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Original article

Synthesis and evaluation of 7-amino-2-(2(3)-furyl)-5-phenylethylamino-oxazolo[5,4-*d*]pyrimidines as potential A_{2A} adenosine receptor antagonists for positron emission tomography (PET)

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Abstract

The brain A_{2A} adenosine receptor (A_{2A}AR) participates with the dopamine D_2 receptor in the control of movement and also might influence behavior. Because PET is an important tool for studying the roles of receptors in disease, a ligand for imaging the brain A_{2A}AR is desirable. This report describes the synthesis and A_{2A}AR antagonist activities of a panel of phenyl-substituted 7-amino-2-(2-furyl)-5-phenylethylamino-oxazolo [5,4-d]pyrimidines, **11aa**–**af**, and their 3-furyl congeners, **11ba**–**bd**. In competitive binding studies all compounds displaced [^{3}H]CGS21680 from the A_{2A}AR with K_i values of 14–33 nM with selectivity for the A_{2A}AR over the A_1AR of 5- to 94-fold. Autoradiography of brain sections showed a high level of unspecific binding that obscured specific binding. Thus, these compounds are not promising PET ligands.

Keywords: A_{2A} adenosine receptor; PET; Antagonist; Oxazolo[5,4-*d*]pyrimidines

1. Introduction

Central nervous system adenosine receptors play important roles in neurotransmission. The adenosine A_1 receptor (A_1AR) is widely distributed in the human brain, the highest densities being in the cerebral cortex, hippocampus, thalamus and striatum. This receptor is strongly neuromodulatory [1], and may initiate the neuroprotective effects of ischemic preconditioning [2]. In comparison autoradiographic studies show that the distribution of the A_{2A}AR is more restricted, the highest densities being in the neurons of the striatum, nucleus accumbens and olfactory tubercle [3]. Many of these neurons coexpress the A_{2A}AR and the D_2 dopaminergic receptor [4]; their reciprocal actions are important in the control of movement. A_{2A}ARs with lower densities in the hippocampus and amygdala may yet importantly affect behavior and may play a role in the pathogenesis of conditions such as attention deficit hyperactivity syndrome, panic disorders and schizophrenia [5].

Various modes of neuroimaging are revealing important new information about the roles of various brain receptors in health and disease. One, positron emission tomography (PET), provides quantitative information about the distribution and density of receptors and how diseases may alter those characteristics [6]. Several A_{2A}AR antagonists have been radiolabeled with carbon-11, but rigorous proof that they were binding specifically to brain A_{2A}ARs is lacking [7]. The present study continues the search for A_{2A}AR ligands for brain imaging that meet several nuclear medicine, pharmacological and radiochemical requirements. The ligand should bind specifically to the A_{2A}AR with high affinity and selectivity. It should penetrate the blood-brain barrier to an extent that supports the imaging of brain receptors. Metabolism particularly by brain should be negligible. Since the A_{2A}AR is a G protein-coupled

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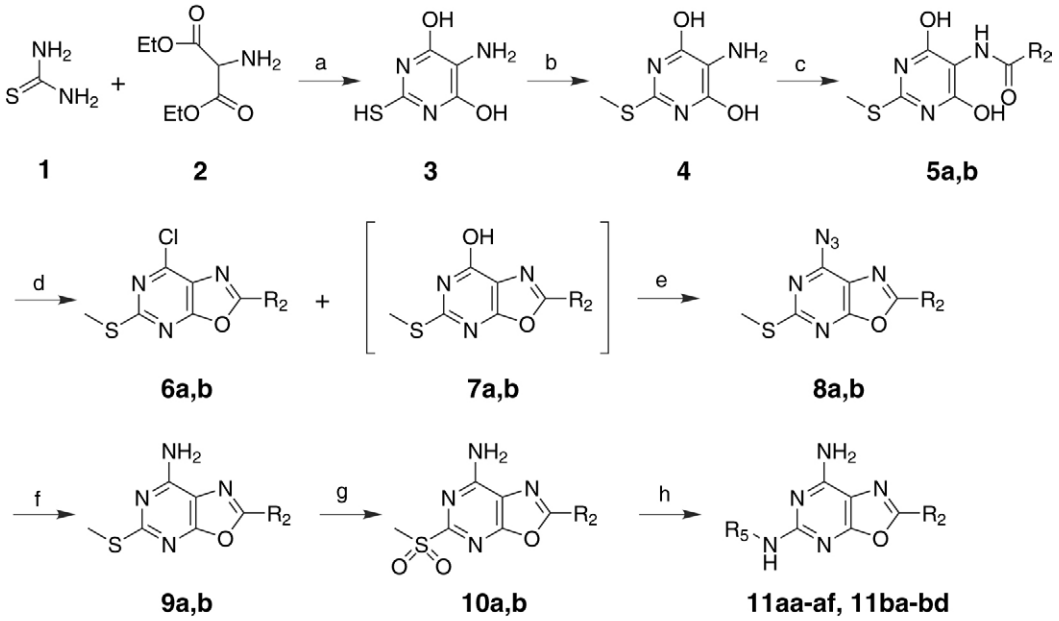
receptor, the ideal radioligand would be an antagonist, because affinity for the receptor would not depend on the degree of receptor-G protein coupling. Because the physical half-life of carbon-11 is so brief ($t_{1/2}$ = 20.4 min) a longer-lived isotope, such as fluorine-18 ($t_{1/2}$ = 109.8 min), that could be dispensed from a central radiopharmacy rather than generated on-site, would be advantageous. Finally, to achieve a high radiochemical yield and in the interest of safety, radiolabeling should occur late in the synthesis, preferably as the final step.

The known A_{2A}AR antagonists are 6:5 bicyclic or 5:6:5 triciyclic heterocycles [8]. CGS15493, the first of the non-xanthine antagonists [9], served as a template for more potent and selective A_{2A}AR antagonists, which differed according to the heterocycle but retained the key exocyclic amino and furyl substituents (or a phenyl group [10]). Although some of those "second generation" antagonists had affinities in the low picomolar range, solubility and bioavailability remained problems which the introduction of hydrophilic groups in the exocyclic substituents did not improve [11]. Our previous work [12] on 6-substituted 4-amino-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalones [10] identified several compounds with good affinity for the A_{2A}AR, but these, too, were poorly water soluble. Accordingly, it seems reasonable that the poor solubility of such molecules owes to the heterocyclic bases themselves, and that the design of new antagonists should consider alternative bases. A patent application [13] described the synthesis of some furyl-substituted purines, oxazolopyrimidines and pteridines. Although these compounds were said to antagonize adenosine, the patent contained little data to support that claim. Nonetheless, we decided to use one class of heterocycles embodied in that patent, the oxazolo[5,4-*d*]pyrimidines, as a lead for developing new A_{2A}AR antagonists potentially suitable for PET.

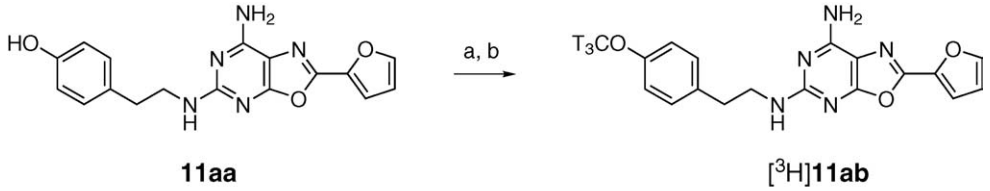
2. Chemistry

Scheme 1 shows the synthetic pathway to the target compounds. The condensation [14] of thiourea, **1**, with diethyl 2-aminomalonate, **2**, generated the starting material, 5-amino-4,6-dioxo-2-methylmercaptopyrimidine, **3**. Alkylation with methyl iodide protected the mercapto group, permitting chemoselective acylation of the 5-amino group of **4** with either 2- or 3-furoyl chloride, forming amides **5a** and **5b**, respectively. Treatment with $POCl_3$ cyclized the amides to form a mixture consisting of about 90–95% of the 7-chlorooxazolo-pyrimidines **6a** and **6b** and 5–10% of 7-oxo-oxazolopyrimidines **7a** and **7b**. This suggests that cyclization of **5a** and **5b** proceeded quantitatively and the succeeding chlorination to an extent of 90–95%. The introduction of the 7-amino group consisted of reacting **6a** and **6b** with NaN_3 and then reducing the 7-azido group of **8a** and **8b** with $SnCl_2$ to obtain **9a** and **9b**. Perborate oxidation [15] of **9a** and **9b** converted the methylmercapto group to a sulfone, a better leaving group for displacement by nucleophiles. The aminolysis of the sulfone with arylethylamines required careful control of the reaction conditions, namely, a 4:1 ratio of amine to **10a**/**b**, 20 ml of acetonitrile/mmol of sulfone and a reaction temperature of 110 °C. Deviations from those parameters led to side reactions, primarily degradative ones. The type of aryl substituent greatly influenced the time required for the reaction to go to completion, which ranged between 24 and 96 hours. The reaction sequence out-



a, $NaOC_2H_5$; b, CH_3I / NaOH; c, R_2COCl, R_2 = 2-furyl (aa-af series), or 3-furyl (ba-bd series); d, $POCl_3$; e, NaN_3 / DMF; f, $SnCl_2$; g, $NaBO_3$; h, R_5-NH_2, R_5 = $Ar(CH_2)_2$-.

Scheme 1. Synthesis of oxazolopyrimidines **11aa**–**af** and **11ba**–**bd**.



a, KOH / DMSO; b, CT_3I.

Scheme 2. Tritiation of **11aa**; synthesis of [^{3}H]**11ab**.

lined above resulted in the formation of 7-amino-2-(2-furyl)-5-phenylethylamino-oxazolo[5,4-*d*]pyrimidines, **11aa**–**af** (a-series), and 7-amino-2-(3-furyl)-5-phenylethylamino-oxazolo[5,4-*d*]pyrimidines, **11ba**–**bd** (b-series) in acceptable overall yields.

For the pharmacological evaluation of these compounds derivative **11ab** was radiolabeled with tritium (Scheme 2) by reacting the potassium salt of **11aa**, formed in situ using KOH in DMSO, with CT_3I.

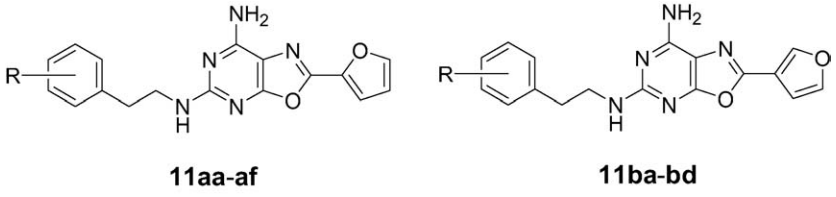
3. Results and discussion

In the literature synthesis [13] the target compounds were generated in four steps, beginning with the condensation of methyl isothiouronium sulfate with 2-(2-furanecarbamoyl)-malonic acid to give 4,6-dihydroxy-5-(2-furoylamino)-2-methylmercaptopyrimidine, **5a**, in one step. Displacement of the methylmercapto group by a 2-arylethylamine followed by simultaneous chlorination/cyclization with $POCl_3$ generated a 5-(2-arylethylamino)-7-chloro-2-(2-furyl)oxazolo[5,4-d]pyrimidine which on reaction with ammonia gave the target compound. Even though this route entailed fewer steps (4 vs. 8 described above) it was not ideal for our purposes for two reasons. First, owing to yields of <20% in the first two steps, overall yields of the literature synthesis were in the range of 1%. Second, a major aim of no-carrier-added (n.c.a.) radiosyntheses is the introduction of the radiolabel at the latest step possible to increase radiochemical yield and promote radiation safety. In this example, the insertion of the arylethylamino group, a favored site for radiolabeling, would occur in the second step, which is early in the synthesis and, moreover, is one of the two low-yield steps. Our synthetic strategy was based on the formation of sulfones **10a** and **10b**, versatile intermediates for nucleophilic displacement reactions. Thus, aminolysis of **10a** and **10b** with various differently substituted phenylethylamines gave the target compounds **11aa**–**af** and **11ba**–**bd** with yields ranging from 35% to 60%.

For the pharmacological evaluation of the synthesized oxazolopyrimidines compound **11aa** was tritiated with commercially available CT_3I under basic conditions. [^{3}H]**11ab** was obtained with a radiochemical yield of 32%, a radiochemical purity exceeding 98% and a specific radioactivity of 3.1 GBq/μmol corresponding to that of the CT_3I used.

In equilibrium binding studies using [^{3}H]**11ab** unspecific binding was very high, accounting for about 95% of total binding, thus making accurate measurements of a K_D value impossible. Tables 1 and 2 summarize the results of competition assays and AR subtype selectivity of **11aa**–**af** and **12ba**–**bd** in

Table 1
A_1 and A_{2A}AR antagonist affinity, selectivity and hydrophobicity factors of compounds **11aa**–**af** and **11ba**–**bd**



Cpd.	R	A_1AR[a]	A_{2A}Ar[b]	A_{2A}/A_1	log *k'w*
11aa	4-OH	360 (165–760)	18 (12–27)	20	3.47
11ab	4-OMe	1570 (120–2080)	22 (19–27)	71	3.50
11ac	3-OMe, 4-OH	1700 (440–5155)	25 (20–30	68	2.97
11ad	3,4-OMe	1270 (465–3470)	14 (8–24)	91	3.81
11ae	3-OMe	440 (265–735)	17 (10–28)	26	4.08
11af	2-OMe	410 (145–1170)	30 (11–78)	14	3.27
11ba	4-OH	340 (37–3040)	29 (22–37)	12	3.53
11bb	4-Ome	435 (95–1965)	33 (26–43)	13	4.20
11bc	3,4-Ome	170 (22–1670)	32 (25–41)	5	3.53
11bd	3-Ome	250 (90–670)	21 (15–28)	12	3.89

[a] K_i (mean and 95% CL) vs. [^{3}H]CPDPX, 2.5 nM.
[b] K_i (mean and 95% CL) vs. [^{3}H]CGS21680, 5 nM.

Table 2

Elemental analyses	
2-Furyl series	
11aa, $C_{17}H_{15}N_5O_3$, Mol. Wt.: 337,33	
calc.: C, 60,53; H, 4,48; N, 20,76;	Found: C, 60,48; H, 4,43; N, 20,84;
11ab, $C_{18}H_{17}N_5O_3$, Mol. Wt.: 351,36	
calc.: C, 61,53; H, 4,88; N, 19,93;	Found: C, 61,57; H, 4,92; N, 19,79;
11ac, $C_{18}H_{17}N_5O_4$, Mol. Wt.: 367,36	
calc.: C, 58,85; H, 4,66; N, 19,06;	Found: C, 58,77; H, 4,62; N, 19,17;
11ad, $C_{18}H_{17}N_5O_3$, Mol. Wt.: 351,36	
calc.: C, 61,53; H, 4,88; N, 19,93;	Found: C, 61,61; H, 4,84; N, 20,01;
11ae, $C_{18}H_{17}N_5O_3$, Mol. Wt.: 351,36	
calc.: C, 61,53; H, 4,88; N, 19,93;	Found: C, 61,50; H, 4,98; N, 19,99;
11af, $C_{19}H_{19}N_5O_4$, Mol. Wt.: 381,39	
calc.: C, 59,84; H, 5,02; N, 18,36;	Found: C, 59,88; H, 4,96; N, 18,47;
3-Furyl series	
11ba, $C_{17}H_{15}N_5O_3$, Mol. Wt.: 337,33	
calc.: C, 60,53; H, 4,48; N, 20,76;	Found: C, 60,54; H, 4,41; N, 20,87;
11bb, $C_{18}H_{17}N_5O_3$, Mol. Wt.: 351,36	
calc.: C, 61,53; H, 4,88; N, 19,93;	Found: C, 61,44; H, 4,96; N, 19,86;
11bc, $C_{18}H_{17}N_5O_3$, Mol. Wt.: 351,36	
calc.: C, 61,53; H, 4,88; N, 19,93;	Found: C, 61,51; H, 4,94; N, 20,04;
11bd, $C_{19}H_{19}N_5O_4$, Mol. Wt.: 381,39	
calc.: C, 59,84; H, 5,02; N, 18,36;	Found: C, 59,91; H, 4,94; N, 18,42;

pig brain membranes. All compounds antagonized binding of [^{3}H]CGS 21680 to the A_{2A}AR with K_i values between 14 and 33 nM; whether the 2-substituent was a 2- or a 3-furyl group was unimportant. The K_i values for antagonism of binding of [^{3}H]CPDPX to A_1ARs were between 170 and 1700 nM, and, because the K_i values for inhibition at the A_{2A}AR varied over a relatively narrow range, affinity for the A_1AR importantly influenced selectivity. Selectivity for the A_{2A}AR over the A_1AR was > 50-fold for compounds **11ab**, **11ac** and **11ad**. Although all contained either a 3- and/or a 4-methoxyphenyl group, these results did not yield unambiguous structure-activity relations. The selectivity of both **11ab** (4-methoxy) and **11ad** (3-methoxy) were >50-fold, but that of **11af** (3,4-dimethoxy) was only 14-fold. By contrast, the selectivity of the 3-furyl derivatives **11ba**–**bd** were rather low, suggesting that the phenyl substituents are not important for selectivity.

Tables 1 and 2 also include measurements of a hydrophobicity parameter (*k'w*). Log k'w varied over a rather narrow range (2.9–4.2) and was unrelated to K_i at either receptor, evidence that hydrophobicity was also not an important determinant of affinity.

In vitro autoradiographic studies showed that neither ligands selective for the A_1AR nor for the A_{2A}AR displaced [^{3}H]**11ab** bound to rat brain slices. Thus, the high degree of unspecific binding obscured specific binding.

In summary, the oxazolopyrimidines examined had affinities for the A_{2A}AR in the low-nanomolar range, and some were quite selective over the A_1AR. Despite a high level of unspecific binding, it was possible to demonstrate specific binding to A_{2A}ARs in equilibrium binding assays of pig brain membranes. However, unspecific binding obscured specific binding in in vitro autoradiographic experiments. For that reason these oxazolopyrimidines are not suitable candidates for imaging brain A_{2A}ARs by PET.

4. Experimental section

General methods. Melting points were measured on an Electrothermal™ apparatus and are uncorrected. The "Zentralabteilung für chemische Analysen" at the Forschungszentrum Juelich performed the elemental analyses, which were within ± 0.4% of the calculated composition. Thin layer chromatography (TLC) employed precoated silica sheets (4 × 8 cm, Polygram™, Macherey-Nagel, Düren) developed with ethyl acetate/hexane: 90:10 (v/v). Mass spectra (MS), ESI (positive), were obtained on a Finnigan Automass III mass spectrometer (Thermo Quest, Dreieich). ^{1}H and ^{13}C NMR spectra were obtained at 200.13 and 50.32 MHz, respectively, by means of a Bruker DPX-200 spectrometer (Avance 200) in ≈ 5% solution in DMSO-d_6 at 25 °C. Chemical shifts are given in δ ppm using the residual proton signal of DMSO-d_6 at 2.52 ppm as a reference. The multiplicity symbols s, d, t and m refer to singlet, doublet, triplet and multiplet, respectively.

Solvents and reagents, which were of the highest grade available, were from Sigma-Aldrich, Deisenhofen, Germany, or Lancaster Synthesis, Muelheim am Main, Germany. N,N-Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were distilled under argon and stored in lightproof containers over 4 Å molecular sieves.

4.1. Chemistry

4.1.1. 5-Amino-2-mercaptopyrimidine-4,6-diol (3) [14]

With exclusion of moisture thiourea (50 g, 657 mmol) and diethyl aminomalonate hydrochloride (100 g, 473 mmol) were added to a mechanically stirred solution of sodium ethoxide formed from sodium (25 g, 1.087 mol) in absolute ethanol (1.5 l). A thick orange paste formed immediately; this was heated for 3 hours at reflux, cooled to room temperature and the solid was collected by filtration through Whatman No. 2 paper. Dissolving the solid material in a minimum amount of water (~ 3 l) and decantation removed traces of insoluble impurities. Acidification with conc. HCl precipitated an orange crystalline solid that was filtered through Whatman No. 2 paper and washed successively with water, ethanol and diethylether. Drying gave **3** (66 g, 88%), m.p. > 300 °C. MS (*m/z*) 160.1 $[M + H]^+$.

4.1.2. 5-Amino-2-methylmercaptopyrimidine-4,6-diol (4) [14]

Iodomethane (66 g, 29 ml, 465 mmol) was added slowly to a solution of **3** (64 g, 407 mmol) in 400 ml 10% NaOH. The solution was stirred for 1 hour at room temperature and then acidified with glacial acetic acid. The precipitated product was collected, washed with water and dried to give 56 g (73%) of solid material, m.p. > 300 °C. ^{1}H NMR: 2.43 (s, 3H, SCH_3), 6.15 (br s, 4H, OH, NH_2). MS (*m/z*): 176.2 $[M + H]^+$.

4.1.3. Furan-2-carboxylic acid-(4,6-dihydroxy-2-methylsulfanyl-pyrimidin-5-yl)-amide (5a)

The addition of 2-furoyl chloride (14.2 g, 10.8 ml, 110 mmol) to an ice-cold solution of **4** (17.3 g, 100 mmol) in

1 N NaOH (250 ml) was followed by stirring for 1 hour at room temperature. The mixture was carefully acidified with conc. HCl and stirred for another 30 min. The solid product was collected by filtration and washed with water, EtOH, and diethylether. After drying the yield was 21 g (78%), m.p. 299–300 °C, darkens at 285 °C. ^{1}H NMR: 2.51 (s, 3H, SCH_3), 6.64 (s, 1H, furyl-*H*-4), 7.23 (s, 1H, furyl-*H*-3), 7.86 (s, 1H, furyl-*H*-5), 9.06 (s, 1H, N*H*), 12.11 (s_{br}, 2H, O*H*). ^{13}C NMR: 13.83 (S*C*H$_3$), 112.73 (furyl-C_4), 114.90 (furyl-C_3), 132.33 (C_5), 141.27 (C_2), 146.09 (furyl-C_5), 148.62 (furyl-C_2), 157.81 (*C*-OH), 160.76 (*C*-OH), 163.81 (*C*=O). MS (*m/z*): 268.1 [M + H]$^+$.

4.1.4. 7-Chloro-2-furan-2-yl-5-methylsulfanyl-oxazolo[5,4-d] pyrimidine (**6a**)

A suspension of **5a** (13.35 g, 50 mmol) in $POCl_3$ (100 ml) was stirred for 3 hours at 120 °C. Excess $POCl_3$ was distilled off (oil bath 140 °C) in vacuo, the residue azeotroped twice with toluene (25 ml), poured into ice/water (100 ml) and stirred vigorously for 30 min. The solid product was dissolved in refluxing ethanol (~ 350 ml) for 15–20 min and filtered to remove insoluble impurities. Upon cooling **6a**, contaminated with some **7a**, precipitated. The solid was filtered off, air-dried and used for the next step without further purification. Yield 19.6 g, 73%.

4.1.5. 7-Azido-2-furan-2-yl-5-methylsulfanyl-oxazolo[5,4-d] pyrimidine (**8a**)

Sodium azide (3 g, 46 mmol) was added to a solution of crude **6a**/**7a** (11.2 g, 42 mmol) in dry DMF (100 ml). After stirring for a few minutes at room temperature solid NaCl began to precipitate and after 12 h the reaction mixture was centrifuged and the supernatant was decanted into water (150 ml). The precipitated product was collected by filtration (**7a**, carried over from the previous step, remained in solution), dissolved in a minimum volume of boiling isopropanol, filtered while hot and cooled to give **8a**, which was pure by TLC. Yield 10.97 g, 95%, m.p. 151–2 °C. ^{1}H NMR: 2.66 (s, 3H, SCH_3), 6.64 (q, 1H, furyl-*H*-4), 7.35 (dd, 1H, furyl-*H*-3), 7.71 (dd, 1H, furyl-*H*-5), ^{13}C NMR: 15.08 (S*C*H$_3$), 113.08 (furyl-C_4), 116.8 (furyl-C_3), 118.92 (C_8), 141.56 (C_9), 147.28 (furyl-C_5), 153.04 (C_2), 153.37 (furyl-C_2), 168.81 ($C_7 + C_5$). MS (*m/z*): 275.2 [M + H]$^+$.

4.1.6. 7-Amino-2-furan-2-yl-5-methylsulfanyl-oxazolo[5,4-d] pyrimidine (**9a**)

The slow addition of **8a** (5.49 g, 20 mmol) in acetone (400 ml) to a solution of $SnCl_2 \times 2\ H_2O$ (9 g, 40 mmol) in MeOH (50 ml) caused immediate gas evolution. After the addition the mixture was heated to 50 °C and stirred for an additional 2 h, concentrated in vacuo to ~ 50 ml, made alkaline with conc. NH_4OH (100 ml) and extracted twice with THF (100 ml). Drying of the extracts over anhydrous Na_2SO_4, filtration and rotary evaporation gave crude **9a** as a yellow solid that was recrystallized from EtOH. Yield 4.2 g, 85%, m.p. 189–90 °C. R_{fazide}: 0.71, R_{famine}: 0.61. ^{1}H NMR: 2.62 (s, 3H, SCH_3), 5.92 (s_{br}, 2H, NH_2), 6.65 (q, 1H, furyl-*H*-4), 7.24 (dd, 1H, furyl-*H*-3), 7.69 (dd, 1H, furyl-*H*-5), ^{13}C NMR: 14.88 (S*C*H$_3$), 112.81 (furyl-C_4), 114.97 (furyl-C_3), 118.01 (C_8), 142.24 (C_9), 146.42 (furyl-C_5), 151.24 (C_2), 155.34 (furyl-C_2), 168.89 ($C_7 + C_5$). MS (*m/z*): 249.2 [M + H]$^+$.

4.1.7. 7-Amino-2-furan-2-yl-5-methylsulfonyl-oxazolo[5,4-d] pyrimidine (**10a**)

Compound **9a** (2.48 g, 10 mmol) was added in one portion to a well-stirred suspension of $NaBO_3 \times H_2O$ (5 g, 55 mmol) in glacial acetic acid (100 ml) preheated to 65 °C (oil bath temperature). The yellow suspension was stirred for 3 h at that temperature, diluted with water (300 ml) and the title compound was collected by filtration (Whatman type 2 paper, no vacuum initially). The sulfone was obtained as pale yellow solid, which was oven dried at 120 °C for 1 hour and kept in a desiccator. Yield 2.72 g (97%) off-white solid, m.p. > 250 °C, darkens at ~ 180 °C. ^{1}H NMR: 3.35 (s, 3H, SO_2CH_3), 6.84 (q, 1H, furyl-*H*-4), 7.47 (dd, 1H, furyl-*H*-3), 8.11 (m, 1H, furyl-*H*-5), 8.53 (s_{br}, 2H, NH_2). ^{13}C NMR: 40.09 (SO_2CH_3), 113.88 (furyl-C_4), 116.69 (furyl-C_3), 118.01 (C_8), 141.74 (C_9), 148.51 (furyl-C_5), 153.31 (C_2), 157.51 (furyl-C_2), 161.39 (C_5), 163.78 (C_7). MS (*m/z*): 282.1 [M + 1]$^+$.

4.1.8. 3-Furoyl chloride [16]

A mixture of 3-furoic acid (33.6 g, 300 mmol) and thionyl chloride (69.3 g, 550 mmol) in 1,2-DCE (350 ml) was refluxed for 12 h. Careful rotary evaporation removed the solvent and excess thionyl chloride; distillation of the residue under reduced pressure gave the acid chloride as a colorless liquid which solidified on standing. Yield 32.5 g, 83%; bp_{50} 62–63 °C (Ref. [16] bp_{47} 65 °C), m.p. 28–29 °C (Ref. [16] m.p. 29 °C).

4.1.9. Furan-3-carboxylic acid-(4,6-dihydroxy-2-methylsulfanyl-pyrimidin-5-yl)-amide (**5b**)

The title amide was prepared as described for **5a**. The yield was 72%, m.p. 191–2 °C, darkens at 175 °C. ^{1}H NMR, δ: 2.53 (s, 3H, –SCH_3), 6.95 (d, 1H, furyl H-4), 7.75 (t, 1H, furyl H-5), 8.28 (s, 1H, furyl H-2), 8.96 (s, 1H, –N*H*), 11.98 (br s, 2H, –O*H*). ^{13}C NMR, δ: 13.81 (–S*C*H$_3$), 110.16 (furyl *C*-4), 123.50 (furyl *C*-3), 132.41 (*C*-5), 144.84 (furyl *C*-5), 144.88 (*C*-2) 146.57 (furyl *C*-2), 160.63 (*C*-OH), 161.75 (*C*-OH), 163.83 (*C*=O). MS calc. for $C_{10}H_9N_3O_4S$, molecular weight 267.26: (*m/z*): 268.1 [M + H]$^+$.

4.1.10. 7-Chloro-2-furan-3-yl-5-methylsulfanyl-oxazolo[5,4-d] pyrimidine (**6b**)

This compound was prepared as described for **6a**. Pyrimidine **6b**, contaminated with some hydroxy compound, **7b**, (~ 5%) was obtained in 73% yield and used for the next step without further purification.

*4.1.11. 7-Azido-2-furan-3-yl-5-methylsulfanyl-oxazolo[5,4-d] pyrimidine (**8b**)*

The azide was prepared as described for **8a**. Pure **8b** was obtained in a yield of 91% after recrystallization from 2-propanol. TLC (R_f 0.71), m.p. 142–3 °C (2-propanol). ^{1}H NMR, δ: 2.88 (s, 3H, –SCH_3), 7.08 (q, 1H, furyl H-4), 7.98 (dd, 1H, furyl H-5), 8.78 (dd, 1H, furyl H-2).^{13}C NMR, δ: 15.08 (–SCH_3), 113.08 (furyl *C*-4), 116.8 (furyl *C*-3), 118.92 (*C*-8), 141.56 (*C*-9), 147.28 (furyl *C*-5), 153.04 (*C*-2), 153.37 (furyl *C*-2), 168.81 (*C*-7 and *C*-5). MS calc. for $C_{10}H_6N_6O_2S$, molecular weight 274.26: (*m/z*): 275.1 $[M + H]^+$.

*4.1.12. 7-Amino-2-furan-3-yl-5-methylsulfanyl-oxazolo[5,4-d]pyrimidine (**9b**)*

Compound **9b** was prepared as described for **9a**. The title compound was purified by recrystallization from EtOH followed by trituration of the crystals with ethyl acetate/hexane 90:10 (v/v) to remove traces of impurities. Yield 95%, m.p. 211–3 °C. TLC: R_{fazide}: 0.71, R_{famine}: 0.61. ^{1}H NMR, δ: 2.49 (s, 3H, –SCH_3); 6.98 (q, 1H, furyl H-4); 7.77 (br s, 2H, –NH_2); 7.94 (t, 1H, furyl H-5); 8.55 (q, 1H, furyl H-2). ^{13}C NMR, δ: 14.57 (–SCH_3), 109.18 (furyl *C*-4), 113.43 (*C*-8), 115.32 (furyl C-3) 144.88 (*C*-9), 145.60 (furyl *C*-2), 146.37 (furyl *C*-5), 153.09 (*C*-2), 165.16 (*C*-5), 167.32 (*C*-7). MS calc. for $C_{10}H_8N_4O_2S$, molecular weight 248.26: (*m/z*): 249.2 $[M + H]^+$.

*4.1.13. 7-Amino-2-furan-3-yl-5-methylsulfonyl-oxazolo[5,4-d]pyrimidine (**10b**)*

Compound **10b** was prepared as described for **10a** in a yield of 91% as an off-white solid, m.p. > 250 °C, darkens at ~ 180 °C. ^{1}H NMR, δ: 3.36 (s, 3H, SO_2CH_3), 7.02 (q, 1H, furyl-*H*-4), 7.97 (t, 1H, furyl-*H*-5), 8.45 (s_{br}, 2H, NH_2), 8.63 (q, 1H, furyl-*H*-2). ^{13}C NMR (DMSO-d_6), δ: 40.08 (SO_2CH_3), 109.28 (furyl-$C_{4'}$), 114.93 (C_8), 117.95 (furyl-$C_{3'}$), 146.62 (furyl-$C_{2'}$ and furyl-$C_{5'}$) 156.59 (C_9), 157.30 (C_2), 161.27 (C_5), 163.86 (C_7). MS calc. For $C_{10}H_8N_4O_4S$, molecular weight 280.26: (*m/z*): 281.1 $[M + H]^+$.

*4.1.14. General procedure for the reaction of **10a** with amines*

A suspension of **10a** (280 mg, 1 mmol) in acetonitrile (20 ml) and the appropriate amine (4 mmol) was refluxed gently (oil bath 105–110 °C) for the specified time. During the reaction with solid amines the suspension changed color from off-white to dark yellow or light brown; liquid amines gave clear solutions that turned orange–brown. Workup (Method A or B, vide infra) depended on the type of amine. Ethyl acetate/hexane 50/50 (v/v) was the solvent for TLC of the products.

4.1.14.1. Solid amines, method A. The hot reaction mixture was diluted with water (20 ml) and poured onto crushed ice (~ 100 g). The solid that separated was taken up in ethyl acetate (100 ml), extracted with 0.5 N HCl (50 ml) and the aqueous layer was reextracted with ethyl acetate (30 ml). The pooled organic phases were washed with water (50 ml), brine (50 ml) and dried over anhydrous Na_2SO_4. Filtration and rotary evaporation at 30–35 °C gave the products (almost pure by TLC). Further purification consisted of taking up the products in acetone (~ 10 ml/100 mg) treatment with ultrasound, separation of insoluble material by filtration and rotary evaporation of the solvent at 30–35 °C. Recrystallization from suitable solvents gave analytical samples.

4.1.14.2. Liquid amines, method B. The hot reaction mixture was poured into ice/water (60 ml), the solid that separated was taken up in ethyl acetate (100 ml) and worked up in the same way as the products of solid amines.

*4.1.15. 2-Furan-2-yl-N^5-[2-(4-hydroxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11aa**)*

Sulfone **10a** reacted with 2-(4-hydroxyphenyl)-ethylamine (tyramine, 550 mg) in 48 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$: 0.48. Method A gave a solid yellow residue for recrystallization from ethanol (reflux, rt, freezer), yield 205 mg (61%), m.p. 211–2 °C (ethanol). ^{1}H NMR, δ: 2.51 (t, 2H, -CH_2Ph); 3.42 (m, 2H, CH_2NH-); 6.69 (d, 2H, phenyl H); 6.75 (q, 1H, furyl H-4); 6.94 (br s, –N*H*–); 7.06 (d, H, 2 phenyl H); 7.17 (d, 2H, furyl-H-3) 7.36 (br s, 2H, –NH_2), 7.96 (m, 1H, furyl H-5), 9.19 (br s, 1H, PhO*H*)). ^{13}C NMR, δ: 35.19, 113.23, 113.32, 115.95, 130.40, 130.65, 142.82, 146.70, 156.43, 160.56, 166.64. MS calc for $C_{17}H_{15}N_5O_3$, molecular weight 337.33: (*m/z*) 338.2 $[M + H]^+$.

*4.1.16. 2-Furan-2-yl-N^5-[2-(4-methoxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11ab**)*

Sulfone **10a** reacted with 2-(4-methoxyphenyl)-ethylamine (605 mg) in 24 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$: 0.63. Method B gave a reddish solid that was dissolved in a minimum amount of hot methoxyethanol, cooled and diluted by volume with water. The product precipitated as an orange solid, yield 197 mg (56%), m.p. 182–3 °C (50% methoxyethanol). ^{1}H NMR, δ: 2.75 (t, 2H, PhCH_2–); 3.41 (q_{br}, 2H, –CH_2NH–); 3.72 (s, 3H, –OCH_3); 6.74 (q, 1H, furyl-*H*-4), 6.84 (d_{br}, 3H, 2Ar*H* + N*H*), 7.19 (d_{br}, 3H, 2Ar*H* + furyl-*H*-3); 7.27 (br s, 2H, –NH_2); 7.96 (s, 1H, furyl H-5). ^{13}C NMR, δ: 35.19 (Ph *C*H_2), 43.96 (–NH*C*H_2–), 113.22 (furyl *C*-4), 113.32 (furyl *C*-3), 115.95 (PhC*H*–); 130.39 (PhC*H*–), 130.65 (*C*-8), 142.82 (*C*-9), 146.67 (furyl-C_5), 156.43 (C_2), 160.56 (furyl-C_2), 161.39 (C_5), 163.78 (C_7). MS calc for $C_{18}H_{17}N_5O_3$, molecular weight 351.36: (*m/z*): 352.1 $[M + H]^+$.

*4.1.17. 2-Furan-2-yl-N^5-[2-(4-hydroxy-3-methoxyphenyl)-ethyl]-oxazolo[4,5-d]pyrimidine-5,7-diamine (**11ac**)*

Dissolving the hydrochloride salt of 2-(4-hydroxy-3-methoxyphenyl)-ethylamine (896 mg, 4.4 mmol) in 32% NH_3 (30 ml) liberated the free base which was obtained in ~ 90% yield by extraction with THF (2 × 30 ml), rotary evaporation and azeotropic drying with CH_3CN (30 ml). The reaction of the amine with **10a** required 168 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$:

0.44. The yellowish solid obtained by method B was triturated with hot acetonitrile, yield 132 mg, 36%, m.p. 196–7 °C. ^{1}H NMR, δ: 2.73 (t, 2H, ArCH_2–); 3.45 (q, 2H, –NHCH_2–); 3.77 (s, 3H, OCH_3); 6.75 (m, 5H, –N*H*–, furyl *H*-4 and 3 phenyl H); 7.18 (dd, 1H, furyl *H*-3); 7.25 (br s, 2H, –NH_2); 796, (q, 1H, furyl *H*-5); 8.71, (s, 1H, PhO*H*). ^{13}C NMR, δ: 35.65, 40.10, 43.83, 56.41, 113.07, 113.30, 113.71, 113.90, 116.21, 116.71, 121.67, 131.45, 141.77, 142.90, 145.58, 146.59, 148.25, 157.16, 157.52, 161.08, 161.41, 163.80, 166.65. MS calc for $C_{18}H_{17}N_5O_4$, molecular weight 367.36: (*m*/*z*) 368.2 $[M + H]^+$.

*4.1.18. 2-Furan-2-yl-N^5-[2-(3-methoxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11ad**)*

2-(3-Methoxyphenyl)-ethylamine (605 mg), reacted with **10a** in 72 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$: 0.59. Method B gave an orange–red solid that was triturated with hot acetonitrile, yield 214 mg, 61%, m.p. 166-7 °C. ^{1}H NMR, δ: 2.83 (t, 2H, PhCH_2–); 3.55 (q, 2H, –NHCH_2–); 3.76 (s, 3H, OCH_3); 6.80 (m, 5H, NH_2, furyl *H*-4, 2 phenyl H); 7.22 (m, 4H, –N*H*–, furyl *H*-3, 2 phenyl H); 7.96, (s, 1H, furyl H-5). ^{13}C NMR, δ:, 55.76, 112.27, 113.08, 113.29, 115.20, 121.82, 130.39, 142.31, 142.90, 144.88, 146.59, 157.17, 160.14, 161.05, 166.64. MS calc for $C_{18}H_{17}N_5O_3$, molecular weight 351.36: (*m*/*z*): 352.1 $[M + H]^+$.

*4.1.19. 2-Furan-2-yl-N^5-[2-(2-methoxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11ae**)*

2-(2-Methoxyphenyl)-ethylamine (605 mg), reacted with **10a** in 28 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$: 0.68. The orange solid obtained by method B was triturated with hot acetonitrile, yield 179 mg, 51%, m.p. 164–5 °C. ^{1}H NMR, δ: 2.85 (t, 2H, PhCH_2–); 3.47 (m, 2H, –NHCH_2–); 3.81 (s, 3H OCH_3); 676 (q, 1H, furyl H-4); 6.90 (m, 2H, furyl H-3 and phenyl H); 7.05 (br s, 1H, –N*H*–); 7.21 (m, 3H, phenyl H); 7.49 (br s, 2H, –NH_2); 7.97 (d, 1H, furyl H-5). ^{13}C NMR, δ: 30.42, 42.15, 56.16, 108.74, 111.49, 113.32, 121.11, 128.32, 128.37, 130.93, 142.76, 146.73, 148.14, 158.13, 166.63. MS calc for $C_{18}H_{17}N_5O_3$, molecular weight 351.36: (*m*/*z*): 352.2 $[M + H]^+$.

*4.1.20. N^5-[2-(3,4-dimethoxyphenyl)-ethyl]-2-furan-2-yl-oxazolo[4,5-d]pyrimidine-5,7-diamine (**11af**)*

Liquid 2-(3,4-dimethoxyphenyl)-ethylamine (725 mg), reacted with **10a** in 72 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$: 0.47. Work-up B gave a yellow solid that was triturated with hot acetonitrile, yield 198 mg, 52%, m.p. 165–6 °C. ^{1}H NMR, δ: 2.78 (t, 2H, ArCH_2); 3.47 (m, 2H, –CH_2NH–), 6.75 (q, 1H, furyl H-4), 6.78 (d, 1H, phenyl H), 6.86 (m, 3H, 2 phenyl H and –N*H*-), 7.06 (d, 2H, 2 phenyl H), 7.17 (dd, 2H, furyl H-3) 7.25 (br s, 2H, –NH_2), 7.96 (m, 1H, furyl H-5). ^{13}C NMR, δ: 35.59, 43.71, 56.26, 56.38, 108.77, 112.76, 113.09, 113.30, 113.44, 133.16, 142.89, 146.59, 14782, 148.01, 149.47, 157.14, 161.06, 166.64, MS calc. for $C_{19}H_{19}N_5O_4$, molecular weight 381.39: (*m*/*z*) 382.2 $[M + H]^+$.

*4.1.21. General procedure for the reaction of **10b** with amines*

Gentle reflux (oil bath 105–110 °C) of a mixture of **10b** (280 mg, 1 mmol) in acetonitrile (20 ml) and the appropriate amine (4 mmol) for the specified time gave the target compound. During the course of the reaction the suspension cleared and changed color from light to dark yellow. Refrigeration usually precipitated crude products, which were separated and crystallized from an appropriate solvent. If cooling did not precipitate the product the reaction mixture was poured onto crushed ice/water (~ 100 g). The solid that separated was taken up in ethyl acetate (100 ml), extracted with 0.5 N HCl (50 ml) and the aqueous layer was back-extracted with ethyl acetate (30 ml). The pooled organic phases were washed with water (50 ml), brine (50 ml) and dried over anhydrous Na_2SO_4. Rotary evaporation at 30–35 °C gave the products that, although pure by TLC (silica, eluent acetone/hexane 50:50 (v/v), required further purification, which consisted of taking up the products in acetone (~ 10 ml/100 mg) treatment with ultrasound, separation of insoluble impurities by filtration and rotary evaporation of the solvent at 30–35 °C. Recrystallization from suitable solvents gave analytical samples.

*4.1.22. 2-Furan-3-yl-N^5-[2-(4-hydroxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11ba**)*

The reaction of **10b** with 2-(4-hydroxyphenyl)-ethylamine, (tyramine, 550 mg) required 96 h. $R_{f\ sulfone}$: 0.56, $R_{f\ product}$: 0.36. After ~ 4 h at 105 °C a solid started to precipitate from the initially homogeneous reaction mixture. The hot mixture was filtered and the filtrate set aside; crude product contaminated with a red impurity precipitated slowly. The precipitate was dissolved in acetonitrile (~ 10 ml) and cooled to room temperature. After some hours the clear yellow solution containing the product was decanted from a solid red impurity. Refrigeration caused the slow crystallization of colorless product. Yield 205 mg (61%), m.p. 143–4 °C (CH_3CN). ^{1}H NMR, δ: 2.51 (t, 2H, ArCH_2), 3.42 (q_{br}, 2H, CH_2NH), 6.69 (d, 2H, 2Ar*H*), 6.75 (q, 1H, furyl-*H*-4), 6.94 (s_{br}, N*H*), 7.06 (d, H, 2Ar*H*), 7.17 (d, 2H, furyl-*H*-3) 7.36 (s_{br}, 2H, NH_2), 7.96 (m, 1H, furyl-*H*-5), 9.19 (s_{br}, 1H, O*H*)). ^{13}C NMR δ: 35.31, 42.91, 109.14, 113.99, 115.64, 130.41, 133.01, 144.27, 146.11, 150.67, 157.14, 158.36, 161.02, 166.84. MS calc for $C_{18}H_{17}N_5O_4$, molecular weight 337.33: (*m*/*z*) 338.1 $[M + H]^+$.

*4.1.23. 2-Furan-3-yl-N^5-[2-(4-methoxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11bb**)*

The reaction of **10b** with liquid 2-(4-methoxyphenyl)-ethylamine (605 mg) required 96 h. $R_{f\ sulfone}$: 0.54, $R_{f\ product}$: 0.61. The reddish solid was triturated with hot acetonitrile and cooled in the refrigerator. Product was filtered off and air dried, yield 197 mg (56%), off-white solid, m.p. 187–8 °C. ^{1}H NMR, δ: 2.82 (t, 2H, -CH_2Ph); 3.43 (m, 2H, –NHCH_2–); 3.73 (s, 3H, –OCH_3); 665 (m, 5H, 3 phenyl H, –N*H*– and furyl H); 7.18 (m, 4H, –NH_2 and 2 phenyl H); 7.90 (m, 1H,

furyl H); 8.43 (m, 1H, furyl H). ^{13}C NMR, δ: 35.18, 43.87, 55.82, 109.06, 114.58, 115.87, 130.48, 132.61, 144.32, 146.06, 15.043, 156.91, 158.45, 160.92, 166.80. MS calc for $C_{18}H_{17}N_5O_3$, molecular weight 351.36: (*m/z*): 352.2 $[M + H]^+$.

4.1.24. 2-Furan-3-yl-N^5-[2-(3-methoxyphenyl)-ethyl]-oxazolo [4,5-d]pyrimidine-5,7-diamine (**11bc**)

The reaction of **10b** with 2-(3-methoxyphenyl)-ethylamine (605 mg) required 96 h. $R_{f\ sulfone}$: 0.56, $R_{f\ product}$: 0.59. The orange–red solid that crystallized from the reaction mixture was recrystallized from acetonitrile, yield 155 mg, 44%, m. p. 146–7 °C, off-white crystals. ^{1}H NMR, δ: 2.87, (t, 2H, C*H*$_2$-Ph); 3.45 (m, 2H, –NHC*H*$_2$–); 3.75 (s, 3H, –OC*H*3); 6.73 (m, 3H, 1 phenyl H, –N*H*– and furyl H); 7.17 (m, 5H, –N*H*$_2$ and 3 phenyl H); 7.88 (m, 1H, furyl H); 8.43 (M, 1H, furyl H). ^{13}C NMR, δ: 34.91, 42.14, 108.92, 111.72, 114.24, 115.72, 120.12, 129.96, 130.11, 144.31, 146.34, 149.17, 151.02, 156.93, 158.41, 160.99, 161.43, 166.82. MS calc for $C_{18}H_{17}N_5O_3$, molecular weight 351.36: (*m/z*): 352.1 $[M + H]^+$.

4.1.25. N^5-[2-(3,4-Dimethoxyphenyl)-ethyl]-2-furan-3-yl-oxazolo[4,5-d]pyrimidine-5,7-diamine (**11bd**)

The reaction of **10b** with 2-(3,4-dimethoxyphenyl)-ethylamine (725 mg) required 72 h. $R_{f\ sulfone}$: 0.56, $R_{f\ product}$: 0.54. The yellow solid was triturated with acetonitrile, yield 210 mg, 55%, m.p. 161–3 °C. ^{1}H NMR δ: 2.78 (t, 2H, –C*H*$_2$Ph), 3.47 (m, 2H, –C*H*$_2$NH–), 6.75 (q, 5H, –N*H*–, furyl H and 3 phenyl H); 7.21 (br s, 2H –N*H*$_2$); 7.90 (m, 1H, furyl H); 8.43 (m, 1H, furyl H). ^{13}C NMR, δ: 35.60, 43.66, 56.28, 56.39, 108.79, 109.06, 112.79, 113.48, 115.83, 121.37, 133.17, 144.36, 146.08, 148.04, 149.49, 150.54, 156.71, 160.69, 166.78. MS calc. For $C_{19}H_{19}N_5O_4$, molecular weight 381.39: (*m/z*) 382.2 $[M + H]^+$.

4.1.26. Radiosynthesis of [^{3}H]**11ab**

CT_3I (85 Ci/mmol, 1 mCi/μl in toluene) was from Amersham Bioscience. The labeling reaction was performed in a conical 1 ml glass vial sealed with a Teflon coated rubber septum and containing a magnetic stirring bar.

The precursor **11aa** (1.7 mg, 5 μmol) was added to a stirred suspension of powdered KOH (1.1 mg, 20 μmol) in dry DMSO (250 μl) and the mixture was stirred at ambient temperature for 1 h. [^{3}H]CH_3I (10 mCi, 10 μl of the toluene stock solution in 240 μl dry DMSO) was added, the mixture was stirred at ambient temperature for 30 min and the reaction stopped by the addition of eluent (250 μl). The product was isolated by semipreparative HPLC, the eluent was evaporated in vacuo at 40 °C and the residue dried by azeotroping with MeCN (2 × 5 ml). For in vitro studies the tritiated compound was taken up in DMSO. Radiochemical yield for [^{3}H]**11ab** was 32%, the radiochemical purity exceeded 98% and the specific activity corresponded to that of the CT_3I used.

Semipreparative HPLC: 2 Columns in series of Multospher 120, RP-18, 5 μm, 250 × 8 mm. Eluent: MeCN/H_2O 50:50 (v/v) + 0.1% Et_3N. Flow 3.5 ml/min. UV at 260 nm. k'$_{\mathbf{11aa}}$ = 1.45, k'$_{\mathbf{11ab}}$ = 5.35.

Quality control: Column LiChrospher 100, RP-18, 5 μm, 250 × 4 mm. Eluent as above. Flow 1 ml/min. UV as above. k'$_{\mathbf{11aa}}$ = 0.96, k'$_{\mathbf{11ab}}$ = 3.88. For continuous measurement of radioactivity the outlet of the UV detector was connected to an in-line scintillation analyzer (Radiomatic™ 515 TR Series, Packard, Dreieich, Germany) and the recorded data were processed by an integrated software system.

Measurement of the specific radioactivity of the tritiated compounds used HPLC, which measured the mass of product by integrating the UV absorption of the product peak and referenced to a standard curve. The product peak was collected and an aliquot counted in a liquid scintillation counter.

4.2. Radioligand binding assays

Corpora striata (for A_{2A}AR assays) and frontal cortices (for A_1AR assays) were dissected from pig brains and the tissue was homogenized for 1 min in 20 volumes of ice-cold 50 mM Tris–HCl buffer, pH 7.4 containing 10 mM $MgCl_2$, soybean trypsin inhibitor (20 μg/ml), bacitracin (200 μg/ml), and benzamidine HCl (160 μg/ml) by means of an Ultra Turrax at 20,000 rpm. The homogenate was centrifuged at 48,000 × *g* for 10 min at 4 °C (Beckmann Optima L, SW41Ti rotor). The pellet was suspended in 20 volumes of Tris–HCl, pH 7.4, containing adenosine deaminase (2 U/ml) and trypsin inhibitor (20 μg/ml), and then was incubated for 30 min at 37 °C. After centrifugation at 48,000 × *g* for 10 min at 4 °C the pellet was diluted in 20 volumes of 50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$. Aliquots of the homogenate (1 ml) were stored at –80 °C.

The assays were performed in triplicate by incubating aliquots of the membrane fractions (68–87 μg protein per assay) in Tris–HCl, pH 7.4, containing adenosine deaminase (2 U/ml), [^{3}H]CPFPX (2.5 nM) and cortical homogenates for the A_1AR or [^{3}H]CGS21680 (5 nM) and striatal homogenates for A_{2A}AR, in a total assay volume of 200 μl. Incubation was carried out at 20 °C for 90 min. Centrifugation at 48,000 × *g* for 6 min at 8 °C separated bound from free ligand. Supernatants were discarded, the pellets washed with ice cold buffer (1 ml) and dissolved by incubating in Solvable™ (500 μl, Canberra-Packard) for 120 min at 50 °C. Aliquots of 450 μl were placed in scintillation vials with scintillation cocktail (10 ml, Ultima Gold XR, Canberra-Packard). Radioactivity was measured in a Liquid Scintillation Analyzer. Protein estimation was performed with a commercial assay (Bio-Rad DC Protein Assay) after solubilization in 15% NH_4OH containing 2% SDS (w/v); human serum albumin served as a standard. A computer-assisted curve-fitting program (GraphPad Prism, version 3.0) calculated IC_{50}, K_i and K_D.

4.3. Measurement of an index of lipophilicity

A method employing HPLC [17] served for estimates of the lipophilicity of all compounds. This method is based on

measurements of the retention times of the test compounds on a 4.6 × 250 mm column of 5 μm Kromasil RP18 eluted at a rate of 1 ml/min with each of the following proportions (v/v) of methanol/water: 90:10, 85:15, 80:20, 75:25, 70:30 and monitoring of the effluent at 260 nm. Converting each datum to a capacity factor k' by the formula $k' = (t - t_o)/t_o$, where t and t_o are the retention time and void time, respectively, and solving the linear regression of log k' on methanol: water ratio for methanol = 0 gave the lipophilicity parameter log k'w.

4.4. Autoradiography

Frozen rat brains (–20 °C) were sectioned at a thickness of 20 μm in the horizontal plane (Leica AG Microsystems, Germany), mounted on gelatine-coated microscope slides (Laboroptik GmbH, Germany), dried at 4 °C and stored at –80 °C until use. Tissue sections were preincubated for 15 min at 4 °C in 50 mM Tris–HCl, pH 7.4, containing 1 mM $MgCl_2$ and 2 U/ml adenosine deaminase, and then for 90 min at 23 °C in the same buffer containing either 0.9 nM [^{3}H]CPDPX, 13 nM [^{3}H]**11ab** or 2.2 nM [^{3}H]ZM241385, alone or with different concentrations of **11ab**. The sections were washed twice for 5 min in 50 mM Tris–HCl at 4 °C, rinsed in deionized water, dried in a stream of cold air and placed on a phosphor image plate (Fuji, Tokyo, Japan) and exposed for 4 days. Laser scanning of the plates employed a phosphor imager BAS 5000 (Fuji) controlled with software provided by the vendor (Raytest Isotopenmeßgeräte, Straubenhardt, Germany, version 2.11a). The advanced image analyzer program (AIDA 3.10, Raytest Isotopenmeßgeräte) processed data from regions of interest (ROIs). Analysis of binding data arrayed in Microsoft Excel 2000 employed a non-linear, curve-fitting analysis program (GraphPad Prism 3.00, GraphPad Software, Inc., San Diego, CA 92130).

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References

[1] T.V. Dunwiddie, Int. Rev. Neurobiol. 27 (1985) 63–139.
[2] C. Heurteaux, I. Lauritzen, C. Widmann, M. Lazdunski, Proc. Natl. Acad. Sci. USA 92 (1995) 4666–4670.
[3] E. Lindström, E. Ongini, B.B. Fredholm, Naunyn Schmiedebergs Arch. Pharmacol. 354 (1996) 539–541.
[4] B. Johansson, V. Georgiev, B.B. Fredholm, Neuroscience 80 (1997) 1187–1207.
[5] J.-L. Moreau, G. Huber, Brain Res. Rev. 31 (1999) 65–82.
[6] M.E. Phelps, Proc. Natl. Acad. Sci. USA 97 (2000) 9226–9233.
[7] M.H. Holschbach, R.A. Olsson, Curr. Pharm. Des. 8 (2002) 99–110.
[8] B. Cacciari, G. Pastorin, G. Spalluto, Curr. Top. Med. Chem. 3 (2003) 403–411.
[9] J.E. Francis, W.D. Cash, S. Psychoyos, G. Ghai, P. Wenk, R.C. Friedman, C. Atkins, V. Warren, P. Furness, J.L. Huyn, G.A. Stone, M. Desai, M. Williams, J. Med. Chem. 31 (1988) 1014–1020.
[10] V. Colotta, D. Catarzi, F. Varano, L. Cecchi, G. Filacchioni, C. Martini, L. Trincavelli, A. Lucacchini, Arch. Pharm. Med. Chem. 332 (1999) 39–41.
[11] P.G. Baraldi, B. Cacciari, R. Romagnoli, G. Spalluto, A. Monopoli, E. Ongini, K. Varani, P.A. Borea, J. Med. Chem. 45 (2002) 115–126.
[12] M.H. Holschbach, D. Bier, W. Wutz, W. Sihver, M. Schüler, R.A. Olsson, Eur. J. Med. Chem. 40 (2005) 421–437.
[13] M.H. Block, A. Harrison, R.B. Hargreaves, Eur. Pat. Appl., 544,445, 2 Jun 93, Chem. Abst. 119 (Imperial Chemical Industries, PLC) (1993) 160314j.
[14] M.R. Harmden, D.T. Hurst, Aust. J. Chem. 43 (1990) 55–62.
[15] A. McKillop, J.A. Tarbin, Tetrahedron 43 (1987) 1753–1758.
[16] H. Gilman, R.R. Burtner, J. Org. Chem. 55 (1987) 2903–2909.
[17] D.J. Minnick, D.A. Brent, J. Frenz, J. Chromatog 461 (1989) 177–191.