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Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 2358–2365

2-Aminothiophene-3-carboxylates and carboxamides as adenosine A_1 receptor allosteric enhancers

George Nikolakopoulos,[a] Heidi Figler,[b] Joel Linden[b] and Peter J. Scammells[a,*]

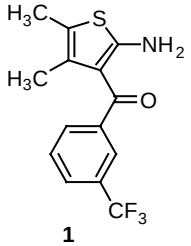
[a]*Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, Vic. 3052, Australia*
[b]*Departments of Medicine (Cardiology) and Pharmacology, University of Virginia, Charlottesville, VA 22908, USA*

Received 13 September 2005; revised 8 November 2005; accepted 8 November 2005
Available online 28 November 2005

Abstract—Three series of 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene and 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophenes with 3-carboxylates and carboxamides have been prepared using the Gewald synthesis and evaluated as A_1AR allosteric enhancers. The structure–activity relationships of these classes of compound are described. A number of compounds, notably **7b**, are more potent and efficacious than PD81,723 (**1**).

1. Introduction

The first allosteric enhancers (AEs) acting at the adenosine A_1 receptor (A_1AR) were reported by Bruns et al. in 1990.[1,2] These compounds were primarily 2-amino-3-benzoylthiophenes and 2-amino-3-benzoyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines and were found to decrease the rate of dissociation of agonist, but not antagonist radioligand from the orthosteric binding site. In addition to allosteric activity, some of these compounds also have weak activity as competitive antagonists of the A_1AR. PD81,723 (**1**) was one of the more potent and effective of the initial series of enhancers and has subsequently been commonly used for benchmarking new AEs. Since this initial discovery, other researchers have directed significant effort to refining the structure–activity relationships of the 3-, 4- and 5-positions of the 2-aminothiophene core.[3–7] The 2-amino and 3-keto groups were found to be crucial for activity, while replacement of the thiophene by benzene resulted in a marked reduction of activity. Substitution at the 4-position increased activity by a factor of 3 (phenyl > methyl > H), while substitution at the 5-position had little effect on activity. The benzoyl group was reported to be important for activity and substituents were identified which further improved activity (e.g., trifluoromethyl, chloro and methoxy). The replacement of the phenyl at the 3-position with a 2- or 3-thienyl group maintained allosteric enhancer activity, but increased competitive antagonist activity of the resulting compounds.[8] Bulky (or hydrophobic) substituents at the *meta*- and *para*-positions of the 3-benzoyl group and also 3-naphthoyl groups greatly enhanced enhancer activity. Thus, the A_1AR is thought to contain an allosteric binding site able to accommodate 3-aroyl substituents that are bulky and/or hydrophobic but not necessarily planar. A second region in the allosteric binding site interacts constructively with alkyl substituents at thiophene C-4 and/or C-5.[6]



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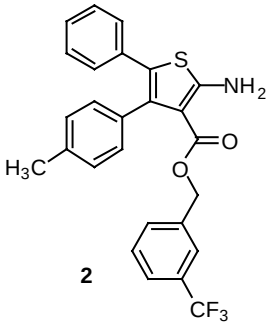

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Relatively few 2-aminothiophenes with esters or amides in the 3-position have been tested as allosteric enhancers. The initial study by Bruns and co-workers included two 2-amino-3-ethoxycarbonyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines.[1,2] Whilst these compounds maintained some enhancing ability, they proved to be

Keywords: Adenosine; Allosteric enhancer; Adenosine A_1 receptor.
* Corresponding author. Tel.: +61 3 9903 9542; fax: +61 3 9903 9582; e-mail: peter.scammells@vcp.monash.edu.au

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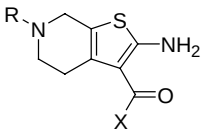
doi:10.1016/j.bmc.2005.11.018

Table 1. 3-Ethoxycarbonyl and 3-benzoyl substituted allosteric enhancers reported by Bruns et al.[1,2]



R	X	Enhancement (%)[a]
Me	OEt	6
Me	Ph	14
CH_2Ph	OEt	66
CH_2Ph	Ph	102

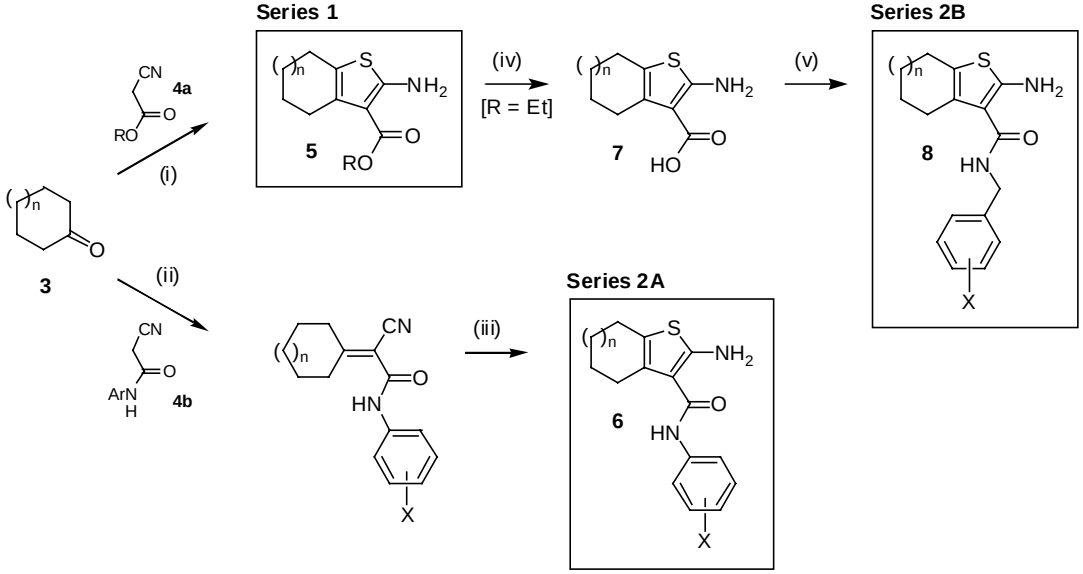
[a] Allosteric enhancement was evaluated using an assay that measured the % increase over control in specific [^{3}H]N^6-cyclohexyladenosine binding to rat brain membranes after addition of 100 μM *R*-PIA for 2 h.

less potent than the corresponding 3-benzoyl substituted 2-amino-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines (see Table 1).

We recently synthesised a series of substituted 2-amino-3-benzoyl-4,5-diphenylthiophene allosteric enhancers. En route to these target compounds we also prepared some novel 2-aminothiophene-3-carboxylates and carboxamides to explore the effect of these substituents on AE activity. Candidate allosteric enhancers were evaluated using a rapid in vitro assay which measures their ability to stabilize the agonist–A_1AR–G protein ternary complex.[9] More specifically, the A_1AR agonist [^{125}I]4-amino-3-iodo-N^6-benzyladenosine 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 adding CPX and GTPγS). Three of the five alkoxycarbonyl compounds that were tested at 50 μM proved to be clearly more effective than PD81,723 (**1**). 3-Trifluoromethylbenzyl 2-amino-4-(4-methylphenyl)-5-phenylthiophene-3-carboxylate (**2**) proved to be the most effective compound in this group with an AE score of 63% (compared with 19% for PD81,723). The sole amide tested recorded a slightly lower AE score than PD81,723. Although the test set was too small to draw many firm conclusions about structure–activity relationships, this investigation demonstrates that 2-aminothiophenes with esters and amides in the 3-position can exhibit substantial AE activity. This current study further explores the structure–activity relationships of this class of compounds as A_1AR allosteric enhancers.

2. Results and discussion

2-Aminothiophene targets were prepared using the Gewald synthesis.[10,11] This synthesis initially involves the Knoevenagel condensation of an aldehyde or ketone with an activated nitrile (such as an α-cyanoketone, cyanoacetate or cyanoacetamide), followed by a sulfur mediated cyclization to afford the desired 2-aminothiophene. There are two common variations on this synthesis: namely (i) a one-pot procedure in which the ketone and activated nitrile react in the presence of sulfur and base, and (ii) a two-step procedure in which the alkene produced by the Knoevenagel condensation is isolated prior to cyclization with sulfur and base. The former is more convenient and has been used for the synthesis of numerous 2-aminothiophenes, while the latter is generally higher yielding.[11] In this study, the one-pot procedure was used to prepare the 2-aminothiophene-3-carboxylates (Series 1, Scheme 1), but gave poor yields of the corresponding phenyl 2-aminothiophene carboxamides. As a result, the two-step variant was employed for the preparation of these compounds (Series 2A, Scheme 1). It was possible



Scheme 1. Reagents: (*n* = 1, 2). (i) Sulfur, NEt_3, DMF; (ii) NH_4OAc, AcOH, C_6H_6; (iii) sulfur, $NHEt_2$, EtOH; (iv) KOH, EtOH [R = Et]; (v) $BnNH_2$, EDC · HCl, DMAP, HOBt, CH_2Cl_2.

to prepare the benzyl 2-aminothiophene-3-carboxamides Series 2B, Scheme 1) in a more convergent fashion from the appropriate ethyl 2-aminothiophene-3-carboxylate in two steps (saponification followed by carbodiimide coupling).

The activity of the new compounds as allosteric enhancers of the human adenosine A_1 receptors was evaluated based on their 'score' to inhibit the kinetics of agonist radioligand dissociation upon the addition of 25 μM GTPγS and 100 μM CPX. As shown in Figure 1, dose–response curves relating score to enhancer concentration (0.1–50 μM) were well fit by hyperbolic equations from which the maximal score could be accurately calculated, provided the ED_{50} was <20 μM. The calculated maximal scores are recorded in Table 2. For compounds with ED_{50} values > 20 μM maximal scores could not be accurately calculated, and the score at 50 μM is recorded as an approximate maximum. Potent compounds (low ED_{50}) were not always highly efficacious. For example, compound **6g** and PD81,723 are potent but have limited efficacy (Fig. 1). In general, the benzyl 2-aminothiophene-3-carboxamides proved to be more efficacious than the corresponding benzyl 2-aminothiophene-3-carboxylates (compare compounds **8a**–**g** with **5a**–**g**). The benzyl 2-aminothiophene-3-carboxamides were also more active than the corresponding phenyl 2-aminothiophene-3-carboxylates (compare **8b**–**g** with **6b**–**g**). Two 2-aminothiophene-3-carboxylic acids (**7a** and **7b**) were also tested. Interestingly, high efficacy and unusually high potency were observed for 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxylic acid (**7b**, Fig. 1).

The effect of further substitution of the phenyl and benzyl of the 2-aminothiophene-3-carboxylates and 3-carboxamides was also investigated. A panel of substituents that had previously been reported to confer high AE activity in a related series of 2-amino-3-aroylthiophenes (primarily halo, methoxy, trifluoromethyl and phenyl) was chosen for this purpose. In this case, no clear correlation between the steric or electronic properties of the substituent and enhancing activity was observed. The benzyl 2-aminothiophene-3-carboxylates and carboxamides (**5a**, **5i**, **8a** and **8k**) were amongst the most effective compounds tested. Thus, in a number of cases further substitution of the benzyl group may lead to unfavourable interaction with the AE binding site.

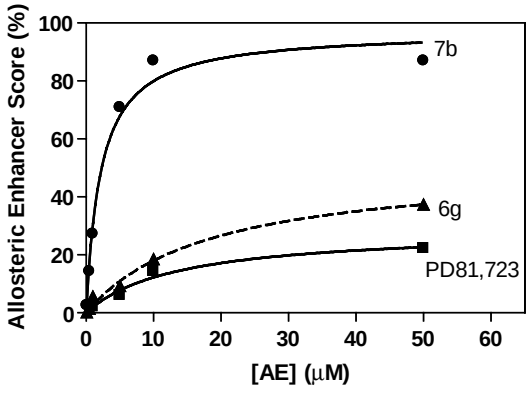


Figure 1. Dose–response curves relating AE score to concentration. Data were fit to rectangular hyperbolae to calculate maximal scores and ED_{50} values listed in Table 2.

The relationship between the size of the C4–C5 polymethylene bridge and enhancing activity was less clear cut for the 2-aminothiophene-3-carboxamides (**8a**–**j** vs **8k**–**t**) than was previously observed for 2-amino-3-aroylthiophenes. 2-Amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamides were more effective than the corresponding 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamides in five cases (3-trifluoromethylbenzyl, 3-methoxybenzyl, 4-chlorobenzyl, 4-bromobenzyl and 4-iodobenzyl). However, the larger polymethylene bridge [–$(CH_2)_5$–] was more effective for the benzyl, 3-chlorobenzyl, 3-nitrobenyl and 4-nitrobenzylcarboxamides.

Since some aminothiophenes have been found to have activity as competitive antagonists of the A_1AR, the antagonist activity of all AEs with ED_{50} values less than 20 μM was measured in a [^{3}H]CPX competitive binding assay. All of the new AEs showed lower antagonist activity than PD81,723 (Table 2). In the case of compound **7b** the % inhibition of [^{3}H]CPX binding was approximately half that of PD81,723 (9% inhibition compared with 19% exerted by PD81,723).

3. Experimental

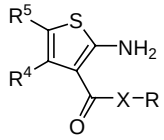
Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. All NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 300.13 and 75.4 MHz, respectively, and, unless stated otherwise, samples were dissolved in $CDCl_3$. Thin-layer chromatography was conducted on 0.2 mm plates using Merck silica gel 60 F_{254}. Column chromatography was achieved using Merck silica gel 60 (particle size 0.063–0.200 μm, 70–230 mesh).

3.1. General procedure for 2-aminothiophene-3-carboxylate formation (5a–k)

The benzyl cyanoacetate (1.0 molar equiv), sulfur (1.1 equiv), ketone (cyclohexanone or cycloheptanone, 1.0 equiv) and triethylamine (2 mL) were dissolved in DMF (2 mL) and stirred at room temperature for 16 h. The reaction mixture was diluted with $CHCl_3$, washed with brine (4 × 25 mL), dried over anhydrous $MgSO_4$ and filtered. The crude product was purified by chromatography using a deactivated SiO_2 column eluted with hexane/ethyl acetate (5:1).

3.1.1. Benzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5a). Yield 69%. ^{1}H NMR δ 1.74–1.80 (m, 4H, 2× CH_2), 2.50–2.51 (m, 2H, CH_2), 2.74–2.77 (m, 2H, CH_2), 5.30 (s, 2H, CH_2Ph), 6.08 (br s, 2H, NH_2), 7.31–7.45 (m, 5H, ArH). HR-ES MS: *m*/*z*, 288.10595 [M+H].

Table 2. AE activity of 2-aminothiophene-3-carboxylates and carboxamides



Compound	X	R^4/R^5	R	ScoreMax[a]	ED_{50} (μM)	% Inhibition[b]
5a	O	$-(CH_2)_4-$	$PhCH_2-$	72.4 ± 1.2%	10.4 ± 1.2	15.2 ± 1.89
5b	O	$-(CH_2)_4-$	3-CF_3PhCH_2-	31 ± 6.5%	>20	
5c	O	$-(CH_2)_4-$	3-OCH_3PhCH_2-	1.5 ± 1.5%	>20	
5d	O	$-(CH_2)_4-$	4-$ClPhCH_2-$	12.0 ± 1.0%	>20	
5e	O	$-(CH_2)_4-$	4-$BrPhCH_2-$	19.5 ± 3.5%	>20	
5f	O	$-(CH_2)_4-$	4-$IPhCH_2-$	6.5 ± 0.5%	>20	
5g	O	$-(CH_2)_4-$	4-CF_3PhCH_2-	21.0 ± 1.0%	>20	
5h	O	$-(CH_2)_4-$	4-$PhPhCH_2-$	25.5 ± 1.5%	>20	
5i	O	$-(CH_2)_5-$	$PhCH_2-$	88.0 ± 1%	>20	
5j	O	$-(CH_2)_5-$	3-CF_3PhCH_2-	59.0 ± 6.0%	>20	
5k	O	$-(CH_2)_5-$	$PhCH_2CH_2-$	48 ± 3.0%	>20	
6b	NH	$-(CH_2)_4-$	3-CF_3Ph-	32.5 ± 3.5%	>20	
6c	NH	$-(CH_2)_4-$	3-OCH_3Ph-	19.0 ± 1.0%	>20	
6d	NH	$-(CH_2)_4-$	4-$ClPh-$	39.5 ± 1.5%	>20	
6e	NH	$-(CH_2)_4-$	4-$BrPh-$	24.5 ± 2.5%	>20	
6f	NH	$-(CH_2)_4-$	4-$IPh-$	50.0 ± 2.0%	>20	
6g	NH	$-(CH_2)_4-$	4-CF_3Ph-	38.5 ± 1.5%	14.2 ± 2.0	7.46 ± 0.56
7a	O	$-(CH_2)_4-$	H	49.5 ± 3.0%	>20	
7b	O	$-(CH_2)_5-$	H	79.5 ± 1.5%	1.35 ± 0.17	9.23 ± 1.20
8a	NH	$-(CH_2)_4-$	$PhCH_2-$	79.5 ± 1.5%	10.4 ± 0.9	6.53 ± 1.10
8b	NH	$-(CH_2)_4-$	3-CF_3PhCH_2-	55.5 ± 3.5%	3.0 ± 0.7	9.47 ± 1.13
8c	NH	$-(CH_2)_4-$	3-OCH_3PhCH_2-	42.5 ± 3.5%	>20	
8d	NH	$-(CH_2)_4-$	4-$ClPhCH_2-$	39.5 ± 1.5%	>20	
8e	NH	$-(CH_2)_4-$	4-$BrPhCH_2-$	50.5 ± 1.5%	>20	
8f	NH	$-(CH_2)_4-$	4-$IPhCH_2-$	70.0 ± 4.0%	>20	
8g	NH	$-(CH_2)_4-$	4-CF_3PhCH_2-	70.0 ± 1.0%	16.5 ± 1.5	10.8 ± 0.73
8h	NH	$-(CH_2)_4-$	3-$ClPhCH_2-$	79.0 ± 3.0%	13.0 ± 1.8	3.67 ± 0.52
8i	NH	$-(CH_2)_4-$	3-NO_2PhCH_2-	1.8 ± 1.8%	>20	
8j	NH	$-(CH_2)_4-$	4-NO_2PhCH_2-	58.5 ± 0.5%	15.0 ± 0.75	8.10 ± 1.18
8k	NH	$-(CH_2)_5-$	$PhCH_2-$	68.5 ± 7.5%	>20	
8l	NH	$-(CH_2)_5-$	3-CF_3PhCH_2-	17.0 ± 3.0%	>20	
8m	NH	$-(CH_2)_5-$	3-OCH_3PhCH_2-	12.5 ± 1.5%	>20	
8n	NH	$-(CH_2)_5-$	4-$ClPhCH_2-$	24.0 ± 3.0%	>20	
8o	NH	$-(CH_2)_5-$	4-$BrPhCH_2-$	22.0 ± 1.0%	>20	
8p	NH	$-(CH_2)_5-$	4-$IPhCH_2-$	23.0 ± 3.0%	>20	
8q	NH	$-(CH_2)_5-$	4-CF_3PhCH_2-	15.0 ± 2.0%	>20	
8r	NH	$-(CH_2)_5-$	3-$ClPhCH_2-$	88.0 ± 4.0%	6.8 ± 0.4	4.51 ± 1.75
8s	NH	$-(CH_2)_5-$	3-NO_2PhCH_2-	85.5 ± 1.5%	6.0 ± 0.22	7.03 ± 1.06
8t	NH	$-(CH_2)_5-$	4-NO_2PhCH_2-	87.0 ± 1.0%	10.3 ± 1.4	4.67 ± 0.89
PD	—	$-CH_3/-CH_3$	3-CF_3Ph-	28 ± 1.1	13.6 ± 2.1	18.8 ± 2.46

[a] For compounds with an ED_{50} < 20 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 > 20 μM the score at 50 μM is recorded.
[b] % inhibition of specific [^{3}H]CPX binding by 10 μM allosteric enhancer, N = 3.

3.1.2. 3-Trifluoromethylbenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5b). Yield 34%. ^{1}H NMR δ 1.71–1.75 (m, 4H, 2× CH_2), 2.48–2.52 (m, 2H, CH_2), 2.69–2.73 (m, 2H, CH_2), 5.31 (s, 2H, CH_2Ph), 6.02 (br s, 2H, NH_2), 7.46–7.68 (m, 4H, ArH). HR-ES MS calcd for $C_{17}H_{17}F_3NO_2S^+$ (M+1) 356.0927, found 356.0921.

3.1.3. 3-Methoxybenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5c). Yield 29%. ^{1}H NMR δ 1.75 (m, 4H, 2× CH_2), 2.49 (m, 2H, CH_2), 2.74 (m, 2H, CH_2), 3.82 (s, 3H, OCH_3), 5.26 (s, 2H, CH_2Ph), 5.99 (br s, 2H, NH_2), 6.86 (d, J = 8.1 Hz, 1H, ArH), 6.98 (d, J = 8.7 Hz, 2H, ArH), 7.30 (d, J = 7.5 Hz, 1H, ArH). HR-ES MS calcd for $C_{17}H_{20}NO_3S^+$ (M+1) 318.1158, found 318.1148.

3.1.4. 4-Chlorobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxylate (5d). Yield 46%. ^{1}H NMR δ 1.70–1.81 (m, 4H, 2× CH_2), 2.48–2.51 (m, 2H, CH_2), 2.67–2.71 (m, 2H, CH_2), 5.23 (s, 2H, CH_2Ph), 5.77 (br s, 2H, NH_2), 7.30–7.37 (m, 4H, ArH). HR-ES MS calcd for $C_{16}H_{17}ClNO_2S^+$ (M+1) 322.0663, found 322.0661.

3.1.5. 4-Bromobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5e). Yield 54%. ^{1}H NMR δ 1.70–1.81 (m, 4H, 2× CH_2), 2.49 (m, 2H, CH_2), 2.69 (m, 2H, CH_2), 5.21 (s, 2H, CH_2Ph), 5.81 (br s, 2H, NH_2), 7.27 (d, J = 8.3 Hz, 2H, ArH), 7.49 (d, J = 8.3 Hz, 2H, ArH). HR-ES MS calcd for $C_{16}H_{17}BrNO_2S^+$ (M+1) 366.0158, found 366.0148.

3.1.6. 4-Iodobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5f). Yield 42%. ^{1}H NMR δ 1.68–1.81 (m, 4H, 2× CH_2), 2.49 (m, 2H, CH_2), 2.68 (m, 2H, CH_2), 5.20 (s, 2H, CH_2Ph), 5.70 (br s, 2H, NH_2), 7.14 (d, J = 8.3 Hz, 2H, ArH), 7.69 (d, J = 8.3 Hz, 2H, ArH). HR-ES MS calcd for $C_{16}H_{17}INO_2S^+$ (M+1) 414.0019, found 414.0010.

3.1.7. 4-Trifluoromethylbenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5g). Yield 53%. ^{1}H NMR δ 1.70–1.82 (m, 4H, 2× CH_2), 2.51 (m, 2H, CH_2), 2.71 (m, 2H, CH_2), 5.33 (s, 2H, CH_2Ph), 5.57 (br s, 2H, NH_2), 7.51 (d, J = 8.1 Hz, 2H, ArH), 7.63 (d, J = 8.1 Hz, 2H, ArH). HR-ES MS calcd for $C_{17}H_{17}F_3NO_2S^+$ (M+1) 356.0927, found 356.0928.

3.1.8. 4-Phenylbenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (5h). Yield 15%. ^{1}H NMR δ 1.70–1.82 (m, 4H, 2× CH_2), 2.51 (m, 2H, CH_2), 2.74 (m, 2H, CH_2), 5.32 (s, 2H, CH_2Ph), 5.97 (br s, 2H, NH_2), 7.33–7.61 (m, 9H, ArH). HR-ES MS calcd for $C_{22}H_{22}NO_2S^+$ (M+1) 364.1366, found 364.1366.

3.1.9. Benzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxylate (5i). Yield 49%. ^{1}H NMR δ 1.60–1.68 (m, 4H, 2× CH_2), 1.77–1.80 (m, 2H, CH_2), 2.54–2.58 (m, 2H, CH_2), 2.98–3.01 (m, 2H, CH_2), 5.30 (s, 2H, CH_2Ph), 5.41 (br s, 2H, NH_2), 7.30–7.41 (m, 5H, ArH). HR-ES MS calcd for $C_{17}H_{20}NO_2S^+$ (M+1) 302.12093, found 302.11954.

3.1.10. 3-Trifluoromethylbenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxylate (5j). Yield 23%. ^{1}H NMR δ 1.57–1.68 (m, 4H, 2× CH_2), 1.77–1.81 (m, 2H, CH_2), 2.56–2.59 (m, 2H, CH_2), 2.96–2.99 (m, 2H, CH_2), 5.33 (s, 2H, CH_2Ph), 5.86 (br s, 2H, NH_2), 7.46–7.68 (m, 5H, ArH). HR-ES MS calcd for $C_{18}H_{19}F_3NO_2S^+$ (M+1) 370.1083, found 370.1094.

3.1.11. Phenethyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxylate (5k). Yield 71%. ^{1}H NMR δ 1.53–1.71 (m, 4H, 2× CH_2), 1.78–1.86 (m, 2H, CH_2), 2.56–2.59 (m, 2H, CH_2), 2.89–2.93 (m, 2H, CH_2), 3.05 (t, J = 6.8 Hz, 2H, CH_2), 4.51 (t, J = 6.8 Hz, 2H, CH_2Ph), 5.55 (br s, 1H, NH_2), 7.22–7.36 (m, 4H, ArH). HR-ES MS calcd for $C_{18}H_{22}NO_2S^+$ (M+1) 316.1366, found 316.1377.

3.2. General procedure for *N*-phenyl 2-aminothiophene-3-carboxamide formation (6b–g)

The appropriate *N*-phenyl cyanoacetamide (1.0 molar equiv), cyclohexanone or cycloheptanone (4.0 equiv), ammonium acetate (1.3 equiv) and glacial acetic acid (3.5 equiv) in benzene (50 mL) were refluxed for 16 h in a Dean–Stark apparatus. The reaction mixture was cooled and then diluted with $CHCl_3$. The mixture was washed with H_2O (100 mL), 10% Na_2CO_3 (100 mL) then H_2O (100 mL) and the organic phase was dried over anhydrous $MgSO_4$ and filtered. Evaporation of the solvent afforded the crude Knoevenagel product, which was used without further purification. This material was dissolved in dry EtOH (11 mL) and prior to the addition of sulfur (4.0 equiv) and *N*,*N*-diethylamine (4.0 equiv). The reaction mixture was stirred for 1.5 h at 40–50 °C and then cooled to 0 °C. After filtration and evaporation of the solvent, the target compound was purified by chromatography on a SiO_2 column eluting with CH_2Cl_2/MeOH/NH_3 (99%:0.5%:0.5%).

3.2.1. 3-Trifluoromethylphenyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (6b). Yield 20%. ^{1}H NMR δ 1.86 (m, 4H, 2× CH_2), 2.57 (m, 2H, CH_2), 2.78 (m, 2H, CH_2), 5.97 (br s, 2H, NH_2), 7.34 (d, 1H, ArH), 7.43 (t, 1H, ArH), 7.71 (t, 2H, ArH), 7.84 (br s, 1H, NH). HR-ES MS calcd for $C_{16}H_{16}F_3N_2OS^+$ (M+1) 341.0930, found 341.0948.

3.2.2. 3-Methoxyphenyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (6c). Yield 28%. ^{1}H NMR δ 1.84–1.85 (m, 2H, 2× CH_2), 2.57 (m, 2H, CH_2), 2.76 (m, 2H, CH_2), 3.82 (s, 3H, OCH_3), 5.95 (br s, 2H, NH_2), 6.65 (d, 1H, ArH), 6.99 (d, 1H, ArH), 7.18–7.31 (m, 2H, ArH), 7.51 (br s, 1H, NH). HR-ES MS calcd for $C_{16}H_{19}N_2O_2S^+$ (M+1) 303.1162, found 303.1150.

3.2.3. 4-Chlorophenyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (6d). Yield 29%. ^{1}H NMR δ 1.82 (m, 4H, 2× CH_2), 2.55 (m, 2H, CH_2), 2.73 (s, 2H, CH_2), 5.94 (br s, 2H, NH_2), 7.25 (d, J = 8.7 Hz, 2H, ArH), 7.46 (d, J = 8.7 Hz, 2H, ArH), 7.57 (br s, 1H, NH). HR-ES MS calcd for $C_{15}H_{16}ClN_2OS^+$ (M+1) 307.0666, found 307.0663.

3.2.4. 4-Bromophenyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (6e). Yield 88%. ^{1}H NMR δ 1.82 (m, 4H, 2× CH_2), 2.54 (m, 2H, CH_2), 2.71 (m, 2H, CH_2), 5.93 (br s, 2H, NH_2), 7.37–7.43 (m, 4H, ArH), 7.58 (br s, 1H, NH). HR-ES MS calcd for $C_{15}H_{16}BrN_2OS^+$ (M+1) 351.0161, found 351.0157.

3.2.5. 4-Iodophenyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (6f). Yield 24%. ^{1}H NMR δ 1.82 (m, 4H, 2× CH_2), 2.55 (m, 2H, CH_2), 2.72 (m, 2H, CH_2), 5.91 (br s, 2H, NH_2), 7.30 (d, J = 8.7 Hz, 2H, ArH), 7.58 (d, J = 8.7 Hz, 2H, ArH). HR-ES MS calcd for $C_{15}H_{16}IN_2OS^+$ (M+1) 399.0023, found 399.0021.

3.2.6. 4-Trifluoromethylphenyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (6g). Yield 29%. ^{1}H NMR δ 1.82 (m, 4H, 2× CH_2), 2.54 (m, 2H, CH_2), 2.74 (m, 2H, CH_2), 5.70 (br s, 2H, NH_2), 7.53 (d,

J = 8.7 Hz, 2H, ArH), 7.64 (d, J = 8.7 Hz, 2H, ArH), 7.79 (br s, 1H, NH). HR-ES MS calcd for $C_{16}H_{16}F_3N_2OS^+$ (M+1) 341.0930, found 341.0947.

3.3. General procedure for 2-aminothiophene-3-carboxylic acid formation (7a,b)

Ethyl 2-aminothiophene-3-carboxylate (1.0 equiv) was dissolved in EtOH (10 mL) and KOH (4.0 molar equiv) in H_2O (10 mL) was added and the reaction was refluxed for ~5 h. After cooling, the reaction mixture was diluted with H_2O and washed with diethyl ether. The aqueous phase was then adjusted to pH 6 with 1 M HCl and a pure solid precipitated, which was collected by suction filtration.

3.3.1. 2-Amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid (7a). Yield 62%. Mp = 116–118 °C. ^{1}H NMR (DMSO-d_6) δ 1.65 (m, 4H, 2× CH_2), 2.39 (m, 2H, CH_2), 2.56 (m, 2H, CH_2), 7.15 (br s, 2H, NH_2). LR-ES MS calcd for $C_9H_{10}NO_2S^-$ (M−1) 196.2, found 196.1.

3.3.2. 2-Amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxylic acid (7b). Yield 77%. Mp = 100–101 °C. ^{1}H NMR (DMSO-d_6) δ 1.50–1.52 (m, 4H, 2× CH_2), 1.70–1.77 (m, 2H, CH_2), 2.49 (m, 2H, CH_2), 2.88–2.92 (m, 2H, CH_2), 7.03 (br s, 2H, NH_2). LR-ES MS calcd for $C_{10}H_{12}NO_2S^-$ (M−1) 210.1, found 210.1.

3.4. General procedure for *N*-benzyl 2-aminothiophene-3-carboxamide formation (8a–t)

A solution of the 2-aminothiophene-3-carboxylic acid **7** (1.0 molar equiv) in CH_2Cl_2 (3 mL) was cooled to 0 °C. The appropriate benzyl amine (1.0 equiv) and a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.0 equiv), 1-hydroxybenzotriazole hydrate (1.0 equiv) and *N*,*N*-(dimethylamino)pyridine (1.0 equiv) in CH_2Cl_2 (3 mL) were added. The mixture was stirred for 16 h at room temperature before being diluted with $CHCl_3$ (10 mL) and washed with brine (3 × 30 mL). After drying (anhydrous $MgSO_4$) and filtration, the solvent evaporated and the crude product was purified by chromatography on a deactivated SiO_2 column eluting with hexane/ethyl acetate (2:1).

3.4.1. Benzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8a). Yield 13%. ^{1}H NMR δ 1.77 (m, 4H, 2× CH_2), 2.53–2.59 (m, 4H, 2× CH_2), 4.58 (d, 2H, CH_2Ph), 5.57 (br s, 2H, NH_2), 6.02 (br s, 1H, NH), 7.24–7.39 (m, 5H, ArH). HR-ES MS calcd for $C_{16}H_{19}N_2OS^+$ (M+1) 287.1213, found 287.1215.

3.4.2. 3-Trifluoromethylbenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8b). Yield 36%. ^{1}H NMR δ 1.79 (m, 4H, 2× CH_2), 2.54–2.61 (m, 4H, 2× CH_2), 4.62 (d, 2H, CH_2Ph), 6.02 (br s, 1H, NH), 6.10 (br s, 2H, NH_2), 7.36–7.56 (m, 4H, ArH). HR-ES MS calcd for $C_{17}H_{18}F_3N_2OS^+$ (M+1) 355.1086, found 355.1082.

3.4.3. 3-Methoxybenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8c). Yield 30%. ^{1}H NMR δ 1.78 (m, 4H, 2× CH_2), 2.53–2.59 (m, 4H, 2× CH_2), 3.80 (s, 3H, OCH_3), 4.55 (d, 2H, CH_2Ph), 5.99 (br s, 2H, NH_2), 6.80–6.94 (m, 3H, ArH), 7.25 (m, 1H, ArH). HR-ES MS calcd for $C_{17}H_{21}N_2O_2S^+$ (M+1) 317.1318, found 317.1314.

3.4.4. 4-Chlorobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8d). Yield 16%. ^{1}H NMR δ 1.78 (m, 4H, 2× CH_2), 2.53–2.58 (m, 2H, 2× CH_2), 4.53 (d, 2H, CH_2Ph), 6.01 (br s, 3H, NH_2 and NH), 7.24 (d, J = 8.4 Hz, 2H, ArH), 7.29 (d, J = 8.4 Hz, 2H, ArH). HR-ES MS calcd for $C_{16}H_{18}ClN_2OS^+$ (M+1) 321.0823, found 321.0839.

3.4.5. 4-Bromobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8e). Yield 16%. ^{1}H NMR δ 1.77 (m, 4H, 2× CH_2), 2.53–2.58 (m, 4H, 2× CH_2), 4.50 (d, 2H, CH_2Ph), 6.01 (m, 2H, NH_2 and NH), 7.18 (d, J = 8.3 Hz, 2H, ArH), 7.44 (d, J = 8.3 Hz, 2H, ArH). HR-ES MS calcd for $C_{16}H_{18}BrN_2OS^+$ (M+1) 365.0317, found 365.0332.

3.4.6. 4-Iodobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8f). Yield 33%. Mp 152–154 °C. ^{1}H NMR δ 1.78 (m, 4H, 2× CH_2), 2.53–2.58 (m, 2H, 2× CH_2), 4.50 (d, 2H, CH_2Ph), 6.01 (br s, 3H, NH_2), 7.06 (d, J = 8.4 Hz, 2H, ArH), 7.64 (d, J = 8.4 Hz, 2H, ArH). HR-ES MS calcd for $C_{16}H_{18}IN_2OS^+$ (M+1) 413.0179, found 413.0158.

3.4.7. 4-Trifluoromethylbenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8g). Yield 34%. ^{1}H NMR δ 1.79 (m, 4H, 2× CH_2), 2.53–2.61 (m, 4H, 2× CH_2), 4.61 (d, 2H, CH_2Ph rotamers), 6.02 (br s, 2H, NH_2), 6.12 (br s, 1H, NH), 7.42 (d, J = 8.1 Hz, ArH), 7.58 (d, J = 8.1 Hz, 2H, ArH). HR-ES MS calcd for $C_{17}H_{18}F_3N_2OS^+$ (M+1) 355.1086, found 355.1083.

3.4.8. 3-Chlorobenzyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxamide (8h). Yield 22%. ^{1}H NMR δ 1.79 (m, 4H, 2× CH_2), 2.53–2.61 (m, 4H, 2× CH_2), 4.55 (d, 2H, CH_2Ph rotamers), 5.89 (br s, 1H, NH), 6.05 (br s, 2H, NH_2), 7.19–7.30 (m, 4H, ArH). HR-ES MS calcd for $C_{16}H_{18}ClN_2OS^+$ (M+1) 321.0823, found 321.0829.

3.4.9. 3-Nitrobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8i). Yield 6%. ^{1}H NMR δ 1.78 (m, 4H, 2× CH_2), 2.52 (m, 2H, CH_2), 2.63 (m, 2H, CH_2), 4.65 (d, 2H, CH_2Ph), 6.02 (br s, 2H, NH_2), 6.23 (br s, 1H, NH), 7.48 (t, J = 7.8 Hz, 1H, ArH), 7.66 (d, J = 7.5 Hz, 1H, ArH), 8.07 (d, J = 7.5 Hz, 1H, ArH), 8.14 (s, 1H, ArH). HR-ES MS calcd for $C_{16}H_{18}N_3O_3S^+$ (M+1) 332.1063, found 332.1064.

3.4.10. 4-Nitrobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8j). Yield 11%. ^{1}H NMR δ 1.81 (m, 4H, 2× CH_2), 2.55 (m, 2H, CH_2), 2.62 (m, 2H, CH_2), 4.67 (d, 2H, CH_2Ph), 6.03 (br s, 2H, NH_2), 6.17 (br s, 1H, NH), 7.47 (d, J = 8.7 Hz, 2H, ArH), 8.18 (d, J = 8.7 Hz, 2H, ArH). HR-ES MS

calcd for $C_{16}H_{18}N_3O_3S^+$ (M+1) 332.1063, found 332.1062.

3.4.11. Benzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8k). Yield 25%. ^{1}H NMR δ 1.59–1.69 (m, 4H, 2× CH_2), 1.76–1.84 (m, 2H, CH_2), 2.57–2.61 (m, 2H, CH_2), 2.72–2.76 (m, 2H, CH_2), 4.57 (d, 2H, CH_2Ph), 5.12 (br s, 2H, NH_2), 5.88 (br s, 1H, NH), 7.25–7.37 (m, 5H, ArH). HR-ES MS calcd for $C_{17}H_{21}N_2OS^+$ (M+1) 301.1369, found 301.1368.

3.4.12. 3-Trifluoromethylbenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8l). Yield 29%. Mp 118–120 °C. ^{1}H NMR δ 1.65–1.68 (m, 4H, 2× CH_2), 1.78–1.82 (m, 2H, CH_2), 2.59–2.62 (m, 2H, CH_2), 2.74–2.78 (m, 2H, CH_2), 4.64 (d, 2H, CH_2Ph), 5.99 (br s, 2H, NH_2), 7.43–7.58 (m, 4H, ArH). HR-ES MS calcd for $C_{18}H_{20}F_3N_2OS^+$ (M+1) 369.1243, found 369.1241.

3.4.13. 3-Methoxybenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8m). Yield 29%. Mp 103–105 °C. ^{1}H NMR δ 1.63–1.66 (m, 4H, 2× CH_2), 1.78–1.80 (m, 2H, CH_2), 2.57–2.60 (m, 2H, CH_2), 2.73–2.76 (m, 2H, CH_2), 3.79 (s, 3H, OCH_3), 4.54 (d, 2H, CH_2Ph), 4.61 (br s, 1H, NH), 5.90 (br s, 2H, NH_2), 6.80–6.91 (m, 3H, ArH), 7.22–7.27 (m, 1H, ArH). HR-ES MS calcd for $C_{18}H_{23}N_2O_2S^+$ (M+1) 331.1475, found 331.1471.

3.4.14. 4-Chlorobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8n). Yield 26%. Mp 100–102 °C. ^{1}H NMR δ 1.63–178 (m, 6H, 3× CH_2), 2.57 (m, 2H, CH_2), 2.71 (m, 2H, CH_2), 4.49 (br s, 2H, CH_2Ph), 5.05 (br s, 2H, NH_2), 6.05 (br s, 1H, NH), 7.25 (m, 4H, ArH). HR-ES MS calcd for $C_{17}H_{20}ClN_2OS^+$ (M+1) 335.0979, found 335.0981.

3.4.15. 4-Bromobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8o). Yield 19%. Mp 145–146 °C. ^{1}H NMR δ 1.60–1.69 (m, 4H, 2× CH_2), 1.77–1.84 (m, 2H, CH_2), 2.58–2.61 (m, 2H, CH_2), 2.71–2.75 (m, 2H, CH_2), 4.51 (d, 2H, CH_2Ph), 4.69 (br s, 2H, NH_2), 5.90 (br s, 1H, NH), 7.20 (d, J = 8.3 Hz, 2H, ArH), 7.45 (d, J = 8.3 Hz, 2H, ArH). HR-ES MS calcd for $C_{17}H_{20}BrN_2OS^+$ (M+1) 379.0474, found 379.0470.

3.4.16. 4-Iodobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8p). Yield 13%. Mp 162–163 °C. ^{1}H NMR δ 1.64–1.66 (m, 4H, 2× CH_2), 1.77–1.81 (m, 2H, CH_2), 2.58 (m, 2H, CH_2), 2.72 (m, 2H, CH_2), 4.51 (d, 2H, CH_2Ph), 5.18 (br s, 1H, NH), 5.87 (br s, 2H, NH_2), 7.08 (d, J = 8.1 Hz, 2H, ArH), 7.66 (d, J = 8.1 Hz, 2H, ArH). HR-ES MS calcd for $C_{17}H_{20}IN_2OS^+$ (M+1) 427.0336, found 427.0342.

3.4.17. 4-Trifluoromethylbenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8q). Yield 22%. Mp 130–132 °C. ^{1}H NMR δ 1.63–1.68 (m, 4H, 2× CH_2), 1.76–1.83 (m, 2H, CH_2), 2.56–2.60 (m, 2H, CH_2), 2.72–2.76 (m, 2H, CH_2), 4.60 (d, 2H, CH_2Ph), 6.10 (br s, 2H, NH_2), 7.41 (d, J = 8.1 Hz, 2H, ArH), 7.57 (d, J = 8.1 Hz, 2H, ArH). HR-ES MS calcd for $C_{18}H_{20}F_3N_2OS^+$ (M+1) 369.1243, 369.1253.

3.4.18. 3-Chlorobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8r). Yield 23%. Mp 121–122 °C. ^{1}H NMR δ 1.65–1.66 (m, 4H, 2× CH_2), 1.79–1.81 (m, 2H, CH_2), 2.58–2.61 (m, 2H, CH_2), 2.73–2.76 (m, 2H, CH_2), 4.55 (d, 2H, CH_2Ph), 5.15 (br s, 2H, NH_2), 5.93 (br s, 1H, NH), 7.21–7.31 (m, 4H, ArH). HR-ES MS calcd for $C_{17}H_{20}ClN_2OS^+$ (M+1) 335.0979, found 335.0986.

3.4.19. 3-Nitrobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8s). Yield 26%. Mp 141–143 °C. ^{1}H NMR δ 1.64 (m, 4H, 2× CH_2), 1.78–1.80 (m, 2H, CH_2), 2.56–2.59 (m, 2H, CH_2), 2.75–2.78 (m, 2H, CH_2), 4.64 (d, 2H, CH_2Ph), 4.88 (br s, 2H, NH_2), 6.24 (br s, 1H, NH), 7.48 (t, J = 8.0 Hz, 1H, ArH), 7.66 (d, J = 7.5 Hz, 1H, ArH), 8.08 (d, J = 8.1 Hz, 1H, ArH), 8.15 (s, 1H, ArH). HR-ES MS calcd for $C_{17}H_{20}N_3O_3S^+$ (M+1) 346.1220, found 346.1233.

3.4.20. 4-Nitrobenzyl 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophene-3-carboxamide (8t). Yield 36%. Mp 155–157 °C. ^{1}H NMR δ 1.67 (m, 4H, 2× CH_2), 1.81–1.82 (m, 2H, CH_2), 2.60–2.63 (m, 2H, CH_2), 2.76–2.78 (m, 2H, CH_2), 4.67 (d, 2H, CH_2Ph), 6.15 (br s, 1H, NH), 7.49 (d, J = 8.1 Hz, 2H, ArH), 8.19 (d, J = 8.1 Hz, 2H, ArH). HR-ES MS calcd for $C_{17}H_{20}N_3O_3S^+$ (M+1) 346.1220, found 346.1228.

3.5. Assay of AE activity[6,7]

The assay of AE activity consisted of three phases: (1) binding to equilibrium of the agonist, ^{125}I-ABA to the A_1AR–G protein ternary complex; (2) stabilization of that complex by adding vehicle or AE for 5 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-ABA 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-ABA and 10 μg 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 50 μL of AE (0.1–50 μM, final) initiated stabilization of the ternary complex (phase 2). Five minutes later 50 μL solution containing 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-ABA. 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.

3.6. Assay of A_1AR 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). 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.

Acknowledgment

The authors thank the Victorian College of Pharmacy, Monash University, for providing a postgraduate scholarship for G.N. The authors also wish to acknowledge financial support from NIH Grant R01 HL56111.

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