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Original article

Synthesis of 2-amino-3-heteroaroylthiophenes and evaluation of their activity as potential allosteric enhancers at the human A_1 receptor

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Abstract

2-Amino-3-benzoylthiophenes are allosteric enhancers of agonist binding to the adenosine A_1 receptor. New compounds bearing an heteroaroyl instead of the benzoyl moiety at the 3-position of the thiophene were synthesized. The phenyl ring was replaced with heterocycles that possess heteroatoms able to form hydrogen bonds (2-furanyl, 2-benzofuranyl, 2-pyridinyl in compounds **2–13**) or with a thienyl moiety as isoster of the phenyl ring (2-thienyl, 3-thienyl and 5-halo-2-thienyl in compounds **14–29**). The effect of several alkyl substituents at positions 4 and 5 of the thiophene ring to increase enhancer activity was determined. The ability of the new molecules to reduce the cAMP content in CHO cells expressing the human adenosine A_1 receptor was evaluated. Compounds **2–13** with hydrogen bond-forming heteroatoms did not show significant activity as allosteric enhancers. On the other hand, compounds **15–16** and **19–20** with an unsubstituted thienyl moiety as replacement for the phenyl ring were nearly as efficacious as PD 81,723, the prototypical A_1 allosteric enhancer. Alkyl substituents at positions 4 and 5 of the thiophene ring were tolerated while a substituted piperidine ring was not tolerated. We conclude that hydrogen bonds could not be formed in the domain of the receptor that accommodates the phenyl ring of 2-amino-3-benzoylthiophene derivatives, indicating that this domain is hydrophobic.

Keywords: Allosteric enhancer; Adenosine A_1 receptor; 2-Amino-3-heteroaroylthiophenes; Cyclic AMP

1. Introduction

Adenosine is cardioprotective during periods of ischemia and a similar effect has been demonstrated in brain. These and other important physiological effects of adenosine on the cardiovascular and central nervous systems are mediated through the interaction with the A_1 receptor subtype [1]. The therapeutic potential of A_1 adenosine receptor agonists is undermined by a poor side effect profile due to the widespread distribution of adenosine receptors throughout the body. One way to avoid these side effects is offered by positive A_1 allosteric adenosine modulators whose action would be limited to times and locations at which significant release of adenosine occurs [2].

A series of 2-amino-3-benzoylthiophene derivatives has been shown to enhance the apparent binding of the radioligand agonist [^{3}H]CHA to A_1 adenosine receptors and to decrease the rate of dissociation of [^{3}H]CHA from the receptor. It has been suggested that these compounds act on an allosteric site, distinct from the orthosteric adenosine binding site, to stabilize the high affinity state of the receptor for agonist binding [3].

Among these compounds, PD 81,723 **(1)** has been proposed as a lead compound and chosen for pharmacological investigations. It increased agonist potencies 4- to 10-fold both in membrane and whole-cell function assays. As a drawback, this compound was also able to inhibit the binding

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of [^{3}H]DPCPX, implying a collateral antagonistic activity [4–8].

Analysis of the structure–activity effects of the first series of 2-amino-3-benzoylthiophene derivatives, reported by Bruns in 1990, indicated that the 2-amino and 3-keto carbonyl groups were both essential for activity. Further, it was suggested that alkyl substitutions at position 4 of the thiophene ring as well as various substitutions on the phenyl ring increased activity. Interestingly, the evaluation of the substitution of the benzoyl group at position 3 of the thiophene with carbamoyl, acetyl and cyclohexyl carbonyl moieties suggested that the phenyl ring could not be replaced [9].

In the series of 2-amino-3-benzoylthiophenes that followed the original paper of Bruns et al., a number of compounds proved to be superior to PD 81,723 **(1)** in enhancing the agonist binding to the A_1 receptor and their collateral antagonistic activity was low [10–13]. These studies gave further information about the structural requirements for enhancement. In particular it was found that a fused ring between positions 4 and 5 of the thiophene led to good activity. Optimally, this fused ring was composed of three or four methylene units or formed *N*-benzyltetrahydropyridine ring.

The aim of the present research was to more thoroughly investigate the nature of the receptor domain that accommodates the phenyl ring of 2-amino-3-benzoylthiophene derivatives. Realizing that the number of compounds considered by Bruns was not sufficient to state that the phenyl ring was the only moiety accepted by the receptor within this domain, we prepared a series of 2-amino-3-heteroaroylthiophenes where we maintained the best substitutions for enhancement at positions 4 and 5 of the thiophene and we replaced the phenyl ring with different heterocycles.

We first replaced the phenyl ring with 2-furanyl, 2-benzofuranyl and 2-pyridyl moieties to test the possibility of hydrogen bond formation within the domain. In addition, especially with pyridine, we could also increase the hydrophilic properties of the molecules, since low water-solubility is one of the major limitations of the 2-amino-3-benzoylthiophene derivatives [3].

In case the domain to which the phenyl ring binds was merely hydrophobic, as Bruns suggested, we thought that replacement of the phenyl ring by an isosteric 2- or 3-thienyl moiety might lead to improved activity. To further increase the lipophilicity of these moieties and further probe the size of this hydrophobic domain, we evaluated the introduction of an halogen (chlorine and bromine) at the 5-position of the thiophene-2-carbonyl moiety.

Our research would confirm or refute the structure–activity relationships initially proposed by Bruns for enhancement of adenosine A_1 receptors and further complete our understanding of these relationships. (Fig. 1)

2. Chemistry

Compounds **2–29** were obtained by a method analogous to that described by Gewald et al. [14–16] (Scheme 1). The appropriate β-ketonitriles **(30–36)** were reacted with the appropriate commercially available ketones (butanone, cyclopentanone, cyclohexanone and 4-benzylpiperidone) in the presence of a base (morpholine) to give the Knoevenagel intermediate. This intermediate, in turn, was reacted with elemental sulfur to provide the desired thiophene ring formation.

Ketonitriles **30–35** were easily synthesized following procedures reported in the literature [17]. Compound **36** was obtained from the corresponding bromo derivative **37** [18] through a reaction of change bromine-cyanide.

3. Pharmacology

The new compounds were evaluated in a functional assay to test their potential A_1 enhancer activity and the putative enhancers identified by this test were then evaluated at the receptor level in equilibrium binding assays.

Allosteric enhancers of the action of adenosine are believed to stabilize a conformation of the A_1 adenosine receptor that has a high affinity for agonists. This effect is manifested as a slowing of the rate of dissociation of an agonist from the receptor [3]. In addition, an allosteric enhancer appears to stabilize an active conformation of the receptor even in the absence of an agonist. Thus, in cells with A_1 adenosine receptors that are active spontaneously in the absence of an agonist (such as the CHO cells used in our study), an allosteric enhancer may increase the number of receptors that are active at any given time, and thus cause a change in cell function. Compounds with the potential to be allosteric enhancers of activation of human A_1 adenosine receptors are expected to decrease the content of cAMP in CHO cells expressing human A_1 adenosine receptor. Not all compounds that decrease cAMP content of CHO cells are allosteric enhancers, however. Compounds that are rapidly toxic to cells, compounds that directly inhibit adenylyl cyclase, and agonists of A_1 adenosine receptors may cause reductions of cAMP content similar to those caused by allosteric enhancers in our assay. Therefore, the results of the experiments reported here can identify compounds as putative allosteric enhancers, but not definitively demonstrate that allosteric enhancement is the mechanism of action.

The cyclic AMP assay was carried out to determine the ability of compounds **2–29** to activate the human A_1 adenosine receptor [19–21]. The experiments were performed using CHO cells expressing recombinant human A_1 receptors. Allosteric enhancement was measured as the action of a test compound at different concentrations (0.01, 0.1, 1 and 10 μM) to reduce the cAMP content in the presence of a sub-optimal (0.01 nM) concentration of an agonist (CPA). The reference compound for comparison was PD 81, 723 **(1)** (Table 1).

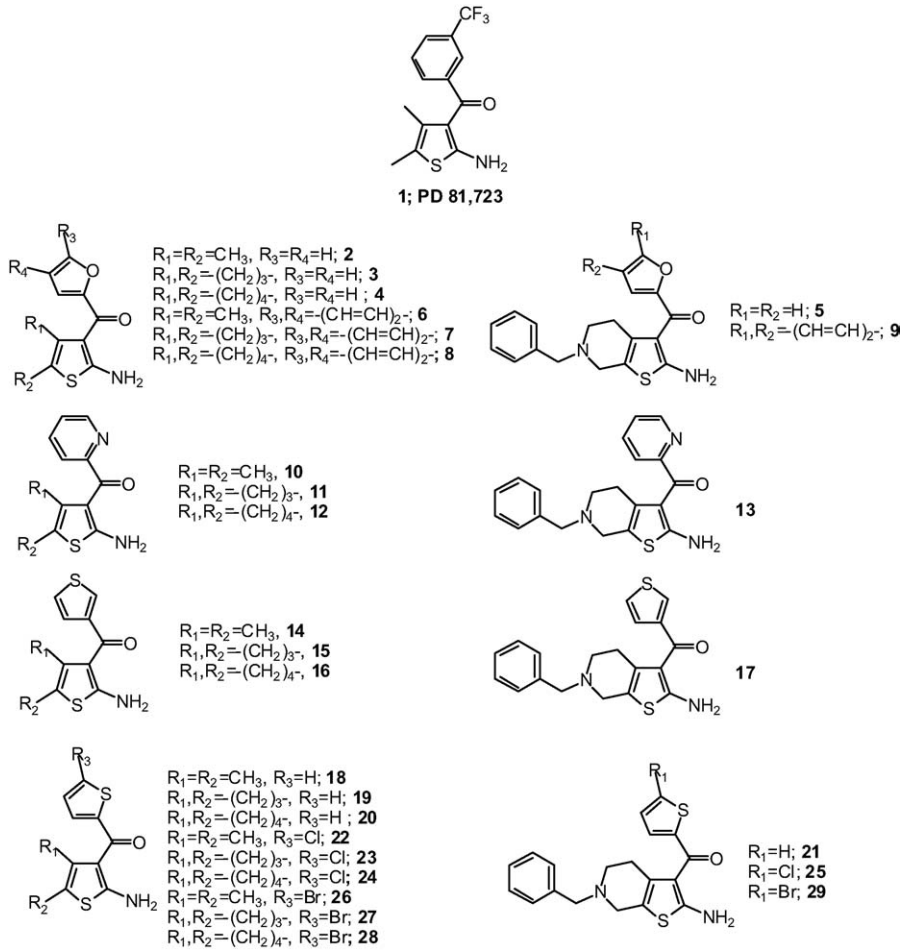


Fig. 1. PD81,723 and structures of compounds **2–29**.

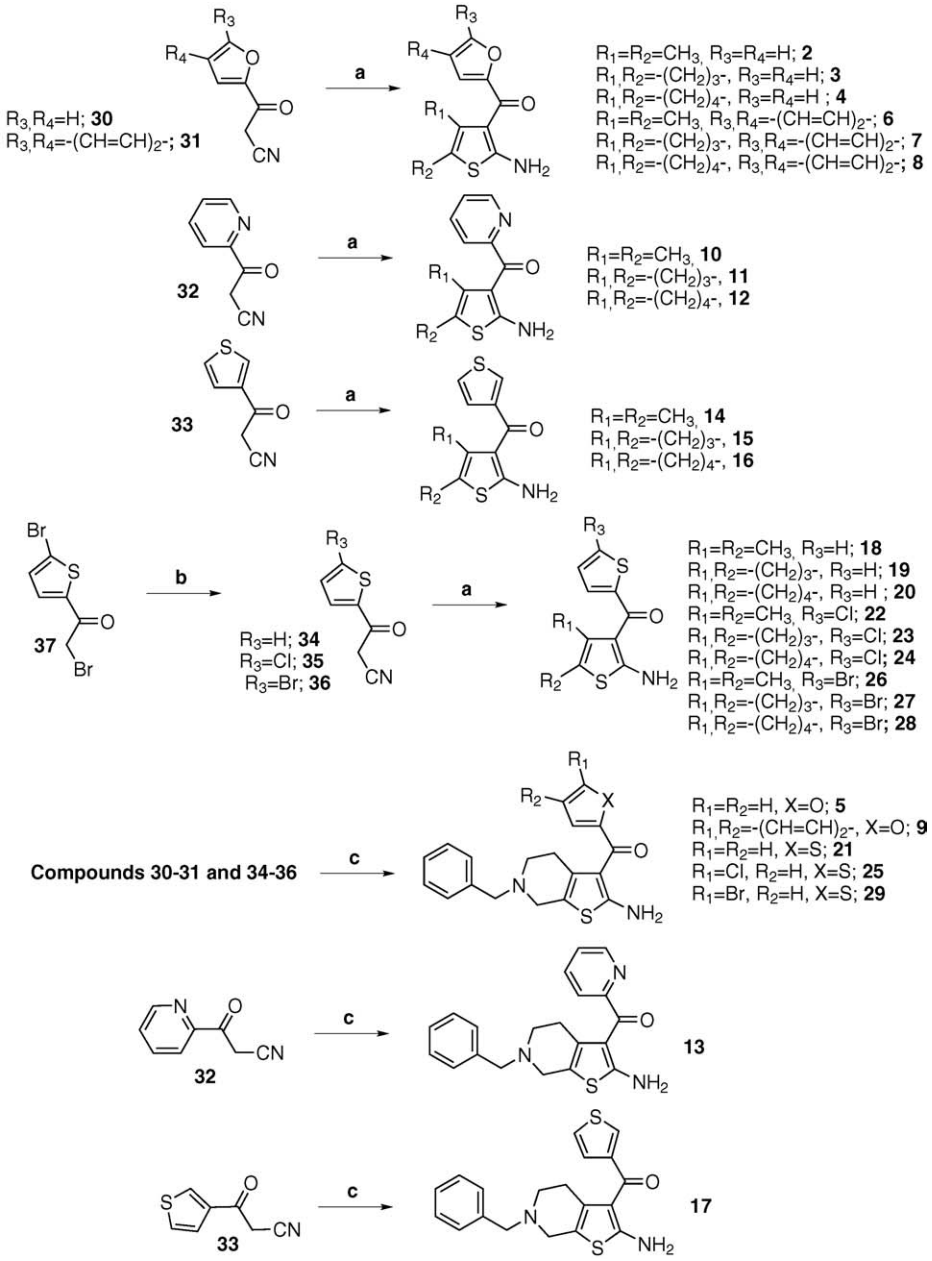
4. Results and discussion

Replacement of the phenyl ring in position 3 of the 2-aminothiophene with heterocycles such as furan **(2–5)**, benzofuran **(6–9)**) and pyridine **(10–13)**, that potentially could form an hydrogen bond with the allosteric site of the A_1 adenosine receptor, led to a significant loss of activity. The most hydrophilic compounds, the pyridine derivatives **10–13**, were inactive as allosteric enhancers at any concentration. Among the furan **(2–5)** and benzofuran **(6–9)** compounds, some showed slight activity **(3–5, 7, 8)** with compound **3** being the most efficacious at concentrations of 0.1, 1 and 10 μM. Furan compounds were more active than benzofuran derivatives bearing the same substitution pattern at the 4 and 5 positions of the 2-aminothiophene ring. This finding suggests that limited steric bulk is preferred at that position.

Replacement of the phenyl ring with a thiophene **(14–21)**, which is electronically similar, was well tolerated. When there was a methylene chain of three or four carbon atoms between positions 4 and 5 of the 2-aminothiophene (compounds **15,16** and **19,20**), enhancer activity was only slightly reduced relative to that of PD 81,723 at 10 μM and was better than PD 81,723 at lower concentrations. The position at which thiophene is connected to carbonyl does not seem important, because 2-thienyl derivatives **18–21** possessed the same activity as the 3-thienyl counterparts **14–17**. Thus, results of functional assay of both the hydrophilic compounds **10–13** and hydrophobic derivatives **14–21**, seem to confirm that the aroyl receptor domain is principally hydrophobic in nature.

To investigate the size of the hydrophobic domain in the receptor to which the phenyl ring at position 3 of the 2-aminothiophene binds, the phenyl group was replaced with 5-chloro- and 5-bromo-2-thienyl moieties **(22–29)**. Compounds **22–29** all proved to be inactive as enhancers, with no significant difference between chloro and bromo derivatives. This suggests that an halogen is not tolerated in that position.

We also evaluated the effects of different substitutions at positions 4 and 5 of 2-aminothiophene. With the exception of compounds **23–24** and **27–28**, enhancement was better when there was a chain of three or four carbons linking the two positions. Compounds bearing a methyl group at positions 4 and 5 **(2, 6, 10, 14, 18, 22, 26)** were weak enhancers and only at some concentrations. Derivatives **5, 9, 13, 17, 21, 25** and **29**, where there was an *N*-benzyl-tetrahydropyridine ring, were the worst enhancers. They mainly showed a reverse value of the cAMP cyclic level (positive change of cAMP content in Table 1), appearing they stabilized the receptor in a conformation that reduced receptor activation.



Scheme 1. Reagents (a) butanone (compounds with R1=R2=CH3), cyclopentanone [compounds with R1,R2=-(CH2)3-], cyclohexanone [compounds with R1,R2=-CH2)4-], S8, morpholine, EtOH, 70 °C for 1 h then 18 h at rt; (b) KCN, EtOH/water, 2 h, rt; (c) *N*-benzylpiperidone, s8, morpholine, EtOH, 70 °C for 1 h then 18 h at rt.

Because the known allosteric enhancers are also A_1-adenosine receptor antagonists at some (usually high) concentration, the fact that compounds **15–16** and **19–20** did not show a greater efficacy than did 10 μM PD 81, 723 could be explained by a possible antagonist property of these compounds. Saturation and inhibition assays were carried out to evaluate the presence of a competitive antagonist effect.

Compounds **16** and **20** were selected for saturation binding experiments that were performed using membranes prepared from CHO:hA_1, rat cortex, and human brain (Table 2).

In the current study PD 81, 723 did not affect the K_D value of the agonist [^{3}H]CCPA to recombinant human A_1 adenosine receptors in CHO cells membranes and to A_1 receptors in human brain and rat cortex membranes but it increased the B_{MAX} value. These findings are in accordance with previous reports [22,23].

The K_D value of [^{3}H]CCPA was slightly increased by the tested compounds. This data indicated that the selected compounds **16** and **20** possessed an adenosine A_1 antagonist activity that partly reversed their allosteric property.

Compounds **15, 16** and **20** were tested at a concentration of 10 μM for their ability to displace the binding of [^{3}H]DPCPX, [^{3}H]ZM 241385, and [^{3}H]MRE 3008F20 to the ligand binding site of CHO:hA1, CHO:hA2A, and CHO:hA3 adenosine receptors, respectively (Table 3).

Table 1
Percentage change in CHO cell cAMP content in presence of compounds **2–29**

Compound	Change in cAMP content from control (mean ± SEM) concentration of test compounds			
	0.01 μM	0.1 μM	1 μM	10 μM
PD 81, 723 (1)	–1 ± 2	–7 ± 2	–13 ± 1	–50 ± 1
2	–4 ± 5	11 ± 5	–7 ± 5	5 ± 10
3	3 ± 4	–14 ± 4	–0.8 ± 3	–23 ± 2
4	–10 ± 2	2 ± 6	–3 ± 6	–10 ± 7
5	–27 ± 3	0.6 ± 4	–15 ± 3	–8 ± 11
6	6 ± 5	–7 ± 4	16 ± 5	7 ± 6
7	–5 ± 3	–13 ± 3	3 ± 5	–4 ± 6
8	–13 ± 5	–3 ± 4	–4 ± 3	–5 ± 4
9	4 ± 3	14 ± 4	–10 ± 2	11 ± 4
10	0.7 ± 3	–8 ± 3	4 ± 4	14 ± 4
11	–3 ± 4	0.8 ± 7	–1 ± 6	2 ± 7
12	–2 ± 4	–8 ± 5	–11 ± 5	8 ± 3
13	2 ± 4	7 ± 5	–0.1 ± 5	31 ± 3
14	–16 ± 4	–20 ± 4	–19 ± 3	3 ± 3
15	–12 ± 4	–5 ± 4	–15 ± 5	–38 ± 3
16	–19 ± 5	–15 ± 6	–16 ± 5	–43 ± 7
17	–8 ± 3	–10 ± 4	14 ± 4	47 ± 4
18	15 ± 5	22 ± 6	26 ± 9	46 ± 10
19	–3 ± 7	–4 ± 6	–6 ± 5	–33 ± 3
20	–11 ± 3	–11 ± 5	–16 ± 3	–40 ± 2
21	–1 ± 5	3 ± 4	23 ± 5	75 ± 4
22	–1 ± 6	–7 ± 5	3 ± 9	–10 ± 6
23	5 ± 5	–15 ± 4	2 ± 5	11 ± 6
24	–14 ± 5	2 ± 4	–2 ± 6	16 ± 5
25	8 ± 4	0.1 ± 4	–10 ± 6	20 ± 6
26	–13 ± 4	–21 ± 8	13 ± 8	0.3 ± 4
27	13 ± 7	1 ± 4	10 ± 9	–5 ± 6
28	14 ± 6	12 ± 4	15 ± 6	28 ± 7
29	6 ± 5	11 ± 7	35 ± 4	92 ± 15

The prototype enhancer PD 81, 723 did not inhibit the binding of a radiolabelled antagonist to A_1 and A_{2A} receptors, but it reduced by 21% the binding of [^{3}H]MRE 3008F20 to the A_3 receptor [21].

None of the selected compounds inhibited binding at the hA2AAR, but they inhibited binding to the hA1AR to some extent (18–35%) and to the hA3AR to similar degree (15–24%).

Table 2
Saturation parameters of [^{3}H]CCPA in the absence (control) and presence of tested compounds (10 μM) on different systems

Compound	K_D control	K_D + compound	B_{MAX} control	B_{MAX} + compound	
PD 81, 723	2.9 ± 0.6	1.9 ± 0.4	111 ± 39	179 ± 50	h-CHO-A1
	0.7 ± 0.6	0.7 ± 0.1	521 ± 52	604 ± 90	RAT CORTEX
	1.7 ± 0.2	1.4 ± 0.1	736 ± 58	957 ± 65	HUMAN BRAIN
16	1.9 ± 0.1	2.1 ± 0.2	1970 ± 69	1872 ± 77	h-CHO-A1
	0.5 ± 0.1	0.8 ± 0.1	642 ± 73	685 ± 59	RAT CORTEX
	1.3 ± 0.1	2.0 ± 0.2	950 ± 107	1007 ± 4	HUMAN BRAIN
20	1.4 ± 0.1	1.7 ± 0.2	2436 ± 23	2099 ± 87	h-CHO-A1
	0.6 ± 0.1	0.9 ± 0.1	679 ± 29	623 ± 30	RAT CORTEX
	1.4 ± 0.1	2.5 ± 0.2	1087 ± 16	1007 ± 105	HUMAN BRAIN

Table 3
Inhibition activity of compounds **15, 16** and **20**

Compound (10 μM)	% A1 antagonism	% A2A antagonism	% A3 antagonism
PD 81, 723	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15	18.0 ± 3.2	0.0 ± 0.0	15.0 ± 3.0
16	23.5 ± 4.6	0.0 ± 0.0	24.0 ± 5.1
20	35.0 ± 5.5	0.0 ± 0.0	23.5 ± 4.6

Inhibition was expressed as percent displacement value (±STD, $n = 3$) of 1 nM of [^{3}H]DPCPX, of 2 nM of [^{3}H]ZM 241385 and of 2 nM [^{3}H]MRE 3008F20 by 10 μM of tested compound.

5. Conclusion

To conclude, among the new compounds reported in the present study, the functional cAMP assay indicated derivatives **15–16** and **19–20** as potential allosteric enhancers at the human A_1 receptor. The replacement of the phenyl at the 3 position of thiophene ring with a 2- or 3-thienyl moiety seemed to maintain the enhancing activity of the reference compound PD 81, 723. However, the substitution added a collateral antagonist A_1 activity that was detected both in saturation and inhibition experiments. This activity possibly reduced the enhancing potential of the compounds.

6. Experimental protocols

6.1. Chemistry

Reactions were monitored by thin-layer chromatography on silica gel (precoated F_{254} Merck plates); the spots were examined with UV light and visualized with aqueous $KMnO_4$. Flash chromatography was performed using Merck silica gel (230–240 mesh). ^{1}H-NMR spectra were recorded on a Bruker AC 200 MHz spectrometer using TMS as internal standard. IR spectra were recorded on a Perkin-Elmer FT-IR Paragon 500 spectrometer. Melting points were determined on a Buchi-Tottoli apparatus and are uncorrected. Elemental analyses were performed by the microanalytical laboratory of Dipartimento di Chimica, University of Ferrara.

*6.1.1. Synthesis of 3-(5-bromo-thiophen-2-yl)-3-oxo-propionitrile **(36)***

A solution of potassium cyanide (15 mmol, 977 mg, 3 eq.) in water (5 ml) was added in one portion at 0 °C to a solution of 2-bromo-1-(5-bromo-thiophen-2-yl)-ethanone **37** (5 mmol, 1.42 g.) in 95% EtOH (15 ml). The mixture was stirred at room temperature overnight and then poured onto a mixture of crushed ice and water and acidified with glacial acetic acid (pH 5–6) to precipitate the nitrile. The pale brown solid was filtered, washed with water and dried, affording 3-(5-bromo-thiophen-2-yl)-3-oxo-propionitrile **36** (yield: 66%) as pale brown solid; mp 159–160 °C (petroleum ether); ^{1}H-NMR ($CDCl_3$) δ: 4.08 (s, 2H), 7.59 (d, J = 2.8 Hz, 1H), 8.16 (d, J = 2.8 Hz, 1H); C_7H_4BrNOS.

*6.1.2. General procedure for the synthesis of 2-amino-3-heteroaroylthiophenes **2–29***

A mixture of the appropriate β-ketonitrile (5 mmol), the appropriate ketone (5 mmol), morpholine (0.44 ml, 5 mmol), and sulfur (164 mg, 5 mmol) was heated at 70 °C for 1 h, and then stirred at room temperature for 20 h. The solvent was evaporated under reduced pressure and the residue was diluted with ethyl acetate (30 ml), washed with water (10 ml), brine (10 ml), dried over Na_2SO_4 and concentrated in vacuo. The crude residue was purified by chromatography on a silica gel column using ethyl acetate–petroleum ether 2:8 as eluant. Final products were recrystallized from petroleum ether.

*6.1.3. (2-Amino-4,5-dimethyl-thiophen-3-yl)-furan-2-yl-methanone **(2)***

2-(2-Furanoyl)acetonitrile **30**, butanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5-dimethyl-thiophen-3-yl)-furan-2-yl-methanone **2**. Yield: 41%; yellow solid; mp 95–97 °C (petroleum ether); IR (KBr) cm^{-1}: 3265, 1573, 1426, 1293, 1021, 750; ^{1}H-NMR ($CDCl_3$) δ: 1.24 (s, 3H), 2.05 (s, 3H), 6.52 (bs, 2H), 7.00 (m, 1H), 7.31 (d, J = 3.4 Hz, 1H), 7.54 (d, J = 3.4 Hz, 1H); $C_{11}H_{11}NO_2S$.

*6.1.4. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-furan-2-yl-methanone **(3)***

2-(2-Furanoyl)acetonitrile **30**, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford 2-amino-5,6-dihydro-4H-cyclopenta[b] thiophen-3-yl)-furan-2-yl-methanone **3**. Yield: 59%; yellow solid; m.p. 149–151 °C (petroleum ether); IR (KBr) cm^{-1}: 3115, 1560, 1476, 1288, 1021, 746; ^{1}H-NMR ($CDCl_3$) δ: 2.30 (m, 2H), 2.73 (m, 4H), 6.53 (m, 1H), 6.81 (bs, 2H), 7.05 (d, J = 3.4 Hz, 1H), 7.55 (d, J = 3.4 Hz, 1H); $C_{12}H_{11}NO_2S$.

*6.1.5. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-furan-2-yl-methanone **(4)***

2-(2-Furanoyl)acetonitrile **30**, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5,6,7-tetrahydro-benzo[b] thiophen-3-yl)-furan-2-yl-methanone **4**. Yield: 40%; yellow solid; m.p. 122 °C (petroleum ether); IR (KBr) cm^{-1}: 3320, 1577, 1433, 1260, 1080, 726; ^{1}H-NMR ($CDCl_3$) δ: 1.63 (m, 2H), 1.82 (m, 2H), 2.28 (m, J = 5.8 Hz, 2H), 2.57 (t, J = 5.8 Hz, 2H), 6.20 (bs, 2H), 6.53 (m, 1H), 6.99 (d, J = 3.4 Hz, 1H), 7.55 (d, J = 3.4 Hz, 1H); $C_{13}H_{13}NO_2S$.

*6.1.6. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c] pyridin-3-yl)-furan-2-yl-methanone **(5)***

2-(2-Furanoyl)acetonitrile **30**, 1-benzyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-furan-2-yl-methanone **5**. Yield: 62%; yellow solid; m.p. 115–117 °C (petroleum ether); IR (KBr) cm^{-1}: 3337, 1579, 1485, 1433, 1287, 734; ^{1}H-NMR ($CDCl_3$) δ: 2.47 (t, J = 5.6 Hz, 2H), 2.61 (t, J = 5.6 Hz, 2H), 3.47 (s, 2H), 3.69 (s, 2H), 6.36 (bs, 2H), 6.52 (m, 1H), 7.01 (d, J = 3.4 Hz, 1H), 7.31 (m, 5H), 7.55 (d, J = 3.4 Hz, 1H); $C_{19}H_{18}N_2O_2S$.

*6.1.7. (2-Amino-4,5-dimethyl-thiophen-3-yl)-benzofuran-2-yl-methanone **(6)***

2-(Benzofuran-2-carbonyl)acetonitrile **31**, butanone, morpholine and sulfur were reacted according to the general procedure to afford 2-amino-4,5-dimethyl-thiophen-3-yl)-benzofuran-2-yl-methanone **6**. Yield: 44%; yellow solid;

m.p. 113–115 °C (petroleum ether); IR (KBr) cm^{-1}: 3305, 1574, 1434, 1257, 750; ^{1}H-NMR ($CDCl_3$) δ: 1.87 (s, 3H), 2.19 (s, 3H), 6.31 (bs, 2H), 7.32 (m, 2H), 7.38 (t, J = 7.8 Hz, 1H), 7.57 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 7.8 Hz, 1H); anal. calcd for $C_{15}H_{13}NO_2S$: C, 66.40; H, 4.83; N, 5.16; found: C, 66.43; H, 4.85; N, 5.14.

*6.1.8. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-benzofuran-2-yl-methanone **(7)***

2-(Benzofuran-2-carbonyl)acetonitrile **31**, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-benzofuran-2-yl-methanone **7**. Yield: 67%; yellow solid; m.p. 128–129 °C (petroleum ether); IR (KBr) cm^{-1}: 3298, 1570, 1422, 1288, 1034, 751; ^{1}H-NMR ($CDCl_3$) δ: 2.33 (m, 2H), 2.74 (m, 4H), 7.03 (bs, 2H), 7.31 (m, 2H), 7.42 (t, J = 7.8 Hz, 1H), 7.57 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 7.8 Hz, 1H); $C_{16}H_{13}NO_2S$.

*6.1.9. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-benzofuran-2-yl-methanone **(8)***

2-(Benzofuran-2-carbonyl)acetonitrile 31, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-benzofuran-2-yl-methanone 8. Yield: 64%; yellow solid; m.p. 88–89 °C (petroleum ether); IR (KBr) cm^{-1}: 3430, 1575, 1430, 752; ^{1}H-NMR (CDCl3) δ: 1.56 (m, 2H), 1.81 (m, 2H), 2.29 (t, J = 5.9 Hz, 2H), 2.59 (t, J = 5.9 Hz, 2H), 6.55 (bs, 2H), 7.32 (m, 2H), 7.42 (t, J = 7.8 Hz, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 7.8 Hz, 1H); $C_{17}H_{15}NO_2S$.

*6.1.10. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-benzofuran-2-yl-methanone **(9)***

2-(Benzofuran-2-carbonyl)acetonitrile **31**, 1-benzyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-benzofuran-2-yl-methanone **9**. Yield: 59%; yellow solid; m.p. 85–87 °C (petroleum ether); IR (KBr) cm^{-1}: 3435, 1580, 1551, 1441, 749; ^{1}H-NMR ($CDCl_3$) δ: 2.45 (t, J = 5.2 Hz, 2H), 2.59 (t, J = 5.2 Hz, 2H), 3.48 (s, 2H), 3.69 (s, 2H), 6.64 (bs, 2H), 7.33 (m, 8H), 7.58 (d, J = 7.8 Hz, 1H), 7.66 (d, J = 7.8 Hz, 1H); $C_{23}H_{20}N_2O_2S$.

*6.1.11. (2-Amino-4,5-dimethyl-thiophen-3-yl)-pyridin-2-yl-methanone **(10)***

2-(Pyridin-2-carbonyl)acetonitrile **32**, butanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5-dimethyl-thiophen-3-yl)-pyridin-2-yl-methanone **10**. Yield: 39%; yellow solid; m.p. 125–127 °C (petroleum ether); IR (KBr) cm^{-1}: 3233, 1565, 1436, 1279, 750; ^{1}H-NMR ($CDCl_3$) δ: 1.46 (s, 3H), 2.12 (s, 3H), 6.76 (bs, 2H), 7.37 (m, 1H), 7.62 (m, 1H), 7.81 (d, J = 7.5 Hz, 1H), 8.63 (d, J = 4.6 Hz, 1H); $C_{12}H_{12}N_2OS$.

*6.1.12. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-pyridin-2-yl-methanone **(11)***

2-(Pyridin-2-carbonyl)acetonitrile **32**, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-pyridin-2-yl-methanone **11**. Yield: 52%; yellow solid; m.p. 145–146 °C (petroleum ether); IR (KBr) cm^{-1}: 3338, 2853, 1560, 1146, 673; ^{1}H-NMR ($CDCl_3$) δ: 2.14 (m, 4H), 2.64 (m, 2H), 7.09 (bs, 2H), 7.36 (m,1H), 7.58 (d, J = 7.6 Hz, 1H), 7.80 (m, 1H), 8.62 (d, J = 4.6 Hz, 1H); $C_{13}H_{12}N_2OS$.

*6.1.13. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-pyridin-2-yl-methanone **(12)***

2-(Pyridin-2-carbonyl)acetonitrile **32**, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-pyridin-2-yl-methanone **12**. Yield: 58%; yellow solid; m.p. 191-193 °C (petroleum ether); IR (KBr) cm^{-1}: 3249, 2949, 1573, 1450, 1286, 676; ^{1}H-NMR ($CDCl_3$) δ: 1.46 (m, 2H), 1.71 (m, 4H), 2.50 (t, J = 6.2 Hz, 2H), 6.99 (bs, 2H), 7.37 (m, 1H), 7.57 (d, J = 7.8 Hz, 1H), 7.81 (m, 1H), 8.62 (d, J = 4.6 Hz, 1H); $C_{14}H_{14}N_2OS$.

*6.1.14. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-pyridin-2-yl-methanone **(13)***

2-(Pyridin-2-carbonyl)acetonitrile 32, 1-benzyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-pyridin-2-yl-methanone 13. Yield: 60%; yellow solid; m.p. 69–71 °C (petroleum ether); IR (KBr) cm^{-1}: 3368, 1578, 1442, 1130, 671; ^{1}H-NMR ($CDCl_3$) δ: 1.89 (t, J = 5.8 Hz, 2H), 2.51 (t, J = 5.8 Hz, 2H), 3.41 (s, 2H), 3.62 (s, 2H), 7.07 (bs, 2H), 7.32 (m, 6H), 7.54 (d, J = 7.8 Hz, 1H), 7.80 (m, 1H), 8.63 (d, J = 4.8 Hz, 1H); $C_{20}H_{19}N_3OS$.

*6.1.15. (2-Amino-4,5-dimethyl-thiophen-3-yl)-thiophen-3-yl-methanone **(14)***

2-(Thiophene-3-carbonyl)acetonitrile **33**, butanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5-dimethyl-thiophen-3-yl)-thiophen-3-yl-methanone **14**. Yield: 38%; yellow solid; m.p. 122–123 °C (petroleum ether); IR (KBr) cm^{-1}: 3337, 1576, 1427, 1265, 719; ^{1}H-NMR ($CDCl_3$) δ: 1.71 (s, 3H), 2.15 (s, 3H), 6.17 (bs, 2H), 7.30 (dd, J = 5.0 Hz, J' = 2.7, 1H), 7.36 (dd, J = 5.0 Hz, J' = 0.9 Hz, 1H), 7.63 (t, J = 2.7 Hz, J' = 0.9 Hz, 1H); $C_{11}H_{11}NOS_2$.

*6.1.16. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-thiophen-3-yl-methanone **(15)***

2-(Thiophene-3-carbonyl)acetonitrile 33, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl-thiophen-3-yl)-methanone 15. Yield: 71%; yellow solid; m.p. 125–127 °C (petroleum

ether); IR (KBr) cm^{-1}: 3330, 3117, 1569, 1435, 715; ^{1}H-NMR ($CDCl_3$) δ: 2.24 (m, 4H), 2.71 (m, 2H), 6.83 (bs, 2H), 7.24 (dd, J = 5.0 Hz, J′ = 2.7, 1H), 7.30 (dd, J = 5.0 Hz, J′ = 0.9 Hz, 1H), 7.56 (t, J = 2.7 Hz, J′ = 0.9 Hz, 1H); $C_{12}H_{11}NOS_2$.

*6.1.17. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-thiophen-3-yl-methanone **(16)***

2-(Thiophene-3-carbonyl)acetonitrile **33**, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-thiophen-3-yl-methanone **16**. Yield: 67%; yellow solid; m.p. 133–135 °C (petroleum ether); IR (KBr) cm^{-1}: 3323, 1576, 1433, 1080, 726; ^{1}H-NMR ($CDCl_3$) δ: 1.55 (m, 2H), 1.77 (m, 2H), 2.01 (t, J = 6.2 Hz, 2H), 2.54 (t, J = 6.2 Hz, 2H), 6.46 (bs, 2H), 7.24 (dd, J = 5.0 Hz, J′ = 2.5, 1H), 7.30 (dd, J = 5.0 Hz, J′ = 0.9 Hz, 1H), 7.58 (t, J = 2.5 Hz, J′ = 0.9 Hz, 1H); $C_{13}H_{13}NOS_2$.

*6.1.18. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-thiophen-3-yl-methanone **(17)***

2-(Thiophene-3-carbonyl)acetonitrile **33**, 1-benzyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-thiophen-3-yl-methanone **17**. Yield: 68%; yellow solid; m.p. 132–133 °C (petroleum ether); IR (KBr) cm^{-1}: 3350, 2805, 1578, 1429, 722; ^{1}H-NMR ($CDCl_3$) δ: 2.14 (t, J = 5.6 Hz, 2H), 2.54 (t, J = 5.6 Hz, 2H), 3.43 (s, 2H), 3.65 (s, 2H), 6.61 (bs, 2H), 7.24 (dd, J = 5.0 Hz, J′ = 2.5, 1H), 7.33 (dd, J = 5.0 Hz, J′ = 1.0 Hz, 1H), 7.58 (t, J = 2.5 Hz, J′ = 1.0 Hz, 1H); $C_{19}H_{18}N_2OS_2$.

*6.1.19. (2-Amino-4,5-dimethyl-thiophen-3-yl)-thiophen-2-yl-methanone **(18)***

2-(Thiophene-2-carbonyl)acetonitrile **34**, butanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5-dimethyl-thiophen-3-yl)-thiophen-2-yl-methanone **18**. Yield: 44%; orange solid; m.p. 191–193 °C (petroleum ether); IR (KBr) cm^{-1}: 3390, 1552, 1429, 1272, 772; ^{1}H-NMR ($CDCl_3$) δ: 1.86 (s, 3H), 2.17 (s, 3H), 5.78 (bs, 2H), 7.07 (dd, J = 4.5 Hz, J′ = 3.5 Hz, 1H), 7.56 (d, J = 3.5 Hz, J′ = 0.7 Hz, 1H), 7.58 (dd, J = 4.5 Hz, J′ = 0.7 Hz, 1H); $C_{11}H_{11}NOS_2$.

*6.1.20. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-thiophen-2-yl-methanone **(19)***

2-(Thiophene-2-carbonyl)acetonitrile **34**, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-thiophen-2-yl-methanone **19**. Yield: 67%; yellow solid; m.p. 133–134 °C (petroleum ether); IR (KBr) cm^{-1}: 3344, 1566, 1435, 1031, 747; ^{1}H-NMR ($CDCl_3$) δ: 2.27 (m, 2H), 2.47 (t, J = 7.2 Hz, 2H), 2.70 (t, J = 7.2 Hz, 2H), 6.55 (bs, 2H), 7.06 (dd, J = 4.6 Hz, J′ = 3.5 Hz, 1H), 7.51 (dd, J = 3.5 Hz, J′ = 1.1 Hz, 1H), 7.53 (dd, J = 4.6 Hz, J′ = 1.1 Hz, 1H); $C_{12}H_{11}NOS_2$.

*6.1.21. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-thiophen-2-yl-methanone **(20)***

2-(Thiophene-2-carbonyl)acetonitrile **34**, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-thiophen-2-yl-methanone **20**. Yield: 56%; orange solid; m.p. 117–118 °C (petroleum ether); IR (KBr) cm^{-1}: 3384, 1576, 1436, 1056, 765; ^{1}HNMR: ($CDCl_3$) δ: 1.59 (m, 2H), 1.81 (m, 2H), 2.20 (m, 2H), 2.54 (m, 2H), 6.10 (bs, 1H), 7.06 (dd, J = 4.5 Hz, J′ = 3.6 Hz, 1H), 7.38 (dd, J = 3.6 Hz, J′ = 0.8 Hz, 1H), 7.60 (dd, J = 4.5 Hz, J′ = 0.8 Hz, 1H); $C_{13}H_{13}NOS_2$.

*6.1.22. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-thiophen-2-yl-methanone **(21)***

2-(Thiophene-2-carbonyl)acetonitrile **34**, 1-benzyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-thiophen-2-yl-methanone **21**. Yield: 78%; yellow solid; m.p. 122–123 °C (petroleum ether); IR (KBr) cm^{-1}: 3412, 2928, 1570, 1485, 1355, 700; ^{1}H-NMR ($CDCl_3$) δ: 2.35 (t, J = 5.4 Hz, 2H), 2.59 (t, J = 5.4 Hz, 2H), 3.47 (s, 2H), 3.67 (s, 2H), 6.26 (bs, 2H), 7.05 (dd, J = 4.0 Hz, J′ = 3.4 Hz, 1H), 7.30 (dd, J = 3.4 Hz, J′ = 0.9 Hz, 1H), 7.35 (m, 5H), 7.53 (dd, J = 4.0 Hz, J′ = 0.9 1H); $C_{19}H_{18}N_2OS_2$.

*6.1.23. (2-Amino-4,5-dimethyl-thiophen-3-yl)-(5-chloro-thiophen-2-yl)-methanone **(22)***

2-(5-Chlorothiophene-2-carbonyl)acetonitrile **35**, butanone, morpholine and sulfur were reacted according to the general procedure to afford 2-amino-4,5-dimethyl-thiophen-3-yl)-(5-chloro-thiophen-2-yl)-methanone **22**. Yield: 46%; red solid; m.p. 125–127 °C (petroleum ether); IR (KBr) cm^{-1}: 3377, 1551, 1424, 1003, 764; ^{1}H-NMR ($CDCl_3$) δ: 1.90 (s, 3H), 2.17 (s, 3H), 5.77 (bs, 2H), 6.89 (d, J = 4.0 Hz, 1H), 7.16 (d, J = 4.0 Hz, 1H); $C_{11}H_{10}ClNOS_2$.

*6.1.24. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-(5-chloro-thiophen-2-yl)-methanone **(23)***

2-(5-Chlorothiophene-2-carbonyl)acetonitrile 35, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-(5-chloro-thiophen-2-yl)-methanone 23. Yield: 69%; red solid; m.p. 170-171 °C (petroleum ether); IR (KBr) cm^{-1}: 3351, 2851, 1550, 1270, 1002, 698; ^{1}H-NMR ($CDCl_3$) δ: 2.28 (m, 2H), 2.52 (t, J = 7.2 Hz, 2H), 2.70 (t, J = 7.2 Hz, 2H), 6.62 (bs, 2H), 6.88 (d, J = 4.0 Hz, 1H), 7.16 (d, J = 4.0 Hz, 1H); $C_{12}H_{10}ClNOS_2$.

*6.1.25. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(5-chloro-thiophen-2-yl)-methanone**(24)***

2-(5-Chlorothiophene-2-carbonyl)acetonitrile **35**, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(5-chloro-thiophen-2-yl)-

methanone **24**. Yield: 76%; yellow solid; m.p. 173–175 °C (petroleum ether); IR (KBr) cm^{-1}: 3349, 2911, 1577, 1430, 765; ^{1}H-NMR ($CDCl_3$) δ: 1.59 (m, 2H), 1.65 (m, 2H), 2.35 (t, J = 5.8 Hz, 2H), 2.55 (t, J = 5.8 Hz, 2H), 6.07 (bs, 2H), 6.88 (d, J = 4.0 Hz, 1H), 7.16 (d, J = 4.0 Hz, 1H); $C_{13}H_{12}ClNOS_2$.

*6.1.26. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-(5-chloro-thiophen-2-yl)-methanone **(25)***

2-(5-Chlorothiophene-2-carbonyl)acetonitrile **35**, 1-benzyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-(5-chloro-thiophen-2-yl)-methanone **25**. Yield: 63%; yellow solid; m.p. 81–83 °C (petroleum ether); IR (KBr) cm^{-1}: 3306, 2807, 1574, 1428, 699; ^{1}H-NMR ($CDCl_3$) δ: 2.39 (t, J = 5.2 Hz, 2H), 2.59 (t, J = 5.2 Hz, 2H), 3.45 (s, 2H), 3.67 (s, 2H), 6.26 (bs, 2H), 6.87 (d, J = 3.9 Hz, 1H), 7.19 (d, J = 3.9 Hz, 1H), 7.35 (m, 5H); $C_{19}H_{17}ClN_2OS_2$.

*6.1.27. (2-Amino-4,5-dimethyl-thiophen-3-yl)-(5-bromo-thiophen-2-yl)methanone **(26)***

2-(5-Bromothiophene-2-carbonyl)acetonitrile **36**, butanone, morpholine and sulfur were reacted according to the general procedure to afford (2-Amino-4,5-dimethyl-thiophen-3-yl)-(5-bromo-thiophen-2-yl)methanone **26**. Yield: 51%; orange solid; m.p. 128–130 °C (petroleum ether); IR (KBr) cm^{-1}: 3348, 1559, 1448, 1263, 769; ^{1}H-NMR ($CDCl_3$) δ: 2.16 (s, 6H), 5.90 (bs, 2H), 7.02 (d, J = 4.0 Hz, 1H), 7.11 (d, J = 4.0 Hz, 1H); $C_{11}H_{10}BrNOS_2$.

*6.1.28. (2-Amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-(5-bromo-thiophen-2-yl)-methanone **(27)***

2-(5-Bromothiophene-2-carbonyl)acetonitrile **36**, cyclopentanone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-5,6-dihydro-4H-cyclopenta[b]thiophen-3-yl)-(5-bromo-thiophen-2-yl)-methanone **27**. Yield: 73%; red solid; m.p. 154–156 °C (petroleum ether); IR (KBr) cm^{-1}: 3349, 1560, 1431, 1269, 975; ^{1}H-NMR ($CDCl_3$) δ: 2.28 (m, 2H), 2.51 (t, J = 7.4 Hz, 2H), 2.70 (t, J = 7.4 Hz, 2H), 6.64 (bs, 2H), 7.02 (d, J = 4.0 Hz, 1H), 7.13 (d, J = 4.0 Hz, 1H); $C_{12}H_{12}BrNOS_2$.

*6.1.29. (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(5-bromothiophen-2-yl)-methanone **(28)***

2-(5-Bromothiophene-2-carbonyl)acetonitrile **36**, cyclohexanone, morpholine and sulfur were reacted according to the general procedure to afford (2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)-(5-bromothiophen-2-yl)-methanone **28**. Yield: 66%; orange solid; m.p. 161–163 °C (petroleum ether); IR (KBr) cm^{-1}: 3379, 2918, 1577, 1432, 761; ^{1}H-NMR ($CDCl_3$) δ: 1.63 (m, 2H), 1.79 (m, 2H), 2.24 (t, J = 5.8 Hz, 2H), 2.55 (t, J = 5.8 Hz, 2H), 6.11 (bs, 2H), 7.02 (d, J = 4.0 Hz, 1H), 7.13(d, J = 4.0 Hz, 1H); $C_{13}H_{12}BrNOS_2$.

*6.1.30. (2-Amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-(5-bromo-thiophen-2-yl)-methanone **(29)***

2-(5-Bromothiophene-2-carbonyl)acetonitrile **36**, 1-ben-zyl-4-piperidone, morpholine and sulfur were reacted according to the general procedure to afford (2-amino-6-benzyl-4,5,6,7-tetrahydro-thieno[2,3-c]pyridin-3-yl)-(5-bromo-thiophen-2-yl)-methanone **29**. Yield: 72%; orange solid; m.p. 87–89 °C (petroleum ether); IR (KBr) cm^{-1}: 3411, 1570, 1411, 978; ^{1}H-NMR ($CDCl_3$) δ: 2.38 (t, J = 5.4 Hz, 2H), 2.59 (t, J = 5.4 Hz, 2H), 3.48 (s, 2H), 3.67 (s, 2H), 6.28 (bs, 2H), 7.01 (d, J = 4.0 Hz, 1H), 7.15 (d, J = 4.0 Hz, 1H), 7.32 (m, 5H); $C_{19}H_{17}BrNOS_2$.

7. Biological evaluation

7.1. Biological materials

[^{3}H]DPCPX (specific activity, 112 Ci/mmol) and [^{3}H]C-CPA (specific activity, 55 Ci/mmol) were obtained from NEN Research Products (Boston, MA); [^{3}H]ZM241385 (specific activity, 17 Ci/mmol) was obtained from Tocris Cookson (Bristol, UK); [^{3}H]MRE 3008F20 (specific activity 67 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). CHO cells transfected with the human recombinant A_1 adenosine receptor (hCHO-A1) were obtained by Prof. K.N. Klotz, University of Wurzburg. Human cerebral cortex was obtained from the University of Ferrara, Medicina Legale Section, with approval of human tissue use protocol. Rat cerebral cortex was harvested from adult male Wistar rats. All tissue culture reagents were obtained from Sigma.

7.2. CHO: huA$_1$ assay

The preparation of Chinese hamster ovary cells expressing human recombinant A_1 adenosine receptors was done as previously described [19]. For experiments, aliquots of cells were placed into 24-well plates with culture medium, serum, and antibiotic for 48 h, by which time the cells had grown to a confluent monolayer. Allosteric enhancement was measured as the action of a test compound at different concentrations (0.01, 0.1, 1 and 10 μM) to reduce the cAMP content of CHO: huA$_1$ cells in the presence of the adenosine A_1 agonist N^6-cyclopentyladenosine (CPA). To begin an experiment, growth medium was removed from the plates and cells were washed once with warm Hanks' buffered saline solution. The wash solution was then removed and replaced with fresh Hanks' solution containing forskolin (1 μM), rolipram (20 μM), (CPA, 0.01 nM), adenosine deaminase (2 U/ml), and the allosteric enhancer to be tested. Forskolin was used to stimulate the activity of adenylyl cyclase, rolipram to inhibit cAMP phosphodiesterase, adenosine deaminase to degrade endogenous adenosine and CPA to cause a small increase of the number of activated adenosine receptors. After 6 min of incubation at 36 °C in the presence of drugs, the incubation solution was removed and hydrochloric acid (final concentration 50 mM) was added to cells to terminate drug action. The content of cAMP in acidified extracts of cells was determined by radioimmunoassay [19]. Because the magnitude of

the effects of allosteric enhancers on CHO:huA$_1$ cells changed subtly with passage number and differed slightly among different aliquots of cells, the actions of the test compounds and the action of the reference compound PD 81, 723 were assayed in each experiment.

7.3. Membrane preparation from CHO-A$_1$ cells

For membrane preparation the culture medium was removed. The cells were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized using a Polytron, and the homogenate was spun for 10 min at 1000*g*. The supernatant was then centrifuged for 30 min at 100 000*g*. The membrane pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4 and incubated with 2 U/ml of ADA for 30 min at 37 °C. Then the suspension was stored at –80 °C. The protein concentration was determined according to a Bio-Rad method with bovine albumin as a standard reference.

7.4. Membrane preparation from rat cortex and human brain

Cerebral cortical tissue from each species was homogenized using a Polytron (setting 6, 20 s) in 20 volumes of ice cold 50 mM Tris–HCl, pH 7.4. This crude membrane homogenate was then centrifuged at 48 000*g* for 15 min at 4 °C. The resulting pellet was resuspended in buffer containing 2 IU/ml ADA to 20 mg/ml original tissue weight and incubated at 37 °C for 30 min to remove endogenous adenosine. This membrane homogenate was recentrifuged at 48 000*g* for 15 min at 4 °C. The resulting membrane pellet was resuspended and recentrifuged at 48 000*g* for 15 min at 4 °C. The final membrane pellets were stored at –80 °C until the time of assay.

7.5. Adenosine receptor binding

To determine the effect of the new series of derivatives of PD 81, 723 on the binding of ligands to A_1, A_{2A}, and A_3 receptors, membranes from CHO: hA_1, hA_{2A}, hA_3, rat cortex, and human brain were incubated in a buffer solution in the absence and presence of test compounds. Test agents were dissolved in DMSO and added to the assay from a 100-fold concentrated solution in DMSO. Control incubations also contained 1% DMSO. Bound and free radioactivities were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Micro-Mate 196 cell harvester (Packard Instrument Company). The filter-bound radioactivity was counted on Top Count Microplate Scintillation Counter (efficiency 57%) with Micro-Scint 20. Binding of 1 nM [^{3}H]CCPA to A_1 receptors in CHO:hA_1, rat, and human brain membranes in the absence and presence of increasing concentrations of test compounds was carried out in triplicate at 25 °C for 90 min in 50 mM Tris–HCl, pH 7.4. Non-specific binding was defined as binding in the presence of 1 μM *R*-PIA.

7.6. Saturation

Saturation binding experiments of [^{3}H]CCPA (0.05–10 nM) to A_1 receptors expressed in CHO:hA_1, rat, and human brain membranes were performed in triplicate at 25 °C for 90 min in 50 mM Tris–HCl, pH 7.4, in the absence and presence of test compounds. Non-specific binding was defined as binding in the presence of 1 μM *R*-PIA.

7.7. Competition assay

Competition experiments of 1 nM [^{3}H]-DPCPX to CHO:hA_1 membranes were performed incubating membranes (100 μg of protein/assay) at 25 °C for 150 min. Competition experiments were performed in duplicate in a final volume of 250 μl in test tubes containing 50 μM Tris–HCl buffer, pH 7.4 and 100 μl of membranes and at least six to eight different concentrations of the tested compounds. Non-specific binding was defined as the binding in the presence of 1 μM DPCPX and was about 25% of total binding. Competition experiments of 2 nM [^{3}H]ZM241385 to CHO:hA_{2A} membranes were performed incubating membranes (100 μg of protein/assay) at 4 °C for 60 min. Competition experiments were performed in duplicate in a final volume of 250 μl in test tubes containing 50 μM Tris–HCl buffer, 10 μM $MgCl_2$, pH 7.4 and 100 μl of membranes and at least six to eight different concentrations of the tested compounds. Non-specific binding was defined as the binding in the presence of 1 μM ZM241385 and was about 30% of total binding. Competition experiments of 2 nM [^{3}H]MRE 3008F20 to CHO:hA3 membranes were performed incubating membranes (100 μg of protein/assay) at 4 °C for 150 min. Competition experiments were performed in duplicate in a final volume of 250 μl in test tubes containing 50 μM Tris–HCl buffer, 10 μM $MgCl_2$, 1 mM EDTA, pH 7.4 and 100 μl of membranes and at least six to eight different concentrations of the tested compounds. Non-specific binding was defined as the binding in the presence of 1 μM MRE 3008F20 and was about 30% of total binding.

7.8. Data analysis

All values are expressed as mean (SEM of three independent experiments. A weighted non-linear least-squares curve fitting program LIGAND was used for computer analysis of saturation and competition experiments. For experiments with two comparison groups, statistical analysis was performed with a two-tailed *t* test. Differences between group mean values were considered significant at P e 0.05.

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