Synthesis and Biological Evaluation of 2,3,5-Substituted [1,2,4]Thiadiazoles as Allosteric Modulators of Adenosine Receptors

Adrianus M. C. H. van den Nieuwendijk,† Daniele Pietra,† Laura Heitman, Anikó Göblyös, and Adriaan P. IJzerman*

Leiden/Amsterdam Center for Drug Research, Division of Medicinal Chemistry, Leiden University, P.O. Box 9502, 2300RA Leiden, The Netherlands

Received April 4, 2003

A number of 2,3,5-substituted [1,2,4]thiadiazole analogues of SCH-202676 (*N*-(2,3-diphenyl-[1,2,4]thiadiazole-5(*2H*)-ylidene)methanamine, **7a**) were synthesized and tested as potential allosteric modulators of adenosine receptors. All compounds were capable of displacing the binding of the radiolabeled agonist [^{3}H]CCPA to human A_1 adenosine receptors, whereas modest and varying effects were observed on the binding of [^{3}H]DPCPX, a radiolabeled antagonist for this receptor subtype. Four compounds, **7a** (SCH-202676), **7k** (LUF5792), **7l** (LUF5794), and **8e** (LUF5789), were selected for more detailed characterization. They all proved allosteric inhibitors of agonist binding, with **7k** being most potent, whereas their effects on antagonist binding were more ambiguous. Subsequently, experiments were done on human adenosine A_{2A} and A_3 receptors. Compounds **7a** and **7l** displayed peculiar displacement characteristics of both radiolabeled agonist and antagonist binding to A_{2A} receptors, whereas **7a** showed some activity on A_3 receptors.

Introduction

Allosteric modulation of G protein-coupled receptors (GPCRs) is a relatively novel and unexplored pharmacological concept. Classically, the mechanism of action for many drugs is either to mimic or to inhibit the action of endogenous signaling molecules, leading to the traditional classification of agonists as well as antagonists/inverse agonists, respectively. Through competition at the binding site for the endogenous neurotransmitter or hormone, the desired effect is exerted.

In contrast, allosteric modulators are thought to act at sites distant from the (primary) ligand binding crevice, potentially leading to a number of benefits. One of these is that an allosteric drug does not have an action per se. It rather modulates the action of the naturally occurring hormone or neurotransmitter when the latter is released. Thus, the temporal and spatial aspects of the natural signaling mechanism may be preserved. A number of recent reviews provide more details regarding the above.[1−3]

Adenosine receptors belong to the superfamily of GPCRs.[4] Three of the four subclasses (A_1, A_{2A}, and A_3, not A_{2B}) have been reported to be allosterically regulated. By far, the most evidence exists for the adenosine A_1 receptor due to the early recognition that PD81,723 (Figure 1, **1**) is capable of enhancing agonist activity at this receptor subtype.[5] PD81,723 and other 2-amino-3-benzoylthiophene derivatives[6−8] act predominantly through decreasing the dissociation rate of the agonist (adenosine or synthetic analogues such as N^6-cyclopentyladenosine (CPA)) from its binding site.[9,10] PD81,723 seems to be fairly selective for adenosine A_1 receptors, since it did not induce similar effects on other GPCRs.[5]

Interestingly, SCH-202676 (Figure 1, *N*-(2,3-diphenyl-[1,2,4]thiadiazole-5(2*H*)-ylidene)methanamine, **7a**) has recently been reported to act as a more promiscuous agent.[11] It appears to modulate many GPCRs, including opioid, muscarinic, adrenergic, and dopaminergic receptors. Its activity on adenosine receptors, however, was not studied. With this in mind, we initiated a project to synthesize SCH-202676 and a number of analogues and characterize their modulatory properties on adenosine receptors.

Chemistry

The synthesis of thiadiazoles such as **7a** has been well-described by Goerdeler and co-workers.[12] Benzamides **2** were used as starting materials that were converted into benzimidoyl chlorides **3** (Scheme 1).[13] Substitution of chlorine by SCN (**4**) followed by addition of an amine afforded thioureas **5** and **6**. Finally, an oxidation with bromine yielded the targeted 2,3,5-substituted [1,2,4]thiadiazoles as their hydrobromide salts (**7** and **8**). The proposed strategy is very flexible, since all of the substituents at positions 2, 3, and 5 of the thiadiazole can be varied.

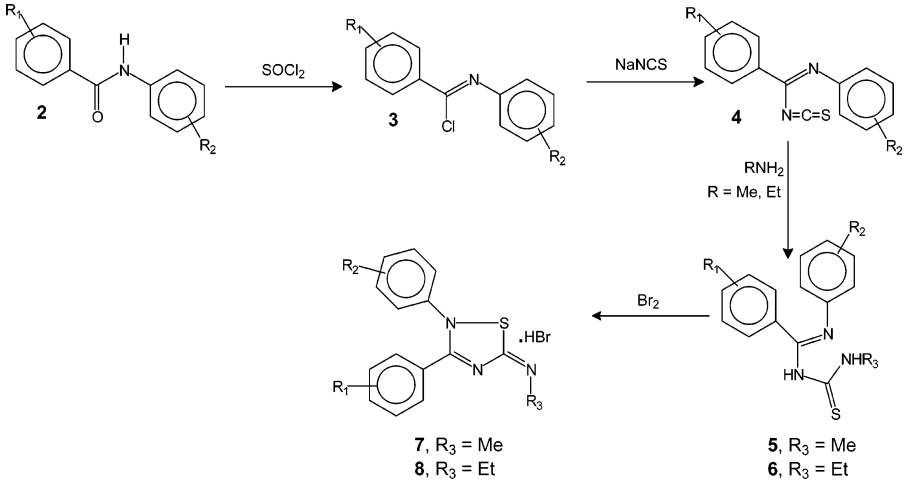
Biology

All compounds were initially characterized in equilibrium radioligand displacement studies on human adenosine A_1 receptors. Both a radiolabeled agonist ([^{3}H]CCPA) and a radiolabeled antagonist ([^{3}H]DPCPX) were used. Subsequently, selected compounds were used for further characterization by, e.g., studying their influence on association and dissociation kinetics of the A_1 receptor radioligands. Similar studies to assess the compounds' modulatory properties were done on human adenosine A_{2A} receptors, with [^{3}H]NECA and [^{3}H]-ZM241385 as the radiolabeled agonist and antagonist, respectively. Finally, only **7a** was studied on human

* To whom correspondence should be addressed. Tel: +31-71-5274651. Fax: +31-71-5274565. E-mail: ijzerman@lacdr.leidenuniv.nl.
† Both authors contributed equally to this work.

10.1021/jm030863d CCC: $27.50
Published on Web 01/06/2004

Scheme 1. Synthetic Route toward 2,3,5-Substituted [1,2,4]Thiadiazoles



adenosine A_3 receptors with [^{125}I]AB-MECA as the radiolabeled agonist and [^{3}H]PSB-11 as the radiolabeled antagonist, respectively.

Results and Discussion

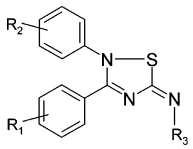
The preparation of the benzamides (**2**) was straightforward. From commercially available anilines and benzoyl chlorides, the amides were prepared in 73−98% yields. Conversion of the amides into benzimidoyl chlorides (**3**) using thionyl chloride appeared to be more difficult. In two cases, bis-3,4-dichlorophenyl amide (**2d**) and bis-4-methoxy-phenyl amide (**2f**), the desired benzimidoyl chlorides could not be obtained. NMR analysis showed mainly starting material in the crude product. Also, efforts using PCl_5 were unsuccessful, although compound **3f** has been reportedly prepared by this method.[14] In all other cases, the benzimidoyl chlorides could be obtained via the protocol described by Von Braun and Pinkernelle.[13] The yields varied from 20 to 80%. These benzimidoyl chlorides were used as such for the next reaction without obvious problems.

The method developed by Goerdeler et al. for the preparation of [1,2,4]thiadiazoles via intermediate thioureas **5** and **6** worked well.[12] Substitution of the chlorine atom in compound **3** by an SCN moiety to form intermediate **4**, followed by addition of an amine, gave the thioureas **5** and **6** in moderate to good yields. The final oxidative formation of thiadiazoles **7** and **8** generally occurred in high yields (65−90%).

Subsequently, all final 2,3,5-substituted [1,2,4]thiadiazoles (see Table 1) were tested on human adenosine A_1 receptors. All compounds at a final concentration of 10 μM significantly inhibited [^{3}H]CCPA binding. In particular **7a**,**k** and **8a**,**k** appeared to be most potent. At the same concentration, derivatives **8b**,**e**,**h**,**k** also significantly inhibited [^{3}H]DPCPX binding but to a lesser extent. On the other hand, compounds **7a**,**b**,**g**,**j**,**l**−**n** and **8a**,**l** appeared to increase [^{3}H]DPCPX binding, while the remaining materials (**7c**,**e**,**h**,**i**,**k** and **8g**,**i**,**j**) did not seem to modulate [^{3}H]DPCPX binding (Table 1). At a lower concentration (1 μM), none of the compounds apparently affected [^{3}H]DPCPX binding.

The methyl-substituted series **7** and the ethyl-substituted series **8** behaved quite similarly, i.e., causing a strong decrease in agonist binding, with relatively little effect on antagonist binding. As compared to **7a** and **8a** (with R_1=R_2=H), only **7k** and **8k** (R_1=H, R_2 = 3-Me) were equipotent or more active in displacing [^{3}H]-CCPA binding.

Table 1. Modulation of [^{3}H]CCPA and [^{3}H]DPCPX Binding to Human A_1 Adenosine Receptors by 2,3,5-Substituted [1,2,4]Thiadiazoles[a]



				% specific binding of radioligand remaining		
				[^{3}H]CCPA[a]	[^{3}H]DPCPX[a]	
compd	R_1	R_2	R_3	compd (10 μM)	compd (1 μM)	compd (10 μM)
PD81,723				225	87	68
7a (SCH-202676)	H	H	Me	10	104	149
7b	4-Cl	4-Cl	Me	20	93	119
7c	3-Cl	3-Cl	Me	36	96	93
7e	4-Me	4-Me	Me	20	104	97
7g	H	4-Cl	Me	33	94	126
7h	H	3-Cl	Me	35	96	108
7i	H	3,4-Cl	Me	33	94	111
7j	H	4-Me	Me	31	99	123
7k (LUF 5792)	H	3-Me	Me	5	99	109
7l (LUF 5794)	H	4-OMe	Me	24	103	132
7m	4-Cl	H	Me	15	106	119
7n	4-OMe	H	Me	35	100	114
8a	H	H	Et	6	103	114
8b	4-Cl	4-Cl	Et	24	95	84
8e (LUF 5789)	4-Me	4-Me	Et	32	100	61
8g	H	4-Cl	Et	30	104	109
8h	H	3-Cl	Et	39	101	86
8i	H	3,4-Cl	Et	35	100	90
8j	H	4-Me	Et	28	101	105
8k	H	3-Me	Et	6	98	75
8l	H	4-OMe	Et	24	103	120

[a] Data are expressed as means from 2 to 3 independent experiments performed in duplicate; individual values varied less than 15%. The results are given as percentage specific binding of radioligand remaining, where control binding is 100% and nonspecific binding is 0%. PD81,723 was used as a reference allosteric modulator for reasons of comparison.

Four compounds (**7a**,**k**,**l** and **8e**) were analyzed in more detail. They inhibited [^{3}H]CCPA binding concentration dependently (Figure 2). Of the four compounds, **7k** proved to be most active with an EC_{50} value of 0.64 μM (Table 2), four times more potent than SCH-202676 (**7a**). The displacement curves were all very steep with pseudo Hill coefficients much larger than unity (Table

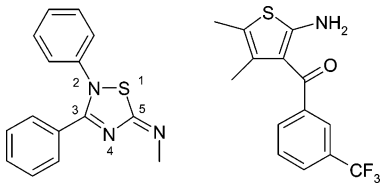


Figure 1. Structures of (*N*-(2,3-diphenyl[1,2,4]thiadiazole-5(2*H*)-ylidene)methanamine (SCH-202676, **7a**) and PD81,723 (**1**).

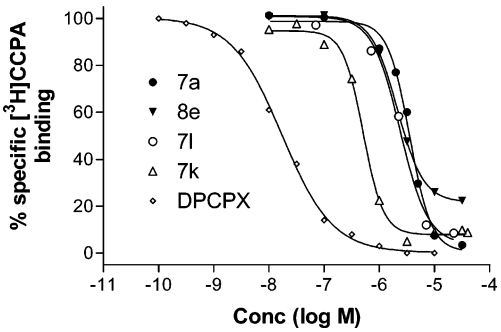


Figure 2. Displacement by **7a**,**k**,**l** and **8e** and the reference competitive antagonist DPCPX of [^{3}H]CCPA binding to human adenosine A_1 receptors. Data are from a representative experiment performed in duplicate ($n = 3$). Radioligand binding is expressed as percent of specific binding.

Table 2. Effects of Various Compounds as Displacing Agents of [^{3}H]CCPA Binding to Human Adenosine A_1 Receptor after 60 Min of Incubation

	[^{3}H]CCPA	
compd	EC_{50} (μM)	slope ($-n_H$)
7a	2.8 (± 0.2)	−6.5 (± 2.8)
8e	1.6 (± 0.3)	−7.9 (± 3.4)
7l	2.4 (± 0.1)	−4.6 (± 2.8)
7k	0.64 (± 0.11)	−2.5 (± 0.2)
DPCPX	0.012 (± 0.002)[a]	−0.97 (± 0.05)

[a] K_i value.

2); full effects were observed within 1−2 concentration log units. Compound **8e** consistently failed to induce entire displacement of [^{3}H]CCPA binding; in all experiments, 20−30% of specific [^{3}H]CCPA binding remained. For reasons of comparison, we also included the "normal" displacement curve of DPCPX in Figure 2 with a pseudo Hill coefficient of approximately unity (Table 2).

Because **7k** did not affect [^{3}H]DPCPX binding, we only tested **7a**,**l** and **8e** more thoroughly on the binding of this radioligand. Figure 3 shows the dose−response curves of **7a**,**l** and **8e**. Bell-shaped curves were observed for **7a**,**l**, whereas **8e** induced a monophasic, but steep, displacement curve ($EC_{50} = 5.1 \pm 0.5$ μM).

To study the mechanism of action of these allosteric modulators, we analyzed the behavior of **7a**,**l** on the association and dissociation kinetics of the radiolabeled agonist [^{3}H]CCPA as well as of the radiolabeled antagonist [^{3}H]DPCPX. The association kinetics of [^{3}H]CCPA were severely affected by the two compounds (Figure 4A; NB the two separate scales on the ordinate) with negligible effect on its dissociation characteristics (Figure 4B and Table 3). The compounds almost fully inhibited [^{3}H]CCPA association, although with slightly different characteristics, i.e., **7a** consistently caused an

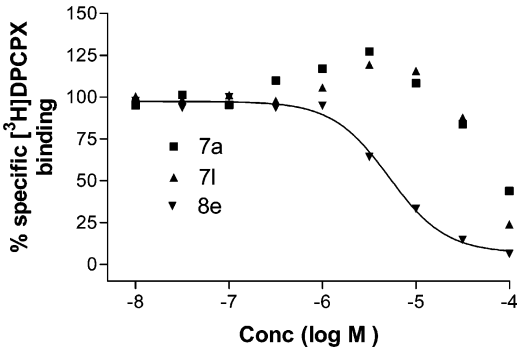


Figure 3. Displacement by **7a**,**l** and **8e** of [^{3}H]DPCPX binding to human adenosine A_1 receptors. Data are from a representative experiment performed in duplicate ($n = 3$). Radioligand binding is expressed as percent of specific binding.

initial small "spike" in [^{3}H]CCPA binding. In contrast, [^{3}H]DPCPX association and dissociation kinetics were both altered by **7a**,**l** (Figure 4C,D and Table 4). Both compounds strongly decreased the [^{3}H]DPCPX dissociation rate, resulting in prolonged $t_{1/2}$ values (Table 4). The apparent K_D values for [^{3}H]DPCPX binding derived from these kinetic experiments (k_{off}/k_{on}) were 0.54 (control), 0.52 (**7a**), and 0.11 nM (**7l**). The latter data suggest **7l** a true allosteric enhancer of antagonist binding to adenosine A_1 receptors. From a more general perspective, their influence on radioligand kinetics defines the compounds as allosteric modulators per se.[3]

We also tested the effects of **7a**,**l** on ligand binding to human adenosine A_{2A} receptors. Compound **7a** inhibited binding of the radiolabeled agonist [^{3}H]NECA at all concentrations tested (Figure 5A). The effect of **7l** on [^{3}H]NECA binding (Figure 5A) showed a maximum increase of [^{3}H]NECA binding at 2.1 μM. At 7 μM, this compound fully inhibited radioligand binding. Moreover, these two compounds inhibited binding of the radiolabeled antagonist [^{3}H]ZM241385 to the adenosine A_{2A} receptor (Figure 5B).

Figure 6 shows the results from dissociation experiments on human adenosine A_{2A} receptors. Compound **7a** increased the dissociation rate of the A_{2A} antagonist [^{3}H]ZM241385 in a concentration-dependent manner (Figure 6A). In the presence of 3 and 10 μM, the dissociation rate decreased 6.1- and 7.3-fold, respectively. The effects of **7l** were also investigated. At 0.7 μM, **7l** increased the dissociation rate of [^{3}H]ZM241385 almost 2-fold, whereas the dissociation rate decreased at 7 μM. Furthermore, there was no change in dissociation as compared to control at 2.1 μM. Allosteric modulation of adenosine A_{2A} receptors has so far only been described for sodium ions and amiloride derivatives.[15] Apparently, the thiadiazoles constitute a second class of small organic molecules that allosterically influence A_{2A} receptor binding.

Compound **7a** was also tested for its effects on ligand binding to human adenosine A_3 receptors. This compound (10 μM) decreased binding of both the A_3 agonist [^{125}I]AB-MECA (64% of specific binding remaining) and the A_3 antagonist [^{3}H]PSB-11 (83% of specific binding remaining).

It has been observed previously that SCH-202676 (**7a**) inhibited antagonist binding to μ, δ, and κ opioid, α_{2a} and β_1 adrenergic, and dopamine D_1 and D_2 receptors. Furthermore, it inhibited agonist binding to α_{2a} adren-

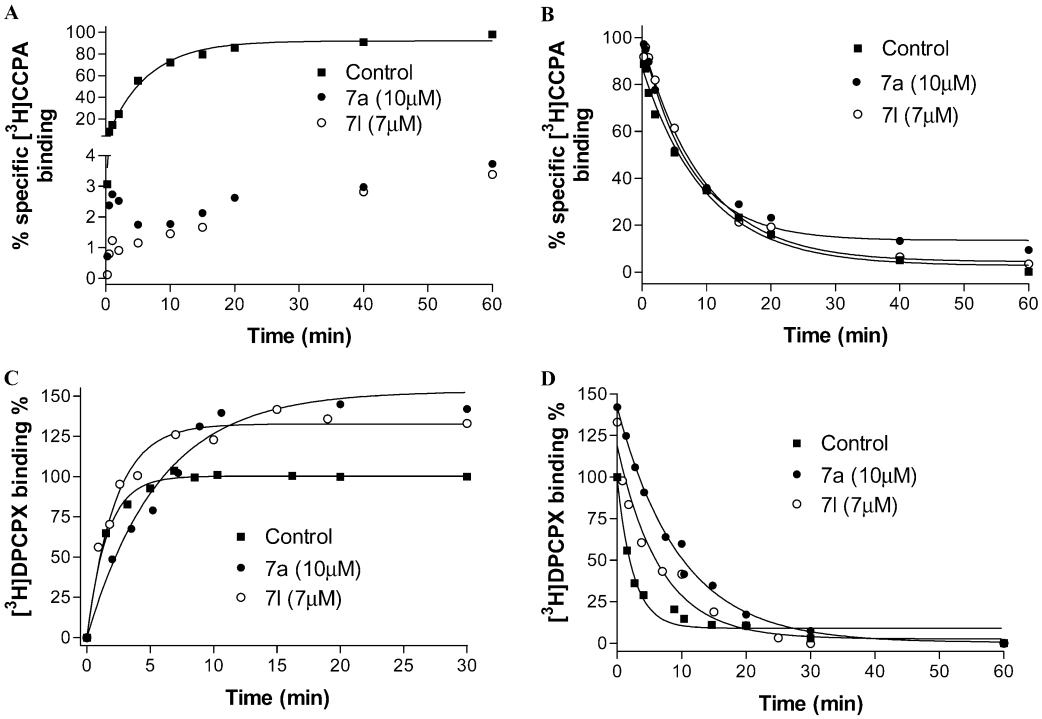


Figure 4. Association (A,C) and dissociation kinetics (B,D) of [^{3}H]CCPA (A,B) and [^{3}H]DPCPX (C,D) binding to and from human adenosine A_1 receptors. Data are from a representative experiment performed in duplicate ($n = 3$). Radioligand binding is expressed as percent of specific binding.

Table 3. Association and Dissociation Kinetic Parameters of [^{3}H]CCPA Binding to Human Adenosine A_1 Receptors in the Presence or Absence of **7a** (10 μM) and **7l** (7 μM)

	association	dissociation	
	k_{on} (nM^{-1} min^{-1})	$t_{1/2}$ (min)	k_{off} (min^{-1})
control	0.048 (± 0.002)	5.88 (± 1.77)	0.12 (± 0.04)
7a		5.37 (± 0.33)	0.13 (± 0.01)
7l		6.48 (± 0.12)	0.11 (± 0.01)

Table 4. Association and Dissociation Kinetic Parameters of [^{3}H]DPCPX Binding to Human Adenosine A_1 Receptors in the Presence or Absence of **7a** (10 μM) and **7l** (7 μM)

	association		dissociation	
	app $t_{1/2}$ (min)	k_{on} (nM^{-1} min^{-1})	$t_{1/2}$ (min)	k_{off} (min^{-1})
control	1.21 (± 0.10)	0.65 (± 0.04)	1.89 (± 0.29)	0.35 (± 0.04)
7a	3.85 (± 0.03)	0.21 (± 0.02)	6.61 (± 0.51)	0.11 (± 0.01)
7l	1.77 (± 0.30)	1.19 (± 0.26)	5.27 (± 0.03)	0.13 (± 0.01)

ergic receptors.[11] The effects appeared to be reversible, since SCH-202676 could be "washed out" in experiments on α_{2a} adrenergic and dopamine D_1 receptors. On adenosine receptors, SCH-202676 displayed a mixed behavior. It displaced agonist binding to A_1 receptors, also in a reversible manner (data not shown), and, similarly, both agonist and antagonist binding to A_{2A} receptors. The displacement curves were very steep (Figures 2, 3, and 5), almost resembling an all-or-none response, quite dissimilar to normal competitive displacement curves spanning approximately 3 concentration log units. Antagonist binding to adenosine A_1 receptors increased at 3 and 10 μM but diminished to control values at 30 μM. All of these data suggest complex interactions between allosteric ligand and receptor, which are not easily explained[16] within the framework of current paradigms such as the extended ternary complex model[17] and the allosteric two state model.[18] It may also be that the system does not reach

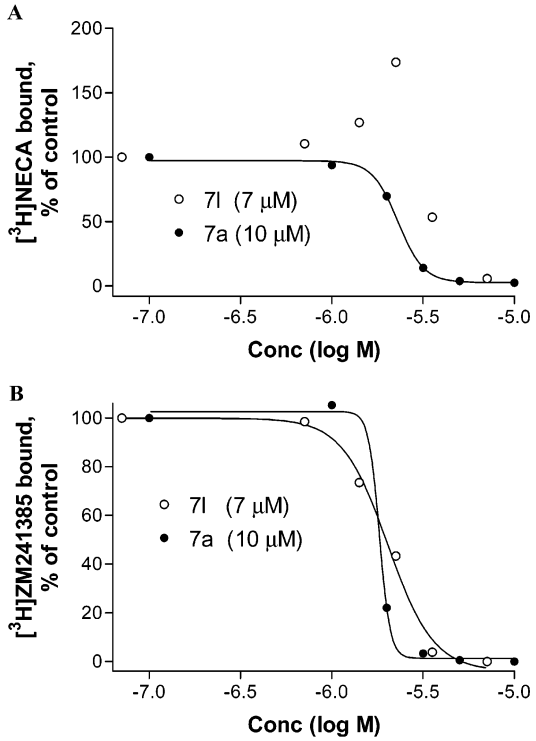


Figure 5. Modulation of [^{3}H]NECA and [^{3}H]ZM241385 binding by **7a** (10 μM) and **7l** (7 μM) on human A_{2A} adenosine receptors. Data are from a representative experiment performed in duplicate ($n = 3$). Radioligand binding is expressed as percent of specific binding.

equilibrium at high concentrations of the allosteric modulator. Similar assumptions have also been brought forward by Proska and Tuček to explain the effects of alcuronium observed at muscarinic receptors, which resemble the observations with **7a**,**l**.[19] In their view, high concentrations of an allosteric inhibitor prevent

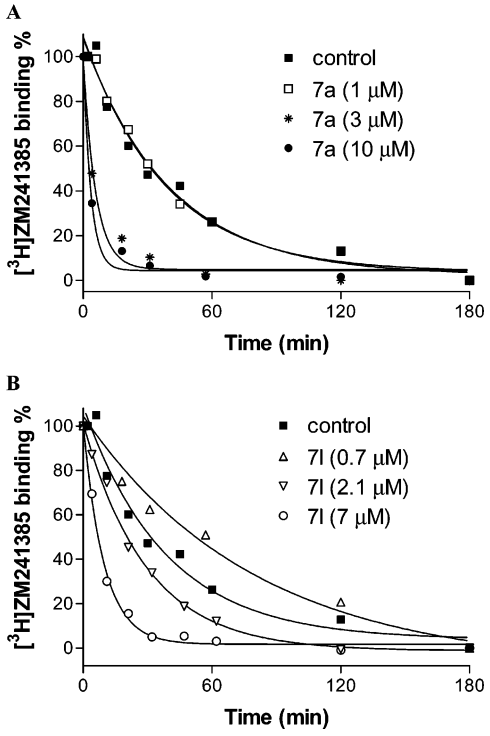


Figure 6. Dissociation of [^{3}H]ZM241385 at 0 °C in the presence or in the absence of various concentrations of **7a** (A) and **7l** (B). Data are from a representative experiment performed in duplicate (n = 3). Radioligand binding is expressed as percent of specific binding.

equilibrium conditions to be reached in the assay. This, in turn, leads to low levels of radioligand binding, easily interpreted as inhibition. Christopoulos and Kenakin[3] provided yet another explanation. They were able to simulate a similar bell-shaped curve by assuming that the allosteric modulator also interacts with the primary ligand binding site on the receptor. In such a case, the compound is not only modulating both its own and the radioligand's binding through an allosteric mechanism but is also competing with the radioligand at the orthosteric site. Last, there are also subtle qualitative differences in the behavior of both **7a** and **7l** besides potency, such as shape of curve and level of inhibition or enhancement, suggesting that even within the class of structurally similar thiadiazoles different sorts of allosteric modulation exist.

Conclusion

A series of substituted thiadiazoles was synthesized and demonstrated to behave as allosteric modulators of adenosine receptor binding. One of the compounds (LUF5792, **7k**) proved to be more active than the reference compound SCH-202676 (**7a**) as an allosteric inhibitor of agonist binding to human adenosine A_1 receptors. Because SCH-202676 has been shown to interact with many more GPCRs, it seems that the compounds in this study may also have value as "universal" small molecule modulators of GPCR binding and function.

Experimental Section

Chemicals. [^{3}H]DPCPX (128 Ci/mmol), [^{3}H]NECA (37 Ci/mmol), and [^{125}I]AB-MECA (2000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. [^{3}H]CCPA (54.9 Ci/mmol) was obtained from NEN. [^{3}H]ZM241385 (17 Ci/mmol) was from Tocris Cookson. [^{3}H]PBS-11 (53 Ci/mmol) was a kind gift of Prof. C. E. Mueller (Bonn University, Germany). DPCPX and PD81,723 were from Sigma-RBI. CPA was synthesized in our laboratory. All other chemicals, including starting materials, were from standard sources and of the highest purity commercially available.

Instruments and Analysis. ^{1}H NMR spectra were measured at 200 MHz with a Bruker AC 200 spectrometer equipped with a PG 200 computer operating in the Fourier transform mode. ^{13}C NMR spectra were measured at 50 MHz. Chemical shifts for ^{1}H and ^{13}C are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard; coupling constants are given in Hz. Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. Combustion analyses of new target compounds (series **7** and **8**) were performed by the analytical department of the Gorlaeus Laboratories, Leiden University (The Netherlands) and are within ±0.4% of theoretical values unless otherwise specified.

General Procedure for the Preparation of Benzamides. The appropriate aniline was dissolved in ethyl acetate (5 mL/mmol) in the presence of triethylamine (1.4 equiv). A solution of the appropriate benzoyl chloride (1.2 equiv) in ethyl acetate (2 mL/mmol) was added dropwise. A precipitate was formed, and the mixture was stirred at room temperature overnight. The next day, most of the solvent was removed in vacuo. Light petroleum was added, and the solids were collected on a filter, washed twice with a mixture of light petroleum/EtOAc (5/1) and three times with water. The residue was dried at 50 °C in vacuo to afford the amide.

4-Chloro-*N*-(4-chlorophenyl)benzamide (2b). Intake: 63 mmol of aniline. Yield: 15.8 g of a white solid (95%). ^{1}H NMR ($CDCl_3$): δ 7.34 (d, 2H, J = 8.8 Hz, arom); 7.47 (d, 2H, J = 8.8 Hz, arom); 7.58 (d, 2H, J = 8.8 Hz, arom); 7.75 (s, 1H, NH); 7.81 (d, 2H, J = 8.8 Hz, arom). ^{13}C NMR (DMSO-d_6): δ 121.8, 127.5, 128.4, 129.6, 133.3, 136.6, 137.9, 164.4.

3-Chloro-*N*-(3-chlorophenyl)benzamide (2c). Intake: 33 mmol of aniline. Yield: 6.01 g of a white solid (75%). ^{1}H NMR (DMSO-d_6): δ 7.17 (d, 1H, J = 8.0 Hz, arom); 7.39 (dd, 1H, J = J = 8.0, arom); 7.64 (m, 3H, arom); 7.96 (m, 3H, arom); 10.49 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 118.4, 120.3, 123.6, 125.9, 127.6, 129.4, 131.2, 133.8, 136.4, 139.9, 164.6.

3,4-Dichloro-*N*-(3,4-dichlorophenyl)benzamide (2d). Intake: 33 mmol of aniline. Yield: 8.36 g of a white solid (76%). ^{1}H NMR (DMSO-d_6): δ 7.80 (m, 4H, arom); 8.14 (s, 1H, arom); 8.22 (s, 1H, arom); 10.64 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 120.2, 121.5, 125.5, 128.0, 129.6, 130.5, 130.7, 130.9, 131.4, 134.4, 134.8, 138.8, 164.5.

4-Methyl-*N*-(4-methylphenyl)benzamide (2e). Intake: 36 mmol of aniline. Yield: 6.03 g of an off white solid (94%). ^{1}H NMR ($CDCl_3$): δ 2.34 (s, 3H, CH_3); 2.42 (s, 3H, CH_3); 7.17 (d, 2H, J = 8.4 Hz, arom); 7.28 (d, 2H, J = 7.3 Hz, arom); 7.52 (d, 2H, J = 8.4 Hz, arom); 7.76 (m, 3H, NH, arom). ^{13}C NMR ($CDCl_3$): δ 20.7, 21.3, 120.4, 120.5, 127.1, 129.0, 129.2, 132.0, 133.7, 135.5, 141.8, 165.9.

4-Methoxy-*N*-(4-methoxyphenyl)benzamide (2f). Intake: 51 mmol of aniline. Yield: 12.8 g of a pale gray solid (98%). ^{1}H NMR (DMSO-d_6): δ 3.75 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 6.93 (d, 2H, J = 8.8 Hz, arom); 7.06 (d, 2H, J = 8.8 Hz, arom); 7.68 (d, 2H, J = 8.8 Hz, arom); 7.97 (d, 2H, J = 8.8 Hz, arom); 10.00 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.1, 55.4, 113.6, 113.7, 122.1, 127.2, 129.5, 132.5, 155.5, 161.8, 164.6.

***N*-(4-Chlorophenyl)benzamide (2g).** Intake: 54 mmol of aniline. Yield: 11.4 g of a white solid (91%). ^{1}H NMR (DMSO-d_6): δ 7.43 (d, 2H, J = 8.8 Hz, arom); 7.57 (m, 3H, arom); 7.83 (d, 2H, J = 8.8 Hz, arom); 7.97 (d, 2H, J = 8.8 Hz, arom); 10.40 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 121.9, 127.4, 127.7, 128.4, 128.5, 131.7, 134.8, 138.2, 165.7.

***N*-(3-Chlorophenyl)benzamide (2h).** Intake: 58 mmol of aniline. Yield: 9.85 g of a white solid (73%). ^{1}H NMR (DMSO-d_6): δ 7.18 (d, 1H, J = 8.0 Hz, arom); 7.40 (dd, 1H, J = J = 8.0 Hz, arom); 7.57 (m, 3H, arom); 7.73 (d, 1H, J = 8.0 Hz,

arom); 7.97 (m, 3H, arom); 10.44 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 118.7, 119.8, 123.3, 127.8, 128.4, 130.2, 131.8, 133.0, 134.6, 140.8, 165.9.

***N*-(3,4-Dichlorophenyl)benzamide (2i).** Intake: 53 mmol of aniline. Yield: 12.4 g of a white solid (88%). ^{1}H NMR (DMSO-d_6): δ 7.60 (m, 4H, arom); 7.78 (d, 1H, J = 8.8 Hz, arom); 7.96 (d, 2H, J = 8.0 Hz, arom); 8.18 (s, 1H, arom); 10.52 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 120.2, 121.4, 125.1, 127.8, 128.5, 130.5, 130.9, 131.9, 134.4, 139.4, 165.9.

***N*-(4-Methylphenyl)benzamide (2j).** Intake: 54 mmol of aniline. Yield: 6.82 g of an off white solid (60%). ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, CH_3); 7.17 (d, 2H, J = 8.0 Hz, arom); 7.56 (m, 3H, arom); 7.68 (d, 2H, J = 8.0 Hz, arom); 7.97 (d, 2H, J = 8.0 Hz, arom); 10.19 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.5, 120.5, 127.7, 128.4, 129.0, 131.4, 132.7, 135.1, 136.8, 165.4.

***N*-(3-Methylphenyl)benzamide (2k).** Intake: 54 mmol of aniline. Yield: 10.7 g of a white solid (93%). ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3); 6.94 (d, 1H, J = 7.3 Hz, arom); 7.25 (dd, 1H, J = 7.3, 8.0 Hz, arom); 7.57 (m, 5H, arom); 9.97 (d, 2H, J = 7.3 Hz, arom); 10.20 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 21.2, 116.4, 117.7, 121.0, 124.4, 127.7, 128.4, 131.5, 135.1, 137.8, 139.2, 165.6.

***N*-(4-Methoxyphenyl)benzamide (2l).** Intake: 54 mmol of aniline. Yield: 9.81 g of a pale gray solid (80%). ^{1}H NMR (DMSO-d_6): δ 3.76 (s, 3H, OCH_3); 6.94 (d, 2H, J = 8.8 Hz, arom); 7.52 (m, 3H, arom); 7.69 (d, 2H, J = 8.8 Hz, arom); 7.96 (d, 2H, J = 8.0 Hz, arom); 10.15 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.2, 137.8, 122.1, 127.7, 128.4, 131.4, 132.3, 135.2, 155.6, 165.3.

4-Chloro-*N*-(phenyl)benzamide (2m). Intake: 50 mmol of aniline. Yield: 11.3 g of a white solid (98%). ^{1}H NMR (DMSO-d_6): δ 7.13 (dd, 1H, J = J = 7.3 Hz, arom); 7.38 (dd, 2H, J = 7.3, 8.0 Hz, arom); 7.63 (d, 2H, J = 8.8 Hz, arom); 7.80 (d, 2H, J = 8.0 Hz, arom); 8.02 (d, 2H, J = 8.8 Hz, arom); 10.34 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 120.5, 123.8, 128.4, 128.6, 129.6, 133.7, 136.4, 139.0, 164.4.

4-Methoxy-*N*-(phenyl)benzamide (2n). Intake: 50 mmol of aniline. Yield: 10.9 g of an off white solid (96%). ^{1}H NMR (DMSO-d_6): δ 3.86 (s, 3H, OCH_3); 7.09 (d, 2H, J = 8.8 Hz, arom); 7.11 (s, 1H, arom); 7.37 (dd, 2H, J = J = 8.0 Hz, arom); 10.12 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 55.4, 113.6, 120.4, 123.4, 127.0, 128.5, 129.6, 139.4, 161.9, 165.0.

General Procedure for the Preparation of the Benzimidoyl Chlorides. The procedure described by Von Braun and Pinkernelle[13] was used. In a 25 mL round-bottomed flask, equipped with a condensor and drying tube (silica blue), a mixture of benzamide and thionyl chloride (1.2 equiv) was heated on an oil bath (130−150 °C) until a homogeneous solution appeared. The mixture was cooled after heating for an additional 2 h. Distillation in vacuo (0.1 mbar) afforded the rapidly solidifying benzimidoyl chlorides.

***N*-(Phenyl)benzimidoyl Chloride (3a).** Intake: 62 mmol of amide. Yield: 10.5 g (79%), bp 122−126 °C, yellow solid. ^{1}H NMR ($CDCl_3$): δ 7.01 (m, 2H, arom); 7.48 (m, 6H, arom); 8.17 (m, 2H, arom). ^{13}C NMR ($CDCl_3$): δ 120.3, 124.9, 128.3, 128.7, 129.3, 131.9, 135.3, 142.9, 147.5.

4-Chloro-*N*-(4-chlorophenyl)benzimidoyl Chloride (3b). Intake: 59 mmol of amide. Yield: 3.8 g (23%), pale yellow solid. ^{1}H NMR ($CDCl_3$): δ 6.96 (d, 2H, J = 8.8 Hz, arom); 7.41 (m, 4H, arom); 8.09 (d, 2H, J = 8.8 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 119.7, 126.3, 126.6, 128.3, 128.4, 130.1, 131.2, 136.3, 140.2, 143.2.

3-Chloro-*N*-(3-chlorophenyl)benzimidoyl Chloride (3c). Intake: 22 mmol of amide. Yield: 4.8 g (76%), bp 175−178 °C, yellow solid. ^{1}H NMR ($CDCl_3$): δ 6.88 (d, 1H, J = 8.0 Hz, arom); 7.02 (s, 1H, arom); 7.19 (d, J = 8.8 Hz, arom); 7.38 (m, 2H, arom); 7.53 (d, 1H, J = 8.4 Hz, arom); 8.04 (d, 1H, J = 8.0 Hz, arom); 8.15 (s, 1H, arom). ^{13}C NMR ($CDCl_3$): δ 118.6, 120.5, 125.4, 127.5, 129.3, 129.6, 129.9, 132.2, 134.5, 134.6, 136.7, 143.0, 148.2.

4-Methyl-*N*-(4-methylphenyl)benzimidoyl Chloride (3e). Intake: 26.7 mmol of amide. Crude imidoyl chloride was dissolved in CH_2Cl_2, and after it was evaporated, a pale brown solid (6.4 g, 98%) remained. This material was used without further purification. ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3); 2.43 (s, 3H, CH_3); 6.93 (d, 2H, J = 8.8 Hz, arom); 7.23 (m, 4H, arom); 8.04 (d, 2H, J = 8.8 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 21.0, 21.4, 120.5, 124.6, 129.0, 129.3, 129.6, 129.7, 132.8, 134.5, 142.4, 145.0.

***N*-(4-Chlorophenyl)benzimidoyl Chloride (3g).** Intake: 47 mmol of amide. Yield: 9.7 g (81%), bp 147−152 °C, yellow solid. ^{1}H NMR ($CDCl_3$): δ 6.96 (d, 2H, J = 8.0 Hz, arom); 7.37 (d, 2H, J = 8.8 Hz, arom), 7.51 (m, 3H, arom); 8.16 (d, 2H, J = 8.0 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 121.9, 128.4, 128.9, 129.4, 130.5, 132.2, 135.1, 144.0, 145.9.

***N*-(3-Chlorophenyl)benzimidoyl Chloride (3h).** Intake: 42 mmol of amide. Yield: 2.1 g (20%). ^{1}H NMR ($CDCl_3$): δ 6.88 (d, 1H, J = 8.0 Hz, arom); 7.01 (dd, 1H, J = J = 2.3 Hz, arom); 7.18 (d, 1H, J = 8.0 Hz, arom); 7.33 (dd, J = J = 8.0 Hz, arom); 7.52 (m, 3H, arom); 8.16 (dd, 2H, J = 8.0, 8.8 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 118.5, 120.3, 124.7, 127.5, 128.2, 129.2, 129.7, 132.1, 134.1, 134.7, 144.2, 148.4.

***N*-(3,4-Dichlorophenyl)benzimidoyl Chloride (3i).** Intake: 25 mmol of amide. Yield: 4.6 g (65%), bp 159−160 °C, black solid. ^{1}H NMR ($CDCl_3$): δ 6.86 (d, 1H, J = 8.8 Hz, arom); 7.14 (s, 1H, arom); 7.50 (m, 4H, arom); 8.14 (d, 2H, J = 8.8 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 120.2, 122.5, 128.5, 129.5, 130.6, 132.5, 134.8, 145.3, 146.9.

***N*-(4-Methylphenyl)benzimidoyl Chloride (3j).** Intake: 32 mmol of amide. Yield: 5.4 g (75%), bp 129−134 °C, yellow solid. ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3); 6.94 (d, 2H, J = 8.8 Hz, arom); 7.23 (d, 2H, J = 8.0 Hz, arom); 7.49 (m, 3H, arom); 8.16 (d, 2H, J = 8.0 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 21.0, 120.5, 121.4, 128.3, 129.3, 131.8, 134.7, 135.5, 142.4, 144.9.

***N*-(3-Methylphenyl)benzimidoyl Chloride (3k).** Intake: 47 mmol of amide. Yield: 5.8 g (53%), bp 140−150 °C, yellow solid. ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, CH_3); 6.83 (s, 2H, arom); 7.02 (d, 1H, J = 8.0 Hz, arom); 7.30 (m, 2H, arom); 7.48 (m, 4H, arom); 8.15 (d, 2H, J = 7.4 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 21.2, 117.2, 120.8, 125.6, 128.2, 128.5, 129.1, 131.7, 135.2, 138.4, 142.4, 147.4.

***N*-(4-Methoxyphenyl)benzimidoyl Chloride (3l).** Intake: 42 mmol of amide. Yield: 4.6 g (45%), bp 150−160 °C, orange solid. ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 3H, OCH_3); 6.95 (d, 2H, J = 8.8 Hz, arom); 7.07 (d, 2H, J = 8.8 Hz, arom); 7.48 (m, 3H, arom); 8.15 (d, 2H, J = 8.0 Hz, arom). ^{13}C NMR ($CDCl_3$): δ 55.3, 114.0, 122.4, 128.3, 129.3, 131.7, 135.9, 140.3, 141.6.

4-Chloro-*N*-(phenyl)benzimidoyl Chloride (3m). Intake: 48 mmol of amide. Yield: 6.2 g (52%), bp 130−140 °C. ^{1}H NMR ($CDCl_3$): δ 7.01 (d, 2H, J = 8.8 Hz, arom); 7.21 (m, 1H, arom); 7.42 (m, 4H, arom); 8.10 (d, 2H, J = 8.8 Hz, arom).

***N*-(4-Methoxyphenyl)benzimidoyl Chloride (3n).** Intake: 45 mmol of amide. Yield: 2.0 g (17%) black oil. ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 3H, OCH_3); 6.95 (m, 4H, arom); 7.33 (m, 2H, arom); 8.11 (m, 2H, Hz, arom). ^{13}C NMR ($CDCl_3$): δ 55.2, 113.4, 120.4, 124.9, 128.6, 131.0, 133.6, 147.5, 162.5.

General Procedure for the Preparation of the Thioureas.[12] The appropriate benzimidoyl chloride was dissolved in acetone p.a. (1 mmol/3 mL). At −15 °C, a solution of NaSCN (1.1 equiv) in acetone (1 mmol/2 mL) was added dropwise. The mixture was stirred on the cooling bath until 0 °C was reached. The precipitate (NaCl) was removed by filtration over a glass filter with Hyflo. The filtrate was stirred on an ice bath, and appropriate amine (1.1 equiv) was added. After it was stirred to room temperature, the solvent was removed by evaporation and the residue was triturated with a diethyl ether/*n*-pentane (usually 1:4) mixture, collected on a glass filter, washed once more, and dried in vacuo to give the thiourea. This product was then used in the next step without further purification.

1-Methyl-3-(phenyl-phenyliminomethyl)thiourea (5a). Scale: 20.9 mmol. Yield: 4.50 g (80%) of an off white solid. ^{1}H NMR ($CDCl_3$): δ 3.26 (d, 3H, J = 4.4 Hz, NCH_3); 6.68 (d, 2H, J = 8.8 Hz, arom); 6.98 (d, 1H, J = 9.5 Hz, arom); 7.47 (m, 7H, arom); 8.06 (s, 1H, NH); 11.84 (broad s, 1H, NH). ^{13}C

NMR ($CDCl_3$): δ 31.7, 121.9, 123.3, 127.8, 128.3, 130.0, 131.5, 146.3, 155.4, 180.7.

1-Ethyl-3-(phenyl-phenyliminomethyl)thiourea (6a). Scale: 9.2 mmol. Yield: 2.30 g (88%) of a pale yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, J = 7.3 Hz, CH_3); 3.76 (m, 2H, CH_2); 6.68 (d, 2H, J = 8.8 Hz, arom); 6.97 (m, 1H, arom); 7.24 (m, 7H, arom); 8.00 (s, 1H, NH); 11.90 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.6, 40.3, 122.2, 123.5, 127.9, 128.5, 130.3, 131.9, 146.4, 155.5, 189.7.

1-[(4-Chlorophenyl)-(4-chlorophenylimino)methyl]-3-methylthiourea (5b). Scale: 6.6 mmol. Yield: 1.65 g (73%) of a pale yellow solid. ^{1}H NMR ($CDCl_3$): δ 3.25 (d, 3H, J = 4.4 Hz, NCH_3); 6.59 (d, 2H, J = 8.8 Hz, arom); 7.14 (m, 4H, arom); 7.30 (d, 2H, J = 8.8 Hz, arom); 8.07 (s, 1H, NH); 11.60 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 32.0, 123.5, 128.8, 129.1, 129.4, 129.8, 136.7, 144.9, 154.9, 180.8.

1-[(4-Chlorophenyl)-(4-chlorophenylimino)methyl]-3-ethylthiourea (6b). Scale: 6.9 mmol. Yield: 1.35 g (57%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 3.75 (m, 2H, CH_2); 6.59 (d, 2H, J = 8.8 Hz, arom); 7.14 (m, 3H, arom); 7.28 (m, 3H, arom); 7.95 (s, 1H, NH); 11.60 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.6, 40.4, 123.5, 128.9, 129.1, 129.4, 136.7, 144.8, 154.9, 179.5.

1-Methyl-3-(4-tolyl-4-tolyliminomethyl)thiourea (5e). Scale: 13.1 mmol. Yield: 2.20 g (57%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 2.24 (s, 3H, CH_3); 2.31 (s, 3H, CH_3); 3.24 (d, 3H, J = 4.7 Hz, NCH_3); 6.57 (d, 2H, J = 8.0 Hz, arom); 6.95 (d, 2H, J = 8.0 Hz, arom); 7.05 (m, 4H, arom); 8.00 (s, 1H, NH); 11.93 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 20.5, 21.1, 31.7, 121.8, 127.8, 129.0, 132.7, 140.3, 143.8, 155.3, 180.9.

1-Ethyl-3-(4-tolyl-4-tolyliminomethyl)thiourea (6e). Scale: 13.0 mmol. Yield: 2.44 g (60%) of a brown solid. ^{1}H NMR ($CDCl_3$): δ 1.31 (t, 3H, J = 7.3 Hz, CH_3); 2.24 (s, 3H, CH_3); 2.31 (s, 3H, CH_3); 3.74 (m, 2H, CH_2); 6.57 (d, 2H, J = 8.0 Hz, arom); 6.95 (d, 2H, J = 8.0 Hz, arom); 7.09 (m, 4H, arom); 7.94 (s, 1H, NH); 12.00 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.5, 20.5, 21.1, 40.2, 121.9, 127.8, 129.1, 132.8, 140.4, 143.8, 155.3, 179.7.

1-[(4-Chlorophenylimino)phenylmethyl]-3-methylthiourea (5g). Scale 6.1 mmol. Yield: 1.23 g (68%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 3.26 (d, 3H, J = 4.4 Hz, NCH_3); 6.62 (d, 2H, J = 8.8 Hz, arom); 7.10 (d, 2H, J = 8.8 Hz, arom); 7.31 (m, 5H, arom); 8.06 (s, 1H, NH); 11.67 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 32.0, 123.6, 128.0, 128.7, 130.0, 131.6, 145.5, 156.0, 181.0.

1-[(4-Chlorophenylimino)phenylmethyl]-3-ethylthiourea (6g). Scale: 6.0 mmol. Yield: 1.75 g (92%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, J = 7.3 Hz, CH_3); 3.76 (m, 2H, CH_2); 6.60 (d, 2H, J = 8.8 Hz, arom); 7.10 (d, 2H, J = 8.0 Hz, arom); 7.31 (m, 5H, arom); 7.99 (s, 1H, NH); 11.72 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.6, 40.4, 123.6, 127.9, 128.8, 130.6, 131.7, 145.1, 156.0, 179.7.

1-[(3-Chlorophenylimino)phenylmethyl]-3-methylthiourea (5h). Scale: 4.2 mmol. Yield: 1.25 g (97%). ^{1}H NMR ($CDCl_3$): δ 3.26 (d, 3H, J = 4.4 Hz, NCH_3); 6.50 (d, 1H, J = 7.3 Hz, arom); 6.76 (s, 1H, arom); 7.00 (m, 2H, arom); 7.29 (m, 5H, arom); 8.08 (s, 1H, NH); 11.63 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 31.9, 120.4, 122.4, 123.5, 127.8, 128.6, 129.5, 130.5, 131.3, 134.0, 147.8, 156.0, 180.8.

1-[(3,4-Dichlorophenylimino)phenylmethyl]-3-ethylthiourea (6i). Scale: 5.3 mmol. Yield: 0.80 g (45%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, J = 7.3 Hz, CH_3); 3.77 (m, 2H, CH_2); 6.46 (d, 1H, J = 8.8 Hz, arom); 6.86 (s, 1H, arom); 7.30 (m, 6H, arom); 8.03 (s, 1H, NH); 11.56 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.5, 40.3, 113.0, 121.7, 124.1, 126.9, 127.8, 128.8, 130.0, 131.1, 132.1, 146.1, 156.5, 179.4.

1-Methyl-3-(phenyl-4-tolyliminomethyl)thiourea (5j). Scale: 6.1 mmol. Yield: 1.72 g (99%). ^{1}H NMR ($CDCl_3$): δ 2.23 (s, 3H, CH_3); 3.26 (d, 3H, J = 5.1 Hz, NCH_3); 6.57 (d, 2H, J = 8.0 Hz, arom); 6.94 (d, 2H, J = 8.0 Hz, arom); 7.29 (m, 5H, arom); 8.03 (s, 1H, NH); 11.91 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 20.6, 31.9, 122.0, 127.9, 128.6, 129.2, 130.2, 132.0, 133.0, 143.8, 155.3, 181.0.

1-Ethyl-3-(phenyl-4-tolyliminomethyl)thiourea (6j). Scale: 6.1 mmol. Yield: 1.70 g (94%). ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 2.24 (s, 3H, CH_3); 3.74 (m, 2H, CH_2); 6.58 (d, 2H, J = 8.0 Hz, arom); 6.94 (d, 2H, J = 8.0 Hz, arom); 7.29 (m, 5H, arom); 7.98 (s, 1H, NH); 11.98 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.5, 20.5, 40.2, 122.0, 127.9, 128.5, 129.1, 130.1, 132.0, 132.9, 143.7, 155.3, 179.6.

1-Methyl-3-(phenyl-3-tolyliminomethyl)thiourea (5k). Scale: 6.9 mmol. Yield: 1.08 g (56%). ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H, CH_3); 3.25 (d, 3H, J = 4.4 Hz, NCH_3); 6.43 (d, 1H, J = 8.0 Hz, arom); 6.56 (s, 1H, arom); 6.78 (d, 1H, J = 7.3 Hz, arom); 7.00 (dd, 1H, J = 7.3, 8.0 Hz, arom); 7.28 (m, 5H, arom); 8.03 (s, 1H, NH); 11.83 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 21.1, 31.9, 119.1, 122.9, 124.4, 128.0, 128.4, 128.6, 130.3, 132.0, 138.4, 146.4, 155.3.

1-Ethyl-3-(phenyl-3-tolyliminomethyl)thiourea (6k). Scale: 7.0 mmol. Yield: 1.52 g (73%). ^{1}H NMR ($CDCl_3$): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 2.21 (s, 3H, CH_3); 3.75 (m, 2H, CH_2); 6.44 (d, 1H, J = 7.3 Hz, arom); 6.56 (s, 1H, arom); 6.78 (d, 1H, J = 7.3 Hz, arom); 7.01 (dd, 1H, J = 7.3, 8.0 Hz, arom); 7.32 (m, 5H, arom); 7.96 (s, 1H, NH); 11.91 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.6, 21.1, 40.3, 119.1, 122.9, 124.3, 127.9, 128.3, 128.5, 130.2, 132.0, 138.4, 146.3, 155.3, 179.7.

1-Methyl-3-(phenyl-4-methoxyphenyliminomethyl)-thiourea (5l). Scale: 9.2 mmol. Yield: 2.52 g (92%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 3.27 (d, 3H, J = 4.4 Hz, NCH_3); 3.72 (s, 3H, OCH_3); 6.64 (m, 4H, arom); 7.27 (m, 5H, arom); 8.05 (s, 1H, NH); 11.94 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 31.7, 55.0, 113.7, 123.0, 127.9, 128.4, 130.0, 131.9, 133.0, 139.4, 155.4, 155.7, 180.8.

1-Ethyl-3-(phenyl-4-methoxyphenyliminomethyl)thiourea (6l). Scale: 4.68 mmol. Yield: 1.32 g (90%) of a yellow solid. ^{1}H NMR ($CDCl_3$): δ 1.33 (t, 3H, J = 7.3 Hz, CH_3); 3.72 (s, 3H, OCH_3); 3.78 (m, 2H, CH_2); 6.67 (m, 4H, arom); 7.32 (m, 5H, arom); 7.94 (s, 1H, NH); 11.99 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 13.6, 40.2, 55.1, 113.8, 123.2, 127.9, 128.6, 130.1, 132.1, 139.4, 155.3, 155.9, 179.7.

1-[(4-Chlorophenyl)phenyliminomethyl]-3-methylthiourea (5m). Scale: 6.4 mmol. Yield: 1.60 g (88%). ^{1}H NMR ($CDCl_3$): δ 3.24 (d, 3H, J = 5.1 Hz, NCH_3); 6.67 (d, 2H, J = 7.3 Hz, arom); 6.99 (dd, 1H, J = J = 7.3 Hz, arom); 7.20 (m, 6H, arom); 8.08 (s, 1H, NH); 11.75 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 31.9, 122.1, 123.7, 128.7, 128.8, 129.5, 130.0, 131.0, 136.4, 146.2, 154.5, 180.9.

General Procedure for the Preparation of [1,2,4]-Thiadiazoles.[12b] The thiourea was dissolved in a minimal amount of CH_2Cl_2 (usually 2−4 mL/mmol) and then diluted with double the volume of ethyl acetate. At room temperature, a 0.5 M solution of bromine in ethyl acetate (2 equiv) was added dropwise. The thiadiazole hydrobromide precipitated. The mixture was left to stand at 5 °C overnight. Filtering, washing (2×) of the residue with pentane/ethyl acetate (3:1), and drying in vacuo afforded the almost pure [1,2,4]thiadiazole. The products can be recrystallized from methanol.

2,3-Diphenyl-5-*N*-methylimino-*2H*-[1,2,4]thiadiazole Hydrobromide (7a). Scale: 16.3 mmol. Yield: 4.50 g (79%), product was recrystallized from methanol giving a white solid: mp 248−250 °C. ^{1}H NMR (MeOD-d_3): δ 3.37 (s, 3H, NCH_3); 7.49 (m, 10H, arom). ^{13}C NMR (DMSO-d_6): δ 31.5, 126.6, 127.6, 128.6, 130.1, 130.6, 132.4, 134.5, 164.6, 175.6. Anal. ($C_{15}H_{13}N_3S$·HBr) C, H, N.

2,3-Diphenyl-5-*N*-ethylimino-*2H*-[1,2,4]thiadiazole Hydrobromide (8a). Scale: 8.1 mmol. Yield: 2.41 g (82%), product was recrystallized from methanol giving a white solid: mp 240−242 °C. ^{1}H NMR (MeOD-d_3): δ 1.40 (t, 3H, J = 7.3 Hz, CH_3); 3.85 (q, 2H, J = 7.3 Hz, NCH_2); 7.48 (m, 10H, arom). ^{13}C NMR (DMSO-d_6): δ 14.1, 40.0, 126.7, 127.7, 128.7, 130.3, 130.7, 132.6, 134.6, 164.9, 175.0. Anal. ($C_{16}H_{15}N_3S$·0.8HBr·1.3H_2O) C, H, N.

2,3-Bis(4-chlorophenyl)-5-*N*-methylimino-*2H*-[1,2,4]thiadiazole Hydrobromide (7b). Scale: 4.85 mmol. Yield: 1.76 g (89%), product was recrystallized from methanol giving a yellow solid: mp 238−239 °C. ^{1}H NMR (MeOD-d_3): δ 3.37 (s, 3H, NCH_3); 7.53 (m, 8H, arom). ^{13}C NMR (DMSO-d_6): δ 31.6,

125.4, 128.9, 129.5, 130.3, 132.1, 133.2, 135.3, 137.4, 164.0, 175.8. Anal. ($C_{15}H_{11}Cl_2N_3S$·1.2HBr) C, H, N.

2,3-Bis(4-chlorophenyl)-5-*N*-ethylimino-*2H*-[1,2,4]thiadiazole Hydrobromide (8b). Scale: 3.69 mmol. Yield: 0.95 g (60%), product was recrystallized from methanol giving a light yellow solid: mp 242−244 °C. ^{1}H NMR (MeOD-d_3): δ 1.39 (t, 3H, J = 7.3 Hz, CH_3); 3.84 (q, 2H, J = 7.3 Hz, NCH_2); 7.53 (m, 8H, arom). ^{13}C NMR (DMSO-d_6): δ 14.0, 39.9, 125.3, 128.8, 129.4, 130.2, 132.0, 133.2, 135.2, 137.4, 163.7, 174.9. Anal. ($C_{16}H_{13}Cl_2N_3S$·HBr·0.7H_2O) C, H, N.

2,3-Bis(3-chlorophenyl)-5-*N*-methylimino-*2H*-[1,2,4]thiadiazole Hydrobromide (7c). Scale: 9.2 mmol. Yield: 1.42 g (42%) based on benzimidoyl chloride, product was recrystallized from methanol giving a white solid: mp 224−226 °C. ^{1}H NMR (DMSO-d_6): δ 3.32 (d, 3H, J = 4.7 Hz, NCH_3); 7.62 (m, 8H, arom); 9.88 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.7, 126.7, 127.6, 128.4, 128.7, 129.9, 130.6, 130.8, 131.7, 132.3, 133.2, 134.0, 135.4, 163.3, 175.9. Anal. ($C_{15}H_{11}Cl_2N_3S$·HBr·0.3H_2O) C, H, N.

2,3-Bis(4-methylphenyl)-5-*N*-methylimino-2*H*-[1,2,4]thiadiazole Hydrobromide (7e). Scale: 7.1 mmol. Yield: 1.87 (70%), product was recrystallized from methanol giving a yellow solid: mp 230−232 °C. ^{1}H NMR (DMSO-d_6): δ 2.32 (s, 3H, CH_3); 2.37 (s, 3H, CH_3); 3.29 (d, 3H, J = 4.8 Hz, NCH_3); 7.46 (m, 8H, arom); 9.85 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 20.9, 21.1, 31.5, 123.8, 127.3, 129.3, 130.3, 130.7, 132.2, 140.6, 143.2, 164.5, 175.4. Anal. ($C_{17}H_{17}N_3S$·HBr·0.2H_2O) C, H, N.

2,3-Bis(4-methylphenyl)-5-*N*-ethylimino-*2H*-[1,2,4]thiadiazole Hydrobromide (8e). Scale: 7.7 mmol. Yield: 2.0 g (66%), product was recrystallized from methanol giving a light yellow solid: mp 238−240 °C. ^{1}H NMR (DMSO-d_6): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 2.33 (s, 3H, CH_3); 2.38 (s, 3H, CH_3); 3.76 (m, 2H, NCH_2); 7.37 (m, 8H, arom); 9.86 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 20.8, 21.1, 39.9, 123.8, 127.4, 129.2, 130.3, 130.7, 132.1, 140.7, 143.2, 165.0, 174.8. Anal. ($C_{18}H_{19}N_3S$·HBr·0.6H_2O) C, H, N.

2-(4-Chlorophenyl)-5-*N*-methylimino-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7g). Scale: 5.0 mmol. Yield: 1.05 g (55%), product was recrystallized from methanol giving a white solid: mp 236−237 °C. ^{1}H NMR (DMSO-d_6): δ 3.30 (d, 3H, J = 4.8 Hz, NCH_3); 7.52 (m, 9H, arom); 9.98 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.6, 126.5, 128.7, 129.6, 130.2, 132.6, 133.5, 135.1, 164.8, 175.8. Anal. ($C_{15}H_{12}ClN_3S$·0.7HBr·1.3H_2O) C, H, N.

2-(4-Chlorophenyl)-5-*N*-ethylimino-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (8g). Scale: 5.5 mmol. Yield: 1.50 g (69%), product was recrystallized from methanol giving a white solid: mp 232−234 °C. ^{1}H NMR (DMSO-d_6): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 3.77 (m, 2H, NCH_2); 7.55 (m, 9H, arom); 10.04 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 40.0, 126.5, 128.7, 129.6, 130.2, 132.6, 133.5, 135.2, 165.1, 175.1. Anal. ($C_{16}H_{14}ClN_3S$·HBr·0.3H_2O) C, H, N.

2-(3-Chlorophenyl)-5-*N*-methylimino-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7h). Scale: 4.1 mmol. Yield: 0.92 g (59%), product was recrystallized from methanol giving a yellow solid: mp 160−161 °C; MS (ESI) m/z 301.9. ^{1}H NMR ($CDCl_3$): δ 3.87 (d, 3H, J = 5.1 Hz, NCH_3); 7.13 (d, 1H, J = 8.1 Hz, arom); 7.44 (m, 8H, arom); 10.22 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.6, 126.5, 127.7, 128.7, 130.2, 130.7, 132.6, 134.0, 135.8, 164.8, 175.7. Anal. ($C_{15}H_{12}ClN_3S$·2.9HBr) C, H, N.

2-(3-Chlorophenyl)-5-*N*-ethylimino-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (8h). Scale: 4.2 mmol. Yield: 0.67 g (40%) based on benzimidoyl chloride, product was recrystallized from methanol giving a white solid: mp 210−211 °C. ^{1}H NMR ($CDCl_3$): δ 1.44 (t, 3H, J = 7.3 Hz, CH_3); 3.83 (m, 2H, NCH_2); 7.14 (d, 1H, J = 8.1 Hz, arom); 7.44 (m, 8H, arom); 10.40 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 39.9, 126.4, 126.6, 127.7, 128.6, 130.2, 130.7, 131.7, 132.6, 134.0, 135.8, 165.0, 175.0. Anal. ($C_{16}H_{14}ClN_3S$·HBr) C, H, N. H: calcd, 3.81; found, 4.60. N: calcd, 10.59; found, 11.09.

2-(3,4-Dichlorophenyl)-5-*N*-methylimino-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7i). Scale: 5.3 mmol. Yield: 0.68 g (31%) based on benzimidoyl chloride, product was recrystallized from methanol giving a light yellow solid: mp 244−245 °C. ^{1}H NMR (DMSO-d_6): δ 3.31 (d, 3H, J = 4.4 Hz, NCH_3); 7.58 (m, 7H, arom); 7.83 (d, 1H, J = 8.8 Hz, arom); 9.85 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.7, 126.4, 128.3, 128.8, 129.9, 130.3, 132.0, 132.4, 132.7, 133.6, 134.4, 165.3, 176.0. Anal. ($C_{15}H_{11}Cl_2N_3S$·HBr) C, H, N. H: calcd, 2.90; found, 3.41. N: calcd, 10.07; found, 10.51.

2-(3,4-Dichlorophenyl)-5-*N*-ethylimino-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (8i). Scale: 2.27 mmol. Yield: 0.92 g (94%), product was recrystallized from methanol giving a white solid: mp 211−213 °C. ^{1}H NMR ($CDCl_3$): δ 1.43 (t, 3H, J = 7.3 Hz, CH_3); 3.83 (m, 2H, NCH_2); 7.08 (d, 1H, J = 8.8 Hz, arom); 7.49 (m, 7H, arom); 10.34 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 40.0, 126.3, 128.3, 128.7, 129.8, 130.3, 131.9, 132.2, 132.6, 133.5, 134.3, 165.2, 175.2. Anal. ($C_{16}H_{13}Cl_2N_3S$·HBr) C, H, N. N: calcd, 9.75; found, 10.27.

5-*N*-Methylimino-2-(4-methylphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole hydrobromide (7j). Scale: 5.8 mmol. Yield: 1.89 g (90%), product was recrystallized from methanol giving a light yellow solid: mp 240−241 °C. ^{1}H NMR (DMSO-d_6): δ 2.36 (s, 3H, CH_3); 3.29 (d, 3H, J = 5.1 Hz, NCH_3); 7.45 (m, 9H, arom); 9.83 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 20.8, 31.5, 126.7, 127.3, 128.7, 130.2, 130.6, 132.0, 132.5, 140.6, 164.6, 175.6. Anal. ($C_{16}H_{15}N_3S$·HBr) C, H, N.

5-*N*-Ethylimino-2-(4-methylphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (8j). Scale: 5.4 mmol. Yield: 1.80 g (89%), product was recrystallized from methanol giving a white solid: mp 242−243 °C. ^{1}H NMR (DMSO-d_6): δ 1.32 (t, 3H, J = 7.3 Hz, CH_3); 2.36 (s, 3H, CH_3); 3.76 (m, 2H, NCH_2); 7.46 (m, 9H, arom); 10.01 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 20.8, 40.2, 126.7, 127.3, 128.6, 130.2, 130.6, 131.9, 132.4, 140.6, 164.8, 174.9. Anal. ($C_{17}H_{17}N_3S$·HBr·0.2H_2O) C, H, N.

5-*N*-Methylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7k). Scale: 3.9 mmol. Yield: 1.25 g (89%), product was recrystallized from methanol giving a white solid: mp 237−238 °C. ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, CH_3); 3.35 (d, 3H, J = 5.1 Hz, NCH_3); 7.00 (m, 1H, arom); 7.12 (s, 1H, arom); 7.34 (m, 4H, arom); 7.53 (m, 3H, arom); 10.21 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 20.7, 31.5, 124.7, 126.6, 127.6, 128.6, 129.9, 130.2, 131.3, 132.5, 134.4, 140.1, 164.5, 175.6. Anal. ($C_{16}H_{15}N_3S$·0.8HBr·1.2H_2O) C, H, N.

5-*N*-Ethylimino-2-(3-methylphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (8k). Scale: 5.1 mmol. Yield: 1.45 g (76%), product was recrystallized from methanol giving a white solid: mp 226−227 °C. ^{1}H NMR ($CDCl_3$): δ 1.43 (t, 3H, J = 7.3 Hz, CH_3); 2.37 (s, 3H, CH_3); 3.81 (m, 2H, NCH_2); 6.98 (m, 1H, arom); 7.13 (s, 1H, arom); 7.33 (m, 4H, arom); 7.53 (m, 3H, arom); 10.33 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 20.7, 39.9, 124.6, 126.6, 127.6, 128.6, 129.9, 130.1, 131.2, 132.5, 134.4, 140.0, 164.4, 174.8. Anal. ($C_{17}H_{17}N_3S$·HBr·0.3H_2O) C, H, N.

5-*N*-Methylimino-2-(4-methoxyphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7l). Scale: 8.36 mmol. Yield: 2.63 g (83%), product was recrystallized from methanol giving a yellow solid: mp 149−150 °C; MS (ESI) m/z 298.0 $(M + H)^{+1}$. ^{1}H NMR (DMSO-d_6): δ 3.29 (d, 3H, J = 4.2 Hz, NCH_3); 3.81 (s, 3H, OCH_3); 7.08 (d, 2H, J = 8.8 Hz, arom); 7.51 (m, 7H, arom); 9.50 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.6, 55.7, 115.3, 126.7, 128.7, 129.1, 130.2, 132.5, 160.6, 165.5, 175.8. Anal. ($C_{16}H_{15}N_3OS$·3.0HBr) C, H, N.

5-*N*-Ethylimino-2-(4-methoxyphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (8l). Scale: 4.68 mmol. Yield: 1.37 g (83%), product was recrystallized from methanol giving a white solid: mp 197−198 °C. ^{1}H NMR (DMSO-d_6): δ 1.31 (t, 3H, J = 7.3 Hz, CH_3); 3.74 (m, 2H, CH_2); 3.80 (s, 3H, OCH_3); 7.07 (d, 2H, J = 8.8 Hz, arom); 7.49 (m, 7H, arom); 9.95 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 14.1, 39.9, 55.7, 115.2, 126.7, 126.8, 128.6, 129.1, 130.2, 132.4, 160.5, 164.9, 174.9. Anal. ($C_{17}H_{17}N_3OS$·0.8HBr·1.2H_2O) C, H, N.

3-(4-Chlorophenyl)-5-*N*-methylimino-2-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7m). Scale: 5.6 mmol. Yield:

1.40 g (65%), product was recrystallized from methanol giving a white solid: mp 252−253 °C. ^{1}H NMR ($CDCl_3$): δ 3.35 (d, 3H, J = 5.1 Hz, NCH_3); 7.30 (m, 4H, arom); 7.48 (m, 5H, arom); 10.24 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.5, 125.5, 127.5, 128.8, 130.2, 130.7, 132.0, 134.3, 137.4, 163.6, 175.6. Anal. ($C_{15}H_{12}ClN_3S$·HBr) C, H, N.

3-(4-Methoxyphenyl)-5-*N*-methylimino-2-phenyl-*2H*-[1,2,4]thiadiazole Hydrobromide (7n). Scale: 7.5 mmol. Yield: 0.37 g (13%) based on benzimidoyl chloride, product was recrystallized from methanol giving a yellow solid: mp 230−231 °C. ^{1}H NMR ($CDCl_3$): δ 3.35 (d, 3H, J = 5.1 Hz, NCH_3); 3.83 (s, 3H, OCH_3); 6.83 (m, 2H, arom); 7.26 (m, 2H, arom); 7.49 (m, 5H, arom); 10.16 (broad, 1H, HBr). ^{13}C NMR (DMSO-d_6): δ 31.4, 55.6, 114.2, 118.5, 127.6, 129.6, 130.3, 130.7, 132.5, 134.9, 162.6, 164.3, 175.1. Anal. ($C_{16}H_{15}N_3OS$·HBr·0.6H_2O) C, H, N.

Radioligand Binding Assays. For displacement experiments, membranes of CHO cells expressing recombinant human adenosine A_1 receptors (40 μg of protein) were incubated at 25 °C for 60 min with ∼1.0 nM [^{3}H]CCPA or ∼1.6 nM [^{3}H]DPCPX, and a fixed concentration or increasing concentrations of the compounds, in a final volume of 0.4 mL of Tris-HCl buffer. Nonspecific binding was measured in the presence of 10 μM CPA (in case of [^{3}H]DPCPX) or 10 μM DPCPX ([^{3}H]CCPA). Binding reactions were terminated by dilution with ice-cold 50 mM Tris-HCl buffer. Samples were then filtered through Whatman GF/B glass fiber filters using a Brandell cell harvester or a Millipore manifold. Filters were washed three times with 2−3 mL of the same buffer. Bound radioactivity was measured in a liquid scintillation counter (LKB Wallac) after the addition of 3.5 mL of scintillation liquid (Emulsifier-Safe, Packard).

In kinetic studies of the interaction between (radiolabeled) antagonist and adenosine A_1 receptors, a single concentration of [^{3}H]DPCPX (0.34−0.36 nM) was used. Membranes (40 μg of protein) were preincubated at 25 °C for 30 min with the compound of interest. Association was started by addition of the radioligand to the membranes. At various time points, the reaction was stopped by rapid dilution. Samples were filtered through Whatman GF/B glass fiber filters, which were washed three times with 2 mL of ice-cold buffer. Then, after 30 min, dissociation was initiated by addition of 100 μM CPA. The dissociation of [^{3}H]DPCPX binding was monitored by sampling at appropriate time intervals. Samples were treated as described above. Similarly, the association of the radiolabeled agonist [^{3}H]CCPA was started by addition of the membranes to the radioligand, in the presence or absence of allosteric modulators. To study dissociation of [^{3}H]CCPA, membranes were preincubated with [^{3}H]CCPA (1 nM) at 25 °C for 60 min. Dissociation of [^{3}H]CCPA was then initiated by the addition of 100 μM 8-cyclopentyltheophylline in the presence or absence of allosteric modulators. Samples were handled as mentioned before.

The conditions for adenosine A_{2A} receptor binding were slightly different. In competition experiments, membranes of CHO cells expressing recombinant human A_{2A} receptors (20 μg of protein) were incubated with ∼10 nM [^{3}H]NECA for 180 min at 25 °C. Otherwise, membranes (40 μg of protein) were incubated with ∼1.6 nM [^{3}H]ZM241385 for 120 min at 25 °C. Nonspecific binding was measured in the presence of 100 μM CPA ([^{3}H]ZM241385) or 10 μM CGS15943 ([^{3}H]NECA).

To study dissociation of [^{3}H]ZM241385, membranes (40 μg of protein) were preincubated with [^{3}H]ZM241385 (1.17−1.39 nM) at 0 °C for 180 min. Dissociation of [^{3}H]ZM241385 was then initiated by the addition of 1 μM CGS15943 in the presence or absence of allosteric modulators. Samples were handled as mentioned before.

For A_3 receptor displacement experiments, membranes of HEK-293 cells expressing recombinant human A_3 receptors (40 μg of protein) were incubated at 37 °C for 60 min with ∼0.1 nM of [^{125}I]AB-MECA, and a fixed concentration of the compound, in a final volume of 0.2 mL of 50 mM Tris/10 mM $MgCl_2$/1 mM EDTA/0.01% CHAPS buffer (pH 8.26 at 5 °C). Nonspecific binding was measured in the presence of 100 μM R-PIA. Binding reactions were terminated by dilution with ice-cold buffer. Samples were then filtered through Whatman GF/B glass fiber filters using a Brandell cell harvester. Filters were washed three times with 2−3 mL of the same buffer. Bound radioactivity was measured in a Minaxi γ Auto-gamma 5000 series γ-counter.

The conditions for [^{3}H]PSB-11 binding to A_3 receptors were essentially as A_1 receptor binding. Membranes of HEK-293 cells expressing recombinant human A_3 receptors (50−80 μg of protein) were incubated at 25 °C for 60 min with ∼4 nM [[3H]PSB-11 in 50 mM Tris-HCl buffer. Nonspecific binding was measured in the presence of 10 μM R-PIA. All membrane preparations for the three adenosine receptor subtypes contained 2 IU/mL of adenosine deaminase.

Statistical Analysis. Binding parameters were estimated by GraphPAD Prism software (GraphPAD, San Diego, CA). Data are expressed as means ± SEM for the number of experiments indicated.

Acknowledgment. We thank Jacobien von Frijtag Drabbe Künzel for performing the adenosine A_3 receptor binding assays.

References

(1) Soudijn, W.; Van Wijngaarden, I.; IJzerman, A. P. Allosteric modulation of G protein-coupled receptors. *Curr. Opin. Drug Discovery Dev.* **2002**, *5*, 749−755.
(2) Christopoulos, A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 198−210.
(3) Christopoulos, A.; Kenakin, T. Protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* **2002**, *5*, 323−374.
(4) Fredholm, B. B.; IJzerman, A. P.; Jacobson, K. A.; Klotz, K.-N.; Linden, J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* **2001**, *53*, 527−552.
(5) Bruns, R. F.; Ferguson, J. H. Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol. Pharmacol.* **1990**, *38*, 939−949.
(6) Van der Klein, P. A. M.; Kourounakis, A. P.; IJzerman, A. P. Allosteric Modulation of the adenosine A_1 receptor. synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding. *J. Med. Chem.* **1999**, *42*, 3629−3636.
(7) Tranberg, C. E.; Zickgraf, A.; Ciunta, B. N.; Luetjens, H.; Figler, H.; Murphree, L. J.; Falke, R.; Fleischer, H.; Linden, J.; Scammells, P. J.; Olsson, R. A. 2-Amino-3-aroyl-4,5-alkylthiophenes: Agonist allosteric enhancers at human A_1 adenosine receptors. *J. Med. Chem.* **2002**, *45*, 382−389.
(8) Baraldi, P. G.; Zain, A. N.; Lampronti, I.; Fruttarolo, F.; Pavani, M. G.; Tabrizi, M. A.; Shryock, J. C.; Leung, E.; Romagnoli, R. Synthesis and biological effects of a new series of 2-amino-3-benzoylthiophenes as allosteric enhancers of A_1-adenosine receptor. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1953−1957.
(9) Bhattacharya, S.; Linden, J. The allosteric enhancer, PD81, 723, stabilizes human A_1 adenosine receptor coupling to G proteins. *Biochim. Biophys. Acta Mol. Cell Res.* **1995**, *1265*, 15−21.
(10) Kollias-Baker, C.; Ruble, J.; Dennis, D.; Bruns, R. F.; Linden, J.; Belardinelli, L. Allosteric enhancer PD81,723 acts by novel mechanism to potentiate cardiac actions of adenosine. *Circ. Res.* **1994**, *7*, 961−971.
(11) Fawzi, A. B.; MacDonald, D.; Benbow, L. L.; Smith-Torhan, A.; Zhang, H.; Weig B. C.; Ho, G.; Tulshian, D.; Linder, M. E.; Graziano, M. P. SCH-202676: an allosteric modulator of both agonist and antagonist binding to G protein-coupled receptors. *Mol. Pharmacol.* **2001**, *59*, 30−37.
(12) (a) Goerdeler, J.; Eggers, W. Herstellung von (*N*-Alkylbenzimidoyl)- und (*N*-Arylbenzimidoyl)carbodiimiden; ihre Umlagerung zu Aminochinazolinen und Dihydro-1,3,5-triazinen. *Chem. Ber.* **1986**, *119*, 3737−3748. (b) Goerdeler, J.; Haag, J.; Löbach, W. Über die Zerfallsprodukte von Imino-Δ^3-1,2,4-thiadiazolinen. *Chem. Ber.* **1979**, *112*, 1288−1296. (c) Goerdeler, J.; Löbach, W. Reaktionen von 5-Imino-Δ^3-1,2,4-thiadiazolinen mit Heteromoleculen (Präparative Gesichtspunkte). *Chem. Ber.* **1979**, *112*, 517−531.
(13) Von Braun, J.; Pinkernelle, W. Thionylchlorid als bequemer Ersatz für Phosphorpentachlorid im Gebiet der Imidchloride. *Berichten* **1934**, *67*, 1218−1220.
(14) Nikonova, I. V.; Koldobskii, G. I.; Zhivich, A. B.; Ostrovskii, V. A. Preparation and properties of 2,3-diphenyl-5-alkyltetrazolium salts. *J. Gen. Chem. USSR* **1991**, *61*, 1951−1957.
(15) Gao, Z. G.; IJzerman, A. P. Allosteric modulation of adenosine A_{2A} receptors by amiloride analogues and sodium ions. *Biochem. Pharmacol.* **2000**, *60*, 669−676.

(16) Tuček, S. Is the R and R* dichotomy real? *TiPS* **1997**, *18*, 414−416.
(17) Samama, P.; Cotecchia, S.; Costa, T.; Lefkowitz, R. J. A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.* **1993**, *268*, 4625−4636.
(18) Hall, D. A. Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of two-state model of receptor activation. *Mol. Pharmacol.* **2000**, *58*, 1412−1423.
(19) Proska, J.; Tuček, S. Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors. *Mol. Pharmacol.* **1994**, *45*, 709−717.

JM030863D