Expedited Articles

Synthesis and Biological Evaluation of the Enantiomers of the Potent and Selective A_1-Adenosine Antagonist 1,3-Dipropyl-8-[2-(5,6-epoxynorbonyl)]-xanthine

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The individual enantiomers **8** and **12** of the potent and highly selective racemic A_1-adenosine antagonist 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)]xanthine (ENX, **4**) were synthesized utilizing asymmetric Diels−Alder cycloadditions for the construction of the norbornane moieties. The absolute configuration of **12** was determined by X-ray crystallography of the 4-bromobenzoate **14**, which was derived from the bridged secondary alcohol **13**. The latter was obtained from **12** by an acid-catalyzed intramolecular rearrangement. The binding affinities of the enantiomers **8** and **12** and the racemate **4** at guinea pig, rat, and cloned human A_1- and A_{2a}-adenosine receptor subtypes were determined. The *S*-enantiomer **12** (CVT-124) appears to be one of the more potent and clearly the most A_1-selective antagonist reported to date, with K_i values of 0.67 and 0.45 nM, respectively, at the rat and cloned human A_1-receptors and with 1800-fold (rat) and 2400-fold (human) subtype selectivity. Both enantiomers, administered intravenously to saline-loaded rats, induced diuresis *via* antagonism of renal A_1-adenosine receptors.

Introduction

Despite extensive efforts over several decades in both academic and industrial laboratories,[1] the only approved adenosinergic agent is the nonselective endogenous agonist adenosine itself, which is used for the treatment and diagnosis of cardiac arrhythmias (Adenocard)[2] and, together with radionuclide imaging, for the diagnosis of ischemic heart disease (Adenoscan).[3] The recent availability of cloned A_1-, A_{2a}-, A_{2b}-, and A_3-adenosine receptors[4] has facilitated the pharmacological screening of ligands for these receptors and has contributed to a new impetus for the search for more potent and selective agonists and antagonists. Among the latter, A_1-selective antagonists related to 8-cycloalkyl-substituted 1,3-dipropylxanthines[5] have recently received particular attention. Representatives of this group are KF-15372 (**1**) and KW-3902 (NAX, **2**, Figure 1), for which diuretic activity and protective effects against acute renal failure have been described.[6,7] In contrast, the chiral oxocyclopentyl derivative KFM 19 (**3**, Figure 1) appears to have therapeutic potential for dementia and related cognitive deficits.[8] Because adenosine receptors are widely distributed both in the vasculature[9] and in the CNS,[10] acute *versus* chronic administration of one of these fairly lipophilic dipropylxanthines could be important in terms of separating peripheral from central effects.[11]

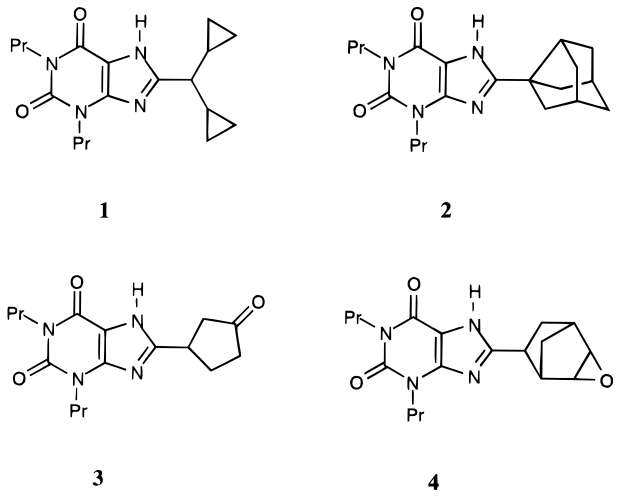

Figure 1. Newer, selective A_1-adenosine antagonists: KF-15372 (**1**), KW-3902 (NAX, **2**), KFM 19 (**3**), and ENX (**4**).

Recently, we described the biological activity of the potent and highly selective A_1-adenosine receptor antagonist 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)]xanthine[12] (ENX, **4**, Figure 1). In this compound, the xanthine ring and the epoxide are respectively situated *endo* and *exo* to the norbornane moiety, and the latter has an asymmetric center at C-2. Due to the marked stereochemical requirements for adenosine receptor affinity exhibited by both agonist and antagonist asymmetric ligands,[13] it became of interest to synthesize the individual enantiomers of **4** and to evaluate their biological activity.

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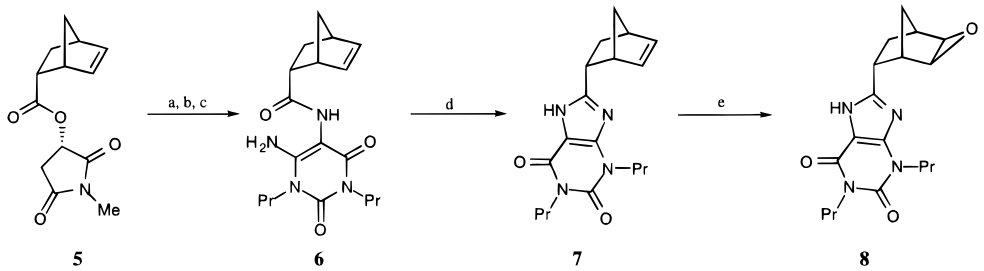
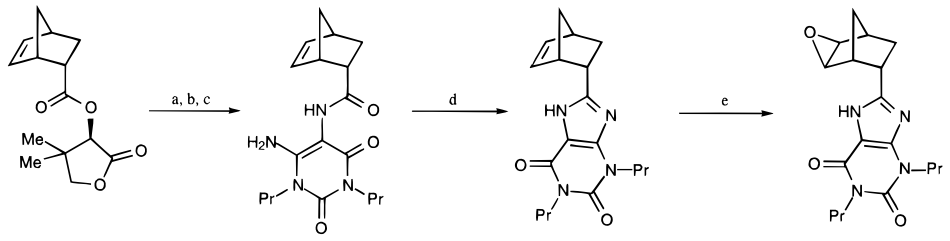
Scheme 1[a]



5 **6** **7** **8**

[a] (a) NaOH; (b) oxalyl chloride; (c) 5,6-diamino-1,3-dipropyluracil, cat. DMAP, pyridine; (d) 2 N NaOH, dioxane; (e) MMPP, aqueous *i*-PrOH.

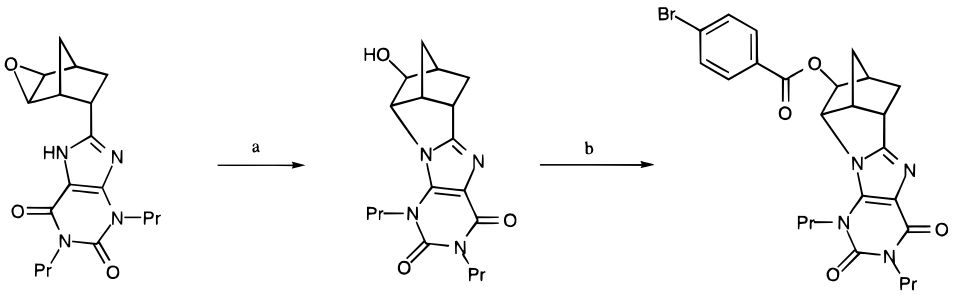
Scheme 2[a]



9 **10** **11** **12**

[a] (a) NaOH; (b) oxalyl chloride; (c) 5,6-diamino-1,3-dipropyluracil, cat. DMAP, pyridine; (d) 2 N NaOH, dioxane; (e) MMPP, aqueous *i*-PrOH.

Scheme 3[a]



12 **13** **14**

[a] (a) 2 N HCl, EtOH; (b) 4-bromobenzoyl chloride, pyridine.

Chemistry

From the many known norbornene syntheses based on asymmetric Diels−Alder cycloadditions with dienophiles containing removable chiral auxiliaries,[14] those utilizing (*S*)-2-hydroxy-*N*-methylsuccinimde[15] and pantolactone[16] were chosen. They provided the required optically active precursors **5** and **9**, respectively, which were treated with aqueous NaOH to remove the chiral auxiliaries (Schemes 1 and 2). The resulting crude carboxylic acids were directly converted into the corresponding acid chlorides, which produced amides **6** and **10** on reaction with 1,3-dipropyluracil-5,6-diamine. Base-induced ring closure provided the *endo*-norbornenyl-substituted xanthines **7** and **11**. The chromatographically distinct *exo*-analog[5c] was not observed, indicating that the cyclization step had occurred without epimerization.

Epoxidation of **7** and **11** with magnesium monoperoxyphthalate[17] resulted stereoselectively in the formation of the desired target *exo*-epoxides **8** and **12**. Since the peracid oxidation of norbornene itself is already highly *exo*-selective[18] (*exo*:*endo* ratio ca. 700:1), it is unlikely that any *endo* epoxidation could take place with either **7** or **11**, because the presence of the xanthine moiety in these compounds should render the *endo*-face of the norbornenyl group even less sterically accessible.

In a transformation mechanistically related to the iodolactonization of *endo*-5-norbornene-2-carboxylic acid,[19] (*S*)-epoxynorbornylxanthine **12** underwent an acid-catalyzed intramolecular cyclization reaction on treatment with HCl in ethanol (Scheme 3). The resulting secondary alcohol **13** was converted into the 4-bromobenzoate **14**, which was subjected to X-ray crystal structure analysis (Figure 2). This conclusively proved that the absolute configuration at C-2 of the norbornane group in **12** is *S* and that the epoxide and the xanthine are respectively situated *exo* and *endo* to the carbocycle.

Results and Discussion

The binding affinities of **4** (ENX, racemate) and its *R*- and *S*-enantiomers **8** and **12** were determined at guinea pig, rat, and cloned human A_1- and A_{2a}-adenosine receptors (see the Experimental Section). While there was little differentiation between the enantiomers at guinea pig and human A_1 receptors, **12** (K_i ca. 0.5 nM at both rat and human receptors) appeared to be approximately 8-fold more potent than **8** in the rat (Table 1). Affinities at the A_{2a} receptor from all three

Table 1. Binding Affinities of Enantiomers **8** and **12**, Racemate **4**, and Other Alkylxanthines at A_1- and A_{2a}-Adenosine Receptors

	A_1K_1 (nM)			$A_{2a}K_i$ (nM)		
compd	guinea pig[a]	rat[a]	human[b]	guinea pig[c]	rat[c]	human[d]
8	3.17 ± 0.005	5.12 ± 1.63	0.80 ± 0.09	250 ± 38	551 ± 173	201 ± 24
4 (ENX)	3.15 ± 0.19	1.43 ± 0.20	0.82 ± 0.05	465 ± 197	309 ± 61	475 ± 66
12	2.56 ± 0.23	0.67 ± 0.14	0.45 ± 0.04	930 ± 30	1250 ± 262	1100 ± 318
2 (KW-3902)	1.10 ± 0.59[e]	12.60 ± 2.90	0.72 ± 0.12	230 ± 75[e]	510 ± 10	108 ± 15
CPX	2.93 ± 0.89[e]	3.77 ± 0.45	2.52 ± 0.05	320 ± 87[e]	260 ± 40	157 ± 53

[a] Inhibition of radioligand binding to membranes from guinea pig and rat forebrain. [b] Inhibition of binding to membranes from HEK-293 cells stably transfected with recombinant human A_1 AdoRs. [c] Inhibition of binding to membranes from guinea pig and rat striatal tissue. [d] Inhibition of binding to membranes from HEK-293 cells stably transfected with recombinant human A_{2a} AdoRs. Radioligands (see the Experimental Section) were used at or below their K_d values. [e] Reference 12.

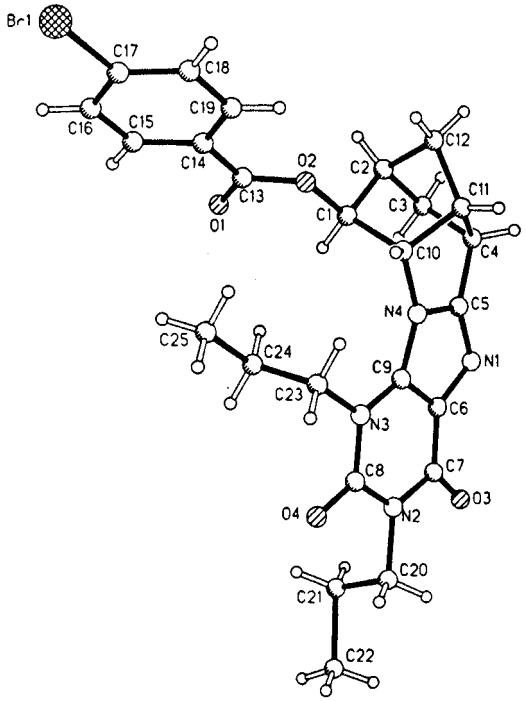


Figure 2. X-ray crystal structure of 4-bromobenzoyl derivative **14**. The atom numbering system used is not conventional.

Table 2. Selectivity Ratios for Enantiomers **8** and **12** and Other Alkylxanthines

		K_i ratio A_{2a}/A_1		
compd	stereochem	guinea pig	rat	human
8	*R*	80	110	250
12	*S*	360	1800	2400
2 (KW-3902)		210	40	150
CPX		110	70	60

species were much lower (K_i values 250−1250 nM). Both enantiomers thus showed good A_1-selectivity across the three species (Table 2), with the *S*-enantiomer **12** being the more selective, particularly at the rat (1800-fold) and the human (2400-fold) adenosine receptors. In comparison, the selectivity of both the noradamantyl derivative **2** and the well-known antagonist CPX (8-cyclopentyl-1,3-dipropylxanthine) was more than 10-fold lower and of the same order of magnitude as that of the racemic *endo*-norbornyl analog lacking the epoxide functionality.[7a] On the basis of these data, the *S*-enantiomer **12** appears to be one of the more potent and clearly the most A_1-selective adenosine receptor antagonist reported to date.

Recently, it was reported that the structurally analogous *S*-enantiomer of N^6-epoxynorbornyladenosine was 10−12-fold more potent as an A_1-agonist than the corresponding *R*-enantiomer.[20] Of the three different binding modes proposed for 8-substituted xanthines with respect to the corresponding N^6-substituted adenosine analogs,[21] these observations decisively support the so-called "N^6−C^8" model first postulated by Peet et al.,[13c] according to which xanthines bind to the receptor in a flipped and rotated orientation with respect to adenosines.

Table 3. Diuretic Effects of (Epoxynorbornyl)xanthines in Saline-Loaded Rats[a]

compd	dose (mg/kg, iv)	urine output (mL/kg per 4 h)	Na^+ output (mmol/kg per 4 h)	K^+ output (mmol/kg per 4 h)
vehicle		20.3 ± 2.8	2.90 ± 0.30	0.89 ± 0.09
8	0.01	26.8 ± 3.3	3.80 ± 0.30	0.97 ± 0.10
	0.1	31.3 ± 2.3	4.56 ± 0.16*	0.89 ± 0.09
	1.0	44.1 ± 4.2*	5.99 ± 0.47*	1.84 ± 0.14*
4 (ENX)	0.01	23.2 ± 2.6	3.54 ± 0.34	1.10 ± 0.09
	0.1	33.0 ± 2.0	5.14 ± 0.28	1.22 ± 0.05
	1.0	43.2 ± 1.3**	6.54 ± 0.08*	1.42 ± 0.16
12	0.01	21.7 ± 2.3	3.02 ± 0.25	0.75 ± 0.15
	0.1	35.1 ± 2.3*	5.29 ± 0.23*	1.34 ± 0.19
	1.0	37.1 ± 1.9*	5.86 ± 0.35*	1.09 ± 0.07

[a] Compounds were administered intravenously to rats (n = six or seven per dose), and urine was collected for 4 h; see the Experimental Section for details. Values are expressed as means ± SEM of vehicle control and drug treatment values, respectively. *p < 0.01. **p < 0.05.

In accordance with the finding that introduction of bulky substituents into the 8-position of 1,3-dialkylxanthines attenuates phosphodiesterase (PDE) inhibitory activity,[22] relatively high concentrations of **12** (EC_{50} values 10−300 μM; data not shown) were required to inhibit PDE isoenzymes types I−V.

The diuretic activity of the test compounds was assessed intravenously in saline-loaded rats (Table 3). Both enantiomers and the racemate increased urine and sodium output in a dose-dependent manner. In keeping with the relatively small affinity difference (<10-fold) between **8** and **12** for the rat A_1-receptor, there was virtually no difference in their potency in vivo.

The *S*-enantiomer **12** (CVT-124) recently completed a phase I study in humans, demonstrating a diuresis profile characterized by dose-dependent increases in the excretion of sodium ion, chloride, and uric acid.[23] Because concomitant increases in potassium ion excretion were not observed, this agent could be useful as a potent and potassium-sparing diuretic for the treatment of edema associated with congestive heart failure.

Experimental Section

Chemistry: General. Melting points were determined on a Mel-Temp II apparatus and are uncorrected. Proton and carbon NMR spectra were recorded in deuteriochloroform on a Varian Gemini 400 spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane (internal standard) and coupling constants (J) in hertz (Hz). Mass

spectra were recorded at the University of Kansas on a Nermag R10-10 quadrupole GC/MS system with SPECTRAL 30 data system. Optical rotations were measured on a JASCO DIP-370 digital polarimeter in chloroform. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Analytical thin-layer chromatography (TLC) was conducted on Analtech silica gel GF plates. Compounds were visualized with UV light, iodine vapor, or ammonium molybdate/sulfuric acid spray. Centrifugally accelerated preparative thin-layer chromatography was performed on a Chromatotron model 7924T (Harrison Research, Palo Alto, CA). Chiral purity of the target compounds was determined on a Waters HPLC system fitted with a Chiralcel OD column under isocratic conditions with a hexane/2-propanol (98:2) mobile phase. Racemic 1,3-dipropyl-8-(5,6-*exo*-epoxy-2-*endo*-norbornyl)xanthine was prepared as described previously.[12]

1,3-Dipropyl-8-[(1*R*,2*R*)-5-norbornen-2-yl]xanthine (7). To a solution of 10.7 g (43 mmol) of the norbornenecarboxylic ester **5**[15] in 100 mL of methanol was added a solution of 8.6 g of NaOH (215 mmol) in 30 mL of water. After being stirred at room temperature for 4 h, the solution was concentrated on a rotary evaporator, cooled in an ice bath, and acidified with 2 N HCl. The resulting mixture was extracted with dichloromethane (3 × 200 mL), and the combined extracts were washed with brine. After being dried over $MgSO_4$, the extracts were filtered and concentrated to ca. 150 mL. DMF (3 drops) was added, followed by a mixture of oxalyl chloride (4.7 mL) and dichloromethane (20 mL) dropwise with stirring. After being stirred at room temperature for an additional 2.5 h, the reaction mixture was evaporated to dryness and then coevaporated twice with dichloromethane (50 mL) to remove traces of oxalyl chloride. The residue was cooled in an ice bath under dry nitrogen for 15 min and then dissolved in pyridine (75 mL). DMAP (500 mg) and 5,6-diamino-1,3-dipropyluracil[24] (10.7 g) were added, and the resulting mixture was stirred at ambient temperature for 2 h, then cooled in an ice bath, and acidified with 6 N HCl. After extraction with AcOEt (2 × 300 mL), and evaporation to dryness, the resulting amide **6** was heated under reflux with 2 N aqueous NaOH (150 mL) in dioxane (150 mL) for 4 h. Concentration on a rotary evaporator to remove most of the dioxane, followed by neutralization with 2 N HCl and extraction with AcOEt, afforded crude **7**, which was purified by filtration through a short column of silica gel (3:1 hexanes/AcOEt) followed by crystallization from CH_2Cl_2/hexanes (final volume ca. 80 mL). White needles of **7** (10.46 g, 74%) with a mp of 140−141 °C were thus obtained: $[\alpha]^{20}_D = +140.8°$ ($c = 2.0$); ^{1}H NMR δ 0.97 (6H, m), 1.46 (1H, d, J = 8 Hz), 1.56 (1H, d, J = 8 Hz), 1.70 (3H, m), 1.81 (2H, m), 2.25 (1H, m), 3.04 (1H, s), 3.45 (1H, s), 3.57 (1H, m), 4.01 (2H, m), 4.08 (2H, m), 5.85 (1H, m), 6.27 (1H, m); ^{13}C NMR δ 11.18, 11.38, 21.34, 31.43, 42.92, 43.23, 45.15, 47.54, 49.82, 106.65, 132.62, 138.25, 148.68, 151.05, 157.97. Anal. ($C_{18}H_{24}N_4O_2$) C, H, N.

1,3-Dipropyl-8-[5,6-*exo*-epoxy-(1*R*,2*R*)-norborn-2-yl]-xanthine (8). To a stirred suspension of **7** (3.6 g, 11 mmol) in 2-propanol (30 mL) and water (15 mL) was added magnesium monoperoxyphthalate (ca. 80%, 7.5 g, ca. 1.1 equiv) in one portion. After the mixture was stirred for 20 h at room temperature, excess peracid was destroyed by successive additions of KI and $Na_2S_2O_3$ to the clear solution, which thereafter was diluted with water (150 mL). The resulting suspension was extracted with AcOEt (2 × 150 mL), and the combined extracts were washed with water, aqueous $NaHCO_3$, and brine. The dried ($MgSO_4$), filtered extracts were evaporated to dryness, and the residue was filtered through a short column of neutral alumina (activity III, CH_2Cl_2). Evaporation of the eluate gave an oil which was crystallized from ether/hexane to afford 2.20 g (58.3%) of **8** as a white solid: mp 143.5-144.5 °C; $[\alpha]^{20}_D = +66.4°$ ($c = 2.0$); ^{1}H NMR δ 0.97 (3H, t, J = 7.5 Hz), 0.99 (3H, t, J = 7.5 Hz), 1.01 (1H, m), 1.56 (1H, m), 1.72 (2H, m), 1.81 (2H, m), 2.07 (2H, m), 2.68 (1H, m), 3.09 (1H, m), 3.11 (1H, m), 3.26 (1H, m), 3.44 (1H, m), 4.03 (2H, t, J = 7.5 Hz), 4.12 (2H, t, J = 7.5 Hz); ^{13}C NMR δ 11.22, 11.44, 21.36, 27.66, 29.58, 37.34, 39.98, 41.66, 43.46, 45.27, 49.40, 51.10, 106.91, 148.97, 150.86, 155.65, 156.04. Anal. ($C_{18}H_{24}N_4O_3$) C, H, N.

1,3-Dipropyl-8-[(1*S*,2*S*)-5-norbornen-2-yl]xanthine (11). The procedure for preparing **7** was followed, except that 100 mL of a THF/MeOH/water mixture (3:3:4) was used for the hydrolytic removal of the chiral auxiliary. Starting with 7.87 g (31.5 mmol) of **9**,[16] 6.5 g (63%) of recrystallized **11** were obtained in this manner: mp 140−141 °C; $[\alpha]^{20}_D = -142.3°$ ($c = 2.0$); ^{1}H NMR δ 0.98 (6H, m), 1.46 (1H, d, J = 8 Hz), 1.57 (1H, d, J = 8 Hz), 1.72 (3H, m), 1.81 (2H, m), 2.25 (1H, m), 3.04 (1H, s), 3.46 (1H, s), 3.56 (1H, m), 4.03 (2H, m), 4.10 (2H, m), 5.84 (1H, m), 6.27 (1H, m); ^{13}C NMR δ 11.17, 11.38, 21.35, 31.22, 38.73, 42.92, 43.25, 45.15, 47.52, 49.78, 132.63, 138.13, 151.05, 155.20, 158.05. Anal. ($C_{18}H_{24}N_4O_2$) C, H, N.

1,3-Dipropyl-8-[5,6-*exo*-epoxy-(1*S*,2*S*)-norborn-2-yl]-xanthine (12). Epoxidation of **11** (8.4 g, 25.6 mmol) with MMPP (15.8 g) as described for the preparation of **8** afforded 5.8 g (66%) of **12** as a white solid: mp 143−145 °C (ether/hexane); $[\alpha]^{20}_D = -67.25°$ ($c = 2.0$); ee = 97.2%; ^{1}H NMR δ 0.96 (3H, t, J = 7.5 Hz), 0.98 (3H, t, J = 7.5 Hz), 1.00 (1H, m), 1.54 (1H, m), 1.72 (2H, m), 1.79 (2H, m), 2.06 (2H, m), 2.67 (1H, m), 3.08 (1H, m), 3.10 (1H, m), 3.25 (1H, m), 3.43 (1H, m), 4.02 (2H, t, J = 7.5 Hz), 4.11 (2H, t, J = 7.5 Hz); ^{13}C NMR δ 11.22, 11.44, 21.38, 27.68, 29.60, 37.36, 39.99, 41.68, 43.46, 45.28, 49.40, 51.09, 106.92, 149.01, 150.87, 155.68, 156.08; MS *m/e* 344 (M^+). Anal. ($C_{18}H_{24}N_4O_3$) C, H, N.

Rearrangement Product 13. A solution of **12** (850 mg, 2.47 mmol) in EtOH (17.5 mL) containing 6 N HCl (2.5 mL) was stirred at room temperature for 30 h and then concentrated to a small volume under vacuum. After dilution with brine (10 mL), the mixture was extracted twice with AcOEt (30 mL). The combined extracts were washed with brine, dried over $MgSO_4$, filtered, and evaporated. The residue was purified on the chromatotron (5% MeOH in CH_2Cl_2) to give 710 mg (83.5%) of **13** as a white foam. An analytical sample was obtained by recrystallization from AcOEt/*i*-Pr_2O: mp 131−133 °C; $[\alpha]^{20}_D = -30.8°$ ($c = 2.0$); ^{1}H NMR δ 0.93 (3H, t, J = 7.5 Hz), 1.02 (3H, t, J = 7.5 Hz), 1.40 (1H, d, J = 13 Hz), 1.66 (2H, m), 1.78 (2H, m), 1.82 (2H, m), 2.04 (1H, m), 2.21 (1H, d, J = 11 Hz), 2.51 (1H, s), 3.04 (1H, m), 3.62 (1H, t, J = 4 Hz), 3.71 (1H, s), 3.80 (1H, m), 3.95 (2H, t, J = 7.5 Hz), 4.15 (1H, m), 4.23 (1H, d, J = 5 Hz); ^{13}C NMR δ 10.77, 11.31, 21.15, 22.27, 33.45, 34.09, 43.27, 45.94, 46.06, 53.60, 67.17, 77.69, 119.38, 136.91, 150.43, 157.28, 158.52; MS *m/e* 344 (M^+). Anal. ($C_{18}H_{24}N_4O_3$) C, H, N.

4-Bromobenzoyl Derivative 14. A solution of **13** (440 mg, 1.28 mmol), 4-bromobenzoyl chloride (420 mg, 1.92 mmol), and DMAP (25 mg) in pyridine (10 mL) was stirred under dry N_2 at 50 °C for 20 h. Water (10 mL) was added, and the mixture was stirred at room temperature for 4 h in order to hydrolyze unreacted acyl chloride. After acidification (2 N HCl), the mixture was extracted twice with ethyl acetate (30 mL). The extracts were washed with 2 N HCl, water, aqueous, $NaHCO_3$, and brine. Evaporation of the dried ($MgSO_4$), filtered extract left an oil (585 mg, 86.8%) which was crystallized from AcOEt/*i*-Pr_2O: mp 140 °C; $[\alpha]^{20}_D = -65.3°$ ($c = 2.0$); ^{1}H NMR δ 0.81 (3H, m), 0.95 (3H, m), 1.13 (2H, m), 1.67 (3H, m), 1.83 (1H, d, J = 12 Hz), 2.18 (2H, m), 2.77 (1H, m), 3.19 (1H, m), 3.73 (1H, m), 3.78 (1H, m), 3.99 (2H, m), 4.10 (1H, m), 4.41 (1H, d, J = 4 Hz), 4.88 (1H, s), 7.64 (2H, m), 7.89 (2H, m). Anal. ($C_{25}H_{27}BrN_4O_4$) C, H, N.

X-ray Structural Analysis of 14. A flat monoclinic crystal of **14** was used for the data collection performed on a R3m Siemens four-circle diffractometer using graphite-monochromated Cu Kα radiation. The cell parameters were obtained by a least-squares fit of 25 reflections with 2θ range 35−45°. A total of 2500 reflections were measured using the $\omega/2\theta$ scan with variable scan speed (2−29°/min) to a maximum $2\theta = 110°$. Three standard reflections were monitored every 97 reflections. No systematic intensity variations were found. No absorption correction was applied. The structure was solved by the direct method (SHELX-86). Hydrogen atoms were introduced geometrically. The structure was refined on F^2 using the full-matrix least-squares technique (SHELX-93) with anisotropic thermal parameters for all non-hydrogen atoms. Final R_1 = 6.2% for 1910 $F_0 > 4\sigma(F_0)$. No peaks greater than 0.37 e/Å^3 were found in the final difference Fourier map. The absolute structure parameter χ converged at −0.06(5).

Radioligand Binding Assays. Binding affinities (K_i) at the A_1- and A_{2a}-adenosine receptors were determined by measurement of the displacement of the binding of the A_1 AdoR antagonist [^{3}H]CPX or the A_{2a} AdoR agonist [^{3}H]CGS-21680 to membranes prepared from guinea pig and rat forebrain (A_1) or striatal tissue (A_{2a}) as described previously[12] and from HEK-293 cells stably transfected with a vector containing the cDNA of the human A_1 or A_{2a} AdoR as described below. Values given are mean ± SEM of triplicate determinations in each of four preparations. All Hill slopes were close to unity.

Cell Culture. HEK-293 cells were obtained from American Tissue Culture Collection. Cells were grown at 37.5 °C in an incubator environment of 5% CO_2 and maintained in Dulbecco's minimal essential medium (DMEM)-F12 supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 μg/mL streptomycin, with or without 0.7 mg/mL G418. HEK-293 cells were grown as monolayers in tissue culture plates or flasks and routinely subcultured every 3 days by a 1:4 dilution in fresh media.

Stable Transfection of HEK-293 Cells. For stable transfections, the cDNAs of human adenosine receptors were subcloned into CLDN10B (a gift from Mitch Reff, SmithKline Beecham), and introduced into HEK-293 cells by means of Lipofectin[25] (GIBCO/BRL). Transfected cells were subcultured at 1:5 dilution into selection medium (normal growth medium supplemented with 0.5 mg/mL G418). Colonies were screened by radioligand binding assays using the agonist [^{125}I]ABA.[26] Selected colonies were maintained in growth medium supplemented with 0.5 μg/mL G418.

Membrane Preparations. HEK-293 cell monolayers were washed with phosphate-buffered saline (PBS, GIBCO/BRL) and harvested in buffer A (10 mM Na-HEPES, 10 mM EDTA, pH 7.4) supplemented with protease inhibitors (10 μg/mL benzamidine, 100 μM PMSF, and 2 μg/mL each of aprotinin, pepstatin, and leupeptin). The cells were homogenized, centrifuged at 30000*g* for 25 min, and washed twice with buffer HE (10 mM Na-HEPES, 1 mM EDTA, pH 7.4, plus protease inhibitors). Final pellets were resuspended in buffer HE supplemented with 10% (w/v) sucrose and protease inhibitors and frozen in aliquots at −80.5 °C. Protein concentration was measured by fluorescamine fluorescence using BSA standards.[27]

Radioligand Binding Assays. Radioligand binding to A_1-adenosine receptors was measured using the agonist [^{125}I]-ABA[26] or [^{3}H]CPX. Binding to A_{2a}-adenosine receptors was measured with [^{125}I]APE[28] or [^{3}H]CGS-21680. Binding was determined in radioligand buffer containing 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, and 1 unit/mL adenosine deaminase. Experiments were performed in triplicate with 10−50 μg of membrane protein in a total volume of 0.1 mL. The incubation time was 2.5 h at 21.5 °C. Nonspecific binding was measured in the presence of 10 μM ABA (A_1), or 100 μM NECA (A_{2a}). Binding assays were terminated by filtration over Whatman GF/C glass fiber filters using a Brandel cell harvester. The filters were rinsed with 3 × 4 mL of ice-cold Tris-Cl (pH 7.4) containing 5 mM $MgCl_2$ at 4.5 °C and counted in a Wallac counter.

Pharmacology. The effects on renal function were assessed in Sprague−Dawley rats (250−350 g). Following a 2 h equilibration period, rats were dosed by tail vein injection with the test compound (0.01, 0.1, and 1.0 mg/kg) or vehicle alone (1.0 mL/kg of 50% saline, 40% polyethylene glycol 550, and 10% ethanol); this was immediately followed by dosing with 40 mL/kg normal saline (po). The animals were placed in metabolic cages, and urine was collected for 4 h after dosing. Urine volumes were measured gravimetrically. Na^+ and K^+ concentrations were assayed by flame photometry. Statistical difference from the vehicle-treated group was determined with a 1-way ANOVA and the Bonferoni multiple comparisons test.

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