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5-Substituted 2-aminothiophenes as A_1 adenosine receptor allosteric enhancers

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Abstract—Two series of 5-substituted 2-amino-4-(3-trifluoromethylphenyl)thiophenes were prepared and evaluated as allosteric enhancers at the A_1 adenosine receptor (A_1AR). In the 3-benzoyl series, a 5-phenyl group was found to confer the greatest potency (**9a**: ED_{50} = 2.1 μM, AE score = 18%). However, the analogue with no 5-substituent (**6b**: ED_{50} = 15.8 μM, AE score = 77%) proved to be the most efficacious. In the 3-ethoxycarbonyl series, the 5-(4-chlorophenyl) analogue was clearly the most potent and efficacious (**9l**: ED_{50} = 6.6 μM, AE score = 57%). The antagonist activity of all compounds was measured using a [^{3}H]CPX competitive binding assay.

1. Introduction

Adenosine is an important endogenous tissue-protective compound released during ischaemia, hypoxia or inflammation which interacts with extracellular G-protein coupled receptors to regulate adenylyl cyclase, and potassium and calcium ion channels.[1] Four receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) have been defined based on pharmacological properties and molecular cloning. Considerable effort has been directed towards developing therapeutic agents targeting these receptors. Adenosine (marketed as Adenocard™) was approved for use in the treatment of supraventricular tachycardia in the early 1990s.[2] A new synthetic A_1 adenosine receptor (A_1AR) agonist, Tecadenoson™, is in clinical development as an anti-arrhythmic agent,[3] while the mixed A_1/A_2AR agonist, AMP-579, reached phase II clinical trials as a cardioprotective agent.[4] Despite these advances, development of adenosine receptor agonists as therapeutic agents has been limited by side-effects associated with global adenosine receptor modulation and the propensity of agonists to cause receptor desensitization upon prolonged exposure. Such problems need to be addressed in order to take advantage of the enormous therapeutic potential of the adenosine receptor system. In this respect, allosteric modulators may prove clinically valuable, with improved therapeutic indices owing to their site- and event-selective actions.[5] Allosteric enhancers bind to an allosteric site, potentiating responses to agonist binding at the principle binding domain (or orthosteric site). Since adenosine production is highest in ischaemic or hypoxic tissue, enhancers which selectively amplify the actions of endogenous adenosine would be selective for ischaemic tissue. Since extracellular adenosine is very rapidly degraded to inactive metabolites, within the circulation and interstitial compartments, amplifying the effects of endogenous adenosine would localize its actions to those cells undergoing ischaemic stress. Agents inducing such site- and event-specific effects clearly offer a therapeutic advantage.

Routine screening for adenosine antagonists by Parke-Davis identified a series of 2-amino-3-benzoylthiophenes that enhanced agonist radioligand binding at A_1ARs.[6,7] The most effective enhancer in this series was PD81,723 (**1**) which proved highly selective for A_1ARs, having no major effect on agonist binding at the other adenosine receptor subtypes or at the other G-protein coupled receptors that were investigated (M_2 muscarinic, α_2 adrenergic or γ-opiate receptors). The initial structure–activity study performed by Parke-Davis established that the amino and ketone groups were important in

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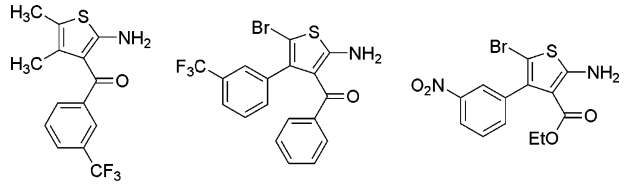
maintaining good activity. More detailed structure–activity relationships of 2-aminothiophene, 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene and 2-amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine cores have subsequently been reported.[8–14]



1 **2** **3**

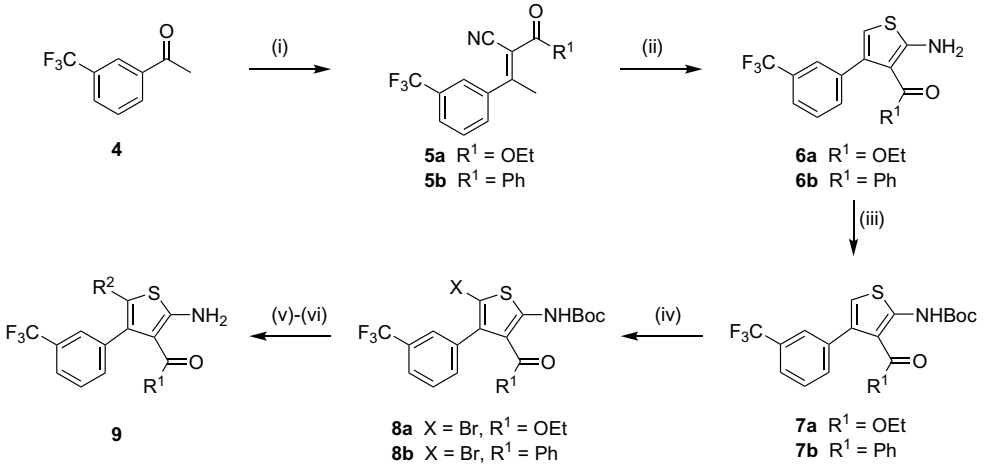
We recently investigated the activity of a series of 2-amino-4,5-diphenylthiophenes and 2-amino-5-bromo-4-phenylthiophenes as A_1AR allosteric enhancers. This work identified compounds that were effective AEs in an in vitro assay that measured the test compounds' ability to stabilize the agonist/A_1AR/G-protein ternary complex, such as [2-amino-5-bromo-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (**2**).[12] 2-Amino-5-bromo-4-(3-nitrophenyl)thiophene-3-carboxylate (**3**) was also found to slow the kinetics of A_1AR agonist dissociation indicating that an ethyl ester in the 3-position can also support AE activity.[12] Herein, we report further investigation of the structure–activity relationships of these classes of interesting lead compounds which focus on the role of the 5-substituent.

2. Results and discussion

2.1. Chemistry

Our previous research on the AE activity of 2-amino-4,5-diphenylthiophenes focused on the structure–activity relationships of the substituent in the 3-position. This work employed a synthetic strategy in which this group was incorporated late in the synthesis, thereby facilitating the preparation of a series of analogues. The 5-substituent was installed in the very first step of this synthesis which is less than ideal for an investigation of the SAR of the 5-position. For this study, an alternative synthetic approach was developed in which the two protected 2-amino-5-bromo-4-phenylthiophenes **8a** and **8b** were employed as key intermediates for the incorporation of substituents in the 5-position via Suzuki–Miyaura cross-coupling reactions[15] prior to a final deprotection step (Scheme 1).

The thiophene core was formed via a two-step Gewald synthesis[16] in which 3′-trifluoromethylacetophenone (**4**) was initially reacted with either benzoylacetonitrile or ethyl cyanoacetate, in the presence of titanium(IV)chloride[17] to afford the corresponding Knoevenagel products **5a** and **5b**, respectively (Scheme 1). These olefins were subsequently cyclised with sulfur under basic conditions to yield the desired 2-aminothiophenes **6a** and **6b** in 91% and 26% yield (over two steps), respectively. Attempts to brominate the 5-position of **6a** or **6b** directly, without first protecting the 2-amino group, resulted in degradation of the starting material to complex mixtures (as evidenced by TLC). Accordingly, compounds **6a** and **6b** were conveniently Boc protected using Boc_2O and catalytic DMAP in dioxane. Heating was required to complete the carbamoylation since no reaction occurred at room temperature. The Boc protected 2-aminothiophenes **7a** and **7b** were able to be smoothly converted to the corresponding 5-bromo analogues **8a** and **8b** using *N*-bromosuccinimide in yields of 43% and 57% (over two steps), respectively. Derivatives **8a** and **8b** were subjected to Suzuki–Miyaura cross-coupling conditions in DMF/H_2O mixtures using tribasic potassium phosphate and catalytic $Pd(PPh_3)_4$ in an inert atmosphere (N_2) with heating (70–80 °C) in a 24-h period or alternatively under microwave irradiation. These conditions provided adequate yields of the Boc protected cross-coupled products after workup. In most cases the cross-coupling reactions did not go to completion even with prolonged reaction times and required the



4 **5a** R^1 = OEt **5b** R^1 = Ph **6a** R^1 = OEt **6b** R^1 = Ph **7a** R^1 = OEt **7b** R^1 = Ph **8a** X = Br, R^1 = OEt **8b** X = Br, R^1 = Ph **9**

Scheme 1. Reagents and conditions: (i) $R^1C(O)CH_2CN$, $TiCl_4$, pyridine, CH_2Cl_2; (ii) sulfur, Et_2NH, EtOH or THF; (iii) Boc_2O, DMAP, dioxane; (iv) NBS, AcOH/CH_2Cl_2; (v) method A: $R^2B(OH)_2$, 3 mol% $Pd[P(Ph)_3]_4$, K_3PO_4, DMF/H_2O. Method B: $R^2B(OH)_2$, 3 mol% $Pd(PPh_3)_2Cl_2$, K_3PO_4, toluene/H_2O, boronic acid, 150 °C MW; (vi) TFA, CH_2Cl_2 or 6 M HCl, EtOH.

addition of more boronic acid and catalyst. The residues obtained were filtered through a silica plug and the resulting crude products were crystallised from MeOH and MeOH/H_2O. Boc deprotection was accomplished by treating cross-coupled products with TFA, CH_2Cl_2 or 6 M HCl, EtOH mixtures. The crude isolated products **9a**–**n** were purified by recrystallisation or column chromatography. Overall yields for the coupling and deprotection sequence ranged from 15% to 67%. Although the final products **9a**–**n** proved to be stable upon standing for long periods when crystalline, varying degrees of degradation were observed in $CDCl_3$ and DMSO-d_6 solutions.

A slightly different route was used for the preparation of compounds **2** and **11**, as the acidic deprotection of the intermediate **8b** resulted in simultaneous removal of bromine in the 5-position (providing **6b**). Phthaloyl protection,[18] bromination and then careful deprotection with hydrazine hydrate at ambient temperature provided the analogues **2** and **11** (Scheme 2). In the case of 5-chloro analogue **11**, there was no observed dehalogenation when treating the intermediate phthaloylated thiophene with hydrazine in excess in a single addition. Yet the 5-bromo derivative **2** could only ever be isolated cleanly when careful addition of hydrazine was employed.

2.2. Pharmacology

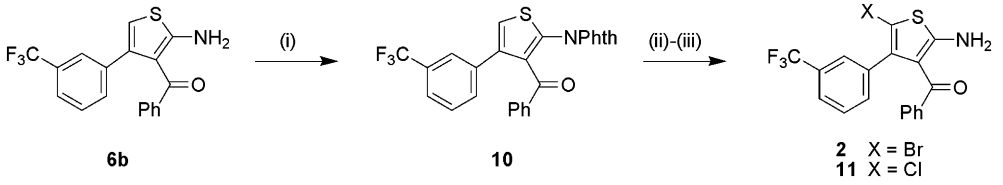
The activity of the target compounds as allosteric enhancers of the human A_1AR was evaluated in a kinetic assay measuring agonist dissociation. In this assay, the A_1AR agonist [^{125}I]4-amino-3-iodo-N^6-benzyladenosine ([^{125}I]IABA) is incubated with membranes from CHO-K1 cells stably expressing the hA_1AR and the ability of the candidate AE to stabilize the agonist–A_1AR–G-protein ternary complex is scored from 0 to 100 as the percentage of the ternary complex remaining after 10 min of dissociation, initiated by the addition of A_1AR antagonist, CPX and GTPγS.[14] A score of 0 corresponds to near complete dissociation of agonist radioligand binding corresponding to the case when no AE is added. A score a 100 would be assigned to an AE that completely blocks radioligand dissociation. Dose–response curves relating this score to enhancer concentration (0.1–50 μM) were fit by hyperbolic equations from which the maximal score, or AE efficacy, was calculated from the fit curve. In cases where the ED_{50} of the AE was >10 μM, the AE efficacy could not be calculated by curve fitting, and the maximum score was estimated by the score at 10 μM AE. Some 2-aminothiophenes have competitive antagonist activity at the orthosteric site that is independent of their allosteric activity. As a result, the antagonist activity of the test compounds was measured relative to [^{3}H]CPX in a competitive binding assay since there is no allosteric effect on antagonist radioligand binding.

Table 1 summarizes the effects of 5-substituted aminothiophenes. The influence of the substituent in the 5-position on AE activity was evaluated in two series, where R^1 is phenyl and ethoxy. In the first series (where R^1 = Ph), the analogue with no 5-substitutent (compound **6b**) proved to be the most effective allosteric enhancer with an AE score of 77%. A range of substituted phenyl groups in the 5-position were also evaluated and all of these compounds had modest maximal AE scores ranging from 5% to 24%. As observed previously, efficacious compounds were not always highly potent. In this series the 5-phenyl analogue **9a** has an ED_{50} value of 2.3 ± 0.8 μM compared with 15.8 ± 2.8 μM for the more efficacious compound **6b**.

A contrasting situation was observed for the second series comprised of ethyl 2-amino-4-(3-trifluoromethylphenyl)thiophene-3-carboxylates (R^1 = OEt). In this series, the analogue with no substituent in the 5-position (compound **6a**) was less effective than all of the 5-phenylthiophenes that were evaluated (compounds **9h**–**m**). The most efficacious and potent AE in this series was compound **9k** which possessed a 4-chlorophenyl group in the 5-position (AE score = 57%, ED_{50} = 6.6 μM). The other 5-phenyl compounds (**9h**–**m**) had only modest AE scores ranging from 10% to 29%. Ethyl 2-amino-4-(3-(trifluoromethyl)phenyl)thiophene-3-carboxylate (**6a**) had a AE score of only 8%, while the 5-pyridyl analogue **9n** was inactive at the test concentration.

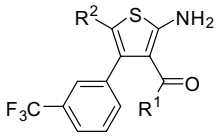
Bromo substitution of the 5-position was more effective than chloro (compare **2** and **11**), though [2-amino-5-bromo-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (**2**) had a lower AE score than observed in our previous study. This compound was found to degrade relatively rapidly when in solution and the variable results obtained in the AE assay may have resulted from differing extents of degradation.

All of these compounds have significant activity as orthosteric antagonists, although there was a wide variation in the ratio of AE activity to antagonist activity. For example, compound **9n** had an AE score of 0% and showed 91% inhibition of [^{3}H]CPX binding, while compound **6b** had an AE score of 77% and at 10 μM showed 61% inhibition of [^{3}H]CPX binding.



Scheme 2. Reagents and conditions: (i) phthalic anhydride, AcOH, reflux; (ii) X = Cl:NCS, AcOH/CH_2Cl_2; X = Br:NBS, AcOH/CH_2Cl_2; (iii) $NH_2NH_2{\cdot}H_2O$, EtOH, rt.

Table 1.



Compound	R^1	R^2	AE score[a] ± SEM	Enhancer ED_{50} (μM)	% Inhibition[b]
6a	OEt	H	7.8 ± 0.7	9.4 ± 1.5	93.0 ± 3.2
6b	Ph	H	76.8 ± 8.5	15.8 ± 2.8	61.1 ± 2.0
9a	Ph	Ph	18.1 ± 0.4	2.3 ± 0.8	82.4 ± 2.2
9b	Ph	3-AcPh	18.5 ± 1.7	>10	33.1 ± 2.0
9c	Ph	4-AcPh	4.8 ± 0.5	>10	25.6 ± 6.8
9d	Ph	4-NO_2Ph	23.7 ± 0.7	>10	34.8 ± 1.4
9e	Ph	4-ClPh	22.5 ± 2.9	>10	59.1 ± 0.4
9f	Ph	4-MeOPh	7.4 ± 0.8	>10	65.2 ± 1.6
9g	OEt	Ph	13.2 ± 0.7	>10	83.6 ± 1.7
9h	OEt	3-AcPh	14.4 ± 1.5	>10	68.9 ± 0.5
9i	OEt	4-AcPh	28.5 ± 2.9	>10	64.6 ± 1.8
9j	OEt	4-NO_2Ph	10.3 ± 1.7	>10	71.4 ± 21.1
9k	OEt	4-ClPh	57.0 ± 5.8	6.6 ± 1.9	58.4 ± 2.3
9l	OEt	4-MeOPh	17.5 ± 0.2	>10	81.5 ± 3.8
9m	OEt	4-CF_3Ph	14.8 ± 1.0	>10	48.5 ± 4.6
9n	OEt	4-pyridyl	0	>10	91.0 ± 0.1
11	Ph	Cl	22.2 ± 1.8	>10	87.1 ± 2.6
2	Ph	Br	53.5 ± 2.2	>10	47.5 ± 0.9
PD (**1**)			28 ± 1.1	13.6 ± 2.1	18.8 ± 2.5

[a] For compounds with an $ED_{50} < 10$ μM this value is based on curve fitting relating AE score to AE concentration using the equation Score = ScoreMax * [AE]/(ED_{50} + [AE]). Data points are means ± SEM, $N = 2–3$. For compounds with ED_{50} values >10 μM the score at 50 μM is recorded.

[b] Orthosteric antagonist activity, % inhibition of specific [^{3}H]CPX binding by 10 μM allosteric enhancer, $N = 3$.

In summary, two series of 5-substituted 2-amino-4-(3-trifluoromethylphenyl)thiophenes were prepared and evaluated as allosteric enhancers at the A_1AR. The first series of compounds possessed a benzoyl group in the 3-position. In this series, a 5-phenyl group was found to confer the greatest potency (**9a**: EC_{50} = 2.1 μM, AE score = 18%). However, the analogue with no 5-substituent (**6b**: EC_{50} = 15.8 μM, AE score = 77%) proved to be the most efficacious. The second series of compounds possessed an ethyl ester in the 3-position. In this series, the 5-(4-chlorophenyl) analogue was clearly the most potent and efficacious (**9k**: EC_{50} = 6.6 μM, AE score = 57%). These values compare favourably with PD81,723 (**1**, EC_{50} of 13.6 μM, AE score = 28%), which has commonly been used for benchmarking new allosteric enhancers. All of the compounds that were evaluated in this study showed greater antagonist activity than PD81,723 in a [^{3}H]CPX competitive binding assay.

3. Experimental

Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. All NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 300.13 and 75.4 MHz, respectively, and, unless stated otherwise, samples were dissolved in $CDCl_3$. Thin-layer chromatography was conducted on 0.2 mm plates using Merck silica gel 60 F254. Column chromatography was achieved using Merck silica gel 60 (particle size 0.063–0.200 μm, 70–230 mesh).

3.1. Ethyl 2-amino-4-(3-(trifluoromethyl)phenyl)thiophene-3-carboxylate (6a)

Compound **4** (0.8 mL, 5.32 mmol) and ethyl cyanoacetate (0.77 mL, 6.42 mmol) were dissolved in dry CH_2Cl_2 (25 mL) in a three-necked flask, fitted with a rubber septum and flushed with N_2 gas. The solution was cooled to 0 °C with an ice bath and neat $TiCl_4$ (1.17 mL, 10.64 mmol) was added in a dropwise fashion. Pyridine (360 μL) was added after 10 min. After the addition was complete, the ice bath was removed and the solution left to stir at room temperature. After 30 min, another aliquot of pyridine (1.08 mL) was added dropwise and stirring was continued overnight. The mixture was diluted with EtOAc (150 mL) and washed with 2 M HCl (2× 100 mL), H_2O (200 mL) and finally with brine. The organic layer was dried ($MgSO_4$), filtered and evaporated to provide 1.49 g of yellow resin that slowly solidified. The resin was taken up in dry THF (10 mL) and elemental sulfur (200 mg) was added followed by *N*,*N*-diethylamine (2 mL) and the solution was stirred at room temperature for 1.5 h. The reaction mixture was then diluted with water and extracted with CH_2Cl_2 (3× 50 mL). The combined organic extracts were washed with water and then brine, dried ($MgSO_4$), filtered and evaporated to dryness. The crude product was chromatographed on silica gel eluting with EtOAc/pet ether (15:85), providing a pale yellow resin that solidified upon standing (1.52 g, 91%). Mp 65–66 °C. ^{1}H NMR δ 7.58–7.40 (m, 4H, ArH), 6.14 (br s, 2H, NH_2), 6.09 (s, 1H, 5-H), 4.04 (q, J = 7.2 Hz, 2H, CH_2CH_3), 0.89 (t, J = 7.2 Hz, 3H, CH_2CH_3). MS (APCI): m/z = 316.2 (100%)

$(M+H)^+$. HR-MS (ESI) Calcd for $C_{14}H_{13}F_3NO_2S^+$: (M+1) 316.0614. Found: 316.0611.

3.2. [2-Amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl]phenyl methanone (6b)

Compound **4** (3 mL, 3.71 g, 19.69 mmol) and benzoylacetonitrile (3.00 g, 20.67 mmol) were dissolved in dry CH_2Cl_2 (75 mL) in a three-necked flask, fitted with a rubber septum and flushed with N_2 gas. The solution was cooled to 0 °C with an ice bath and neat $TiCl_4$ (4.6 mL, 42.27 mmol) was added in a dropwise fashion. Pyridine (1.41 mL) was added after 10 min. After the addition was complete, the ice bath was removed and the solution left to stir at room temperature. A further aliquot of pyridine (4.23 mL) was added after 1 h and the reaction mixture was stirred overnight. The reaction mixture was diluted with EtOAc (250 mL) and washed with 2 M HCl (2× 200 mL), H_2O (300 mL) and finally with brine. The organic layer was dried ($MgSO_4$), filtered and evaporated to provide an orange resin (7.42 g). This resin was dissolved in dry THF (40 mL), elemental sulfur (667 mg) and *N*,*N*-diethylamine (4 mL) were added and the reaction mixture was stirred at room temperature overnight. The solution was diluted with water and extracted with CH_2Cl_2 (3× 100 mL). The combined organic extracts were washed with water and then brine, dried ($MgSO_4$), filtered and concentration to a red/brown residue (8.03 g). The crude product was chromatographed on silica gel eluting with EtOAc/pet ether (20:80), supplying an orange resin that was triturated with *sec*-butanol/H_2O (80:20) to afford orange crystals (1.81 g, 26%). Mp 135–138 °C. ^{1}H NMR δ 7.29–7.26 (m, 2H, ArH), 7.21–7.18 (m, 3H, ArH), 7.13–7.06 (m, 2H, ArH), 7.00–6.95 (m, 2H, ArH), 6.6 (br s, 2H, NH_2), 6.21 (br s, 1H, 5-H). MS (APCI): *m*/*z* = 348.1 (100%) $(M+H)^+$. HR-MS (ESI) Calcd for $C_{18}H_{13}F_3NOS^+$: (M+1) 348.0664. Found: 348.0660.

3.3. Ethyl 5-bromo-2-(*tert*-butoxycarbonylamino)-4-(3-(trifluoromethyl)phenyl)thiophene-3-carboxylate (8a)

Compound **6a** (3.84 g, 12.18 mmol) was dissolved in dioxane (45 mL) and Boc_2O (2.79 g, 12.79 mmol) was added to the solution followed by DMAP (148 mg, 10 mol%). The homogeneous solution was heated on an oil bath at 70–80 °C for 2 h. After cooling, the solution was diluted with ether and washed with water (2×) and then brine. The organic layer was dried ($MgSO_4$), filtered and evaporated to give a reddish brown oil (4.9 g). This oil was chromatographed on silica gel column using EtOAc/pet ether (5:95) as an eluent to afford a clear colourless resin (2.27 g, 45%). The resin (1.93 g, 4.65 mmol) was dissolved in 25 mL of CH_2Cl_2/AcOH (1:1) and cooled in an ice salt bath (∼−16 to −18 °C). NBS (0.91 g, 5.11 mmol) was added and the reaction mixture was stirred for 0.5 h on an ice/salt bath. The solution was diluted with water (200 mL) and extracted with ether. The organic phase was washed with saturated $NaHCO_3$ solution (until gas evolution ceased) and finally with brine. The organic layer was dried ($MgSO_4$), filtered from insoluble material and concentrated to a solid, that was recrystallised from MeOH/H_2O (95:5) to afford a white solid (2.2 g, 96%). ^{1}H NMR δ 10.43 (br s, 1H, NH), 7.63–7.61 (m, 1H, ArH), 7.53–7.48 (m, 2H, ArH), 7.41–7.39 (m, 1H, ArH), 3.95 (q, *J* = 7.2 Hz, 2H, CH_2CH_3), 1.49 (br s, 9H, $C(CH_3)_3$), 0.78 (t, *J* = 7.2 Hz, 3H, CH_2CH_3). MS (APCI): *m*/*z* = 492.0 (48%), 494.0 (100%) $(M-H)^-$. HR-MS (ESI) Calcd for $C_{19}H_{19}BrF_3NNaO_4S^+$: (M+Na) 516.0062. Found: 516.0070.

3.4. [5-Bromo-2-(*tert*-butoxycarbonylamino)-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl]phenyl methanone (8b)

Compound **6b** (1.00 g, 2.88 mmol) was dissolved in dioxane (20 mL) and Boc_2O (0.66 g, 3.02 mmol) was added to the solution followed by DMAP (35 mg, 10 mol%). The homogeneous solution was heated on an oil bath at 70–80 °C for 2 h. After cooling, the solution was diluted with ether and washed with water (2×) and then brine. The organic layer was dried ($MgSO_4$), filtered from insoluble material and evaporated to give an orange oil (1.84 g). This oil was chromatographed on silica gel column using EtOAc/pet ether (5:95) as an eluent to afford a pale yellow resin (0.75 g, 58%). The resin (494 mg, 1.10 mmol) was dissolved in 7 mL of CH_2Cl_2/AcOH (1:1) and cooled in an ice salt bath (∼−16 to −18 °C). NBS (216 mg, 1.21 mmol) was added and the reaction mixture was stirred for 0.5 h on an ice/salt bath. The solution was diluted with water (100 mL) and extracted with ether. The organic phase was washed with saturated $NaHCO_3$ solution (until gas evolution ceases) and finally with brine. The organic layer was dried ($MgSO_4$), filtered from insoluble material and concentrated to afford a pale yellow solid. This solid was recrystallised from MeOH/H_2O (95:5) resulting in a white solid (0.58 g, 99%). ^{1}H NMR δ 10.55 (br s, 1H, NH), 7.26–7.22 (m, 5H, ArH), 7.18–7.12 (m, 2H, ArH), 7.02–6.97 (m, 2H, ArH), 1.53 (br s, 9H, $C(CH_3)_3$). HR-MS (ESI) Calcd for $C_{23}H_{19}BrF_3NNaO_3S^+$: (M+Na) 548.0113. Found: 548.0120.

3.5. General procedure for the preparation of compounds (9a–n)

3.5.1. Method A. Compound **8a** or **8b** (100 mg) was dissolved in 4:1 DMF/2 M K_3PO_4 (1 mL, previously sonicated for 30 min) under an atmosphere of N_2. The appropriate boronic acid (1.2 equiv) and $Pd(PPh_3)_4$ (6.6. mg, 5.70 μmol, 3 mol%) were added and the reaction mixture was heated at 70–80 °C for 24 h. The solution was cooled and diluted with EtOAc (20 mL) and washed with water. The aqueous layer was extracted with EtOAc and the combined organics were washed with water (3×) and finally with brine. The organic layer was dried ($MgSO_4$), filtered from insoluble material and concentrated to a residue. The residue was filtered through a silica gel plug eluting with $CHCl_3$ and concentrated to a residue which was subjected to silica gel chromatography.

3.5.2. Method B. Compound **8a** or **8b** (200 mg) was dissolved in 4:1 DMF/2 M K_3PO_4 (2 mL, previously sonicated for 30 min) in a sealed vial (microwave pressure

tube). The appropriate boronic acid (1.2 equiv) and $Pd(PPh_3)_2Cl_2$ (8.0 mg, 11.40 μmol, 3 mol%) were added and the mixture was heated with stirring to 150 °C for 15 min. If the reaction was incomplete (by TLC), a further equivalent of boronic acid and 3 mol% of catalyst were added and microwave heating was continued for 10 min at 150 °C. The solution was cooled and the toluene layer was filtered through a silica gel plug eluting with EtOAc and finally chloroform and concentrated to a residue.

3.5.2.1. [2-Amino-5-phenyl-4-(3-trifluoromethylphenyl)-thiophen-3-yl]phenyl methanone (9a). *Method B:* The residue obtained was sufficiently pure for the subsequent deprotection step. It was dissolved in CH_2Cl_2 (1 mL), TFA (1 mL) was added and the reaction mixture was stirred at room temperature. After 3 h the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to give a reddish brown residue. The residue was chromatographed on a silica gel column eluting with EtOAc/pet ether (15:85) to provide a light yellow resin which slowly crystallised upon standing at 4 °C. Complete crystallisation was induced by sonicating in a minimum of methanol to afford a yellow powder (74 mg, 45%). Mp 86–89 °C. ^{1}H NMR δ 7.23–6.93 (m, 14H, ArH), 3.15 (br s, 2H, NH_2). HR-MS (ESI) Calcd for $C_{24}H_{17}F_3NOS^+$: (M+1) 424.0983. Found: 424.0982.

3.5.2.2. [5-(3-Acetylphenyl)-2-amino-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (9b). *Method A:* The crude product was chromatographed on silica gel eluting with EtOAc/pet ether (5:95) to provide 40 mg of clear pale yellow resin that was triturated with MeOH/water. The resultant solid was filtered and washed with H_2O providing pale yellow powder (38 mg, 35%). This cross-coupled product (30 mg, 53.04 μmol) was dissolved in CH_2Cl_2 (1 mL) and TFA (400 μL) was added at room temperature. After 1.5 h, the mixture was diluted with $CHCl_3$ (10 mL), evaporated and chromatographed on silica gel eluting with $CHCl_3$ to provide 26 mg of yellow resin. The resin was slowly crystallised from MeOH with careful addition of water to give a pale brown powder (15 mg, 61%). Mp 160–164 °C. ^{1}H NMR δ 7.77–7.74 (m, 1H, ArH), 7.56 (m, 1H, ArH), 7.27–6.95 (m, 11H, ArH), 2.35 (s, 3H, Ac), 1.88 (br s, 2H, NH_2). HR-MS (ESI) Calcd for $C_{26}H_{19}F_3NO_2S^+$: (M+1) 466.1083. Found: 466.1081.

3.5.2.3. [5-(4-Acetylphenyl)-2-amino-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (9c). *Method A:* The residue obtained was chromatographed on silica gel eluting with EtOAc/pet ether (5:95) to provide a clear pale yellow resin that was crystallised from MeOH/H_2O (90:10). The solid obtained was filtered and washed with H_2O to give a pale yellow powder (54 mg, 50%). This cross-coupled product (40 mg, 70.72 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (300 μL) was added and the reaction mixture was stirred at room temperature. After 2 h the mixture was diluted with $CHCl_3$ (10 mL), evaporated and chromatographed on silica gel eluting with $CHCl_3$ providing a yellow powder (19 mg, 58%). Mp 190–196 °C. ^{1}H NMR δ 7.74 (d, J = 8.4 Hz, 2H, ArH), 7.20–6.91 (m, 11H, ArH), 2.52 (s, 3H, Ac), 1.90 (br s, 2H, NH_2). HR-MS (ESI) Calcd for $C_{26}H_{19}F_3NO_2S^+$: (M+1) 466.1083. Found: 466.1081.

3.5.2.4. [2-Amino-5-(4-nitrophenyl)-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (9d). *Method A:* The residue obtained was dissolved in a minimum of MeOH and crystallised upon the addition of water. The resultant solid was filtered and washed with H_2O to provide a yellow powder (75 mg, 69%). This cross-coupled product (50 mg, 87.94 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (1 mL) was added and the reaction mixture was stirred at room temperature. After 2 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a solid that was filtered and washed with water (43 mg). The solid was chromatographed on silica gel eluting with EtOAc/pet ether (20:80) to afford a semi-solid that was triturated with 80% MeOH/H_2O to give an orange solid (22 mg, 41%). Mp 221–229 °C. ^{1}H NMR δ 8.01 (d, J = 8.7 Hz, 2H, ArH), 7.19–7.17 (m, 3H, ArH), 7.12–7.04 (m, 6H, ArH), 6.98–6.93 (m, 2H, ArH), 2.31 (br s, 2H, NH_2). HR-MS (ESI) Calcd for $C_{24}H_{16}F_3N_2O_3S^+$: (M+1) 469.0828. Found: 469.0828.

3.5.2.5. [2-Amino-5-(4-chlorophenyl)-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (9e). *Method A:* The residue obtained was crystallised from MeOH. The solid was filtered and washed with ice-cold MeOH to provide a tan coloured powder (51 mg, 48%). This cross-coupled product (46 mg, 82.44 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (200 μL) was added and the reaction mixture was stirred at room temperature. After 1.5 h, the mixture was diluted with $CHCl_3$ (10 mL) and evaporated to give a reddish brown residue. The residue was triturated with a minimum amount of ice-cold MeOH and the resultant solid was filtered and washed with MeOH/H_2O (60:40) to afford a yellow powder (31 mg, 82%). Mp 185–194 °C. ^{1}H NMR δ 7.20–6.90 (m, 13H, ArH), 5.21 (br s, 2H, NH_2). MS (APCI): m/z = 60.0 (40%), 458.0 (100%) $(M+H)^+$. HR-MS (ESI) Calcd for $C_{24}H_{16}ClF_3NOS^+$: (M+1) 458.0588. Found: 458.0591.

3.5.2.6. [2-Amino-5-(4-methoxyphenyl)-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (9f). *Method B:* The residue obtained was sufficiently pure for the next step. It was dissolved in CH_2Cl_2 (1 mL), TFA (1 mL) was added and the reaction mixture was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$ (10 mL) and evaporated to give a reddish brown residue. Chromatography on a silica gel column, eluting with 15:85 EtOAc/pet ether (15:85), yielded a light yellow resin. This material was crystallised by sonicating in a minimum of methanol to afford a yellow powder (36 mg, 22%). Mp 68–71 °C. ^{1}H NMR δ 7.21–7.19 (m, 2H, ArH), 7.09–6.91 (m, 9H, ArH), 6.77–6.72 (m, 2H, ArH), 3.74 (br s, 3H, CH_3O) 2.25 (br s, 2H, NH_2). HR-MS (ESI) Calcd for $C_{25}H_{19}F_3NO_2S^+$: (M+1) 454.1089. Found: 454.1090.

3.5.2.7. Ethyl 2-amino-5-phenyl-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9g). *Method B:* The residue obtained was sufficiently pure for the next step. It was dissolved in CH_2Cl_2 (1 mL), TFA (1 mL) was added

and the reaction was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$ (10 mL) and evaporated to give a reddish brown residue. This residue was chromatographed on a silica gel column eluting with EtOAc/pet ether (15:85) to provide a pink resin (141 mg), which solidified upon standing. Complete crystallisation was induced by sonicating in 50% aqueous methanol to yield a pink powder (76 mg, 48%). Mp 113–116 °C. ^{1}H NMR δ 7.53 (br s, 2H, ArH), 7.38–7.30 (m, 2H, ArH), 7.20–6.95 (m, 5H, ArH), 4.00 (br s, 2H, NH_2), 3.95 (q, J = 7.2 Hz, 2H, CH_2CH_3), 0.80 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{20}H_{17}F_3NO_2S^+$: (M+1) 392.0932. Found: 392.0929.

3.5.2.8. Ethyl 5-(3-acetylphenyl)-2-amino-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9h). *Method A:* The residue obtained was crystallised from MeOH. The solid was filtered and washed with ice-cold MeOH to give an off-white powder (75 mg, 69%). This cross-coupled product (75 mg, 140.57 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (200 μL) was added and the reaction mixture was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a brown/red residue. The residue was chromatographed on a silica gel column eluting with EtOAc/pet ether (15:85) to provide a brown semi-solid (35 mg). The solid was dissolved in a minimum of MeOH and precipitated by the addition of water, yielding an off-white powder (33 mg, 54%). Mp 164–170 °C. ^{1}H NMR δ 7.70 (m, 1H, ArH), 7.56–7.54 (m, 3H, ArH), 7.41–7.31 (m, 2H, ArH), 7.26–7.23 (m, 2H, ArH), 4.12 (br s, 2H, NH_2), 3.95 (q, J = 7.2 Hz, 2H, CH_2CH_3), 2.32 (s, 3H, Ac), 0.79 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{22}H_{19}F_3NO_3S^+$: (M+1) 434.1032. Found: 434.1031.

3.5.2.9. Ethyl 5-(4-acetylphenyl)-2-amino-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9i). *Method A:* The residue obtained was crystallised from MeOH. The solid was filtered and washed with ice-cold MeOH to give a peach coloured powder (100 mg, 93%). This cross-coupled product (100 mg, 187.42 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (400 μL) was added and the reaction mixture was stirred at room temperature. After 2 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a black/green residue. The residue was chromatographed on a silica gel column eluting with $CHCl_3$, providing a yellow resin (54 mg) that solidified upon standing. The solid was recrystallised from MeOH to afford a yellow powder (13.5 mg, 16%). Mp 167–170 °C. ^{1}H NMR δ 7.71 (d, J = 8.4 Hz, 2H, ArH), 7.58–7.53 (m, 2H, ArH), 7.41–7.30 (m, 2H, ArH), 7.05 (d, J = 8.4 Hz, 2H, ArH), 3.93 (q, J = 7.2 Hz, 2H, CH_2CH_3), 2.51 (s, 3H, Ac), 0.79 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{22}H_{19}F_3NO_3S^+$: (M+1) 434.1032. Found: 434.1030.

3.5.2.10. Ethyl 2-amino-5-(4-nitrophenyl)-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9j). *Method A:* The residue obtained was crystallised from MeOH. The solid was filtered and washed with ice-cold MeOH to give a brownish yellow powder (75 mg, 69%). This cross-coupled product (75 mg, 139.79 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (200 μL) was added and the reaction mixture was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a reddish brown residue. The residue was chromatographed on a silica gel column eluting with EtOAc/pet ether (15:85), providing a reddish brown resin (82 mg). The resin was crystallised from MeOH/water to afford a reddish brown powder (33 mg, 54%). Mp 178–184 °C. ^{1}H NMR δ 7.97 (d, J = 8.7 Hz, 2H, ArH), 7.60 (d, J = 7.8 Hz, 1H, ArH), 7.53 (s, 1H, ArH), 7.42 (t, J = 7.7 Hz, 1H, ArH), 7.32 (d, J = 7.5 Hz, 1H, ArH), 7.08 (d, J = 8.7 Hz, 2H, ArH), 3.95 (q, J = 7.2 Hz, 2H, CH_2CH_3), 0.79 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{20}H_{16}F_3N_2O_4S^+$: (M+1) 437.0777. Found: 437.0781.

3.5.2.11. Ethyl 2-amino-5-(4-chlorophenyl)-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9k). *Method A:* The residue obtained was crystallised from MeOH. The solid was filtered and washed with ice-cold MeOH to give a white powder (102 mg, 96%). This cross-coupled product (100 mg, 190.13 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (1 mL) was added and the reaction mixture was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a reddish brown residue. The residue was chromatographed on a silica gel column eluting with EtOAc/pet ether (10:90) to give a pink resin (70 mg). The resin was recrystallised from EtOH/water to afford a pink powder (57 mg, 70%). Mp 127–129 °C. ^{1}H NMR δ 7.55–7.48 (m, 2H, ArH), 7.39–7.26 (m, 2H, ArH), 7.16–6.91 (m, 4H, ArH), 3.94 (q, J = 7.2 Hz, 2H, CH_2CH_3), 0.78 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{20}H_{16}ClF_3NO_2S^+$: (M+1) 426.0537. Found: 426.0552.

3.5.2.12. Ethyl 2-amino-5-(4-methoxyphenyl)-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9l). *Method B:* The residue obtained was sufficiently pure for the next step. It was dissolved in CH_2Cl_2 (1 mL), TFA (1 mL) was added and the reaction mixture was stirred at room temperature. After 3 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a reddish brown residue. The residue was chromatographed on a silica gel column eluting with EtOAc/pet ether (15:85) to provide a light purple resin which slowly solidified upon standing. Complete crystallisation was induced by sonication in 50% aqueous methanol, giving a light purple powder (91 mg, 49%). Mp 112–116 °C. ^{1}H NMR δ 7.51–7.49 (m, 2H, ArH), 7.37–7.27 (m, 2H, ArH), 7.10–6.65 (m, 4H, ArH), 3.94 (q, J = 7.2 Hz, 2H, CH_2CH_3), 3.73 (br s, 3H, CH_3O) 0.79 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{21}H_{19}F_3NO_3S^+$: (M+1) 422.1038. Found: 422.1031.

3.5.2.13. [2-Amino-4-(3-trifluoromethylphenyl)-5-(4-trifluoromethylphenyl)thiophen-3-yl]phenyl methanone (9m). *Method A:* The residue obtained was crystallised from MeOH. The solid was filtered and washed with ice-cold MeOH to give a light brown powder (100 mg, 88%). This cross-coupled product (100 mg, 178.73 μmol) was dissolved in CH_2Cl_2 (1 mL), TFA (400 μL) was added and the reaction mixture was stirred at room tempera-

ture. After 2 h, the mixture was diluted with $CHCl_3$ (10 mL) and concentrated to a reddish brown residue. The residue was chromatographed on a silica gel column eluting with $CHCl_3$, providing a brown solid (55 mg). The solid was triturated with a minimum of ice-cold 80% MeOH/H_2O to yield a brown powder (35 mg, 43%). Mp 135–140 °C. ^{1}H NMR δ 7.57–7.53 (m, 2H, ArH), 7.41–7.36 (m, 3H, ArH), 7.32–7.29 (m, 1H, ArH), 7.08 (d, J = 8.1 Hz, 2H, ArH), 3.94 (q, J = 7.2 Hz, 2H, CH_2CH_3), 2.04 (br s, 2H, NH_2), 0.79 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{21}H_{16}F_6NO_2S^+$: (M+1) 460.0800. Found: 460.0800.

3.5.2.14. Ethyl 2-amino-5-(4-pyridyl)-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (9n). *Method A:* The residue obtained was chromatographed on silica gel eluting with EtOAc/pet ether (15:85) to give a clear colourless resin (32 mg). The resin was crystallised by dissolving in MeOH and precipitating with water. The solid was washed with water to provide a white powder (26 mg, 26%). This cross-coupled product (25 mg, 50.76 μmol) was dissolved in EtOH (1 mL), 6 M HCl (200 μL) was added and the reaction mixture was stirred at room temperature. After 2 h, the mixture was heated on an oil bath at 50–60 °C for 24 h. The solution was concentrated to a green/yellow solid. The solid was recrystallised from EtOH/ether to afford a green solid as the hydrochloride salt (21 mg, 96%). Mp 164–170 °C. ^{1}H NMR (DMSO) δ 8.44 (d, J = 6.0 Hz, 2H, ArH), 8.36 (br s, 2H, NH_2), 7.84 (d, J = 7.5 Hz, 1H, ArH), 7.71–7.66 (m, 2H, ArH), 7.56 (d, J = 7.5 Hz, 1H, ArH), 7.11 (d, J = 6.3 Hz, 2H, ArH), 3.86 (m, 2H, CH_2CH_3), 0.72 (t, J = 7.2 Hz, 3H, CH_2CH_3). HR-MS (ESI) Calcd for $C_{19}H_{16}F_3N_2O_2S^+$: (M+1) 393.0879. Found: 393.0868.

3.6. [2-Amino-5-chloro-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl]phenyl methanone (11)

Compound **6b** (0.65 mg, 1.86 mmol) and phthalic anhydride (0.33 g, 2.23 mmol) were dissolved in 17 mL of glacial acetic and refluxed overnight. The solution was concentrated and the resultant solid was recrystallised from *sec*-butanol/H_2O (80:20) to give compound **10** as a pale yellow solid (661 mg). This crude product (200 mg, 0.42 mmol) was dissolved in 50:50 $CHCl_3$/AcOH (4 mL) and NCS (67 mg, 0.50 mmol) was added. After 10 min, DMF (0.5 mL) was also added to solubilise all solids. The homogeneous solution was stirred at room temperature for 3 h. An additional portion of NCS (67 mg, 0.50 mmol) was added and the reaction mixture was stirred overnight. The solution was diluted with water and extracted with EtOAc. The organic phase was washed with dilute sodium bicarbonate solution and then water. The organic layer was dried ($MgSO_4$), filtered and concentrated to a yellow resin. The resin was filtered through a silica gel plug eluting with diethyl ether to give a pale yellow foam that was crystallised from MeOH (142 mg, 66%). The resultant solid (140 mg, 0.27 mmol) was dissolved in 2 mL of dioxane/EtOH (1:1) and vigorously stirred at room temperature. Hydrazine hydrate (18.6 μL, 0.38 mmol) was added and stirring was continued for 2.5 h. The mixture was concentrated and chromatographed on silica gel eluting with $CHCl_3$ to afford a yellow resin (60 mg). This material was dissolved in a minimum of MeOH and precipitated with water (21 mg, 20%). Mp 139–141 °C. ^{1}H NMR δ 7.24–7.06 (m, 9H, ArH and NH_2), 6.99–6.93 (m, 2H, ArH). HR-MS (ESI) Calcd for $C_{18}H_{12}ClF_3NOS^+$: (M+1) 382.0275. Found: 382.0276.

3.7. [2-Amino-5-bromo-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl]phenyl methanone (2)

Compound **10** (60 mg, 0.13 mmol) was dissolved in 50:50 CH_2Cl_2/AcOH (1 mL) and cooled to 0 °C with ice bath. NBS (27 mg, 0.15 mmol) was added and the reaction mixture was stirred at room temperature for 1.5 h. The solution was concentrated to a residue and then taken up in EtOAc. The EtOAc solution was washed with 5% sodium bicarbonate solution (2×), water and brine. The organic layer was dried ($MgSO_4$), filtered and concentrated to give a pale yellow solid (65 mg, 93%). HR-MS (ESI) Calcd for $C_{26}H_{14}BrF_3NO_3S^+$: (M+1) 555.9824. Found: 555.9824. The crude product (50 mg, 0.09 mmol) was dissolved in 0.5 mL of EtOH and vigorously stirred at room temperature. Hydrazine hydrate (6.0 μL, 0.124 mmol) was added slowly and dropwise. After stirring at room temperature for 20 min, dioxane (0.5 mL) was added to solubilise all materials and was left to stir overnight. The next day a further 5 μL of hydrazine hydrate was added and after 1 min a precipitate formed. The solution was immediately concentrated and the residue obtained was filtered through a silica gel plug eluting with 50:50 EtOAc/pet ether to provide a pale yellow resin that solidifies upon standing at 4 °C. Complete crystallisation was induced by dissolving in a minimum of MeOH and adding water to afford a green solid (37 mg, 96%). Mp 122–124 °C. ^{1}H NMR δ 7.25–7.06 (m, 7H, ArH), 6.98–6.93 (m, 2H, ArH), 6.35 (br s, 2H, NH_2). MS (APCI): m/z = 426.0 (90%), 428.0 (100%) $(M+H)^+$. HR-MS (ESI) Calcd for $C_{18}H_{12}BrF_3NOS^+$: (M+1) 425.9775. Found: 425.9772.

3.8. Assay of AE activity

The assay of AE activity consisted of three phases: (1) binding to equilibrium of the agonist, [^{125}I]IABA, to the A_1AR–G-protein ternary complex; (2) stabilization of that complex by adding vehicle or AE for 30 min and (3) dissociation of the complex by adding a combination of an A_1AR antagonist, 100 μM BW-1433, and 25 μM GTPγS for 10 min. Compounds were scored between 0% (no different than AE vehicle) and 100% (complete abolition of [^{125}I]IABA dissociation). The assay employed membranes from CHO-K1 cells stably expressing the hA_1AR. For agonist binding to equilibrium (phase 1) the buffer consisted of 10 mM Hepes, pH 7.2, containing 0.5 mM $MgCl_2$, 1 U/mL adenosine deaminase, 0.5 nM [^{125}I]IABA and 10 μg of membrane protein in a final volume of 100 μL applied to 96-well Millipore GF/C glass fibre filter plates. After 90 min at room temperature the addition of 50 μL of AE (0.1–50 μM, final) initiated stabilization of the ternary complex (phase 2). Thirty minutes later 50 μL solution con-

taining BW-1433 and GTPγS was added to initiate the dissociation of the ternary complex. Ten minutes later membranes were filtered, washed, dried and counted for residual [^{125}I]IABA. The percentage of specifically bound agonist remaining after 10 min of dissociation served as an index of AE activity:

$$\%\ \text{AE score} = 100 \times (B - B_o)/(B_{eq} - B_o)$$

where B is the residual binding (cpm) bound at the end of 10 min of dissociation in the presence of an AE; B_o is the residual binding (cpm) at the end of 10 min of dissociation in the absence of an AE and B_{eq} is the cpm bound at the end of 90 min of equilibrium binding.

The percentage of specific binding remaining after 10 min of dissociation constitutes an index of AE activity for ranking candidate compounds. A score of 100% means no dissociation and a score of zero means complete dissociation.

3.9. Assay of A_1R antagonist activity

CHO-K1 membranes expressing human A_1 adenosine receptors were resuspended at 400 μg/mL in HE buffer containing 1 U/mL adenosine deaminase. Fifty microlitres of membrane solution was added to 50 μL HE buffer containing [^{3}H] CPX (2 nM). One hundred microlitres of HE buffer with either vehicle, enhancer or NECA (to define non-specific) was added. The final drug concentrations were 10 μM for enhancer and 100 μM for NECA. Samples were incubated for 90 min at room temperature, filtered and counted on a liquid scintillation counter. Binding was performed in triplicate and expressed as % inhibition as compared to control binding.

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