Preparation, Properties, Reactions, and Adenosine Receptor Affinities of Sulfophenylxanthine Nitrophenyl Esters: Toward the Development of Sulfonic Acid Prodrugs with Peroral Bioavailability

Luo Yan and Christa E. Müller*

University of Bonn, Pharmaceutical Chemistry Poppelsdorf, Pharmaceutical Sciences, Bonn, Germany

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Many currently known antagonists for P2 purinergic receptors are anionic molecules bearing one or several phenylsulfonate groups. Among the P1 (adenosine) receptor antagonists, the xanthine phenylsulfonates are a potent class of compounds. Due to their high acidity, phenylsulfonates are negatively charged at physiologic pH values and do not easily penetrate cell membranes. The present study was aimed at developing lipophilic, perorally bioavailable prodrugs of sulfonates by converting them into chemically stable nitrophenyl esters. Initial stability tests at different pH values using nitrophenyl tosylates as model compounds showed that *m*-nitrophenyl esters were stable over a wide pH range, while the ortho and para isomers were less stable under strongly acidic or basic conditions. A series of *m*- and *p*-nitrophenyl esters of *p*-sulfophenylxanthine derivatives were synthesized as model compounds. The target xanthine derivatives were obtained in high yields by condensation of the appropriate 5,6-diaminouracils with 4-(nitrophenoxysulfonyl)benzoic acids in the presence of a carbodiimide, followed by ring closure with polyphosphoric acid trimethylsilyl ester. The chemical and enzymatic stability of the *m*-nitrophenyl esters was investigated in vitro by means of capillary electrophoresis. High stability in aqueous solution, in artificial gastric acid, and in serum was observed. However, compound **5d**, used as a prototypic xanthine *m*-nitrophenylsulfonate, was hydrolyzed by rat liver homogenate indicating an enzymatic pathway of hydrolysis. Thus, nitrophenyl esters of sulfonic acids have a potential as peroral prodrugs of drugs bearing a sulfonate group. The nitrophenyl esters of sulfophenylxanthines were additionally investigated for their adenosine receptor affinities. They showed high affinity at A_1, A_{2A}, and A_{2B}, but not at A_3 ARs. One of the most potent compounds was 1-propyl-8-[4-[[*p*-nitrophenoxy]sulfonyl]-phenyl]xanthine (**9d**), a mixed A_1/A_{2B} antagonist (K_iA_1 3.6 nM, K_iA_{2B} 5.4 nM) selective versus the other subtypes. As a further result of this study, the *m*-nitrophenoxy group was found to be a suitable protecting group for sulfonates in organic synthesis due to its high lipophilicity and stability; it can be split off under strongly basic conditions. This new protection strategy allowed for the upscaling of the synthesis of 1-propyl-8-*p*-sulfophenylxanthine (PSB-1115), a selective A_{2B} antagonist.

Introduction

Since the late 1970s, when Burnstock proposed the subclassification of purinergic receptors into P1 and P2 families,[1] the development of potent and selective antagonists for these two classes of receptors as pharmacological tools and as potential drugs has been an active area of research.[2,3] P2 receptors are activated by nucleotides such as ATP, ADP, UTP, or UDP, which bear phosphate groups that are negatively charged at physiological pH values.[3] The vast majority of P2 receptor antagonists known to date are also anionic molecules, e.g. suramin, Reactive Blue 2, PPADS, and derivatives thereof[3] (Figure 1). All of them share a common partial structure containing one or several phenylsulfonate moieties. The negatively charged phosphate groups of P2 agonists and the negatively charged

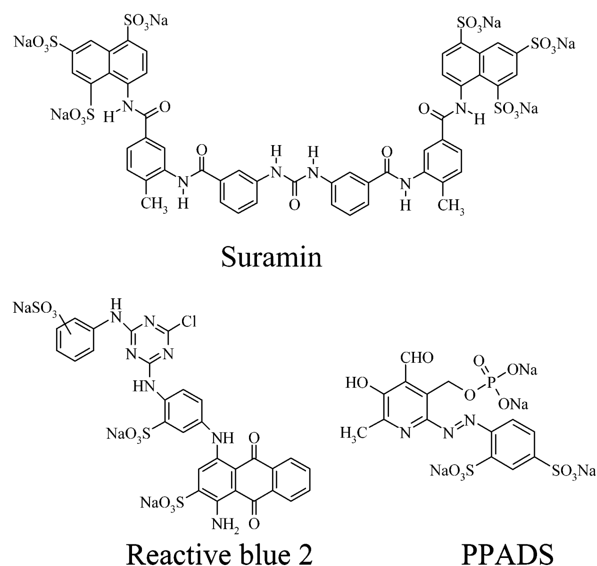

Figure 1. Examples of P2 receptor antagonists with phenylsulfonate structure.

* Address correspondence to Dr. Christa E. Müller, Pharmazeutisches Institut, Pharmaceutical Sciences Bonn, Pharmazeutische Chemie Poppelsdorf, Kreuzbergweg 26, D-53115 Bonn, Germany. Phone: +49-228-73-2301. Fax: +49-228-73-2567. E-mail: christa.mueller@uni-bonn.de.

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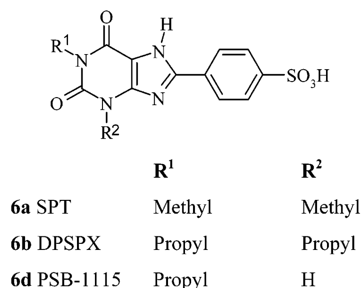


	R^1	R^2
6a SPT	Methyl	Methyl
6b DPSPX	Propyl	Propyl
6d PSB-1115	Propyl	H

Figure 2. Examples of P1 (adenosine) receptor antagonists with phenylsulfonate structure: 8-*p*-sulfophenylxanthine derivatives.

sulfonate groups of P2 antagonists are believed to bind to positively charged amino acid residues which are typically found in the ligand binding pocket of P2 receptors.[3,4]

In the P1 antagonist field, sulfophenyl derivatives have also been described, including 8-*p*-sulfophenyltheophylline (SPT) and 1,3-dipropyl-8-*p*-sulfophenylxanthine (DPSPX), which were introduced by Daly et al. because of their high water-solubility.[5,6] Recently, 1-propyl-8-*p*-sulfophenylxanthine (PSB-1115) was developed as an A_{2B}-selective adenosine receptor (AR) antagonist[7] (Figure 2). The sulfonic acid group appears to contribute to the high selectivity of the compound at A_{2B} ARs versus the other AR subtypes, especially versus A_1 ARs.[7]

Due to the low pK_a value of free sulfonic acid groups (pK_a < 1),[8] sulfophenylxanthine derivatives and P2 antagonists with phenylsulfonate structure are deprotonated under physiological conditions. This means that they do not penetrate into the central nervous system (CNS) and thus are only peripherally active.[9-11] However, to be able to investigate CNS effects in vivo, e.g. a potential neuroprotective effect of P2 antagonists,[12] penetration into the brain is essential. Furthermore, because of their polar character, sulfonates are not readily able to cross cell membranes and thus can hardly be absorbed from the gut if they are perorally applied. Therefore, they are only parenterally applicable, if a systemic effect is desired. However, the preferred way of drug application is peroral.

Our approach to overcome these obstacles was the development of prodrugs of these pharmacologically active aryl sulfonic acid derivatives. The prodrugs should be more lipophilic than their parent drugs in order to be able to cross cell membranes and to be absorbed from the gastrointestinal tract. The compounds should be stable in the strongly acidic medium of the stomach and stay intact before being absorbed and reaching the target cells. They should release the parent active drug in vivo, preferably by an enzymatic mechanism.

Ester prodrugs of compounds bearing acidic groups have been described. Such prodrugs are well-known for carboxylic and phosphoric acids, but not for sulfonic acids.[13,14] Two kinds of sulfonic acid derivatives are conceivable as potential prodrugs: amides of sulfonic acids (sulfonamides) or sulfonic acid esters. Sulfonamides are highly stable in vivo and therefore are unsuitable as sulfonate prodrugs.[14] In contrast, sulfonic acid esters are relatively unstable since they may react with nucleophiles in vitro and in vivo. Reactive sulfonic

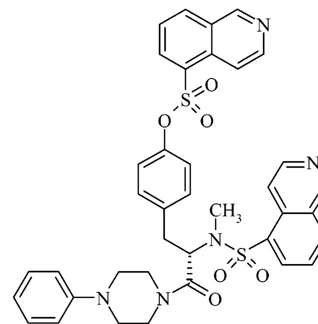


Figure 3. Structure of the $P2X_7$ receptor antagonist KN62.

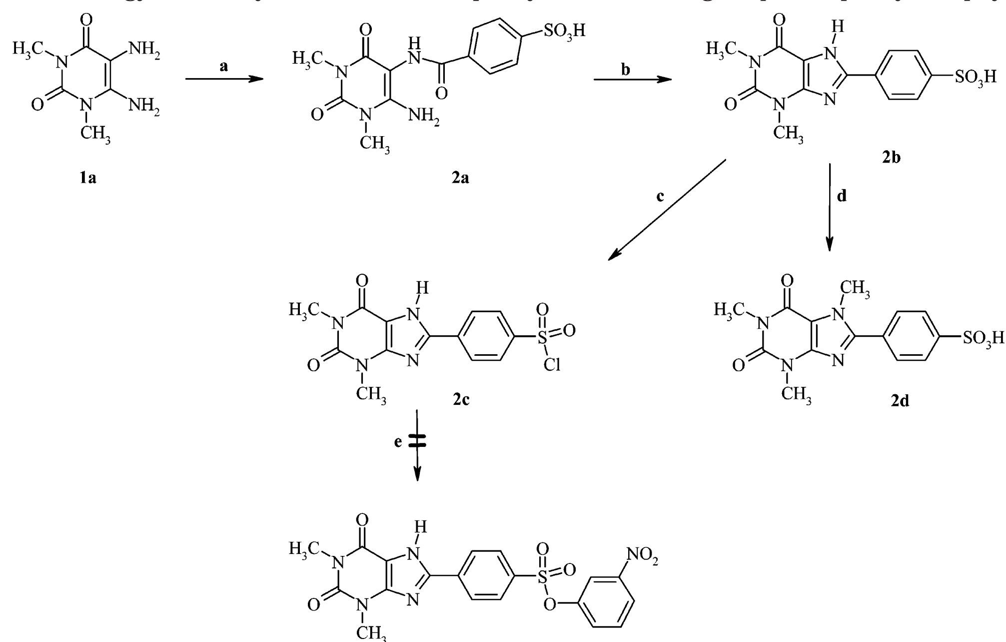
acid esters are used in synthetic organic chemistry (e.g. mesylates, tosylates) and as anticancer drugs (busulfane, treosulfane) due to their alkylating properties.[15] However the stability of sulfonic acid esters greatly depends on the substitution pattern. Highly stable esters can be obtained if suitable, electron-withdrawing, deactivating substituents are introduced. Examples of obviously stable sulfonic acid esters are the 1-*N,O*-bis-1,5-isoquinolinesulfonyl-*N*-methyl-L-tyrosyl-4-phenylpiperazine (KN 62) (Figure 3), which was introduced as a $P2X_7$ antagonist, and related compounds.[16] In the present study we synthesized nitrophenyl esters of a series of sulfophenylxanthine derivatives in order to test our hypothesis that chemically stable sulfonic acid esters may be suitable sulfonate prodrugs. The stability, reactivity, and enzymatic hydrolysis of the sulfonate esters was investigated, and adenosine receptor affinities of the new xanthine derivatives were also determined.

Results and Discussion

Chemistry. Nitrophenyl esters were selected as potential lipophilic prodrugs of sulfonic acid drugs due to their generally high chemical stability.[17-22] Initial stability studies were performed with nitrophenyl esters of tosylate, namely *o*-, *m*-, and *p*-nitrophenyl tosylate as model compounds, which were synthesized as previously described.[23] Subsequently, nitrophenyl esters of *p*-sulfophenylxanthine derivatives, potent adenosine receptor antagonists, were prepared.

Synthesis of Xanthine Sulfonic Acid Nitrophenyl Esters. 1,3-Dimethyl-8-*p*-sulfophenylxanthine was prepared according to published procedures.[5,6,24] 5,6-Diamino-1,3-dimethyluracil **1a** was used as a starting compound (Scheme 1). For the preparation of the carboxybenzamidouracil **2a**, the diaminouracil **1a** was reacted with *p*-sulfobenzoic acid potassium salt using the water-soluble *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) as a condensing agent. Ring closure of **2a** to xanthine **2b** was performed in aqueous sodium hydroxide solution. Methylation yielded the corresponding caffeine derivative **2d** as previously described.[24] Esterification of **2b** was initially attempted by transformation of the *p*-sulfophenylxanthine to the corresponding chlorosulfonylphenylxanthine derivative using thionyl chloride, followed by esterification with nitrophenol under basic conditions. In fact, chlorination of the *p*-sulfophenyl xanthine could successfully be performed by refluxing compound **2b** with thionyl chloride, as confirmed by mass spectrometry of product **2c** (Scheme 1). However, the subsequent

Scheme 1. Initial Strategy for the Synthesis of *m*-Nitrophenyl Ester Prodrugs of *p*-Sulfophenyltheophylline[a]



[a] Reagents: (a) potassium *p*-sulfobenzoate, EDC, H_2O, rt, 1 h; (b) 2.5 N NaOH, 70 °C, 10 min; (c) $SOCl_2$, reflux, 2 h; (d) MeI, DMF, K_2CO_3, rt, overnight; (e) *m*-nitrophenol or sodium *m*-nitrophenoxide.

esterification of the chlorosulfonylphenylxanthine with an excess of *m*-nitrophenol or sodium *m*-nitrophenoxide could not be achieved under various reaction conditions, e.g. in the presence of triethylamine or pyridine as a base at different temperatures ranging from −30 to 30 °C.

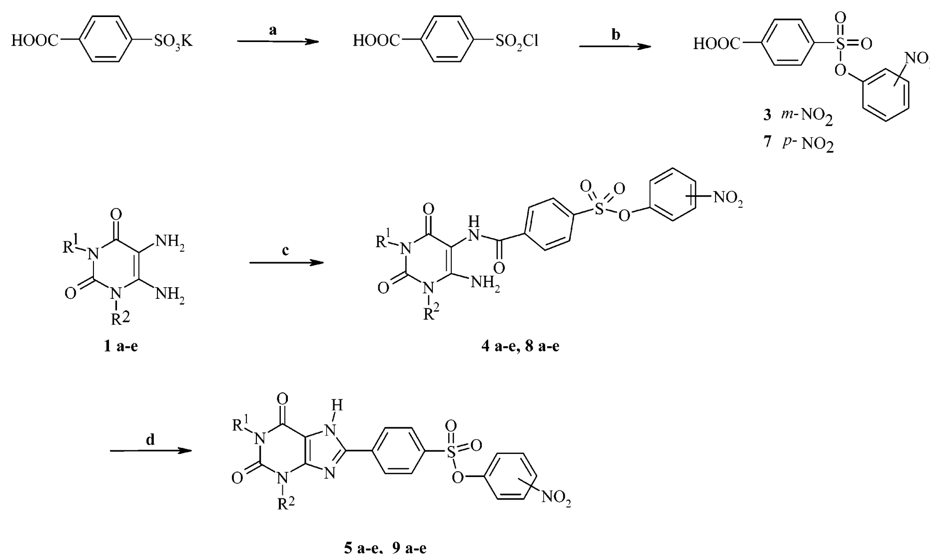
As a consequence, an alternative synthetic pathway was developed (Scheme 2). First *p*-sulfobenzoic acid potassium salt was chlorinated at low temperature using chlorosulfonic acid.[26,27] The resulting sulfonyl chloride was converted to 4-(*m*-nitrophenoxysulfonyl)-benzoic acid **3**, or 4-(*p*-nitrophenoxysulfonyl)benzoic acid **7**, respectively, under Schotten−Baumann conditions.[28] Compounds **3** and **7** were condensed with 5,6-diamino-uracils **1a**−**e** using *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC) as condensing agent to obtain 6-amino-5-[4-[[*m*-nitrophenoxy]sulfonyl]benz-amido]uracil derivatives **4a**−**e** or 6-amino-5-[4-[[*p*-nitro-phenoxy]sulfonyl]benzamido]uracil derivatives **8a**−**e**. Ring closure of **4a**−**e** and **8a**−**e** to 8-[4-[[*m*-nitrophen-oxy]sulfonyl]phenyl]xanthine derivatives **5a**−**e** and the corresponding *p*-nitrophenyl-substituted derivatives **9a**−**e** were achieved by heating of the compounds with poly-phosphoric acid trimethylsilyl ester (PPSE), a powerful but mild condensing agent, at 170 °C for 1.5−2 h[25,29] (Scheme 2).

Since the nitrophenoxysulfonylphenylxanthine derivatives could easily be obtained, isolated, and purified in large quantities by this method, we subsequently tested whether the nitrophenoxy group might be used as a protecting group in the synthesis of phenylsulfonic acid derivatives.

***m*-Nitrophenoxy as a Protecting Group for Phenylsulfonic Acids: New, Improved Synthesis of 1-Substituted 8-*p*-Sulfophenylxanthine Derivatives.** The synthesis of 1-substituted 8-*p*-sulfophenyl-xanthine derivatives, such as 1-propyl- or 1-butyl-8-*p*-sulfophenylxanthine, has been reported.[7,25] However, the described syntheses are difficult to perform on a large, multigram scale. Monosubstituted carboxamido-uracil derivatives possess low reactivity, and the ring closure to obtain 1-substituted 8-*p*-sulfophenylxanthines requires harsh reaction conditions, e.g. reflux with hexamethyldisilazane (HMDS) for more than 50 h.[7] If the reported PPSE method was applied for ring closure, isolation of the desired products was difficult, since PPSE as well as the products are well soluble in water and methanol. 1-Substituted 8-*p*-sulfophenylxanthines have considerable pharmaceutical potential because of their good water-solubility and their high selectivity for A_{2B} ARs.[7] Therefore gram amounts were required for extended pharmacological investigations including animal experiments.[30] Since large amounts were difficult to obtain by the reported methods, we tried to develop an improved synthesis using the nitrophenyl esters as protecting groups. *m*-Nitrophenyl esters of 1-methyl- and 1-propyl-8-*p*-sulfophenylxanthine (compounds **5c** and **5d**) could easily be obtained, isolated, and purified in multigram amounts (Scheme 2). Subsequent alkaline hydrolysis using 2 N aqueous sodium hydroxide solution yielded the desired sulfophenylxanthine derivatives **6c** and **6d** within 30 min in almost quantitative yields, isolated yields ranging between 80 and 90% (Scheme 3). The products were recrystallized from H_2O as reported.[7] Alternatively, preparative HPLC was applied, which proved to be an excellent method for the purification even of large amounts (several grams) of 1-substituted-8-*p*-sulfophenylxanthines. For the quick determination of the purity of the synthesized xanthine derivatives **6c** and **6d**, a fast, convenient capillary electrophoresis ultraviolet (CE-UV) method was developed. Figure 4 shows a typical electropherogram of compound **6d**, confirming the high purity of 1-propy-8-*p*-sulfophenylxanthine.

Thus, hydrolysis of *m*-nitrophenyl esters of 1-substituted sulfophenylxanthine derivatives is a convenient method to obtain 1-substituted 8-*p*-sulfophenylxanthine derivatives, especially when gram amounts of compound

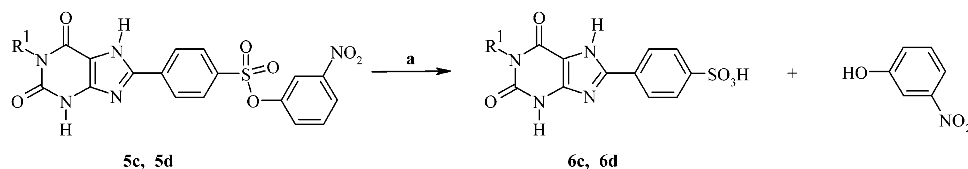
Scheme 2. Synthesis of Nitrophenyl Ester Prodrugs of *p*-Sulfophenylxanthine Derivatives[a]



Compd.	R^1	R^2	Nitro
5a	methyl	methyl	*m*-
5b	propyl	propyl	*m*-
5c	methyl	H	*m*-
5d	propyl	H	*m*-
5e	butyl	H	*m*-
9a	methyl	methyl	*p*-
9b	propyl	propyl	*p*-
9c	methyl	H	*p*-
9d	propyl	H	*p*-
9e	butyl	H	*p*-

[a] Reagents: (a) 1. chlorosulfonic acid, rt overnight, 2. chipped ice; (b) *m*-nitrophenol or *p*-nitrophenol, THF, pH = 8−9, 4 h; (c) **3** or **7,** EDC, MeOH, rt; (d) PPSE, 120 °C, 10 min, 170 °C, 1.5−2 h. **6c**: R^1 = methyl. **6d**: R^1 = propyl.

Scheme 3. Preparation of 1-Substituted 8-*p*-Sulfophenylxanthine Derivatives by Hydrolysis of the *m*-Nitrophenyl Esters[a]



6c: R^1 = methyl. **6d**: R^1 = propyl

[a] Reagent: (a) 2 N NaOH; 70 °C, 30 min.

are needed. On the other hand, the hydrolysis reaction showed that these sulfonic acid esters are relatively stable since complete hydrolysis lasted for about 20 min at 70 °C in 2 N aqueous sodium hydroxide solution. A considerable amount of starting compound was still detectable after 10 min of hydrolysis under these conditions. Due to the balanced stability−reactivity profile of *m*-nitrophenyl esters of phenylsulfonic acids, *m*-nitrophenyl is a suitable protecting group for sulfonic acid derivatives cleavable under strongly basic conditions.

Analytical Data. In Table 1, yields, melting points, and analytical data of all final and most intermediate products are collected. ^{1}H and ^{13}C NMR data of all intermediate and final products were recorded; they were in accordance with the proposed structures. Complete NMR data are available as Supporting Information.

Stability of Simple Sulfonic Acid Esters as Model Compounds. Investigations of the hydrolysis and stability of different sulfonic acid esters have previously been performed.[17−22] Nitrophenoxy sulfonates belong to the most stable esters among differently substituted sulfonic acid esters. However, there are no further reports on a comparison of the hydrolysis constants of *o*-, *m*-, and *p*-nitrophenyl benzene sulfonates at different pH values. Therefore syntheses and stability tests of model compounds, i.e., *o*-, *m*-, and *p*-nitrophenyl tosylate, were initially performed.

Since these esters are not water-soluble, acetonitrile was added for solubilization, the ratio of acetonitrile: buffer being 10:90 or 5:95, respectively. Different aceto-

Table 1. Yields, Melting Points, and Analytical Data of the Synthesized Compounds

compd	formula	MW	anal.[a]	yield [%]	mp [°C]
2b	$C_{13}H_{12}N_4O_5S{\cdot}2H_2O$	372.37	C, H, N	65	>300 (lit. mp > 300)[7]
2d	$C_{14}H_{14}N_4O_5S{\cdot}2.5H_2O$	395.41	C, H,[b] N	35	>300
3	$C_{13}H_9NO_7S$	323.28	C, H, N	45	213.5
7	$C_{13}H_9NO_7S$	323.28	C, H, N	41	218–219
4a	$C_{19}H_{17}N_5O_8S{\cdot}0.5H_2O$	484.45	C,[c] H, N	60	239
4b	$C_{23}H_{25}N_5O_8S{\cdot}0.5H_2O$	540.57	C,[d] H, N[e]	55	245
4c	$C_{18}H_{15}N_5O_8S{\cdot}0.5H_2O$	470.42	C, H, N	55	268
4d	$C_{20}H_{19}N_5O_8S$	489.47	C, H, N	57	252
4e	$C_{21}H_{21}N_5O_8S{\cdot}0.5H_2O$	512.50	C, H, N	42	253
5a	$C_{19}H_{15}N_5O_7S$	457.42	C, H, N	40	>300
5b	$C_{23}H_{23}N_5O_7S{\cdot}0.5H_2O$	522.54	C, H, N	36	>300
5c	$C_{18}H_{13}N_5O_7S{\cdot}0.4H_2O$	450.61	C, H, N	65	>300
5d	$C_{20}H_{17}N_5O_7S{\cdot}0.5H_2O$	480.46	C, H, N	50	>300
5e	$C_{21}H_{19}N_5O_7S$	485.48	C,[f] H, N	88	>300
6c	$C_{12}H_9N_4O_2SNa{\cdot}2.5H_2O$	389.33	C,[g] H,[h] N	87	>300
6d	$C_{14}H_{13}N_4O_5SNa{\cdot}2.5H_2O$	417.39	C, H, N	81	>300 (lit. mp > 300)[7]
8a	$C_{19}H_{17}N_5O_8S$	475.44	n.d.[i]	71	>250
8b	$C_{23}H_{25}N_5O_8S{\cdot}0.5H_2O$	531.55	n.d.	68	>250
8c	$C_{18}H_{15}N_5O_8S$	461.41	n.d.	60	>250
8d	$C_{20}H_{19}N_5O_8S$	489.47	n.d.	57	>250
8e	$C_{21}H_{21}N_5O_8S$	503.49	n.d.	66	>250
9a	$C_{19}H_{15}N_5O_7S{\cdot}H_2O$	475.44	C, H, N	38	>300
9b	$C_{23}H_{23}N_5O_7S{\cdot}0.5H_2O$	522.54	C, H, N	31	>300
9c	$C_{18}H_{13}N_5O_7S{\cdot}1.5H_2O$	470.43	C, H, N	93	>300
9d	$C_{20}H_{17}N_5O_7S$	471.45	C,[j] H,[k] N	65	>300
9e	$C_{21}H_{19}N_5O_7S$	485.48	C, H, N	76	>300

[a] Elemental analyses were within ±0.4% of calculated values, unless otherwise noted. [b] Calcd, 4.81; found, 4.3. [c] Calcd, 47.06; found 47.52. [d] Calcd, 51.06; found 51.55. [e] Calcd, 12.95; found, 12.45. [f] Calc, 51.96; found, 52.57. [g] Calcd, 36.98; found, 36.44. [h] Calcd, 3.59; found, 2.90. [i] n.d, not determined (intermediate products). [j] Calcd, 50.95; found, 50.31. [k] Calcd, 3.63; found, 4.39.

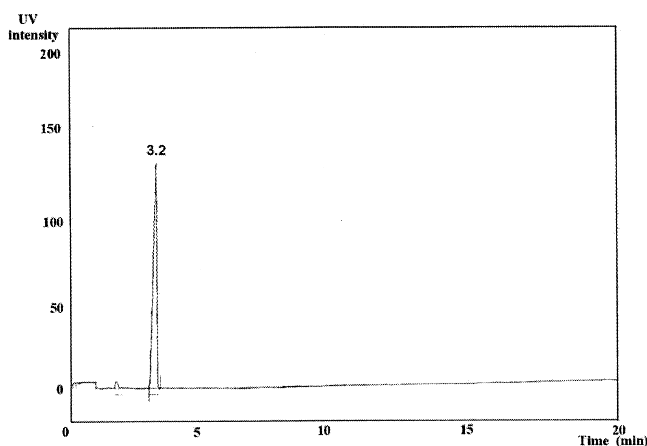


Figure 4. Purity determination of compound **6d** by capillary electrophoresis; running buffer: phosphate 20 mM (pH 7.4), voltage: 10 kV.

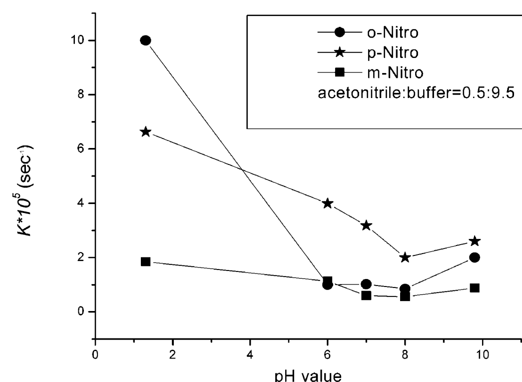


Figure 5. Hydrolysis of *o*-, *m*-, and *p*-nitrophenyl tosylate in acetonitrile: buffer (5:95) as a function of pH at 25 °C (K = hydrolysis rate constant).

nitrile concentrations were used in order to investigate whether the organic solvent had any influence on the hydrolysis rate. Hydrolysis at five different pH values was investigated, i.e., pH 1, 6, 7, 8, and 9.8, ranging from strongly acidic to alkaline conditions. Hydrolysis constants (K) were measured spectrophotometrically by detecting the absorption of released *o*-, *m*-, or *p*-nitrophenol. It was found that all hydrolysis reactions followed a pseudo-first-order kinetic. Plots of $\log(A_t - A_0)$ (A_t: absorption at a certain time; A_0: absorption at zero time) versus time were linear for three half-lives, the slopes yielding the rate constants. Figure 5 shows the hydrolysis rate constants (K) of the three sulfonate esters at five different pH values in acetonitrile: buffer (5:95). We found that the addition of acetonitrile did not have significant effects on the hydrolysis of sulfonic acid esters, since the K values were almost identical in the presence of 5% acetonitrile and 10% acetonitrile. *m*-Nitrophenyl tosylate was the most stable ester, while *p*-nitrophenyl tosylate was the least stable ester among these three differently substituted sulfonate esters. *m*-Nitrophenyl tosylate was quite stable over a large pH range, while *o*- and *p*-nitrophenyl tosylate exhibited lower stability at basic (pH 9.8) and particularly at strongly acidic (pH 1) pH values. Therefore, *m*-nitrophenyl esters of xanthine phenylsulfonic acids were selected as potential prodrugs.

Stability Studies of Sulfophenylxanthine *m*-Nitrophenyl Esters as Potential Sulfonate Prodrugs. An effective oral prodrug must be (i) lipophilic enough to be absorbed from the gastrointestinal tract, (ii) stable enough toward any hydrolysis that might occur before reaching the bloodstream, and (iii) capable of releasing the active parent drug in the body e.g. through enzymatic biotransformation. Therefore, it was necessary to study the chemical and enzymatic in vitro stability of the synthesized putative prodrugs.

The most frequently used analytical method for stability tests is HPLC. However, because the synthesized xanthine sulfonic acid esters are very lipophilic,

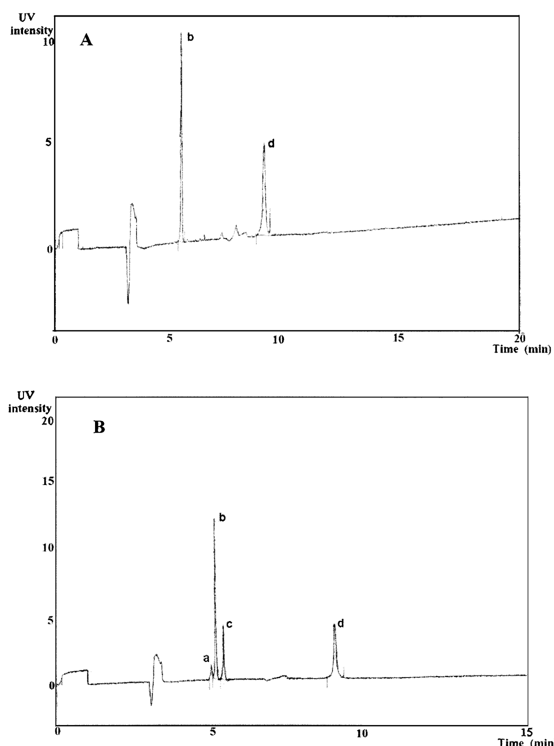


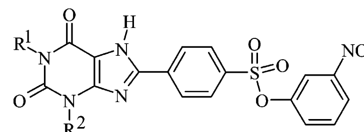
Figure 6. Electropherograms of 1-propyl-8[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5d** before incubation (A) and after 26 h of incubation in 0.001 N NaOH (B). CE conditions: voltage, 10 kV; running buffer, 100 mM borate buffer, 50 mM SDS (pH 8); detection at 320 nm; a: *m*-Nitrophenol, 4.9 min; b: Internal standard (diclofenac-Na), 5.1 min; c: 1-Propyl-8-(*p*-sulfophenyl)xanthine, 5.3 min; d: 1-Propyl-8-[4-[[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine, 8.8 min.

they are insoluble in most common HPLC solvents, such as methanol, acetonitrile, H_2O, etc. Therefore, capillary electrophoresis (CE) with a diode array detector (DAD) was used for the analyses. Compared with HPLC, CE is advantageous in many regards including speed, versatility, low running costs, high separation efficiency, and the requirement of only very small sample volumes. The application field of CE comprises not only the separation of proteins, DNA fragments, and carbohydrates, but also drug metabolism studies.[31] In biological applications CE has the advantage of not requiring sample pretreatment; the incubation solution can directly be injected without prior removal of proteins.

At first, the chemical stability of the *m*-nitrophenyl esters was investigated. Stock solutions (2 mM) in dimethyl sulfoxide (DMSO) were prepared, since the esters were very well soluble in DMSO, but less soluble in water. To reduce or eliminate the influence of DMSO, stock solutions were diluted into aqueous buffer solutions at a ratio of 1:100. The decomposition of the esters **5a**–**e** in 0.001 N aqueous sodium hydroxide solution (pH 11) was studied at 37 °C in order to determine the chemical stability of the compounds. Since we know that the esters are less stable in alkaline media than in neutral solutions, it is likely that, if they are stable in alkaline medium, they should also be stable at neutral pH value.

Expected hydrolysis products of these sulfonic acid esters are the respective 8-*p*-sulfophenylxanthine derivatives and *m*-nitrophenol. This could be confirmed by CE/UV analyses based on the migration times and

Table 2. Stability of Sulfophenylxanthine *m*-Nitrophenyl Esters 5a–e toward 0.001 N Aqueous Sodium Hydroxide Solution at 37 °C Determined by Capillary Electrophoresis with UV Detection at 254 nm



compd	R^1	R^2	$t_{1/2}$ (h)[a]	K (s^{-1})[a]
5a	methyl	methyl	20.8	9.2×10^{-6}
5b	propyl	propyl	21.2	9.1×10^{-6}
5c	methyl	H	20.2	9.6×10^{-6}
5d	propyl	H	23.8	8.1×10^{-6}
5e	butyl	H	19.1	10.1×10^{-6}

[a] Values represent means of two to three separate experiments.

the UV spectra of the compounds formed. Figure 6 shows the CE electropherograms of compound **5d** before incubation and after 26 h of incubation in 0.001 N aqueous sodium hydroxide solution at 37 °C. The hydrolysis products 1-propyl-8-*p*-sulfophenylxanthine and *m*-nitrophenol could clearly be detected showing migration times of 5.3 and 4.9 min, respectively. It was found that the chemical hydrolysis by hydroxide ions of these sulfonic acid esters followed a first-order kinetic. In Table 2, the hydrolysis constants (K) and the half-lives ($t_{1/2}$) of the esters are collected. It was shown that the xanthine sulfonate esters were very stable toward chemical hydrolysis by 0.001 N aqueous sodium hydroxide solution with half-lives of about 20 h.

Because the chemical structure of all investigated *m*-nitrophenyl sulfonate esters is similar, their stability should be comparable. This was confirmed by the chemical stability tests, in which no significant differences between the half-lives of esters **5a**–**e** were observed. Therefore only one representative compound was selected for performing further in vitro stability tests.

Compound **5d**, 1-propyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine, was selected as an example to investigate its biological stability at 37 °C in (i) fetal calf serum, (ii) simulated gastric acid, and (iii) rat liver homogenate. These various media were thought to be valid in vitro models for the conditions that may affect the potential prodrugs during oral application.

For the biological in vitro stability tests, different CE separation conditions were applied than for the chemical stability tests because of the protein content in the biological samples, which does not impede CE analysis but influences the migration and separation by CE. Thus, voltage and SDS concentration had to be adjusted. Detection in biological samples was at 320 nm in order to exclude interference from biological molecules, some of which show absorption at 254 nm. The internal standard had to be chosen accordingly (diclofenac sodium salt for the chemical hydrolysis experiments, *p*-aminosalicylate sodium salt for the biological experiments). Table 3 summarizes the separation conditions used in chemical and biological stability tests.

The stability test in simulated gastric acid was performed for 4 h since drugs usually do not stay in the stomach any longer. Compound **5d** was found to be very stable under these conditions and degradation was negligible. After 4 h of incubation in simulated gastric acid, 94% of **5d** could still be detected.

Table 3. Separation Conditions in Capillary Electrophoresis for the Investigation of Hydrolysis of Compound **5d**

incubation medium	ce buffer	voltage (kV)	internal standard	wavelength (nm)
0.001 N aq NaOH solution	100 mM borate buffer + 50 mM SDS (pH 8)	10	diclofenac sodium salt	254
fetal calf serum	100 mM borate buffer + 100 mM SDS (pH 8)	15	*p*-aminosalicylate sodium salt	320
simulated gastric acid	100 mM borate buffer + 100 mM SDS (pH 8)	15	*p*-aminosalicylate sodium salt	320
rat liver homogenate	100 mM borate buffer + 150 mM SDS (pH 8)	12	*p*-aminosalicylate sodium salt	320

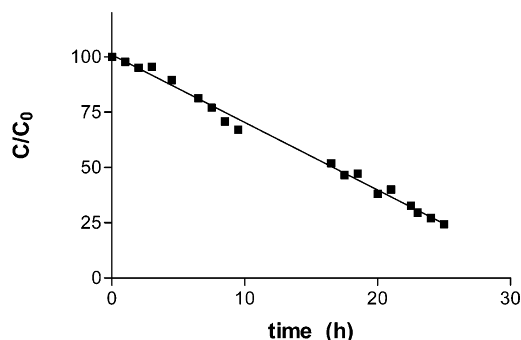


Figure 7. Stability test of 1-propyl-8-[4-[[*m*-nitrophenoxy]-sulfonyl]phenyl]xanthine **5d** in fetal calf serum at 37 °C (C = concentration of compound, C_0 = concentration of compound at time zero).

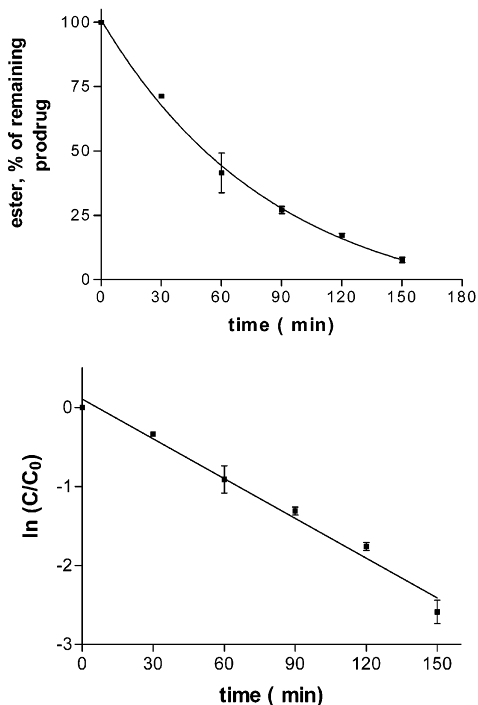


Figure 8. Hydrolysis of 1-propyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine **5d** by rat liver homogenate (protein concentration: 16 mg/mL, concentration of **5d**: 2×10^{-5} M) at 37 °C; $t_{1/2}$ = 41 min (C = concentration of compound, C_0 = concentration of compound at time zero).

In the next step, hydrolysis of **5d** in fetal calf serum was measured as an indicator for the stability of the *m*-nitrophenyl sulfonate esters in blood serum. The hydrolysis of **5d** in fetal calf serum was also very slow with a half-life ($t_{1/2}$) of 16 h following a zero-order kinetic (Figure 7).

Finally, metabolism of **5d** by liver enzymes was investigated. The hydrolysis of compound **5d** in freshly prepared rat liver homogenate could be described as a first-order kinetic reaction (Figure 8). Its half-life ($t_{1/2}$) was 40 min under the applied conditions. It is currently unknown which enzyme(s) may be responsible for the metabolism of the sulfonic acid *m*-nitrophenyl esters

Thus, the ester was very stable in simulated gastric acid as well as in serum. However, it was readily hydrolyzed by incubation with rat liver homogenate, indicating an enzymatic pathway of hydrolysis.

In contrast to the chemical stability tests (see above), hydrolysis products could not be detected in the electropherograms of the biological in vitro stability tests. The only change that could be observed was the decrease of the peak area of the ester with increased incubation time. The reason no hydrolyzed product could be detected by CE analyses is not clear. One possible explanation would be that both hydrolysis products interacted with the proteins in the biological media forming neutral, insoluble compounds; thus they were not detectable by CE anymore. As a matter of fact we observed the precipitation of insoluble material during incubation with rat liver homogenate. To prove that the ester was actually cleaved to release the parent drug by enzymatic hydrolysis, thin-layer chromatography (TLC) was used as a supporting analytical method. It was found that after ca. 2 h of incubation of **5d** in rat liver homogenate at 37 °C, a large amount of 1-propyl-8-*p*-sulfophenylxanthine could be detected by TLC on silica gel using dichloromethane:methanol (4:1) as the mobile phase, while the educt had almost quantitatively disappeared. Thus it was proven that the active parent drug was actually released presumably by enzymatic biotransformation.

From the chemical and biological in vitro stability tests, it can be concluded that the synthesized sulfophenylxanthine *m*-nitrophenyl esters are relatively stable, since their half-lives in 0.001 N aqueous sodium hydroxide solution solution at 37 °C reach 20 h on average. In biological media, the prototypic compound **5d** was not degraded by simulated gastric acid within 4 h of incubation at 37 °C. This means that it will be stable in the gastrointestinal tract and should cross the gastrointestinal barrier reaching the blood stream without prior degradation. The compound will remain as an intact ester in the circulation since its half-life in fetal calf serum was ca. 16 h. Finally, the ester will be hydrolyzed in the liver by enzymatic biotransformation.

Adenosine Receptor Affinity. To investigate the effects of esterification of 8-(*p*-sulfophenyl)xanthine derivatives with nitrophenols on adenosine receptor affinity, all compounds were investigated in radioligand binding studies at rat brain A_1, A_{2A}, and human A_3 ARs. [^{3}H]-2-Chloro-N^6-cyclopentyladenosine (CCPA), [^{3}H]-3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine (MSX-2), and [^{3}H]-2-phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one (PSB-11), respectively, were used as A_1, A_{2A}, and A_3 radioligands. Selected compounds were also tested at human A_{2B} ARs using [^{3}H]8-((4-(2-hydroxyethylamino)-2-oxo-ethoxy)phenyl)-1-propylxanthine (PSB-

Table 4. Adenosine Receptor Affinities of 8-Phenylxanthines, 8-*p*-Sulfophenylxanthines and Corresponding Sulfonic Acid Nitrophenyl Esters

5a -5e, 9a - 9e **6a - 6e** **10a - 10e**

compd	R^1	R^2	K_i ± SEM [nM] (or % inhibition of radioligand binding at 10 μM)			
			A_1-affinity rat [^{3}H]CCPA (n = 3)	A_{2A}-affinity rat [^{3}H]MSX-2 (n = 3)	A_{2B}-affinity human [^{3}H]PSB-298 (n = 2)	A_3-affinity human [^{3}H]PBS-11 (n = 2)
			m-Nitrophenyl Esters of Sulfophenylxanthines			
5a	methyl	methyl	21 ± 3	117 ± 22	n.d.[a]	(25% ± 1)
5b	propyl	propyl	4.4 ± 0.3	28 ± 29	43 ± 3.5	4300 ± 230 (66% ± 3)
5c	methyl	H	18 ± 2	63 ± 29	n.d.	(11% ± 4)
5d	propyl	H	6.5 ± 0.9	54 ± 5.2	13 ± 3.6	(14% ± 3)
5e	butyl	H	5.7 ± 0.1	21 ± 2.6	23 ± 6	(21% ± 1)
			p-Nitrophenyl Esters of Sulfophenylxanthines			
9a	methyl	methyl	7.7 ± 0.5	99 ± 21	n.d.	(16% ± 9)
9b	propyl	propyl	2.7 ± 0.1	16 ± 1.5	n.d.	(44% ± 2)
9c	methyl	H	6.3 ± 7	34 ± 12	n.d.	(5% ± 3)
9d	propyl	H	3.6 ± 0.03	74 ± 2	5.4 ± 4.3	(47% ± 9)[f]
9e	butyl	H	2.6 ± 0.3	78 ± 18	26 ± 2.5	(9% ± 9)
			8-Phenylxanthines			
10a	methyl	methyl	89[32,b]	850[32,b]	415[33,d]	n.d.
10b	propyl	propyl	10[32,b]	190[32,b]	18.9[33,d]	n.d.
10c	methyl	H	260[25,b]	2200[25,b]	n.d.	n.d.
10d	propyl	H	67[25,b]	1900[25,b]	4.7[7,e]	n.d.
10e	butyl	H	40[7]	642[7]	11.8[7,e]	n.d
			8-*p*-Sulfophenylxanthines			
6a (SPT)	methyl	methyl	14000[32,b]	14000[32,b]	1330[33,d]	11000[34,c]
6b (DPSPX)	propyl	propyl	210[32,b]	1400[32,b]	250[35]	183[34,c]
6c (PSB-1115)	methyl	H	1238	11800	n.d.	n.d.
6d	propyl	H	2200[25,b]	24000[25,b]	53.4[7,e]	14%[7]
6e	butyl	H	475[7]	8070[7]	70[7,e]	39%[7]

[a] n.d.: not determined. [b] [^{3}H]PIA was used as A_1-radioligand and [^{3}H]NECA as A_{2A}-radioligand. [c] Sheep A_3 receptor. [d] [^{125}I]ABOPX binding at human HEK-293 A_{2B} receptor. [e] [^{3}H]ZM241385 binding to recombinant human A_{2B} receptor. [f] N = 3.

298). Native brain tissues could be used for A_1 (cortex) and A_{2A} (striatum) assays due to the high density of those receptor subtypes in the brain. A_{2B} and A_3 receptors are expressed in much lower density, and thus artificial recombinant systems had to be used. It has previously been shown that species differences for rat and human A_1, A_{2A}, and A_{2B} ARs are moderate, only for the A_3 ARs large species differences have been described, antagonists being in most cases considerably less potent at rat as compared to human A_3 ARs.[2] Therefore we have chosen to determine the affinity at human A_3 ARs as being more relevant for drug development. However, rat data for A_1 and A_{2A} affinity were thought to be sufficient for obtaining a rough estimation of the compounds' selectivity even though data from different species were compared. Determined affinities of the synthesized nitrophenyl esters are collected in Table 4. Data of 1,3-substituted 8-phenylxanthines (**10a**−**e**) and 1,3-substituted 8-*p*-sulfophenylxanthines (**6a**−**e**) are given for comparison.

The nitrophenyl esters of *p*-sulfophenylxanthine derivatives (**5a**−**e**, **9a**−**e**) showed high affinities for A_1, A_{2A}, and A_{2B} adenosine receptors. Figure 9 illustrates the effects on A_1 and A_{2A} AR affinity of the esters in comparison with the corresponding 8-phenylxanthine and 8-*p*-sulfophenylxanthine derivatives. It had been observed earlier that a *p*-sulfonate group results in a decrease in A_1 and A_{2A} AR affinity as compared with the corresponding 8-phenylxanthine derivatives.[24,25] We have now found that esterification with *m*- or *p*-nitrophenol not only restored but even increased A_1 and A_{2A} affinity in comparison with the corresponding 8-phenylxanthine derivatives. Thus, the esterified compounds are no prodrugs in a classical sense since they show enhanced affinity. A similar trend, but less pronounced, was observed for the A_{2B} AR with a selected set of compounds (Table 4). All nitrophenyl esters showed weak to negligible binding to human A_3 ARs. The most potent compound at A_3 ARs was the 1,3-dipropyl-substituted *m*-nitrophenyl ester **5b** with a K_i value of 4.3 μM confirming the previously observed favorable effects of 1,3-dipropyl substitution of xanthines on A_3 affinity.[7] However, all compounds, including **5b**, were much more potent at the other AR subtypes than at A_3 receptors.

All investigated nitrophenyl esters were more potent at A_1 than at A_{2A} ARs (3.5−30-fold). 1-Propyl- and 1-butyl-substituted *p*-nitrophenyl esters (**9d**, **9e**) showed the highest A_1 versus A_{2A} selectivity (20- and 30-fold) respectively, which was, however, lower versus the A_{2B} receptor subtype.

At A_1 ARs *p*-nitro-substitution (**9a**−**e**) was superior to a *m*-nitro substitution (**5a**−**e**, see Table 4) resulting in about 2-fold lower K_i values ranging from 2.6 to 7.7 nM. At A_{2A} ARs, 1,3-disubstituted and 1-methyl-substituted xanthine *p*-nitrophenyl sulfonates also exhibited higher affinities than the corresponding xanthine *m*-nitrophenyl sulfonates, but the 1-propyl- and

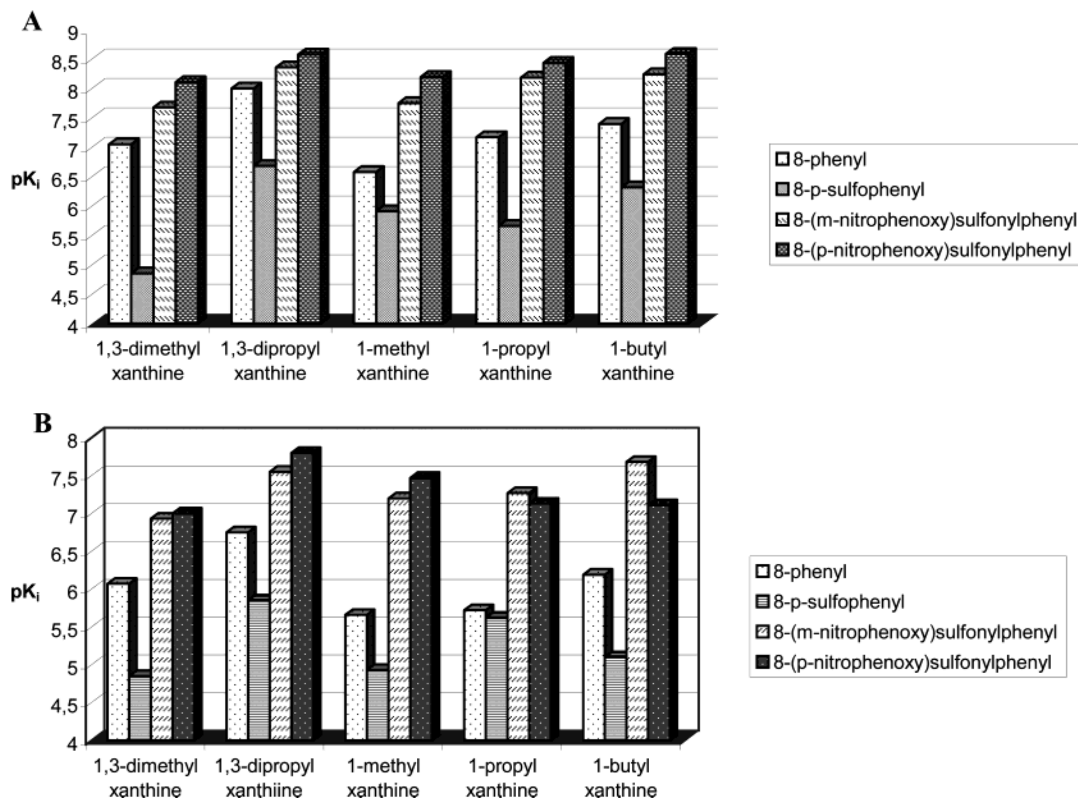


Figure 9. Effects of sulfonate and nitrophenoxysulfonate groups in 8-phenylxanthine derivatives on rat A_1 (A) and A_{2A} (B) adenosine receptors. Affinities determined in radioligand binding assays are given as pK_i values ($-\log K_i$ values) [M].

1-butyl-substituted xanthine derivatives showed the opposite effect. The influence of the 1,3-substitution pattern on A_1 and A_{2A} affinity was altogether moderate. N^1-Monoalkylated compounds were similarly potent as 1,3-disubstituted derivatives. At A_1 ARs the rank order of potency in the *p*- and *m*-nitrophenyl series was very similar: 1,3-dipropyl ≈ 1-butyl ≈ 1-propyl > 1-methyl ≈ 1,3-dimethyl. At A_{2A} ARs the rank order of potency was similar as above for the *m*-nitrophenyl esters, but somewhat different for the *p*-nitrophenyl derivatives; in the latter series, the 1-methyl-substituted compound was surprisingly potent with the following rank order of potency: 1,3-dipropyl > 1-methyl > 1-propyl ≈ 1-butyl > 1,3-dimethyl.

The 1,3-dipropyl-substituted *p*-nitrophenyl ester **9b** belonged to the most potent A_1 and A_{2A} ligands of the present series, with a K_i value of 2.7 nM at A_1 and 16 nM at A_{2A} ARs, whereas the 1,3-dimethyl-substituted *m*-nitrophenyl ester **5a** had the lowest affinity with a K_i value of 21 nM at A_1 and 117 nM at A_{2A} ARs. Thus, it can be concluded that the introduction of a second propyl group (in the 3-position) of 1-propylxanthine nitrophenyl sulfonates increased the affinity to A_1, A_{2A}, and A_3 ARs, while the introduction of a second methyl group into the 3-position of 1-methylxanthine nitrophenyl sulfonates decreased the affinity to A_1 and A_{2A} ARs.

Compounds **5b**, **5d**, **5e**, **9d**, and **9e** were selected for additional testing at A_{2B} ARs. Table 4 shows that all compounds investigated exhibited relatively high affinity for A_{2B} ARs. There is not much difference between *p*- and *m*-nitrophenyl-substituted derivatives (compare **5d/9d** and **5e/9e**). 3-Unsubstituted xanthines which had been somewhat less potent or similarly potent to 1,3-dipropyl-substituted derivatives at A_1 and A_{2A} ARs (compare **5b/5d** and **9b/9d**) were more potent at A_{2B} ARs (compare **5b/5d**). This appears to be a general trend and is also true for 8-phenyl-, 8-*p*-sulfophenyl-, and 8-cycloalkylxanthine derivatives.[7] The most potent A_{2B} antagonist of the present series was 1-propylxanthine *p*-nitrophenylsulfonate **9d** with a K_i value of 5.4 nM at human A_{2B} ARs; the compound showed similarly high affinity for A_1 ARs and therefore was nonselective. Compared with the corresponding 8-*p*-sulfophenylxanthines, it can be observed that the introduction of a nitrophenyl substituent increased A_{2B} affinity greatly, but the selectivity of A_{2B} versus other AR subtypes tended to decrease. Compared with the corresponding 8-phenylxanthines, the introduction of a bulky nitrophenoxysulfonyl group decreased the A_{2B} affinity slightly, and A_{2B} selectivity decreased as well.

Conclusions

In conclusion, nitrophenyl esters of sulfophenyl xanthine derivatives, that are potent adenosine receptor antagonists, have been synthesized. The chemical and enzymatic stability of the esters was determined in vitro. The compounds showed high stability in aqueous solution, in artificial gastric acid, and in serum. However, compound 1-propyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]-phenyl]xanthine **5d**, investigated as a prototypic ester, was readily cleaved by incubation with rat liver homogenate. Thus, nitrophenyl esters of sulfonic acids have a potential as peroral prodrugs of drugs bearing a sulfonate group. Besides, the nitrophenyl esters of 8-*p*-sulfophenylxanthine derivatives showed high potency at A_1, A_{2A}, and A_{2B} ARs. 1-Butyl-8-[4-[[*p*-nitrophenoxy]-sulfonyl]phenyl]xanthine (**9e**) was the most potent and selective A_1 AR antagonist of the present series with a K_i value of 2.6 nM at rat A_1 ARs and 30-fold selectivity

versus rat A_{2A}, and 10-fold versus human A_{2B} receptors, the compound was inactive at human A_3 ARs. The corresponding 1-propylxanthine derivative (**9d**) was a very potent mixed A_1/A_{2B} antagonist (K_iA$_1$ 3.6 nM, K_iA$_{2B}$ 5.4 nM). *m*-Nitrophenol has been shown to be a suitable protecting group for phenylsulfonic acids, which can be cleaved off in alkaline solution.

Experimental Section

Chemical Synthesis. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance 500 MHz spectrometer. The chemical shifts of the remaining protons of the deuterated solvent served as internal standards for spectra recorded in DMSO-d_6: δ ^{1}H: 2.49, δ ^{13}C: 39.1. Tetramethylsilane was used as internal standard for spectra recorded in acetone-d_6 and D_2O. All compounds were checked for purity by TLC on silica gel 60 F_{254} (Merck) aluminum plates, using dichloromethane: methanol (4:1) or dichloromethane:methanol (8:1) as the mobile phase. Melting points were determined on a Buechi 530 melting point apparatus and were uncorrected. Elemental microanalyses were performed at the Pharmaceutical Institute, Pharmaceutical Chemistry Endenich, University of Bonn. Mass spectra were recorded on an MS-50 spectrometer at the Chemical Institute, University of Bonn.

o-, *m*-, and *p*-Nitrophenyl tosylates were synthesized by reacting toluenesulfonyl chloride with *o*-, *m*-, or *p*-nitrophenol in dichloromethane in the presence of triethylamine (TEA) as described.[23]

1,3-Disubstituted-5,6-diaminouracils (**1a**, **1b**) were prepared from 1,3-disubstituted urea and cyanoacetic acid followed by ring closure, nitrosation and reduction as described.[36,37] 3-Substituted 5,6-diaminouracils **1c**−**e** were prepared from 6-aminouracil via regioselective alkylation followed by nitrosation and reduction as described.[38,39]

6-Amino-1,3-dimethyl-5-(4-sulfobenzamido)uracil (2a). A solution of 0.9 g (5.3 mmol) of 1,3-dimethyl-5,6-diaminouracil, 1.3 g (5.4 mmol) of *p*-sulfobenzoic acid potassium salt, and 1.0 g (5.2 mmol) of *N*-(3-(dimethylamino)propyl)-*N*-ethylcarbodiimide hydrochloride (EDC) in 20 mL of water was stirred for 1 h at rt. The solvent was removed by evaporation, a small amount of methanol was added, and the residue was collected by filtration. The filtrate was cooled to complete the precipitation of further product.

1,3-Dimethyl-8-*p*-sulfophenylxanthine (2b). Carboxamide derivative **2a** (0.91 g, 0.26 mmol) was dissolved in 25 mL of 2.5 N NaOH and heated at 70 °C for 10 min. After cooling to 0 °C, the product was precipitated by the addition of concd HCl to pH 6. The precipitate was washed with cold 1 N HCl. The product was recrystallized by dissolution in aqueous NaOH solution followed by acidification with aqueous HCl solution.

1,3-Dimethyl-8-(*p*-chlorosulfonylphenyl)xanthine (2c). Compound **2b** (59 mg, 1.16 mmol) was refluxed with 10 mL of thionyl chloride for 2 h, and then remaining thionyl chloride was completely removed in vacuo. A white solid was obtained, which did not have to be further purified.

8-*p*-Sulfophenyl-1,3,7-trimethylxanthine (2d). 1,3-Dimethyl-8-*p*-sulfophenylxanthine **2b** (100 mg, 0.3 mmol) was dissolved in 10 mL of DMF, K_2CO_3 (8.2 mg, 0.6 mmol) and MeI (0.37 mL, 6 mmol) were added, and the mixture was stirred at rt overnight. The solution was filtered and diethyl ether (20 mL) was added to the filtrate to precipitate the product. The white precipitation was filtered off and washed with diethyl ether.

4-[[*m*-Nitrophenoxy]sulfonyl]benzoic Acid (3), 4-[[*p*-Nitrophenoxy]sulfonyl]benzoic Acid (7). General Procedure. Chlorosulfonic acid (50 mL) was slowly added to 5 g (0.021 mol) of *p*-sulfobenzoic acid potassium salt, while the temperature was kept below 30 °C. The mixture was stirred overnight. A crystalline precipitate was obtained when chipped ice was carefully added to the above mixture. After filtration, the residue was washed with cold water and dried to yield 4-chlorosulfonylbenzoic acid.[26,27] *m*-Nitrophenol or *p*-nitrophenol (1.99 g, 0.014 mmol) was dissolved in 50 mL of THF and 50 mL of Tris-HCl buffer (50 mM, pH 9). A solution of 4-chlorosulfonylbenzoic acid (3.4 g, 0.014 mol) in 50 mL of THF was added slowly to the above solution. The pH value of the mixture was kept at 8−9 by the addition of 2.5 N NaOH. After stirring for about 4 h at rt, no more phenol could be detected by TLC. Then the solution was brought to pH 7 by the addition of 1 N HCl, and the THF was removed in vacuo. The product precipitated when the aqueous solution was acidified with 1 N HCl to pH 1. The formed product was recrystallized from acetone/cyclohexane.

Compound **3**: Yield 45% (calcd. from *p*-sulfobenzoic acid); mp 213.5 °C; ^{1}H NMR (acetone-d_6) δ (ppm): 7.56 (m, 1H, aromatic), 7.74 (t, 1H, J = 8.35 Hz, aromatic), 7.93 (t, 1H, J = 2.2 Hz, aromatic), 8.09 (d, 2H, J = 8.51 Hz, aromatic), 8.25 (m, 1H, aromatic), 8.3 (d, 2H, J = 8.51 Hz, aromatic). ^{13}C NMR (acetone-d_6) δ (ppm): 118.57, 123.32, 129.7, 129.76, 131.67, 132.19, 137.27, 139.38, 150.38 (aromatic), 165.99 (COOH). Anal. ($C_{13}H_9NO_7S$) C, H, N.

Compound **7**: Yield 41% (calcd. from *p*-sulfobenzoic acid); mp 218−219 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 7.36 (d, 2H, J = 9.14 Hz, armatic), 8.02 (d, 2H, J = 8.82 Hz, aromatic), 8.18 (d, 2H, J = 8.82 Hz, aromatic), 8.26 (d, 2H, J = 9.14 Hz, aromatic). ^{13}C NMR (DMSO-d_6) δ (ppm): 123.81, 126.26, 129.06, 131.03, 137.12, 137.54, 146.62, 153.3 (aromatic), 166.11 (COOH). Anal. ($C_{13}H_9NO_7S$) C, H, N.

6-Amino-1,3-dimethyl-5-[4-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (**4a**), 6-amino-1,3-dipropyl-5-[4-[[*m*-nitrophenoxy]-sulfonyl]benzamido]uracil (**4b**), 6-amino-1-methyl-5-[4-[[*m*-nitrophenoxy]sulfonyl]benzamido]uracil (**4c**), 6-amino-1-propyl-5-[4-[[*m*-nitrophenoxy] sulfonyl]benzamido]uracil (**4d**), 6-amino-1-butyl-5-[4-[[*m*-nitrophenoxy]sulfonyl] benzamido]uracil (**4e**), 6-amino-1,3-dimethyl-5-[4-[[*p*-nitrophenoxy]sulfonyl]benzamido] uracil (**8a**), 6-amino-1,3-dipropyl-5-[4-[[*p*-nitrophenoxy]-sulfonyl]benzamido]uracil (**8b**), 6-amino-1-methyl-5-[4-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (**8c**), 6-amino-1-propyl-5-[4-[[*p*-nitrophenoxy]sulfonyl]benzamido]uracil (**8d**), 6-amino-1-butyl-5-[4-[[*p*-nitro- phenoxy]sulfonyl]benzamido]uracil (**8e**). **General Procedure.** To a suspension of 1,3-substituted-5,6-diaminouracil **1a**−**e** (7.2 mmol) in methanol was added an equimolar amount of 4-[[*m*-nitrophenoxy]sulfonyl]benzoic acid **3** or 4-[[*p*-nitrophenoxy]sulfonyl]benzoic acid **7**, and an equimolar or slightly excessive amount of EDC. The mixture was stirred at rt. The color of the solution turned yellow, and a precipitate began to form. The reaction was stopped when no more diaminouracil could be detected by TLC after several hours. The precipitate was filtered off and washed with cold water. Products were recrystallized by dissolving them in DMF followed by dropwise addition of water.

1,3-Dimethyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]xanthine (**5a**), 1,3-dipropyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]-xanthine (**5b**), 1-methyl-8-[4-[[*m*-nitrophenoxy]sulfonyl] phenyl]-xanthine (**5c**), 1-propyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]-xanthine (**5d**), 1-butyl-8-[4-[[*m*-nitrophenoxy]sulfonyl]phenyl]-xanthine (**5e**), 1,3-dimethyl-8-[4-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (**9a**), 1,3-dipropyl-8-[4-[[*p*-nitrophenoxy]sulfonyl]phenyl]xanthine (**9b**), 1-methyl-8-[4-[[*p*-nitrophenoxy]-sulfonyl]phenyl]xanthine (**9c**), 1-propyl-8-[4-[[*p*-nitrophenoxy]-sulfonyl]phenyl]xanthine (**9d**), 1-butyl-8-[4-[[*p*-nitrophenoxy] sulfonyl]phenyl]xanthine (**9e**). **General Procedure.** A mixture of carboxamido derivative **4a**−**e** or **8a**−**e**, (0.32 mmol) and 4 g of polyphosphoric acid trimethylsilyl ester (PPSE) was first heated at 120 °C for 10 min and then at 170 °C for 1.5−2 h. After cooling, 20 mL of methanol was added to the mixture and the formed precipitate was collected by filtration and washed with methanol. The products were finally recrystallized by dissolving them in DMF and subsequent dropwise addition of water.

1-Methyl-8-*p*-sulfophenylxanthine (6c), 1-Propyl-8-*p*-sulfophenylxanthine (6d). General Procedure. Compound **5c** or **5d** (6 mmol) was dissolved in 60 mL of 2 N NaOH. The solution was heated at 70 °C for 30 min. After cooling to rt, the pH was carefully adjusted to 7 by concd HCl, and the solution was extracted with CH_2Cl_2 serveral times to remove

the hydrolyzed *m*-nitrophenol. The product was precipitated by careful acidification to pH 4 with HCl, filtered off, and recrystallized from water or purified by preparative HPLC (see below).

Hydrolysis Measurements of Model Compounds. Hydrolysis rates of *o*-, *m*-, and *p*-nitrophenyl tosylates were measured by following UV absorbance changes using an HP 8452A spectrophotometer equipped with a diode array detector. All reactions were carried out under pseudo-first-order conditions while the buffer concentration was maintained in large excess over that of the esters. Acetonitrile was used as a cosolvent (10% v/v or 5% v/v) to keep esters in solution. Five buffer solutions were used: 50 mM KCl−HCl buffer (pH 1), 50 mM KH_2PO_4 buffer (pH 6, pH 7, pH 8), and 50 mM H_3BO_3−KCl buffer (pH 9.8). Rate constants were measured by following the appearance of the product continuously at fixed wavelengths. Absorbance readings were made at the λ_{max} of the products: *p*-nitrophenol (400 nm), *m*-nitrophenol (376 nm), *o*-nitrophenol (400 nm). Hydrolysis rate constants (K) were calculated as the slope of a plot of $\log(A_t - A_0)$ versus time.

Preparative HPLC. The HPLC system consisted of a WellChrom Preparative HPLC pump K-1800 (Knauer GmbH, Berlin, Germany), a spectrophotometer K-2600 (Knauer GmbH, Berlin, Germany), and a computer equipped with the Eurochrom 3.05 preparative software (Knauer GmbH, Berlin, Germany). The preparative column and precolumn used were Eurospher 100, C_{18}, 10 μm, 250 × 20 mm and Eurospher 100, C_{18}, 5 μm, 30 × 20 mm, respectively. The elution system was as follows: eluent A, 35% methanol and 65% water; eluent B, water. Sample (10 mg/mL, 10 mL) was injected into the HPLC system at a speed of 10 mL/min. Then a linear gradient at the speed of 20 mL/min from 50% eluent A + 50% eluent B to 100% eluent A over 20 min was used. The retention time of compound **6d** was 11 min.

Capillary Electrophoresis. Apparatus. A P/ACE 5500 CE instrument (Beckman Coulter Instruments, Fullerton, CA) equipped with a DAD-UV detection system using a fused silica capillary (30/37 cm long; 75 μm diameter) was used for the purity, stability, and degradation studies. An electrokinetic injection for 5 s was applied for introducing the sample. The capillary temperature was kept constant at 25 °C.

Purity Determination by Capillary Electrophoresis (CE). The buffer used for the purity determination was 20 mM phosphate buffer (pH 7.4) at a voltage of 10 kV. The migration time of compound **6d** was 3.2 min.

Stability and Degradation Studies with Capillary Electrophoresis. Materials. Fetal calf serum was bought from Sigma (F 7524). Simulated gastric acid was made according to lit:[40] To 3.20 g of pepsin, 2.0 g of NaCl, and 80 mL of HCl (1 mol/L) was added distilled water to obtain 1000 mL, and this solution was stored at 4 °C. Rat liver homogenate was prepared as described with slight modifications:[41,42] Fresh rat liver (6.5 g) was homogenized in Dulbecco's phosphate-buffered saline (DPBS) (consisting of 132.5 mg of $CaCl_2 \cdot 2H_2O$, 100 mg of $MgCl_2 \cdot 6H_2O$, 200 mg of KCl, 200 mg of KH_2PO_4, 8000 mg of NaCl, and 1500 mg of Na_2HPO_4 in a total volume of 1000 mL, pH 7.2, freshly prepared) and centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant was carefully decanted to obtain the rat liver homogenate. The protein concentration was 16 mg/mL as determined by the method of Bradford.[43] The rat liver homogenate was kept at −80 °C before use.

Method and Conditions. Borate buffer (pH 8.0, 100 mM), containing various amounts of SDS, and different voltages were used according to the different biological media. In degradation studies with 0.001 N NaOH solution, 10 kV voltage was applied and 50 mM SDS was used, while in studies with fetal calf serum and simulated gastric acid media, 5 kV voltage and 100 mM SDS gave better results; in studies with rat liver homogenate, 12 kV and 150 mM SDS was used.

Chemical Stability. Stock solutions (1 mL, 2 mM in DMSO) of **5a**−**e** were incubated with 99 mL of 0.001 N NaOH at 37 °C in a water bath. At certain time intervals, 4.8 mL of incubation solution was withdrawn and mixed with 0.1 mL of 1 mg/mL diclofenac sodium salt as internal standard for analysis by capillary electrophoresis. The initial concentration of the test compound in the analytical sample was 2×10^{-5} M.

Biological Stability. A stock solution of **5d** (30 μL, 50 mM in DMSO; 150 μL, 2 mM in DMSO; 10 μL, 20 mM in DMSO) was added to fetal calf serum (1470 μL), simulated gastric acid (9850 μL), and rat liver homogenate (9850 μL), respectively. The mixture was incubated in a water bath at 37 °C with a rotation speed of 900 rpm. At certain time intervals, 100 μL of fetal calf serum incubation solution was withdrawn and diluted with 4.7 mL of H_2O and 50 μL of a solution of *p*-aminosalicylate sodium salt (1 mg/mL) as internal standard; 400 μL of simulated gastric acid incubation solution was withdrawn and mixed with 100 μL of a solution of *p*-aminosalicylate sodium salt (0.1 mg/mL) as internal standard; 50 μL of rat liver homogenate incubation solution was withdrawn and added to 100 μL of 0.1% TFA, 250 μL of Dulbecco's phosphate-buffered saline (DPBS), and 100 μL of a solution of *p*-aminosalicylate sodium salt (0.1 mg/mL) as internal standard. The solutions were analyzed by CE after short vortexing. The initial concentration of the test compound was 2×10^{-5} M.

During the stability experiment in simulated gastric acid, the migration time of the prodrug was delayed for ca. 2−3 min after each test, probably due to proteins absorbed to the capillary. Since an alkaline borate buffer (pH 8) was applied as the running buffer, pepsin in simulated gastric acid may have precipitated in the capillary, inducing a change of the capillary's surface. The resulting prolongation of the migration time of the compounds did not influence the results.

The half-lives were determined by the curve fitting program GraphPad Prism, version 2.0 (GraphPad, San Diego, CA).

Radioligand Binding Assays. Materials. Radioligands were obtained from the following sources: [^{3}H]CCPA was from NEN Life Sciences (54.9 Ci/mmol), [^{3}H]MSX-2 (85 Ci/mmol), [^{3}H]PSB-11 (53 Ci/mmol), and [^{3}H]PSB-298 (124 Ci/mmol) were from Amersham. The nonradioactive precursors of [^{3}H]-MSX-2 (MSX-1), [^{3}H]PSB-11 (PSB-10), and [^{3}H]PSB-298 (PSB-297) were synthesized in our laboratory.[7,44,45]

Membrane Preparations. Frozen rat brains obtained from Pel Freez, Rogers, AR, were dissected to obtain cortical membrane preparations for A_1 assays, and striatal membrane preparations for A_{2A} assays, as described.[46,47] Membranes from Chinese hamster ovary (CHO) cells stably transfected with the human A_{2B} and A_3 AR were prepared as described.[48]

Radioligand Binding Assays. Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO); the final concentration of DMSO in the assay was 2.5%. The radioligands and their concentrations were as follows: [^{3}H]-CCPA,[49] 0.5 nM (rat A_1), [^{3}H]MSX-2,[44] 1 nM (rat A_1), [^{3}H]PSB-298 (manuscript in preparation), 5 nM (human A_{2B}); [^{3}H]PSB-11,[45] 0.5 nM (human A_3). Binding assays were performed essentially as described.[44,45,48,49] About 70 μg/mL of protein was used in the A_1 and A_{2A} assays; ca. 50−100 μg/mL of protein was used in the human recombinant A_{2B} and A_3 assay, respectively. Membranes were preincubated for 20 min with 0.12−0.22 IU/mL of adenosine deaminase in order to remove endogenous adenosine. Curves were determined using 6−7 different concentrations of test compounds spanning 3 orders of magnitude. At least two to three separate experiments were performed, each in duplicate or triplicate. Data were analyzed using GraphPad Prism, version 2.0 (GraphPad, San Diego, CA). For nonlinear regression analysis, the Cheng−Prusoff equation and K_D values of 0.2 nM (rat A_1) for [^{3}H]CCPA, 8 nM (rat A_{2A}) for [^{3}H]MSX-2, 56 nM (human A_{2B}) for [^{3}H]PSB-298, and 4.9 nM (human A_3) for [^{3}H]PSB-11 were used to calculate K_i values from IC_{50} values.

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Supporting Information Available: Additional ^{1}H and ^{13}C NMR data of synthesized compounds. This material is available free of charge in the Internet at http://pubs.acs.org.

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