hz, 2H, CH_2), 7.52 (t, J = 8.0 Hz, 1H, arom H), 7.78 (d, J = 8.4 Hz, 1H, arom H), 7.89 (d, J = 7.8 Hz, 1H, arom H), 8.40–8.44 (m, 1H, arom H), 8.65 (d, J = 2.0 Hz, 1H, arom H), 8.92 (d, J = 2.0 Hz, 1H, arom H), 10.15 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{12}BrN_3OS$ 361.9958, found 361.9960.

*9.1.3.1.2. 3-(3-Acrylylamidophenyl)-6-bromoisothiazolo[4,3-b] pyridine (**6b**).* This compound was obtained using the precursor 5a and acryloyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a light yellow solid in 85% yield (100 mg, 0.28 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 5.81 (dd, J = 10.0, 2.1 Hz, 1H, H double bond), 6.31 (dd, J = 17.0, 2.1 Hz, 1H, H double bond), 6.49 (dd, J = 17.0, 10.0 Hz, 1H, H double bond), 7.56 (t, J = 8.0 Hz, 1H, arom H), 7.84–7.89 (m, 1H, arom H), 7.93 (d, J = 7.8 Hz, 1H, arom H), 8.48–8.52 (m, 1H, arom H), 8.67 (d, J = 2.1 Hz, 1H, arom H), 8.93 (d, J = 2.1 Hz, 1H, arom H), 10.46 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{10}BrN_3OS$ 359.9801, found 359.9793.

*9.1.3.1.3. 3-(3-Isobutyrylamidophenyl)-6-bromoisothiazolo[4,3-b] pyridine (**6c**).* This compound was obtained using the precursor 5a and isobutyryl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a light yellow solid in 86% yield (105.4 mg, 0.28 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 1.12 (s, 3H, CH_3), 1.14 (s, 3H, CH_3), 2.60–2.70 (m, 1H, CH), 7.52 (t, J = 7.9 Hz, 1H, arom H), 7.80 (d, J = 7.8 Hz, 1H, arom H), 7.91 (d, J = 7.1 Hz, 1H, arom H), 8.44 (bs, 1H, arom H), 8.67 (d, J = 1.9 Hz, 1H, arom H), 8.93 (d, J = 1.9 Hz, 1H, arom H), 10.14 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{14}BrN_3OS$ 376.0114, found 376.0093.

*9.1.3.1.4. 3-(3-Cyclopropylamidophenyl)-6-bromoisothiazolo[4,3-b]pyridine (**6d**).* This compound was obtained using the precursor 5a and cyclopropanecarbonyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a beige solid in 87% yield (105.9 mg, 0.28 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 0.79–0.90 (m, 4H, 2 x CH_2), 1.78–1.89 (m, 1H, CH), 7.53 (t, J = 7.9 Hz, 1H, arom H), 7.77 (d, J = 8.2 Hz, 1H, arom H), 7.90 (d, J = 7.9 Hz, 1H, arom H), 8.43 (s, 1H, arom H), 8.67 (d, J = 1.9 Hz, 1H, arom H), 8.93 (d, J = 1.8 Hz, 1H, arom H), 10.51 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{12}BrN_3OS$ 373.9958, found 373.9949.

*9.1.3.1.5. 3-(3-Cyclopentylamidophenyl)-6-bromoisothiazolo[4,3-b]pyridine (**6e**).* This compound was obtained using the precursor 5a and cyclopentanecarbonyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a beige solid in 82% yield (107.4 mg, 0.26 mmol). 1H NMR (500 MHz, DMSO) δ: 1.49–1.62 (m, 2H, CH_2), 1.63–1.81 (m, 4H, 2 x CH_2), 1.82–1.93 (m, 2H, CH_2), 2.75–2.93 (m, 1H, CH), 7.47–7.56 (m, 1H, arom H), 7.75–7.82 (m, 1H, arom H), 7.87–7.94 (m, 1H, arom H), 8.43 (s, 1H, arom H), 8.65 (s, 1H, arom H), 8.92 (s, 1H, arom H), 10.15 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{16}BrN_3OS$ 402.0271, found 402.0260.

*9.1.3.1.6. 3-(4-Propylamidophenyl)-6-bromoisothiazolo[4,3-b] pyridine (**8a**).* This compound was obtained using the precursor 5b and propionyl chloride. The reaction mixture was stirred at reflux during 6h. The crude residue was purified by silica gel flash column

chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a beige solid in 73% yield (86.3 mg, 0.24 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 1.10 (t, J = 7.5 Hz, 3H, CH_3), 2.38 (q, J = 7.5 Hz, 2H, CH_2), 7.82 (d, J = 8.6 Hz, 2H, arom H), 8.19 (d, J = 8.6 Hz, 2H, arom H), 8.62 (d, J = 1.9 Hz, 1H, arom H), 8.89 (d, J = 1.9 Hz, 1H, arom H), 10.21 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{12}BrN_3OS$ 361.9958, found 361.9955.

*9.1.3.1.7. 3-(4-Acrylylamidophenyl)-6-bromoisothiazolo[4,3-b] pyridine (**8b**).* This compound was obtained using the precursor 5b and acryloyl chloride. The reaction mixture was stirred at reflux during 6h. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and acetone (in a ratio of 8:2) as mobile phase, affording the title compound as an orange solid in 71% yield (83.3 mg, 0.23 mmol). ^{1}H NMR (300 MHz, DMSO) δ: 5.82 (dd, J = 10.0, 1.9 Hz, 1H, double bond), 6.31 (dd, J = 16.9, 1.8 Hz, 1H, double bond), 6.48 (dd, J = 16.9, 10.0 Hz, 1H, double bond), 7.89 (d, J = 8.7 Hz, 2H, arom H), 8.22 (d, J = 8.6 Hz, 2H, arom H), 8.62 (d, J = 2.0 Hz, 1H, arom H), 8.90 (d, J = 2.0 Hz, 1H, arom H), 10.49 (s, 1H, NH) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{10}BrN_3OS$ 359.9801, found 359.9797.

9.1.3.2. General procedure B. To a solution of the precursor 3-(3-aminophenyl)-6-bromoisothiazolo[4,3-*b*]pyridine 5a (1 equiv) in dry 1,4-dioxane or dry DMF (15 mL), dry triethylamine (2 equiv) and DMAP (1% w/w) were added. The reaction mixture was cooled at 0 °C and then the appropriated acid chloride (1.2 equiv) was added. The resulting mixture was stirred at 100 °C overnight. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was purified by silica gel, yielding the corresponding compounds. The following compounds were made according to the general procedure B.

*9.1.3.2.1. 3-(3-N-Pyrrolidinylamidophenyl)-6-bromoisothiazolo [4,3-b]pyridine (**6f**).* This compound was obtained using 1-pyrrolidinecarbonyl chloride and dry DMF as solvent. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 6:4) as mobile phase, affording the title compound as a light yellow solid in 73% yield (96.1 mg, 0.23 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.90–2.02 (m, 4H, 2 x CH_2), 3.43–3.54 (m, 4H, 2 x NCH_2), 6.43–6.55 (m, 1H, NH), 7.36–7.47 (m, 1H, arom H), 7.57–7.65 (m, 1H, arom H), 7.69–7.78 (m, 1H, arom H), 8.19–8.23 (m, 1H, arom H), 8.24–8.30 (m, 1H, arom H), 8.71–8.78 (m, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{15}BrN_4OS$ 403.0223, found 403.0216.

*9.1.3.2.2. 3-(3-N-Morpholinylamidophenyl)-6-bromoisothiazolo [4,3-b]pyridine (**6g**).* This compound was obtained using 4-morpholinecarbonyl chloride and dry 1.4-dioxane as solvent. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 6:4) as mobile phase, affording the title compound as a light yellow solid in 71% yield (97.1 mg, 0.23 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.46–3.54 (m, 4H, 2 x NCH_2), 3.76–3.68 (m, 4H, 2 x OCH_2), 6.74 (s, 1H, NH), 7.43–7.50 (m, 1H, arom H), 7.55–7.60 (m, 1H, arom H), 7.73–7.79 (m, 1H, arom H), 8.18 (t, J = 1.8 Hz, 1H, arom H), 8.27 (d, J = 2.0 Hz, 1H, arom H), 8.74 (d, J = 2.0 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{15}BrN_4O_2S$ 419.0172, found 419.0154.

*9.1.4. Synthesis of the Boc-protected 3-(N-piperidinyl)isothiazolo [4,3-b]pyridines (**10a-c**, **14** and **20a-b**)*

General Procedure: To a solution of 3,6-dibromoisothiazolo[4,3-*b*]pyridine (1 equiv) in absolute ethanol (20 mL) the appropriate modified piperidine (1.5 equiv) and triethylamine (1.5 equiv) were added. The resulting mixture was stirred at reflux overnight. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was purified by silica gel, yielding the corresponding compounds. The following compounds were made according to this procedure.

*9.1.4.1. tert-Butyl (1-(6-bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)carbamate (**10a**).* This compound was obtained using 3-(Boc-amino)piperidine as a racemic mixture. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 91% yield (127.8 mg, 0.37 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.45 (s, 9H, 3 x CH_3), 1.66–2.05 (m, 4H, 2 x CH_2), 3.69–3.85 (m, 2H, CH_2), 3.85–3.97 (m, 1H, CH), 3.97–4.12 (m, 2H, CH_2), 4.71–4.85 (m, 1H, NH), 7.92 (d, J = 2.1 Hz, 1H, arom H), 8.28 (d, J = 2.1 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{21}BrN_4O_2S$ 413.0642, found 413.0609.

*9.1.4.2. tert-Butyl ((1-(6-bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)carbamate (**10b**).* This compound was obtained using 3-(Boc-aminomethyl)piperidine as a racemic mixture. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 90% yield (130.4 mg, 0.31 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (s, 9H, 3 x CH_3), 1.67–1.82 (m, 2H, CH_2), 1.82–1.94 (m, 2H, CH_2), 1.95–2.05 (m, 1H, CH), 3.06–3.37 (m, 3H, CH, CH_2), 3.58–3.70 (m, 1H, CH), 4.09–4.29 (m, 2H, CH_2), 5.30–5.39 (m, 1H, NH), 7.91 (d, J = 2.0 Hz, 1H, arom H), 8.27 (d, J = 1.7 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{23}BrN_4O_2S$ 427.0798, found 427.0793.

*9.1.4.3. tert-Butyl (2-(1-(6-bromoisothiazolo[4,3-b]pyridin-3-yl) piperidin-3-yl)ethyl)carbamate (**10c**).* This compound was obtained using 3-(2-Boc-aminoethyl)piperidine as a racemic mixture. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 87% yield (130.5 mg, 0.29 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.17–1.42 (m, 2H, CH_2), 1.45 (s, 9H, 3 x CH_3), 1.49–1.58 (m, 2H, CH_2), 1.74–2.01 (m, 3H, CH_2, CH), 2.96–3.09 (m, 1H, CH), 3.15–3.37 (m, 3H, CH_2, CH), 4.18–4.30 (m, 1H, CH), 4.71–4.82 (m, 1H, CH), 4.91–5.02 (m, 1H, NH), 7.90 (d, J = 2.0 Hz, 1H, arom H), 8.29 (d, J = 2.0 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{25}BrN_4O_2S$ 441.0955, found 441.0946.

*9.1.4.4. tert-Butyl ((1-(6-bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-4-yl)methyl)carbamate (**14**).* This compound was obtained using 4-(Boc-aminomethyl)piperidine as a racemic mixture. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 89% yield (129.3 mg, 0.30 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.37–1.54 (m, 11H, tBuO, CH_2), 1.83–1.93 (m, 3H, CH_2, CH), 3.08 (t, J = 6.2 Hz, 2H, CH_2), 3.19 (td, J = 12.7, 2.4 Hz, 2H, CH_2), 4.63–4.72 (m, 3H, CH_2, NH), 7.91 (d, J = 2.1 Hz, 1H, arom H), 8.27 (d, J = 2.1 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{23}BrN_4O_2S$ 427.0798, found 427.0620.

*9.1.4.5. tert-Butyl (S)-((1-(6-bromoisothiazolo[4,3-b]pyridin-3-yl) piperidin-3-yl)methyl)carbamate (**20a**).* This compound was obtained using (*R*)-3-(Boc-aminomethyl)piperidine. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 89% yield (129.2 mg, 0.30 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.48 (s, 9H, 3 x CH_3), 1.70–1.84 (m, 1H, CH), 1.84–2.09 (m, 4H, 2 x CH_2), 3.08–3.40 (m, 3H, CH, CH_2), 3.58–3.73 (m, 1H, CH), 4.11–4.31 (m, 2H, CH_2), 5.34–5.44 (m, 1H, NH), 7.91 (d, J = 2.0 Hz, 1H, arom H), 8.28 (bs, 1H,

arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{17}H_{23}BrN_4O_2S$ 427.0798, found 427.0790.

*9.1.4.6. tert-Butyl (R)-((1-(6-bromoisothiazolo[4,3-b]pyridin-3-yl) piperidin-3-yl)methyl)carbamate (**20b**).* This compound was obtained using (*S*)-3-(Boc-aminomethyl)piperidine. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 87% yield (126.4 mg, 0.29 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (s, 9H, 3 x CH_3), 1.68–1.82 (m, 1H, CH), 1.84–2.07 (m, 4H, 2 x CH_2), 3.07–3.38 (m, 3H, CH, CH_2), 3.57–3.70 (m, 1H, CH), 4.10–4.31 (m, 2H, CH_2), 5.37–5.49 (m, 1H, NH), 7.91 (d, $J = 2.0$ Hz, 1H, arom H), 8.27 (bs, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{17}H_{23}BrN_4O_2S$ 427.0798, found 427.0790.

*9.1.5. Acidic removal of Boc-protecting group (**11a-c**, **15** and **21a-b**)*

General Procedure: To a solution of the corresponding Boc-amino precursor 10a-c, 14 and 20a-b (1 equiv) in 1,4-dioxane (5 mL) at 0 °C, hydrogen chloride solution 4.0 M in 1,4-dioxane (10 mL) was added slowly. The resulting mixture was stirred at room temperature for 4–5 h. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was co-evaporated several times with dichloromethane and precipitate with a mixture of dichloromethane and diethyl ether (in a ratio 1:4), yielding the hydrochloric salt of the corresponding amino derivative. The following compounds were made according to this procedure.

*9.1.5.1. 1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-ammonium chloride (**11a**).* This compound was obtained using the Boc-amino precursor 10a, affording the title compound as an orange solid in 96% yield (81.2 mg, 0.23 mmol). ^{1}H NMR (300 MHz, DMSO) *δ:* 1.64–1.81 (m, 2H, CH_2), 1.85–1.99 (m, 1H, CH), 2.02–2.13 (m, 1H, CH), 3.35–3.56 (m, 2H, CH_2), 3.35–3.56 (dd, $J = 12.7$, 9.2 Hz, 1H, CH), 4.19–4.29 (m, 1H, CH), 4.51–4.62 (m, 1H, CH), 8.17 (d, $J = 1.8$ Hz, 1H, arom H), 8.38 (d, $J = 1.8$ Hz, 1H, arom H), 8.43 (bs, 3H, $^+NH_3$) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{11}H_{13}BrN_4S$ 313.0118, found 313.0138.

*9.1.5.2. (1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl) methanammonium chloride (**11b**).* This compound was obtained using the Boc-amino precursor 10b, affording the title compound as an orange solid in 94% yield (80 mg, 0.22 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 1.25–1.46 (m, 1H, CH), 1.48–1.73 (m, 1H, CH), 1.75–1.97 (m, 2H, CH_2), 1.97–2.15 (m, 1H, CH), 2.65–2.93 (m, 2H, CH_2), 3.14–3.40 (m, 2H, CH_2), 4.17–4.34 (m, 1H, CH), 4.52–4.69 (m, 1H, CH), 8.08 (s, 1H, arom H), 8.34 (s, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{15}BrN_4S$ 327.0274, found 327.0280.

*9.1.5.3. 2-(1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl) ethan-1-ammonium chloride (**11c**).* This compound was obtained using the Boc-amino precursor 10c, affording the title compound as an orange solid in 90% yield (77.1 mg, 0.20 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 1.16–1.39 (m, 1H, CH), 1.47–1.69 (m, 3H, CH_2, CH), 1.72–1.93 (m, 3H, CH_2, CH), 2.76–3.00 (m, 2H, CH_2), 3.19–3.41 (m, 2H, CH_2), 4.27–4.42 (m, 2H, CH_2), 7.76–7.98 (m, 3H, $^+NH_3$), 8.07 (s, 1H, arom H), 8.33 (s, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{13}H_{17}BrN_4S$ 341.0431, found 341.0420.

*9.1.5.4. 2-(1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl) ethan-1-ammonium chloride (**15**).* This compound was obtained using the Boc-amino precursor 14, affording the title compound as an orange solid in 93% yield (79.1 mg, 0.22 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 1.34–1.51 (m, 2H, CH_2). 1.87–2.04 (m, 3H, CH_2, CH), 2.68–2.81 (m, 2H, CH_2), 3.31 (t, $J = 11.8$ Hz, 2H, CH_2), 4.52–4.62 (m, 2H, CH_2), 8.11 (d, $J = 2.0$ Hz, 1H, arom H), 8.18 (bs, 3H, $^+NH_3$), 8.36 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{15}BrN_4S$ 327.0274, found 327.0241.

*9.1.5.5. (S)-(1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methanammonium chloride (**21a**).* This compound was obtained using the (*S*)-Boc-amino precursor 20a, affording the title compound as an orange solid in 92% yield (70.1 mg, 0.21 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 1.27–1.49 (m, 1H, CH), 1.48–1.76 (m, 1H, CH), 1.77–2.01 (m, 2H, CH_2), 2.02–2.16 (m, 1H, CH), 2.73–2.94 (m, 2H, CH_2), 3.19–3.38 (m, 2H, CH_2), 4.19–4.28 (m, 1H, CH), 4.65–4.76 (m, 1H, CH), 8.12 (d, $J = 2.0$ Hz, 1H, arom H), 8.19 (bs, 3H, $^+NH_3$), 8.36 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{15}BrN_4S$ 327.0274, found 327.0276.

*9.1.5.6. (R)-(1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methanammonium chloride (**21b**).* This compound was obtained using the (*R*)-Boc-amino precursor 20b, affording the title compound as an orange solid in 89% yield (68.1 mg, 0.21 mmol.). ^{1}H NMR (300 MHz, DMSO) *δ*: 1.28–1.47 (m, 1H, CH), 1.50–1.73 (m, 1H, CH), 1.72–1.98 (m, 2H, CH_2), 1.98–2.13 (m, 1H, CH), 2.69–2.91 (m, 2H, CH_2), 3.18–3.37 (m, 2H, CH_2), 4.23–4.33 (m, 1H, CH), 4.64–4.49 (m, 1H, CH), 8.06 (d, $J = 2.0$ Hz, 1H, arom H), 8.34 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{12}H_{15}BrN_4S$ 327.0271, found 327.0276.

*9.1.6. Synthesis of 3-amidopiperidinyl-6-bromoisothiazolo[4,3-b] pyridines (**12a-d**, **16a-b**, **18a-h** and **23a-b**)*

General procedure: To a solution of the precursor 3-(*N*-aminopiperidinyl)-6-bromoisothiazolo[4,3-*b*]pyridine (1 equiv) in dry dichloromethane (15 mL), dry triethylamine (2 equiv) was added. The reaction mixture was cooled at 0 °C and then the appropriated acid chloride, sulfonyl chloride or isocyanate (1.2 equiv) was added. The resulting mixture was stirred at room temperature overnight or at 35 °C for compounds 18e, 18f and 18g. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was purified by silica gel, yielding the corresponding compounds. The following compounds were made according to this procedure.

*9.1.6.1. N-(1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl) propionamide (**12a**).* This compound was obtained using the precursor 11a and propionyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 83% yield (87.6 mg, 0.24 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ:* 1.12 (t, $J = 7.6$ Hz, 3H, CH_3), 1.73–1.98 (m, 4H, 2 x CH_2), 2.19 (q, $J = 7.6$ Hz, 2H, CH_2), 3.70–3.83 (m, 1H, CH), 3.85–4.04 (m, 3H, CH, CH_2), 4.17–4.27 (m, 1H, CH), 5.73–5.85 (m, 1H, NH), 7.91 (d, $J = 2.0$ Hz, 1H, arom H), 8.28 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{14}H_{17}BrN_4OS$ 369.0380, found 369.0374.

*9.1.6.2. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl) methyl)propionamide (**12b**).* This compound was obtained using the precursor 11b and propionyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase, affording the title compound as a yellow solid in 81% yield (85.3 mg, 0.22 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 1.22 (t, $J = 7.6$ Hz, 3H, CH_3), 1.41–1.56 (m, 1H, CH), 1.67–1.84 (m, 2H, CH_2), 1.84–1.95 (m, 1H, CH), 1.97–2.09 (m, 1H, CH), 2.28 (q, $J = 7.6$ Hz, 2H, CH_2), 3.13–3.26 (m, 1H, CH), 3.28–3.49 (m, 2H, CH_2), 3.74 (dd, $J = 13.0$, 7.9 Hz, 1H, CH), 3.90–4.03 (m, 1H, CH), 4.29 (dd, $J = 13.0$,

3.4 Hz, 1H, CH), 6.19–6.27 (m, 1H, NH), 7.92 (d, $J = 2.0$ Hz, 1H, arom H), 8.24 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{19}BrN_4OS$ 383.0536, found 383.0525

*9.1.6.3. N-(2-(1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)ethyl)propionamide (**12c**).* This compound was obtained using the precursor 11c and propionyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 75% yield (79.0 mg, 0.20 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.15 (t, $J = 7.6$ Hz, 3H, CH_3), 1.28–1.38 (m, 1H, CH), 1.55 (q, $J = 7.1$ Hz, 2H, CH_2), 1.74–1.91 (m, 3H, CH_2, CH), 1.93–2.01 (m, 1H, CH), 2.19 (q, $J = 7.6$ Hz, 2H, CH_2), 3.07 (dd, $J = 12.8$, 10.3 Hz, 1H, CH), 3.25–3.45 (m, 3H, CH_2, CH), 4.23–4.29 (m, 1H, CH), 4.70–4.76 (m, 1H, CH), 5.69 (bs, 1H, NH), 7.90 (d, $J = 2.1$ Hz, 1H, arom H), 8.26 (d, $J = 2.1$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{21}BrN_4OS$ 397.0693, found 397.0679.

*9.1.6.4. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)acrylamide (**12d**).* This compound was obtained using the precursor 11b and acryloyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase, affording the title compound as a yellow solid in 81% yield (84.9 mg, 0.22 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.43–1.60 (m, 1H, CH), 1.66–1.82 (m, 1H, CH), 1.82–1.98 (m, 2H, CH_2), 1.98–2.15 (m, 1H, CH), 3.16–3.39 (m, 2H, CH_2), 3.42–3.60 (m, 1H, CH), 3.71–3.86 (m, 1H, CH), 3.88–4.02 (m, 1H, CH), 4.23 (dd, $J = 13.1$, 3.3 Hz, 1H, CH), 5.70 (dd, $J = 10.0$, 1.5 Hz, 1H, double bond), 6.17 (dd, $J = 17.0$, 10.0 Hz, 1H, double bond), 6.32 (dd, $J = 17.0$, 1.5 Hz, 1H, double bond), 6.64 (bs, 1H, NH), 7.90 (d, $J = 1.9$ Hz, 1H, arom H), 8.23 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{17}BrN_4OS$ 381.0380, found 381.0375.

*9.1.6.5. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-4-yl)methyl)propionamide (**16a**).* This compound was obtained using the precursor 15 and propionyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.1) as mobile phase, affording the title compound as a yellow solid in 92% yield (95.8 mg, 0.25 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.18 (t, $J = 7.6$ Hz, 3H, CH_3), 1.38–1.57 (m, 2H, CH_2), 1.82–1.99 (m, 3H, CH, CH_2), 2.24 (q, $J = 7.6$ Hz, 2H, CH_2), 3.08–3.27 (m, 4H, 2 x CH_2), 4.57–4.78 (m, 2H, CH_2), 5.53–5.69 (m, 1H, NH), 7.91 (d, $J = 2.1$ Hz, 1H, arom H), 8.27 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{19}BrN_4OS$ 383.0536, found 383.0534.

*9.1.6.6. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-4-yl)methyl)acrylamide (**16b**).* This compound was obtained using the precursor 15 and acryloyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase, affording the title compound as a yellow solid in 89% yield (93.2 mg, 0.24 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.42–1.59 (m, 2H, CH_2). 1.85–1.99 (m, 2H, CH_2), 3.20 (td, $J = 12.7$, 2.4 Hz, 2H, CH_2), 3.30 (t, $J = 6.3$ Hz, 2H, CH_2), 4.68 (d, $J = 12.8$ Hz, 2H, CH_2), 5.68 (dd, $J = 10.2$, 1.4 Hz, 1H, double bond), 5.72–5.81 (m, 1H, NH), 6.10 (dd, $J = 16.9$, 10.2 Hz, 1H, double bond), 6.31 (dd, $J = 16.9$, 1.4 Hz, 1H, double bond), 7.90 (d, $J = 2.1$ Hz, 1H, arom H), 8.26 (d, $J = 2.1$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{17}BrN_4OS$ 381.0380, found 381.0372.

*9.1.6.7. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)-3-methylbutanamide (**18a**).* This compound was obtained using the precursor 11b and isovaleryl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 77% yield (86.9 mg, 0.21 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.99 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.43–1.58 (m, 1H, CH), 1.70–1.84 (m, 1H, CH), 1.85–1.96 (m, 2H, CH_2), 1.99–2.21 (m, 4H, 2 x CH_2), 3.14–3.26 (m, 1H, CH), 3.29–3.40 (m, 1H, CH), 3.41–3.54 (m, 1H, CH), 3.80 (dd, $J = 13.1$, 7.9 Hz, 1H, CH), 3.95–4.10 (m, 1H, CH), 4.24 (dd, $J = 13.2$, 3.6 Hz, 1H, CH), 6.17–6.25 (m, 1H, NH), 7.94 (d, $J = 2.1$ Hz, 1H, arom H), 8.26 (d, $J = 2.1$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{23}BrN_4OS$ 411.0849, found 411.0849.

9.1.6.8. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanecarboxamide (18b). This compound was obtained using the precursor 11b and cyclopropanecarbonyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 87% yield (94.4 mg, 0.24 mmol). 1H NMR (500 MHz, CDCl3) δ: 0.76–0.84 (m, 2H, CH2), 0.98–1.04 (m, 2H, CH2), 1.39–1.45 (m, 1H, CH), 1.48–1.57 (m, 1H, CH), 1.72–1.84 (m, 1H, CH), 1.87–1.96 (m, 2H, CH2), 1.99–2.09 (m, 1H, CH), 3.17–3.26 (m, 1H, CH), 3.32–3.40 (m, 1H, CH), 3.41–3.51 (m, 1H, CH), 3.79 (dd, $J = 13.0$, 7.9 Hz, 1H, CH), 3.97–4.07 (m, 1H, CH), 4.28 (dd, $J = 13.0$, 3.5 Hz, 1H, CH), 6.31–3.37 (m, 1H, NH), 7.94 (d, $J = 2.1$ Hz, 1H, arom H), 8.27 (d, $J = 2.1$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{19}BrN_4OS$ 395.0536, found 395.0528.

*9.1.6.9. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopentanecarboxamide (**18c**).* This compound was obtained using the precursor 11b and cyclopentanecarbonyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 69% yield (80.1 mg, 0.19 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.38–2.09 (m, 12H, 6 x CH_2), 2.51–2.66 (m, 1H, CH), 3.12–3.48 (m, 4H, 2 x CH_2), 3.64 (dd, $J = 13.0$, 8.4 Hz, 1H, CH), 3.97–4.19 (m, 1H, CH), 4.34 (dd, $J = 13.0$, 3.3 Hz, 1H, CH), 6.11–6.23 (m, 1H, NH), 7.92 (d, $J = 2.0$ Hz, 1H, arom H), 8.23 (d, $J = 2.0$ Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{18}H_{23}BrN_4OS$ 423.0849, found 423.0841.

*9.1.6.10. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)benzamide (**18d**).* This compound was obtained using the precursor 11b and benzoyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 88% yield (104.1 mg, 0.24 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.58–1.71 (m, 1H, CH), 1.73–1.87 (m, 1H, CH), 1.89–2.02 (m, 2H, CH_2), 2.11–2.24 (m, 1H, CH), 3.35–3.47 (m, 2H, CH_2), 3.64–3.89 (m, 2H, CH_2), 4.08 (dd, $J = 12.9$, 6.9 Hz, 1H, CH), 4.32 (dd, $J = 13.2$, 3.0 Hz, 1H, CH), 6.98–7.06 (m, 1H, NH), 7.45–7.62 (m, 3H, arom H), 7.80–7.88 (m, 2H, arom H), 7.92 (d, $J = 2.0$ Hz, 1H, arom H), 8.06 (d, $J = 2.0$ Hz, 1H, arom H). HR-MS m/z $[M+H]^+$ calcd for $C_{19}H_{19}BrN_4OS$ 431.0536, found 431.0524.

*9.1.6.11. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)pyrrolidine-1-carboxamide (**18e**).* This compound was obtained using the precursor 11b and 1-pyrrolidinecarbonyl chloride. The reaction was stirred at 35 °C overnight. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase, affording the title compound as a yellow solid in 63% yield (73.3 mg, 0.17 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.40–1.50

(m, 1H, CH), 1.71–1.84 (m, 1H, CH), 1.85–1.93 (m, 2H, CH_2), 1.93–1.98 (m, 4H, 2 x CH_2), 1.99–2.07 (m, 1H, CH), 3.15–3.22 (m, 1H, CH), 3.33–3.42 (m, 6H, 3 x CH_2), 3.55 (dd, J = 13.0, 8.8 Hz, 1H, CH), 4.05–4.23 (m, 1H, CH), 4.41 (dd, J = 12.9, 3.6 Hz, 1H, CH), 4.62 (t, J = 5.8 Hz, 1H, NH), 7.90 (d, J = 2.1 Hz, 1H, arom H), 8.20 (d, J = 2.1 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{22}BrN_5OS$ 424.0802, found 424.0776.

*9.1.6.12. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)morpholine-4-carboxamide (**18f**).* This compound was obtained using the precursor 11b and 4-morpholinecarbonyl chloride. The reaction was stirred at 35 °C overnight. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase, affording the title compound as a yellow solid in 77% yield (93.2 mg, 0.21 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) *δ:* 1.41–1.53 (m, 1H, CH), 1.72–1.81 (m, 1H, CH), 1.87–1.95 (m, 2H, CH_2), 2.00–2.07 (m, 1H, CH), 3.15–3.24 (m, 1H, CH), 3.32–3.47 (m, 6H, 3 x CH_2), 3.65 (dd, J = 13.0, 8.3 Hz, 1H, CH), 3.69–3.76 (m, 4H, 2 x CH_2), 3.99–4.09 (m, 1H, CH), 4.36 (dd, J = 13.1, 3.3 Hz, 1H, CH), 5.02 (t, J = 5.7 Hz, 1H, NH), 7.91 (d, J = 2.1 Hz, 1H, arom H), 8.18 (d, J = 2.0 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{17}H_{22}BrN_5O_2S$ 440.0751, found 440.0741.

*9.1.6.13. N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanesulfonamide (**18 g**).* This compound was obtained using the precursor 11b and cyclopropanesulfonyl chloride. The reaction was stirred at 35 °C overnight. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 71% yield (84.1 mg, 0.19 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ:* 0.86–1.03 (m, 2H, CH_2), 1.07–1.24 (m, 2H, CH_2), 1.58–1.71 (m, 1H, CH), 1.74–1.85 (m, 1H, CH), 1.87–2.01 (m, 2H, CH_2), 2.13–2.22 (m, 1H, CH), 2.31–2.45 (m, 1H, CH), 3.20–3.46 (m, 3H, CH_2, CH), 3.66–3.80 (m, 1H, CH), 3.85–3.98 (m, 1H, CH), 4.23–4.39 (m, 1H, CH), 6.19–6.34 (m, 1H, NH), 8.36 (d, J = 2.0 Hz, 1H, arom H).7.97 (d, J = 2.0 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{15}H_{19}BrN_4O_2S_2$ 431.0206, found 431.0208.

*9.1.6.14. 1-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)-3-cyclopropylurea (**18h**).* This compound was obtained using the precursor 11b and cyclopropyl isocyanate. The crude residue was purified by flash chromatography using a mixture of hexane and acetone (in a ratio of 3:2) as mobile phase, affording the title compound as a yellow solid in 79% yield (89.1 mg, 0.22 mmol). ^{1}H NMR (300 MHz, DMSO) *δ*: 0.29–0.43 (m, 2H, CH_2), 0.50–0.69 (m, 2H, CH_2), 1.14–1.39 (m, 1H, CH), 1.51–1.71 (m, 1H, CH), 1.71–1.95 (m, 3H, CH_2, CH), 2.34–2.43 (m, 1H, CH), 2.99 (t, J = 6.2 Hz, 2H, CH_2), 3.05–3.20 (m, 1H, CH), 4.24–4.34 (m, 1H, CH), 4.49–4.60 (m, 1H, CH), 6.03–6.10 (m, 1H, NH), 6.20 (bs, 1H, NH), 8.08 (d, J = 2.1 Hz, 1H, arom H), 8.31 (d, J = 2.1 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{20}BrN_5OS$ 410.0645, found 410.0641.

*9.1.6.15. (S)-N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanecarboxamide (**22a**).* This compound was obtained using the precursor 21a and cyclopropanecarbonyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 84% yield (91.3 mg, 0.23 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.74–0.86 (m, 2H, CH_2), 0.94–1.05 (m, 2H, CH_2), 1.36–1.46 (m, 1H, CH), 1.46–1.58 (m, 1H, CH), 1.70–1.84 (m, 1H, CH), 1.83–1.98 (m, 2H, CH_2), 1.97–2.10 (m, 1H, CH), 3.16–3.28 (m, 1H, CH), 3.30–3.41 (m, 1H, CH), 3.41–3.52 (m, 1H, CH), 3.80 (dd, J = 13.0, 7.9 Hz, 1H, CH), 3.94–4.08 (m, 1H, CH), 4.27 (dd, J = 12.7, 3.2 Hz, 1H, CH), 6.29–6.42 (m, 1H, NH), 7.94 (d, J = 2.0 Hz, 1H, arom H), 8.27 (d, J = 2.0 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{19}BrN_4OS$ 395.0536, found 395.0653.

*9.1.6.16. (R)-N-((1-(6-Bromoisothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanecarboxamide (**22b**).* This compound was obtained using the precursor 21b and cyclopropanecarbonyl chloride. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as mobile phase, affording the title compound as a yellow solid in 82% yield (89.1 mg, 0.22 mmol). ^{1}H NMR (300 MHz, $CDCl_3$) *δ*: 0.72–0.84 (m, 2H, CH_2), 0.96–1.06 (m, 2H, CH_2), 1.36–1.46 (m, 1H, CH), 1.46–1.62 (m, 1H, CH), 1.67–1.84 (m, 1H, CH), 1.85–1.98 (m, 2H, CH_2), 1.98–2.14 (m, 1H, CH), 3.16–3.28 (m, 1H, CH), 3.30–3.53 (m, 2H, CH_2), 3.79 (dd, J = 13.0, 7.9 Hz, 1H, CH), 3.94–4.08 (m, 1H, CH), 4.27 (dd, J = 12.7, 3.2 Hz, 1H, CH), 6.29–6.42 (m, 1H, NH), 7.93 (d, J = 2.0 Hz, 1H, arom H), 8.26 (d, J = 2.0 Hz, 1H, arom H) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{16}H_{19}BrN_4OS$ 395.0536, found 395.0528.

*9.1.7. Suzuki reaction at 6 position of isothiazolo[4,3-b]pyridines (**7a-g**, **9a-b**, **13a-d**, **17a-b**, **19a-h** and **23a-b**)*

General Procedure: A solution of the appropriate 3-aryl- or 3-piperidinyl-6-bromoisothiazolo[4,3-*b*]pyridines (1 equiv) in a mixture of 1,4-dioxane/water (ratio 9:1) was degassed with argon and subsequently 4-amino-3-methoxyphenylboronic acid pinacol ester (1.2 equiv), $Pd(PPh_3)_4$ (0.02 equiv) and K_2CO_3 (2 equiv) were added. The mixture was degassed a second time, filled with argon and stirred at 85 °C overnight. After completion of the reaction as monitored by TLC, the volatiles were evaporated to dryness and the resulting residue was purified by silica gel and subsequently, was precipitated with diethyl ether yielding the corresponding compounds. The following compounds were made according to this general procedure.

*9.1.7.1. N-(3-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)propionamide (**7a**).* This compound was obtained using the precursor 6a. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 3:2) as a mobile phase, affording the title compound as an orange solid in 82% yield (45.5 mg, 0.11 mmol). Mp = 119.1–121.3 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 1.27 (t, J = 7.6 Hz, 3H, CH_3), 2.43 (q, J = 7.5 Hz, 2H, CH_2), 3.95 (s, 3H, OCH_3), 4.04 (bs, 2H, NH_2), 6.83 (d, J = 8.0, 1H, arom H), 7.12 (d, J = 1.9 Hz, 1H, arom H), 7.17 (dd, J = 8.0, 2.0 Hz, 1H, arom H), 7.48 (t, J = 7.9 Hz, 1H, arom H), 7.53 (bs, 1H, NH), 7.79 (d, J = 8.0 Hz, 1H, arom H), 7.83 (d, J = 7.7 Hz, 1H, arom H), 8.11 (d, J = 2.2 Hz, 1H, arom H), 8.39 (s, 1H, arom H), 9.09 (d, J = 2.2 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) *δ:* 9.6 (CH_3), 30.8 (CH_2), 55.6 (OCH_3), 109.3 (CH), 115.0 (CH), 119.1 (CH), 120.5 (CH), 124.0 (CH), 121.0 (CH), 124.2 (CH), 126.9 (C), 129.9 (CH), 131.0 (C), 136.1 (C), 137.3 (C), 138.9 (C), 143.1 (C), 147.7 (C), 151.6 (CH), 157.1 (C), 161.5 (C), 172.2 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{20}N_4O_2S$ 405.1380, found 405.1372.

*9.1.7.2. N-(3-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)acrylamide (**7b**).* This compound was obtained using the precursor 6b. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as a mobile phase and a second time using dichloromethane and methanol (10:0.1), affording the title compound as an orange solid in 83% yield (46.3 mg, 0.12 mmol). Mp = 159.2–161.3 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 3.96 (s, J = 9.4 Hz, 3H, OCH_3), 4.04 (bs, 2H, NH_2), 5.82 (dd, J = 10.2, 1.1 Hz, 1H, double bond), 6.29 (dd, J = 16.8, 10.2 Hz, 1H, double bond), 6.49 (dd, J = 16.8, 1.1 Hz, 1H, double bond), 6.84 (d, J = 8.0 Hz, 1H, arom

H), 7.13 (d, $J = 1.9$ Hz, 1H, arom H), 7.19 (dd, $J = 8.0$, 2.0 Hz, 1H, arom H), 7.51 (t, $J = 8.0$ Hz, 1H, arom H), 7.54 (bs, 1H, NH), 7.82–7.90 (m, 2H, arom H), 8.13 (d, $J = 2.1$ Hz, 1H, arom H), 8.46 (s, 1H, arom H), 9.11 (d, $J = 2.1$ Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) δ: 55.6 (OCH_3), 109.3 (CH), 115.1 (CH), 119.3 (CH), 120.5 (CH), 121.2 (CH), 124.2 (CH), 124.4 (CH), 126.9 (C), 128.3 (CH_2 double bond), 130.1 (CH), 131.0 (CH double bond), 131.2 (C), 136.1 (C), 137.3 (C), 138.6 (C), 143.2 (C), 147.7 (C), 151.7 (CH), 157.1 (C), 161.3 (C), 163.5 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{18}N_4O_2S$ 403.1223, found 403.1216.

*9.1.7.3. N-(3-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)isobutyramide (**7c**).* This compound was obtained using the precursor 6c. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as a mobile phase, affording the title compound as an orange solid in 78% yield (43.1 mg, 0.10 mmol). Mp = 203.1–204.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.28 (s, 3H, CH_3), 1.29 (s, 3H, CH_3), 2.50–2.60 (m, 1H, CH), 3.95 (s, 3H, OCH_3), 4.04 (bs, 2H, NH_2), 6.83 (d, $J = 8.0$ Hz, 1H, arom H), 7.12 (d, $J = 1.4$ Hz, 1H, arom H), 7.18 (dd, $J = 8.0$, 1.7 Hz, 1H, arom H), 7.46–7.51 (m, 2H, arom H, NH), 7.79 (d, $J = 7.8$ Hz, 1H, arom H), 7.85 (d, $J = 7.7$ Hz, 1H, arom H), 8.11 (d, $J = 2.0$ Hz, 1H, arom H), 8.41 (s, 1H, arom H), 9.09 (d, $J = 2.0$ Hz, 1H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ: 19.6 (CH_3), 36.8 (CH), 55.6 (OCH_3), 109.3 (CH), 115.0 (CH), 119.1 (CH), 120.5 (CH), 121.0 (CH), 124.0 (CH), 124.2 (CH), 126.9 (C), 129.9 (CH), 131.0 (C), 136.0 (C), 137.3 (C), 139.0 (C), 143.1 (C), 147.7 (C), 151.6 (CH), 157.1 (C), 161.5 (C), 175.4 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{23}H_{22}N_4O_2S$ 419.1536, found 419.1523.

*9.1.7.4. N-(3-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)cyclopropanecarboxamide (**7d**).* This compound was obtained using the precursor 6d. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as a mobile phase, affording the title compound as an orange solid in 79% yield (43.7 mg, 0.11 mmol). Mp = 175.3–177.1 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.83–0.90 (m, 2H, CH_2), 1.10–1.15 (m, 2H, CH_2), 1.50–1.58 (m, 1H, CH), 3.95 (s, 3H, OCH_3), 4.03 (bs, 2H, NH_2), 6.84 (d, $J = 8.0$ Hz, 1H, arom H), 7.13 (d, $J = 1.7$ Hz, 1H, arom H), 7.18 (dd, $J = 8.0$, 1.8 Hz, 1H, arom H), 7.47 (t, $J = 8.0$ Hz, 1H, arom H), 7.69–7.78 (m, 2H, arom H, NH), 7.84 (d, $J = 7.6$ Hz, 1H, arom H), 8.11 (d, $J = 2.1$ Hz, 1H, arom H), 8.41 (s, 1H, arom H), 9.09 (d, $J = 2.1$ Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ: 8.1 (CH_2), 15.9 (CH), 55.6 (OCH_3), 109.3 (CH), 115.0 (CH), 119.0 (CH), 120.4 (CH), 120.9 (CH), 123.9 (CH), 124.2 (CH), 126.9 (C), 129.9 (CH), 131.0 (C), 136.1 (C), 137.3 (C), 139.0 (C), 143.1 (C), 147.7 (C), 151.6 (CH), 157.1 (C), 161.5 (C), 172.1 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{23}H_{20}N_4O_2S$ 417.1380, found 417.1373.

*9.1.7.5. N-(3-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)cyclopentanecarboxamide (**7e**).* This compound was obtained using the precursor 6e. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 4:1) as a mobile phase, affording the title compound as a yellow solid in 83% yield (45.6 mg, 0.10 mmol). Mp = 219.2–221.7 °C. ^{1}H NMR (500 MHz, DMSO) δ: 1.52–1.61 (m, 2H, CH_2), 1.64–1.82 (m, 4H, 2 x CH_2), 1.82–1.95 (m, 2H, CH_2), 2.84 (p, $J = 8.0$ Hz, 1H, CH), 3.92 (s, 3H, OCH_3), 5.17 (s, 2H, NH_2), 6.78 (d, $J = 8.1$ Hz, 1H, arom H), 7.33 (dd, $J = 8.1$, 1.9 Hz, 1H, arom H), 7.37 (d, $J = 1.8$ Hz, 1H, arom H), 7.51 (t, $J = 8.0$ Hz, 1H, arom H), 7.77 (dd, $J = 8.3$, 0.9 Hz, 1H, arom H), 7.98 (d, $J = 8.0$ Hz, 1H, arom H), 8.31 (d, $J = 2.1$ Hz, 1H, arom H), 8.48–8.51 (m, 1H, arom H), 9.27 (d, $J = 2.1$ Hz, 1H, arom H), 10.13 (s, 1H, NH) ppm. ^{13}C NMR (126 MHz, DMSO) δ: 25.8 (CH_2), 30.2 (CH_2), 45.4 (CH), 55.6 (OCH_3), 109.6 (CH), 113.9 (CH), 118.4 (CH), 120.5 (CH), 120.7 (CH), 122.4 (CH), 122.7 (CH), 123.4 (C), 129.7 (CH), 130.3 (C), 135.7 (C), 139.2 (C), 140.3 (C), 142.3 (C), 146.9 (C), 151.9 (CH), 157.1 (C), 160.9 (C), 174.8 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{25}H_{24}N_4O_2S$ 445.1693, found 445.1682.

*9.1.7.6. N-(3-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)pyrrolidine-1-carboxamide (**7f**).* This compound was obtained using the precursor 6f. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 6:4) as a mobile phase, affording the title compound as a yellow solid in 75% yield (41.4 mg, 0.08 mmol). Mp = 160.5–162.6 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.95–1.99 (m, 4H, 2 x CH_2), 3.47–3.52 (m, 4H, 2 x NCH_2), 3.95 (s, 3H, OCH_3), 4.04 (bs, 2H, NH_2), 6.48 (s, 1H, NH), 6.83 (d, $J = 8.0$ Hz, 1H, arom H), 7.12 (d, $J = 1.8$ Hz, 1H, arom H), 7.17 (dd, $J = 8.0$, 1.9 Hz, 1H, arom H), 7.45 (t, $J = 7.9$ Hz, 1H, arom H), 7.68 (dd, $J = 8.2$, 1.3 Hz, 1H, arom H), 7.79 (d, $J = 7.8$ Hz, 1H, arom H), 8.10 (d, $J = 2.1$ Hz, 1H, arom H), 8.26–8.28 (m, 1H, arom H), 9.08 (d, $J = 2.1$ Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ: 25.6 (CH_2), 45.8 (CH_2), 55.6 (OCH_3), 109.3 (CH), 115.0 (CH), 118.8 (CH), 120.4 (CH), 120.9 (CH), 122.6 (CH), 124.1 (CH), 126.9 (C), 129.7 (CH), 130.8 (C), 135.9 (C), 137.2 (C), 140.2 (C), 143.1 (C), 147.7 (C), 151.4 (CH), 153.8 (C), 157.0 (C), 162.0 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{24}H_{23}N_5O_2S$ 446.1645, found 446.1633.

*9.1.7.7. N-(3-(6-(4-Amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)morpholine-4-carboxamide (**7g**).* This compound was obtained using the precursor 6 g. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 6:4) as a mobile phase, affording the title compound as an orange solid in 71% yield (39.0 mg, 0.08 mmol). Mp = 227.1–228.9 °C. ^{1}H NMR (500 MHz, DMSO) δ: 3.45–3.51 (m, 4H, 2 x NCH_2), 3.59–3.68 (m, 4H, 2 x OCH_2), 3.92 (s, 3H, OCH_3), 5.16 (s, 2H, NH_2), 6.78 (d, $J = 8.1$ Hz, 1H, arom H), 7.33 (dd, $J = 8.1$, 1.7 Hz, 1H, arom H), 7.37 (d, $J = 1.3$ Hz, 1H, arom H), 7.47 (t, $J = 8.0$ Hz, 1H, arom H), 7.64 (d, $J = 7.4$ Hz, 1H, arom H), 7.93 (d, $J = 7.7$ Hz, 1H, arom H), 8.26–8.35 (m, 2H, arom H), 8.80 (s, 1H, NH), 9.26 (d, $J = 2.0$ Hz, 1H arom H) ppm. ^{13}C NMR (126 MHz, DMSO) δ: 44.3 (NCH_2), 55.6 (OCH_3), 66.1 (OCH_2), 109.5 (CH), 113.9 (CH), 119.0 (CH), 120.5 (CH), 121.3 (CH), 121.7 (CH), 122.4 (CH), 123.4 (C), 129.4 (CH), 130.0 (C), 135.6 (C), 139.2 (C), 141.4 (C), 142.3 (C), 146.9 (C), 151.8 (CH), 155.2 (C), 157.2 (C), 161.2 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{24}H_{23}N_5O_3S$ 462.1594, found 462.1589.

*9.1.7.8. N-(4-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)propionamide (**9a**).* This compound was obtained using the precursor 8a. The crude residue was purified by silica gel flash chromatography using a mixture of dichloromethane and acetone (in a ratio of 95:5) as a mobile phase. After purification, the compound was precipitated with methanol and then with diethyl ether, affording the title compound as a yellow solid in 56% yield (31.2 mg, 0.08 mmol). Mp = 253.2–254.6 °C. ^{1}H NMR (600 MHz, DMSO) δ: 1.11 (t, $J = 7.6$ Hz, 3H, CH_3), 2.38 (q, $J = 7.5$ Hz, 2H, CH_2), 3.92 (s, 3H, OCH_3), 5.17 (s, 2H, NH_2), 6.78 (d, $J = 8.1$ Hz, 1H, arom H), 7.33 (dd, $J = 8.1$, 2.0 Hz, 1H, arom H), 7.37 (d, $J = 2.0$ Hz, 1H, arom H), 7.80–7.84 (m, 2H, arom H), 8.23–8.27 (m, 2H, arom H), 8.28 (d, $J = 2.2$ Hz, 1H, arom H), 9.25 (d, $J = 2.2$ Hz, 1H, arom H), 10.17 (s, 1H, NH) ppm. ^{13}C NMR (150 MHz, DMSO) δ: 9.6 (CH_3), 29.7 (CH_2), 55.6 (OCH_3), 109.5 (CH), 113.9 (CH), 119.4 (CH), 120.5 (CH), 122.4 (CH), 123.4 (C), 124.6 (C), 128.6 (CH), 135.6 (C), 139.2 (C), 141.1 (C), 142.1 (C), 146.9 (C), 151.4 (CH), 157.1 (C), 160.7 (C), 172.5 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{20}N_4O_2S$ 405.1380, found 405.1369.

*9.1.7.9. N-(4-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)phenyl)acrylamide (**9b**).* This compound was obtained

using the precursor 8b and DMF as a solvent. The reaction mixture was stirred at 95 °C overnight. The crude residue was purified by silica gel flash chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.1) as a mobile phase. After purification the compound was precipitated with methanol and then with diethyl ether, affording the title compound as a yellow solid in 79% yield (44.1 mg, 0.11 mmol). Mp = 281.9–283.2 °C. ^{1}H NMR (600 MHz, DMSO) δ: 3.92 (s, 3H, OCH_3), 5.16 (s, 2H, NH_2), 5.81 (dd, J = 10.1, 1.8 Hz, 1H, double bond), 6.32 (dd, J = 17.0, 1.8 Hz, 1H, double bond), 6.48 (dd, J = 16.9, 10.1 Hz, 1H, double bond), 6.78 (d, J = 8.1 Hz, 1H, arom H), 7.33 (dd, J = 8.1, 2.0 Hz, 1H, arom H), 7.37 (d, J = 1.9 Hz, 1H, arom H), 7.87–7.93 (m, 2H, arom H), 8.27–8.32 (m, 3H, arom H), 9.25 (d, J = 2.2 Hz, 1H, arom H), 10.45 (s, 1H, NH) ppm. ^{13}C NMR (150 MHz, DMSO) δ: 55.6 (OCH_3), 109.5 (CH), 113.9 (CH), 119.8 (CH), 120.5 (CH), 122.4 (CH), 123.4 (C), 125.2 (C), 127.6 (CH_2 double bond), 128.7 (CH), 131.7 (CH double bond), 135.6 (C), 139.2 (C), 140.7 (C), 142.2 (C), 146.9 (C), 151.5 (CH), 157.1 (C), 160.5 (C), 163.5 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{18}N_4O_2S$ 403.1223, found 403.1215.

*9.1.7.10. N-(1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)propionamide (**13a**).* This compound was obtained using the precursor 12a. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and ethyl acetate (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 79% yield (44.1 mg, 0.11 mmol). Mp = 161.1–162.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.14 (t, J = 7.6 Hz, 3H, CH_3), 1.80–1.88 (m, 2H, CH_2), 1.90–1.99 (m, 2H, CH_2), 2.21 (q, J = 7.5 Hz, 2H, CH_2), 3.79–3.90 (m, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.95 (d, J = 3.0 Hz, 1H), 3.99 (m, 2H, NH_2), 4.02–4.06 (m, 1H, CH), 4.25–4.30 (m, 1H, CH), 6.02 (d, J = 6.7 Hz, 1H, NH), 6.80 (d, J = 7.9 Hz, 1H, arom H), 7.08 (s, 1H, arom H), 7.12 (d, J = 7.9 Hz, 1H, arom H), 8.63 (s, 1H, arom H), 7.81 (s, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) δ: 9.8 (CH_3), 21.9 (CH_2), 29.0 (CH_2), 29.7 (CH_2), 44.9 (CH), 51.4 (CH_2), 55.1 (CH_2), 55.5 (OCH_3), 109.2 (CH), 115.0 (CH), 120.2 (CH), 123.8 (CH), 127.4 (C), 133.6 (C), 135.9 (C), 136.9 (C), 144.6 (CH), 147.6 (C), 156.5 (C), 173.4 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{21}H_{25}N_5O_2S$ 412.1802, found 412.1786.

*9.1.7.11. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)propionamide (**13b**).* This compound was obtained using the precursor 12b. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase and a second purification using dichloromethane and acetone (in a ratio of 9:1), affording the title compound as a yellow solid in 75% yield (41.5 mg, 0.10 mmol). Mp = 193.3–194.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.24 (t, J = 7.6 Hz, 3H, CH_3), 1.54–1.61 (m, 1H, CH), 1.74–1.83 (m, 1H, CH), 1.87–1.98 (m, 2H, CH_2), 2.03–2.10 (m, 1H, CH), 2.32 (q, J = 7.6 Hz, 2H, CH_2), 3.17–3.26 (m, 1H, CH), 3.35–3.42 (m, 1H, CH), 3.50–3.57 (m, 1H, CH), 3.88–3.92 (m, 1H, CH), 3.93 (s, 3H, OCH_3), 4.03 (dd, J = 13.0, 7.1 Hz, 1H, CH), 4.16 (dd, J = 13.0, 3.2 Hz, 1H, CH), 6.57–6.61 (m, 1H, NH), 6.82 (d, J = 8.0 Hz, 1H, arom H), 7.07 (d, J = 1.8 Hz, 1H, arom H), 7.11 (dd, J = 8.0, 1.9 Hz, 1H, arom H), 7.82 (d, J = 2.0 Hz, 1H, arom H), 8.58 (d, J = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) δ: 10.1 (CH_3), 22.9 (CH_2), 27.5 (CH_2), 30.0 (CH_2), 35.5 (CH), 40.8 (CH_2), 52.8 (CH_2), 53.2 (CH_2), 55.6 (OCH_3), 109.3 (CH), 115.0 (CH), 120.2 (CH), 124.3 (CH), 127.5 (C), 133.2 (C), 136.0 (C), 137.0 (C), 144.0 (CH), 147.6 (C), 156.5 (C), 173.3 (C), 173.9 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{27}N_5O_2S$ 426.1958, found 426.1963.

*9.1.7.12. N-(2-(1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)ethyl)propionamide (**13c**).* This compound was obtained using the precursor 12c. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 3:2), affording the title compound as a yellow solid in 81% yield (44.6 mg, 0.10 mmol). Mp = 146.2–147.4 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.14 (t, J = 7.6 Hz, 3H, CH_3), 1.29–1.39 (m, 1H, CH), 1.50–1.61 (m, 2H, CH_2), 1.77–1.91 (m, 3H, CH, CH_2), 1.92–2.00 (m, 1H, CH), 2.18 (q, J = 7.6 Hz, 2H, CH_2), 3.06 (dd, J = 12.7, 10.3 Hz, 1H, CH), 3.26–3.40 (m, 2H, CH_2), 3.42–3.51 (m, 1H, CH), 3.93 (s, 3H, OCH_3), 3.98 (bs, 2H, NH_2), 4.21–4.26 (m, 1H, CH), 4.83–4.89 (m, 1H, CH), 5.89–5.94 (m, 1H, NH), 6.81 (d, J = 8.0 Hz, 1H, arom H), 7.08 (d, J = 1.8 Hz, 1H, arom H), 7.13 (dd, J = 8.0, 1.9 Hz, 1H, arom H), 7.79 (d, J = 2.1 Hz, 1H, arom H), 8.59 (d, J = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ: 9.9 (CH_3), 24.3 (CH_2), 29.7 (CH_2), 30.6 (CH_2), 33.2 (CH_2), 33.5 (CH), 36.9 (CH_2), 52.5 (CH_2), 55.6 (CH_2), 55.6 (OCH_3), 109.2 (CH), 115.0 (CH), 120.2 (CH), 123.9 (CH), 127.5 (C), 133.2 (C), 135.8 (C), 136.9 (C), 143.8 (CH), 147.6 (C), 156.6 (C), 172.8 (C), 173.8 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{23}H_{29}N_5O_2S$ 440.2115, found 440.2111.

*9.1.7.13. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)acrylamide (**13d**).* This compound was obtained using the precursor 12b. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile phase, affording the title compound as an orange solid in 87% yield (48.3 mg, 0.14 mmol). Mp = 119.2–122.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 1.55–1.65 (m, 1H, CH), 1.74–1.81 (m, 1H, CH), 1.89–1.97 (m, 2H, CH_2), 2.06–2.16 (m, 1H, CH), 3.25–3.33 (m, 1H, CH), 3.36–3.43 (m, 1H, CH), 3.59–3.69 (m, 1H, CH), 3.83–3.89 (m, 1H, CH), 3.93 (s, 3H, OCH_3), 4.01 (bs, 2H, NH_2), 4.14–4.06 (m, 2H, CH_2), 5.69 (dd, J = 10.2, 1.3 Hz, 1H, double bond), 6.23 (dd, J = 17.0, 10.2 Hz, 1H, double bond), 6.34 (dd, J = 17.0, 1.3 Hz, 1H, double bond), 6.81 (d, J = 8.0 Hz, 1H, arom H), 6.99–7.03 (m, 1H, NH), 7.06 (d, J = 1.6 Hz, 1H, arom H), 7.11 (dd, J = 8.0, 1.8 Hz, 1H, arom H), 7.81 (d, J = 2.0 Hz, 1H, arom H), 8.58 (d, J = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) δ: 22.8 (CH_2), 27.4 (CH_2), 35.4 (CH), 40.7 (CH_2), 52.9 (CH_2), 53.1 (CH_2), 55.6 (OCH_3), 109.3 (CH), 114.9 (CH), 120.2 (CH), 124.3 (CH), 126.2 (CH_2 double bond), 127.4 (C), 131.2 (CH double bond), 133.2 (C), 136.0 (C), 137.0 (C), 144.1 (CH), 147.6 (C), 156.5 (C), 165.8 (C), 173.3 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{25}N_5O_2S$ 424.1802, found 424.1801.

*9.1.7.14. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-4-yl)methyl)propionamide (**17a**).* This compound was obtained using the precursor 16a. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 3:2) as mobile phase, affording the title compound as a yellow solid in 83% yield (46.0 mg, 0.11 mmol). Mp = 174.3–175.4 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.17 (t, J = 7.6 Hz, 3H, CH_3), 1.45–1.58 (m, 2H, CH_2), 1.84–1.92 (m, 3H, CH_2, CH), 2.23 (q, J = 7.6 Hz, 2H, CH_2), 3.15–3.25 (m, 4H, 2 x CH_2), 3.92 (s, 3H, OCH_3), 3.99 (bs, 2H, NH_2), 4.68–4.73 (m, 2H, CH_2), 5.74–5.78 (m, 1H, NH), 6.80 (d, J = 8.0 Hz, 1H, arom H), 7.08 (s, 1H, arom H), 7.12 (dd, J = 8.0, 1.6 Hz, 1H, arom H), 7.79 (d, J = 1.9 Hz, 1H, arom H), 8.61 (d, J = 1.9 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ: 9.9 (CH_3), 29.1 (CH_2), 29.7 (CH_2), 35.8 (CH), 44.6 (CH_2), 50.6 (CH_2), 55.5 (OCH_3), 109.2 (CH), 114.9 (CH), 120.1 (CH), 123.7 (CH), 127.6 (C), 133.4 (C), 135.8 (C), 136.8 (C), 144.0 (CH), 147.5 (C), 156.5 (C), 173.0 (C), 174.0 (C) ppm. HR-MS m/z $[M+H]^+$ calcd for $C_{22}H_{27}N_5O_2S$ 426.1958, found 426.1955.

*9.1.7.15. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-4-yl)methyl)acrylamide (**17b**).* This compound was obtained using the precursor 16b. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.2) as mobile

phase and a second purification using dichloromethane and acetone (7:3), affording the title compound as a yellow solid in 79% yield (43.8 mg, 0.10 mmol). Mp = 129.8–131.4 °C. ^{1}H NMR (500 MHz, DMSO) *δ*: 1.34–1.43 (m, 2H, CH_2), 1.78–1.86 (m, 3H, CH, CH_2), 3.06–3.14 (m, 2H, CH_2), 3.18–3.30 (m, 2H, CH_2), 3.88 (s, 3H, OCH_3), 4.62 (d, *J* = 12.6 Hz, 2H, CH_2), 5.06 (s, 2H, NH_2), 5.59 (dd, *J* = 10.2, 2.2 Hz, 1H, double bond), 6.09 (dd, *J* = 17.1, 2.2 Hz, 1H, double bond), 6.25 (dd, *J* = 17.1, 10.2 Hz, 1H, double bond), 6.74 (d, *J* = 8.1 Hz, 1H, arom H), 7.20 (dd, *J* = 8.1, 2.0 Hz, 1H, arom H), 7.25 (d, *J* = 1.9 Hz, 1H, arom H), 7.84 (d, *J* = 2.1 Hz, 1H, arom H), 8.17 (t, *J* = 5.8 Hz, 1H, NH), 8.71 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, DMSO) *δ:* 28.9 (CH_2), 35.3 (CH), 43.9 (CH_2), 50.2 (CH_2), 55.5 (CH_3), 109.3 (CH), 113.8 (CH), 120.1 (CH), 122.1 (CH), 124.1 (C), 125.1 (double bond CH_2), 131.8 (double bond CH), 132.5 (C), 135.2 (C), 138.7 (C), 143.7 (CH), 146.8 (C), 156.1 (C), 164.8 (C), 172.3 (C) ppm. HR-MS *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{25}N_5O_2S$ 424.1802, found 424.1777.

*9.1.7.16. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)-3-methylbutanamide (**19a**).* This compound was obtained using the precursor 18a. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3), affording the title compound as a yellow solid in 81% yield (44.3 mg, 0.10 mmol). Mp = 102.1–104.3 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ:* 0.97–1.04 (m, 6H, 2 x CH_3), 1.50–1.62 (m, 1H, CH), 1.72–1.82 (m, 1H, CH), 1.84–1.97 (m, 2H, CH_2), 2.01–2.10 (m, 1H, CH), 2.13–2.24 (m, 3H, CH, CH_2), 3.15–3.27 (m, 1H, CH), 3.33–3.42 (m, 1H, CH), 3.51–3.60 (m, 1H, CH), 3.88–3.96 (m, 4H, OCH_3, CH), 4.00 (bs, 2H, NH_2), 4.05–4.08 (m, 2H, CH_2), 6.72–6.77 (m, 1H, NH), 6.81 (d, *J* = 8.0 Hz, 1H, arom H), 7.07 (d, *J* = 1.8 Hz, 1H, arom H), 7.11 (dd, *J* = 8.0, 1.9 Hz, 1H, arom H), 7.82 (d, *J* = 2.1 Hz, 1H, arom H), 8.59 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ*: 22.6 (CH_3), 22.6 (CH_3), 22.8 (CH_2), 26.3 (CH), 27.4 (CH_2), 35.5 (CH), 40.6 (CH_2), 46.5 (CH_2), 52.8 (CH_2), 53.3 (CH_2), 55.5 (OCH_3), 109.2 (CH), 115.0 (CH), 120.1 (CH), 124.2 (CH), 127.4 (C), 133.2 (C), 135.9 (C), 137.0 (C), 144.0 (CH), 147.6 (C), 156.5 (C), 172.6 (C), 173.4 (C) ppm. HR-MS *m*/*z* $[M+H]^+$ calcd for $C_{24}H_{31}N_5O_2S$ 454.2271, found 454.2261.

*9.1.7.17. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanecarboxamide (**19b**).* This compound was obtained using the precursor 18b. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 3:2), affording the title compound as a yellow solid in 77% yield (42.3 mg, 0.10 mmol). Mp = 193.9–195.8 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 0.73–0.81 (m, 2H, CH_2), 0.97–1.05 (m, 2H, CH_2), 1.41–1.49 (m, 1H, CH), 1.51–1.61 (m, 1H, CH), 1.74–1.82 (m, 1H, CH), 1.87–1.98 (m, 2H, CH_2), 2.02–2.11 (m, 1H, CH), 3.20–3.28 (m, 1H, CH), 3.33–3.43 (m, 1H, CH), 3.50–3.58 (m, 1H, CH), 3.93 (s, 3H, OCH_3), 3.96–4.08 (m, 4H, CH_2, NH_2), 4.15 (dd, *J* = 12.9, 3.2 Hz, 1H, CH), 6.70–6.74 (m, 1H, NH), 6.81 (d, *J* = 8.0 Hz, 1H, arom H), 7.07 (d, *J* = 1.8 Hz, 1H, arom H), 7.11 (dd, *J* = 8.0, 1.9 Hz, 1H, arom H), 7.82 (d, *J* = 2.0 Hz, 1H, arom H), 8.59 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ:* 7.1 (CH_2), 7.2 (CH_2), 15.0 (CH), 23.0 (CH_2), 27.6 (CH_2), 35.7 (CH), 41.1 (CH_2), 52.8 (CH_2), 53.4 (CH_2), 55.6 (OCH_3), 109.4 (CH), 115.0 (CH), 120.2 (CH), 124.4 (CH), 127.6 (C), 133.3 (C), 136.0 (C), 137.0 (C), 144.1 (CH), 147.6 (C), 156.6 (C), 173.4 (C), 173.6 (C) ppm. HR-MS *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{27}N_5O_2S$ 438.1958, found 438.1948.

*9.1.7.18. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopentanecarboxamide (**19c**).* This compound was obtained using the precursor 18c. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3), affording the title compound as a yellow solid in 83% yield (44.5 mg, 0.10 mmol). Mp = 173.3–175.1 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.45–1.55 (m, 1H, CH), 1.55–1.65 (m, 2H, CH_2), 1.72–1.82 (m, 3H, CH_2, CH), 1.82–1.98 (m, 6H, 3 x CH_2), 1.99–2.09 (m, 1H, CH), 2.63 (p, *J* = 8.1 Hz, 1H, CH), 3.16–3.26 (m, 1H, CH), 3.30–3.39 (m, 1H, CH), 3.42–3.51 (m, 1H, CH), 3.85 (dd, *J* = 12.9, 7.7 Hz, 1H, CH), 3.92 (s, 3H, OCH_3), 3.95–4.07 (m, 3H, CH, NH_2), 4.22 (dd, *J* = 12.9, 3.1 Hz, 1H, CH), 6.53–6.58 (m, 1H, NH), 6.81 (d, *J* = 8.0 Hz, 1H, arom H), 7.06 (d, *J* = 1.7 Hz, 1H, arom H), 7.10 (dd, *J* = 8.0, 1.8 Hz, 1H, arom H), 7.80 (d, *J* = 2.0 Hz, 1H, arom H), 8.57 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ*: 23.1 (CH_2), 25.9 (CH_2), 27.6 (CH_2), 30.5 (CH_2), 30.5 (CH_2), 35.7 (CH), 41.0 (CH_2), 46.1 (CH), 52.6 (CH_2), 53.4 (CH_2), 55.5 (OCH_3), 109.2 (CH), 115.0 (CH), 120.1 (CH), 124.1 (CH), 127.4 (C), 133.2 (C), 135.9 (C), 136.9 (C), 143.9 (CH), 147.6 (C), 156.5 (C), 173.2 (C), 176.3 (C) ppm. HR-MS *m*/*z* $[M+H]^+$ calcd for $C_{25}H_{31}N_5O_2S$ 466.2271, found 466.2254.

*9.1.7.19. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)benzamide (**19d**).* This compound was obtained using the precursor 18d. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 3:2) as mobile phase and a second purification using dichloromethane and methanol (in a ratio of 10:0.1), affording the title compound as a yellow solid in 72% yield (39.4 mg, 0.08 mmol). Mp = 121.0–123.2 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ:* 1.67–1.75 (m, 1H, CH), 1.76–1.85 (m, 1H, CH), 1.90–2.01 (m, 2H, CH_2), 2.15–2.23 (m, 1H, CH), 3.36–3.49 (m, 2H, CH_2), 3.67–3.74 (m, 1H, CH), 3.77–3.85 (m, 1H, CH), 3.91 (s, 3H, OCH_3), 4.11 (dd, *J* = 13.1, 2.9 Hz, 1H, CH), 4.38–4.44 (m, 1H, CH), 6.80 (d, *J* = 8.0 Hz, 1H, arom H), 7.00 (d, *J* = 1.8 Hz, 1H, arom H), 7.03 (dd, *J* = 8.0, 1.8 Hz, 1H, arom H), 7.43–7.54 (m, 3H, arom H), 7.57–7.62 (m, 1H, NH), 7.81 (d, *J* = 2.0 Hz, 1H, arom H), 7.88–7.92 (m, 2H, arom H), 8.41 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ:* 22.6 (CH_2), 27.4 (CH_2), 35.4 (CH), 40.9 (CH_2), 52.8 (CH_2), 53.4 (CH_2), 55.5 (OCH_3), 109.0 (CH), 115.0 (CH), 120.1 (CH), 124.1 (CH), 127.2 (CH), 128.5 (CH), 131.3 (CH), 133.2 (C), 135.5 (C), 135.8 (C), 136.9 (C), 144.3 (CH), 147.6 (C), 156.6 (C), 168.2 (C), 173.3 (C) ppm. HR-MS *m*/*z* $[M+H]^+$ calcd for $C_{26}H_{27}N_5O_2S$ 474.1958, found 474.1951.

*9.1.7.20. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)pyrrolidine-1-carboxamide (**19e**).* This compound was obtained using the precursor 18e. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 1:1), affording the title compound as a yellow solid in 72% yield (39.4 mg, 0.08 mmol). Mp = 190.7–192.6 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.43–1.55 (m, 1H, CH), 1.73–1.84 (m, 1H, CH), 1.85–1.99 (m, 6H, 3 x CH_2), 2.01–2.10 (m, 1H, CH), 3.17–3.27 (m, 1H, CH), 3.34–3.47 (m, 6H, 3 x CH_2), 3.71 (dd, *J* = 12.9, 8.3 Hz, 1H, CH), 3.93 (s, 3H, OCH_3), 4.00 (bs, 2H, NH_2), 4.10–4.16 (m, 1H, CH), 4.35 (dd, *J* = 12.8, 3.2 Hz, 1H, CH), 4.79 (t, *J* = 5.7 Hz, 1H, NH), 6.80 (d, *J* = 8.0 Hz, 1H, arom H), 7.06 (d, *J* = 1.7 Hz, 1H, arom H), 7.10 (dd, *J* = 8.0, 1.8 Hz, 1H, arom H), 7.79 (d, *J* = 2.0 Hz, 1H, arom H), 8.54 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ:* 23.4 (CH_2), 25.6 (CH_2), 27.8 (CH_2), 36.4 (CH), 42.7 (CH_2), 45.6 (CH_2), 52.3 (CH_2), 53.7 (CH_2), 55.5 (OCH_3), 109.2 (CH), 114.9 (CH), 120.1 (CH), 124.0 (CH), 127.6 (C), 133.3 (C), 135.8 (C), 136.9 (C), 143.7 (CH), 147.6 (C), 156.6 (C), 156.8 (C), 173.2 (C) ppm. HR-MS *m*/*z* $[M+H]^+$ calcd for $C_{24}H_{30}N_6O_2S$ 467.2224, found 467.2217.

*9.1.7.21. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)morpholine-4-carboxamide (**19f**).* This compound was obtained using the precursor 18f. The crude

residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 1:1), affording the title compound as a yellow solid in 76% yield (41.5 mg, 0.09 mmol). Mp = 120.2–122.9 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 1.50–1.60 (m, 1H, CH), 1.73–1.83 (m, 1H, CH), 1.87–1.96 (m, 2H, CH_2), 2.02–2.11 (m, 1H, CH), 3.20–3.28 (m, 1H, CH), 3.37–3.50 (m, 6H, 3 x CH_2), 3.69–3.76 (m, 4H, 2 x OCH_2), 3.90–3.97 (m, 5H, OCH_3, CH_2), 4.00 (bs, 2H, NH_2), 4.24 (dd, *J* = 12.9, 3.3 Hz, 1H, CH), 5.29 (t, *J* = 5.7 Hz, 1H, NH), 6.81 (d, *J* = 8.0 Hz, 1H, arom H), 7.05 (d, *J* = 1.9 Hz, 1H, arom H), 7.08 (dd, *J* = 8.0, 1.9 Hz, 1H, arom H), 7.80 (d, *J* = 2.1 Hz, 1H, arom H), 8.50 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) *δ:* 23.0 (CH_2), 27.7 (CH_2), 36.0 (CH), 42.5 (CH_2), 44.1 (CH_2), 52.7 (CH_2), 53.2 (CH_2), 55.5 (OCH_3), 66.5 (CH_2), 109.1 (CH), 115.0 (CH), 120.0 (CH), 124.1 (CH), 127.4 (C), 133.2 (C), 135.8 (C), 137.0 (C), 143.8 (CH), 147.6 (C), 156.6 (C), 158.0 (C), 173.3 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{24}H_{30}N_6O_3S$ 483.2173, found 483.2166.

*9.1.7.22. N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanesulfonamide (**19g**).* This compound was obtained using the precursor 18 g. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 74% yield (40.5 mg, 0.08 mmol). Mp = 110.1–111.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 0.82–0.90 (m, 1H, CH), 0.92–0.99 (m, 1H, CH), 1.04–1.11 (m, 1H, CH), 1.15–1.23 (m, 1H, CH), 1.64–1.71 (m, 1H, CH), 1.74–1.80 (m, 1H, CH), 1.88–1.98 (m, 2H, CH_2), 2.17–2.23 (m, 1H, CH), 2.32–2.38 (m, 1H, CH), 3.22–3.29 (m, 1H, CH), 3.38–3.46 (m, 2H, CH_2), 3.56–3.63 (m, 1H, CH), 3.74 (dd, *J* = 13.3, 2.8 Hz, 1H, CH), 3.93 (s, 3H, OCH_3), 4.60 (dd, *J* = 13.3, 4.4 Hz, 1H, CH), 6.80 (d, *J* = 8.0 Hz, 1H, arom H), 7.06–7.11 (m, 2H, arom H, NH), 7.13 (dd, *J* = 8.0, 1.9 Hz, 1H, arom H), 7.85 (d, *J* = 2.0 Hz, 1H, arom H), 8.68 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) *δ:* 4.9 (CH_2), 5.5 (CH_2), 22.1 (CH_2), 30.1 (CH_2), 26.8 (CH), 35.3 (CH), 43.7 (CH_2), 52.8 (CH_2), 53.2 (CH_2), 55.6 (OCH_3), 109.2 (CH), 114.9 (CH), 120.3 (CH), 124.4 (CH), 127.0 (C), 132.9 (C), 136.3 (C), 137.1 (C), 144.7 (CH), 147.6 (C), 156.4 (C), 173.4 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{22}H_{27}N_5O_3S_2$ 474.1628, found 474.1631.

*9.1.7.23. 1-((1-(6-(4-Amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)-3-cyclopropylurea (**19h**).* This compound was obtained using the precursor 18h. The crude residue was purified by silica gel flash column chromatography using a mixture of dichloromethane and methanol (in a ratio of 10:0.3) as mobile phase and a second purification using hexane and acetone (in a ratio of 3:2), affording the title compound as an orange solid in 69% yield (38.1 mg, 0.08 mmol). Mp = 134.0–136.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$) *δ*: 0.62–0.68 (m, 2H, CH_2), 0.75–0.82 (m, 2H, CH_2), 1.40–1.46 (m, 1H, CH), 1.76–1.86 (m, 1H, CH), 1.87–1.98 (m, 2H, CH_2), 2.04–2.13 (m, 1H, CH), 2.49–2.55 (m, 1H, CH), 3.22–3.29 (m, 1H, CH), 3.33–3.43 (m, 3H, CH, CH_2), 3.93 (s, 3H, OCH_3), 4.25–4.31 (m, 1H, CH), 4.57 (dd, *J* = 12.8, 3.5 Hz, 1H, CH), 4.79 (s, 1H, NH), 5.33–5.38 (s, 1H, NH), 6.80 (d, *J* = 8.0 Hz, 1H, arom H), 7.07 (d, *J* = 1.9 Hz, 1H, arom H), 7.10 (dd, *J* = 8.0, 1.9 Hz, 1H, arom H), 7.79 (d, *J* = 2.1 Hz, 1H), 8.55 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) *δ*: 7.7 (CH_2), 22.4 (CH), 23.8 (CH_2), 27.9 (CH_2), 36.5 (CH), 42.8 (CH_2), 52.0 (CH_2), 54.1 (CH_2), 55.5 (OCH_3), 109.3 (CH), 115.0 (CH), 120.1 (CH), 123.9 (CH), 127.6 (C), 133.3 (C), 135.8 (C), 136.9 (C), 143.8 (CH), 147.6 (C), 156.6 (C), 159.0 (C), 173.2 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{23}H_{28}N_6O_2S$ 453.2067, found 453.2064.

*9.1.7.24. (S)-N-((1-(6-(4-Amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanecarboxamide (**23a**).* This compound was obtained using the precursor 22a. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 75% yield (41.5 mg, 0.09 mmol). Mp = 128.2–129.8 °C. $[\alpha]_D$ −14.8 (*c* 0.50, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 0.73–0.80 (m, 2H, CH_2), 0.98–1.03 (m, 2H, CH_2), 1.40–1.48 (m, 1H, CH), 1.51–1.59 (m, 1H, CH), 1.73–1.82 (m, 1H, CH), 1.86–1.95 (m, 2H, CH_2), 2.02–2.10 (m, 1H, CH), 3.19–3.27 (m, 1H, CH), 3.34–3.40 (m, 1H, CH), 3.48–3.55 (m, 1H, CH), 3.93 (s, 3H, OCH_3), 3.95–4.02 (m, 4H, CH_2, NH_2), 4.16 (dd, *J* = 13.0, 3.3 Hz, 1H, CH), 6.76 (t, *J* = 5.7 Hz, 1H, NH), 6.81 (d, *J* = 8.0 Hz, 1H, arom H), 7.06 (d, *J* = 1.9 Hz, 1H, arom H), 7.10 (dd, *J* = 8.0, 1.9 Hz, 1H, arom H), 7.81 (d, *J* = 2.1 Hz, 1H, arom H), 8.58 (d, *J* = 2.1 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) *δ*: 7.1 (CH_2), 7.1 (CH_2), 14.9 (CH), 23.0 (CH_2), 27.6 (CH_2), 35.7 (CH), 41.1 (CH_2), 52.7 (CH_2), 53.4 (CH_2), 55.5 (OCH_3), 109.3 (CH), 114.9 (CH), 120.2 (CH), 124.3 (CH), 127.5 (C), 133.2 (C), 136.0 (C), 136.9 (C), 144.0 (CH), 147.6 (C), 156.5 (C), 173.3 (C), 173.6 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}N_5O_2S$ 438.1958, found 438.1949.

*9.1.7.25. (R)-N-((1-(6-(4-amino-3-methoxyphenyl)isothiazolo[4,3-b]pyridin-3-yl)piperidin-3-yl)methyl)cyclopropanecarboxamide (**23b**).* This compound was obtained using the precursor 22b. The crude residue was purified by silica gel flash column chromatography using a mixture of hexane and acetone (in a ratio of 7:3) as mobile phase, affording the title compound as a yellow solid in 78% yield (43.1 mg, 0.1 mmol). Mp = 129.7–131.2 °C. $[\alpha]_D$ +14.8 (*c* 0.50, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$) *δ:* 0.74–0.81 (m, 2H, CH_2), 0.97–1.05 (m, 2H, CH_2), 1.42–1.48 (m, 1H, CH), 1.52–1.59 (m, 1H, CH), 1.74–1.82 (m, 1H, CH), 1.87–1.96 (m, 2H, CH_2), 2.03–2.10 (m, 1H, CH), 3.19–3.26 (m, 1H, CH), 3.34–3.42 (m, 1H, CH), 3.49–3.57 (m, 1H, CH), 3.93 (s, 3H, OCH_3), 3.95–4.05 (m, 4H, CH_2, NH_2), 4.16 (dd, *J* = 13.0, 3.1 Hz, 1H, CH), 6.71–6.76 (m, 1H, NH), 6.81 (d, *J* = 8.0 Hz, 1H, arom H), 7.06 (d, *J* = 1.7 Hz, 1H, arom H), 7.10 (dd, *J* = 8.0, 1.8 Hz, 1H, arom H), 7.81 (d, *J* = 2.0 Hz, 1H, arom H), 8.58 (d, *J* = 2.0 Hz, 1H, arom H) ppm. ^{13}C NMR (150 MHz, $CDCl_3$) *δ*: 7.1 (CH_2), 7.1 (CH_2), 14.9 (CH), 23.0 (CH_2), 27.6 (CH_2), 35.7 (CH), 41.1 (CH_2), 52.7 (CH_2), 53.4 (CH_2), 55.5 (OCH_3), 109.3 (CH), 114.9 (CH), 120.2 (CH), 124.3 (CH), 127.5 (C), 133.2 (C), 136.0 (C), 136.9 (C), 144.0 (CH), 147.6 (C), 156.5 (C), 173.3 (C), 173.6 (C) ppm. HR-MS *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}N_5O_2S$ 438.1958, found 438.1954.

9.2. GAK LanthaScreen™ Eu binding assay

The compounds were subjected to a LanthaScreen™ binding assay in which 10 titrations of dissolved test compound in DMSO are transferred to a 384-well plate. Sequential addition of the kinase buffer (50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl and 1 mM EGTA), the 2X kinase antibody (Eu Anti GST) mixture and the 4X Tracer 222 solution was performed. After shaking for 30 s and a 1 h incubation period at room temperature, the plate was read on a fluorescence plate reader. When the bound tracer in the active site was displaced by the test compound, fluorescence was not observed. The collected data were then compared to a 0% displacement control with pure DMSO and a 100% displacement control with staurosporine, a known inhibitor of GAK and plotted against the logarithmic concentration parameter. The IC_{50} was subsequently extracted.

9.3. Antiviral assays

The compounds were tested for antiviral activity in human hepatoma (Huh7) cells infected with dengue virus. The cells were pretreated for 1 h and then infected with DENV2 expressing Renilla luciferase marker. The effect of the compounds on the overall

infection was measured at 48 h post-infection via luciferase assays and the half-maximal effective concentration (EC_{50}) values were calculated [19,30].

9.3.1. Virus construct

Renilla reporter plasmid encoding full length DENV2 (New Guinea C strain) [44,45] was used for the antiviral assays (a gift from Pei-Yong Shi (The University of Texas Medical Branch).

9.3.2. Cells

Huh7 (Apath LLC) cells were grown in DMEM (Mediatech) supplemented with 10% FBS (Omega Scientific), nonessential aminoacids, 1% L-glutamine, and 1% penicillin-streptomycin (ThermoFisher Scientific) and maintained in a humidified incubator with 5% CO_2 at 37 °C.

9.3.3. Virus Production

DENV2 RNA was transcribed *in vitro* using mMessage/mMachine (Ambion) kits. DENV was produced by electroporating RNA into BHK-21 cells, harvesting supernatants on day 10 and titering *via* standard plaque assays on BHK-21 cells. Viral titers were determined *via* standard plaque assay on Vero76 cells.

9.3.4. Infection assays

Huh7 cells were infected with DENV in replicates (n = 3–10) for 4 h at multiplicity of infection (MOI) of 0.05. Overall infection was measured at 48 h by standard luciferase assays.

9.3.5. Viability assays

The effect of the compounds on cell viability was assessed using AlamarBlue® reagent (Invitrogen) according to the manufacturer's protocol in the DENV-infected Huh7 cells. Fluorescence was detected at 560 nm on an InfiniteM1000 plate reader (Tecan) and the reads were used to calculate the half-maximal cytotoxic concentration (CC_{50}) values.

9.3.6. Quantification and statistical analysis

All data were analyzed with GraphPad Prism software. Fifty percent effective concentrations (EC_{50}) were measured by fitting of data to a 3-parameter logistic curve.

9.4. Protein expression and purification

Plasmid encoding N-terminally His_6-tagged GAK kinase domain was electroporated into Rosetta strain BL21 *E. coli* cells using Gene Pulser Xcell™ Electroporation Systems (Bio-Rad) with 1800V. Protein expression was induced with 0.5 mM IPTG at 20 °C overnight. Cells were then harvested and resuspended in lysis buffer composed of 50 mM HEPES (pH 7.5), 500 mM NaCl, 5 mM imidazole, 5% glycerol, and 0.5 mM TCEP [tris-(2-carboxyethyl)phosphine]. Following sonication and spinning at 48,400×*g* at 4 °C for 60 min the supernatant was harvested. GAK was purified *via* Ni-affinity followed by TEV protease digestion to remove the HIS_6 tag. GAK was further purified by size-exclusion chromatography *via* Superdex S75 10/60 column on AKTA pure (GE Lifesciences). GAK protein was concentrated *via* 10 kDa Amicon centrifugal filters (Merck) and suspended in storage buffer composed of 10 mM HEPES (pH 7.5), 300 mM NaCl, 5% glycerol, and 0.5 mM TCEP at −80 °C.

9.5. Kinase selectivity assay

Compound **23b** was screened against a diverse panel of 97 kinases (scanEDGE kinase assay panel, DiscoverX, KinomeScan) at a concentration of 10 μM [21]. The results are reported as the percentage of kinase/phage remaining bound to the ligands/beads, relative to a control. High affinity compounds have % of control values close to zero, while weaker binders have higher % control values.

9.6. Mass spectrometry analysis

To test covalent inhibitor binding, the purified GAK protein (0.725 mg/mL) was incubated with a well-known irreversible inhibitor of GAK ((5Z)-7-oxozeaenol) and test compounds at a final concentration of 100 μM for 30 min at 4 °C. 2 μl of protein was denatured by the addition of 48 μl of 1% formic acid and analyzed by electrospray ionization mass spectrometry (ESI-MS) on the Agilent 1260 HPLC and Bruker MicroTOF-Q II. The column was a BioResolve RP mAb Polyphenyl 450 A, 2.7 μ 100 × 2.1 mm from Waters, the temperature was 50 °C, and the flow rate was 0.3 mL/min. The injection volume was 2 μL. Data was collected in full scan MS mode with a mass range of 400–4000 Da. The collision RF setting was 800 Vpp.

9.7. Docking method

The cyclin-G associated kinase structure (GAK, PDB entry 4Y8D) [29] was used in all docking experiments. The reference compound **1** and water molecules were removed from the file. The 2D structure of the inhibitors were drawn in ChemBioDraw Ultra and the 3D structure was generated and optimised in ChemBio3D Ultra in the mm2 force field, and saved as pdb files [46]. We used then Autodocktools to add polar hydrogens and Gasteiger charges to the receptor structure 4Y8D and the potential inhibitor files [47]. The files were then converted into pdbqt files. Autodock vina [48] was used for non-covalent docking of **7a**, **13b**, **23a** and **23b**. The docking region on the enzyme for the docking was centered at the CB atom of Ala67.b and having the shape of a cubic box of 22.5 Ang.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113158.

Abbreviations

AP	adaptor protein complex
BOC	*tert*-butyloxycarbonyl
CC_{50}	half-maximal cytotoxic concentration
CCV	clathrin-coated vesicle

DCM	dichloromethane
DENV	Dengue virus
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
EC_{50}	half-maximal effective concentration
EGFR	epidermal growth factor receptor
GAK	cyclin G-associated kinase
HCV	hepatitis C virus
IC_{50}	half maximal inhibitory concentration
K_D	dissociation constant
SAR	structure-activity relationship
TR-FRET	time-resolved fluorescence resonance energy transfer
WHO	World Health Organization

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