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Original article

Efficient synthesis of 6-(hetero)arylthieno[3,2-*b*]pyridines by Suzuki–Miyaura coupling. Evaluation of growth inhibition on human tumor cell lines, SARs and effects on the cell cycle

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ABSTRACT

A wide variety of new bi(hetero)aryl derivatives of the thieno[3,2-*b*]pyridine skeleton was obtained in high to excellent yields (65–91%) by Suzuki–Miyaura cross-coupling of the methyl 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate, recently reported by us, with aryl or heteroaryl pinacolboranes or potassium trifluoroborates.

The coupling products obtained were evaluated for their growth inhibitory effect on three human tumor cell lines, representing different tumor models, MCF-7 (breast adenocarcinoma), A375-C5 (melanoma) and NCI-H460 (non-small cell lung cancer). Some of the compounds showed an interesting activity against the tested cell lines, with GI_{50} values in the μM range, and it was possible to establish some structure–activity relationships (SARs). Several compounds presented GI_{50} values below 15 μM, particularly a bithiophene and an *o*-aniline thienopyridine derivative. The first presented selectivity for MCF-7 and NCI-H460 cell lines, with very low GI_{50} values (0.7–1.0 μM), while the latter was active against the three cell lines tested in this study, also presenting very low GI_{50} values (2.5–4.2 μM). The effect of these two compounds on cell cycle progression was analyzed in the NCI-H460 cell line. Results showed that both compounds interfered with the normal cell cycle distribution.

1. Introduction

Recently the thienopyridine skeleton has shown interesting biological activities. (3-Amino-6-thien-2-yl-thieno[2,3-*b*]pyridin-2-yl)arylmethanones showed selectivity against a tumorogenic cell line, in very low EC_{50} values [1]. Diarylamine derivatives of thieno[3,2-*b*]pyridines [2], substituted thieno[3,2-*c*]pyridine ureas [3] and diarylether derivatives of thieno[3,2-*b*]pyridine phenylacetylthioureas [4] are inhibitors of the vascular endothelial growth factor receptor (VEGFR-2) which mediates the biological function of the vascular endothelial growth factor (VEGF), related to angiogenesis and metastasis.

Herein we report the synthesis of a wide variety of 6-(hetero)arylthieno[3,2-*b*]pyridines by Suzuki–Miyaura cross-coupling of the methyl 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate with several (hetero)arylboronated compounds. The potential of the bi(hetero)aryl compounds obtained as antitumor agents was evaluated through the study of their *in vitro* growth inhibitory effect on three human tumor cell lines, representing different tumor models, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and A375-C5 (melanoma), and it was possible to establish some structure–activity relationships. For the most active compounds, a study of their effects on normal cell cycle distribution was also performed in the NCI-H460 cell line.

2. Results and discussion

2.1. Chemistry

The 6-bromothieno[3,2-*b*]pyridine **1** recently reported by us [5], was used as the brominated component in the palladium-catalyzed Suzuki–Miyaura cross-coupling [6], with several (hetero)aryl pinacolboronates [7] or potassium trifluoroborates [8]. These boron compounds are easier to handle than the boronic acids due to their

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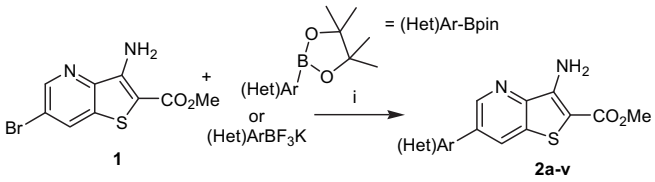
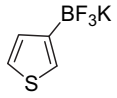
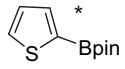
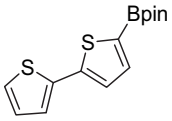
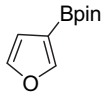
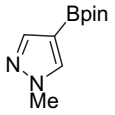
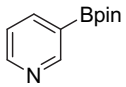
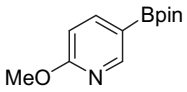
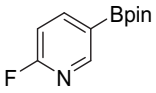
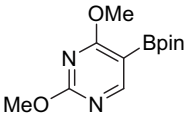
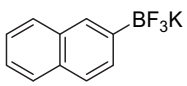
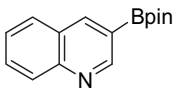
insensitivity to air and moisture. Furthermore the corresponding boronic acids did not react with compound **1** under the conditions used.



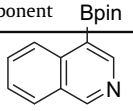
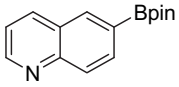
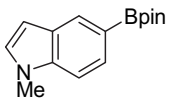
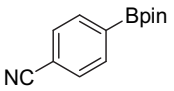
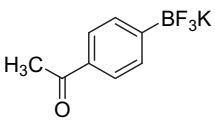
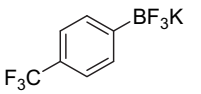
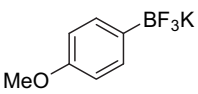
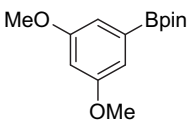
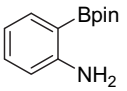
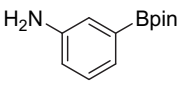
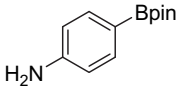
i) $PdCl_2(dppf).CH_2Cl_2$ (1:1) (2 mol%), K_2CO_3 (6 equiv.), DME/H_2O (3:1), 1-3 h, 90 °C.

Table 1
Boronated coupling components, 6-(hetero)arylthieno[3,2-*b*]pyridines **2a-v** product yields and GI_{50} values presented by compounds **2** in three human tumor cell lines.

Boronated component	Biheteroaryl (η)	GI_{50} (μM)[a]		
		MCF-7	A375-C5	NCI-H460
	**2a**, 80%	12.7 ± 2.0	12.1 ± 1.7	12.4 ± 2.2
	**2b**, 70%	26.9 ± 2.7	> 150.0	18.0 ± 5.0
	**2c**, 72%	1.0 ± 0.1	> 75.0[b]	0.7 ± 0.03
	**2d**, 76%	> 120.0[b]	> 120.0[b]	> 120.0[b]
	**2e**, 72%	> 150.0	> 150.0	> 150.0
	**2f**, 88%	> 112.3[b]	> 112.3[b]	> 112.3[b]
	**2g**, 90%	> 37.5[b]	> 37.5[b]	> 37.5[b]
	**2h**, 82%	ND[c]	ND[c]	ND[c]
	**2i**, 88%	> 75.0[b]	> 75.0[b]	> 75.0[b]
	**2j**, 88%	50.9 ± 2.3	95.6 ± 12.8	43.5 ± 1.7
	**2k**, 91%	9.8 ± 1.7	9.1 ± 1.7	9.5 ± 1.7

(*continued on next page*)

Table 1 (*continued*)

Boronated component	Biheteroaryl (η)	GI_{50} (μM)[a]		
		MCF-7	A375-C5	NCI-H460
	**2l**, 81%	10.7 ± 1.8	10.1 ± 2.7	7.7 ± 1.1
	**2m**, 80%	> 150.0	> 150.0	> 150.0
	**2n**, 82%	23.0 ± 2.5	21.2 ± 4.4	20.3 ± 3.0
	**2o**, 80%	> 75.0[b]	> 75.0[b]	> 75.0[b]
	**2p**, 90%	> 150.0	> 150.0	> 150.0
	**2q**, 75%	> 150.0	> 150.0	> 150.0
	**2r**, 70%	> 150.0	> 150.0	> 150.0
	**2s**, 70%	56.6 ± 3.2	82.0 ± 3.7	31.8 ± 5.2
	**2t**, 84%	4.2 ± 0.1	2.5 ± 0.2	3.8 ± 1.1
	**2u**, 75%	> 150.0	> 150.0	> 150.0
	**2v**, 65%	107.8 ± 2.7	80.8 ± 11.1	37.1 ± 4.4

*This compound was obtained as an intermediate in the step 1 of Scheme 1.

[a] The lowest concentrations causing 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 48 h, expressed as means ± SD of three independent experiments performed in duplicate.

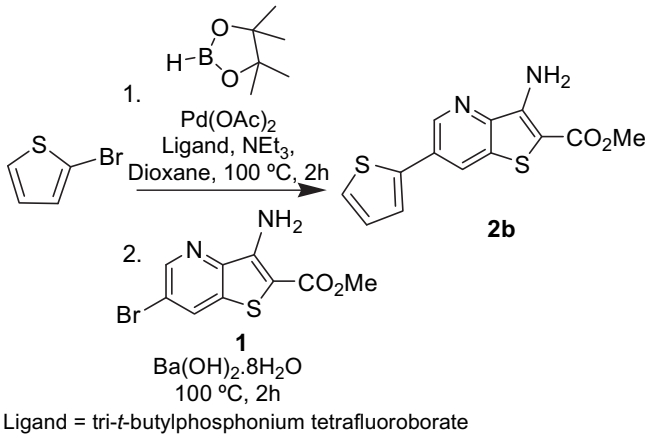
[b] Due to limited solubility of the compound in DMSO, the maximum concentration tested was lower than 150 μM.

[c] The compound precipitated when a solution in DMSO was added to the cell culture medium. Doxorubicin was used as positive control (GI_{50}: MCF-7 = 43.3 ± 2.6 nM; A375-C5 = 130.2 ± 10.1 nM; NCI-H460 = 35.6 ± 1.6 nM).

The 6-(hetero)arylthieno[3,2-*b*]pyridines **2a**–**v** were obtained in high to excellent yields (65–91%) using the reaction conditions depicted in Table 1. We have already successfully applied these conditions to the synthesis of dehydroamino acid derivatives by Suzuki–Miyaura cross-couplings of β,β-dibromodehydroalanine and β-bromodehydrophenylalanine derivatives with (hetero)aryl boronated compounds [9,10]. Thus the conditions are rather general due to the different types of brominated substrates that can be used in the Suzuki–Miyaura coupling.

For the synthesis of compound **2b** neither the 2-thienyl pinacolborane ester nor the corresponding potassium trifluoroborate

salt are commercially available so it was decided to perform a one pot two steps, borylation with pinacolborane followed by Suzuki coupling (BSC) with the brominated thieno[3,2-*b*]pyridine **1** (Scheme 1). Due to the electron-rich character of the thiophene ring, compound **2b** was only obtained using tri-*t*-butylphosphonium tetrafluoroborate (*t*-Bu_3PHBF_4) [11] as the ligand, in 70% yield (Scheme 1). To our knowledge it is the first time that this ligand was used in a BSC reaction.



Scheme 1. Synthesis of compound **2b** by a BSC reaction.

2.2. Effect on the growth of human tumor cell lines

The *in vitro* growth inhibitory activity of all the compounds prepared was evaluated using three human tumor cell lines, representing different tumor models, namely breast adenocarcinoma (MCF-7), melanoma (A375-C5), and non-small cell lung cancer (NCI-H460). For all cell lines, the results were obtained after 48 h of continuous exposure to the compounds. The results are summarized in Table 1.

From the analysis of Table 1 it is possible to establish some structure–activity relationships (SARs). Comparing the thienopyridines **2a**–**2e**, bearing five-member rings, compound **2a** (with a thiophene ring linked at the 3-position) shows a low GI_{50} (around 12 μM) for all the tumor cell lines tested, while compound **2b** (with a thiophene ring linked at the 2-position) shows a moderately low GI_{50} only for MCF-7 and NCI-H460 cell lines. Compound **2c** (with a 2,2′-bithiophene system) presents the lowest GI_{50} values selectively for MCF-7 and NCI-H460 cell lines (1.0 and 0.7 μM, respectively). In turn, the thienopyridines bearing a furan linked at the 3-position, **2d**, and a *N*-methylpyrazole linked at the 4-position, **2e**, did not show any activity at the tested concentrations.

Assays with the pyridine and pyrimidine derivatives **2f**–**2i** were technically difficult to perform due to problems related to solubility in DMSO or, in the case of **2h**, related to precipitation when the DMSO solution was added to the aqueous cell culture medium. These compounds did not show any activity at the tested concentrations.

In the series of the thienopyridines substituted with naphthalene, quinolines and an isoquinoline (**2j**–**2m**), interesting results were obtained. The naphthalene derivative **2j** presents GI_{50} values approaching 50 μM for MCF-7 and NCI-H460 cell lines, but a lower activity against A375-C5 cells was registered. The quinoline derivative **2k** (linked by the 3-position of the quinoline to the thienopyridine moiety) and the isoquinoline derivative **2l** (linked by the 4-position) both present low GI_{50} values against all the cell lines studied (below 10 μM). This was in striking contrast to what was observed for the quinoline derivative **2m** (linked by the 6-position of the quinoline), for which no activity was found at the maximum tested concentration. The position of the C–C linkage in the nitrogenated system of these compounds seems to be important for the antitumoral activity. The *N*-methylindole **2n** (linked by the 5-position of the indole) was active against the three cell lines, presenting moderately low GI_{50} values (slightly over 20 μM).

The *p*-substituted phenyl derivatives, either with electron-donating or electron-withdrawing groups, **2o**–**r**, were not active at the concentrations tested. The dimethoxy derivative **2s**, in turn, showed activity against the MCF-7 and NCI-H460 cell lines, with GI_{50} values from 30 to 60 μM, but were less active against A375-C5 cells (GI_{50} over 80 μM). Among the aryl derivatives tested, the 2-aminophenyl derivative **2t** presented the lowest GI_{50} values (below 5 μM) against all the tumor cell lines tested, while the 3-aminophenyl derivative **2u** was not active at the tested concentrations. The 4-aminophenyl derivative **2v** showed only moderately low GI_{50} values against NCI-H460 cell lines (around 37 μM).

In addition, analysis of the growth inhibition curves allowed to conclude that all the active compounds inhibited the growth of the human tumor cell lines in a dose-dependent manner (data not shown).

2.3. Cell cycle analysis

The most active compounds, **2c** and **2t**, were selected to be further studied regarding their influence on the cell cycle distribution of the NCI-H460 cells. With this purpose, cells were incubated for 48 h with the previously determined GI_{50} concentrations of the compounds (from Table 1) and also with twice the GI_{50} concentration. The reason for using both these concentrations was to increase the possibility of detecting the effect on the cell cycle, given the fact that the GI_{50} was determined with an assay that detects alterations in protein content, not in cellular viability. Flow cytometry results showed that both compounds interfere with the normal cell cycle distribution of this cell line. When analyzing the effect of the highest concentration tested (twice the GI_{50}), compound **2c** caused 15% increase in the percentage of cells in G1 and 15% reduction in the percentage of cells in the S-phase of the cell cycle (Fig. 1). Upon incubation with compound **2t** (with twice the GI_{50}) there was a reduction in the percentage of cells in the S-phase of about 10% and an increase of cells in the G2/M phases of about 12% (Fig. 1). Therefore, compound **2c** seems to induce a cell

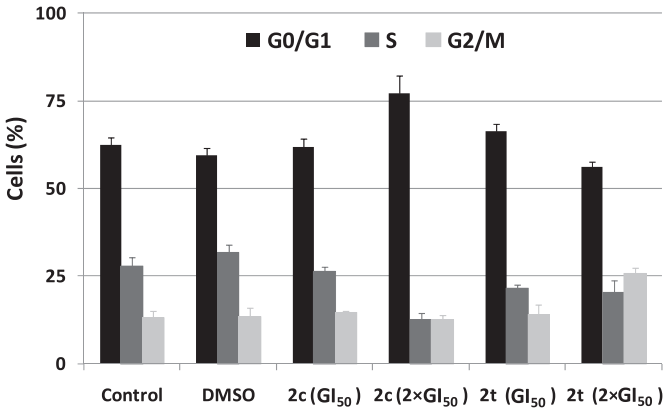


Fig. 1. Cell cycle analysis of NCI-H460 cells treated for 48 h with compounds 2c or 2t at their GI_{50} or 2 × GI_{50} concentrations (0.7 or 1.4 μM and 3.8 or 7.6 μM, respectively). Untreated cells were used as the control and a solvent control was also included (DMSO). Results are the mean ± SD of three independent experiments performed in duplicate.

cycle arrest on phases G0/G1, whereas compound **2t** appears to cause a G2/M cell cycle arrest. According to these findings, the compounds tested probably present different mechanisms of action.

3. Conclusions

We were able to synthesize a large variety of new methyl 6-(hetero)aryl-3-aminothieno[3,2-*b*]pyridine-2-carboxylates in high to excellent yields (65–91%) by Suzuki–Miyaura cross-couplings of the methyl 6-bromo-3-aminothieno[3,2-*b*]pyridine-2-carboxylate with (hetero)aryl pinacolborane esters or potassium trifluoroborates using mild and general conditions. The growth inhibitory effect of the coupling products was evaluated against three human tumor cell lines and some SARs were established. The most active compounds were shown to be: **2c** (a 2,2′-bithiophene derivative) with selectivity against MCF-7 and NCI-H460 cell lines presenting very low GI_{50} values (1 and 0.7 μM, respectively), and the *o*-aminophenyl derivative **2t**, which displays GI_{50} values below 5 μM for the three cell lines studied. Other compounds presented very good activity, such as the quinoline and isoquinoline derivatives, **2k** and **2l** respectively, with the C–C bond in the nitrogenated ring and with GI_{50} values between 6 and 12 μM, and the thiophene derivative **2a**, with GI_{50} values between 10 and 14 μM. The influence of the most active compounds, **2c** and **2t**, on the cell cycle distribution was evaluated in the NCI-H460 cell line. Compound **2c** caused a cell cycle arrest in the G0/G1 phases, while compound **2t** caused a G2/M arrest, thus pointing out to different cellular mechanisms of action. Based on the current results it is worthwhile to pursue the lead **2c** for further optimization.

4. Experimental

4.1. Synthesis

Melting points (°C) were determined in a SMP3 Stuart apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Plus at 300 and 75.4 MHz, and on a Bruker Avance at 400 and 100.6 MHz, respectively. Heteronuclear correlations, $^1H-^{13}C$, HMQC or HSQC and HMBC were performed to attribute some signals. MS (EI) and HRMS data were recorded by the mass spectrometry service of the University of Vigo, Spain. Elemental analysis was performed on a LECO CHNS 932 elemental analyser. The reactions were monitored by thin layer chromatography (TLC). Dry flash was performed on Macherey–Nagel silica gel 150–230 mesh. Petroleum ether refers to the boiling range 40–60 °C. Ether refers to diethylether. DME refers to 1,2-dimethoxyethane. The catalyst $PdCl_2(dppf).CH_2Cl_2$ (1:1) refers to 1,1′-bis(diphenylphosphino)ferrocene dichloropalladium (II), complex with dichloromethane (1:1) and was purchased from Aldrich.

4.1.1. General procedure for the Suzuki–Myaura coupling (except for **2b**)

In a round flask, DME (3 mL) and water (1 mL), the 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate (**1**) (hetero)aryl boronic acid pinacol ester or potassium trifluoroborate (1.2 equiv.), $PdCl_2(dppf).CH_2Cl_2$ (1:1) (2 mol%), K_2CO_3 (6 equiv.) were added and the mixture was heated with stirring at 90 °C for some hours (following the reaction by TLC). After cooling, the solvents were evaporated under reduced pressure giving an oily solid which was submitted to a dry flash column chromatography (silica 150–230 mesh) using ethyl acetate.

*4.1.1.1. Methyl 3-amino-6-(thien-3-yl)thieno[3,2-b]pyridine-2-carboxylate (**2a**).* Compound **1** (150 mg, 0.540 mmol), potassium 3-thiophenetrifluoroborate (123 mg, 0.648 mmol) and heating for 3 h gave compound **2a** as a yellow solid (123 mg, 80%), m.p. 159–161 °C, after some washes with petroleum ether. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.93 (3H, s, OMe), 6.23 (2H, broad s, NH_2), 7.44–7.51 (2H, m, Ar–H), 7.61–7.62 (1H, m, Ar–H), 8.19 (1H, d, $J = 2.0$ Hz, Ar–H), 8.88 (1H, d, $J = 2.0$ Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.66 (OMe), 99.74 (C), 122.14 (CH), 126.02 (CH), 127.28 (CH), 127.92 (CH), 130.62 (C), 134.63 (C), 138.43 (C), 145.30 (CH), 147.55 (C), 165.33 (C=O) ppm. Anal. calcd. for $C_{13}H_{10}N_2O_2S_2$: C, 53.77; H, 3.47; N, 9.65; S, 22.10%; found: C, 53.73; H, 3.51; N, 9.56; S, 22.50%.

*4.1.1.2. Methyl 3-amino-6-(thien-2-yl)thieno[3,2-b]pyridine-2-carboxylate (**2b**).* A dried Schlenk tube was charged under argon with dry dioxane (3 mL), 2-bromothiophene (150 mg, 0.1 mL, 0.900 mmol), $Pd(OAc)_2$ (5 mol%), tri-*t*-butylphosphonium tetrafluoroborate (20 mol %), pinacolborane (3 equiv.) NEt_3 (4 equiv.). The mixture was left stirring for 2 h at 100 °C. After cooling compound **1** (1 equiv.) and $BaOH.8H_2O$ (3 equiv.) were added. The mixture was left stirring for more 2 h at 100 °C giving compound **2b** as a yellow solid (102 mg, 70%), after the dry flash referred in the general procedure, m.p. 231–233 °C, after some washes with petroleum ether. ^1H NMR (DMSO-d_6, 300 MHz): δ 3.82 (3H, s, OMe), 6.90 (2H, br s, NH_2), 7.20–7.24 (1H, m, Ar–H), 7.69–7.72 (1H, m, Ar–H), 7.77–7.79 (1H, m, Ar–H), 8.65 (1H, d, $J = 2.0$ Hz, Ar–H), 8.99 (1H, d, $J = 2$ Hz, Ar–H) ppm. ^{13}C NMR (DMSO-d_6, 75.4 MHz): δ 51.54 (OMe), 97.36 (C), 126.00 (CH), 127.38 (CH), 127.69 (CH), 128.88 (CH), 128.99 (C), 134.03 (C), 139.30 (C), 144.14 (CH), 145.06 (C), 147.88 (C), 164.50 (C=O) ppm. MS (EI): *m/z* (%) 290 (M^+, 100), 258 (80), 230 (27). HRMS M^+ calcd. for $C_{13}H_{10}N_2O_2S_2$ 290.0184, found 290.0194.

*4.1.1.3. Methyl 3-amino-6-(2,2′-bithenyl-3-yl)thieno[3,2-b]pyridine-2-carboxylate (**2c**).* Compound **1** (150 mg, 0.500 mmol), 2,2′-bithiophene-5-boronic acid pinacol ester (190 mg, 0.648 mmol) and heating for 2 h gave compound **2c** as a yellow solid (144 mg, 72%), m.p. 218–219 °C, after some washes with petroleum ether. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.93 (3H, s, OMe), 6.29 (2H, broad s, NH_2), 7.05–7.08 (1H, m, Ar–H), 7.22 (1H, d, $J = 4.0$ Hz, Ar–H), 7.25–7.29 (2H, m, Ar–H), 7.38 (1H, d, $J = 4.0$ Hz, Ar–H), 8.19 (1H, d, $J = 2.0$ Hz, Ar–H), 8.87 (1H, d, $J = 2.0$ Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.74 (OMe), 100.28 (C), 124.34 (CH), 124.90 (CH), 125.17 (CH), 125.74 (CH), 127.22 (CH), 128.03 (CH), 129.20 (C), 134.87 (C), 136.65 (C), 138.21 (C), 138.93 (C), 143.53 (CH), 144.65 (C), 147.15 (C), 165.16 (C=O) ppm. Anal. calcd. for $C_{17}H_{12}N_2O_2S_3$: C, 54.82; H, 3.25; N, 7.52; S, 25.83%; found: C, 54.44; H, 3.23; N, 7.31; S, 26.23%.

*4.1.1.4. Methyl 3-amino-6-(fur-3-yl)thieno[3,2-b]pyridine-2-carboxylate (**2d**).* Compound **1** (150 mg, 0.500 mmol), 3-furanboronic acid pinacol ester (126 mg, 0.648 mmol) and heating for 2 h gave compound **2d** as a yellow solid (112 mg, 76%), m.p. 170–171 °C, after some washes with petroleum ether. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.92 (3H, s, OMe), 6.24 (2H, broad s, NH_2), 6.77–6.78 (1H, m, Ar–H), 7.56–7.57 (1H, m, Ar–H), 7.87–7.88 (1H, m, Ar–H), 8.09 (1H, d, $J = 2.0$ Hz, Ar–H), 8.76 (1H, d, $J = 2.0$ Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.66 (OMe), 99.60 (C), 108.55 (CH), 123.12 (C), 127.40 (CH), 127.55 (C), 134.70 (C), 139.43 (CH), 144.41 (CH), 144.55 (C), 145.08 (C), 147.47 (C), 165.29 (C=O) ppm. Anal. calcd. for $C_{13}H_{10}N_2O_3S$: C, 57.00; H, 3.67; N, 10.21; S, 11.69%; found: C, 57.10; H, 3.89; N, 10.12; S, 12.10%.

*4.1.1.5. Methyl 3-amino-6-(1-methyl-1H-pyrazol-4-yl)thieno[3,2-b]pyridine-2-carboxylate (**2e**).* Compound **1** (150 mg, 0.500 mmol), 1-methylpyrazole-4-boronic acid pinacol ester (135 mg, 0.648 mmol) and heating for 2 h gave compound **2e** as a yellow solid (112 mg, 72%), m.p. 231–233 °C, after some washes with petroleum ether. ^1H NMR ($CDCl_3$, 400 MHz): δ 3.92 (3H, s, OMe), 4.00 (3H, s, NMe), 6.22

(2H, broad s, NH_2), 7.50 (1H, s, Ar–H), 7.86 (1H, s, Ar–H), 8.08 (1H, d, J = 2.0 Hz, Ar–H), 8.75 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 39.30 (NMe), 51.64 (OMe), 99.28 (C), 119.51 (C), 126.69 (CH), 127.47 (CH), 127.84 (C), 134.83 (C), 137.02 (CH), 144.47 (CH), 144.72 (C), 147.59 (C), 165.36 (C=O) ppm. MS (EI): *m*/*z* (%) 288 (M^+, 100), 256 (69), 228 (35). HRMS M^+ calcd. for $C_{13}H_{12}N_4O_2S$ 288.0681, found 288.0682.

*4.1.1.6. Methyl 3-amino-6-(pyrid-3-yl)thieno[3,2-b]pyridine-2-carboxylate (**2f**).* Compound **1** (150 mg, 0.540 mmol), 3-pyridineboronic acid pinacol ester (133 mg, 0.648 mmol) and heating for 3 h gave compound **2f** as a yellow solid (135 mg, 88%), m.p. 210–212 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.94 (3H, s, OMe), 6.26 (2H, broad s, NH_2), 7.44–7.48 (1H, m, Ar–H), 7.94–7.97 (1H, m, Ar–H), 8.23 (1H, d, J = 2.0 Hz, Ar–H), 8.69–8.71 (1H, m, Ar–H), 8.84 (1H, d, J = 2.0 Hz, Ar–H), 8.92 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.76 (OMe), 100.44 (C), 123.87 (CH), 129.26 (CH), 132.50 (C), 133.31 (C), 134.59 (C), 134.71 (CH), 145.45 (CH), 146.24 (C), 147.35 (C), 148.40 (CH), 149.58 (CH), 165.24 (C=O) ppm. MS (EI): *m*/*z* (%) 285 (M^+, 100), 253 (71), 225 (34). HRMS M^+ calcd. for $C_{14}H_{11}N_3O_2S$: 285.0572, found 285.0571.

*4.1.1.7. Methyl 3-amino-6-(6-methoxypyrid-3-yl)thieno[3,2-b]pyridine-2-carboxylate (**2g**).* Compound **1** (150 mg, 0.500 mmol), 2-methoxypyridine-5-boronic acid pinacol ester (150 mg, 0.648 mmol) and heating for 1 h gave compound **2g** as a yellow solid (154 mg, 90%), m.p. 224–226 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.93 (3H, s, OMe), 4.02 (3H, s, 6′-OMe), 6.34 (2H, broad s, NH_2), 6.90 (1H, d, J = 9.0 Hz, 5′-H), 7.84 (1H, dd, J = 9.0 and 2.4 Hz, 4′-H), 8.19 (1H, d, J = 2.0 Hz, Ar–H), 8.46 (1H, dd, J = 2.4 and 0.4 Hz, 2′-H), 8.79 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.77 (OMe), 53.80 (6′-OMe), 100.31 (C), 111.54 (5′-CH), 126.19 (C), 129.11 (CH), 132.77 (C), 134.91 (C), 137.48 (4′-CH), 144.44 (CH), 144.79 (C), 145.47 (2′-CH), 147.01 (C), 164.41 (C–O), 165.17 (C=O) ppm. Anal. Calcd for $C_{15}H_{13}N_3O_3S$: C, 57.13; H, 4.16; N, 13.33; S, 10.17%; found: C, 56.73; H, 3.82; N, 13.10; S, 9.96%.

*4.1.1.8. Methyl 3-amino-6-(2-fluoropyridin-5-yl)thieno[3,2-b]pyridine-2-carboxylate (**2h**).* Compound **1** (150 mg, 0.500 mmol), 2-fluoropyridine-5-boronic acid pinacol ester (143 mg, 0.648 mmol) and heating for 1 h gave compound **2h** as a yellow solid (135 mg, 82%), m.p. 232–234 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.94 (3H, s, OMe), 6.26 (2H, broad s, NH_2), 7.11 (1H, m, Ar–H), 8.01 (1H, m, Ar–H), 8.20 (1H, d, J = 2.0 Hz, Ar–H), 8.51–8.52 (1H, m, Ar–H), 8.80 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.80 (OMe), 100.66 (C), 110.12 (CH, d, J = 37.0 Hz), 129.34 (CH), 130.42 (C), 134.64 (C), 140.02 (CH, d, J = 9.0 Hz), 145.08 (CH), 146.13 (C), 146.28 (CH, d, J = 16.0 Hz), 147.20 (C), 163.72 (CF, d, J = 240.0 Hz), 165.18 (C=O) ppm. Anal. Calcd. for $C_{14}H_{10}FN_3O_2S$: C, 55.44; H, 3.32; N, 13.85; S, 10.57%; found: C, 55.60; H, 3.22; N, 13.72; S, 10.81%.

*4.1.1.9. Methyl 3-amino-6-(2,4-dimethoxypyrimid-5-yl)thieno[3,2-b]pyridine-2-carboxylate (**2i**).* Compound **1** (150 mg, 0.500 mmol), 2,4-dimethoxypyrimidine-5-boronic acid pinacol ester (147 mg, 0.648 mmol) and heating for 1 h gave compound **2i** as a yellow solid (161 mg, 88%), m.p. 239–241 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.93 (3H, s, OMe), 4.07 (6H, s, 2 × OMe), 6.24 (2H, broad s, NH_2), 8.18 (1H, d, J = 2 Hz, Ar-H), 8.36 (1H, s, 6′-H) 8.73 (1H, d, J = 2.0 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.69 (CO_2*Me*), 54.38 (OMe), 55.09 (OMe), 100.11 (C), 112.68 (C), 128.31 (C), 130.75 (CH), 134.14 (C), 145.57 (C), 146.61 (6′-CH), 147.39 (C), 157.93 (CH), 165.21 (C), 165.30 (C), 168.25 (C) ppm. Anal. Calcd for $C_{15}H_{14}N_4O_4S$: C, 52.02; H, 4.07; N, 16.18; S, 9.26%; found: C, 52.32; H, 4.01; N, 15.98; S, 9.65%.

*4.1.1.10. Methyl 3-amino-6-(naphthalen-2-yl)thieno[3,2-b]pyridine-2-carboxylate (**2j**).* Compound **1** (150 mg, 0.500 mmol), potassium 2-naphthalenetrifluoroborate (152 mg, 0.648 mmol) and heating for 2 h gave compound **2j** as a yellow solid (159 mg, 88%), m.p. 161–163 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.94 (3H, s, OMe), 6.34 (2H, broad s, NH_2), 7.53–7.58 (2H, m, Ar–H), 7.76 (1H, dd, J = 8.6 and 2.0 Hz, Ar–H), 7.89–7.95 (2H, m, Ar–H), 7.99 (1H, d, J = 8.6 Hz, Ar–H), 8.11 (1H, d, J = 2.0 Hz, Ar–H), 8.35 (1H, d, J = 2.0 Hz, Ar–H), 8.98 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.71 (OMe), 100.17 (C), 124.98 (CH), 126.66 (CH), 126.75 (CH), 126.80 (CH), 127.73 (CH), 128.29 (CH), 129.12 (CH), 129.60 (CH) 133.01 (C), 133.55 (C), 134.49 (C), 134.79 (C), 135.79 (C), 145.04 (C), 145.60 (CH), 147.30 (C), 165.27 (C=O) ppm. MS (EI): *m*/*z* (%) 334 (M^+, 100), 302 (65), 274 (31). HRMS M^+ calcd. for $C_{19}H_{14}N_2O_2S$ 334.0776, found 334.0777.

*4.1.1.11. Methyl 3-amino-6-(quinolin-3-yl)thieno[3,2-b]pyridine-2-carboxylate (**2k**).* Compound **1** (150 mg, 0.500 mmol), quinoline-3-boronic acid pinacol ester (165 mg, 0.648 mmol) and heating for 1 h, gave compound **2k** as a yellow solid (164 mg, 91%), m.p. 222–224 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.93 (3H, s, OMe), 6.27 (2H, broad s, NH_2), 7.63–7.66 (1H, m, Ar–H), 7.77–7.81 (1H, m, Ar–H) 7.93 (1H, d, J = 8.0 Hz, Ar–H), 8.19 (1H, d, J = 8.0 Hz, Ar–H), 8.35 (1H, d, J = 2.0 Hz, Ar–H), 8.40 (1H, d, J = 2.0 Hz, Ar–H), 8.96 (1H, d, J = 2.0 Hz, Ar–H), 9.22 (1H, broad s, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.74 (OMe), 100.47 (C), 127.52 (CH), 127.77 (C), 128.08 (CH), 129.34 (CH), 129,36 (CH), 130.22 (CH), 130.34 (C), 132.53 (C), 134.10 (CH), 134.63 (C), 145.66 (CH), 146.15 (C), 147.37 (C), 147.67 (C), 149.16 (CH), 165.23 (C=O) ppm. MS (EI): *m*/*z* (%) 335 (M^+, 100), 303 (75), 275 (31). HRMS M^+ calcd. for $C_{18}H_{13}N_3O_2S$ 335.0728, found 335.0732.

*4.1.1.12. Methyl 3-amino-6-(isoquinolin-4-yl)thieno[3,2-b]pyridine-2-carboxylate (**2l**).* Compound **1** (150 mg, 0.500 mmol), isoquinoline-4-boronic acid pinacol ester (165 mg, 0.648 mmol) and heating for 1 h, gave compound **2l** as a yellow solid (155 mg, 81%), m.p. 213–215 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.95 (3H, s, OMe), 6.31 (2H, broad s, NH_2), 7.73–7.80 (1H, m, Ar–H), 7.87 (1H, d, J = 8.0 Hz, Ar–H) 8.14 (1H, d, J = 8.0 Hz, Ar–H), 8.21 (1H, d, J = 1.6 Hz, Ar–H), 8.56 (1H, broad s, Ar–H), 8.77 (1H, d, J = 1.6 Hz, Ar–H), 9.39 (1H, broad s, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.78 (OMe), 100.47 (C), 124.12 (CH), 128.04 (CH), 128.34 (C), 128.48 (CH), 129.95 (C), 131.47 (C), 131.84 (CH), 132.12 (CH), 134.30 (C), 134.34 (C), 142.22 (CH), 146.30 (C), 147.42 (C), 147.56 (CH), 152.43 (CH), 165.28 (C=O) ppm. Anal. Calcd for $C_{18}H_{13}N_3O_2S$: C, 64.46; H, 3.91; N, 12.53; S, 9.56%; found: C, 64.17; H, 3.90; N, 12.29; S, 9.32%.

*4.1.1.13. Methyl 3-amino-6-(quinolin-6-yl)thieno[3,2-b]pyridine-2-carboxylate (**2m**).* Compound **1** (150 mg, 0.500 mmol), quinoline-6-boronic acid pinacol ester (165 mg, 0.648 mmol) and heating for 2 h, gave compound **2m** as a yellow solid (145 mg, 80%), m.p. 174–175 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.94 (3H, s, OMe), 6.27 (2H, broad s, NH_2), 7.48–7.52 (1H, m, Ar–H), 8.02–8.10 (2H, m, Ar–H), 8.26–8.35 (3H, m, Ar–H), 8.97–8.99 (2H, m, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 51.74 (OMe), 100.21 (C), 121.90 (CH), 126.37 (CH), 128.47 (C), 128.83 (CH), 129.40 (CH), 130.55 (CH), 134.59 (C), 134.94 (C), 135.65 (C), 136.40 (CH), 145.89 (C), 145.94 (CH), 147.45 (C), 147.83 (C), 151.00 (CH), 165.29 (C=O) ppm. MS (EI): *m*/*z* (%) 335

(M^+, 100), 303 (71) 275 (28). HRMS M^+ calcd. for $C_{18}H_{13}N_3O_2S$ 335.0728, found 335.0727.

*4.1.1.14. Methyl 3-amino-6-(1-methyl-1H-indol-5-yl)thieno[3,2-b] pyridine-2-carboxylate (**2n**).* Compound **1** (150 mg, 0.500 mmol), 1-methylindole-5-boronic acid pinacol ester (167 mg, 0.648 mmol) and heating for 2 h, gave compound **2n** as a yellow solid (153 mg, 82%), m.p. 197–198 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.86 (3H, s, NMe), 3.94 (3H, s, OMe), 6.26 (2H, broad s, NH_2), 6.58–6.59 (1H, m, Ar–H), 7.14 (1H, d, J = 2.8 Hz, Ar–H), 7.44–7.53 (2H, m, Ar–H), 7.90–7.91 (1H, m, Ar–H), 8.25 (1H, d, J = 2.0 Hz, Ar–H), 8.93 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 33.00 (NMe), 51.62 (OMe), 99.26 (C), 101.58 (CH), 110.01 (CH), 120.02 (CH), 121.24 (CH), 128.79 (CH), 129.16 (C), 130.04 (C), 134.70 (C), 136.71 (C), 137.40 (C), 144.80 (C), 146.40 (CH), 147.74 (C), 165.47 (C=O) ppm. Anal. Calcd for $C_{18}H_{15}N_3O_2S$: C, 64.10; H, 4.48; N, 12.45; S, 9.50%; found: C, 63.70; H, 4.67; N, 12.10; S, 9.46%.

*4.1.1.15. Methyl 3-amino-6-(4-cyanopheny)thieno[3,2-b]pyridine-2-carboxylate (**2o**).* Compound **1** (150 mg, 0.500 mmol), 4-cyanophenylboronic acid pinacol ester (148 mg, 0.648 mmol) and heating for 1 h, gave compound **2o** as a yellow solid (133 mg, 80%), m.p. 285–286 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.95 (3H, s, OMe), 6.33 (2H, broad s, NH_2), 7.80 (2H, d, J = 8.4 Hz, 2 × Ar–H), 7.83 (2H, d, J = 8.4 Hz, 2 × Ar–H), 8.28 (1H, d, J = 2.0 Hz, HetAr–H), 8.85 (1H, d, J = 2.0 Hz, HetAr–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.85 (OMe), 101.14 (C), 112.38 (C), 118.38 (C), 128.10 (2 × CH), 129.97 (CH), 133.04 (2 × CH), 133.77 (C), 134.73 (C), 141.78 (C), 144.90 (CH), 146.99 (C), 165.09 (C=O) ppm. MS (EI): m/z (%) 309 (M^+, 100), 277 (99) 249 (33), HRMS M^+ calcd. for $C_{16}H_{11}N_3O_2S$ 309.0572, found 309.0580.

*4.1.1.16. Methyl 6-(4-acetylphenyl)-3-aminothieno[3,2-b]pyridine-2-carboxylate (**2p**).* Compound **1** (150 mg, 0.500 mmol), potassium 4-acetylphenyltrifluoroborate (146 mg, 0.648 mmol), and heating for 2 h, gave compound **2p** as a yellow solid (158 mg, 90%), m.p. 214–216 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.67 (3H, s, Me), 3.94 (3H, s, OMe), 6.35 (2H, broad s, NH_2), 7.75 (2H, d, J = 7.2 Hz, 2′ and 6′-H), 8.10 (2H, d, J = 7.2 Hz, 3′ and 5′-H), 8.30 (1H, broad s, HetAr–H), 8.88 (1H, broad s, HetAr–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 26.70 (Me), 51.79 (OMe), 100.79 (C), 127.60 (2′ and 6′-CH), 129.26 (3′and 5′-CH), 129.88 (CH), 134.50 (C), 134.73 (C), 136.81 (C), 141.65 (C), 145.13 (CH), 145.50 (C), 147.04 (C), 165.12 (C=O), 197.38 (C=O) ppm. Anal. Calcd for $C_{17}H_{14}N_2O_3S$: C, 62.56; H, 4.32; N, 8.58; S, 9.82%; found C, 62.39; H, 4.20; N, 8.53; S, 9.71.

*4.1.1.17. Methyl 3-amino-6-[4-(trifluoromethyl) phenyl]thieno[3,2-b] pyridine-2-carboxylate (**2q**).* Compound **1** (150 mg, 0.500 mmol), potassium 4-(trifluoromethyl)phenyltrifluoroborate (163 mg, 0.648 mmol) and heating for 2 h, gave compound **2q** as a yellow solid (132 mg, 75%), m.p. 162–164 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.94 (3H, s, OMe), 6.28 (2H, broad s, NH_2), 7.74–7.80 (4H, m, Ar–H), 8.24 (1H, d, J = 2.0 Hz, Ar–H), 8.85 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.76 (OMe), 100.59 (C), 124.00 (CF_3, q, J = 273.0 Hz) 126.19 (2 × CH, q, J = 4.0 Hz), 127.80 (2 × CH), 129.53 (CH), 130.56 (C, q, J = 33.0 Hz) 134.31 (C), 134.58 (C), 140.90 (C), 145.44 (CH), 146.03 (C), 147.24 (C), 165.20 (C=O), ppm. MS (EI): m/z (%) 352 (M^+, 100), 320 (88), 292 (34), HRMS M^+ calcd. for $C_{16}H_{11}F_3N_2O_2S$ 352.0493, found 352.0493.

*4.1.1.18. Methyl 3-amino-6-(4-methoxyphenyl)thieno[3,2-b]pyridine-2-carboxylate (**2r**).* Compound **1** (150 mg, 0.500 mmol) and potassium 4- methoxyphenyltrifluoroborate (139 mg, 0.648 mmol), heating for 2 h gave compound **2r** as a yellow solid (115 mg, 70%), m.p. 174–176 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.89 (3H, s, OMe), 3.93 (3H, s, OMe), 6.35 (2H, broad s, NH_2), 7.05 (2H, d, J = 8.8 Hz, 3′ and 5′-H), 7.59 (2H, d, J = 8.8 Hz, 2′ and 6′-H), 8.21 (1H, d, J = 2 Hz, Ar–H), 8.83 (1H, d, J = 2 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.71 (OMe), 55.43 (OMe), 99.89 (C), 114.79 (3′ e 5′-CH), 128.57 (2′ e 6′-CH), 128.93 (CH), 129.48 (C), 132.46 (C), 134.90 (C), 135.61 (C), 144.33 (C), 144.95 (CH), 147.21 (C), 147.96 (C), 160.18 (C), 165.27 (C=O) ppm. MS (EI): m/z (%) 314 (M^+, 100), 282 (61), 254 (29); HRMS M^+ calcd. for $C_{16}H_{14}N_2O_3S$ 314.0727, found 314.0725.

*4.1.1.19. Methyl 3-amino-6-(3,5-dimethoxyphenyl)thieno[3,2-b]pyridine-2-carboxylate (**2s**).* Compound **1** (150 mg, 0.500 mmol) and 3,5- dimethoxyphenylboronic acid pinacol ester (171 mg, 0.648 mmol), heating for 2 h gave compound **2s** as a yellow solid (120 mg, 70%), m.p. 182–184 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.88 (6H, s, 2 × OMe), 3.93 (3H, s, OMe), 6.28 (2H, broad s, NH_2), 6.55 (1H, t, J = 2.1 Hz, 4′-H), 7.76 (2H, d, J = 2.1 Hz, 2′ and 6′-H), 8.20 (1H, d, J = 2.0 Hz, Ar–H), 8.83 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 51.71 (OMe), 55.51 (2 × OMe), 100.02 (C), 100.21 (4′-CH), 105.73 (2′ e 6′-CH), 129.30 (CH), 134.52 (C), 135.85 (C), 139.43 (C), 145.52 (C), 145.68 (CH), 147.38 (C), 161.38 (2 × C), 165.30 (C=O) ppm. MS (EI): m/z (%) 344 (M^+, 100), 312 (63), 284 (25); HRMS M^+ calcd. for $C_{17}H_{16}N_2O_4S$ 344.0831, found 344.0829.

*4.1.1.20. Methyl 3-amino-6-(2-aminophenyl)thieno[3,2-b]pyridine-2-carboxylate (**2t**).* Compound **1** (150 mg, 0.500 mmol) and 2-aminophenylboronic acid pinacol ester (142 mg, 0.648 mmol) heating for 2 h gave compound **2t** as a green solid (130 mg, 84%), m.p. 154–156 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.92 (5H, appt s, OMe and 2′-NH_2), 6.25 (2H, broad s, NH_2), 6.84 (1H, dd, J = 8.0 and 1.2 Hz, 3′-H), 6.92 (1H, appt dt, J = 7.6 and 1.2 Hz, 5′-H), 7.18 (1H, dd, J = 7.6 and 1.6 Hz, 6′-H), 7.22–7.27 (1H, m, 4′-H), 8.18 (1H, d, J = 2.0 Hz, Ar–H), 8.72 (1H, d, J = 2.0 Hz, Ar–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.68 (OMe), 99.93 (C), 116.58 (3′-CH), 119.73 (C), 123.82 (1′-C), 129.70 (4′-CH), 130.74 (6′-CH), 131.39 (CH), 134.10 (C), 134.41 (C), 142.90 (C), 145.92 (C), 147.25 (CH), 147.40 (C), 165.31 (C=O) ppm. Anal. Calcd. for $C_{15}H_{13}N_3O_2S$: C, 60.18; H, 4.38; N, 14.04; S, 10.71%; found C, 60.30; H, 4.34; N, 13.78; S, 10.49%.

*4.1.1.21. Methyl 3-amino-6-(3-aminophenyl)thieno[3,2-b]pyridine-2-carboxylate (**2u**).* Compound **1** (150 mg, 0.500 mmol) and 3-aminophenylboronic acid pinacol ester (142 mg, 0.648 mmol) heating for 2 h gave compound **2u** as a yellow solid (105 mg, 75%), m.p. 198–200 °C, after some washes with petroleum ether. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.82 (3H, s, OMe), 5.24 (2H, br s, 3′-NH_2), 6.65 (1H, dd, J = 8.0 and 1.2 Hz, Ar–H), 6.90–6.96 (4H, m, Ar–H and 3-NH_2), 7.16 (1H, apparent t, J = 7.6 Hz, 5′-H), 8.52 (1H, d, J = 1.6 Hz, Ar–H), 8.85 (1H, d, J = 1.6 Hz, Ar–H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.49 (OMe), 97.11 (C), 112.37 (CH), 114.09 (CH), 114.79 (CH), 128.88 (CH), 129.73 (5′-CH), 133.88 (C), 135.83 (C), 137.26 (C), 145.14 (C), 145.41 (CH), 147.92 (C), 149.36 (C), 164.56 (C=O) ppm. MS (EI): m/z (%) 299 (M^+, 100), 267 (70), 239 (35); HRMS M^+ calcd. for $C_{15}H_{13}N_3O_2S$ 299.0728, found 299.0732.

*4.1.1.22. Methyl 3-amino-6-(4-aminophenyl)thieno[3,2-b]pyridine-2-carboxylate (**2v**).* Compound **1** (150 mg, 0.500 mmol) and 4-aminophenylboronic acid pinacol ester (142 mg, 0.648 mmol) heating for 2 h gave compound **2v** as a yellow solid (94 mg, 65%), m.p. 179–181 °C, after some washes with petroleum ether. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.81 (3H, s, OMe), 5.44 (2H, br s, 4′-NH_2),

6.68 (2H, d, $J = 8.8$ Hz, 3′ and 5′-H), 6.86 (2H, br s, 3-NH_2), 7.54 (2H, d, $J = 8.8$ Hz, 2′ and 6′-H), 8.47 (1H, d, $J = 2.0$ Hz, Ar–H), 8.89 (1H, d, $J = 2.0$ Hz, Ar–H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.41 (OMe), 96.23 (C), 114.28 (3′and 5′-CH), 123.18 (C), 126.72 (CH), 127.90 (2′ and 6′-CH), 134.12 (C), 135.56 (C), 143.88 (C), 144.74 (CH), 148.12 (C), 149.51 (C), 164.61 (C=O) ppm. MS (EI): *m/z* (%) 299 (M^+, 100), 267 (64), 239 (33); HRMS M^+ calcd. for $C_{15}H_{13}N_3O_2S$ 299.0728, found 299.0740.

4.2. In vitro antitumor evaluation

4.2.1. Material and methods

4.2.1.1. Reagents. Fetal bovine serum (FBS), L-glutamine, phosphate buffered saline (PBS) and trypsin were purchased from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was purchased from Cambrex (New Jersey, USA). Acetic acid, dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin, ethylene diamine tetraacetic acid (EDTA), sulforhodamine B (SRB) and trypan blue were purchased from SigmaChemical Co. (Saint Louis, USA). Trichloroacetic acid (TCA) and Tris were sourced from Merck (Darmstadt, Germany).

4.2.1.2. Solutions of the compounds. Stocks solutions of the tested compounds were prepared in DMSO and kept at − 70 °C. Appropriate dilutions were freshly prepared in the test medium just prior to the assays. The effect of the vehicle solvent (DMSO) on the growth of the cell lines was evaluated by exposing untreated control cells to the maximum concentration of DMSO used in the assays (0.25%). No influence was found (data not shown).

4.2.1.3. Cell cultures. Three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) were used. MCF-7 and A375-C5 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK), and NCI-H460 was kindly provided by the National Cancer Institute (NCI, Bethesda, USA). Cell lines were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 5% heat-inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL), at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were obtained by plating 1.5×10^5cells/mL for MCF-7 and 0.75×10^5 cells/mL for A375-C5 and NCI-H460, followed by a 24 h incubation.

4.2.1.4. Growth inhibition assay. The *in vitro* effects on the growth of human tumor cell lines were evaluated according to the procedure adopted by the NCI (USA) in their "*In vitro* Anticancer Drug Discovery Screen", using the protein-binding dye sulforhodamine B (SRB) to assess cell growth [12,13]. Briefly, exponentially growing cells were exposed for 48 h, in 96-well microtiter plates, to five serial dilutions of each test compound, starting from a maximum concentration of 150 μM (when possible). Following this exposure period, adherent cells were fixed *in situ*, washed and stained with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (Bio-tek Instruments Inc., Powerwave XS, Wincoski, USA). Dose-response curves were obtained for each test compound and cell line, and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of the compounds that inhibited 50% of the net cell growth was calculated as described elsewhere [13]. Doxorubicin was tested following the same protocol and used as a positive control.

4.2.1.5. Cell cycle analysis and detection of sub-G1 peak. For cell cycle distribution analysis, H460 cells were plated at 0.75×10^5cells/mL in 6-well plates and left incubating for 24 h. Cells were then incubated with complete medium only (blank), medium with the compound's solvent (control DMSO) or with compounds **2c** or **2t** at their respective GI_{50} or $2 \times GI_{50}$ concentrations (previously determined by the SRB assay). Following 48 h incubation, cells were harvested, fixed in 70% ethanol and subsequently resuspended in PBS containing 0.1 mg/mL RNase A and 5 μg/mL propidium iodide, prior to analysis [14].

Cellular DNA content, for cell cycle distribution analysis, and presence of sub-G1 peaks (suggestive of apoptosis induction) were analyzed by flow cytometry using an Epics XL-MCL Coulter flow cytometer plotting 20,000 events per sample. The percentage of cells in the G1/G0, S, and G2/M phases of the cell cycle was subsequently determined using FlowJo 7.2 software (Tree Star, Inc.).

Three independent experiments were performed in duplicate and results are presented as the mean ± SD of the results obtained.

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